Identification and characterization of *Quercus robur* ectomycorrhiza in relation to heavy metal contamination

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Content

Abbreviations

ab.	abundances
AM	vesicular-arbuscular mycorrhiza
AMD	acid mining drainage
a_t	around trees
Av.	average
AvCVSp	average of coefficient of variation for all species
BP	Berger-Parker index
b_t	between trees
CCA	canonical correspondence analyses
CMNs	common mycorrhizal networks
CPVS	cumulated percentages of explained species variance
CV	coefficient of variation
DCA	detrended correspondence analyses
DCCA	detrended canonical correspondence analyses
ECM	ectomycorrhiza
EDX	energy-dispersive X-ray spectroscopy
E.V.	environmental variables
F	extracted factor
hDCCA	hybrid detrended canonical correspondence analyses
IGS	intergenic spacer
ITS	internal transcribed spacer
LMOA	low molecular organic acids
PCA	principal component analyses
PCR	polymerase chain reaction
PIXE	particle-induced X-ray emission
rDNA	ribosomal deoxyribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SD	standard deviation
sp.	species
TDM	trophic dynamic module
λ	eigenvalues

1 Introduction

1.1 Soils as habitats

Many of the soils of the world are affected by acidity, a problem resulting from mining, heavy fertilization with certain nutrients, acid rain, and weathering of sulfide minerals. The acidity can lead to protein denaturation and enzyme inhibition. Aside from the problems directly associated with low pH, acidification causes increased metal mobility. The ecological effects of such environmental stresses include loss of biodiversity and the impairment of live support functions such as decomposition and nutrient cycling. The ecological importance of biodiversity is complicated to determine, but it is commonly suggested that for ecosystem functioning under changing environmental conditions, it is preferable to try to maintain as high diversity as possible (Heinonsalo, 2004). Soils are heterogenic environments and provide a wide variety of niches for living organisms due to differences in physical, chemical and biological parameters (Rajala, 2008). The vegetation, microbes and animals in turn alter the soil through a wide range of biological activities. Microorganisms are useful indicators for environmental monitoring and ecological risk assessment because they are present in high amounts in all kinds of environments and play key roles in food webs and element cycles (Bloem *&* Breure, 2003). In their terrestrial environment, fungi are of fundamental importance as decomposers and plant symbionts (mycorrhizas), playing important roles in mineralization and other biogeochemical cycles. They are often dominant under acidic conditions and in soil they can comprise the largest pool of biomass. Their filamentous explorative growth habit and high surface area to mass ratio, leads to close interactions with soil particles and dissolved components. Fungus-metal interactions are an integral component of environmental cycling processes. The interactions of metals and their derivatives with fungi depend on the metal species, organisms and environment, while fungal metabolic activities can also influence speciation and mobility (Gadd *&* Sayer, 2000).

Surface mineral extraction creates many substrates for primary succession and already covers approx. 1% of the Earth’s land. Mining has always been a part of

civilization and is a crucial part of the global economy. Mining removes vegetation and soils and creates mine pits, stockpiles of topsoil, tailings and slurry lagoons. An example for primary succession is the study site heap site at Kanigsberg in Thuringia a former uranium mining area. Additionally, surface and ground water as well as pollution results from mining activities. The unearthing of geological formations with its subsequent weathering and chemical alteration of minerals can cause the generation of acidic seepage waters, which percolate through soil and are distributed vertically and horizontally into adjacent habitats. Acid mining drainage (AMD) is often involved in such contamination (Kothe *et al*., 2005). In order to prevent AMD formation, remediation actions try to minimize pyrite (FeS_2) oxidation. Some of these methods involve the control of the microflora, since the microbial community has been shown to be the single largest cause for AMD formation. Covers are used to limit the access of oxygen to the mine waste. This, in turn, will limit both the biological and chemical oxidation of the sulfides, which will substantially reduce the production of AMD. There are several types of soil covers: organic matter, forest or grassland vegetation and till. Vegetation has the ability to enhance the stability of mine wastes and decreases erosion (Fig. 1A.). At the same time, soil enhances microbial activity, especially at the surface of roots. The plant-root system is an effective way of introducing added energy supplies and microorganisms, and the use of the rhizosphere is being investigated as a treatment technology (Ernst, 2005). Plant roots may be linked by shared or common mycorrhizal networks (CMNs) that constitute pathways for the transfer of resources among plants. The movement of water by CMNs is potentially important to plant survival during drought, and that the functional ecophysiological traits of individual mycorrhizal fungi may be a component of this mechanism (Egerton-Warburton *et al*., 2007). In addition, fungi have capabilities for the disposal of recalcitrant soil contaminants and their fruitbodies are often found in mining areas (Fig. 1B.,C.). Increased attention to ecological interactions in soil could reduce costs and improve the efficacy of restoring a vegetation cover to land impacted by heavy metals or other disturbances. This is not only true for grassland ecosystems, but also for forests.

Forest management and harvesting operations often provide disturbance, and the revegetation initiates secondary succession (Smith *&* Read, 1997; van Schöll *et al*., 2008). One example is the study site near Greiz, in Eastern Thuringia (Fig. 2A.),

Figure 1: **A. The former heap site at Kanigsberg (Thuringia) after waste rock removal. Fruitbodys of ECM fungi found at the site: B. *Pisolithus tinctorius* and C. *Paxillus involutus*.**

where intensive silvicultural practices and cutting were followed by reforestation with a mixed forest including oak (*Quercus robur*) and some birches (*Betula pendula*) (Engler,1998). The soil is a podzol characterized by four distinct soil horizons: a dark-coloured organic (O) horizon underlayed by a white/ash-coloured eluvial (E) horizon, overlying a usually dark coloured illuvial (B) horizon on top of the unaltered parent (C) material (van Schöll *et al*., 2008) (Fig. 2B). Samples taken from 0-10 cm depth correspond to organic horizons noted with H and from 10-20 cm depth from the inorganic horizon were noted with A. Podzol soils are typical for relatively poor sites where concentrations of available soil nutrients are highly limited and the pH is low. Soil acidification may be viewed as a decrease in the base saturation of the soil: in other words, a decrease in the proportion of the cation exchange capacity satisfied by basic cations and a corresponding increase in the proportion of exchangeable hydrogen and aluminum. However, some trees are adapted to these conditions and have developed mechanisms to survive and successfully compete in these environments. The foundation of this success is that such plants allocate photosynthetically fixed carbon compounds to root symbiotic fungi, which help the plants to mobilize nutrients in these recalcitrant soils.

Discussions of succession and resource availability generally involve semantic difficulties. It is recommended to distinguish between primary and secondary succession as well as between resource supply and demand.

Figure 2: **A. *Quercus robur* forest at Greiz (Thuringia) after clear cut of coniferous forest and reforestation of mixed oak forest. B. Podzol soil profile: O=organic horizon, E=eluvial, B=illuvial horizon, C=parent material.**

As a control, uncontaminated land without disturbance at Jenzig forest, closed to Jena, was used as a third study site (Fig. 3). All three sites were mixed oak forests providing a habitat for ectomycorrhizal fungi, which were investigated for impact on disturbances.

Figure 3: ***Quercus robur* forest at Jenzig (Jena, Thuringia).**

1.2 Ectomycorrhiza

The mycorrhizal symbiosis is a common association between plant roots and fungi; in nature the majority of terrestrial plant roots are colonized by symbiotic fungi forming mycorrhizas (Smith *&* Read, 1997). The term “mycorrhiza” was coined by Frank in 1885. In mutual mycorrhizal symbiosis, the fungus takes up nutrients effectively from the soil and translocates parts of them to the host plant. In return, the host plant supports mycorrhizal fungi by delivering up to 39% photosynthesized carbohydrates (Smith *&* Read, 1997).

Ectomycorrhiza (ECM) is of special importance for boreal woodlands. ECM is formed between c. 5000-6000 species of fungi in the subphylum Basidiomycotina (Kendrick, 1992, Molina *et al*., 1992), some Ascomycotina (e.g. members of *Tuberales*) and two members from Zygomycotina with the fine roots of c. 2000 species of plants, including important components of forest ecosystems worldwide, e.g. members of the *Pinaceae*, *Fagaceae*, *Betulaceae* and *Myrtaceae* (Kendrick, 1992), and also some monocotyledons and ferns (Wilcox, 1996). Usually, the fungal hyphae grow between root cortical cells producing a netlike structure called the Hartig’ net (Fig. 4). Many ECM also have a sheath, or mantle, of fungal tissue that may completely cover the short root. Contiguous with the mantle are hyphal strands that extend into the soil. Often the hyphal strands will aggregate to form rhizomorphs that may be visible to the unaided eye. These hyphae function in the absorption and translocation of inorganic nutrients and water, but also release nutrients from litter layers by production of enzymes involved in mineralization of organic matter or low molecular organic acids involved in weathering of minerals.

The diagnostic feature of ECM is a heterorrhizic system, comprised of two kinds of roots; long roots of potentially unlimited extension and short roots with restricted growth and life span (Fig. 4A.). Root hairs are absent and these roots are completely enveloped by a fungal mantle along the length and apex. Mycorrhizal structures are not static organs but change ontogenetically from a juvenile to a mature state and finally senescence (Smith *&* Read, 1997).

The different structures of ectomycorrhiza can be used to classify and identify the mycorrhizal fungi. The features may differ between different host associations. The mantle may be smooth or with radiating hyphae, which enter into soil to increase the

absorptive surface of short roots. Different ECM fungi form distinctive mantles of varying thickness, texture and colour. For example, the mantle formed by *Lactarius subericatus* ECM consists of a pseudoparenchymatous outer mantle and a plectenchymatous inner mantle with branched laticifers while *Cenococcum geophilum* mantle consists of horizontally arranged palisade cells, alternating with groups of small pseudoparenchymatous cells (Fig. 4D.). When the hyphae composing the mantle can not any longer be recognized, the structure is called a pseudoparenchyma or synnema. But when hyphae maintain their identity and can still be recognized, the structure is called plectenchyma or prosenchyma. Mantle surface can range from thin to profuse and texture can vary from smooth, cottony, velvety or warty to granular. The hyphae radiating from the mantle surface may be simple or branched, bearing simple or clamped septae; the colour of these hyphae may be hyaline, black or orange, yellow or brown. The colour of mantle is mainly due to coloured radiating hyphae. In general, the fine structure of the mantle is similar to that of basidiomata hyphae, helping the identification of ECM types.

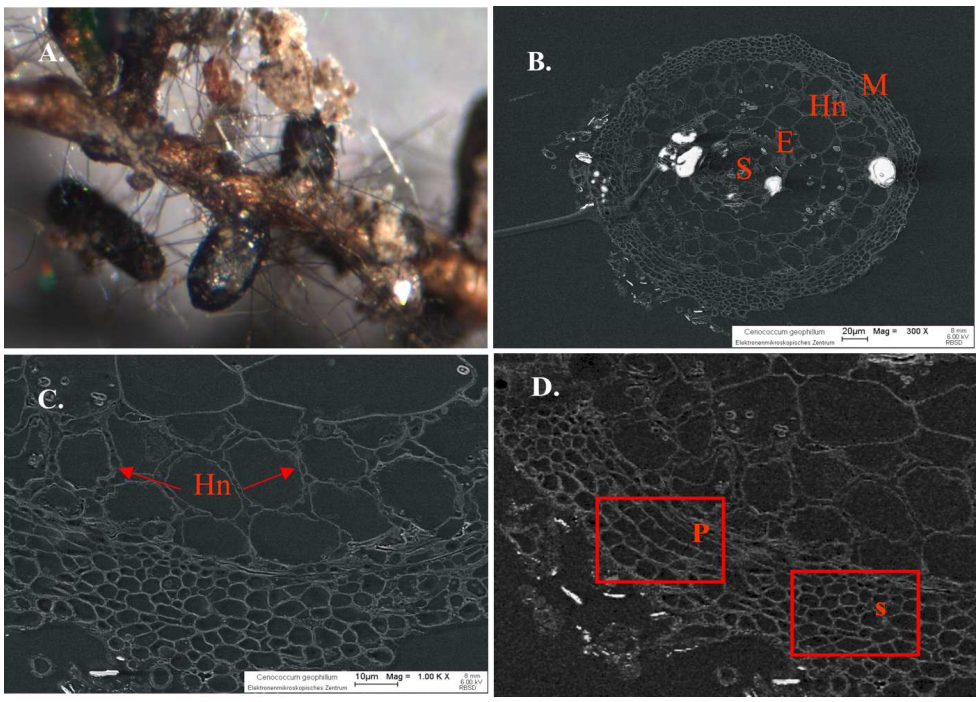

Figure 4: **Cenoccocum geophilum + Quercus robur ECM. A. Heterorrhizic system with short roots. B. Cross-section with: hyphal mantle (M), intercellular hyphae of the fully developed Hartig' net (Hn), endodermis (E), and central stele (S). C. Lobed Hartig' net hyphae. D. Micrograph of the mantle formed by C. geophilum consisting of horizontally arraged palisade cells (P) alternating with groups of small pseudoparenchymatous cells (s) and lobed Hartig' net hyphae.**

Hartig' net development starts when hyphae come in contact with unsuberized living cortical or epidermal cells and is characterized by changes of hyphal growth and morphology. The diameter of the hyphae may be greater or smaller than those of the hyphal sheath. Hyphae are oriented transversely to the root axis and begin to branch out irregularly. The hyphae penetrate in the direction of the endodermis-growth and longitudinal direction through intercellular spaces is rather restricted. The transversal growth direction is surley an advantage for nutrient transport between the endodermis and the hyphal mantle exploiting the shortest way between both. The transversal growth direction also ensures hyphal establishment in the intercellular spaces of cortex cells of a suitable developmental space. Hartig' net has been described to be "lames fungique" and examination of its structure by electron microscopically reveals that it consists of complicated fan-like, palmetti or labyrinth systems which provide a very large surface of contact between cells of the two symbionts (Tarkka, 2000).

Hormone production by ECM is responsible for the club-shaped roots that are typical of this association. The soil surrounding ECM roots, mycorrhizosphere, has a rich microbe flora. The microbial diversity depends on plant and fungal partners of the ECM association. The ECM associated bacteria affect mycorrhizal functioning in several ways including the regulation of fungal growth, host root-symbiotic fungus recognition events, nutrient mineralization, and protection against pathogens (Duponnois *et al.*, 1991). The interplay between symbiotic fungi and soil positively influences the biology of the fungus and the host plant. This leads to selection of plant beneficial bacterial strains in mycorrhizas, and the mycorrhizosphere. Enhanced mycorrhiza formation and the beneficial bacteria have profound positive influences on plant nutrition, pathogen-, and stress resistance (Lehr *et al*., 2007).

For further ecological investigations, Agerer (2002) proposed a coding system with reference to ecologically important features, to ensure comparability of different mycorrhizal studies. These characters include colour, occurrence and abundance of cystidia or lactifers, emanating hyphae and rhizomorphs, shape and size of cells in mantle layers, shape and diameter of emanating hyphae, cystidia, clamp connections and rhizomorphs, and thickness of their cell walls.

1.3 Interaction of ectomycorrhizal fungi with their environment

ECM fungi occur in forests where low litter quality and low decomposition and mineralization rates cause N and P limitation (Baxter *&* Dighton, 2005). Plants provided with sufficient amount of inorganic nutrients are able to grow without mycorrhizae, but in the field, non-mycorrhizal seedlings do not survive as well as mycorrhizal seedlings (Marx *et al*., 1977). ECM fungi increase uptake of dissolved nutrients, and can also mobilize nutrients (mainly N, P, but also micronutrients) by extracellular enzymes, from organic to inorganic sources. Through excretion of organic acids they capture nutrients (Wallander, 2000; Landeweert *et al*., 2001; van Schöll, 2008), aided by plant carbohydrates to enhance metabolic activity.

In addition, mycorrhizal fungi contribute to amelioration of stress experienced by their plant hosts, including metal toxicity, oxidative stress, water stress, and effects of soil acidification (Colpaert, 2008; Finlay *et al*., 2008; van Schöll *et al*., 2008). Mechanical protection and antibiotic production could play a role to fulfil this function. Fluctuations in the availability of water commonly occur in natural environments and drought periods can cause considerable stress symptoms for trees (Garbaye, 2000). Mycorrhizal root tips, which are typically ensheathed by mantle and which often are connected to extensive network of extraradical or extramatrical mycelium with or without rhizomorphs, are much less sensitive to dry soil conditions. The mycorrhizal association results in altered roots, where the presence of the thick external sheath affords physical protection. Thus, ECM also protect host plant roots against root pathogens and root herbivorous soil microfauna. Nutrient deficiencies change ECM seedling exudation patterns of organic acids and thus their potential to mobilize cations from minerals (van Schöll *et al*., 2008). Al is a major component of most soil mineral grains. Consequently, mineral weathering will release Al in the soil solution of the mineral soil horizons. In soils with a pH below 4, a situation typical for podzol soils, dissolved Al is present mainly in the form of the phytotoxic Al^{3+} (Kinraide, 1991), and increased weathering will contribute to Al toxicity. In such soils, the Al toxicity, as well as of other toxic metals, may be reduced by increased production of chelating agents.

Mechanisms of metal tolerance have been reviewed by Colpaert (2008). Extracellular mechanisms such as chelation and cell-wall binding as well as cellular mechanisms

such as binding to (non)-protein thiols and transport into intracellular compartments play a role in metal homeostasis of ECM fungi (Fig. 5). Reduced uptake of metals into the cytosol might be achieved by extracellular chelation or precipitation of metals with organic compounds, mostly acids such as citrate and oxalate.

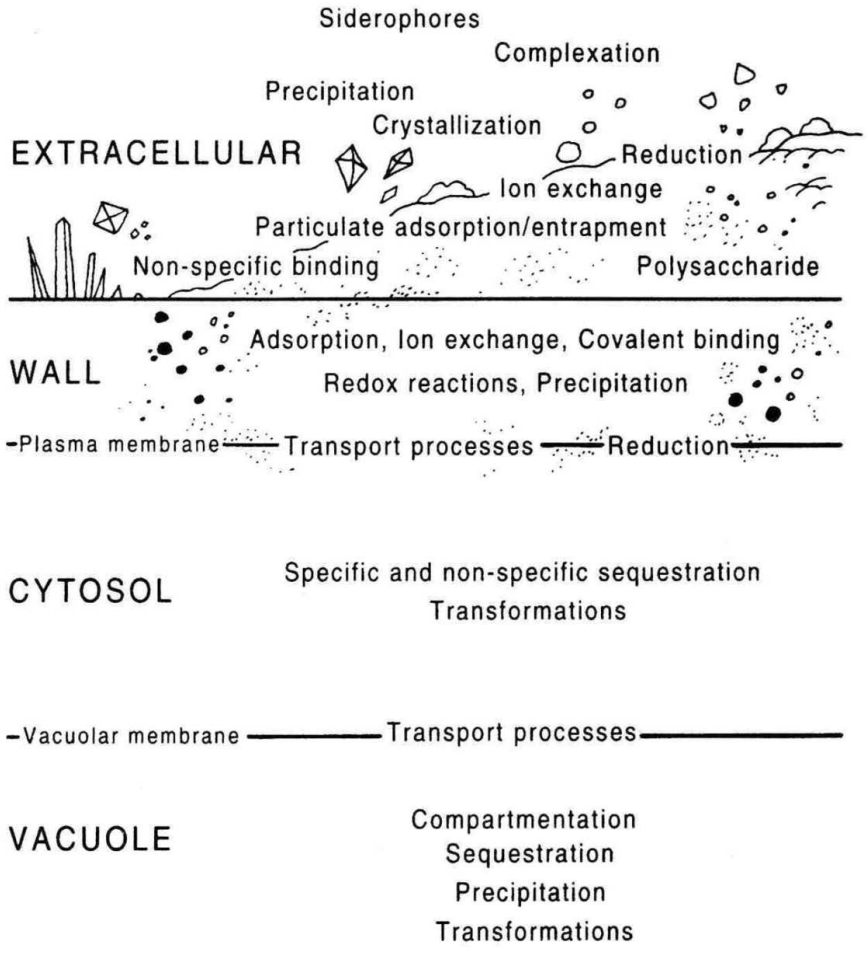

Figure 5: **Mechanisms and cellular location of key fungal transformations of metals and metalloids. The list of interactions is not exhausive, and considerable differences may occur between different species and strains. The location of some processes, especially certain sequestration and tranformation reactions, is still uncertain, and this diagram does not include the possible involvement of other organelles, e.g., mitochondria, endoplasmic reticulum, and nucleus, in metal homeostasis and compartmentation (Gadd *&* Sayer, 2000).**

Fomina *et al*. (2005) investigated solubilization of toxic metal minerals and metal tolerance by ericoid and ECM fungi. Both oxalic acid and chelation are involved in the dissolution of depleted uranium corrosion products and transformation of metallic uranium into meta-autunite minerals, which are capable of long-term uranium retention (Fomina *et al.*, 2008). Lanfranco *et al*. (2002) showed that changes in hyphal morphology occur when an ericoid mycorrhiza-forming ascomycete is treated

with millimolar concentrations of Zn. This led to apical swellings and increased branching in the subapical parts.

Toxic metals may cause oxidative stress and several studies of mycorrhizal fungal responses suggested that the fungi may be able to regulate genes providing protection against reactive oxygen species (ROS). Lanfranco *et al*. (2005) present evidence of a functional arbuscular mycorrhizal CuZn superoxide dismutase which may provide protection against localized host defence responses involving ROS. Other studies (Schützendübel *&* Polle, 2002) suggest that ECM fungi improve protection against toxic metal-induced oxidative stress through strongly induced glutathione synthesis.

1.4 Diversity of ectomycorrhizal fungi

ECM fungi occur in remarkably species-rich assemblages. ECM fungal communities mostly comprise few, frequently occurring species and many more rare species (Taylor, 2002; Buée *et al*., 2005; Koide *et al*., 2005). Species may spatially partition the forest floor (Dickie *et al*., 2002; Genney *et al*., 2006). Many ECM community studies have focussed on mycorrhizas in the humus horizon. In recent years some community-level studies in boreal forests used modern molecular methodology highlighted the vertical distribution of ECM (Dickie *et al*., 2002; Rosling *et al*., 2003). ECM communities are found to be vertically distributed and some ECM fungal species are restricted to the mineral soil (Landeweert *et al*., 2001; Rosling *et al*., 2003; Tedersoo *et al*., 2003). Numerous factors may affect the diversity of an ECM community.

Formation of an ECM symbiosis in forest soil depends on host species, age and vigour of the trees, edaphic and environmental conditions, availability of fungal inoculum, competition, microflora and microfauna. Anthropogenic stress, site history, habitat size and degree of isolation are also likely to affect the ECM community structure (Rajala, 2008). Ishida *et al.* (2007) made a significant contribution to our understanding of niche differentiation by showing that cooccurring host species have distinct mycorrhizal communities, reflecting both host taxonomy and, arguably, successional status. Increased plant diversity is likely to create more heterogeneous

litter input, which may create opportunities for niche differentiation by ECM fungi. Bruns (1995) hypothesized that in a monoculture forest stand, ECM diversity might result from resource partitioning, soil disturbance and competitive interactions between ECM fungal species. Mycorrhizal fungi compete for two general classes of resources: host-derived carbon and soil or detritus derived mineral nutrients. Both types of resources are heterogeneous in space (e.g., soil depth, distance from tree) and time (e.g., season, host successional series). Some species seem to be distributed accordingly, but the question of how widespread these patterns are remains largely unanswered. Small-scale natural disturbances that sever roots, mix soil horizons and litter horizons, or change local pH and nutrient availability, are likely to create additional habitats for ECM fungi.

During the last decades, a number of reports have documented a decrease in species diversity and abundance of sporocarps of ECM fungi in northwestern and central Europe. Especially in forests on poor soils, while disappearance of specialized species has locally been noted, some generalist species remained. Hydnaceous, chantarelloid fungi and species of the genera *Cortinarius* and *Tricholoma* have been most sensitive, which suggests that the decline of ECM fungi is not only a consequence of decreasing tree vitality. The most important cause for this dramatic decline has been found to be atmospheric nitrogen deposition, with an additional role for acidication (Lilleskov *et al.*, 2001).

From the standpoint of ecosystem function, taxonomic diversity is only relevant if it reflects functional diversity. Functional differences between ECM fungi include differences in nutrient cycling, symbiotic capabilities, ability to proliferate, and to tolerate drought and heavy metal stress. Different morphologies and physiologies of ECM fungi sum up to the total benefit of symbiosis for the host plant, depend on the infection pattern of its total root system and the extend of infection by individual species. The ECM fungi, in turn, are sensitive to variation in soil nutrient status, and the reduced mycorrhiza formation makes them more vulnerable to environmental stress and pathogens. Interpretation of the functional significance of a change in ECM community diversity to the host plants and to the entire ecosystem is currently constrained by the lack of knowledge concerning the functional capabilities under field conditions of most ECM taxa.

1.5 Functional morphology: hyphae, rhizomorphs and dispersal

Hyphae extend from the mantle to facilitate nutrient solubilization and transport. In the hyphae, transport occurs by motile tubular vacuoles that can move material across long distances (Tarkka, 2000). In addition to normal, morphologically undistinct hyphae, most of the basidiomycete ECM fungi can also form rhizomorphs, linear aggregates of fungal hyphae containing large central “vessel” hyphae that may represent significant extensions to the root system (Duddrigde *et al*., 1980; Rousseau *et al*., 1994). At the onset of rhizomorph formation, the leading hyphae grow in parallel approaching each other, they form linear aggregates, and allow the formation of branches and intercellular bridges (Cairney *&* Burke, 1994). After the hight tubular aggregate of hyphae is formed, cellular contents of the central hyphae disappears and septal cross-walls break down, leading to vessel hypha formation (Agerer, 1992). The vessel hyphae have been implicated for acropetal C transport, and the living cortical hyphae for transport of P and other nutrients (Cairney *&* Burke, 1994). Studies by Wallander *et al*. (2002) using PIXE analysis of element contents of fungal rhizomorphs also suggested that *Rhizopogon* species had the ability to mobilize significant amounts of P and K from the minerals apatite and biotite and probably play a significant role in transporting these to the trees. The development and differentiation of the extramatrical mycelium may represent important predictive features relevant to the ecological classification of ECM.

Agerer *et al*. (2001) suggested that in some cases form and function of ECM could be related: smooth mantles and uptake of organic nutrients; rhizomorphs and water–uptake under dry conditions, etc. Five different exploration types of ECM are distinguished based on the amount of emanating hyphae or the presence and differentiation of rhizomorphs (Fig. 6). Field evidence for such differences was obtained by Dighon *et al*. (1990) for mycorrhizal fungi of birch (*Betula pendula* and *B. pubescens*). They showed that three taxa of ECM fungi differed in phosphorus uptake rate, with a species of *Hebeloma* sp. being much more efficient than species of *Laccaria* sp*.* or *Lactarius* sp.. However, it is not clear how many species have unique or overlapping physiological roles.

ECM fungi can disperse and form new mycorrhizae either *via* living hyphal connections, special resting structures like sclerotia, or spores (Brundrett, 1991).

Living hyphae, which form parts of the plant-supported hyphal network, are probably the most common source of inoculum (Dahlberg *&* Stenlid, 1995). Within a few years after clear-cut logging, the remaining stumps and dying roots have been shown to support living hyphae of ECM fungi (Hagerman *et al*., 1999).

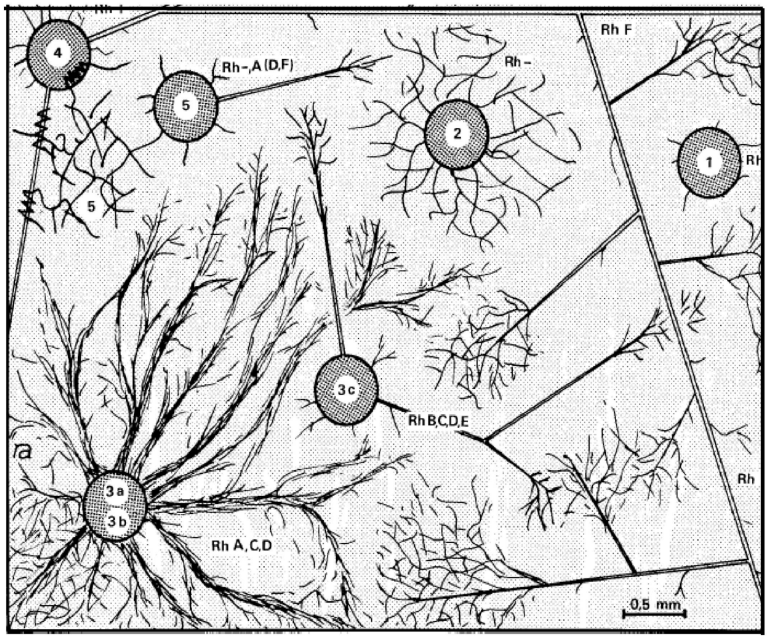

Figure 6: **Schematic drawings of different exploration strategies, represented by cross-sections of ECM and the extramatrical mycelium. (1) Contact exploration, (2) short distance exploration, (3a, b) medium distance- fringe and -mat exploration, (3c) medium-distance smooth exploration, (4) long-distance exploration, (5) pick-a-back exploration, shown as mycorrhiza and as soil hyphae in contact and intruding into rhizomorphs and ECM of a long distance exploration type ECM. All figures are to scale (Rh rhizomorph, – rhizomorph lacking, A–F organization types of rhizomorphs (Agerer, 2001).**

Spores and sclerotia can survive in soil for a very long time and can be activated by suitable conditions, e.g. increased soil moisture, heat shock after forest fire or stimulatory compounds in root exudates. Spore activation is followed by spore germination. The emanating primary monokaryotic mycelium of a basidiospore may already be capable of forming mycorrhizae (Kope *&* Fortin, 1990), but normally mycorrhiza formation take place with dikaryotic mycelium, formed after mating of two sexually compatible primary mycelia. Little is known about forms of mycelium responsible of mycorrhiza formation by Ascomycetes, which are numerous in ECM communities (e.g. Tedersoo *et al*., 2003). Some fungal species are known to spread successfully *via* spores while others form mycorrhizae preferentially *via* living connections or sclerotia (Brundrett, 1991). Early species are characterized mainly by

reproduction by spores, while late species by clonal expansion (Sarah *et al*., 2002). Mainly, medium and long distance types of exploration are found by early species, while mainly contact and short distance types are found by late species (Agerer, 2001).

1.6 Investigation of ectomycorrhizal fungi

ECM fungal identification has traditionally been based on observations on the morphological characters of mycorrhizal root tips (e.g. Agerer, 1987-2006). Additionally, sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) is commonly used for identification of ECM fungi (Horton *&* Bruns, 2001). The ITS region between the 18S rDNA and 28S rDNA genes consists of two noncoding spacers, ITS1 and ITS2, which are separated by the 5.8S rDNA gene (Fig. 7). The interspecific variation in the ITS region is high and variation among individual rDNA repeats can sometimes be observed. Two standard primers ITS1+ITS4 (White *et al.*, 1990), several taxon-specific primers have been described that allow selective amplification of fungal sequences.

Figure 7: **The scheme of ITS region of rDNA.**

Sampling strategies are of essential importance to ECM community investigation (Taylor, 2002). Sound sampling strategy, statistical analyses and multiple hypothesis testing greatly improve the quality of biodiversity studies (Morris *et al*., 2002). In addition, the inherent difficulties in defining the identity of ‘species’, ‘individual’ and ‘population’ or ‘community’ in the case of ECM fungi complicate matters (Taylor, 2002). Temporal variation in ECM community structures (Kjøller, 2006; Koide *et al*., 2007), spatially heterogenity (Tedersoo *et al*., 2003; Genney *et al*., 2006) imply that the number and size of collected soil samples may have an impact on the observed

ECM community structure (Menkis *et al*., 2005). Commonly, the investigation of ECM communities is restricted to the organic soil horizon. Although organic soil, along with the uppermost mineral horizon, usually has the highest living fine root density and biomass (Rosling *et al*., 2003), mineral soil may also contain many root tips. As ECM communities are found to be vertically distributed and some ECM fungal species are restricted to the mineral soil (Landeweert *et al*., 2001; Rosling *et al*., 2003; Tedersoo *et al*., 2003), sampling mycorrhizas also from mineral soil reveals a more complete picture of the ECM community. Only such broad investigations will allow to develop saturated databases of fungal community composition, structure and spatio-temporal dynamics in relation to variable resources and conditions. This requires the development of quantitative models of species–environment relationships built on several key elements: appropriate study designs, community data, environmental data and models (Lilleskov *&* Parrent, 2007). The value of an indicator is affected not only by stress factors, but also by soil type, land-use and vegetation.

1.7 Aims of the study

Mycological studies are rare in post mining landscapes, and it is necessary to improve our knowledge about the succession of ECM fungi on reclamation sites (Gebhardt *et al.*, 2007; Buscot *et al*., 2000). In order to understand the functional differences between different fungal symbionts and the reasons for changes in ECM community structure, the relationship between ECM diversity and exploration types of ECM was investigated. In a succession gradient, three different *Quercus robur* test sites with high heterogeneity of soil parameters (heavy metals, essential nutrients and pH) and land use (uranium mining, forest harvesting and reforestation) were examined. The sites represented different stages of succession and were investigated at tree, community and ecosystem level. The sites Kanigsberg-Wismut (a former uranium mining area, primary succession young forest grown on naturally charged land), Greiz (secondary succession young forest grown on contaminated land after clear-cut and reforestation), and Jenzig-Jena (uncontaminated area, primary succession used as control) were selected to answer four main questions:

1) Which ECM communites can be observed in a mining area? Are ECM fungi that grow in primary succession able to grow on contaminated land? What is the situation of ECM communities in secondary succession?

2) Does the heavy metal distribution explaine the heterogeneity in ECM communities? Does the communitiy composition change with heavy metals concentrations? Can the environmental available fraction of esential and heavy metals be used as a tool to assess correlation with ECM?

3) Can the classification of ECM in exploration types be used as a functional tool in experiments at large scales?

4) It is possible to develop a general predictive model of ECM fungal community-environment relationships?

2 Material and methods

2.1 Sampling sites

Three young *Quercus robur* forests in Eastern Thuringia in different stages of succession have been selected.

One of the sampling sites is Kanigsberg, a former uranium mining site, situated near Ronneburg, Thuringia, Germany (50 49 48 4; 12 09 358; 334 m Gauss-Krüger coordinates, Potsdam-date). At Kanigsberg, remediation required removal of the heap and covering with 30-40 cm top soil. The site was reforested as a mixed forest including *Quercus robur, Fagus* sp., *Fraxinus* sp. and *Larix* sp. Multi-heavy metal contamination, low pH, sulphate, drought, lower weathering rates are lead to ecosystem disturbance.

The second forest, situated close to the city of Greiz (45 12 8 37; 56 15 900; 305 m), was established after clear-cutting a spruce forest. A mixed *Quercus robur, Fagus* sp., forest was established providing the initiation of secondary succession. The acidic pH and high concentration of manganese are common and represent typical sites on black schist (Engler, 1998).

The third location, Jenzig, is situated near Jena, Thuringia, Germany (44 74 28 5 ; 56 44 93 0; 357 m) and consists of a mixted forest (*Fagus* sp. and *Quercus robur*) on limestone and serves as a non-contaminated control area (primary succession).

2.2 Soil sampling and morphotyping

The fungal community structure associated with the root system was determined by morphotyping, strain isolation and sequencing of ITS. Direct DNA extraction from soil samples was taken from extensive and intensive sampled probes of root tips. Extensive sampling was performed with two samples of 4-10 root tips from each of 10-11 *Quercus robur* trees an all samplig sites in August, November 2004 and in March 2005. The intensive sampling was performed with 100 samples around 19 selected *Quercus robur* trees respectively in Kanigsberg and Jenzig in June-July 2006 and in

Kanigsberg and Greiz in November 2006.

To obtain ECM samples representative for the major rooting zone, soil cores (50 cm diameter, 20 cm depth) from the organic H horizons and the inorganic A-horizon were taken on a half circle along the radius of the crown diameter of each tree. Samples were taken more than 2 m distant from one another in order to avoid autocorrelation (Tedersoo *et al*., 2006). The samples were stored in plastic bags at 5℃ in the dark until processing. Cleaning was perf ormed by soaking the samples in tap water (24 h, 5℃), followed by selecting oak ro ots. After washing in tap water, 20 single roots per sample were cut into segments of 30 cm in length and root tips chosen at random using coordinates derived from random number tables present in the target squares were selected. Living root tips and ECM morphotypes of each subsample were isolated, categorized and separated based on their morphology. Their total abundance was determined with the aid of a dissecting microscope (Stemi, 2000-C) following Agerer (1987-2006). A subsample of each ECM morphotype was fixed in cacodylat buffer containing 2% glutaraldehyde and 4% formaldehyde. Mantle, hyphae and rhizomorph preparations were used to identify the ECM to genus or, if possible, to species level, using a light microscope (Axioplan, Carl Zeiss). Subsequently, ECM were classified into exploration types (Agerer, 2001; 1987-2006). The morphotypes which did not match any of those present in the reference were termed unidentified, grouped accordingly to morphological characters and given a descriptive name (e.g. “brown”, “orange”, etc.). Following the examination, up to five mycorrhizal root tips of each morphotype were taken from each root system and placed separately in 1.5-ml centrifuge tubes, labelled and stored at −80℃ for direct ITS identification. To e valuate the specific distribution of exploration types within soil horizons, genera with more than one species have been grouped by their genus, and others by species. The relative abundance (number of living ECM per meter of the finest root length) and the relative genus/species abundance in a specific soil horizon (proportion of ECM expressed as percentage of each genus/species per total number of living mycorrhizal root tips) were recorded.

2.3 Identifying ECM fungi by DNA-based methods

To further facilitate the investigation of community structure, sequencing of ITS was

used. The isolation of fungal cultures was performed from individual mycorrhizal root tips collected during 2004-2005 and included multiple representatives of morphotypes recognized. For rare morphotypes represented by only one or two root tips, ITS sequencing was performed. For isolation, root tips were sterilized in 33% hydrogen peroxide for 15 to 60 s, rinsed three times in sterile deionised water, plated onto modified Melin Norkrans medium (Kottke *et al*., 1987) and incubated at room temperature in the dark. Dishes were checked daily and emerging cultures were transferred onto fresh agar medium. Fungal mycelia were examined using a Stemi 2000C stereomicroscope, equipped with 5 fold magnification. The DNA of growing mycelia was extracted (Cenis, 1992) and ITS fragments were amplified by PCR using specific primers ITS 1 and ITS 4 (White *et al*., 1990). The ITS fragments of 600-800 bp (pDrive vector, Qiagen) were cloned using *Escherichia coli* K12DH5α (GibroLife Technologies, Karlsruhe). The sequences of the cloned fragments were compared to GenBank entries to identify the species (http://www.ncbi.nlm.nih.gov/ and http://unite.zbi.ee). For direct sequencing, DNA of individual root tips was extracted from one to five ectomycorrhizal root tips (frozen and kept in liquid nitrogen) using the Power Soil DNA Isolation Kit (Mobio, USA). ITS PCR was performed and the resulting ITS fragments of 600-800 bp were extracted from the gel, cloned and sequenced.

2.4 Soil composition

Soil from each horizon was dried at room temperature, and sieved (< 2 mm). 25 ml of 1 M KCl were given to 10 g of soil, thoroughly stirred, incubated for 1 h, and the pH of the supernatant was measured (inoLab pH 720, WTW GmbH, Weilheim, Germany). Soil pH and organic matter have been determined by usual methods (Neagoe *et al*., 2008). Heavy metal and essential elements (As, Ba, Cd, Co, Cr, Cs, Cu, Ni, P, Pb, Rb, S, Sr, U and Zn) were analyzed for 23 selected soil samples using ICP-MS (PQ3-S-Option, VG Elemental, UK) or ICP-OES (Spectroflame, Spectro, Germany) after sequential extraction (after Zeien *&* Brümmer, 1989) (Tab. 1).

For sequential extraction the reagents were used with 2 g of soil. During each extraction, an aliquot of soil suspension was taken. After extraction, the suspension

was centrifuged and the resulting supernatant solution was decanted and filtered.

Table 1: **Sequential extraction procedure for heavy metal associations used in the present study (after Zeien *&* Brümmer, 1989)**

Step	Fraction	Extraction reagent	Extraction conditions
I	Mobile	1 M NH_4NO_3	Shaken at RT for 24 h
II	Exchangeable fraction	1 M CH_3COONH_4 (pH 6)	Shaken at RT for 24 h
III	Mn oxides	0.1 M NH_2OH–HCl + 1 M CH_3COONH_4 (pH 6)	Shaken at RT for 30 min
IV	Organic	0.025 M NH_4–EDTA (pH 4.6)	Shaken at RT for 90 min
V	Amorphous Fe oxides	0.2 M $NH_4OOCCOONH_4$ (pH 3.25)	Shaken at RT in the dark for 4 h (twice)
VI	Crystalline Fe oxides	0.1 M ascorbic acid in 0.2 M $NH_4OOCCOONH_4$ (pH 3.25)	Shaken at 96 ℃ in the dark for 30 min (twice)
VII	Residual	Total element content of the residual fraction detected by aqua regia extraction	

The summation of the mobile (1^{st}) and the exchangeable (2^{nd}) fractions can be used to assess the environmentally available components. The fractions bound to Mn oxides (3^{rd}) and to organic material (4^{th}) are supposed to represent the potentially mobile component under changing environmental conditions, while the more stable associations with Fe oxides (5^{th} and 6^{th}) represent both anthropogenic and geogenic components, the residual (7^{th}) fraction is geogenic.

2.5 Data processing

The relative abundance needed for computing the diversity indices was based on unit of density on a unit of root length at α and β level, and based on density on unit of soil volume at γ level (in order to allow comparison with other studies at γ level, notably Staudenrausch *et al*., 2005; Iordache *&* Bodescu, 2005).

For each sampled tree, separately for humic and inorganic horizons were calculated the number of ECM species/tree, the Berger Parker index (maximum number of morphotypes in one species/total number of morphotypes) and the CV (coefficient of variation defined as the ratio of the standard deviation and average) of species abundances around trees, and its average for all species present at a tree (AvCVSp). For each forest, additionally, was computed the average of AvCVSp using the code **a**round **t**rees and its CV. CVa_t reflects how different individual trees are with respect to the heterogeneity of ECM species distribution around them. It was also calculated the CV of ECM abundances between trees (b_t) for each fungal species, and the average of the CV of ECM abundance between trees and its CV. CVb_t reflect how different are the species from the point of view of the distribution between trees (larger it is, more different are the species from the point of view of the pattern of distribution between trees). The Soerensen coefficient was used for comparing the similarity between trees.

To compare the ECM richness between forests rarefaction (Richard *et al*., 2005; Tedersoo *et al*., 2006; Midgley *et al*., 2007) and diversity indices were used (Ishida *et al.*, 2007). Mao Tau estimates of the observed species, three estimators of species richness Chao2, Jacknife2 and bootstrap at forest level (humic and inorganic horizons pooled) were computed using the program EstimateS 8.0 (Colwell, 2005). The same program was used for computing Fisher's alpha and Shannon index of the diversity separately for the humic and inorganic horizons at forest level. To reduce the number of control variables, standing for metals, multivariate techniques, mainly principal component analyses (PCA) (e.g. Astorga Espana *et al*., 2007; Loska *&* Wiechula, 2003) were executed. In this study for reduction of metals dimensionality the PCA (Canoco for Windows version 4.54) were used, after verifying its applicability with Barttlet's sphericity test (Statistica software '99 edition). PCA was applied to several combinations of metal concentrations with different mobilities. For testing similarity of the distribution of metals in soil a hierarchical cluster analysis was performed according to Ward's method (Ward, 1983) using Statistica software '99 edition. The ECM data were analyzed for the underlying gradient under the assumption of unimodal model by detrended correspondence analyses (DCA, in two variants: detrending by first order polynomials and by segments, log transformation of species data). The underlying gradient restricted by the metals (previously reduced

dimensionally by PCA) was inspected by hybrid detrended canonical correspondence analyses (hDCCA, detrending by second order polynomials, Hill's scaling focused on inter-species distances, log transformation of species data, Monte Carlo permutation test with 499 unrestricted permutations under full model. The first two eigenvalues reported are canonical, the others no). At Greiz, canonical ordination techniques (CCA) was designed to detect the patterns of variation in data that can be explained best by the observed environmental variables. The resulting ordination diagram expresses not only a pattern of variation in species composition but also the main relationships between the species and each of the environmental variables (Jongman *et al*., 1995). These multivariate analyses were performed with Canoco for Windows version 4.54.

2.6 Heavy metal tolerance

To investigate the effects of heavy metals on fungi, an experiment was performed with different concentrations of heavy metals. The heavy metals chromium, copper, nickel and lead in concentrations of 0 mM, 1.5 mM, 3 mM, 4 mM, 5 mM and 6 mM were selected. For each heavy metal and for each concentration, three parallel samples were used. *Hymenoscyphus* sp. isolate, originating from the Kanigsberg, was selected to be used in this experiment.

3 Results

3.1 Improved framework for the ecosystem approach

A key issue for ensuring the success of the ecosystem approach is to identify the ecosystem's structure at the appropriate time and space scale. For instance, if there is one ECM community in a forest, then it is meaningful to estimate ECM richness and diversity directly at forest level (α diversity), but if there is an assemblage of ECM communities, then the diversity of forest at three levels should be characterized (α, β and γ). In the first case, the succession processes take place directly at forest level, in the second case the dynamic of diversity at forest level reflects succession processes occurring in elementary communities, propagating bottom-up at higher hierarchical levels, and constrained top-down by meta-community level processes (e.g. dispersion mechanisms as controlled by forest level vegetation dynamic or contamination).

A methodologically relevant definition of the basic unit at which to consider diversity (a development from the elementary community notion) is provided by Pahl-Vostl (1995) under the name of 'trophic-dynamic module' (TDM). A TDM is defined as the groups of biological populations having 1) rates of biomass cycling (inversely correlated with lifetime of the individuals) of the same order of the magnitude, 2) the same location in space and time and 3) the role of each species in the food web. Application of criterion 1 leads to dynamic classes, further application of criteria 2 leads to dynamic modules, which, by criterion 3 are split in TDMs. The above definition can be amended (Iordache *&* Bodescu 2005) with the remark that some populations can be included in more TDMs at the same time, because of their internal structural diversity. For instance, decidious tree populations have parts with very different rates of biomass cycling, like leaves and wood – criterion 1, as well as parts with different location in space like below vs. above grounds – criterion 2; so they will belong to at least 3 TDMs, two above ground and one below ground; frog populations have parts – tadpoles and adults – differing both in space and their role in food webs. The notions of "same order of magnitude", "same location in space and time", and "same role in food web" are to be defined by the researcher, and can be

applied more stringent or relaxed. In the most stringent application, they will lead to a model identical with the “reality” (isomorphic model). If relaxed too much they will lead to a model too aggregated and having lost the key characteristics of the real system (simplistic model). Only at an appropriate intermediate level, they will lead to a model simple enough for explanatory value, but keeping the basic characteristic of the system (homomorphic model).

While succession in ecosystems is a process taking place at the level of the networks of TDMs, mechanisms may be analyzed for a group (e.g. fungi) at TDM level. The scale of the TDMs varies hugely, which implies that this is not one “true” scale for ecosystem processes or a simple, nested hierarchy of ecosystems. In Fig. 8 the linear model (dotted line) assumes that there is linear appearance of new emergent properties when increasing the scale of analyses, without need to privilege a certain scale (this model is preferred by those considering that ecosystems are methodological concepts applicable at any scale). The nested hierarchy model (continuous line) (see Fig. 8) assume that at certain scales there are jumps of emergent properties allowing the identification of an ecosystem level, then these ecosystem interact over a range of intermediary scales and at another point there is another jump, and so on (this model is preferred by those considering the ecosystems are “real” entities).

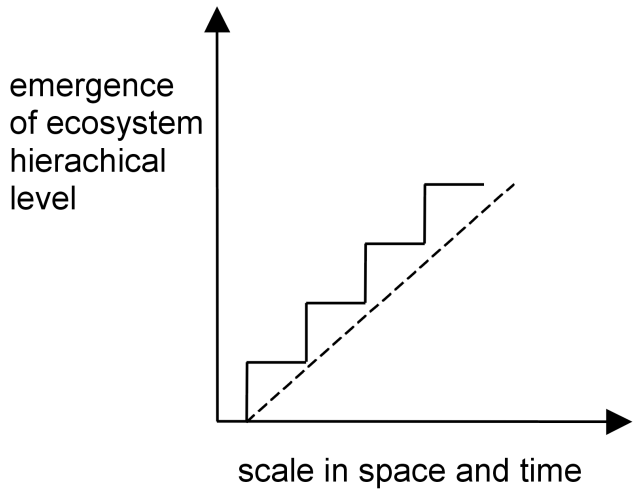

Figure 8: **Two simplistic models of the relationship between space-time scale of analyses and the emergence of ecosystem hierarchical levels.**

Rather, emergence of new structural (e.g. new TDMs) and functional (e.g. increase in overall biological productivity, or changes in the rates of biogeochemical processes) properties should be defined and used to drive the mathematical function that links scale and emergence of new properties in different areas and in different periods of time.

As ECM fungi have more or less the same rate of biomass cycling and the same role in food-webs, one cannot expect the separation of ectomycorrhizal TDMs based on these criteria. The scale of separation, howewer, can be very different.

Applying the separation of TDMs to early versus late-stage ECM communities at the same tree, TDMs can be distinguisched depending on the age of the roots (two TDMs per tree), the net differences in communities structure with depth (humic horizons vs. inorganic horizon, two TDMs per tree) and the clonal development of late-stage (potentially allowing the same population to occupy more than one tree).

Based on these considerations we propose a model of ECMs community structure in a forest (Fig. 9). Using this theoretical framework, we are able to identify the following components of ECM structural diversity in a tree island: number of TDMs, species richness inside each TDM (α), at tree (β) and at forest level (γ), and finally evenness inside each TDM.

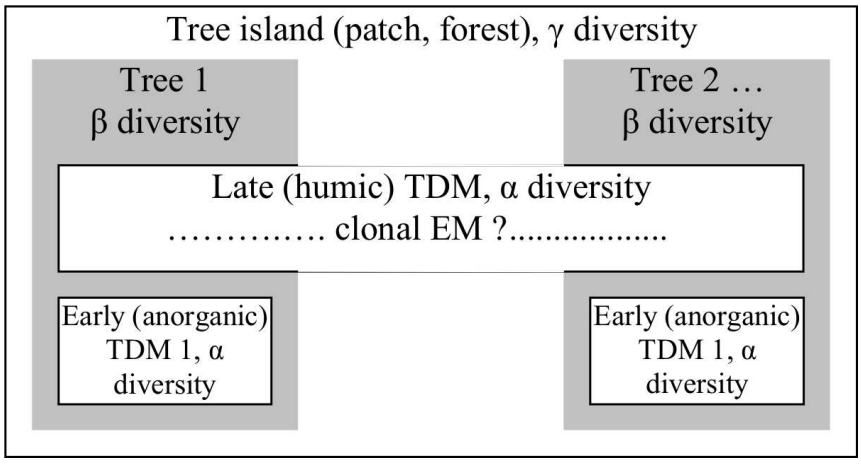

Figure 9: **Model of ECMs community structure in a forest.**

3.2 Implementing the improved framework

In order to make these theoretical ideas approachable, an experimental design should be set up in which 1) different sites are investigated at tree, community and ecosystem level, 2) appropriate data processing procedure is devised, and 3) results are interpreted at all hierarchical levels. Here, data of field study were used to implement the framework set up in the previous chapters (Fig. 10).

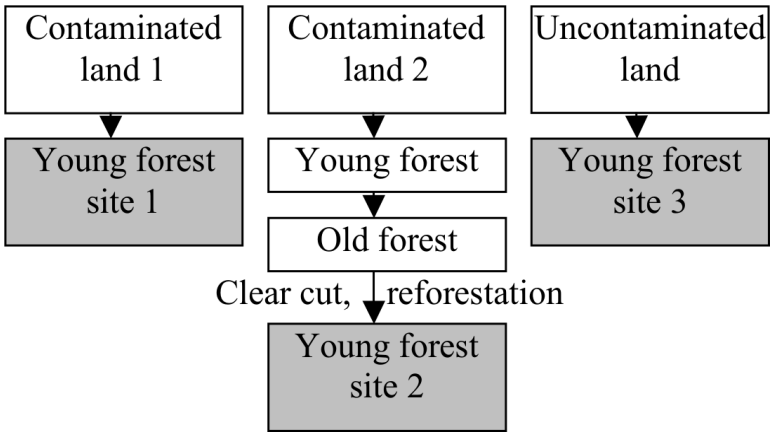

Figure 10: **Relative positions of the sites from succession point of view.**

We assume that the general successional pattern of ECM communities at TDM level in the first phases is an increase in richness and evenness (Dighton *&* Mason, 1985), and coupling this with the selection pressure of contamination on early stage colonizing fungi, will be attenuated by the development of an organic soil horizon. Based on these, we would predict that the richness and evenness of ECM communities at tree level in secondary succession young forests grown on contaminated lands is larger than in primary succession young forests grown on contaminated lands in the upper TDM (humic horizon), but smaller in the lower TDM (inorganic horizon).

A structure of data processing and interpretation is presented in Figure 11. The relative abundance needed for computing the diversity indices has to be based on density per root length at α and β level, and based on density per soil volume at γ level. This will allow comparison with other studies at γ level, notably Staudenrausch *et al*. (2005).

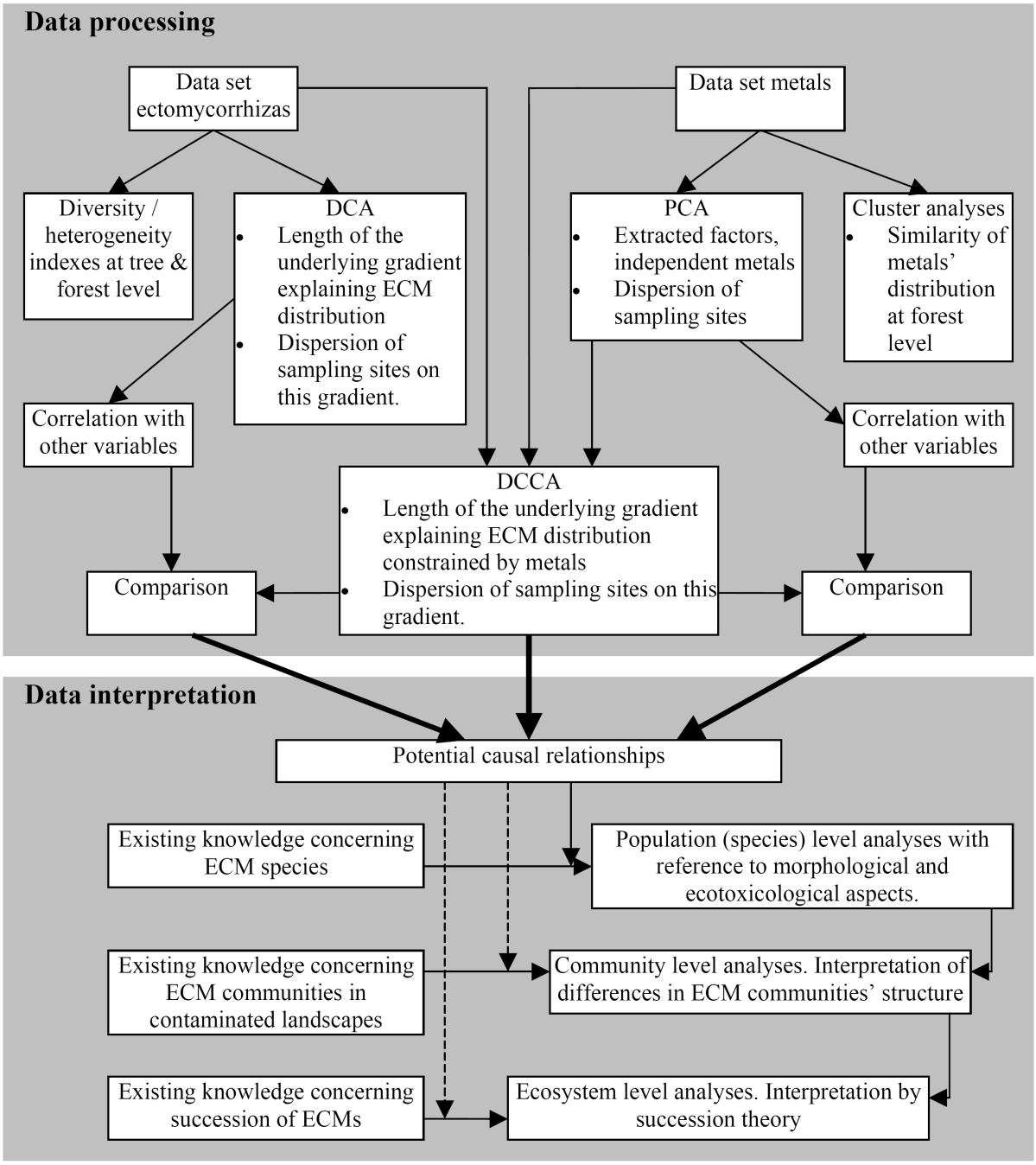

Figure 11: **Schematic diagram showing the structure of data processing and interpretation in thesis.**

3.3 Morphotyping and identification of *Quercus* ECM

From the three study sites Jenzig, Greiz and Kangisberg along an increasing contamination gradient, both intensive and extensive sampling of oak roots were performed and the ectomycorrhiza was identified by ECM typing and ITS sequencing. Each morphotype was assigned to an exploration type, based on the

occurrence of structural differences (Fig. 12).

At Jenzig, 11 frequent ECM morphotypes were found at all investigated trees and in both horizons with twice the abundance of occurrence in the organic H horizon as compared to the inorganic A horizon. Using direct sequencing of ITS, one *uncultured ectomycorrhizal fungus* was additionally identified and the resulting sequence was deposited with the accession number EU700262 at NCBI database. The 11 fungal types: three *Lactarius* sp., rose morphotype, *Cenococcum geophilum,* orange-yellow morphotype, *Thelephoraceae* sp., *Xerocomus* sp., *Xerocomus porosporus, Pisolithus tinctorius* and *yellow rhizomorphs*, covered all proposed exploration types (Fig. 12A).

The contact exploration type is represented by ectomycorrhizae belonging to *Lactarius* with a smooth mantle with the characteristic laticiferous hyphae and only a few or without emanating hyphae. *Lactarius* sp. presents gelatinized mantle. The *rose* morphotype does not have emanating hyphae. The second identifyed *Lactarius sp.* and *orange_yellow* morphotypes show some fine emanating hyphae. All these types are hydrophilic, and often, the few emanating hyphae are in close contact with the surrounding substrates (Agerer, 2001). *Cenococcum geophilum* Fr. belongs to the short distance type. It was present at all trees and showed characteristic black emanating hyphae, hydrophilic and unramified. The resulting ITS sequence has the accesion number EU700264 in the above mentionated database. The medium distance exploration type of the *fringe subtype* was represented by *Thelephoraceae sp.* (EU700263)*. Thelephoraceae* sp. type presents brown colour, monopodial pinnate ramification and the corresponding thelephoroid rhizomorphs. The *mat subtype* was found with *Xerocomus* sp*.*, where the rhizomorphs present some hyphae out-grow from the margin that seem to be different than *Xerocomus porosporus* type, with higher differentiated rhizomorphs. The *smooth subtype* was represented by monopodial pyramidal ramification of *Lactarius* sp. with laticiferous hyphae in the mantle and the rhizomorphs belonging to the medium distance exploration type. The long distance exploration type presented by *Xerocomus porosporus* forming white hyphal fans, which could be identified by ITS (see EU700259), by *Pisolithus tinctorius* with gold-yellow rhizomorphs and yellow rhizomorphs.

Seven morphotypes were found in Greiz: *Lactarius quietus*, *Russula ochroleuca*,

Laccaria amethystina, Cenococcum geophilum, Paxillus involutus, *Pisolithus tinctorius* and brown rhizomorphs (Fig. 12B). The morphotypes were found at only few trees while most trees show little or no mycorrhization in the inorganic A horizon. The identified morphotypes belong to the contact exploration type (*Lactarius quietus* sequenced as ITS EU700258 with a hydrophilic, gelatinous mantle and white colour; *Russula ochroleuca* with yellow colour, the typical greenish-yellow patches and soil particles adherent to the mantle and identified ITS (EU700257)), the short distance type with *Cenococcum geophilum* in lower abundance, the medium distance exploration type (the *fringe subtype* is represented by *Laccaria amethystina*. This ECM shows a characteristic colouration of laticifers which is similar to the violet colour in the fruitbody). The long distance exploration type with smooth ectomycorrhizae and few, but highly differentiated rhizomorphs was represented by *Paxillus involutus, Pisolithus tinctorius*, brown rhizomorphs. *Pisolithus tinctorius* forms golden yellow mycelial strands. The brown-white ECM of *Paxillus involutus* (Batsch) Fr. has characteristic sclerotia that adhere to rhizomorphs. The brown rhizomorphs present smooth margins (Agerer, 1987-2006).

At Kanigsberg, only 5 morphotypes were isolated: *Cenococcum geophilum*, *Tomentella sublilacina, Scleroderma* sp., *Paxillus involutus,* and brown rhizomorphs (12C). Additional, by direct sequencing of ITS, 2 different *Scleroderma* species were identified: *Scleroderma areolatum* (EU700255) and *Scleroderma* sp. (EU700256). *Helotiales* sp. (EU700254) and *Hymenoscyphus* sp. (EU700265) were identified by indirect isolation in pure culture. Fruitbodies of *Pisolithus tinctorius* and *Paxillus involutus* were found at the site, indicating its existence in the roots. The morphotypes are heterogeneously distributed, *Cenococum geophilum* being the predominant morphotype, while the brown rhizomorphs could also be found in each sample and horizon. For the short distance type, *Cenococcum geophilum* was present. The long distance exploration types with smooth ectomycorrhizae and few, but highly differentiated rhizomorphs could be observed by *Paxillus involutus, Pisolithus tinctorius*, brown rhizomorphs and *Tomentella sublilacina* (EU700253). *Pisolithus tinctorius* forms golden yellow mycelial strands. The brown-white ECM of *Paxillus involutus* has characteristic sclerotia that adhere to rhizomorphs (Agerer, 1987-2006).

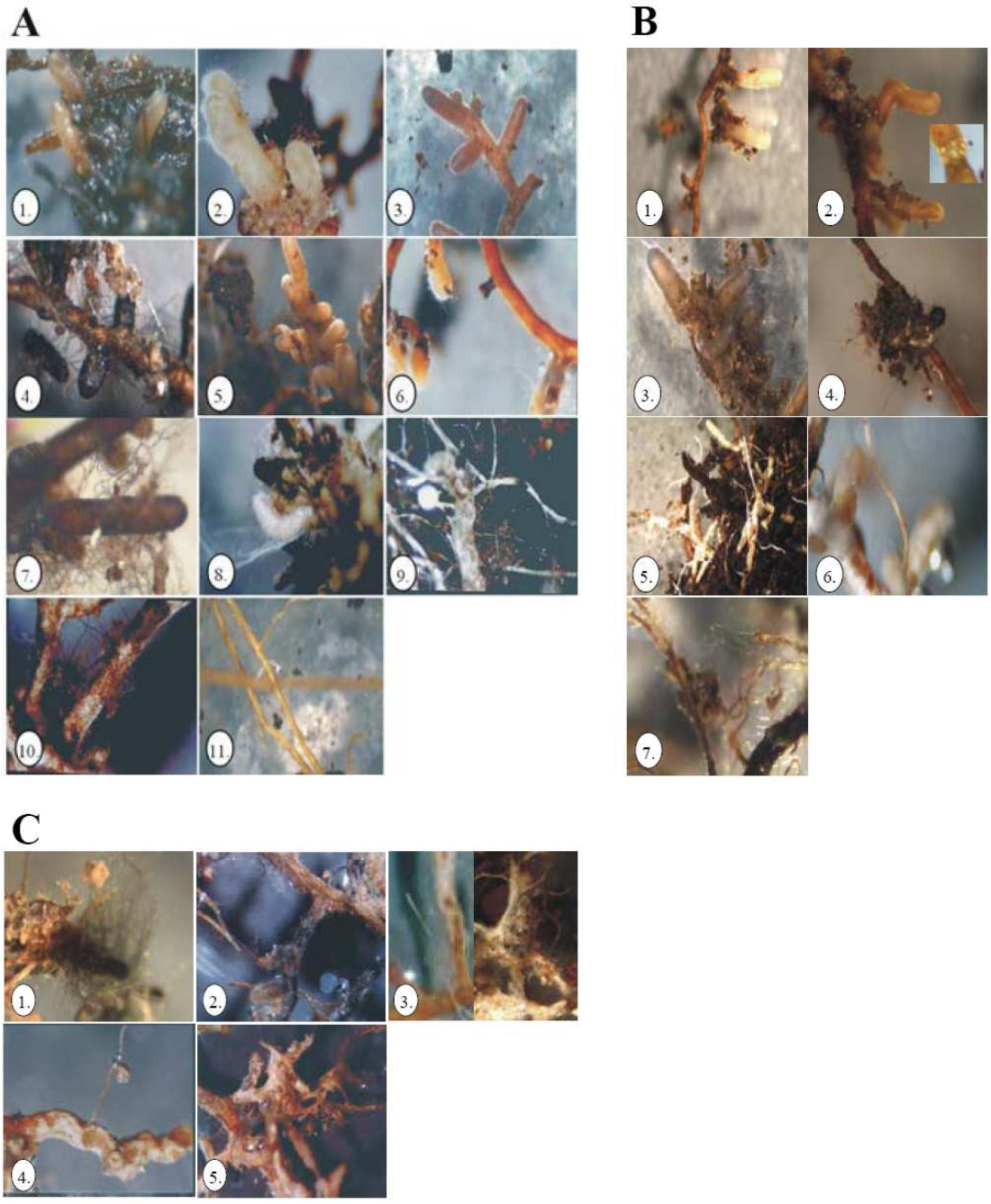

Figure 12: **Exploration types of ECM found at the three study sites:**

A) Jenzig: contact types of *Lactarius* sp. (1.), *Lactarius* sp. (2.), rose morphotype (3.); short distance exploration type *Cenococcum geophilum* (4.); medium distance types of monopodial *Lactarius* sp. (5.), orange-yellow morphotype (6.), *Thelephoraceae sp.* (7.) *Xerocomus* sp. (8.) and long exploration type with *Xerocomus porosporus* (9.), *Pisolithus tinctorius* (10.) and *yellow rhizomorphs* (11.).

B) Greiz: contact types *Lactarius quietus* (1.); *Russula ochroleuca* (2.); medium distance types of *Laccaria amethystina* (3.); short distance exploration type *Cenococcum geophilum* (4.); and long exploration type with *Pisolithus tinctorius* (5.), M. brown *(Paxillus involutus)* (6.) and *rhizomorphs brown* (7.).

C) Kanigsberg: short distance exploration type *Cenococcum geophilum* (1.) and long exploration type with *Tomentella sublinacina* (2.), *Scleroderma areolatum* (3.), *Paxillus involutus* (4.) and rhizomorphs brown (5.).

3.4 Comparison of ECM communities

The highest average number of species per tree was at the uncontaminated site, Jenzig, for both horizons (Tab. 2). At Kanigsberg, the average richness was higher in the A horizon than at Greiz, but lower in the H horizon. The richness per tree and horizon was most heterogenous between trees at Greiz, and at Kanigsberg. The evenness per tree and horizon was highest at Jenzig in both horizons (Tab. 2, Berger-Parker index), and lowest at Kanigsberg in both horizons, while it was also low in the A horizon at Greiz. The highest similarity between trees (Soerensen index) was found at Jenzig, followed by Greiz, and lowest at Kanigsberg, for both horizons. The CVb_t index indicates that the pattern of species distribution between trees was most similar at Jenzig in both horizons, followed by Kanigsberg in both horizons, and Greiz in H horizon. The particular situation of Greiz in the A horizon was due to the fact that two trees showed most of the morphotypes of all types, with very low numbers of all types in all other samples. The same CVb_t index indicates that ECM distribution around the trees (by horizon) is most similar for Jenzig (both horizons) and Greiz (H horizon), followed by Greiz and Kanigsberg (both A horizons), with highest heterogeneity of the samples around trees in Kanigsberg (H horizon).

In Greiz was immediately obvious that some roots presented high abundance of mycorrhizae, and even sometimes a diverse selection (Fig. 13), whereas other roots had no ECM. This indicates a highly heterogenous distribution of ECM in this 15 year old oak forest.

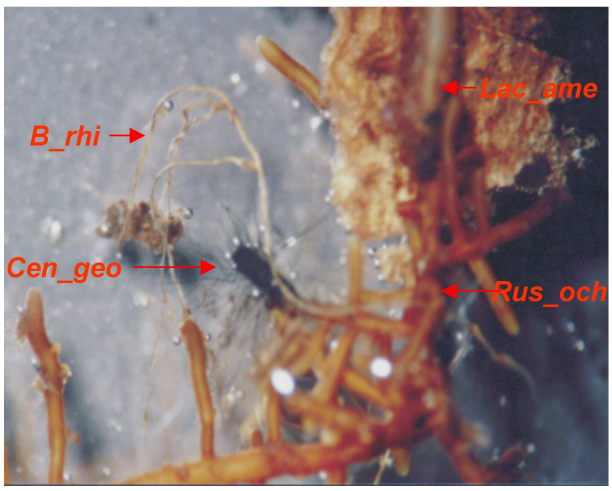

Figure 13: **Root containing *Cenococcum geophilum* (*Cen_geo*), *Russula ochroleuca* (*Rus_och*), *Laccaria amethystina* (*Lac_ame*) and unidentified brown rhizomorphs (*B_rhi*) found to the second tree (sample 2QH).**

Table 2: **Parameters and indexes characterizing the distribution of ECM around trees and between trees.**

Site	No. of trees	Soil horizon	No. of ECM sp.	No. sp./tree		BP		Av CV of ECM ab.				Soerensen b_t		CV b_t pH
				Av	CV	Av	CV	a_t	CVa_t	b_t	CVb_t	classic	Chao	
Kanigsberg	9	H	4	1.3	38	0.93	14	80	72	207	31	0.38	0.35	20
		A	6	1.8	47	0.82	23	123	39	218	32	0.32	0.34	13
Greiz	6	H	7	2.7	61	0.67	46	124	23	181	34	0.43	0.45	9
		A	4	1.3	61	0.90	27	114	45	245	0	0.53	0.54	3
Jenzig	4	H	10	5.5	35	0.55	35	114	23	154	12	0.61	0.83	12
		A	11	5.5	43	0.58	40	110	18	163	18	0.57	0.77	18

The abundance of ECM was higher in the organic H compared to the inorganic horizon. In addition, the distribution was very heterogeneous and differed from 0 to 38 ECM types per sample. Generally, the abundance was high only for the second and third investigated tree, where ECM were present in the inorganic horizon (Fig. 14).

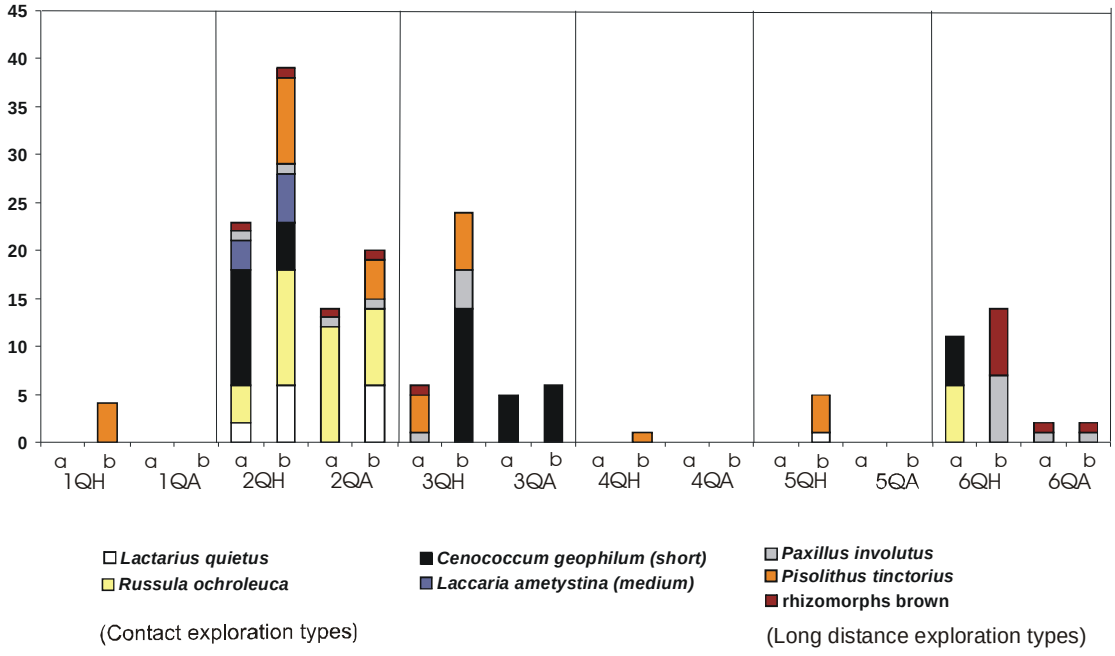

Figure 14: **Numerical abundance of ectomycorrhiza for six randomly selected *Quercus robur* trees at Greiz (H-humic horizon; A-inorganic horizon; a, b- two collected probes).**

To evaluate functional characteristics, the identified ECM were grouped into contact, short, medium and long distance exploration types (Fig.12). The two most frequent ECM types, *Lactarius quietus* Fr. and *Russula ochroleuca* (Pers.), belong to the contact exploration type**.** They were found on tree number 2, in both horizons; and in the organic H horizon of tree number 5. *Russula ochroleuca* was found on trees number 2 and 6.

The short distance exploration type was abundantly represented by *Cenococcum geophilum* (Agerer, 1987-2006) present on trees number 2, 3 and 6, mostly in the organic H horizon, but occasionally also found in the inorganic horizon. The medium distance exploration type was represented by *Laccaria amethystina* (Agerer, 1987-

2006) and was found only in the organic H horizon of the second tree.

The long distance exploration type included ECM characterized by rather smooth ectomycorrhizae with few, but highly differentiated rhizomorphs and was heterogeneously distributed. Representatives of this group include *Paxillus* and the brown rhizomorphs of unknown fungal origin found on tree number 6. *Pisolithus* was present on all trees with exception of tree number 6.

Only one tree (number 2) presented all four exploration types of ECM but only in the organic H horizon, with *Lactarius quietus, Russula ochroleuca, Cenococcum geophilum, Laccaria amethystina, Pisolithus tinctorius* and brown rhizomorphs. In all other samples, the low abundance and highly heterogeneous distribution of ECM along the six trees investigated indicated that ECM had been repressed, most likely by ecotoxicologically elevated heavy metal concentrations. This seemed especially probable because of the low pH measured in most samples.

Fig. 15 allows the comparison of the estimators of diversity indices in each horizon in the studied forest (average and SD). At the minimum number of the studied trees, Fisher's alpha index (reflecting mostly the species richness) is highest at Jenzig, both in the H and in the A horizons. Greiz diversity appears higher than at Kanigsberg in the H horizon, but lower in the A horizon. The Shannon index of diversity (putting an equal accent on richness and evenness) is lowest at Kanigsberg in the H horizon, and apparently highest at Greiz in the H horizon. No significant effects were detected between sites in the A horizon. One can notice also the very high CV of this index in the A horizon at Greiz.

The total number of morphotypes (abundance at the sites) was highest in Jenzig, followed by Kanigsberg and Greiz (Fig. 15). Most morphotypes of the A horizons were also present in the H horizons, with the exception of the orange-yellow morphotype at Jenzig (where it was present only in the A horizon), *Pisolithus tinctorius* and gray mycelium of *Scleroderma* sp. at Kanigsberg (present only in the A horizon). These morphotypes were not dominant in the A horizon.

The results of the estimations of the total number of species at forest level (pooling the data for H and A horizons, and including only the samples with non-zero number of morphotypes) are presented in Fig. 16. At Jenzig, the estimated richness is highest in all variants (Chao2, Jacknife2 and Bootstrap estimators). Chao2 and

Jacknife2 suggest no significant differences between the real richness at forest level in Greiz and Kanigsberg. The number of species observed at Greiz and Kanigsberg is probably not significant (large overlap of confidence boundaries for MaoTau). The only estimator indicating larger species richness at Greiz, than at Kanigsberg, is the bootstrap estimator.

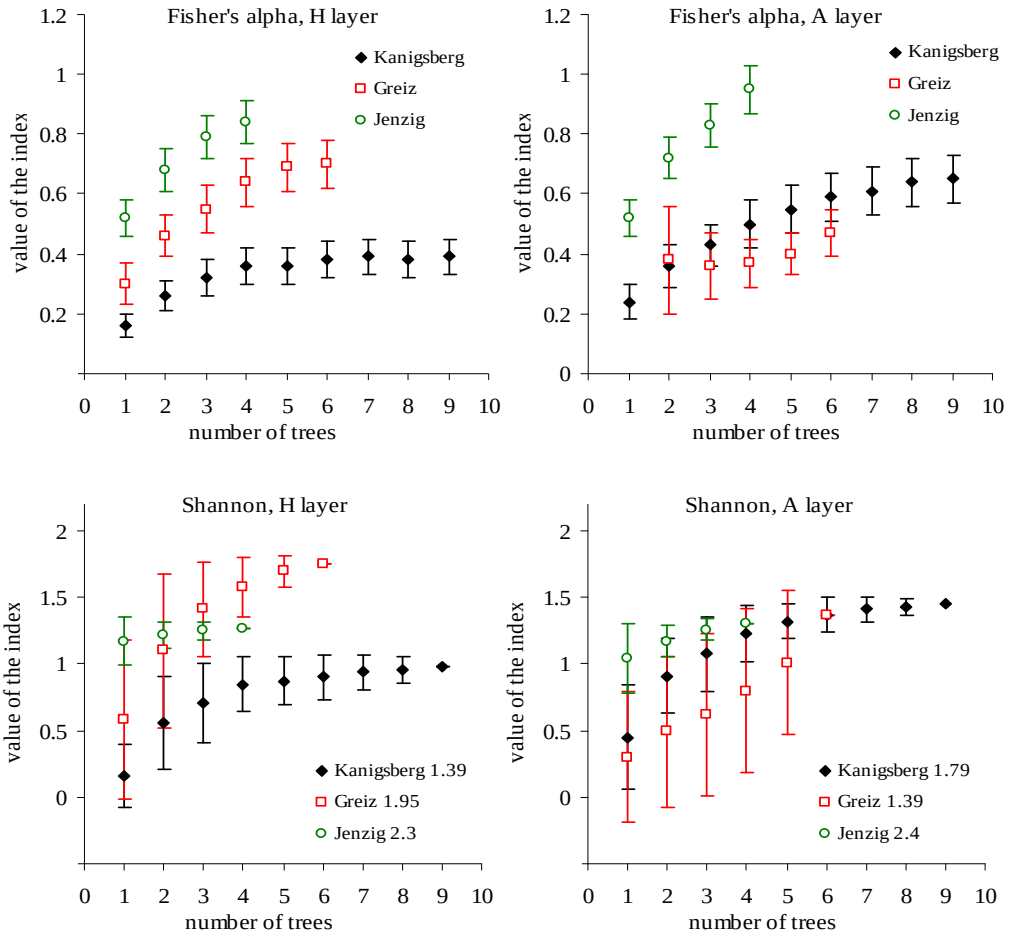

Figure 15: **Estimators of diversity indices at the studied sites. Values near site names indicate maximum possible values of the Shannon index. The indices are plotted after 50 randomizations.**

Information concerning the relative abundances of the species at forest level (pooled data), and implicitly, about evenness at this level is provided in Fig. 17. In contrast to results at tree level, at forest level the lowest evenness is observed in Jenzig and the highest is in Greiz, with Kanigsberg having intermediary values. The situation at Jenzig is due to the peculiar distribution of morphotypes between trees. At one Jenzig tree a large number of *Cenococcum geophilum* morphotypes and a consistent number of morphotypes of other species was found, thus the evenness was not very

low at tree level. But the species present with *Cenococcum geophilum* at that tree had low abundance values at other trees, being replaced by other locally abundant species.

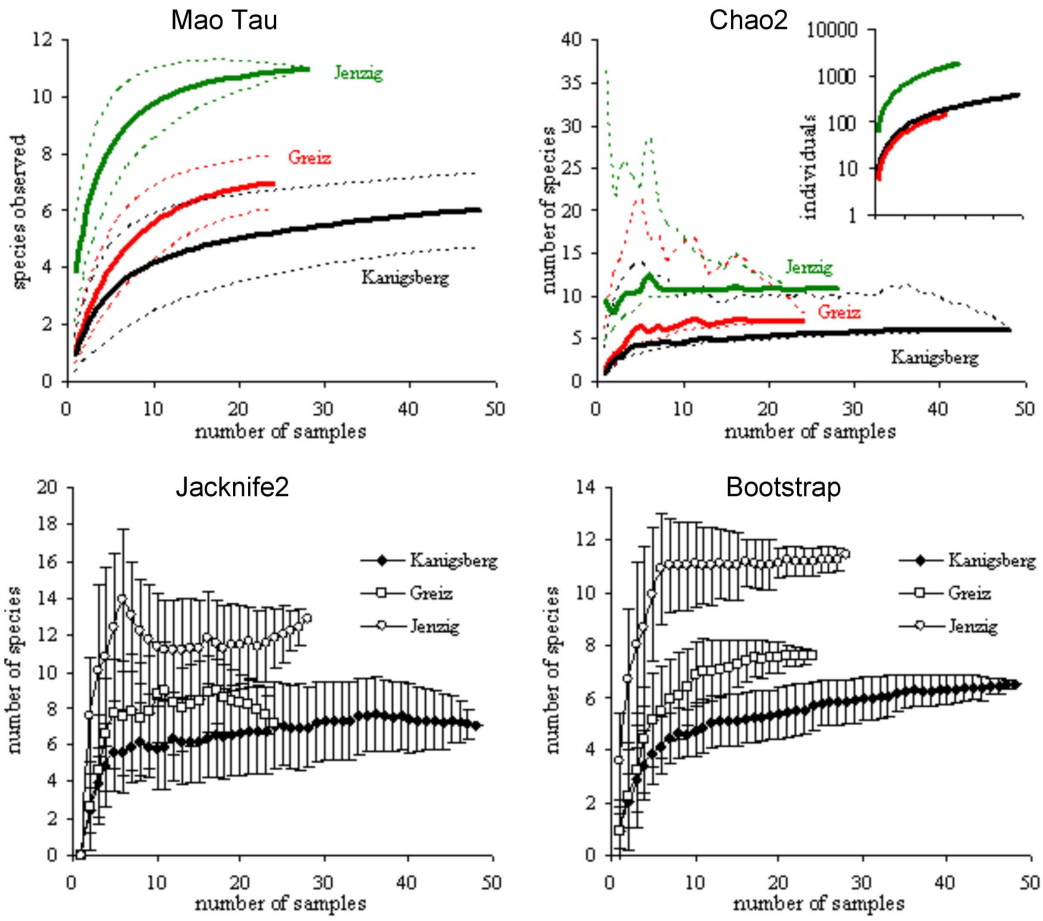

Figure 16: **Rarefaction curves (Mao Tau method, with upper and lower 95% confidence boundaries) and three estimators of the species richness at the investigated sites (Chao2 with upper and lower 95% confidence boundaries, Jacknife2 and Bootstrap with SD). Inserted graph: sampled number of morphotypes (individuals) as the number of samples has increased. The cumulative number of species at each site is plotted after 50 randomizations.**

While *Cenococcum geophilum* was still locally abundant at other trees, at forest level, *Cenococcum geophilum* became highly dominant. Another interesting issue is that there are many site specific species, and, in particular, that most of the minority species present at the non contaminated site Jenzig are not present at Kanigsberg or Greiz. Greiz, on the other hand, is characterized by large numbers of *Pisolithus tinctorius*, *Russula ochroleuca* and *Lactarius quietus*, while Kanigsberg is dominated by *Scleroderma areolatum*, *Tomentella sublilacina*, and brown rhizomorphs.

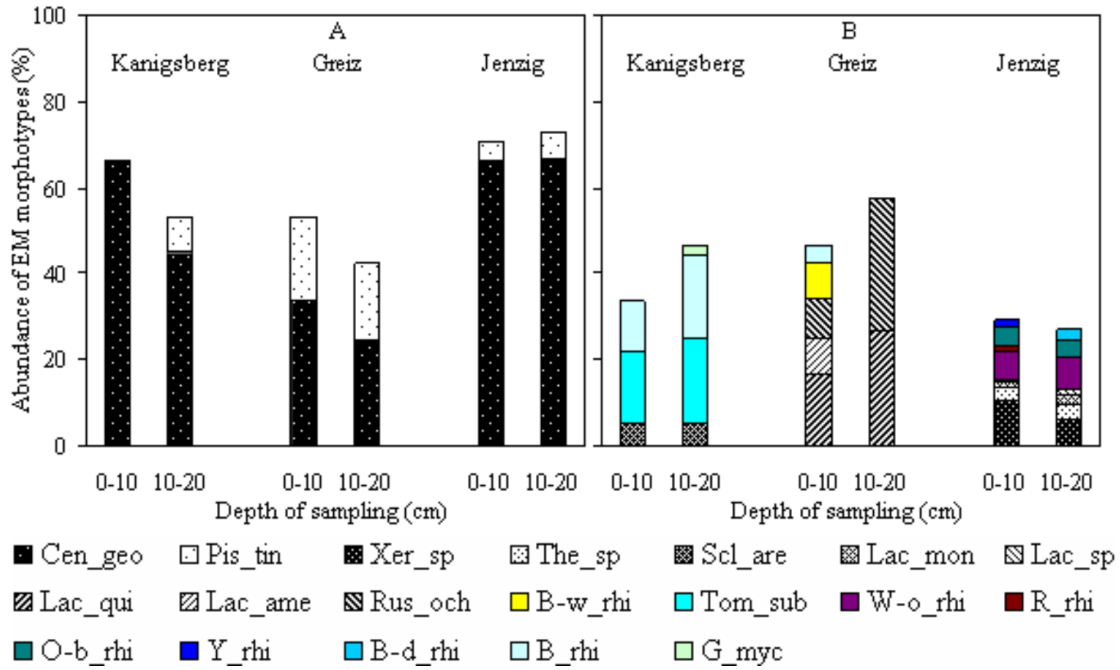

Figure 17: **Abundance of different ECM morphotypes. A) Abundance of morphotypes which occurred at all three sites. B) Abundance of morphotypes restricted to one or two sites.**

(*Cen_geo=Cenococcum geophilum; Pis_tinc=Pisolithus tinctorius; Xer_sp=Xerocomus* sp*; The_sp=Thelephoraceae* sp.*; Scl_are=Scleroderma areolatum; Lac_mon=Lactarius* sp. monopodial; *Lac_sp.=Lactarius* sp.; *Lac_qui=Lactarius quietus; Lac_ame=Laccaria amethystina, Rus_och=Russula ochroleuca; B-W_rhiz=Paxillus involutus* rhizomorphs*; Tom_sub=Tomentella sublinacina; W-o_rhi=White morphotype without rhizomorphs ; R_rhiz=Rose_morphotype; O-b_rhiz=Orange braun morphotype*; *Y_rhiz=yellow rhizomorphs; B-d_rhiz=Beige drop morphotype; B_rhi= Brown rhizomorphs; G_myc=Gray mycelium of Scleroderma* sp.*).*

3.5 Heavy metal distribution

The average total concentration of metals, P and pH in the studied soils (Tab. 3) shows high U at Kanigsberg and Jenzig and high Pb for Greiz, while most other metals do not exceed the limits for soil protection.

However, sequential extraction could show the ecotoxicological relevant fractions of metals (Tab. 4). Results revealed, that, e.g., U at Jenzig is unavailable and not relevant in ecotoxicological terms while U concentrations at Kanigsberg and Greiz, are bioavailable with up to 16% (fractions 1 + 2). While P availability was low at Kanigsberg, in contrast to the high S availability, very high available fractions of Al, Cd, Mn and Sr at Greiz could be shown. These pattern warranted multivariate analyses to gain a holoistic view of these distribution patterns.

Table 3: **Average concentration of elements (µg/g d.w.) and pH in the soil of the studied ecosystems.**

	Kanigsberg		Greiz		Jenzig	
	Av.	**SD**	**Av.**	**SD**	**Av.**	**SD**
Al	14499	2433	10639	2528	20404	1912
As	47	27	44	8	20	3
Cd	0.3	0.1	0.5	0.4	0.8	0.2
Co	14	10	10	3	9	3
Cr	46	18	19	4	30	6
Cs	3	1	3	1	2	1
Cu	79	19	26	6	31	19
Fe	65453	16181	36151	9368	17751	1119
Mn	135	22	1337	668	432	16
Ni	47	19	23	4	31	18
P	758	142	1112	303	1686	16
Pb	23	5	218	67	53	15
S	1276	260	1207	351	1376	255
Sr	36	15	25	10	322	190
U	12	8	2	1	65	48
Zn	84	22	125	26	56	95
pH	4.01	0.67	3.08	0.22	6.03	0.90

Table 4: **Percent of easily extractable fractions from total metals in soil samples (K – Kanigsberg, G – Greiz, J – Jenzig).**

	Fraction 1			Fraction 2			Fraction 3			Fraction 4		
	K	**G**	**J**	**K**	**G**	**J**	**K**	**G**	**J**	**K**	**G**	**J**
Al	1.26	5.22	0.01	0.08	0.43	0.05	0.07	0.37	0.09	0.23	3.08	0.27
As	0.62	0.20	1.99	0.59	0.22	2.44	0.55	0.45	1.56	0.80	0.99	2.54
Cd	10.85	26.63	2.03	3.23	6.43	4.10	3.69	5.57	3.79	2.82	2.35	14.88
Co	3.86	5.82	0.79	0.58	1.08	0.87	6.26	1.14	1.47	0.51	1.54	10.88
Cr	0.21	0.87	0.11	0.39	2.95	0.27	0.77	0.74	0.36	0.38	3.14	0.32
Cs	7.93	1.30	1.11	0.87	0.27	0.06	0.18	0.06	0.04	0.17	0.07	0.04
Cu	4.09	0.16	1.32	1.41	0.41	0.61	1.03	0.14	0.06	3.07	10.15	15.92
Fe	0.01	0.16	0.01	0.005	0.22	0.10	0.13	0.89	0.19	0.36	5.86	0.46
Mn	7.85	26.70	3.10	0.64	4.98	0.20	0.77	5.92	1.25	0.26	1.22	0.32
Ni	4.23	4.28	0.57	0.63	0.10	0.13	0.90	0.10	0.13	0.41	2.02	11.85
P	0.40	1.70	1.22	0.55	2.53	3.20	0.54	1.57	1.32	1.44	6.55	3.21
Pb	0.42	4.84	0.16	0.39	7.53	0.86	0.49	7.29	0.87	2.18	16.32	14.71
S	15.77	5.09	4.02	6.77	4.13	2.00	1.96	2.12	1.17	1.96	6.39	2.48
Sr	3.61	29.06	11.14	0.55	4.14	2.95	0.15	0.44	0.38	0.39	0.36	0.28
U	1.25	1.53	0.06	14.89	4.69	0.31	3.22	3.09	0.31	1.60	1.66	0.74
Zn	3.49	9.43	0.88	0.57	1.05	5.01	0.69	0.79	5.36	0.61	0.89	35.03

Principal component analyses was applied to toxic element concentrations in fraction 1 (variant A), the sum of fractions 1 and 2 (variant B), the sum of fractions 1-4 (variant C), and the percent of fractions 1 to 4 from the total concentration (variant

Table 5: **Positive or negative of PCA factors with: clusters of metal concentrations in the extracted fractions, their eigenvalues (λ) and cumulated percents of explained variance in metals.**

	Variant A			Variant B				Variant C			Variant D		
	F.1	F.2	F.3	F.1	F.2	F.3	F.4	F.1	F.2	F.3	F.1	F.2	F.3
Al	+			+						+	+		
As		not included			not included				not correlated			+	
Cd			+			+		+				not correlated	
Co		not correlated					-		not correlated				+
Cr	+			+						+	+		
Cs		-			-				not correlated			not correlated	
Cu		-			-				+			+	
Fe	+			+						+	+		
Mn			+			+		+			+		
Ni		-					-		+				+
Pb	+			+						+	+		
Sr		+			+				not correlated		+		
U		not correlated					-		+			+	
Zn			+			+		+			+		
λ	5.32	2.53	1.59	6.02	3.08	1.42	1.03	5.23	2.11	1.35	6.81	2.52	1.74
CPVM	48	71	86	46	70	81	89	52	73	87	49	67	79
Roots density	-0.46 p.053	0.69 p.002		-0.48 p.044	0.64 p.004		-0.43 p.078			-0.47 p.048			-0.43 p.071
pH	-0.64 p.004	0.60 p.009		-0.66 p.003	0.59 p.010					-0.63 p.005	-0.45 p.060		-0.46 p.054

Table 6: **Correlation and p level of significance between non correlated elements, essential elements and other soil parameters.**

	Variant A			Variant B		Variant C				Variant D			
	P	S	Co	P	S	P	S	Cs	Sr	P	S	Cd	Cs
Roots density		-0.487 p.040	-0.63 p.005		-0.59 p.009		-0.75 p.000	-0.42 p.086	0.84 p.000		-0.73 p.001		-0.46 p.057
Depth	0.516 p.028			0.42 p.080									
pH						-0.46 p.054	-0.53 p.024		0.79 p.000	-0.57 p.014	-0.61 p.007	-0.54 p.021	

D)(see Tab. 5). Most metals group in 3-4 factors in all variants of the analyses. The extracted factor explain 86% of the variance in metals concentrations in variant A, 89% for the variant B, 87% for the variant C and 79% for the variant D. The most stable group of metals is Al, Cr, Fe and Pb which is highly correlated with factor 1 in variant A and B, and with factor 3 in variant C. Another stable group is Cs, Cu and Sr (correlated with factor 2 in variant A and B), and a third is Cd, Mn and Zn (correlated with factor 3 in variant A and B, and with factor 1 in variant C). The number of metals, not correlated with the extracted factor ranges from 1 to 4, being lowest in variant B (As). Interestingly, variant B explaining the highest percent of variation in concentration, had the lowest number of non-correlated elements, and reflected the sum of the highest bioavailable concentrations (Tab. 5).

The extracted factors and other soil parameters (like root density and pH) show a significant (or nearly significant, at alpha 0.05) correlation coefficient (R). While there is a negative correlation between the Al, Cr, Fe, Pb, Cs and Ni concentrations to root density and pH, a positive correlation is found between Sr and the same parameters. The highest concentrations of Sr at Jenzig, where also the pH and root density was highest (Tab. 5). S availability is clearly negatively correlated with pH and root density, while easily available P is positively correlated with depth and inversely correlated with the organic matter concentrations present in the top soil (Tab. 6).

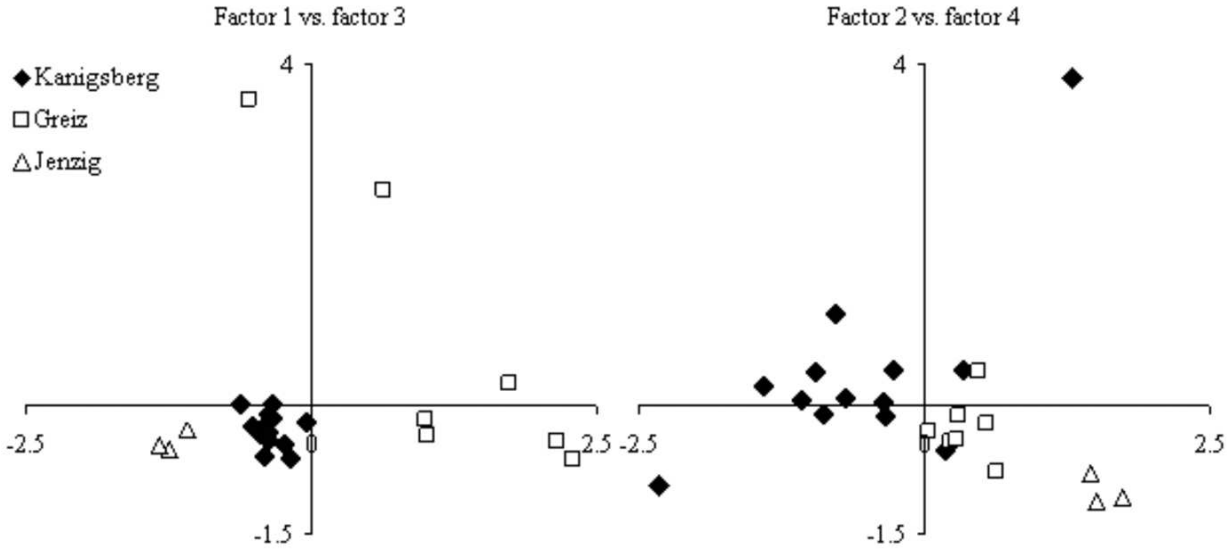

Figure 18: **Plot of sample scores after PCA of cumulated heavy metal of variant B.**

The distribution of elements showed different pattern for the three sites. Fig. 18 presents the sample scores plotted on the factors extracted in variant B. The samples from Jenzig were homogenous with respect to all metals, the samples from Greiz were heterogenous with respect to factors 1 and 2 (Al, Cr, Fe, Pb, Cd, Mn and Zn), while the samples from Kanigsberg were heterogenous with respect to the factors 3 and 4 (Cs, Cu, Sr, Co, Ni and U).

Soil analysis was done at Greiz, where samples were taken from two horizons at each tree (numbers 1, 2, 6; see Tab. 8) and analyzed by sequential extraction. The pooled environmentally available fractions (1+2) showed elevated Al, Mn, Fe, Pb concentrations distributed heterogeneous between trees. Most heavy metals and essential nutrients were found at higher concentrations in the organic compared to the inorganic A horizons, except of Al, which was present in higher concentrations in the inorganic horizon. Pb was uniformly distributed between both horizons. Two subsequent time points for collection of soil samples revealed that the availability of metals was constant (Tab. 7 and Tab. 8).

Table 7: **Environmental available element content (µg/g; mean±SD) in the Q soil sample at Greiz 2005.**

Greiz, 2005	Elements	Q
essential elements	Ca	230.28±2.78
	K	98.31±5.23
	Mg	33.37±0.11
trace elements with potential toxicity	Co	0.76±0.01
	Cu	0.21±0.02
	Fe	53.68±0.85
	Mn	225.10±1.14
	Ni	0.42±0.02
	Zn	5.73±0.07
toxic elements	Al	693.71±12.98
	Cd	0.04±0.00
	Cr	0.12±0.03
	Cs	0.07±0.00
	Pb	13.70±0.05
	Sr	2.03±0.02
	U	0.07±0.00

Table 8: **Environmental available element content (µg/g; mean±SD) and pH in three of the six randomly selected trees at humic and inorganic horizons (Greiz, 2006).**

	elements	1QH	1QA	2QH	2QA	6QH	6QA
essential	P	57.34±2.54	31.53±2.90	67.84±2.32	20.51±1.94	86.47±6,48	18.71±3.01
elements	S	122.67±13	101,69±18	147.09±14.21	26.99±9.45	127.81±22.65	116.67±19.87
trace elements	Co	0.68±0.02	0.69±0.03	0.48±0.02	0.27±0.01	1.11±0.04	0.75±0.01
with potential	Cu	0.25±0.02	0.19±0.03	0.22±0.02	0.00±0.00	0.09±0.02	0.03±0.00
toxicity	Fe	117.49±3.26	170.70±1.43	82.54±0.65	91.36±1.64	166.14±1.95	194.37±2.10
	Mn	291.78±3.26	137.91±2.12	1001.98±3.99	285.03±5.33	577.22±6.84	246.77±1.04
	Ni	1.54±0.04	1.26±0.03	1.24±0.05	0.39±0.03	1.34±0.12	0.61±0.06
	Zn	13.48±0.16	9.09±0.10	26.43±0.24	6.06±0.16	24.21±0.45	6.95±0.10
toxic elements	Al	700,66±21	866.22±10.21	329.07±1.24	539.56±4.91	473.09±4.62	696.76±7.17
	Cd	0.12±0.02	0.09±0.01	0.40±0.03	0.06±0.01	0.36±0.03	0.06±0.01
	Cr	1.09±0.21	0.75±0.18	0.59±0.12	0.64±0.27	0.57±0.20	1.09±0.23
	Cs	0.06±0,00	0.15±0.01	0.05±0.00	0.00±0.00	0.00±0.00	0.01±0.00
	Pb	33.80±0.13	36.23±0.20	26.04±0.24	21.08±0.14	31.83±0.24	26.13±0.12
	Sr	10.50±0.39	6.18±0.02	15.16±0.12	4.68±0.04	15.22±0.09	4.96±0.06
	U	0.05±0	0.44±0.08	0.06±0.00	0.03±0.00	0.05±0.00	0.06±0.00
pH soil		3±0.23	2.96±0.05	3.21±0.2	2.99±0	3.4±0.6	3.15±0

The constant availability of metals is highly relevant for correlation with ECM distribution and therefore it was verified by cluster analysis (Fig. 19). Different sites separate well with respect to metal concentrations (best in variant B). The linkage distance, an indicator of heterogeneity at site level, between the Jenzig samples in variants A and B is smaller than the distance between Greiz or Kanigsberg sites. The variant B (sum of fractions 1 and 2) groups best the samples according to sites. Thus, both Greiz and Kanigsberg sites are heterogenous in the distribution of metals in soil, but with respect to different elements.

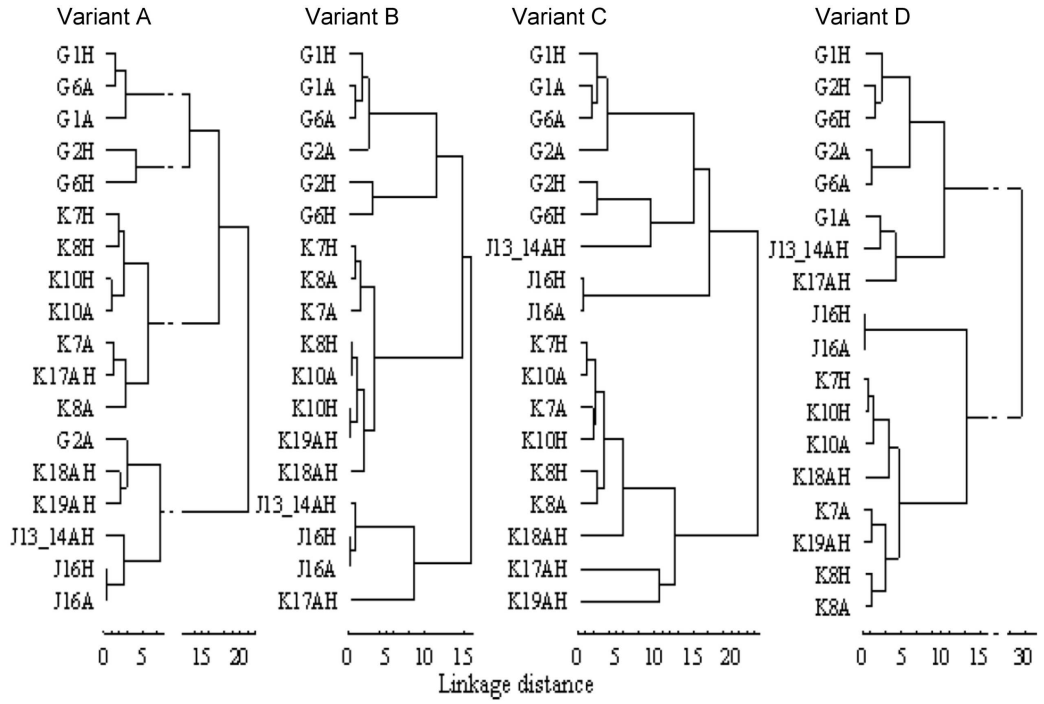

Figure 19: **Cluster diagrams (Ward`s method, City-block (Manhattan) distances) of the samples built using sample scores on extracted factors, and standardized values of P, S and not correlated elements. (variant A=fraction 1, variant B=the sum of fractions 1 and 2, variant C=the sum of fractions 1-4 and variant D=the percent of fractions 1 to 4 from the total concentration of sequencial extraction)**

3.6 Ecological implications of metals on ECM diversity

The relationship between ECM distribution and the ecological context was investigated primarily based on the average values of the parameters at each tree and depth. Table 9 and 10 presents the summary of the multivariate analyses performed under the assumption of unimodal distributions of the ECMs along

environmental gradients. Detrended correspondence analyses followed by correlation of the extracted factors (gradients) with independent environmental parameters (P, S, pH and density of roots). Hybrid detrended canonical correspondence analyses based on the environmental factors extracted by PCA and independent of metals where the metals were not correlated with the PCA extracted factors. As expected, the total variance in ECM abundance explained by the factors extracted in the unrestricted analyses (DCA) is smaller than the restricted variant (DCCA): 53.6% vs 57.7-60.8%, depending of the PCA factors used – variants A, B, or C (Tab. 9). In a visual form, this can be seen in the length of the gradients extracted, which are larger in DCA than in the hDCCA. An important aspect is that only the first two factors of the canonical analyses are, in fact canonical; the other two factors are not correlated with the included PCA factor and independent metals. Thus, the total explained variance in species data which can be attributed to metals is only corresponding to variant B (28.6% for the metals in variant A, 32.1% percent for the metals in variant B, and 30.8% for the metals in variant C). The sum of most available metals in soil (variant B) is the best variant for explaining the distribution of ECMs as correlated with metals (Tab. 9).

Table 9: **Summary of multivariate analyses. a) Eigenvalues (λ) and cumulated percentages of explained species variance (CPVS) of the extracted factors (axes 1 to 4) after detrended correspondence analyses (detrending by first order polonomials and by segments) and detrended canonical correspondence analyses using hDCCA and detrending by second order polinomials.**

	DCA		hDCCA Variant A		hDCCA Variant B		hDCCA Variant C	
Axes	λ	CPVS	λ	CPVS	λ	CPVS	λ	CPVS
1	0.72(0.72)	20.9(20.9)	0.59	20.0	0.62	20.7	0.61	21.5
2	0.63(0.46)	39.1(34.3)	0.25	28.6	0.34	32.1	0.26	30.8
3	0.35(0.30)	49.2(43.0)	0.55	47.5	0.53	50.0	0.61	52.5
4	0.15(0.10)	53.6(45.8)	0.30	57.7	0.29	59.8	0.23	60.8

Taken separately, none of the extracted axes are significant at alpha 0.05 (p value ranges between 0.114 and 0.246), but considering all canonical axes together, their relation between the distributions of all considered metals and ECMs distribution result as statistically significant (Tab. 10).

Table 10: **Canonical coefficients for standardized environmental variables (E.V.) and p level of significance of the canonical axes (first axes and all canonical axes).**

Variant A			Variant B			Variant C		
E. V.	**Axes 1**	**Axes 2**	**E. V..**	**Axes 1**	**Axes 2**	**E. V.**	**Axes 1**	**Axes 2**
Co	0.31	25.3	Factor 1	0.85	0.15	Sr	-16.03	0.21
U	-0.54	-0.12	Factor 2	-0.51	12.05	Factor 1	-11.61	36.41
Factor 1	0.26	-0.18	Factor 3	0.7	0.44	Factor 2	0.79	-0.68
Factor 2	-13.5	23.29	Factor 4	0.09	11.49	Factor 3	-10.32	19.18
Factor 3	0.19	11.34						
p MonteCarlo			p MonteCarlo			p MonteCarlo		
axes 1	0.246		axes 1	0.12		axes 1	0.114	
can. axes	0.042		all axes	0.026		all axes	0.014	

In the Fig. 20 the directions of the arrows indicate an increase in the value of a parameter. The relative importance of an environmental parameter is proportional with the length of the arrow. The degree of correlation of an environmental variable with axes is proportional with the projection of the environmental arrow on the axes. Preference of a species for a parameter value (average in origin, larger values in the arrow direction) can be assessed by projecting the species point on the environmental arrow. Average value of the parameter in a site-horizon can be assessed by projecting the site-horizon point (centroid) on the environmental arrow. The centroid of the sampled sites is surrounded by species most characteristic at that site. The species show clear differentiation along the extracted factors, which in turn are highly correlated with the environmental variables (Fig. 20).

The clearest separation of community structure with depth is found at Kanigsberg, followed by Jenzig. The central position of *Cenococcum geophilum* reflects its ubiquitous distribution. The B variant of PCA explains the highest percentage of ECM variance, and thus this variant was used for representative analysis (Fig. 21). The correlated separation of trees *versus* horizons and sites by ECM and metals shows that not one, single metal is responsible. Rather, the overall contamination (relevant to ECMs) seems to be higher at Greiz than at Kanigsberg, at least for the metals retained in the factors extracted by PCA in variant B. One tree at Kanigsberg is atypical in terms of ECM species composition and correlated with low concentrations of the analyzed soil elements.

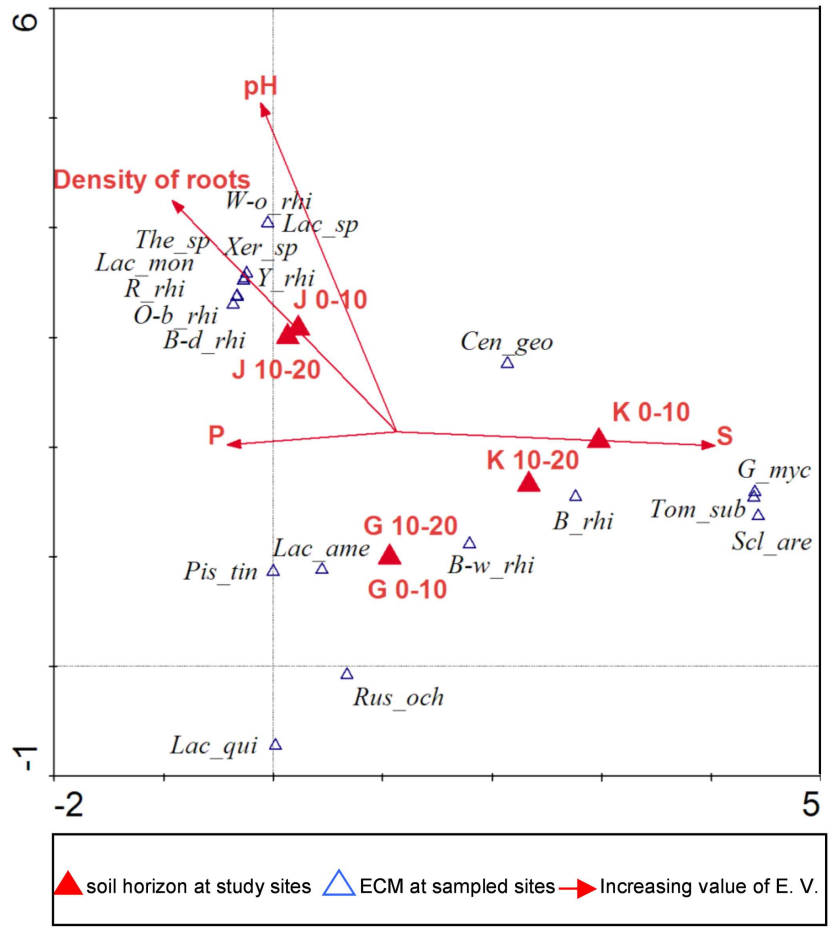

Figure 20: **DCA plot of species, environmental variables and centroids of sampled sites (detrending by segments).**
(*Cen_geo=Cenococcum geophilum; Pis_tinc=Pisolithus tinctorius; Xer_sp=Xerocomus* sp*; The_sp=Thelephoraceae* sp.*; Scl_are=Scleroderma areolatum; Lac_mon=Lactarius* sp. monopodial; Lac_sp.=Lactarius* sp.**; Lac_qui=Lactarius quietus; Lac_ame=Laccaria amethystina, Rus_och=Russula ochroleuca; B-W_rhiz=Paxillus involutus* rhizomorphs*; Tom_sub=Tomentella sublinacina; W-o_rhi=White morphotype without rhizomorphs ; R_rhiz=Rose_morphotype; O-b_rhiz=Orange braun morphotype*; *Y_rhiz=Yellow rhizomorphs; B-d_rhiz=Beige drop morphotype; B_rhi= Brown rhizomorphs; G_myc=Gray mycelium of Scleroderma* sp.**).**

The separation for the non-canonical axes (not dependent on the analysed metals; Fig. 22) is due to several atypical samples, two at Kanigsberg, one at Jenzig, and one at Greiz. Since As was not correlated with the PCA factors in variant B, these samples were double-checked with respect to As concentrations. The samples from Kanigsberg and Jenzig plotted separate correlating with available As concentrations (varaint B). Knowing that P can be protective towards As toxicity, the distribution of available P was also checked. The samples with atypical distribution indeed showed high available As and, at the same time, low available P concentrations. Consequently, there are indications that As (antagonized by P) plays an important

role in the separation of ECM communities along the non-canonical axes extracted by hDCCA.

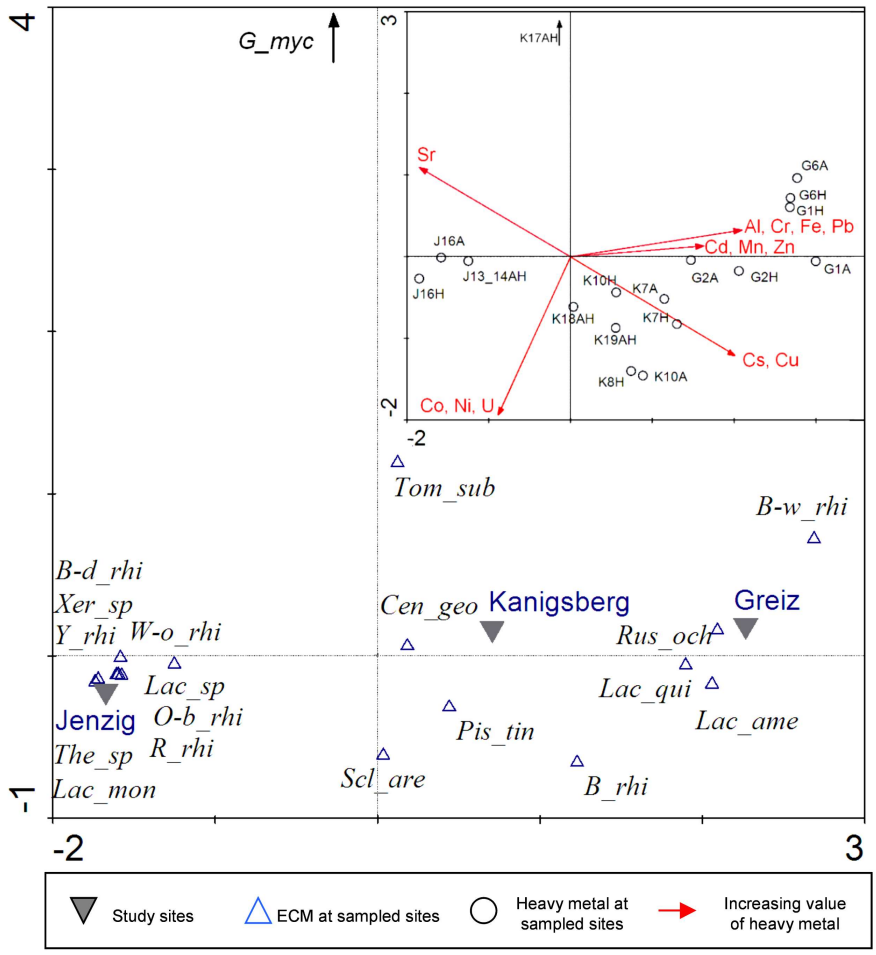

Figure 21: **hDCCA plots on canonical axes 1 and 2 non canonical axes 3 and 4. (*Cen_geo=Cenococcum geophilum; Pis_tinc=Pisolithus tinctorius; Xer_sp=Xerocomus* sp*; The_sp=Thelephoraceae* sp.*; Scl_are=Scleroderma areolatum; Lac_mon=Lactarius* sp. monopodial; *Lac_sp.=Lactarius* sp.; *Lac_qui=Lactarius quietus; Lac_ame=Laccaria amethystina, Rus_och=Russula ochroleuca; B-W_rhiz=Paxillus involutus* rhizomorphs*; Tom_sub=Tomentella sublinacina; W-o_rhi=White morphotype without rhizomorphs ; R_rhiz=Rose_morphotype; O-b_rhiz=Orange braun morphotype*; *Y_rhiz=Yellow rhizomorphs; B-d_rhiz=Beige drop morphotype; B_rhi= Brown rhizomorphs; G_myc=Gray mycelium of Scleroderma* sp.*).***

In order to identify the best possible correlations with high, low and no ECM diversity at Greiz, samples from oak tree number 1, 2 and 6, respectively, were selected (see Fig. 14).

In Fig. 23, Canonical correspondence analysis (CCA) revealed that samples 1 and 2 (belonging to tree number 1), and sample 6 (from the inorganic horizon of tree number 6) show similar abiotic stressors. Sample 5 (from the organic horizon of tree

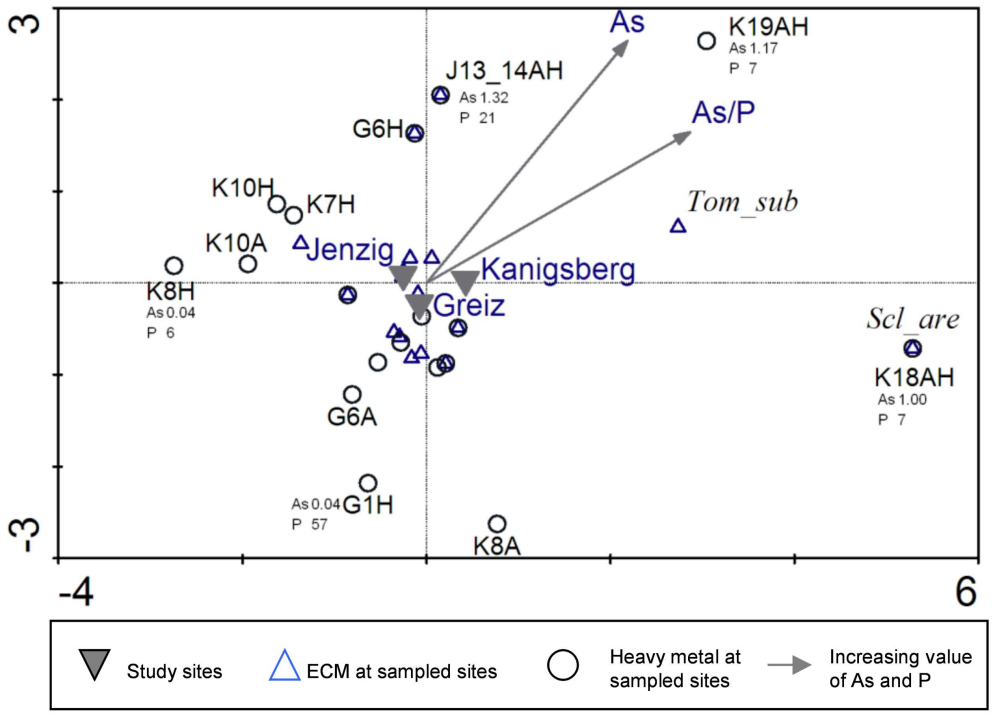

Figure 22: **hDCCA plots on non canonical axes 3 and 4. Concentrations of As an P (µg/g d.w., extracted in fractions 1 and 2) are indicated below selected samples. (*Tom_sub=Tomentella sublinacina; Scl_are= Scleroderma areolatum*).**

number 6) clusters opposite, indicating different environmental variables. Samples belonging to tree number 2 (samples 3 and 4) cluster separately. According to CCA, four groups of elements were the strongest contributors to the ordination axis: Al, Cr, Cs, U strongly correlate with each other and represent group I (Eigenevalue 0.111); Fe, Pb, Cu form group II; Co and Ni group together with the essential elements S and P as well as low pH and represent group III; Mn, Cd, Zn, Sr cluster with P and low pH in group IV. These groups form a gradient of correlation: group I correlates well with group II, which also correlates with group III, and group III strongly correlates with group IV. Group I, made up of heavy metals is negatively correlated with group IV including the essential elements.

Introducing the four exploration types of ECM into this matrix of heavy metals showed a second level of clustering: with the first group of heavy metals, the medium exploration type, *Laccaria amethystina,* and the long exploration type, represented by *Paxillus involutus* and the brown rhizomorphs, strongly correlate. The latter was

absent from tree number 1 (samples 1, 2) and represented by few mycorrhizae in the inorganic horizon of tree number 6 (in sample 6), the sample with the highest concentration of Al and Cr. The inorganic horizon of tree number 1 (sample 2) was the sample with the highest U concentration in the environmentally available fraction. Thus, U seems detrimental to the long exploration type ECM. The contact morphotype, *Russula ochroleuca,* is negatively correlated with group I (heavy metals) and clusters to group IV, closely correlated to Mn. *Lactarius quietus*, the second contact morphotype, clusters near tree number 2 (samples 3 and 4). This ECM also clusters to group IV and includes the essential element P. The short exploration type of ECM, *Cenococcum geophilum,* clusters to group III and strongly correlates with Ni, Co, and with the essential elements S and P.

The contact types *Lactarius quietus* and *Pisolithus tinctorius* are negatively correlated with group II. Sample 5 (organic H horizon) clusters separately from group I and presents a low concentration of Al, higher concentration of Cd and the highest Ni, Co, and S concentrations, but also P. This sample is correlated with *Cenococcum geophilum*. The highest concentration of toxic metals belonging to group I is correlated with low ECM diversity on trees number 1 and 6 (in samples 1, 2 and 6). *Russula ochroleuca* and *Cenococcum geophilum* are correlated with low pH and toxic metals but also with the essential element P. The relation between environmental factors and ECM types shows a clustering of the highest ECM diversity with group IV and the strongest disagreement with groups I and II.

Thus, it can be concluded that the availability of P positively affects ECM and group I has neither a negative or a positive effect. The presence of nutrients, specifically P, in the inorganic horizon of tree number 2 could explain the higher ECM abundance and diversity in the inorganic horizon of this tree.

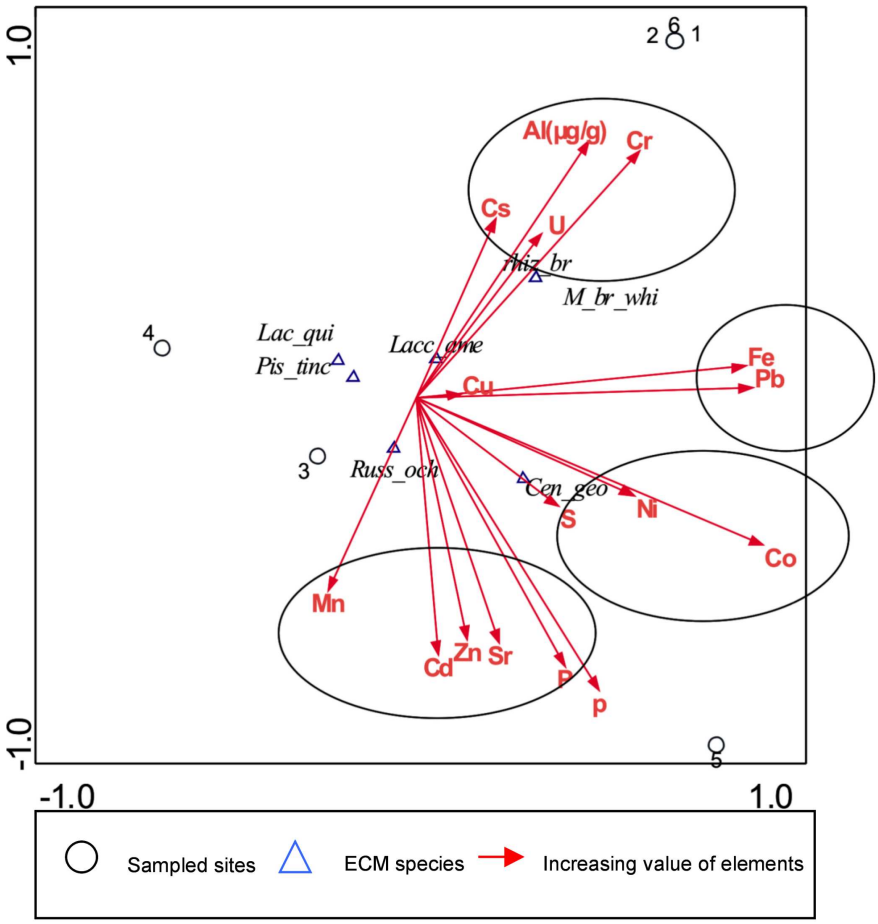

Figure 23: **Canonical correspondence analysis shows ECM type distribution in relation to environmental variables. (1-6: sample number, odd number represent the organic horizon and even number the inorganic horizon from tree number Q1, Q2 and Q6).**

(*Cen_geo=Cenococcum geophilum; Lacc_ame=Laccaria amethystina; Lac_qui=Lactarius quietus; M_br_wh=Paxillus involutus*; *Pis_tinc=Pisolithus tinctorius; rhiz_br=Brown rhizomorphs; Russ_och=Russula ochroleuca*).

3.7 Heavy metal tolerance of ECM fungi

Pisolithus tinctorius types collected on 1 Nov., 2006 at Greiz seems to accumulate heavy metals in the mantle (Fig. 24) in contrast to *Laccaria amethystina* (Fig. 25).

Heavy metal tolerance and host protection in ectomycorrhizal fungi were investigated in a *Hymenoscyphus* sp. (Ascomycota) isolated from the metal-polluted soil in Kanigberg in 2005. The heavy metals Cr, Cu, Ni and Pb were used. The experiment shows that the *Hymenoscyphus* sp. strain protect oak-seedlings against Pb and Cr-toxicity. The strain shows no resistence to Ni at 3 mM and 6 mM, but grows very well

in the contol. In the case of Cu, the strain grows well in the control, and tolerates concentration of 3 mM, but shows no resistance for Cu at 6 mM (Fig. 26).

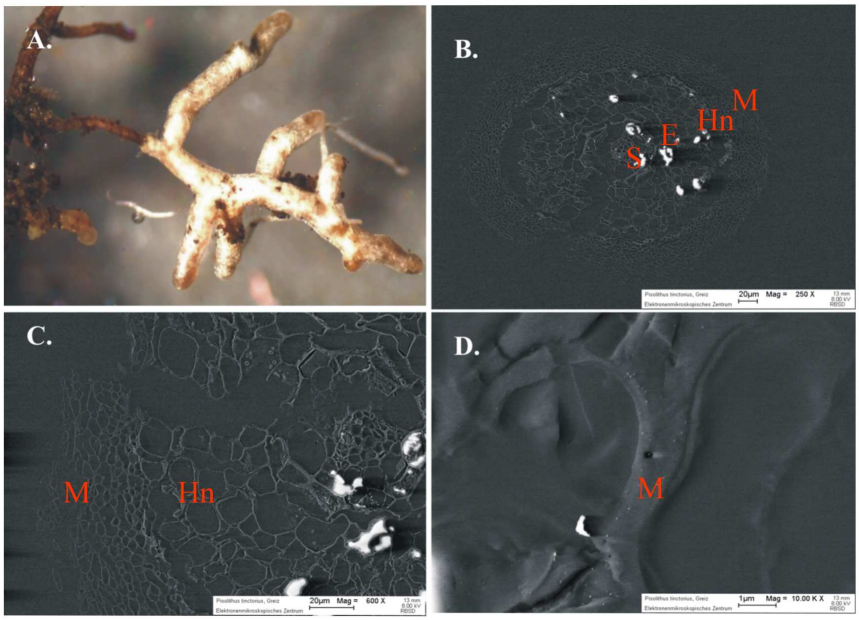

Figure 24: ***Pisolithus tinctorius* + *Quercus robur* ECM. A. Heterorrhizic system with monopodial short root. B. Cross-section with hyphal mantle (M), intercellular hyphae of the fully developed Hartig' net (Hn), endodermis (E), and central stele (S). C. Magnification of the mantle formed by *P. tinctorius* consisting of 8-10 fungal cells layers. D. Magnification of the mantle hyphae with accumulating heavy metals.**

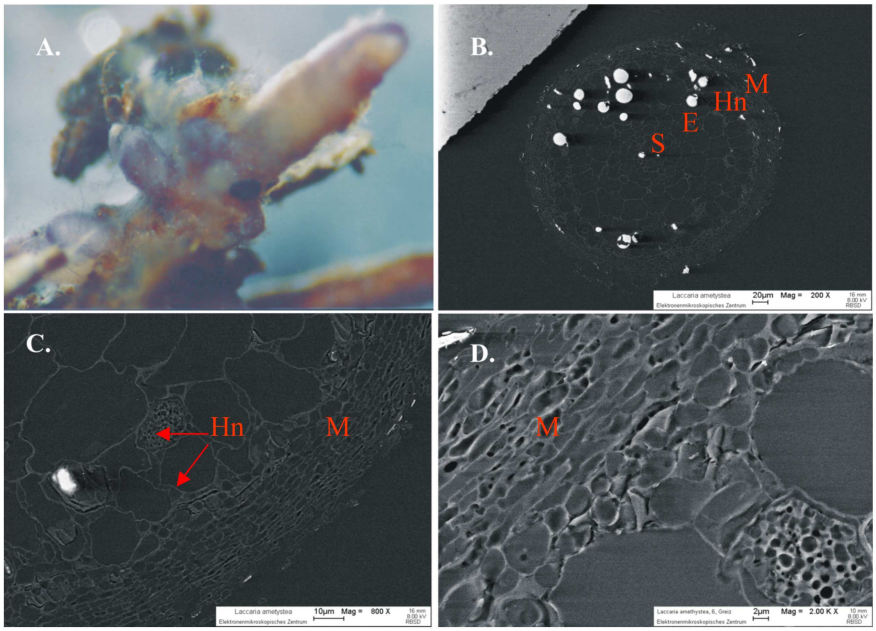

Figure 25: ***Laccaria amethystina* + *Quercus robur* ECM. A. Heterorrhizic system with monopodial pinat short root . B. Cross-section with hyphal mantle (M), intercellular hyphae of the fully developed Hartig' net (Hn), endodermis (E), and central stele (S). C. Magnification of the mantle formed by *L. amethystina* with 10-12 fungal layers . D. Magnification of the mantle and lobed Hartig'net hyphae without accumulating heavy metals.**

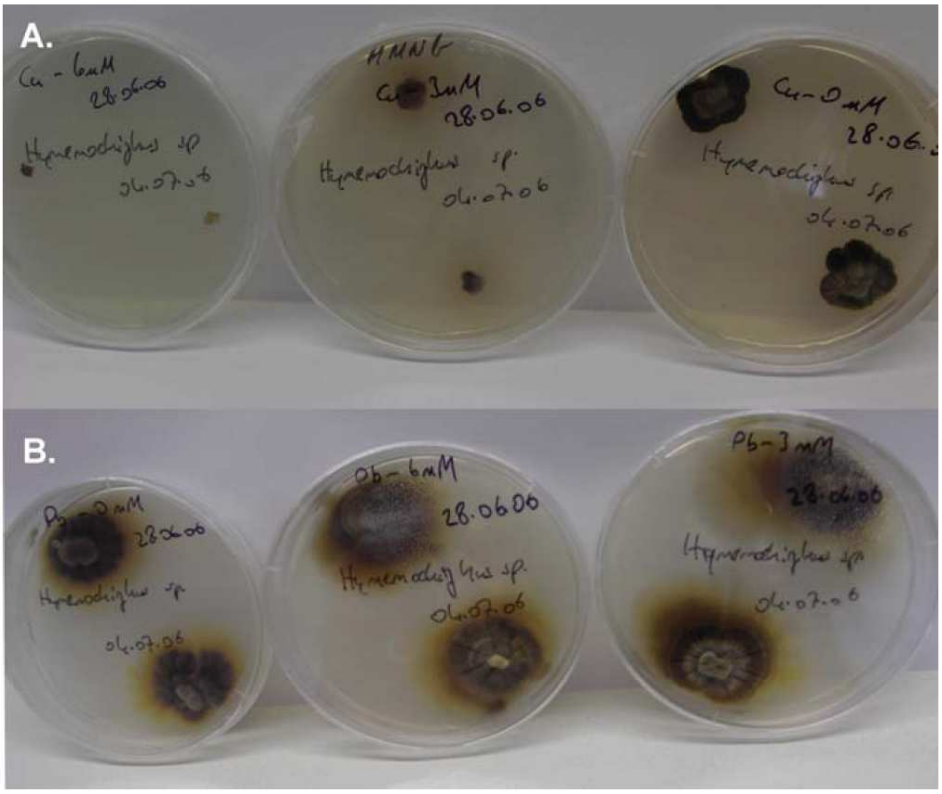

Figure 26: ***Hymenoscyphus* sp. isolated from the metal-polluted soil Kanigsberg in 2005. Heavy metal tolerance and host protection in ectomycorrhizal fungi investigated for A. Cu (left to right: 6 mM, 3 mM, 0 mM) and B. Pb (left to right: 0 mM, 6 mM, 3 mM).**

4 Discussion

4.1 Methodological considerations: Investigation of ectomycorrhizal communities

The application of molecular methods combined with morphotyping in ECM community studies have been proposed by several authors (Horton *&* Bruns, 2001; Menkins *et al*., 2005; Staudenrausch *et al*., 2005). Therefore, the results of morphotyping and ITS-PCR fingerprinting can be compared in our studies due to identical experimental set-up and the reliability of this approach can be evaluated. Morphotyping is a good method to go through large numbers of root tips and is suggested to give reliable diversity estimates of the individual seedlings and classification of ECM in exploration types. However, often it is difficult to identify similar ECM by morphotyping, because one root tip may be colonized by two ECM fungi. ITS-PCR fingerprinting, on the other hand, allows a better identification, but does not identify all ectomycorrhiza. However, the definition of ECM species by sequence similarities is complicated by the fact that the level of intraspecific ITS sequence variation differs between ECM fungi. Contaminations and artifacts are always risks in PCR amplification. Identification of sequences by comparing them to the sequences deposited in public nucleotide databases may be problematic, since taxonomic coverage of databases is limited. This was clearly highlighted in the comparable analysis of our ITS-taxa and sequences. Probably the most serious drawbacks of public databases are mistakes in sequences and in their identifications (Nilsson *et al*., 2006). To help overcome these problems in mycorrhizal studies, the UNITE database (http://unite.ut.ee) was created comprising 2511 well-annotated fungal ITS sequences from 118 genera as of January 2008 (Rajala, 2008). The combination of both morphotyping of defined sample units as a stratifying sampling method and subsequent PCR-ITS fingerprinting was found to be a reliable and relatively cost-efficient method for ectomycorrhizal species identification in large scale experiments, like this study.

4.2 Ecological implications of metals on ECM diversity

Environmental risk analaysis in cases of sediment and soil pollution is generally based on chemical analysis of selection of xenobiotic compounds. Many metals are essential nutrients (Cu, Co, Fe, Mo, Mn, Ni, and Zn) if they are present in apropiate concentrations, and often toxic if they are present at higher levels. Others, (Cd or Pb) have no known physiological activity. Usually, the total concentrations are used to predict the degree of pollution and the potential risks. However, the bioavailability of contaminants varies strongly with the properties of the environment. In soils and sediments the availability and toxicity of heavy metals is inversely related to pH, organic matter content and clay content. Ecotoxicological studies generally focus on toxic effects of pure chemicals on single species. When results of such studies are extrapolated to the field situation in order to predict ecological effects, interactions between populations and communities are not taken into account and food chain effects are neglected. In addition, toxicity tests generally have (sub) lethal toxicity endpoints and are based on relatively short-term test periods. Limiting ecological relevance in labor, contaminants may be adsorbed to soil organic matter and mineral soil particles, and soil organisms may be adapted to the heavy metals (Bloem *&* Breure, 2003). The often complex mixture of contaminants present in the field long-term determin effectes like reduced decomposition. Therefore, sensitive biological indicators are needed to detect changes in ecosystem. Multilevel experiments or gradient studies are necessary for determining the shape of a response curve. As the scale of investigation expands, additional variables, such as temperature, precipitation and biogeographic constraints (e.g. endemism), will probably emerge as significant variables. Thus variable choice affect model quality (Lilleskov *&* Parrent, 2007).

If pH is low enough Al^{3+} and heavy metals (explanatory variables) may be leached from soil particles. This may damage soil microbial life (ECM in the role of response variable) as well as reduce nutrients availability to trees (P, S in the role of covariables). Additionally covariables were used as root density, depth of the roots, organic matter.

As reviewed in detail by Colpaert, 2008, woody plants often are not considered as primary colonisers of metal-polluted soils, but on a number of sites pioneer tree

species, such as willows, poplars, birches and pines, are able to build up small pioneer populations. In trees with their long reproductive cycles, the adaptive potential for metal tolerance seems to be low (Meharg *&* Cairney, 2000) and even if there is a selection for individuals with a higher tolerance, it may take many decades before a reasonable tolerant population establishs. In addition, woody pioneers rely much more on their ECM fungi than herbaceous pioneer plants rely on their AM mycobionts, irrespective of any soil pollution (Ashkannejhad *&* Horton, 2006). After severe disturbances, when mycorrhizal propagules are scarce, ECM plants are slow colonisers. Therefore, it can be concluded that trees resist extreme metal toxicity through a large phenotypic plasticity and through their association with a small guild of well-adapted ECM fungi (Wilkinson *&* Dickinson, 1995).

For ECM fungi, species diversity seems to be lower on the most polluted areas (Staudenrausch *et al*., 2005). Colpaert *et al*. (2004) focused on the occurrence of ECM fungi in pioneer pine forests along the Zn pollution gradient. Only on the most polluted area the very low number of four ECM morphotypes was found on roots of 25 yr-old pine trees and the frequent occurrence show the dark septate ascomycetous *Hymenoscyphus ericae*. Similar ascomycetes are also present on pioneer pine trees that colonise Cu mine spoil in Norway (Vrålstad *et al*., 2002). Whether this ericoid mycorrhizal fungus can improve fitness of an ECM host under metal stress remains unclear. There are indications that particular ascomycetes are more stress-resistant than basidiomycetes and show up more frequently in mycorrhizal communities facing harsh environmental conditions or after severe disturbances (Baar *et al*., 1999; Trowbridge *&* Jumpponen, 2004). In the uranium mining area we have also isolated *Cenococcum geophilum*, *Hymenoscyphus ericae* and *Helotiales* sp.. *Cenococcum geophilum* is a ubiquitere species but remarkable is the frequent occurrence of a *Hymenoschyphus ericae* and *Helotiales* aggregate, which was absent from the control plots.

In general, we have only a very incomplete view on the biodiversity of mycorrhizal fungi in metal-polluted environments especially for the belowground community (Colpaert, 2008). Studies based on aboveground sporocarp observations show that apart from the dark ascomycetes, the metalliferous sites had also a basidiomycete ECM fungus in common: *Suillus luteus*, a typical mycobiont of young pine trees that

is very common in primary successions (Rühling *et al*., 1984, Rühling *&* Söderström, 1990). Although molecular studies regularly find a considerable lack of correspondence between the above- and belowground communities of ECM colonizers (Gardes *&* Bruns, 1996), the field studies of Rühling suggest that particular mycorrhizal species disappear with increasing metal stress. Some basidiomycete taxa that have been frequently found on heavily polluted soils include *Hebeloma* sp., *Pisolithus tinctorius* (Turnau *et al*., 1988), *Rhizopogon* sp. (Turnau *et al*., 1996), *Scleroderma* sp. (Jones *&* Hutchinson, 1986), and the Cd-accumulating *Amanita muscaria* (Kalač *et al*., 1991).

We could define the belowground ECM in a primary succession established in a uranium mining area. Apart from the dark septate ascomycetous fungi we have isolated basidiomycetous fungi like *Tomentella sublinacina*, *Pisolithus tinctorius*, *Paxillus involutus* and two strains of *Scleroderma* sp. (*Scleroderma verrucosum* and *Scleroderma* sp). *Tomentella sublinacina* was reported as early- and late-stage fungus by Bellei *et al*. (1992). The identified basidiomycetous fungi at Kanigsberg belong to the long exploration type of mycorrhiza. Long distance ECM fungi can alleviate low nutrient stress which presents a major challenge at the poluted site at Kanigsberg. Their external mycelia contribute largely to the nutrient uptake and transfer to the host. However, it proved to be quite difficult to demonstrate unequivocally that metal toxicity exhibited selection pressure on fungal communities and populations. It seems likely that in a primary succession in contaminated land the particular mycorrhizal guilds may have a high constitutive metal tolerance so that evolution for higher tolerance is simply not necessary.

The colonization of ECM is completely different in secondary successions where tree seedlings rapidly recruit ECM fungi, more often specialists, from dormant spore banks or other resistant propagules (Nara, 2006b; Izzo *et al*., 2006). Mycorrhizal fungi that colonize podzolic acidic soils can be exposed to high levels of toxic metals such as aluminium, iron and manganese. For a successfull symbiosis, both partners must be able to withstand the metal toxicity during all stages of colonization events. We have assessed the distribution of ECM in a 15-year old oak (*Quercus robur*) mixed forest on podzol with acidic pH of 2.85 to 3.40. Our results show that the observed abundance of ECM types was lower than on alkaline or slightly acidic

substrates and differed among the soil horizons: the organic horizon contained more ECM than the mineral horizon. The investigation by CCA indicated that a group of the heavy metals, including Al, Cr, Fe and Pb, strongly contributes to the reduction of ECM abundance. Although present at a low level, the medium distance exploration type fungus *Laccaria amethystine* and the long distance exploration type fungus *Paxillus involutus* as well as the observed brown rhizomorphs correlated with this group. The contact ECM types *Russula ochroleuca*, *Lactarius quietus* and a long distance exploration type fungus *Pisolithus tinctorius* were in sharp disagreement with these heavy metals but correlated positively with Mn, Cd, Zn, Sr and with the essential element P. The short distance exploration type *Cenococcum geophilum* was strongly correlated with Ni, Co and Cd, followed by a cluster of Fe, Pb and Cu, and also by the essential elements P and S. These findings indicate that the toxicity of heavy metals may be ameliorated by the availability of P.

It is likely that in this secundary succession (with reforestation of oak replacing a spruce forest) mycorrhizal guilds have no high constitutive metal tolerance so that evolution for higher tolerance is necessary.

4.2.1 Succession in primary versus secundary contamination

Factors responsible for patterns of successional stage and distribution of ECM roots are forest management and metal contamination. Discussed in terms of managing systems in order to maximize tree growth and form while effectively restoring soil water balance. The presence of ECM contact types in the higly heterogenous secundary succession area of Greiz is most likely due to preexisting spores or living mycelia. Horewer, the long distance exploration type seems to prevail over time. In conditions, where the heavy metal pollution is so severe that there are consistent detrimental effects on metabolism, organisms are subjected to selective pressure for increased resistance to toxic metals and species with the least efficient detoxification systems will disappear from the ecosystem. There has been a long debate whether mycorrhizal fungi have evolved adaptive tolerance against particular heavy metals (Hartley *et al*., 1997). A major reason was the lack of sufficient data from different populations from sites with high and low levels of pollution. Measuring and comparing metal tolerance can only be achieved by screening a significant number of individuals

from one or more species. It is necessary to analyze the intra- and interpopulation variation in metal tolerances. This is far easier for plants than for their fungal partners. The isolation and axenic cultivation of large numbers of mycorrhizal fungi can be quite troublesome. Nevertheless, inter-species comparisons *in vitro* can still be misleading because some fungi might be much more sensitive to stress *in vitro* than in symbiosis in contrast to grow equally well *in vitro* as in nature. Intraspecific comparisons are probably less susceptible to such confounding factors. Ideally once tolerance identified *in vitro*, it should also be verified in a plant experiment. Selection for adaptive metal tolerance in mycorrhizal fungi has been discovered in only a few higher fungi. *Pisolithus tinctorius* isolated from an old coal mining site had higher Al tolerance than isolates from rehabilitated and forest sites (Egerton-Warburton *&* Griffin, 1995). In the pioneer forests around several Zn smelters in Belgium, adaptive Zn tolerance was found in *Suillus luteus*, *S. bovinus* and *Rhizopogon luteolus*, but not in *Paxillus involutus* (Colpaert *et al.*, 2004).

From a system's ecology perspective, succession is a process occurring at ecosystem level (community and its environment), so it is *a priori* not appropriate to search for reductive species level understanding of succession, while it is meaningful to look for species level mechanisms supporting succession in a community and ecosystem context. The search for indicator species of successional stages has been, however, a part of the reductive paradigm, both in plant ecology and in fungal ecology. Consequently, much of the literature dealing with ECM succession is dedicated to the concept of early and late succession species, as label species for certain succession stages.

4.2.2 Early- and late-stage species approach

Mason *et al*. (1982, 1983) coined the term early and late stage fungi for groups of species identified based on basidiocarp production around *Betula* trees. The early and late succesional dominants could be affected by resource availability (Gibson *&* Deacon, 1990; Lilleskov *&* Bruns 2003). Early species are characterized mainly by reproduction by spores, while late species by clonal expansion. Removal of forest floor increased both the fungal species richness and abundance of fruiting bodies, but increase in fruiting body production occurred mainly in early succession fungal

species (Bigg, 2000). Large and persistent genets formed by clonal expansion in some ECM species (*Suilus* sp.*, Xerocomus* spp.) were shown to possess stress tolerant adaptive characteristics (mycelial cords or strands) that facilitate their competitive ability in mature forests (Bergemann *&* Miller, 2002), while for other species (e.g. *Russula* spp.) growth results only from mycelia radiation in multiple directions. Sarah *et al*. (2002) found the persistence of a genotype of *Russula* over an 11 years sampling period. The clonal behavior may have consequences on the colonization of new trees: disturbing the tree roots of existing plants changed the fungal species that formed mycorrhizas on roots of planted seedlings adjacent to existing plants (Bigg, 2000). In addition the disturbance at the tree root, the distribution of early and late species are influenced by the tree species, by differences in the life cycle of the tree, or by litter type. Early stage species for instance were found with *Quercus* up to 20 years, while with *Betula*, up to 6 years have been described. This apparently correlated with the life time of the tree species (Keizer *&* Arnolds, 1994). Different litter types below trees also have been found to induce different ectomycorrhizal communites to develop, linked possibly to functional differences like P cycling (Conn *&* Dighton, 2000).

Another influencing factor is the overall environment of the tree. Air pollution, e.g. can influence the nutritional status of the tree and indirectly the quantity of organic exudates available for ECM, leading to unfavorable conditions for late ECM species (Keizer *&* Arnolds, 1994). And finally, the age of the roots is a biotic determinant of ECM types. Bigg (2000) found that the youngest roots were populated with early succession species, while older parts of the root system were associated with later stage species. Thus, a habitat separation between early and late stage ECM communities are be seen in the same forest, suggesting that processes of ECM succession are either infra-ecosystem (if we accept that the forest is the ecosystem), or that the forest is an assemblage of (micro)ecosystems (if we accept that the fungal community supports a micro-ecosystem).

For lines of arguments are found which limited the concept of early and late stage mycorrhizal fungi. The first direction of criticism provides exceptions to the characteristics shortly mentioned above (1). Another criticism points out the major role of the dispersion and other biological mechanisms in regulating the communities'

structure over succession (2). A third line puts the accent on the influence of the environment on the succession mechanisms (3). An integrative approach is based on an ecological perspective (4).

(1) For instance Fiore-Donno (2001) demonstrates that in a mature forest two late stage species have contrasting colonization patterns: one by clonal growth, the other one by sexual spore propagation. Consequently, one can not expect necessarily higher genetic diversity in early-stage communities.

(2) Newton (1992) proposed a functional classification of fungi based on epidemiological, dispersion characteristics (the relative ability of different fungi to colonize and spread from different sources of inoculum) in search for more appropriate classification criteria than early and late successional. The different morphs (emanating hyphae or the presence and differentiation of rhizomorphs, mantel type, laticifers, cystidia, sclerotia, the hydrophoby) were used to classifyd ectomycorrhiza in a wide range of potential exploration groups extending from the contact to the long distance exploration types (Raidl, 1997; Agerer, 2001). Mycelia that remain non rhizomorphic are thought to reflect a limited ability to explore surrounding soil, while mycelia that comprise highly differentiated rhizomorphs are regarded as more adapted to long-distance exploration (Agerer, 2001). To some extent, Agerer's classification is relevant also for the early versus late stage classification, as one can expect to have long distance exploration types especially when the nutrients are scarce, i.e. in early stage communities. Buscot *et al*. (2000) also suggested that it should be possible to re-classify the species involved into a small number of groups biological and ecological traits were used therein, ranging from dispersal and foraging abilities to stress tolerance and nutrient mobilization and uptake. These parameters for classification rely on „ecological strategies" as described for fungi by Pugh (1980).

(3) Keizer and Arnolds (1994) studied the relationship between *Quercus* tree age and numbers of ectomycorrhizal species and sporocarps, and found that changes in species composition and diversity showed much variation correlated to different environmental conditions, and also that succession in later stages cannot be explained by root extension alone (after 30 years, the soil was entirely occupied by

fine roots). The crucial role of soil factors in the course of succession had been previously suggested (e. g. Mason *et al*.,1987).

(4) From an ecological perspective it became obvious that both environmental variables and dispersal were important factors shaping mycorrhizal communities. This stresses the importance of using a metacommunity approach when dealing with the diversity and succession processes of a certain community (Lekberg *et al*., 2007). In particular, the distance to other tree islands (Peay *et al*., 2007) resulted to be a key factor controlling EM diversity at tree islands (forest) level.

What can be retained from the early/late-stage distinction is synthesized in Tab. 11 and is well summarized by Keizer and Arnolds (1994):"The concepts of early and late stage fungi are primarily based on physiological characteristics of species and indeed are useful to unterstand early phases of primary forest succession. However, they are not appropriate to describe ectomycorrhizal succession under field conditions over a longer period since: 1) some early-stage fungi are restricted to young trees but others are maintained on the root systems of old trees; 2) some late-stage fungi appear already with young trees; 3) seedlings near mature trees may be infected by late-stage fungi; 4) late-stage fungi are dominant during some 90-95% of the lifetime of a tree and can be divided into several groups."

Table 11: **Comparison of early- and late-stage EM species characteristics.**

Species / Characteristic	**Reproduction**	**Genetic diversity**	**Requirement of C, N, P**	**Exploration types**
Early	primarily by spores	higher	small	mainly medium and long distance
Late	primarily by clonal expansion	lower	greater	mainly contact and short distance
Source	Sarah *et al*., 2002			Our hypothesis

4.2.3 Application of the ecosystem approach to fungal succession

The ecosystem concept was used to explain the high diversity of ECM communities, and the distribution and dynamic of this diversity: the high diversity of ECM was explained by referring to the concept of niche, fundamental in ecosystem theory.

Dickie (2007), for instance, points out that: “ectomycorrhizal fungi encounter a highly variable environment with myriad possible niche dimensions. Many of these niche dimensions are relatively narrow in breadth. Nonetheless, dimension breadth is relatively unimportant compared with dimension numbers (*n*), as available niche space in a community, i.e. the ‘*n* dimensional hypervolume’, increases multiplicatively with niche breadth but, exponentially with increasing dimension numbers”.

The differences in ECM diversity from one ecosystem to another in space and time were explained by correlating them to the abiotic characteristics of the ecosystem or by attempting to build an ecosystem level succession theory. This kind of work seems to have started with Christensen (1969) who investigated 36 ecosystems and used classification, ordination and regression techniques to describe the species composition of the fungal communities. During the International Biological Program, there was a vogue for comparing fungal succession on different types of litter (Frankland, 1998). For the particular case of ECM fungi, Bigg (2000) showed, that usually young stands have few, very abundant fungal species, with other species present in low to very low quantities. Over time, the community changes to more species present, but roots are still dominated by relatively few species. So species richness would increase with succession, but the evenness will remain more or less the same. Dighton and Mason (1985) had previously developed a three stages model in which species richness increases from young to medium-aged stands, then strongly decreases in old stands, to reach a very low final level (following the vegetation pattern in *Fagus* forests, for instance), apparently contradictory to Bigg’s (2000) model. Because both models reflect correct data sets, it seems that there is no unique diversity pattern in the dynamics of ECM with succession. Twieg *et al*. (2007) for instance stated explicitly that simple categories such as ‘early stage’, ‘multi stage’, and ‘late stage’ were insufficient to describe fungal species’ successional patterns and that ECM fungal succession may be best described in the context of stand development, without the need for a universal explanation theory.

From the above short overview it can be seen that, until now, the application of ecosystem concept in the study of ECM diversity patterns in space and time had more a heuristic than a quantitative explanatory value. This situation arose from the fact that the ecosystem approach seems to be applied especially in the interpretation

phase of the research programs dealing with ECM, and to a lesser extent in the design phase. This short coming can only be rescued by specific design of experiments.

The ecosystem model proposed in this thesis suggest that diversity patterns of ECMs communities in forests can be investigated at three hierarchical levels: number of TDMs, species richness inside each TDM (α), at tree (β) and at forest level (γ), and finally evenness inside each TDM.

Based on review of the literature data it can be concluded that the scale of separation in TDMs can be very different. In one example, species richness of ectomycorrhizal fungi was investigated, on tree islands of constant age and host composition that range in size from <10 to >10000 m^2 showing that ectomycorrhizal species richness is significantly reduced on smaller and more isolated tree islands, and the species–area slope that we observed (0.20–0.23) is similar to average slopes reported for macro-organisms. Species occurrence patterns across tree islands and investment trends in fungal fruit bodies suggested that a trade-off between competition and dispersal could play an important role in structuring ectomycorrhizal assemblages (Peay *et al*., 2007). Another impact on TDM separation is seen with on sampling effort. Appropriate estimation of diversity was found to be difficult task because of the large number of samples needed and the heterogenous distribution of ECM in forest floor. By constructing species area curves for data published in previous studies, Horton & Bruns (2001) demonstrated that usually insufficient samples were analyzed to have covered the diversity of ECM taxa present. Anderson & Cairney (2007) show that is necessary to take cores at least 3 m apart, in order to achieve the greatest sampling efficiency, but point out that community composition is variable at much finer scale (5-20 cm), with a complete change in ECM community composition occurring in some cases at a scale of 50 cm. In the vertical dimension, different fungi tipically occupy different horizons (Anderson *&* Cairney, 2007).

The model proposed is a way of conceptualizing the structural diversity allowing a functional interpretation. For instance, changes in microbial diversity did not always correspond to changes in functional redundancy (Yin *et al*., 2000). The reason for this is that diversity is usually characterized unstructured, at tree island level, which mixes the diversity of different TDMs. As functional redundancy of species occurs

only at infra-TDM level, an increase of overall diversity (across pooled TDMs) does not reflect functional redundancy. E.g., decrease of redundancy in one TDM coupled with an increase in another TDM (or appearance of new TDMs) would load to similar results. The approach of defining structural diversity, in contrast, allows to quantification of the role of each species in the production of ecosystem services by investigating the influence of each species on the rate of relevant processes occurring at functional group (TDM) level (Luck *et al*., 2003; Kremen *et al*., 2005). At the same time, the extent to which ECMs contribute to the resource partitioning by physiological connections between trees is shown (Egerton-Warburton *et al*., 2007).

The study could show a decreasing number of exploration types along a contamination gradient from Jenzig, to Greiz, to Kanigsberg. Especially at the former mining site Kanigsberg, the abundance of ectomycorrhiza is reduced, and the contact morphotype is missing. Beside the interpretation at ecosystem and community level, the interpretation at population/species level is a must in order to underline the mechanisms supporting the higher level patterns. In this respect we suggest the use of a cluster of species with different characteristics, such as:

Cenococcum is a versatile species of ectomycorrhizal fungi

Cenococcum geophilum is not host-specific and forms tiny, jet-black, hairy and unbranched ectomycorrhizas with a wide range of tree species in all ectomycorrhizal forests worldwide but displays additional characters that may contribute to drought tolerance, such as the accumulation of melanin and thick, microfibrillar and gelatinous cell walls (di Piedro *et al*., 2007) and this character appear to be distinct from other mechanisms involved in water supply and conservation by ectomycorrhizas and which cannot be tested on excised root tips, such as uptake and conduction by rhizomorphs (Lamhamedi *et al*., 1992).

It is possible that the *Cenococcum geophilum* at such disturbed sites is genetically highly hetorogenous. The high functional heterogeneity of *Cenococcum geophilum* is remarked by Horton *&* Bruns (2001) and high genetic diversity was proved by Jany *et al.* (2002). Recombination and genetic differentiation in the mycorrhizal fungus *Cenococcum geophilum* between two populations indicated that there was genetic differentiation (LoBuglio *&* Taylor, 2002). Such genetic variation was documented for other species in areas contaminated with metals. The genetic variation in the

population of *S. luteus* from an unpolluted site was considerably larger than that observed at a polluted site (Colpaert *et al*., 2000). With increasing distance from Zn smelters, the frequency of Zn tolerant genotypes decreases (Colpaert *et al*., 2004). Addition of small concentration of metals to isolates of *Aspergillus niger* from mine surroundings can even stimulate the production of biomass, compared to isolates from not contaminated areas (Buckova *et al*., 2007).

Tomentella as an example of a "late stage" versatile species

Tomentella sublinacina, a species characterizing Kanigsbergs' community, may be based upon slower colonization rates and greater competitive ability (Lilleskov, 2003) and it is characterized as late stage (Visser, 1995). Despite the fact that the capability to colonize roots from spores has eventually dispersed by soil invertebrates (Lileskov *&* Bruns, 2005). This may be an important detail in the context of soil application for remediation porposes. Also, *Tomentella* species are well known from other mining areas (Danielson, 1991), despite the fact that *T. sublilacina* is not the most ubiquitous of the group (Koljag *et al*., 2000). Belling *&* Abler (2004) confirms that *T. sublilacina* prefers habitats with little or no organic matter, a fact underlined also by Baar *et al*. (1999) who found viable propagules of *T. sublilacina* on mineral soils. Early stage and late stage ECM fungi were also reported (Bellei *et al.*, 1992). It is possible, however, that the same fungus could act as an 'early-stage fungus' as well as a 'late-stage fungus' depending on host species and habitat.

Pisolithus and Paxillus

Pisolithus tinctorius and *Paxillus involutus* ectomycorrhizae have been reported on various tree species and on numerous types of adverse sites, such as exhibitig high soil temperature, extreme acidity, drought, low fertility or high levels of toxic metals. *Paxillus involutus* is tolerant specially for Ni (Blaudez *et al.*, 2000). Growth of vegetative mycelium on fly ash variants and metal accumulation data indicated that *Pisolithus tinctorius* ECM-1290 was very tolerant towards many metals (Ray *et al*., 2005). Therefore, *Pisolithus* sp. shows potential for application at degraded sites because of its adaptation to ecologically diverse and adverse conditions.

Scleroderma sp.

Scleroderma sp. on *Eucalyptus globulus* with long distance exploration type

ectomycorrhizae revealed a higher growth increase, at least in a homogenized, sterile mineral soil, than some species with a medium distance, smooth exploration type. The proximal parts of hyphae and rhizomorphs are relatively hydrophobic. Therefore, the formation of rhizomorphs will shift the zone of uptake from the direct vicinity of the mycorrhizae to more remote areas (Unestam *&* Sun, 1995).

Helotiales sp. and *Hymenoscyphus* sp.

In addition, *Helotiales* sp. and *Hymenoscyphus* sp. were isolated from metal poluted areas (Vralstad *et al*., 2002). This implies *Hymenoscyphus* sp. as a fungus that might be able to tolerate higher metal concentrations.

Gebhardt *et al*. (2007) suggested that most ECM morphotypes from reclamation sites are not adapted to well-developed organic horizons, and should be present in the inorganic horizon of reference sites. However, in our case, none of the species present in the inorganic horizon at Jenzig or Greiz was present at Kanigsberg except for *Cenococcum geophilum.* The fact that most species were present in both horizons (H and A) at a site could be an argument for only one TDM corresponding to the two horizons. The fact that most of the minority species present at Jenzig are not present at Kanigsberg or Greiz cannot be attributed to incomplete sampling, because the estimators of diversity show only very limited differences between the number of species observed and estimated at the sites. A point of convergence between the results of the two studies is the larger heterogeneity in ECM distribution in the contaminated sites compared to the controls.

The richness and evenness of ECM communities at tree level in Greiz a secondary succession young forest grown on contaminated land was larger than at Kanigsberg a primary succession young forest grown on contaminated land in the upper TDM (humic horizon), but smaller in the lower TDM (inorganic horizon). Thus, the ecosystem level hypotheses of this study could be generally validated. Another interesting finding was the relationship between the diversity at TDM level and the diversity at stand level. A reversal of community evenness patterns took place between sites, with highest evenness in TDMs at Jenzig site, but lowest evenness of the overall community in Jenzig at forest scale.

From the perspective of relating diversity patterns with the metals' contamination, an interesting result is that not a single element was responsible for the pattern of ECM

distribution. We found a typical case of multiple stressors, and the ECMs probably experienced synergistic multimetal effects. Another finding is related to the heterogeneity in metals' distribution. The distribution of metals at small scale is important in controlling the rates of microbial processes in contaminated areas (e.g., Bringmark *&* Bringmark, 2001). Local heterogeneity of abiotic paramaters may favor an increased diversity by increasing the number of niches. We found a larger heterogeneity in metals' distribution both at Kanigsberg and at Greiz (with respect to different clusters of metals) than at Jenzig, and this was associated with an increased evenness in the forest, but with lower species richness. The heterogeneity in the distribution of ECM species around trees was indeed higher at the contaminated sites, and in particular in the top horizon at Kanigsberg.

From a successional perspective our results show that the distinction early versus late stage species is not meaningfully applicable in the case of contaminated areas. The diversity in successionally more advanced contaminated systems such as Greiz (secondary succession) was higher at tree level in the humic horizon than in contaminated areas under primary succession (Kanigsberg), but lower in the inorganic horizon. Overall, at site level the community was more diverse at Greiz than at Kanigsberg, but very heterogeneously distributed between trees. This indicates that the distinction between primary and secondary succession is a good basis to predict ECM development at disturbed sites over time. The ecosystem concept, as a basis for modeling ECM community structures in a forest allows development of predictive tools and can be adopted for further studies.

5 Conclusion

Mycorrhizal symbioses play fundamental roles in shaping terrestrial ecosystems and the characteristic forest plant communities that dominate the major terrestrial biomes of the world do so because selection has favoured different types of ECM association that are functionally adapted to the prevailing situation of edaphic and climatic conditions characterizing different environments. The significance of mycorrhizal fungi is that they connect the trees as primary producers of the ecosystems, to the heterogeneously distributed nutrients required for growth.

The experimental design used in this thesis took into account variables at tree, forest and ecosystem level. This enabled us to assess the possibility of model approaches that might provide a realistic evaluation of the roles played by mycorrhizae in natural communities. The ecosystem concept used in this thesis explain the high diversity of ECM communities and the distribution and dynamics of this diversity. The high diversity of ECM was explained by referring to the concept of niche, fundamental to ecosystem theory. The differences in ECM diversity between the investigated ecosystems could be explained by correlation to abiotic characteristics of the ecosystem and an ecosystem level succession theory was formulated. Our results show that the distribution and diversity of ECM was significantly correlated with specific clusters of metals. The correlation of long distance ECM type with heavy metals clusters indicate that the toxicity of heavy metals may be ameliorated by the availability of P. The absence of the contact types of ECM could be used as sensitive biological indicators to detect changes in ecosystem. It seems likely that in a primary succession on contaminated land, the particular mycorrhizal guilds may have a high constitutive metal tolerance. Selection among fungi in secundary succession is based on fungal propagules preexisting in the area bevor disturbance (acidification/heavy metal bioavailability). Here, adaptation towards metal tolerance is required. The ecosystem level hypotheses of this study could be generally validated. It is suggested that 1) classification of ECM species should be done relative to a well defined succession series; the differention early and late stage ECM fung is not helpful; 2) the heterogeneity within the ecosystem can be used as indicator for ecosystems disturbance and succession stage. Elucidating the diversity of mechanisms involved, the range of interactions with other organisms, and the ways in which these are

regulated remains the ultimate challenge in understanding the role of these fungi in biogeochemical cycles. Comparative analysis of different systems improve our understanding of responses to environmental and climatic perturbations. This new knowledge is an important prerequisite for future, sustainable management of terrestrial ecosystems. In conclusion, this study stresses the imporance of investigating the ECM at ecosystem level. As concluded also by Read (2002), two factors have contributed to show progress in analysis of ECM communities. The first is simply, that the essentially short-term structure of science funding, is not compatible with the need to investigate processes at the ecosystem level many of which, by their very nature, are long term phenomena. The second, is that progress towards understanding of ecosystem level processes requires interdisciplinary collaborations like such betwen soil scientists, microbiologists and mycologists. The investigated sites here contributed to form a more realistic picture of ECM function during primary and secondary succession in oak forests.

6 Summary

Mycorrhizal symbiosis plays an important role for forest establishment, tree growth and nutrition. Precise information is available on mycorrhizal function under experimental conditions, but little reliable information on the extent to which these functions are expressed under relevant, essentially multi-factorial circumstances of the kind that prevail in nature. The aim of this thesis was to understand the functional differences between different fungal symbionts and the reasons for changes in ECM community structure. The relationship between the diversity and exploration types of ECM was investigated in tree different *Quercus robur* forests with high heterogeneity of soil parameters (heavy metals, esential nutrients and pH) and land use (mining, forest harvesting and reforestation). Different succession stages at tree, community and ecosystem level were investigated to allow modeling of ecosystem development after disturbance.

Surface mineral extraction creates many substrates for primary succession, ideal sites to investigate the influence of metals on ECM community. ECM in primary succession was investigated at the former uranium mining heap site Kanigsberg, Thuringia, Germany, that has been covered with 30-40 cm top soil and was then planted with a mixed forest including *Quercus robur, Betula* sp.*, Fraxinus* sp. and *Larix* sp.*.* At the study site near Greiz, Eastern Thuringia, a mixed forest including oak (*Quercus robur*) and some birches (*Betula* sp.) was planted and the reforestation initiated a secundary succession. Jenzig (Jena, Germany) was used as unpolluted control site.

Two field campaigns have been performed: an extensive one for qualitative description of the fungal diversity, and an intensive one focusing on estimation of abundances around 19 selected trees. The fungal community structure was quantitatively determined in 100 samples by classification in types of exploration, direct DNA isolation and strain isolation followed by morphological identification as well as sequencing of ITS. The use of ECM typing of defined sample units as a stratifying sampling method for PCR-ITS fingerprinting was found to be a reliable and relatively cost-efficient method for ECM species identification and functional characterization. Diversity ECM indexes were highest at Jenzig, and had low, similar values at Kangisberg and Greiz. At Jenzig, the diversity was homogenous, while at

Kanigsberg and Greiz the diversity was highly heterogeneous, both between trees and around trees.

To compare metal contamination, 17 elements were determined in 23 selected soil samples after a seven step sequential extraction. The three chosen ecosystems are well individualized by metals distribution in soil and by other soil covariables. Highly correlated metals grouped in 3-4 clusters, depending on the extracted fraction. The bioavailable fraction was a good estimator explaining 89% of diversity. Kanigsberg and Greiz had a heterogeneous distribution of metals, in contrast to Jenzig with a homogenous distribution. Relations between environmental variables and genus/species abundance of ECM fungi were analyzed by means of multivariate ordination techniques. After removing the ECM variability due to P and organic matter/depth, several groups of metals could be identified as explaining most of the data variability. Clusters of Al-Cr-Fe-Pb and Cd-Mn-Zn separated well the ECM composition of Jenzig, Kanigsberg and Greiz ecosystems, with highest concentrations of metals at Greiz, leading even to trees without mycorrhiza in three samples. Clusters of Cs-Cu and Co-Ni-U separated Greiz from Kanigsberg, with highest metals concentrations in Kanigsberg. Further heterogeneity of ECM distribution at Kanigsberg (and in particular the presence of *Tomentella sublilacina*) was correlated with high concentrations of As, coupled to relatively lower concentrations of other metals and P in soil.

In primary succession, particular mycorrhizal guilds may be found, with occurrence of dark ascomycetes. In the uranium mining area, we isolated *Cenococcum geophilum*, *Hymenoscyphus ericae* and *Helotiales* sp.. *Cenococcum geophilum* is a ubiquitous species while the frequent occurrence of a *Hymenoschyphus ericae* and *Helotiales*, which was rare or absent from the controle area, is remarkable. Through our study, we complement the information on belowground ECM in a primary succession established in a mining area. Apart from the dark, septate ascomycetous fungi, we isolated basidiomycetous fungi like *T. sublinacina*, *Pisolithus tinctorius*, *Paxillus involutus* and two strains of *Scleroderma* sp (*Scleroderma areolatum* and *Scleroderma* sp.). *Tomentella sublinacina* was reported both as early- and late-stage fungus. It is possible, however, that the same fungus could act as an ‘early-stage fungus’ as well as a ‘late-stage fungus’ depending on host species and habitat. The

identified basidiomycetous fungi at Kanigsberg belong to the long exploration type of mycorrhiza, compared with Jenzig where the 11 fungal types covered all proposed exploration types.

The colonization of ECM is completely different in secondary succession where tree seedlings rapidly recruit ECM fungi, more often specialists, from dormant spore banks or other resistant propagules. Seven frequent ECM types were identified: *Laccaria amethystina*, *Russula ochroleuca*, *Lactarius quietus*, *Cenococcum geophilum*, *Paxillus involutus*, *Pisolithus tinctorius* and brown rhizomorphs of unknown fungal origin. The long distance exploration type seems to be selected over time, indicating that the toxicity of heavy metals may be ameliorated by the availability of P. The absence of contact types of ECM could be used as sensitive biological indicators to detect changes in ecosystems.

The diversity and evenness of ECM communities in both sampled horizons of the uncontaminated site was larger than in the contaminated forests when computed at tree level, but the evenness was lowest in the uncontaminated site when computed at forest level. The richness and evenness of ECM communities at tree level in Greiz was larger than at Kanigsberg in the upper TDM. The distribution and diversity of ECM was significantly correlated with clusters of metals.

The ecosystem concept was used to explain the high diversity of ECM communities and the distribution and dynamics of this diversity. The high diversity of ECM was explained by referring to the concept of niche. The differences in ECM diversity from one ecosystem to another in space and time were explained by correlation to abiotic characteristics of the ecosystem. This leads to an ecosystem level succession theory.

7 Zusammenfassung

Die Mykorrhiza spielt eine wesentliche Rolle in der Überlebensstrategie verschiedener Pflanzen. Obwohl es aufschlussreiche Informationen über die Funktion von ECM an unbelasteten Standorten gibt, exisitieren nur wenige Informationen darüber, wie diese unter relevanten, essentiell multifaktoriellen Umständen in belasteten Gebieten beschaffen sind. Das Ziel dieser Arbeit war es, funktionelle Unterschiede zwischen verschiedenen Mykorrhizapilzen und die Gründe für die Veränderungen der ECM bezüglich der Bodenparameter zu analysieren und darzustellen. Der Zusammenhang zwischen der Diversität und den Ausbreitungsstrategien wurde in drei verschiedenen *Quercus robur*-Wäldern mit hoher Heterogenität der Boden-Parameter (Schwermetalle, Nährstoffe und pH) und der Landnutzung (Uran- Bergbau, Waldrodung und Aufforstung) untersucht. Die drei gewählten Standorte befinden sich in verschiedenen Sukzessionsstadien. Um ein Ökosystem-Modell entwerfen zu können, wurden die Baum-, Stand- und Waldebenen untersucht.

Das ehemalige Uranbergbaugebiet bei Kanigsberg in Thüringen, Deutschland, wurde als belastetes Gebiet untersucht. Es ist ein idealer Standort für die Untersuchung der Ektomykorrhiza in Abhängigkeit von Schwermetallen in einem primärem Sukzessionstadium. Die untersuchte Fläche wurde durch die Wismut GmbH saniert und mit einem Mischwald aus *Quercus robur, Betula* sp., *Fraxinus* sp. und *Larix* sp. aufgeforstet. Am Standort Greiz (Forst, Ost-Thüringen) wurde das Gebiet nach der Waldrodung mit Mischwald aufgeforstet, sodass hier eine sekundäre Sukzession ausgelöst wurde. Als unbelastetes Waldgebiet wurde das Areal um den Jenzig bei Jena, untersucht.

Zur Untersuchung des Bodens und der ECM-Pilze wurden Bodenproben in zwei verschiedenen Strategien entnommen: eine extensive Probenentnahme wurde zur qualitativen Beschreibung der ECM-Pilze durchgeführt, während eine intensive Probenentnahme um 19 ausgewählte Bäume zur Auswertung der Abundanz angewendet wurde. Um detaillierte Aussagen über die Diversität der Ektomykorrhiza machen zu können, wurden morphologische Analysen der Ektomykorrhiza-Kurzwurzeln sowie eine molekularbiologische Bestimmung der Pilzheterogenität von 100 Bodenproben durchgeführt. Es zeigte sich, dass die ECM-Ausbreitungsstrategie

eine zuverlässige und kostengünstige Methode darstellt, um ECM-Pilze auf einer funktionellen Basis zu charakterisieren.

Nur im Untersuchungsgebiet Jenzig wurde im Gegensatz zu Kanigsberg und Greiz eine hohe ECM-Diversität gefunden. Das Untersuchungsgebiet Jenzig wies eine homogene Diversität auf. In Kanigsberg und Greiz war Sie heterogen. Die Untersuchungen wurden zwischen und im Umkreis der Bäume durchgeführt.

Zur Bestimmung der Schwermetallbelastung wurden 17 Elemente aus 23 ausgewählten Bodenproben mit Hilfe der sequenziellen Extraktion gemessen. Die drei ausgewählten Ökosysteme unterschieden sich im Schwermetallgehalt und in weiteren Bodenparametern. Durch statistische Verfahren konnten stark korrellierte Schwermetalle in 3-4 Cluster zusammengefasst werden. Es konnte gezeigt werden, dass die bioverfügbare Fraktion 89% der Diversität erklärt. Dabei zeigten wieder Kanigsberg und Greiz eine heterogene Verteilung der Schwermetalle. Durch multivariate Statistiken konnten Aussagen über die Anpassungen an abiotische Stressfaktoren, wie die Schwermetallbelastung gewonnen werden. Nach Abzug der ECM-Variabilität durch P und C org konnten einige Schwermetalle, zusammengefaßt in 3-4 Gruppen, die Variabilität größtenteils erklären. Die Gruppen Al-Cr-Fe-Pb und Cd-Mn-Zn zeigten deutlich unterschiedliche die ECM in den drei Ökosystemen Jenzig, Kanigsberg und Greiz, wobei in Greiz die höchsten Konzentrationen der Schwermetalle vorlagen. Besonders hohe Belastungen wurden an Bäumen ohne Mykorrhizen gefunden. Der höhere Gehalt der Schwermetalle Cs-Cu und Co-Ni-U grenzen das Ökosystem Kanigsberg vom Ökosystem Greiz ab, mit einem höheren Gehalt dieser Schwermetalle in Kanigsberg. Des Weiteren korrelierte die heterogene ECM-Verteilung am Kanigsberg (z.B. die Anwesenheit von *Tomentella sublilacina*) mit den höheren As- und geringere P-sowie anderen Schwermetallkonzentrationen.

Die primäre Sukzession wird vorrangig durch Ascomyceten-ECM bestimmt. Aus Proben des Uranbergbaugebiet es wurden *Cenococcum geophilum*, *Hymenoscyphus ericae* und *Helotiales* sp. isoliert. Obwohl *Cenococcum geophilum* eine weltweit häufigere Art ist, werden *Hymenoscyphus ericae* und *Helotiales* sp. ebenfalls oft nachgewiesen. In unbelasteten Gebieten fehlen sie jedoch oder kommen sehr selten vor. Außer Ascomycetenpilzen wurden Basydiomyceten wie beispielsweise *Tomentella sublinacina*, *Pisolithus tinctorius* und *Paxillus involutus*

sowie zwei Stämme von *Scleroderma* sp. (*Scleroderma verrucosum* und *Scleroderma* sp.) isoliert. Die identifizierten Basidiomyceten am Kanigsberg zeichnen sich durch eine Ausbreitungsstrategie aus, die große Distanzen überwinden kann, indem die glatten Ektomykorrhizakurzwurzeln mit wenigen, aber hoch differenzierten Rhizomorphen verbunden sind. Dagegen wurden im unbelasteten Ökosystem Jenzig alle Ausbreitungsstrategien vorgefunden.

Die ECM-Besiedlung verläuft in der sekundären Sukzession anders, weil die Bäume ECM-Pilzen und deren Überdauerungsformen aus der Umgebung rekrutieren können. Sieben häufige ECM wurden identifiziert: *Laccaria amethystina*, *Russula ochroleuca*, *Lactarius quietus*, *Cenococcum geophilum*, *Paxillus involutus*, *Pisolithus tinctorius* und braune Rhizomorphen unbestimmter Zugehörigkeit. Die Ausbreitungsstrategie über große Distanzen überwiegt mit der Zeit, sodass die Schwermetallbelastung über P-Lieferbarkeit verbessert werden kann. Es scheint, dass die ECM-Pilze im Ökosystem Greiz eine Schwermetallanpassung benötigen. Die Abwesenheit der Pilze mit Kontaktausbreitung könnte als sensitiver biologischer Indikator benutzt werden, um Änderungen im Ökosystemen nachzuweisen.

Die Diverstät und die Äquität der ECM-Pilze in beiden Horizonten des unbelasteten Gebietes war höher als in den belasteten Gebieten, wenn Populationen an Bäumen direkt betrachtet wurden. Jedoch war die Äquität im unbelasteten Gebiet geringer, wenn der Wald betrachtet wurde. Der Reichtum und die Äquität der ECM-Gemeinschaften auf Baumebene im Ökosystem Greiz war größer als im Ökosystem Kanigsberg in der oberen TDM, beispielsweise im H-Horizont. Die Ausbreitung und die Diversität der ECM korrelierten im Wesentlichen mit den Schwermetallgruppen.
Das Ökosystem-Konzept wurde benutzt, um die höhere Diversität der ECM-Gemeinschaften, die Ausbreitung und Dynamik dieser Diversität zu erklären. Die höhere Diversität der ECM wurde durch das Nischenkonzept erklärt. Die Unterschiede zwischen der ECM-Diversität eines Ökosystems in Raum und Zeit wurde mit Hilfe der Korrelation abiotischer Faktoren des Ökosystems dargestellt. Das konnte zu einer Ökosystemtheorie zusammengeführt werden.

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10 Eigenständigkeitserklärung

Ich erkläre, dass ich die Dissertation “ IDENTIFICATION AND CHARACTERIZATION OF *QUERCUS ROBUR* ECTOMYCORRHIZA IN RELATION TO HEAVY METAL CONTAMINATION” selbständig und nur mit der darin angegebenen Hilfe verfasst habe. Die Dissertation wurde in keiner anderen Fakultät oder Universität eingereicht.

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Felicia Gherghel

11 Curriculum vitae

PERSONAL DATA:	**Felicia Gherghel**
Nationality	Romanian
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EDUCATION:

2004-Present	Ph.D. student at the Friedrich Schiller University, Jena, Germany, Institute of Microbiology, in the Professor Erika Kothe's group; associate of the International Max Plank Research School (IMPRS), Institute for Chemical Ecology: The Exploration of Ecological Interactions with Molecular and Chemical Techniques
2002-2004	Research associate at Al. I. Cuza University, Iasi, Romania, Department of Botany, in Professor C. Toma's group
2001-2002	M.Sc. student at Al. I. Cuza University, Iasi, Romania, research in the field of ecological conservation
2000-2001	Socrates Erasmus exchange program at Friedrich Schiller University in Jena, Germany
1997-2001	Diploma in Biology: *The structure, function, diversity and evolution of biological systems* at Al. I. Cuza University, Iasi, Romania
1993-1997	Roman-Voda Senior High School, Division of Chemistry and Biology, Roman, Romania
1986-1993	Primary school, Harghita, Romania

HONORS AND EMPLOYMENT:

2008	Employed at the Friedrich-Schiller-University, Jena, Institute of Microbiology
2004-09	Doctoral fellowship, F.G. from the state of Thuringia, Germany and DFG-Gk1257. Work on *Identification and Characterization of* ***Quercus robur*** *ectomycorrhiza in dependence of relation to heavy metal contamination*. Supervisor Professor Erika Kothe
2004-04	Postgraduate scholarship, issued by the Romanian Government for study at the Friedrich-Schiller-University, Jena, Institute of Microbiology

TEACHING:

Recurrent	Training/supervision of M.Sc. students and international student interns under the German Academic Exchange Service DAAD andthe International Association for the Exchange of Students for Technical Experience IAESTE student trainee exchange program
2007	Supervision of the Diploma thesis German equivalent of a Masters thesis of Steffi Formann: “Diversity of Ectomycorrhizal Fungi at Jenzig”, Jena; August, 2007
2005-03	Supervision of 2 weeks of Mycorrhiza practical courses at the Institute for Microbiology, Microbial Phytopathology, Friedrich Schiller University, Jena

RESPONSIBILITIES:

2007-Present	Scientific Coach of Project OPSIS, Optimization of Professional Support for International Students, Supervision of International Students, International Office, Friedrich Schiller University, Jena
2005-2008	Representative of students at the Max Plank Institute for Chemical Ecology International Max Plank Research School IMPRS, Jena, Germany

PROJECTS:

2003	*The morphology and anatomy of ectomycorrhiza of **Fagus sylvatica** in dependence on environmental stress factors*. CNSIS nr. 40213/4.11.2003. Romania. Supervisor Professor C. Toma.

LANGUAGE: English , German and Romanian

12 Publications

Dimkpa C., **Gherghel F.,** Haferburg G., Reinicke M., Schindler F., Schlunk I., Schmidt A., Schütze E., Zeggel L., Merten D., Büchel G., Kothe E., 2008. *The effect of acid mine drainage on soil microbiology*. Jabalpur Reviews, Behl RK ed. In press.

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