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**The effect of forest tree species change on decomposer
diversity :A case study of the Breuil research site
(Burgundy, France)**

Ariana Kulhánková Kubartová

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Ecole Doctorale : RP2E

Department of Botany, Faculty of Sciences, Charles University, Prague
Faculty of Sciences, Henri Poincaré University, Nancy
(co-tutelle)

**The effect of forest tree species change on
decomposer diversity :
A case study of the Breuil research site
(Burgundy, France)**

**Effet des essences forestières sur la diversité des
décomposeurs : étude du site atelier de Breuil
(Bourgogne, France)**

Ariana Kubartová
(Kulhánková)

Thesis committee (Jury)

Tomas HERBEN	Professor Charles University (examineur, President)
Monique CARNOL	Professor Liège University (B) (rapporteur)
Hana SANTRUCKOVÁ	Professor South Bohemia University (examineur)
Károly MÁRIALIGETI	Professor University Budapest (rapporteur)
Jacques RANGER	Directeur de recherche INRA (examineur)
Jacques BERTHELIN	Directeur de Recherche CNRS (examineur)

Doctoral thesis

November 23rd 2007

University Charles - PRAGUE



Laboratoire des Interactions Microorganismes,
Minéraux, Matière Organique dans les Sols UMR 7137
BP 239 - 54506 Vandoeuvre-les-Nancy



UR 1138
Biogéochimie des Ecosystèmes Forestiers
54280 Champenoux

Preface

The thesis was presented under the form of published or submitted or to be submitted papers. A first part (pages 5 to 31) is a large synthesis of the whole thesis work with an introduction and a conclusion. Then the chapters are constituted by or made of the different papers.

Extended abstract

Kubartová A., 2007: The effect of forest tree species change on decomposer diversity: A case study of the Breuil research site (Burgundy, France). Doctoral thesis

Introduction and objectives: This thesis summarizes the results of studies regarding microbial colonization of organic substrates (cellulose, wood, tree litters) *in situ* in forest ecosystems, and the relation of microbial communities to the decomposition rate of the substrates. The main objectives were to determine the patterns mainly of fungal (and bacterial in selected samples) colonization of the studied substrates during *in situ* decomposition in different forest stands, to detect the role of environmental conditions and to identify the link between the decomposer diversity and decomposition rate of the substrates. Microbial diversity was determined in the samples by TGGE of amplified ITS (or 16S) rDNA fragments.

Results and conclusions: Substitution of tree species in the research site caused changes in the composition of fungal and bacterial community compositions for all studied substrates. Similarly, the communities were changing during the incubation period. Any significant decrease of species richness was detected for any substrate or tree species. Most of the decomposer species were detected in all tree species and in all the substrates. Nevertheless, their distribution was affected by the vegetation cover, the substrate and its stage of decomposition. The decomposition was the fastest in young broadleaved plantations, then in the native forest and was the slowest in conifers. Leaf-needle mixed litters were found to sustain fungal diversity and to increase the decomposition rate. Overall, the differences in the decomposition rate did not seem to be influenced by a species loss, but rather by a change in the microclimatic and nutrient conditions of the tree stands. Modification in the environmental conditions and seasonality were important for the development of the

microorganism communities. During summer months, species richness tended to decrease and community composition changed, as documented both for wood and cellulose samples. Common species were replaced by infrequent ones, able to tolerate dry and hot summer conditions. The results underlined an effect of fungal biomass and cellulase activity on decomposition rate and indicated that the variables reached their highest values during the summer months, despite lower species richness and presence of infrequent species. Hence, the biotic interactions and/or actual species activity might be more relevant for decomposition rate than the community structure. Further, it was shown that the results were partly influenced by the methodological approach used, i.e. when comparing TGGE and T-RFLP technique in wood decomposition experiment. In the future, it would be interesting to compare functional gene expression and variability or enzymatic activity. The diversity of other functional groups might be also targeted.

Keywords: decomposers, biodiversity, temperate forests, litter, molecular techniques, decomposition rate

Résumé étendu

Kubartová A. 2007: Effet des changements de peuplements forestiers sur la diversité des décomposeurs microbiens : Etude du site de Breuil (Bourgogne, France)

Introduction et Objectifs: Ce travail de thèse présente les résultats d'études concernant la colonisation microbienne de substrats organiques (cellulose, bois, litières forestières) placés in situ, sous les humus de sols de divers écosystèmes forestiers et les relations entre les communautés microbiennes et la décomposition de ces substrats organiques. Les objectifs principaux visaient à (1) déterminer des schémas ou diagrammes de la colonisation fongique (mais aussi bactérienne pour certains échantillons sélectionnés) de substrats organiques placés in situ dans différents peuplements forestiers localisés sur un même site (2) définir le rôle de paramètres environnementaux associés au changement d'espèces forestières (3) identifier les relations entre la diversité des décomposeurs et leur activité caractérisée essentiellement par la vitesse de décomposition et éventuellement d'autres variables (e.g. biomasse fongique..) pour des échantillons particuliers (cas de la cellulose).

Principaux résultats et conclusions: La substitution de la forêt native (hêtre et chêne) par diverses espèces forestières, hêtre, chêne, épicéa, Douglas, entraîne des changements dans la composition des communautés bactériennes et fongiques pour tous les substrats étudiés. Les communautés changent aussi au cours de la période d'incubation in situ. Aucune diminution significative de la richesse en espèces microbiennes n'est observée tant pour les substrats que pour les essences forestières. Toutefois leur distribution est modifiée sous l'influence de ces mêmes paramètres ainsi que par le stade de décomposition des substrats . La décomposition est la plus rapide dans les jeunes plantations de feuillus puis dans la forêt native et la plus lente pour les résineux comme l'épicéa. Les litières mixtes e.g. chêne – épicéa favorise la diversité fongique et le taux de décomposition. La vitesse de décomposition n'est pas influencée par la perte d'espèces microbiennes, mais plutôt par les modifications de conditions microclimatiques et nutritionnelles de la plantation, au niveau de la parcelle considérée. Pendant l'été la richesse en espèces tend à diminuer et la composition de la communauté à changer comme on l'observe avec la décomposition du bois et de la cellulose. Les espèces communes sont remplacées par des espèces moins fréquentes qui toléreraient mieux l'accroissement de température et la sécheresse. Les résultats soulignent aussi une relation entre la biomasse fongique, l'activité cellulosique et la vitesse de décomposition. Ils indiquent que ces paramètres atteignent leur valeurs les plus élevées pendant l'été, alors que la richesse en espèces est la plus faible et la présence d'espèces inhabituelles plus fréquente. Les interactions biotiques –abiotiques et / ou l'activité des espèces sont des paramètres plus importants que la structure de la communauté microbienne. Ces résultats peuvent être sensiblement influencés par l'approche méthodologique utilisée comme le suggère la comparaison des méthodes TGGE et T- RFLP dans une étude de la décomposition du bois. Pour l'avenir , il serait intéressant de comparer l'expression de gènes d'activité et la variabilité des activités enzymatiques . La connaissance de la diversité de divers groupes fonctionnels pourrait aussi être un objectif à développer. La hiérarchisation des paramètres abiotiques serait à préciser ainsi que la présence d'espèces indicatrices. .

Mots clés: décomposeurs, biodiversité, forêts tempérées, litières, techniques moléculaires, vitesse de décomposition, biodégradation.

Author's address: Ariana Kubartová, Department of botany, Faculty of Sciences, Charles University, Benátská 2, Prague 2, 128 01, Czech Republic

E-mail: arikul@centrum.cz

The PhD thesis was prepared in cooperation among (co-tutelle):

Department of botany, Faculty of Sciences, Charles University, Benátská 2, Prague 2, 128 01, Czech Republic

&

LIMOS - Laboratoire des Interactions Microorganismes - Minéraux - Matière Organique dans les Sols, CNRS UHP UMR 7137, Université Henri Poincaré, Vandoeuvre-les-Nancy, France

Supervisor: Prof. Jacques Berthelin

&

Unité Biogéochimie des Écosystèmes Forestiers, INRA, Centre de Nancy, Champenoux, France

Supervisor: Dr. Jacques Ranger, Directeur de Recherches à l'INRA

&

Department of Ecology and Hydrobiology, Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, České Budějovice, 370 05, Czech Republic

Supervisor: Prof. Hana Šantrůčková, CSc.

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List of papers

The present thesis is based on following papers, which will be referred to by their Roman numerals:

Papers I – V

- I. Microbial diversity during cellulose decomposition in different forest stands: I. Microbial communities and environmental conditions; Kubartova A, Moukoumi J, Beguiristain T, Ranger J, Berthelin J; *Microbial Ecology*, 54: 393-405
- II. Microbial diversity during cellulose decomposition in different forest stands: II. Functioning and role of diversity; Kubartova A, Moukoumi J, Munier-Lamy C, Ranger J, Berthelin J, Beguiristain T. (submitted)
- III. Spatial and temporal diversity of wood decomposer communities in different forest stands, determined by ITS rDNA targeted TGGE; Kulhankova A, Beguiristain T, Moukoumi J, Berthelin J, Ranger J; *Annals of Forest Science*, 63: 547-556, 2006.
- IV. Link of fungal diversity and wood decay in temperate forests; Kubartová A, Székely A, Beguiristain T, Moukoumi J, Ranger J. (to be submitted)
- V. Diversity and decomposing ability of saprophytic fungi from temperate forest litters; Kubartová A, Moukoumi J, Ranger J, Berthelin J, Beguiristain T. (to be submitted)

Paper not included in the thesis:

Competition of *Scleroconidioma sphagnicola* with fungi decomposing spruce litter needles; Koukol O, Mrnka L, Kulhánková A, Vosátka M; *Canadian Journal of Botany*, 84: 469-476, 2006.

Extended synthesis of the thesis manuscript

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1 Introduction

Temperate forests in Europe have been affected by human activities for centuries; even-aged plantations and coniferous species were often favoured (Spiecker 2003). Such large changes in forest structure affect the whole ecosystem, including decomposition, through influencing i) initial litter quality, notably the concentration of nutrients and recalcitrant organic compounds like lignin and tannins, and ii) the microenvironment in which litter decomposition takes place (Augusto *et al.* 2002). These can in consequence affect the abundance, composition and activity of the decomposer communities. By return, decomposing microorganisms, have via decomposition impact on other ecosystem functions such as nutrient cycling, primary productivity, carbon storage, organic matter accumulation and humification or even resistance to perturbations and resilience after disturbances. Nevertheless, the influence of above plant diversity on belowground microbial community and its role in ecosystem processes are still largely unexplored (Deacon *et al.* 2006).

Fungal decomposition of complex organic substrates is a good example of niche complementarity (Setälä & McLean 2004). Fungi replace one another as their community dynamic alters in space and time; each species is adapted for occupation of various niches, including use of particular substrates. The general features of fungal succession on dead plant material involve associations of weak pathogens during and after the litter fall, followed by primary saprotrophs and later by decomposers of more recalcitrant substrates (Adl 2003). Although most substrates can be decomposed by many fungal species, the enzyme production and thus decomposing ability of each species varies depending on environmental conditions, substrate availability and also on interactions with other fungi, bacteria and fauna.

Higher niche variability in all-aged, species-heterogeneous forests is believed to increase microbial richness and activity due to diversity of carbon sources in more variable litter and root exudates, higher primary production, variable microclimate, presence of dead wood or spatial and age variability. Higher microbial richness is expected i) to reduce the spatio-temporal variation of the activity of the functional community ii) to mitigate the risk of functional losses following extreme environmental conditions, and thereby iii) to increase the average rates of relating ecological processes (Ekschmitt *et al.* 2001). On the other hand, many decomposer species are reported to be functionally redundant (Setälä & McLean 2004) and thus can be lost with a little effect on the structure and functioning of the whole community (Deacon *et al.* 2006). An overlap in potential functions also suggests potential capacity for inter-specific competition, when the effect of diversity can be weak due to

saturation at low species richness (Cox *et al.* 2001; Setälä & McLean 2004) or even negative due to over-saturation and enhanced competition (Wardle & van der Putten 2003).

Traditional soil microbiological methods determining community structure provided information only on the fraction of cultivable bacteria and fungi. They were time consuming and did not allow to analyze large part of the communities. The expansion of molecular fingerprinting methods during the last decade facilitated the detection and identification or at least discovery of microorganisms and enabled identification of uncultivable microorganisms, analyses of bulk soil sample and comparison of numerous samples (Leckie 2005). However, the spatial variability and temporal dynamics of microbial communities in complex environment are not easy to describe. Similarly, behavior of a simple community in microcosm might not reflect the behavior in a natural environment. For that reason, experiments have been done with incubation of model-substrates, i.e. cellulose and wood, as well as tree-litter (Moukouri 2006) in order to compare the colonization and decomposition of the simple and complex organic compounds in natural conditions. Such incubations were performed in even-aged monoculture plantations in order to identify the effects of a particular tree species, as well as in a native forest, characteristic by variability of tree species, age and structure.

The present thesis deals mainly with fungal colonization of organic materials and the links to decomposition rate in forest ecosystems. Three different types of substrates were incubated *in situ*: i) cellulose and ii) wood ('lignin'), as the model materials and main components of plant litter; and iii) natural tree litter. These substrates were colonized and decomposed in broadleaved and coniferous plantations and in a native forest as a reference plot. Molecular tools were used to describe the decomposer communities in order to answer the following aims.

1.1 Objectives

The overall aim of this study was to expand the current knowledge on factors controlling the microbial (mainly fungal) colonization of organic materials decomposing in temperate forest sites. The second main task was to link the microbial diversity to decomposition rate of the colonized substrates in order to identify the consequences of tree species substitution to forest ecosystem processes.

Specific tasks were:

- a) to improve the method of ITS1F and ITS2 targeted TGGE and other methods used
- b) to determine the patterns of fungal ingrowths in decomposing cellulose strips, wood samples and tree litters in several forest tree stands in order to detect effect of the vegetation cover and the stage of decomposition
- c) to identify the relationship between decomposer species richness and diversity with their decomposing rate in order to approach their activity
- d) to detect the role of environmental conditions or seasonality
- e) to study bacterial diversity in selected samples
- f) to compare two fingerprinting methods, TGGE and T-RFLP
- g) to define the effect of changing tree species versus the native forest

The thesis is based on the five papers listed in the List of papers. Each of the papers has more detailed objectives inside.

2 State of the art

2.1 Decomposition

2.1.1 General features

Decomposition of plant litter is a key process in recycling of nutrients and formation of humus in forest ecosystems. It involves a complex set of processes including chemical, physical and biological parameters acting upon a wide variety of organic substrates that are themselves constantly changing (Berg 2000; Berg & McLaugherty 2003).

The plant litter consists of varying amounts of several major classes of organic compounds. The relative proportions of these compounds vary with plant part, age, growing conditions and between species. The major groups can be classified according to their chemical structure and properties, functional groups molecular weight and solubility, and primary constituents. Soluble and very soluble compounds, sugars, low molecular weight phenolics and some N compounds are mostly lost in the beginning, during first days of decomposition. Larger macromolecules, including cellulose, hemicelluloses and lignin, are degraded more slowly, in time scale of months and years. During decay, condensation of phenolics and lignin degradation products together with other constituents, results in the net accumulation of newly formed, so called recalcitrant, substances, i.e. humic substances. The final product of microbial decay of the litter is humus, which becomes slowly mixed with the mineral soil in the A horizon. Humus is a complex mixture of polymeric phenolic residues, mainly derived from plants, combined with carbohydrates and heterogeneous materials of plant, animal and microbial origin, with high number recalcitrant molecules (Berg & McLaugherty 2003). The rate of further decay is slow, and humus can accumulate as an H layer.

The dominant decomposers in soils, including temperate forest ecosystems, are the microorganisms, encompassing both fungi and bacteria. Both these main groups can degrade cellulose, hemicellulose and the different lignins. Still, the fungi are generally considered the most important group. Their biomass is usually two fold higher than bacterial biomass, they are generally more acid tolerant and complete degradation of lignin appears to be carried out mainly by the fungi (Virzo De Santo *et al.* 2002). Microbial succession on plant debris follows continual change of plant debris quality, together with interaction of the organisms themselves. The process typically begins with colonization by bacteria, Ascomycetes, and imperfect fungi (Deuteromycetes) that consume the less recalcitrant components. The cellulose present in non-lignified tissues can be also attacked by some of these organisms. Subsequently, the remaining lignified litter is colonized mainly by Basidiomycetes that degrade ligno-cellulose complexes and/or aromatic rings. The rest becomes part of the soil organic matter (Cadish & Giller 1997).

2.1.2 Decomposition dynamics and environmental conditions

The decomposition rate may decrease from ca. 10^{-1} per day in fresh litter to ca. 10^{-6} per day or lower in more decomposed material (Berg 2000), following a negative exponential curve for every substrate. The retardation may be so strong that the decomposition can be estimated to reach a limit value for total mass loss. This is due to changes in organic matter quality as the recalcitrant components become enriched, together with the succession of microorganisms able to compete for the substrate with a given chemical composition. In the early stage, decomposition of soluble, unshielded cellulose and hemicelluloses takes place,

being influenced by climate and concentrations of major nutrients, such as N or P. In a later phase the decay is ruled by the lignin concentration, while the influence of climate gradually decreases to nothing (Berg & McClaugherty 2003). Physical protection, limiting accessibility, is also of major importance. In addition to the succession in microbial community that occurs along with decay, there are also seasonal changes reflecting mainly temperature, moisture and litter input quality.

The most important environmental factors, influencing microorganisms and the decomposition in earlier stages, involve moisture and temperature, soil type and structure, pH, aeration and redox potential, interacting together. The soil water content affects not only the moisture available to microorganisms but also the aeration status, the nature and amount of soluble materials, the osmotic pressure and pH of the solution. Temperature affects the physiological reaction rates of cells as well as most of the physicochemical characteristics of the environment (Paul & Clark 1996). Other important parameters are the quality and quantity of the litter (Berg & McClaugherty 2003). All plant litter contains essential nutrients, but their concentration varies with the litter species. Once decomposition and microbial ingrowths have started, distribution and concentration of nutrients changes, becoming highly different from the fallen litter (Berg & McClaugherty 2003).

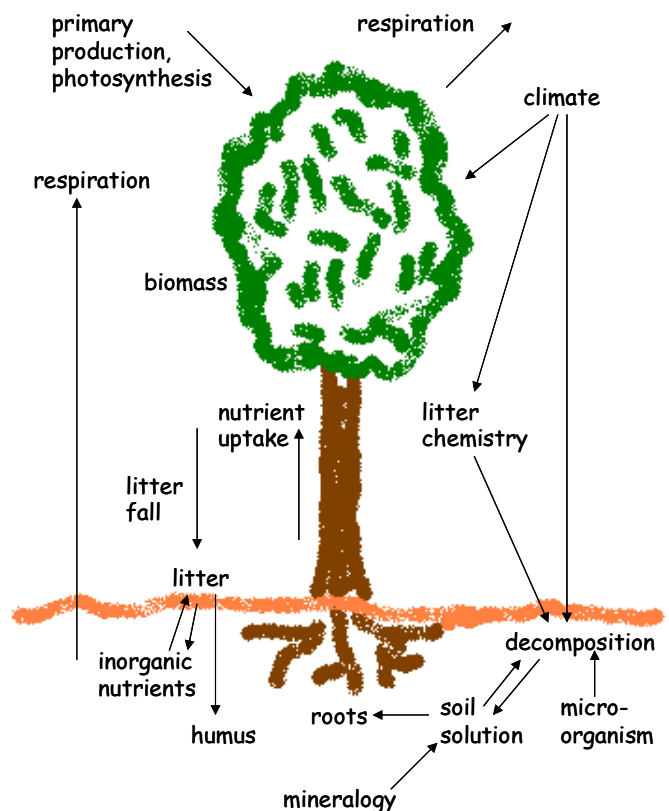


Fig. 1. Generalized schema of the decomposition and related processes in forests

2.1.3 Degradation of the main groups of compounds in litter

Plant litter material is composed of soluble and insoluble organic compounds. There are four principal groups of **soluble organic materials** in litter: sugars, low weight phenolics and aliphatic hydrocarbons, N compounds and glycerides. Most of them are readily lost from litter by dissolution and leaching combined with the consumption by rapidly growing microorganisms. The phenolics are highly variable in their solubility and many of them

condense into less soluble forms or react with larger molecules. N, P and other nutrients are mostly in organic forms and small part is soluble and easily available for biodegradation (Paul & Clark 1996).

Cellulose is the most abundant, insoluble organic compound on the planet and therefore represents a huge source of energy for microorganisms. It is made up of glucose units connected with β -1-4 bonds forming long chains of organic molecules organized into fibers and can constitute 10-50% of litter mass. Its decomposition may take place under both aerobic and anaerobic conditions. The ability to degrade cellulose aerobically is widespread among fungi and is especially well presented among the Asco- and Basidiomycota. It is also known for several soil bacteria species in both filamentous (e.g. *Streptomyces*, *Micromonospora*) and non-filamentous (e.g. *Bacillus*, *Cellulomonas*) genera. Aerobic cellulolytic fungi and bacteria produce freely diffusible extracellular cellulase enzyme systems of endoglucanases, exoglucanases and β -glucosidases that act synergically in the conversion of cellulose to glucose (Paul & Clark 1996). It is assumed that by far the most cellulose is degraded by fungi (Boer *et al.* 2005). This is probably due to the chemical nature of cellulose, which is embedded in a matrix of other structural polymers, in particular hemicelluloses and lignin, and has crystalline structure in cellular walls resistant to enzymatic hydrolysis. The hyphal growth of fungi and cellulolytic Actinomycetes appears to allow a better access the cellulose fibers. Non-filamentous bacteria seem to be confined to easily accessible cellulose, due to their restricted ability to penetrate solids. Many other soil bacteria appear to have incomplete cellulolytic system, c.f. (Boer *et al.* 2005).

Hemicelluloses, the second most abundant insoluble polysaccharide in nature, have heterogeneous composition of various sugar units. They are usually classified according to the main sugar residues in the backbone of the polymer. They are non-fibrillar in organization and are an integral part of all plant cell walls, linked to cellulose by weak hydrogen bonding. The hydrolysis of hemicelluloses requires more complex enzyme systems than are needed for cellulose; it occurs by the concerted action of exo-, endo- and ancillary-enzymes (Aro *et al.* 2005). Fungi do not degrade hemicelluloses equally, due to their chemical diversity. Common fungi may degrade xylan more actively than other hemicelluloses (e.g. pectin, carboxymethylcellulose), but all wood-rotting Basidiomycetes and many Ascomycetes or imperfect fungi degrade a wider variety of hemicelluloses (Dix & Webster 1995).

The structure of **lignin** is based on the phenyl propanoid unit, which consist in an aromatic ring and a three-carbon side chain. It is not formed by a specific enzyme but in chemical polycondensation involving phenols and free radicals and therefore does not show a specific order. It encrusts the cellulose and hemicellulose matrix to create lignocellulitic complex. It remains relatively intact for a long time, when more easily degradable compounds are decomposed so that its concentration increases along the time. A portion of lignin entering the soil does not undergo complete decomposition but reacts with microbial products in the formation of soil organic matter (Paul & Clark 1996). Despite the widespread distribution of lignin (15-40% litter mass) and its early appearance in terrestrial live, its decomposition is restricted to relatively small spectrum of microorganisms. Lignin decomposition is largely, but not exclusively, found in certain genera of Basidiomycota collectively named white-rot fungi (e.g. genera *Trametes*, *Polyporus*, *Polystictus*) (Paul & Clark 1996). They are able to split aromatic rings and degrade all components to CO₂ and H₂O, although only in presence of some other readily degradable substrate as the primary energy source. The breakdown of lignin is mediated by enzymes such as laccases and peroxidases, and occurs strictly under aerobic conditions (Cadish & Giller 1997). Another group of fungi, brown-rot fungi (*Poria*, *Gleophyllum*) are also able to modify lignin by removing the CH₃ subgroups and side chains (R-O-CH₃), but only to a limited extent and decompose rather crystalline cellulose (Paul & Clark 1996). Similar strategy is known for soft-rot fungi (*Chaetomium* or *Preussia*), which are

important in wet conditions and appear to degrade hardwood lignin more effectively than softwoods (Paul & Clark 1996). Bacterial lignin degradation appears to be negligible comparing to the activity of white rot fungi. However, both filamentous and non-filamentous bacteria (e.g. *Azobacter* or *Pseudomonas*) can colonize lignin-like compounds and lower the molecular size. Several Actinomycetes, such as *Streptomyces* and *Nocardia*, are also able to solubilise lignin to gain access to cellulose, c.f. (Clausen 1996; Boer *et al.* 2005).

2.1.4 Wood decay

Wood decays slowly because of the major limitation to microbial growth supposed by the presence of lignin in the cell walls of the tissues and by their structure. Lignin coats cell wall polysaccharides and chemically combines with them to form lignocellulose, a substance which is very resistant to microbial degradation. This is due to the strong and random bonding between the phenol-propane units and to chemical diversity of the lignin of different plants. Lignin therefore protects the cell wall polysaccharides from microbial hydrolysis except in the case of those organisms that can first chemically modify or degrade lignin and thus can initiate any significant decay of wood (Dix & Webster 1995).

To grow on wood, fungi also need to be able to tolerate certain chemical and physical stresses, typically high level of tannins, phenols and other aromatics. Another adverse feature of wood is its low nitrogen and phosphorus content. An adaptation of wood decay fungi is that they can grow at very low nitrogen and phosphorus concentrations. They need a very efficient recycling of nitrogen within the mycelium, high rates of intracellular protein turnover and ability of N and P transfer from surrounding (c.f. Dix & Webster 1995). Bacteria can act synergically to the breakdown of wood by affecting the permeability, attacking wood structure, or as passive colonizers (Adl 2003).

2.1.5 Decomposer microorganisms

Bacteria obtain their nutrients from the soil solution by osmotrophy. They rely on nutrients and minerals already dissolved from litter. Most species secrete small extracellular enzymes that can pass through the bacterial membranes, or that assemble on the outer membrane. These enzymes digest specific chemical bonds of substrate compounds in the soil environment and the soluble molecules become accessible for uptake by bacterial cell. Some species have single substrate requirement for growth, others require more than one source or may need cofactors. Most species can growth on a variety of substrate molecules. In these species, when a preferred substrate is present, it inhibits the uptake of alternative substrates. There can be correlation that are active together on one substrate and cooperate by syntrophy to digest more effectively complex molecules (Adl 2003). Some bacteria have evolved the potential to utilize products released from complex substrates as a result of fungal exoenzyme activity (Boer *et al.* 2005). Bacteria will probably be out-competed by fungal hyphae in lignin decay (Horner-Devine *et al.* 2003).

Fungi are also osmotrophic and release exoenzymes, similarly to bacteria. But as Eucaryotes, they can synthesize larger proteins for secretion in much greater quantities and thus the impact on microenvironment is supposed to be far greater than the effect of bacteria (Adl 2003). Unlike many bacteria, fungal cells can secrete enzymes for several substrates at the same time. Many common saprophytic fungi are ubiquitous, non-resource specific, utilizing a large but relatively restricted range of carbon sources. They are rather opportunistic decomposers and may act as pioneer species. Typically, they grow quickly and have short reproductive cycles. Characteristically, they exploit disturbed environments where competition is typically low (including litter fall). They invest significant part of their biomass

in spores, and often rest spores, that remain as the units of survival. Often, they are stress-tolerant (i.e. low water potential or phenols in litter) (c.f. Dix and Webster 1995). Other, more specialist saprophytic species have more restricted types of distribution and can be highly resource-specific. They metabolize a wide range of organic carbon sources, including lignin in many cases. They are slow to reproduce and devote more energy to the production of fruit bodies. Reproduction is usually seasonal and coincides with conditions maximizing the success of progeny establishment. They are characteristically highly competitive and antagonistic, with tendency to replace other fungi in the community, taking over their territory and accumulated resources (Dix & Webster 1995).

The phylloplane fungi can persist on fallen litter several months after the litter fall, even though most appear to be poorly adapted for sustained growth in litter and their decomposing ability is may be rather slight. The common soil fungi such as *Penicillium*, *Humicola*, *Trichoderma*, *Fusarium*, *Gliocladium*, *Doratomyces*, quickly colonize fallen litter and are widely believed to be responsible for its degradation, having the cellulolytic capacity (Dix & Webster 1995). Associated with the final stages of decay are the litter inhabiting basidiomycetes, mainly agarics, including the genera *Mycena*, *Marasmius*, *Collybia*, *Clitocybe* (Dix & Webster 1995). These fungi strongly hydrolyze plant cell wall polysaccharides and most also degrade lignin. The ability to degrade lignocellulose efficiently is thought to be associated with a mycelium growth which allows the fungus to transport scarce nutrients over a distance into the nutrient poor lignocellulose substrate that constitutes its carbon source (Dix & Webster 1995).

The majority of saprophytic fungi grow on wide range of plant litter within different habitats. The majority of deciduous woodland species can appear in conifers as well, but may show preferences for one or the other. Only a few species are considered to be taxon specific, e.g. *Clitocybe odora* or *Collybia dryophila* are two examples that are associated almost exclusively to beech leaf litter (Dix & Webster 1995). Accordingly, common species of conifer woods also decay deciduous litter only with few exceptions, such as *Micromphale perforans* and *Marasmius androsaceus* (Dix & Webster 1995).

2.1.6 Decomposer communities

Soil communities are among the most species-rich components of terrestrial ecosystems (Jones & Bradford 2001; Setälä 2002). Density and survival of a decomposer population depends on the duration that adequate growth conditions persist. It also depends on the niche diversity and the interactions with other species. Many species represented in each soil sample are inactive because the conditions are not suitable or their substrate is absent (Adl 2003).

Different saprophytic species can coexist on a substrate, particularly when nutrients are not limiting. Another possible interaction is symbiotic facilitation of colonization by a former species for later species, when one species may remove a physical or chemical barrier or facilitate the substrate digestion by providing enzymes missing in another species. Lastly, they can be competitors, e.g. for an essential nutrient. All these interactions occur at the same time between different species (Dix & Webster 1995).

A richer microbial community might correspond to higher rates of decomposition, since the greater the genetic diversity, the greater the enzyme diversity (Dix & Webster 1995). However, it seems that many decomposer species are functionally redundant (Setälä & McLean 2004) and thus could be lost with little effect on the structure and functioning of the whole community (Deacon *et al.* 2006). Such an overlap in potential functions would suggest that potential capacity for inter-specific competition can be large and that functionally equivalent infrequent taxa are waiting to replace dominant taxa. In such case the effect of

diversity could be also weak caused by saturation at low species richness or by functional redundancy interactions (Cox *et al.* 2001; Setälä & McLean 2004) or even negative due to over-saturation and enhanced competition (Wardle & van der Putten 2003).

An alternative view is, that communities of decomposers are relatively disordered and stochastic due to constant changes in physical and biotic conditions. It might provide no time for competitive exclusion and explain how an assemblage of species requiring the same resources could coexist (Deacon *et al.* 2006). Another hypothesis says, that striking spatial heterogeneity of soils could reduce resource competition between species and thus slowing down competition-induced specialization, which would also lead to high degree of generalism (Setälä 2002).

Intra-specific variation in function can be high, i.e. there is variability between the proportions of cellulose, hemicelluloses and lignin which are digested by different strains of one species, and even by the same strain through various regions of the substrate (Dix & Webster 1995; van der Heijden *et al.* 2004). Further, a key-species with respect to diversity may not necessarily be key-species for processes or resistance and resilience. No significant relationship between frequency of occurrence and decomposing ability was found (Osono & Takeda 1999; Deacon *et al.* 2006). Community composition is likely to be more important than species richness (Jones & Bradford 2001; Bradford *et al.* 2002), but is more complicated to describe and test all the interactions among numerous species.

It is necessary to well choose the appropriate temporal and spatial scales in each study. Temporal heterogeneity may pose limits on microbial activity, and flushes or resource inputs from litter or root exudates may allow largely dormant populations to become active. (Leckie 2005) Thus, there can be a rapid transition among rare and common species and their turnover ought to be included in the analysis (Schwartz *et al.* 2000). The spatial scale is mainly related to the aims of the study (Jones & Bradford 2001). It can vary from soil microhabitats through vertical stratification within soil horizons to e.g. fragmented habitats on landscape level (Torsvik & Ovreas 2002; Nannipieri *et al.* 2003).

2.1.7 Links between decomposers and vegetation cover

The vegetation influences the microbial community by climate and litter properties and their changes over time. The community composition may, in turn, influence the vegetation via decomposition (Bengtsson *et al.* 2000; Griffiths *et al.* 2001a). However, knowledge of the links between aboveground and belowground diversity and processes is still scanty (Schwartz *et al.* 2000; Srivastava 2002).

Increase of tree diversity and stand structure in forests should contribute to increase microbial biomass and activity by higher niches variability (e.g. diversity of carbon sources in more variable litters and root exudates, higher primary production, variable microclimate, presence of dead wood or spatial and age variability) (Setälä 2002). Deciduous litter should be more favorable for microbial decomposers than the coniferous one, by its higher concentration of nutrients and lower amount of inhibitors (Berg & McLaugherty 2003).

Even if soil biodiversity would be unimportant in ecosystem functioning under static conditions, it may significantly affect the stability in response to disturbance through buffering new conditions and process rates (Deacon *et al.* 2006). Diverse ecosystem would be more likely to contain species able to tolerate a disturbance than poor systems and infrequently taxa can act as an insurance effect (Jones & Bradford 2001).

2.2 Molecular approach

2.2.1 General features

Traditional approaches of microbial diversity have relied on laboratory cultivation of isolates from natural environments and identification by classical techniques, including analysis of morphology, physiological characteristics and biochemical properties. However, it has long been known that about 90% of microorganisms existing in the nature are not amenable to currently available cultural methods (Ward *et al.* 1990). To overcome these limitations, attention has been focused on molecular biological techniques, providing cultivation independent options for analysis of complex communities and giving a more realistic view of species richness and distribution (Marshall *et al.* 2003). Hence, the molecular approaches should lead to increase knowledge of microbial ecology within the habitat studied and to reveal the existence of unknown species.

The first step of the molecular approach is cell lyses and DNA extraction from complex samples. Then PCR (polymerase chain reaction) amplification is carried out, using the particular primers. Then two possibilities exist i) cloning of PCR product, which is probably the most successful way, but time consuming and laborious, or ii) more rapid and better suited fingerprinting techniques, such as TGGE or T-RFLP, which provide a pattern based on sequence or length polymorphism. They allow the simultaneous analysis of multiple samples which makes possible to compare the genetic diversity of the communities from different habitats, or to study behavior of individual communities over time (Muyzer 1999b). As with any technique used to study microbial communities in the environment, there are limitations to molecular approach that need to be taken into account, as reviewed e.g. by Anderson & Cairney (2004), Kirk *et al.* (2004) or Leckie (2005).

2.2.2 DNA extraction

There are two to three basic steps in DNA extraction. The cell must be lysed (broken open) to release the nucleus (eucaryotic cells) or directly DNA (procaryotic cells). The nucleus (if present) must also be opened to release the DNA. At this point the DNA must be protected from enzymes that will degrade it, causing shearing. Once the DNA is released, it must then be precipitated in alcohol. A number of rapid and flexible extraction techniques are available (Anderson & Cairney 2004) as well as commercial extraction kits. Because the analysis is based on extraction of community DNA from environmental samples, it is important that the DNA recovered is representative of the habitat. To achieve this, consideration must be given to several factors, including an appropriate sampling strategy, size of the sample or efficient lysis of cells (Anderson & Cairney 2004, Leckie 2005).

2.2.3 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) amplifies a particular region of nucleic acid so that enough material is available for subsequent analyses. It provides highly sensitive means of amplifying even small quantities of DNA in a short time. The reaction is done on an automated cycler that can heat and cool the tubes with the reaction mixture. The mixture have to contain: targeted nucleic acid, particular primer pair, dNTP (free bases), PCR buffer with magnesium ions and *Taq* polymerase. The polymerase is stable at the high temperatures need to perform the amplification, whereas other DNA polymerases become denatured. Three major steps are repeated for 25 or 35 cycles: denaturation, annealing and extension. After the

denaturation, the bases (dNTP) are added by the polymerase complementarily to the template between the two primers attached to the beginning and the end of the template. Because both strands are copied during PCR, there is an exponential increase of the number of copies. Once amplified, PCR products can be used in different procedures, including DNA fingerprinting.

Biases may be introduced by the PCR. These can occur for a number of reasons, for example preferential or selective amplification of different species might occur as well as sequencing errors. Humic acids and other contaminants, which are co-extracted with nucleic acids from organic-rich samples, such as litter or soil, can inhibit Taq-polymerase during the PCR. To circumvent the problem, PCR additives, such as BSA, are used in PCR reactions of soil DNA, in order to avoid laborious DNA purification procedures that can result in the loss of template (Anderson & Cairney 2004).

2.2.4 Primers

The presence of DNA regions with different degrees of conservation enables the identification of sequences that are common to specific phylogenetic groups or species and design of specific primers. Several variables must be taken into account when designing PCR primers. Among the most critical are: primer length, melting temperature (T_m), specificity or G/C content. The use of highly variable regions of the 16S rDNA gene as molecular indicator for bacterial populations in environmental samples was introduced by (Muyzer *et al.* 1993). The particular primer pair, 1401r and 968f was firstly published by Heuer & Smalla (1997). The internal transcribed spacer (ITS) region is now perhaps the most widely sequenced DNA region in fungi. It includes the entire ITS1-5.8S-ITS2 region of the nuclear rDNA. The 5.8 gene sequence is highly conserved and is useful for verifying the identity of a sequence. ITS1 and ITS2, which are un-translated insertions, benefit from a fast rate of evolution and they are much more variable. However, ITS2 has sub-regions of fairly high conservation, as compared with ITS1 (Coleman 2003). The use of ITS primers to amplify fungal ITS of rDNA was originally described by White *et al.* (1990) and pioneered by Gardes & Bruns (1993). Studies based on the ITS region show that the intraspecific variation is very small when studying fungal isolates from the same geological area, while the interspecific variation is large enough to separate different species (Karén *et al.* 1997). Moreover, ITS region analyze obtains finer scale information and greater specificity than analyze of small subunit rDNA (18S) (Lord *et al.* 2002; Anderson & Cairney 2004) which was utilized earlier in fungal diversity studies.

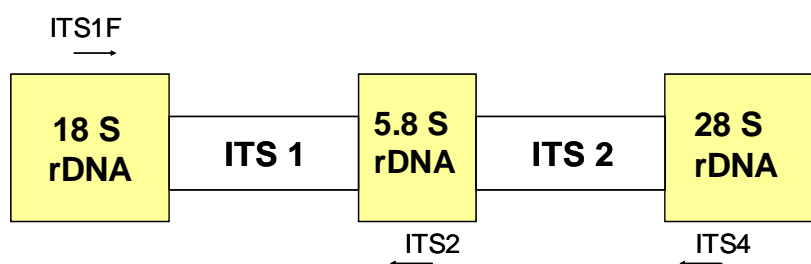


Fig. 2 Fungal rDNA fragment amplified in this study using fungal specific primer ITS1F and universal ITS2 (for TGGE) or ITS4 (in case of T-RFLP).

2.2.5 TGGE

The TGGE (temperature gradient gel electrophoresis) is a fingerprinting technique which can separate DNA fragments of the same length but with different sequences. It exploits two physical properties of DNA: the effect of base pair sequence on the temperature induced melting transition, and the influence of partial denaturation on electrophoretic mobility (Wartell *et al.* 1998). In a partially denaturing acrylamide gel, double stranded DNA is subjected to the conditions that will melt it in discrete so-called melting domains, stretches of base-pairs with an identical melting temperature, T_m . When the T_m of the lowest melting domain is reached at a particular position in the gel, a transition of a helical to branched molecule occurs. This partial melting reduces its mobility and its migration will practically halt. The melting point depends upon the proportion and position of C and G bases. This sequence variation within the domains causes the melting temperatures to differ, making possible to separate DNA fragments. To avoid complete denaturation of the helix, attachment of a GC-rich sequence to the 5'-end of one of the primers is needed. This GC-clamp is co-amplified and thus introduced into the amplified DNA fragments and it acts as a high melting temperature domain preventing this section from dissociate into single strands (Muyzer & Smalla 1998). After the procedure, the gels are stained by e.g. ethidium bromide, silver nitrate, SYBR Green or Gold to visualize the bands. It has been shown that the technique can be used for semi-quantitative comparison (Muyzer *et al.* 1993; Fromin *et al.* 2002; Dilly *et al.* 2004). Bands of interest can be excised from the gels to be sequenced, i.e. the precise sequence of nucleotides in a sample DNA can be determined. The obtained sequences can be compared to the sequences published in a database, such as GenBank.

This method has been introduced by (Rosenbaum & Riesner 1987) and applied more often to bacterial communities (Muyzer & Smalla 1998; Muyzer 1999a). Another relevant technique based on the same principle is DGGE (denaturing gradient gel electrophoresis) which requires a chemical denaturing gradient. Thus the data produced by TGGE should be more reproducible and the gel preparation is less complicated. Reproducibility of whole analysis depends on all analytical steps from the sampling to the extraction and amplification procedures as well as to the gels themselves. The use of reference patterns, loading of precise amounts and the precision of gel staining are required to obtain identical patterns (Fromin *et al.* 2002).

Fungal communities have been sometimes investigated with TGGE, but most often 18S analyses were used in the papers, e.g. in following systems: forest soil (Agnelli *et al.* 2004), polluted soil (van Elsas *et al.* 2000), wine yeasts (Hernan-Gomez *et al.* 2000), wheat rhizosphere (Smit *et al.* 1999) or rhizosphere of *Ammofila arenaria* (Kowalchuk *et al.* 1997).

2.2.6 T-RFLP

Terminal restriction fragment length polymorphism analysis is based on restriction enzyme digestion of PCR-amplified DNA that has been fluorescently labeled at one end. Fragments are resolved by size using an automated analyzer with laser detection of the terminally labeled products, producing a highly reproducible fingerprint of the community (Osborn *et al.* 2000). The technique allows detecting the terminal fragment only. This simplifies the banding pattern without reducing the diversity. However, the inability to generate sequence information from T-RFLP peaks makes the identification of unknown species in a sample difficult. This method has been used and discussed in numerous studies, as reviewed e.g. by Anderson & Cairney (2004) or Kirk *et al.* (2004).

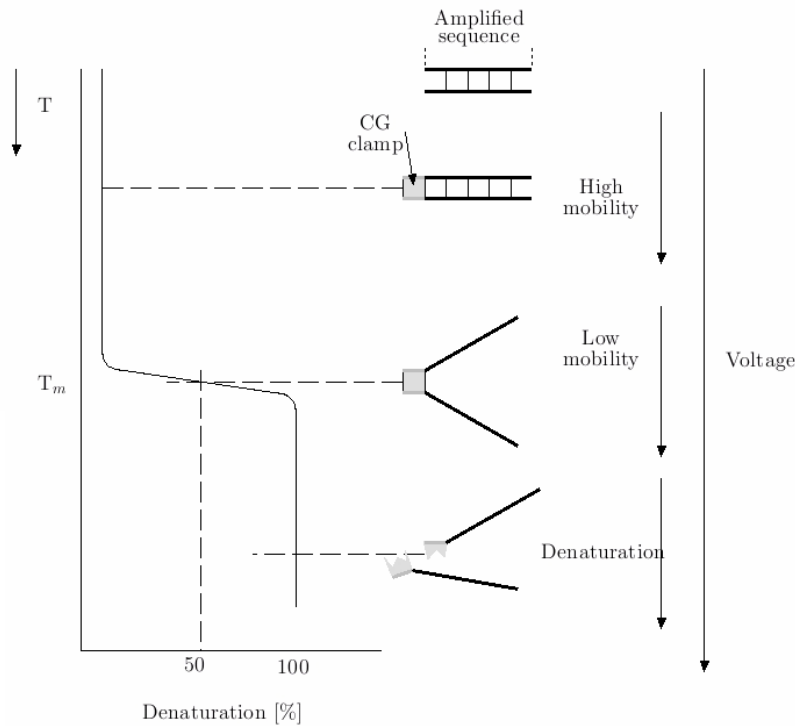


Fig. 3 Schema of DNA migration and denaturation during the TGGE on acrylamide gel.

3 Materials and methods

The study site was in Breuil experiment forest site, Morvan, Burgundy, France (47°18'10"N, 4°4'44"E). The site is situated on a shelf with a slight slope of NW orientation, elevation is 638m above sea level. It was established in 1976; the semi-natural beech-oak forest was clear-cut and replaced by monospecific plantations of diverse broadleaves and conifers in 1000m² plots each. The sandy acid soil (pH 4-4.5, sand 60%) derived from the granitic substrate. The annual mean air temperature reaches 9°C, precipitation about 1300mm and evapotranspiration of 640mm (Ranger *et al.* 2004). Five different tree plots were chosen for the treatment: four young plantations, namely beech (*Fagus sylvatica* L.), oak (*Quercus sessiliflora* Smith.), Norway spruce (*Picea abies* Karst.) and Douglas fir (*Pseudotsuga menziesii* Franco.) and the native forest (*Fagus sylvatica* L., *Quercus sessiliflora* Smith, associated with *Betula verrucosa* Ehrh. and *Corylus avelana* L.) as a reference plot. All the differences in chemical, physical and biological characteristics were assumed to be caused by dominant tree species. The main characteristics of the plots were described in Article III and in the study site report (Ranger J. *et al.* 2004).

The decomposition was measured using litter bag test. Two model organic materials, cellulose and lignin (represented by beech wood), as the main components of plant litter, were

installed in the field. Further, leave and needle litter of the plantations, two litter-mixtures (leaves-needles) and samples from L and F layer of the forest floor were also targeted. Cellulose strips were incubated for 10 months period, from February to December 2004 and sampled monthly. Wood samples were incubated 2 years, from November 2002 to November 2004 and sampled after 4, 8, 12 and 24 months of the incubation. Litter samples were incubated as the wood ones and analyzed after 4, 10 and 24 months (Moukoui 2006). The 10 months samples were not possible to amplify and were not included in further analyses. Forest floor samples were sampled in June 2004. All samples were stored frozen until analyses.

All the samples (using three replicates) were analyzed for the fungal diversity using TGGE molecular approach. The cellulose samples and 24 months lignin samples were also targeted for bacterial diversity. The DNA was extracted using the Dneasy plant mini kit (Quiagen), then amplified by PCR using higher-fungi specific ITS1F and universal clamped ITS2 primers for fungi, copying a ± 300 bp fragment of ITS rDNA. A primer pair targeting 18S gene of rDNA was also tested, but gave lower quality results. In case of bacteria, the eubacterial primer set 1401r and clamped 968f, giving a 475bp fragment of 16S rDNA, was used. PCR products were checked on agarose gel and separated on acrylamide gels by TGGE. The gels were stained by ethidium bromide in case of cellulose and wood and by silver nitrate in case of litters and forest floor layer samples. Matrices of species distribution within the samples were calculated and used for statistical analyses of species richness and community composition. Twenty important bands were sequenced to be compared to sequences available in the Genebank database of the NCBI. The wood samples of all sampling times were also analyzed by T-RFLP using fluorescently labeled ITS1F primer and ITS4. Data from consecutive studies were used to link the decomposer diversity to the decomposition process and environmental conditions. These data included: decomposition rates of all studied samples and substrates; fungal biomass, cellulase activity and nitrogen concentration in cellulose samples (Moukoui 2006) and the environmental data from the plots (soil temperature and moisture, litter composition and others, (Ranger *et al.* 2004)).

For all methodological details, see the papers I-V.

4 Discussion and conclusion of the result

4.1 Molecular approach optimization

The molecular methods were successfully optimised and tested for reproducibility before their common use in analyzes of the samples. DNA extraction using beat beating and CTAB procedure was tested, but DNA extracted by the commercial kit and liquid nitrogen mechanical lysis was of a higher quality and easier to amplify. PCR conditions were optimised using high quality DNA extracted from the mycelium on the wood samples. In case of cellulose and litter samples, BSA was added to PRC mix in order to bound the inhibitors and to increase the yields. A primer pair amplifying a part of fungal 18S rDNA was tested. There were less bands on the TGGE profile comparing to the ITS primers and the bands were less intensive. In T-RFLP approach (Art. IV), two universal primers, ITS2 and ITS4, were tested with ITS1F. The fragments amplified with ITS2 were too short for the restriction, while it performed well with ITS4.

The acrylamide gel composition and the conditions of TGGE were optimised to give the most pronounced bands. Several samples were re-amplified and re-electroforesed, giving the same banding pattern all the time and showing the approach to be reproducible. Ideally, one species yields one band but in some strains two or more bands have been previously detected caused by primer degeneracy or induced by heteroduplex molecules. On the other hand, closely related or even phylogenetically unrelated strains have similar electrophoretic mobilities, resulting in the band from one species being hidden behind the band of another species (Muyzer 1999b). Moreover, only the main species representing more than 1% of relative proportion appear on the patterns (Muyzer & Smalla 1998). Several bands of the same position on the gel were sequenced, as well as the bands of several re-amplified samples. The sequences were the same in all cases. All sequences, amplified by ITS1F-ITS2 belonged to fungi.

The gels were stained in ethidium bromide in case of the wood and cellulose samples. The DNA of the litters samples was of lower quality and contained more inhibitors, even after purification. Hence, the gels containing litter-DNA bands have to be stained in more sensitive silver nitrate. Later, the SYBR Gold staining was successfully used for different samples of forest litter and gave even better results than the silver nitrate, being a good option for further analyzes. Only dominant bands of the wood samples were sequenced. The cellulose bands were not intensive enough for sequencing and the litter bands were not possible to re-amplify due to silver nitrate staining. Certain limitation imposed by existing

databases also appeared. Therefore sequence blasting provided only approximated identification and rarely complete determination to species level. Methodology was further discussed in Art. III.

4.2 Fungal diversity in decomposing substrates

Any highly significant differences of species richness values were not detected, either between tree stands or between studied materials (Art. I, III, V). The place of tree species according to species richness of microorganisms differed according to the material tested in the incubation experiment; e.g. in case of wood, the native forest showed the highest values of species richness, followed by broadleaved and then by coniferous plantations. Nevertheless, the order was different for cellulose or litter material, often with minor differences. The only evident increase of species richness over time was found for wood samples. In litters, the SR was rather similar in the former and later sampling, except the mixed litters with the clear increase. In cellulose, the species richness was more strongly influenced by effect of season rather than by time of incubation, mainly during the summer months. The same trend was found for wood samples in the late summer sampling.

All tree stands displayed characteristic community structures in every studied substrate. Effect of tree species and duration of incubation was always significant within whole data set for a given substrate. When a tree stand or a sampling date were tested individually, the effect of time or stand might not be significant, but that happened rather rarely. For wood samples, the native forest displayed the most distant community structure from all stands at the end of the incubation period. The broadleaved plantations displayed similar communities as well as the conifers did (Art. III). The native forest was the most diverse also overall in the cellulose strips, comparing to the beech and spruce stands (Art. I). Noticeable was the shift of community structure during summer months, both for wood and cellulose samples (Art. I, III). The abundant species were replaced by infrequent ones and the overall SR declined, probably as a consequence of drought stress.

Fungal diversity in L and F forest floor layer (Art. V) was also targeted. Fungal species richness did not significantly differed between the tree species either L and F layer, while the community composition did, similarly to substrates decomposed in litter bags.

Almost all species were detected in all studied substrates and in all tree stands. However, their abundances and temporal patterns could highly differ, as documented e.g. in Art. II. Tab. 1. shows the presence of sequenced species (Art. III) or TGGE bands of the same position in particular substrate or tree stand, indicating also the percentage of samples in

which the species was present. Bands from cellulose samples were not intense enough to be sequenced and silver nitrate staining disallowed sequencing of litter or layer bands. *Megacollybia platyphylla* was the only species which sequence showed 100% agreement with the GeneBank sequence. It was also detected as the most abundant species within all substrates and tree stands. Other sequencing results showed lower similarity to the sequences published in the GeneBank database and often were not determinable even to genus level. It documented the high number of unknown, or at least unsequenced, species in the forest soils and potential to further research. (Lejon *et al.* 2005) also documented the differences between fungal and bacterial communities within soil profiles and tree stands in the same study site, using ARISA approach. There have been many other investigations of microbial diversity in forest (see references in Art. I, III, V), but they are difficult to compare. They considered different forest ecosystems located in different sites, studied the diversity at different scales and were often affected also by other factors than only the tree cover.

Tab. 1 Abundances of twenty sequenced species (Art. III), given in the percentage of all samples of a substrate/tree stand, where the species was present. Species n. indicates the band position in the TGGE profile, which was then used for the species indication in text and graphs. Identity means the closest Blast result, which percentage of similarity is also shown, after the Blast match. A – Ascomycetes, B – Basidiomycetes. Black colour indicates the present of the given species in more than 50% of the samples, dark grey: 25-50%, light grey: 10-25%, white: 0-10%.

Species n.	Identity	Blast match	Similarity	Cellulose	Lignin	Litters	Layers	Native f.	Beech	Oak	Spruce	Douglas	
6	<i>Leaf litter ascomycete strain its408 isolate</i>	A	AF502889	96	60	45	78	50	45	60	54	50	63
7	<i>Megacollybia platyphylla</i>	B	AF498289	100	57	62	72	73	69	54	83	56	71
10	<i>unpublished species</i>	-	-	-	15	32	36	50	26	31	25	17	54
12	<i>Epacris microphylla root associated fungus</i>	AY268197	98	17	53	42	27	33	35	46	13	42	42
14	<i>uncultured fungus clone B1c from forest</i>	AY324159	98	10	27	25	40	29	15	29	21	25	25
17	<i>leaf litter ascomycete strain its356</i>	A	AF502859	97	15	38	22	43	29	21	21	31	42
19	<i>Trametes versicolor</i>	B	AY673076	93	13	28	25	10	12	23	38	15	17
21	<i>Gerronema strombodes</i>	B	U66433	92	22	45	14	20	26	40	25	19	33
23	<i>Phlebia albida</i>	B	AY219368	94	36	27	28	37	50	29	8	42	21
25	<i>Dactylaria sp. P24</i>	A	AY265332	88	21	37	14	43	29	38	25	21	38
26	<i>Phanerochaete sordida</i>	B	AY219383	96	44	58	31	17	40	52	46	40	25
28	<i>Fungal endophyte WMS13</i>	A	AY063309	94	25	43	47	37	29	33	42	38	46
30	<i>Cryphonectria sp. CMW 11302</i>	A	AY214324	98	8	42	17	23	19	27	21	23	25
33	<i>Epacrid root endophyte E4-5-5</i>	A	AF148952	91	10	17	22	17	7	17	17	13	13
35	<i>Menispora tortuosa</i>	A	AF178558	92	8	15	17	20	7	13	13	19	25
37	<i>Contosporium apollinis</i>	A	AJ244271	90	10	17	19	30	17	17	17	13	33
39	<i>Helotiales sp. sd2aN4b(A)</i>	A	AY465452	95	11	28	8	17	19	23	8	13	17
41	<i>ascomycete sp. olrim349</i>	A	AY354279	95	8	27	22	40	12	21	33	15	21
42	<i>Woollisia root associated fungus XVIII</i>	A	AY230788	90	11	38	14	30	29	19	25	19	33
46	<i>Chaetosphaeria pulviscula</i>	A	AF178544	94	26	18	3	37	14	19	25	29	25

4.3 Effect of fungal diversity to decomposition rate

The young broadleaved plantations showed the highest values of mass loss at the end of each experiment. Both wood and litters decomposed the fastest in oak, followed by beech stand. Oak stand was not included in cellulose treatment and thus beech showed the highest values. In the case of wood, the native forest occupied the middle position between

broadleaved and conifers. The cellulose decomposed faster in the native forest than in spruce during the initial eight months, but had lower mass loss in the end of the experiment, after ten months. There were no data for the native forest about litter decay. The mixed litters ML were higher than mass loss of both simple litters.

Fungal species richness did not generally appear to be the main factor affecting the decomposition rate of materials decomposed in the present study (Art. II, IV, V). In a few cases, there was found really higher decomposition rate together with higher species richness, e.g. in mixed litters (Art. V). However, there were not a clear relationship the most often and even negative effects could be detected, e.g. the high decomposition rate values during summer months with low species richness of cellulose or certain wood samples (Art. II, IV).

None or even negative effects of species richness in species rich communities were already documented in previous studies, e.g. (Griffiths *et al.* 2000; Cox *et al.* 2001; Griffiths *et al.* 2001b; Wilkinson *et al.* 2002; Setälä & McLean 2004; Deacon *et al.* 2006). It might be due to high degree of functional redundancy in fungal assemblages and resulting competition in the communities (Setälä & McLean 2004; Deacon *et al.* 2006). Habitat utilization seemed to be regulated rather by the total abundance of the community, where the effect of species losses can be compensated by population growth of the remaining species (Ekschmitt *et al.* 2001). Hence, loss of biodiversity would have greater functional implications in poor and/or fluctuating environments (Jones & Bradford 2001) and maintaining diversity might be necessary for long-term sustainability by sustaining the disturbances (Bengtsson *et al.* 2000).

The actual species composition of the fungal assemblage is likely to be more important than species richness, but it is not easy to test this effect on the soil functioning for a large range of particular species in the field. The results showed differences in community composition between the tree species. However, we could not exactly identify the role of the variability in community structure and separate it from the effects of various environmental conditions and species interactions. Additionally, the intra-specific variation in function can be high and there might be no relationship between taxonomic and functional diversity (van der Heijden *et al.* 2004; Deacon *et al.* 2006). Further, the decomposition rate would respond in a relative manner, i.e. occur slower or faster, but not cease completely when community composition changed. As an illustration, the community composition was diverse in beech litter, comparing to the others, in 4 months, together with high species richness, but the decomposition rate was low (Art. V). On the contrary, the diverse communities in beech and oak coincided with high decomposition rate in wood samples (Art. IV). In the cellulose experiment, the community composition effect was probably minor, as it is a simple, easily

decomposable substrate accessible for majority of species (Art. II). Other variables concerning cellulose samples, described by Moukoui (2006), e.g. fungal biomass (Art. I), cellulase activity or nitrogen accumulation (Art. II) were also related to decomposer diversity (Art. I, II). Different species affected the overall dynamics of decomposition at a biochemical level in recent studies (Cox *et al.* 2001; Dilly *et al.* 2001). In the study of Osono *et al.* (2003), Basidiomycota caused loss of lignin and carbohydrates in variable proportions, while Ascomycota attacked carbohydrates without need of delignification Osono *et al.* (2003), although the mass loss of studied Japanese larch litter was not significantly different between diverse Asco- and Basidiomycota. Fungi imperfecti isolates (*Fusarium*, *Penicillium*) were able to attack the hemicellulosic, cellulosic and also lignin fractions of wheat straw but were variously powerful lignin-degraders (Rodriguez *et al.* 1996). The same study suggested the significant role of imperfect fungi in carbon turnover in soil, where occurrence of white-rot Basidiomycetes was very sparse comparing to the imperfect fungi. Similarly, Basidiomycetes reached a peak after 6 months of decomposition of pine litter and become less important in later stages, when soil fungi (*Mucor*, *Penicillium*, *Trichoderma*) were the most frequent (Virzo De Santo *et al.* 2002). Nevertheless, the effects of interactions of specific fungi might be even more important than the functional competence of communities (Cox *et al.* 2001). Moreover, they are possibly not the same in the field as in cultures in the laboratory, where the majority of species-function studies were ruled.

4.4 Role of environmental conditions

The ecological conditions of the studied stands differed. Nevertheless, all these differences were assumed to be caused by the dominant tree species, because geology and meso-climate conditions were very homogeneous and site histories were the same in the research site of Breuil (Art. I, III). All plantations were made after clear cutting the semi-native broadleaf forest 30 years ago, in 1976. The inorganic and organic composition of the falling litter was diverse between the tree species (Art. V). The forest floor properties were also divergent (Art. III, V). Generally, the broadleaved were richer in nitrogen and other nutrients, had lower C/N ratio and were warmer and wetter than coniferous. These parameters should make these stands more favorable for the decomposers, comparing to conifers (Berg & McClaugherty 2003). The organic chemical, nitrogen and phosphorus dynamics were previously suggested to be related to the ingrowths, substrate utilization and succession of the xylariaceous Ascomycota and the Basidiomycota during the decomposition of beech leaves (Osono & Takeda 2001).

The effects of soil temperature and moisture to decomposer communities were tested individually in cellulose and wood samples. They were found to be significant to community composition in both cases. In the cellulose study, both temperature and moisture were more important than the incubation time or the tree species. Time of exposure and months of sampling were important as well. The role of temperature and moisture was higher during the summer months. The species richness decreased during that period and the frequent species were replaced by others, infrequent or even rare species (Art. II, III). Nevertheless, those changes were often accompanied by an enhanced activity (Art. II, IV) and higher overall biomass (Art. I, II), which was the most evident in the beech stand during the cellulose incubation. A possible explanation might be decreased competition between less species in the community together with dry, but favorite temperature conditions and possibly with higher amounts of nutrients released from surrounding litter or root exudates. Deacon *et al.* (2006) also documented the infrequent species to be feeble competitors, but potentially even more active than abundant species. These occasional species might increase the resilience of the ecosystem, waiting to replace the abundant taxa in case of a disturbance or condition change. Further, this finding demonstrated the importance of repeated samplings to record the whole species pool.

4.5 Bacterial diversity

The bacterial diversity was studied in all cellulose samples and in the last sampling of the wood decomposition experiment. As for fungi, there was not any important decrease of species richness between tree species. Similarly, the community composition differed within tree species (Art. I, III). Both species richness and community composition developed during the incubation of cellulose strips. The effect of seasonality was also evident, with the decrease of species richness and change of community composition during the summer months (Art. I). The decomposition rate of cellulose samples during the eight months period was slightly positively related to bacterial species richness in the native forest and spruce plots, while a negative relation was detected in the beech stand (Art. II).

4.6 T-RFLP

TGGE and T-RFLP approaches were compared, using DNA of the wood samples (Art. III, IV), since particular molecular methods can differ in results (e.g. (Anderson & Cairney

2004; Leckie 2005). Although the results of the two methods were partially different, the main trends were shown by both of them (Art. IV).

4.7 Native forest versus plantations

The native forest evolution was historically marked by three periods: i) native species with a rather extensive management, ii) intensive harvest for fuel wood during the 18th and 19th century, iii) very extensive management on the broadleaved part of the forest in the 20. century, but a massive introduction of conifers in large areas justifying the initial experimentation to study the effect of such species changes. The coppice with standards treatment is no more active and the so called native broadleaved forest slowly evolved towards a high forest as it is often the case in France. It is dominated by European beech and sessile oak, associated with other tree species like European birch and hazel nut tree. The native forest differed from the plantations by several points: i) age (150 vs. 30 years), ii) mixed species vs. monocultures, iii) age differentiation, coppice with standards vs. even-aged high forest, iv) no soil preparation for plantations after the clear-cut (no windrowing), v) greater amount of dead wood on the forest floor, vi) lower density, more sun energy can reach the soil surface.

Numerous studies were made on soil solid phase and solutions, showing i) rather net differentiation of the soil geochemistry after the 30 years, ii) very clear differences between the N-mineralization rate and forms of mineral-N produced (no nitrification in the native forest, very limited in spruce, medium in beech, rather high in oak and very high in Douglas-fir), iii) very significant difference in soil solutions (no nitrates in the native forest, very low in spruce, intermediate in beech and oak, high in Douglas-fir). All those parameters converge to indicate that the forest tree species strongly controlled the C and N cycles and more specifically the nitrification rate. However, the fungal and bacterial communities were found to be more involved by moisture and temperature conditions than by different concentrations of mineral-N forms (Art. II, III).

Previous study compared the sporocarp diversity of both saprophytic and ectomycorrhizal fungi in the same research site (Le Tacon *et al.* in Ranger *et al.* 2004). It was the highest in the native forest and the lowest in the oak and beech stands. The native forest also displayed the highest microbial biomass (Lejon *et al.* 2005). It produced 4882 kg of litter.ha⁻¹.year⁻¹, i.e. less than oak stand, but more than spruce, Douglas and beech (Ranger *et al.* 2004). In this study, the native forest displayed slightly higher species richness of wood

samples than the plantations, but average species richness on the cellulose strips and in the forest floor layers. There was the most specific community composition colonizing the cellulose in the native forest, when compared to beech and oak stands, both for fungi and bacteria (Art. I). In the case of wood, it had most fungal species in common with the oak stand, followed by beech, when analyzed all sampling dates together (Art. III). On the contrary, it shared some species with each of the plantations in the F layer of forest floor, i.e. in the late stages of the litter decay (Art. V). The decomposition was found to run slower in the native forest than in the broadleaved plantations (Art. II, IV).

Indeed, all of the fungal species detected should originate in the native forest. They might be suppressed or favored by the change of tree species after the mono-specific plantation. The fungal communities have already changed, but the species richness remained similar. Possibly, the 30 years were not enough to reduce the fungal diversity more markedly. It would be interesting to know the effects after longer period or even after several generations of plantations. Further, community changes during the re-plantation and clearing stage might be still influential until present. Mixed litters, composed of 50% of leaves and 50% of needles, were decomposed on the borders of adjoining particular tree stands. They displayed significantly high species richness accompanied by presence of characteristic species after two years of the incubation (Art. V). Both of them decomposed faster than appropriate simple litters in both sampling times. Thus, the litter mixing maintained the fungal diversity and increased the decomposition rate.

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Photos

Fig. 1 Tree plantations and the native forest; a) Douglas-fir b) spruce c) oak d) beech e-f) the native forest

Fig. 2 Samples in different stages of the experiment; a) litter bags prepared for sampling b) cellulose sample after sampling c) TGGE in progress

Fig. 3 Examples of the substrates after the incubation; a-c) cellulose d-e) wood f) oak-Douglas mixed litter g) spruce needles

Fig. 4 DNA in different steps of the molecular approach; a) extracted DNA b) DNA amplified by PCR c) bands separated on acrylamide gel by TGGE.



Fig. 1 Tree plantations and the native forest; a) Douglas-fir b) spruce c) oak d) beech e-f) the native forest

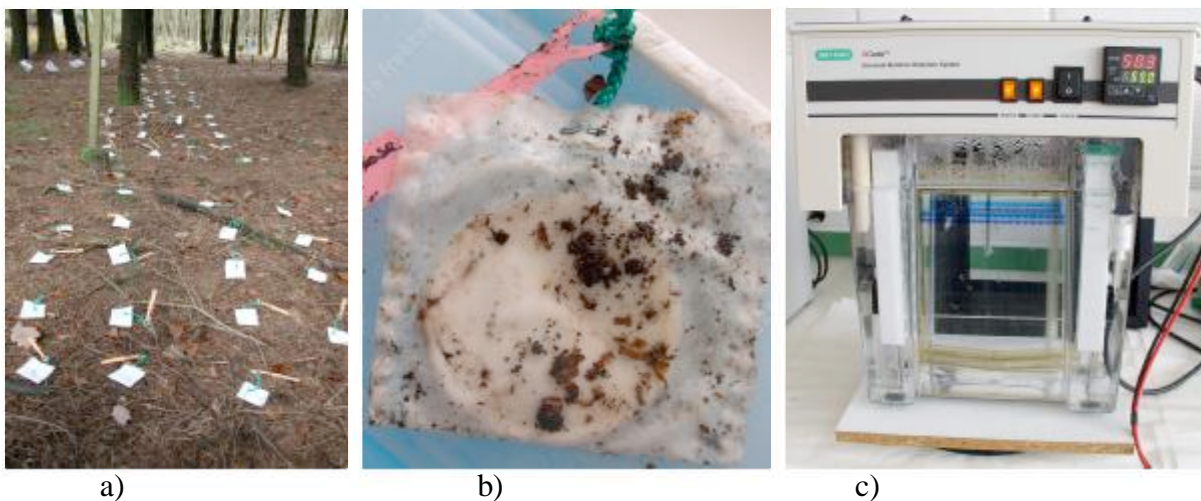


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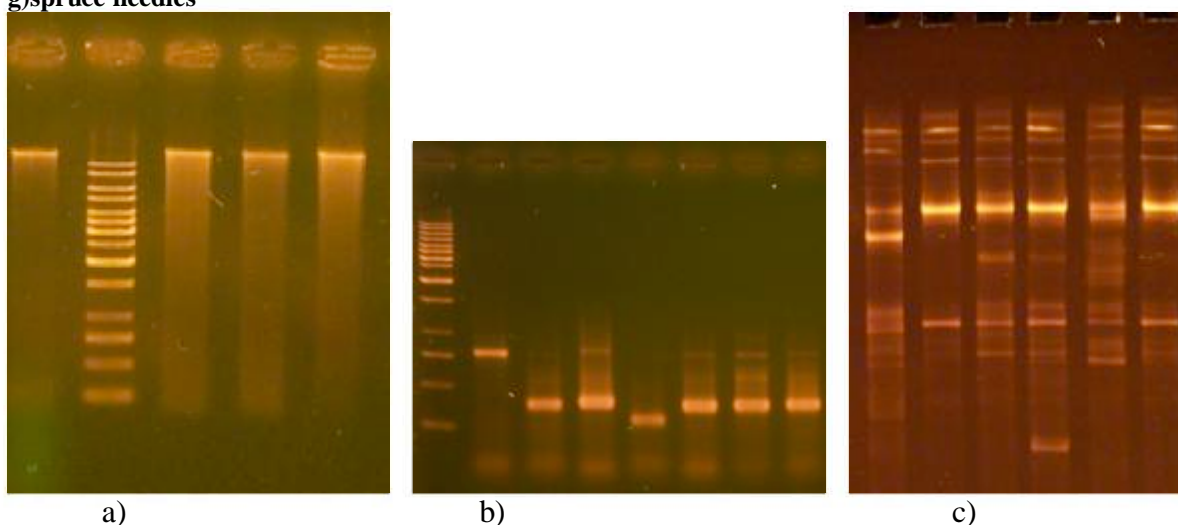


Fig. 4 DNA in different steps of the molecular approach; a) extracted DNA b) DNA amplified by PCR c) bands separated on acrylamide gel by TGGE

Papers I - V

Microbial Diversity During Cellulose Decomposition in Different Forest Stands: I. Microbial Communities and Environmental Conditions

Ariana Kubartová · Judicaël Moukoui ·
Thierry Béguiristain · Jacques Ranger ·
Jacques Berthelin

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Abstract We studied the effect of forest tree species on a community of decomposers that colonize cellulose strips. Both fungal and bacterial communities were targeted in a native forest dominated by beech and oak and 30-year-old beech and spruce plantations, growing in similar ecological conditions in the Breuil-Chenue experimental forest site in Morvan (France). Microbial ingrowths from the 3rd to 10th month of strip decomposition (May to December 2004) were studied. Community composition was assessed using temperature gradient gel electrophoresis with universal fungal (ITS1F, ITS2) and bacterial (1401r, 968f) primers. Soil temperature and moisture as well as fungal biomass were also measured to give additional information on decomposition processes. Changing the dominant tree species had no significant influence in the number of decomposer species. However, decomposer community composition was clearly different. If compared to the native forest, where community composition highly differed, young monocultures displayed similar species structure for fungi and bacteria. Both species numbers and community composition evolved during the

decay process. Time effect was found to be more important than tree species. Nevertheless, the actual environmental conditions and seasonal effect seemed to be even more determining factors for the development of microbial communities. The course and correlations of the explored variables often differed between tree species, although certain general trends were identified. Fungal biomass was high in summer, despite that species richness (SR) decreased and conversely, that high SR did not necessarily mean high biomass values. It can be concluded that the growth and development of the microbiological communities that colonized a model material *in situ* depended on the combination of physical and biological factors acting collectively and interdependently at the forest soil microsite.

Introduction

Changing forest species is a common practice in European forestry, especially when the forest production is favored. Such a change in plant diversity has implications for belowground ecosystem functioning, including microorganism diversity [4]. Decomposer communities may in turn affect the process of decay of raw organic material and thus the mineralization of nutrients strongly relevant for nutrient bioavailability, especially in acid soil conditions. This may cause feedback effects on the plant community that modify net primary production, root exudation, the forest-floor diversity and the abundance, and activity and composition of the communities [5].

Decomposition of plant litter is a key process in nutrient recycling and humus formation in forest ecosystems. The combined effects of resource quality (chemical and physical

A. Kubartová (✉)
Department of Botany, Faculty of Sciences,
Charles University,
Benátská 2,
128 01 Praha 2, Czech Republic
e-mail: arikul@centrum.cz

J. Moukoui · J. Ranger
INRA, Centre de Nancy, Unité Biogéochimie
des Ecosystèmes Forestiers,
54 280 Champenoux, France

J. Moukoui · T. Béguiristain · J. Berthelin
LIMOS, CNRS UHP UMR 7137,
Université Henri Poincaré, BP 239,
54 506 Vandoeuvre-les-Nancy, France

characteristics of the litter), physical–chemical environment (e.g., moisture, pH, radiation, and temperature), and the occurrence and activity of decomposer organisms regulate decomposition processes. The community structure and activity of decomposers in holorganic layers changes throughout the decay process as the quality of the substrate changes. In addition, there are seasonal changes reflecting temperature and moisture conditions [6]. Microbial activity is supposed to be depressed by low temperature in the cold period and by aridity in the warm period. It is therefore essential to study the relationships between microorganisms, vegetation, and environment to better understand forest ecosystem processes and to predict possible turnover caused by a disturbance.

Decomposition is usually initiated by generalist primary colonizers involving a diverse community of fungi and bacteria that utilize simple sugars, oligosaccharides, and other low molecular weight compounds. After this initial flush of microbial activity, specialist secondary colonizers that are less competitive are involved in the decay of more recalcitrant polymers such as lignocellulose complexes [9]. Both bacteria and fungi play typical roles in decomposition processes in forests. They are seen to compete for simple plant-derived substrates and have developed antagonistic strategies. For more recalcitrant substrates both competitive and mutualistic strategies appear to have developed. Bacteria can produce growth factors required by fungi, regulate fungal enzymatic activity by utilizing the breakdown products, or increase accessibility of substrates to fungi producing their own cellulases and pectinases, as discussed by Boer *et al.* [8].

The dynamics of complex systems are not easy to describe. The use of model material decomposing in tree monocultures could be a possible way to control variability by decreasing the effects of total natural complexity. Cellulose strips were chosen as a model material to simplify the complexity of the studied system because they decompose quickly and permit frequent sampling and the study of early and late decay stages and seasonal effect. However, most of the plant-derived cellulose is physically protected from enzymatic hydrolysis by lignin and thus its colonization and degradation is more complicated. We studied the decay of the cellulose strips during 10 months and their colonization by fungi and bacteria in different tree stands. Different parameters have been investigated along time: cellulase decomposition (mass loss, fungal biomass, cellulase activity, and carbon and nitrogen dynamics); diversity of bacterial and fungal communities colonizing and degrading the cellulose strips; and environmental field parameters (moisture and temperature in the profile, nitrate and ammonium concentrations in soil solution). Possible correlations between microbial community diversity and environmental parameters are presented in the first article.

The decomposition parameters in relation with microbial diversity will be discussed in a second paper.

The present study aims were (1) to determine the differences in pattern of fungal and bacterial ingrowths in decomposing cellulose strips in three forest stands with attention to the succession of species, (2) to determine the role of soil moisture and temperature or season effect in the processes of community dynamics, (3) to find relationships between microorganisms and their environment, and (4) to identify a general trend of microbial colonization of decomposing organic substrate, if it exists. To answer these questions, we investigated polymerase chain reaction/temperature gradient gel electrophoresis (PCR-TGGE) as culture-independent molecular fingerprinting approach, which helped to determine the dominant fungal and bacterial species patterns in decomposed samples.

Methods

Site Description

The experimental site of Breuil forest is situated in the Morvan highlands NE of Central Massif, France (altitude 640 m, latitude 47°18'10", longitude 4°4'44", mean annual temperature 9°C, precipitation 1280 mm, evapotranspiration 640 mm). The soil developed on granite is a coarse-textured acid Alocrisoil (pH 4–4.5). The previous coppice with standard forest dominated by European beech (*Fagus sylvatica* L.) and secondarily by sessile oak (*Quercus petraea* Liebl.) in the high forest strata and by birch (*Betula pendula* Roth) and hazel (*Corylus avellana* L.). It was replaced by various monospecific plantations in 1000-m² plots 30 years ago [25]. Breuil study site is homogenous; the tree species plots should not differ in substrate or mesoclimatic conditions. Hence, differences should be caused only by trees dominant over the last 30 years.

In Situ Experiments

The cellulose decomposition experiment was completed in a native forest and in two plantations (Norway spruce, *Picea abies* Karst. and European beech, *F. sylvatica* L.) from late February 2004 to early December 2004. Five round cellulose strips (Whitman, Ø 47 mm, total weight ±0.9 g) were put into a polyamide porous bag (mesh size 5 µm). The distance between two replicates was 0.5 and 1.5 m between the blocks. Seven bags were collected from each stand regularly at 4-week intervals. Three bags from the 3rd to the 10th (May to early December) sampling dates were analyzed for fungal and bacterial diversity. Soil temperature (Campbell data logger CR10 and probes 107, Shephed, England) and moisture (TDR Trase system BE, Soil Moisture Equipment Corp.,

Santa Barbara, CA, US) were continuously measured every 4 h in the studied plots (except the native forest because of technical reasons) and their average values during the 4-week period between samplings were used for analyses. Ammonium and nitrate concentrations were continuously monitored in soil solutions collected under forest floor in the three stands using zero-tension thin plate lysimeters [25]. Fungal biomass in samples was estimated using the ergosterol method described in Ruzicka *et al.* [26] and Zhao *et al.* [31], based on ergosterol concentration measures in the membrane of fungal cells. Ergosterol was extracted from an aliquot of powdered cellulose with pure methanol (7 mL) in the presence of potassium hydroxides (0.44 g). The mixture was maintained at 70°C for 90 min. Then 2 mL of hexane was added and all was shaken before extracting the hexane. Hexane extracts were nitrogen-dried, then stored at -20°C before analysis. For analysis, samples were dissolved in 500 µL of methanol then treated in high performance liquid chromatography HPLC (with a C18 column, detector at 282 nm, and methanol [95%] as mobile phase). The calibration curve was established with the commercial ergosterol (Sigma-Aldrich). Ergosterol data were converted to biomass of fungal hyphae as 7 mg ergosterol/1 g hyphae [1].

Molecular Analyses

Total genomic DNA was extracted from the samples using the DNeasy plant mini kit (Qiagen) following the manufacturer's protocol without any modification and amplified using PCR. Concerning fungi, ITS1F and clamped ITS2 primers were used giving partial ITS rDNA sequence of ±280-bp-length. The 50-µL of PCR assays contained 2 µL of template, 5 µL of PCR buffer (Sigma, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂), 1 µL of diethylnitrophenyl thiophosphate (10 mM), 1 µL of each primer (20 µM), 0.5 µL of *Taq* polymerase (5 U/µL, Sigma), and 1 µL of GC-rich solution (Sigma); the amplification regime was as follows: initial cycle of denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45s, annealing at 55°C for 45 s, and extension at 72°C for 1 min 15 s, ending with a final elongation at 72°C for 8 min. In bacterial treatment, 16S gene of rDNA from the same extraction was targeted using 1401r and a clamped 968f primer pair giving a 450-bp segment [15] and a similar reaction mixture with 2 µL of bovine serum albumin (3%) added. The PCR regime differed slightly: denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 40s, annealing at 56°C for 30 s, and extension at 72°C for 1 min, and the final elongation step at 72°C for 5 min. All PCR reactions were performed with iCycler Thermal cycler (BioRad). PCR products were verified on agarose gel stained with ethidium bromide. TGGE was performed with a Dcode Universal Mutation Detection system (BioRad). For fungi, the polyacrylamide

gels were prepared with 8% of acrylamide (w/v), 8 M of urea, 1.25× Tris-acetate-EDTA (TAE) and 0.2% of glycerol (v/v) and 300 µL of ammonium persulfate (10%) and 30 µL of TEMED as polymerization agents. Ten microliters of amplified DNA samples with the same volume of loading buffer were separated using electrophoresis in 1.25× TAE at a constant voltage of 145 V with a temperature gradient from 50 to 55°C with temperature increment of 1°/h and stained with ethidium bromide. For bacteria, TGGE differed in that we used 7% acrylamide gels (w/v) and a temperature gradient from 58 to 63°C with the same temperature increment. Fungal and bacterial species were represented by the TGGE bands. There were no obvious traces of strip colonization during the first 2 months after the placement. Hence, the molecular analyses covered a period from the 3rd to the 10th month of incubation, i.e., from May to December. Twenty of the most important fungal bands were sequenced previously [17]. However, the species are presented only as numbers because their identification to genus level was not possible. For more details, see Kulhankova *et al.* [17].

Statistics

The gel profiles were analyzed using the Quantity One software (BioRad). A matrix of species distribution and relative abundance was obtained both for fungi and bacteria. Variables targeted were: (1) total number of species, i.e., species pool present in all involved samples, e.g., in the three repetitions from a tree plot in a given sampling time or overall in a plot depending on circumstances; (2) species richness (SR below), i.e., average number of species per sample; (3) Shannon-Wiener diversity index (SW below), based on decimal logarithm; and (4) community composition of fungi or bacteria. These variables were first analyzed together within all tree plots and sampling dates. Then they were tested separately in a particular tree plot or a sampling time to obtain a clearer picture of the importance of the studied variable. The data were tested by computing ANOVA using the Statistica software (StatSoft) and multivariable analyses using Canoco (Microcomputer Power). Principal component analysis (PCA, or unimodal detrended correspondence analysis, DCA, if needed) was used to assess the general differences between band patterns. Redundancy discriminate analysis (RDA, or unimodal canonical correspondence analysis, CCA, if needed) then provided more information because it also took into account environmental factors such as tree species, time, and soil properties related to the sample and gave the proportion of variation explained [20]. The Monte Carlo permutation test was used to calculate the significance of a given environmental factor and thus its relevance for community structure. Application of the multivariable methods was reviewed by Fromin *et al.* [12]. The correla-

tions were described by Pearson's correlation coefficient: correlation $\rho_{X,Y}$ between the variables X and Y with values μ_X and μ_Y respectively, and standard deviations σ_X and σ_Y were defined as: $\rho_{X,Y} = \text{cov}(X, Y) / (\sigma_X \sigma_Y)$ where $\text{cov}(X, Y) = E((X - \mu_X)(Y - \mu_Y))$, in which E is the expected value of the variable and cov means covariance.

Results

Species Richness and Diversity, Fungal Biomass

Forty-seven different fungal species (presented by numbers) were detected from the potentially detectable number of 50 within all studied TGGE profiles, i.e., within all cellulose samples (Table 1). The total number of species per sample varied from 4 to 16 with the average SR equal to 9.1 within all samples. Among the tree species, the highest average was found for Norway spruce followed by the native forest and beech (9.5, 8.9, and 8.8, respectively). The same order was also observed for the SW diversity index. There were no large differences in the total numbers of species detected between the three studied tree plots, ranging from 44 species present in Norway spruce, 42 in beech, and 40 in the native forest. The tree species effect for fungal SR within the whole data set was not significant. SW diversity differed significantly between tree stands. The SR differed for individual

sampling times whereas SW diversity did not. In individual stands no richness or diversity changed significantly within/throughout time and both seemed to be more affected by the current ecological conditions. Similarly the tree species effect on SR was only weak in some given sampling times.

For the whole dataset, i.e., for all tree plots together, the least total number of fungal species in all samples were found after 4 months of incubation in June (27). The largest total number, 34 species, was detected after 3 and 10 months, in May and December, which were the first and last samplings dates. Differences appeared for individual tree plots (Fig. 1a), e.g., there was no decrease in the total species number in late summer (September) in spruce, comparing to beech and to the native forest.

Generally, the average SR per sample increased until August, then decreased in September before reaching its highest value in October. It then decreased continuously until winter (Table 1). There were no strong differences between tree plots except that the changes appeared earlier in beech than in spruce and in the native forest (Fig. 1c). The SW diversity index followed the same trend as SR for all tree species taken as a whole and if considered individually (Fig. 1e).

Fungal biomass in all tree plots as a whole increased continuously from May to September, when it reached its highest value. Then it decreased notably in October and maintained the same value until the end of the incubation

Table 1 Results of the multivariable analyses, total species number, SR, and SW diversity index values in fungal communities

Data set	Tested effect	Species number	Indirect 1st axe	Direct anal. canonical axes	<i>p</i> value	<i>F</i> ratio	SR	SW index of diversity
All matrix	Time ^a	47	10.7	4.7	0.002	3.331	9.1	0.802
	Stand ^a			3.9	0.044	1.385	4–16 ^b	0.28–1.21 ^b
3 months	Stand ^c	34	21.6	22.3	0.770	0.863	8.4	0.814
4 months		27	26.1	44.2	0.002	2.377	7.1	0.765
5 months		28	24.6	29.8	0.132	1.274	8.7	0.824
6 months		32	26.4	33.1	0.048	1.485	10.6	0.828
7 months		33	24.8	26.8	0.264	1.097	8.0	0.684
8 months		33	25.7	36.5	0.002	1.721	11.1	0.887
9 months		29	31.2	35	0.052	1.616	9.8	0.856
10 months		34	21	31.5	0.034	1.378	9.0	0.754
Native forest	Time	40	16.4	9.4	0.004	2.272	8.9	0.810
Spruce		44	15.2	8.5	0.006	2.047	9.5	0.857
Beech		42	14.4	5.7	0.102	1.324	8.8	0.738
Spruce and beech	Moisture			5.1	0.002	2.472		
	Temperature			5.2	0.002	2.539		

The first two lines show scores where all the data set was analyzed together to detect time and tree stand effect, respectively; 3–10 months: effect of tree stand was tested in particular sampling dates; Native forest–Beech: effect of time was tested separately in the tree stands; lastly, moisture and temperature effect was tested in the beech and spruce stand together.

Variability explained by the first axis of indirect analysis and by all canonical axes; results of Monte Carlo permutation tests can be found.

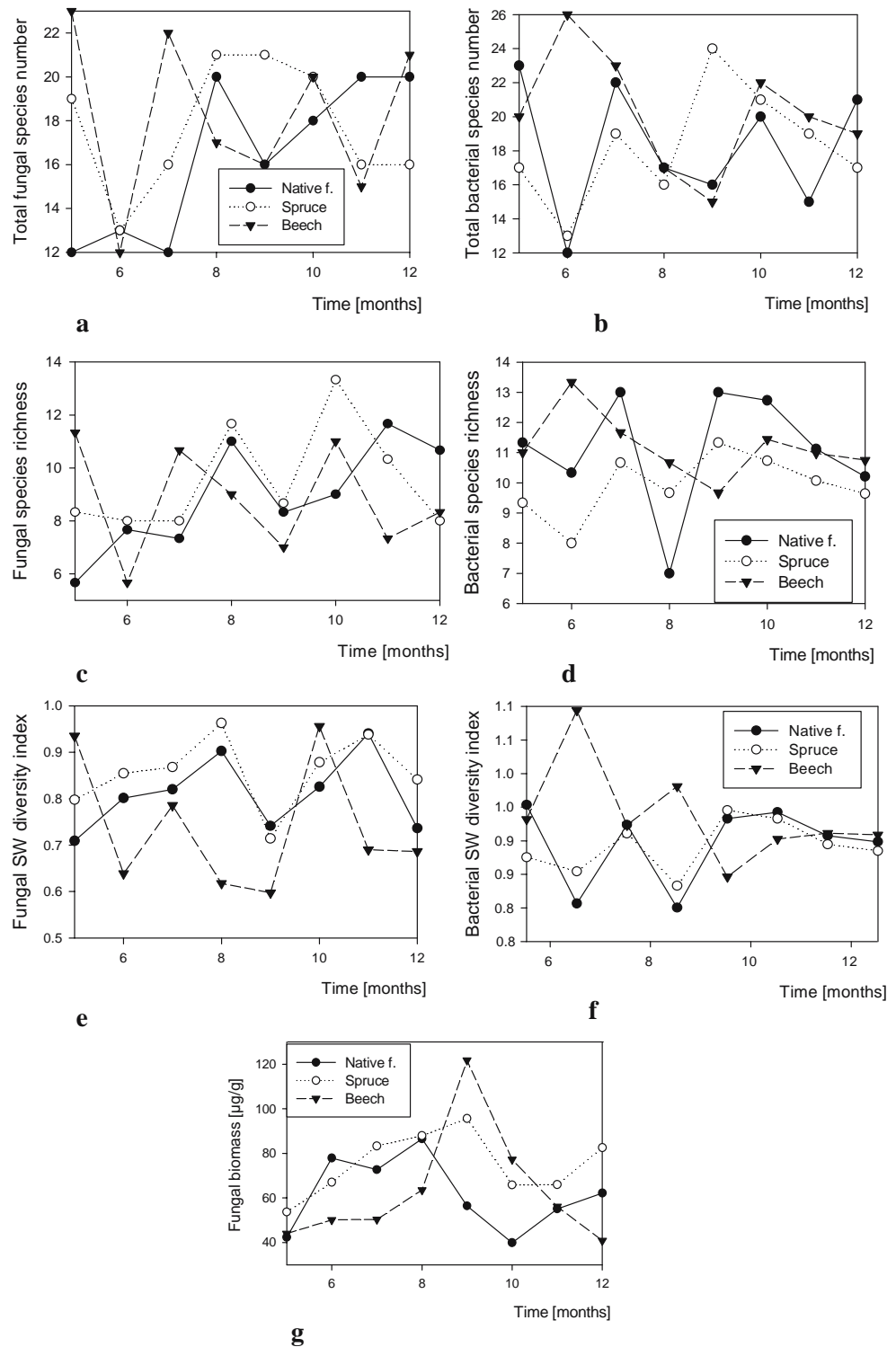
3 months corresponded to May and 10 months to December samplings.

^a Second effect (time or tree stand) used as covariable

^b Total range of values

^c Unimodal analysis (DCA and CCA) had to be used instead of linear analysis (PCA and RDA)

Figure 1 (a–g) Curves of variables along time, from the sampling in May 2004 (3 months of incubation) to the last sampling in December 2004 (10 months of incubation). Numbers on x-axis correspond to months (6 = June, 12 = December). Total number (species pool) of different species detected within all replicates for fungi and bacteria (a, b); SR (average species number per sample) of fungi and bacteria (c, d); SW diversity index of fungi and bacteria (e, f); fungal biomass (g)



period (Fig. 1g). In individual tree plots, spruce and the native forest showed a similar trend except that there was an earlier peak and decline of biomass in the native forest during the summer. In both native forest and spruce, the biomass increased again in the last 2 months. The trend for beech was different; the value heightened sharply in

September from the lowest values of the tree species to the highest for the whole group, and later decreased to the lowest values again. The biomass did not significantly differ between stands, but did within the sampling times.

Forty-six different bacterial species were detected in the 8-month study period (Table 2). The average SR was found

Table 2 Results of the multivariable analyses, total species number, SR, and SW diversity index values in bacterial communities

Data set	Tested effect	Species number	Indirect 1st axe	Direct anal. canonical axes	<i>p</i> value	<i>F</i> ratio	SR	SW index of diversity	
All matrix	Time ^a	46	13.7	4.9	0.002	3.873	10.7	0.908	
	Stand ^a			4.1	0.038	1.427	6–17 ^b	0.63–1.22 ^b	
3 months	Stand	29	24.4	25.4	0.440	1.021	10.6	0.920	
4 months		32	33.7	41.3	0.018	2.108	10.6	0.918	
5 months		33	23	31.5	0.050	1.378	11.8	0.919	
6 months		25	26.4	37.9	0.016	1.827	9.1	0.872	
7 months		33	24.4	41.4	0.002	2.123	11.3	0.908	
8 months		32	23.7	39.6	0.017	1.742	11.6	0.926	
9 months		33	25.9	40.1	0.009	1.964	10.7	0.904	
10 months		30	22.4	36.2	0.027	1.598	10.2	0.897	
Native forest		Time	40	29.5	9.8	0.002	2.849	11.1	0.896
Spruce			38	26.2	8.7	0.034	2.076	9.9	0.891
Beech		43	25.4	6.3	0.002	2.684	11.2	0.937	
Spruce and beech	Moisture			6.4	0.002	2.142			
	Temperature			5.3	0.002	1.724			

See the legend of Table 1.

to be 10.7 species per sample with a total range from 6 to 17 species. Among tree species, the highest richness was found in beech followed by the native forest and the spruce plantation (11.2, 11.1, and 9.9 species per sample, respectively). The same order was obtained for the average SW diversity (0.937, 0.896, and 0.891, respectively) and for the total number of bacterial species detected within all samples from a tree plot (43, 40, and 38, respectively). Neither time nor stand effects were significant for SR or for SW diversity index for all trees together or taken individually.

Generally, the total number of bacterial species detected within all tree plots together increased until July, fell markedly in August, then reached a high value again that was maintained until winter. In individual tree species (Fig. 1b), bacterial SR in beech reached its highest value in June, whereas at that time the SR values were the lowest in spruce and the native forest. In September, beech and the native forest showed lower SR values after a decrease in August, but overall the greater number of species for all dates was detected in spruce.

The bacterial average species number per sample, i.e., SR, was highest in July (11.8 overall), then decreased to the lowest value in August (9.1) and increased again in September followed by a slow decline until winter. There was a certain distinctness in the trend between the tree species effect (Fig. 1d). The decline in July and the increase in September were most evident in the native forest.

The bacterial SW in tree species as a whole remained similar throughout the period, except for a moderate decrease in August (0.87, the lowest value). It reached its highest rate in October (0.93). Spruce and the native forest showed similar courses: two declines in diversity in June and August and otherwise balanced values, only gently

declining until winter. On the contrary, beech presented the highest values in June and August and the lowest values in July and September (Fig. 1f).

Community Structure

Multivariable analyses of community structure showed clear discrimination because of tree species both for fungi and bacteria. Similarly, each sampling date was characterized by a specific pattern.

The first PCA component expressed 10.7% of the variability of the fungal community composition for the whole dataset. In RDA, time and tree species explained only 8.2% of the total variability. Time effect appeared a little stronger than tree species effect (4.7 or 3.9% with tree species or time as the covariable; Table 1). Both time and tree species effect were significant when analyzing with the second factor (tree species or time, respectively) as the covariable. The native forest was the most discriminating toward community structure, regarding the forward selection results. Spruce and beech were less discriminating. They were clearly separated from the native forest in the RDA ordination plot (Fig. 2a).

Community composition was also tested separately for each sampling date and for individual tree species to more precisely identify the changes. The tree species effect was detected as significant in half of sampling times and explained from 22.3 to 44.2% of the species variability (Table 1). Very low tree effect was found in the first sampling in May and quite low again in September. The time effect was important for the native forest and for spruce, whereas the beech fungal community was less affected by the duration of incubation. The variability

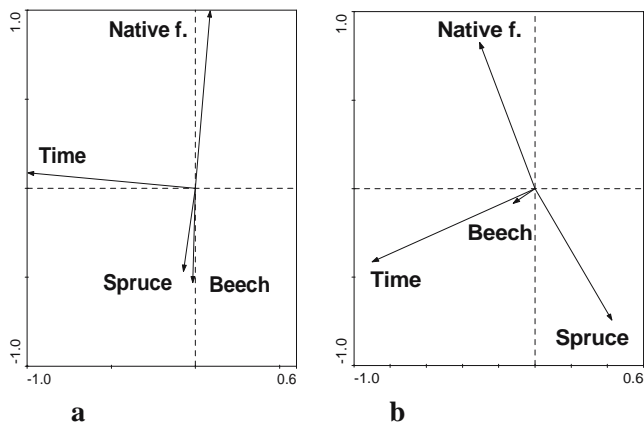


Figure 2 (a, b) RDA plots showing tree species position in ordination space according to community composition development along time. Time was a more determining variable than tree species and thus is closer to the *x*-axis. Spruce and beech, selecting more resembling communities, would be more distinctly separated on the third axis, whereas the native forest is well discriminated in the upper part of the plot; **(a)** fungal communities, **(b)** bacterial communities

expressed by the time effect in individual tree species was 9.4% for the native forest, 8.5% for spruce, and 5.7% for beech.

Concerning bacterial communities, the first PCA axis explained 13.7% of variability for the whole data set (Table 2). As for fungi, time and tree species effect were significant only with the second variable of tree species or time as covariable. Time and tree species effect explained together 8.7% of the total variability for the community structure. Time was more relevant (4.9% with tree species as covariable) than tree species, responsible for 4.1% of variability (time as covariable). The forward selection showed the native forest to be the most discriminating to bacterial community structure, whereas beech was the least. The native forest and spruce were well discriminated in RDA ordination plot whereas beech took approximately a central position (Fig. 2b).

At particular sampling times, tree species effect explained from 25.4 to 41.4% of the variability and was significant every time except for the first sampling. Time effect was found to be significant also in individual stands and explained the highest proportion of the variability for the community in the native forest, less in spruce and the least in beech (9.8, 8.7, and 6.3%, respectively).

Species Patterns

Three of 47 detected fungal species were presented only once in all 72 TGGE profiles, and 8 species were very frequent (present in more than two third of the samples). Thirty-five species were present in all tree stands whereas only ten species were found in the eight sampling times. Nine of them were common to all stands. For bacteria, 4

species from the 46 detected appeared only once in all TGGE profiles, 11 of them were very frequent. Thirty-four species occurred in all stands. Thirteen species were present at all sampling times, 11 of them in all tree species. Besides the generalists, other species were typical, had higher abundances or avoided particular tree species, both for fungi and bacteria. No fungal species, detected at least two times, was present only in one tree plot. Four species avoided native forest, three beech, two spruce.

For bacteria, one species was detected only in beech and one only in the native forest. Spruce was avoided by four fungal species, followed by native forest and beech (two and one nondetected species). Similarly, certain species preferred early or conversely later stages of colonization. However, most species were dependent rather on seasonal fluctuation of soil conditions and appeared either in summer months or both in the early and the late sampling dates. Several species appeared only rarely or randomly.

Fungal species distribution within the tree species after 6 months, in August, is shown in the RDA ordination plot in Fig. 3. August was chosen as the month representing the middle period of incubation with favorable conditions, allowing full development of colonizing species. The tree species effect on community structure was significant and

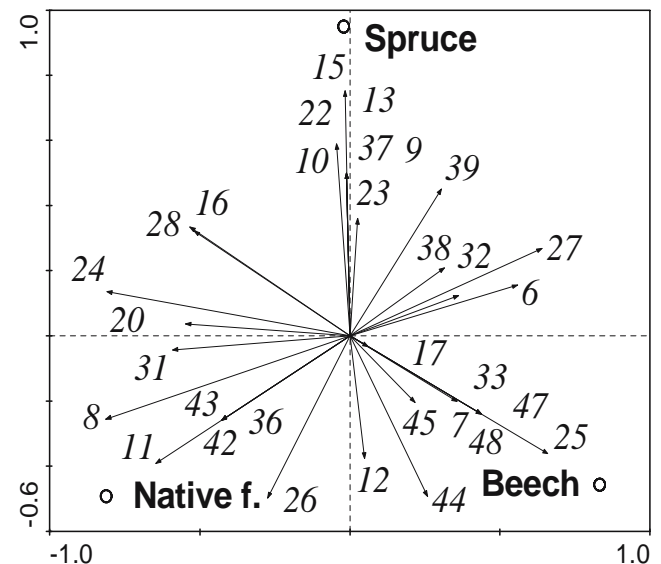


Figure 3 RDA plot illustrating tree species positions in ordination space according to fungal species distribution along the two first canonical axes in August, after 6 months of decay. The tree species effect on community structure was significant and SR and diversity were high that month. The beech and native forest were clearly separated along the *x*-axis. Spruce took a central position on the *x*-axis, but was markedly discriminated on the second, *y*-axis from both beech and native forest, whose position on that axis did not differ. Arrows with numbers represent fungal species detected in the sampling. The length of the arrow indicates relative importance of the species in explaining variability in samples whereas the angle between arrows indicates the degree to which they are correlated

SR and diversity were high in the months of August. Beech and native forest were clearly separated along the x -axis. Spruce took a central position on the x -axis, but was markedly discriminated on the second, y -axis, from both beech and the native forest, whose position on that axis did not differ. The plot shows that the distribution of trees on the RDA ordination plot did not always follow the general trend (Fig. 3) in certain sampling dates. Correlation coefficients of particular fungal species to tree species were evident when analyzing sampling dates separately (Fig. 3); that was not so clear when analyzing all sampling dates together. Species correlations to time when analyzing the tree species together are shown in Fig. 4. The plot shows that there were no species strictly correlated with time. Some species had no seasonal specificity and appeared in the central part of the ordination space, others were present either in the middle time (negative part of y -axis) or both in the beginning and the end (positive part of y -axis) of the study period, as mentioned above.

Environmental Conditions

Soil temperature and volumetric moisture were continuously measured in spruce and beech forest and their role within the seasons was tested more in detail. Beech was found to be

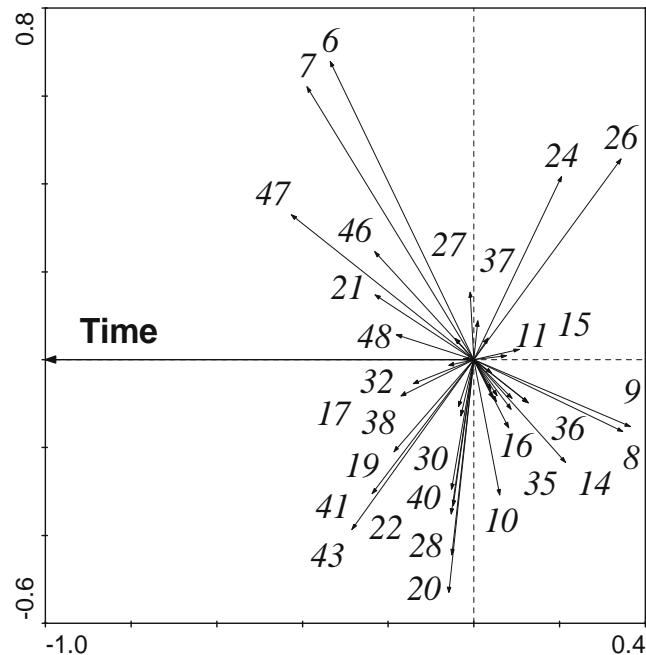


Figure 4 RDA plot showing correlation of fungal species, represented by arrows with species numbers, to time (x -axis) within all tree species stands together. There were no species strictly correlated to time. Certain species had no preferences and were plotted in the central part of the ordination space, others were present either in summer months (negative part of y -axis) or both in the beginning and the end (positive part of y -axis) of the study period

warmer and drier than the spruce forest. Temperature ranged from 8.4 to 16.3°C in beech whereas in spruce it ranged only from 5.7 or 14.1°C with a minimum in spring and maximum in late summer. Moisture reached the highest values in late autumn during the last sampling (20.7% in beech and 23.0% in spruce) whereas the lowest in late summer (9.9 and 13.4% of soil volume, respectively; Fig. 5a).

Fungal community changes showed a higher relation to these two variables than to the duration of incubation. Tree species effect was minor compared to the other variables. Both temperature and moisture effects were significant to fungal community composition and responsible for 5.2 and 5.1% of the species structure variability in beech and spruce together (Table 1). The trend was the same when regarding bacterial community structure in the two forests; 6.4 and 5.3% of the variability were explained by these variables in beech and spruce together (Table 2). The values were slightly higher when testing tree stands individually, namely, for spruce.

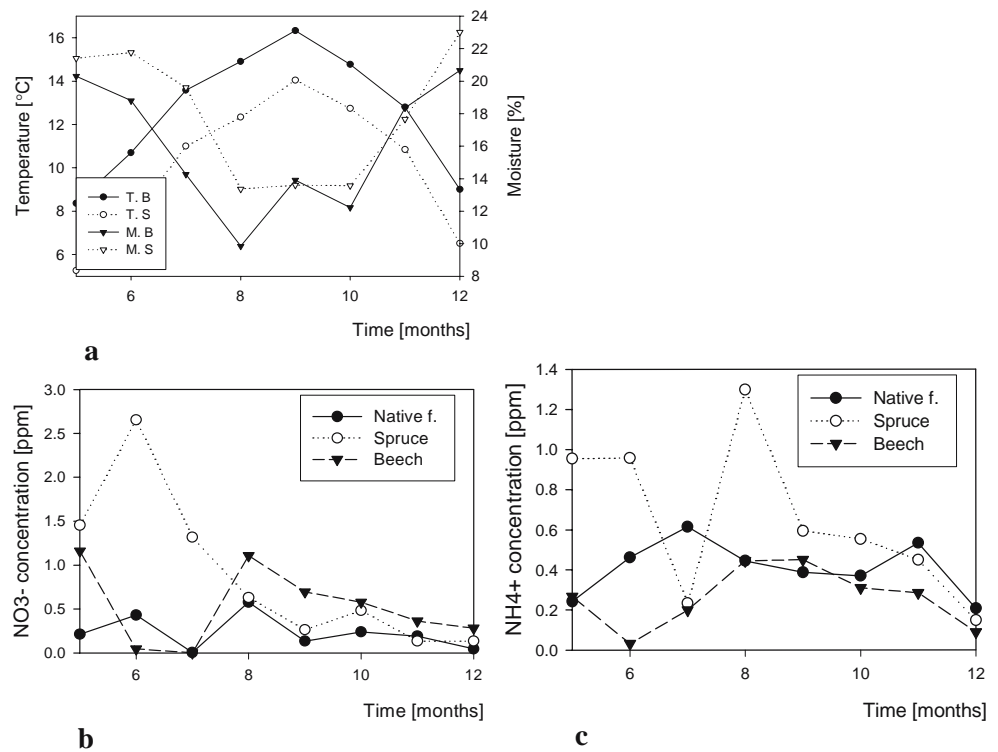
In addition, inorganic nitrogen, both ammonium and nitrates, concentrations in soil solution were collected in all tree stands under the forest floor. The evolution of the concentrations is given in Fig. 5b, c. The concentration of NO_3^- was the highest in the spruce plot until July, and in the beech plot starting with August. Ammonium concentration was mostly the highest in spruce and the lowest in the beech plot. Both ion concentrations explained 5.3% of the whole fungal matrix variability. Nitrate concentration was more relevant than ammonium and its effect on community composition was significant, whereas ammonium was not. However, no species were strongly related to nitrogen ion concentrations.

In individual stands, only the nitrate concentration effect in the spruce plot was significant for fungal community composition, explaining 11.4% of its variability. Hence, NO_3^- concentration was an even more determining factor than time, temperature, or moisture effect in spruce. In other cases, only from 3.5 to 5.7% of the variability were explained by NO_3^- or NH_4^+ concentration and the effect was not important. A similar relation was also found for bacterial diversity.

Correlations of the Variables

Correlation coefficients between studied parameters are given in Table 3, both for individual tree species and for all together with average values as general trends. Fungal total species number, SR, and SW diversity were not necessarily well correlated together. Although their time trends basically matched, there were certain irregularities, mainly in certain periods and in the spruce plot (Fig. 1a–f), e.g., the total number of fungal species present in the spruce plot was still high in September, whereas the SR per sample and SW diversity index fell. The total number decreased in other tree plots at the same time. A month later, in October,

Figure 5 (a–c) Curves of environmental variables along time, from the sampling in May 2004 (3 months of incubation) to the last sampling in December 2004 (10 months of incubation). Numbers on x-axis correspond to months (6 = June, 12 = December). Average soil volumetric moisture (M) and temperature (T) values in beech (B) and spruce (S) forest **(a)**; nitrate and ammonium concentrations in tree plots **(b, c)**



the total number of fungal species present in the spruce plot started to decrease but SR per sample and SW increased; so there was no relation of these variables in spruce. The total number of bacterial species detected within samples, SR per sample, and SW diversity index were better correlated than in the case of fungi.

Fungal biomass was not correlated with total species number, SR, or with SW diversity index. Biomass gradually increased until August despite fluctuations in SR. The main differences occurred in September. Biomass declined in the native forest according to the SR. In spruce, it increased a little and reached a very high value in beech,

Table 3 Correlation coefficients between species parameters, biomass, soil moisture, temperature and time, both for fungi and bacteria (except biomass), in individual tree species and general trend in all trees together

		Fungi				Bacteria			
		Native forest	Spruce	Beech	All	Native forest	Spruce	Beech	All
Total sp. number	SR	0.95	0.54	0.89	0.34	0.94	0.95	0.96	0.86
	SW diversity	0.49	-0.17	0.69	-0.14	0.81	0.90	0.67	0.72
	Biomass	0.04	0.30	-0.29	-0.03	-	-	-	-
	Moisture	-0.22	-0.78	0.07	-0.02	0.21	-0.60	0.29	0.25
	Temperature	0.25	0.54	-0.29	-0.09	0.00	0.67	-0.37	0.15
SR	Time	0.85	0.06	-0.05	0.27	0.00	0.39	-0.35	0.18
	SW diversity	0.67	0.52	0.83	0.73	0.80	0.81	0.84	0.83
	Biomass	0.25	-0.09	-0.08	-0.07	-	-	-	-
	Moisture	-0.21	-0.75	-0.21	-0.55	0.12	-0.62	0.25	-0.01
	Temperature	0.28	0.57	-0.07	0.38	0.16	0.72	-0.35	0.25
SW diversity	Time	0.81	0.32	-0.19	0.48	0.02	0.41	-0.42	0.00
	Biomass	0.36	-0.16	-0.09	-0.55	-	-	-	-
	Moisture	-0.44	-0.12	0.09	-0.23	0.19	-0.33	0.15	0.32
	Temperature	0.44	0.09	-0.26	0.02	-0.03	0.48	-0.34	-0.18
Biomass	Time	0.26	0.19	-0.21	0.04	0.15	0.34	-0.51	-0.29
	Moisture	-0.28	-0.53	-0.40	-0.64	-	-	-	-
	Temperature	0.15	0.78	0.58	0.78	-	-	-	-
	Time	-0.18	0.31	0.16	0.14	-	-	-	-

although it was relatively low before. In October, biomass decreased in all tree species whereas SR increased again. In general, fungal biomass was higher in warmer conditions and lower in humid, the most markedly in spruce again, independent of SR.

Highly negative correlation of total species number and SR per sample to moisture was found in spruce compared to other tree plots for both kinds of organisms. Simultaneously, the most positive correlation to temperature was found in spruce plot, possibly too cold and humid environment for microorganisms. Conversely, the native forest fungal diversity was lower in colder and more humid months. More fungal and bacterial species survived in spruce in September compared to beech and the native forest, probably because of the drought. Another extreme could be found in beech, where SR and SW were slightly negatively correlated to temperature and neutrally to moisture. Maybe it was too warm or dry for certain species in beech in the summer. The coefficient values also showed that bacteria should be more moisturized or rather less drought-tolerant than fungi, which also coincided to the earlier decline of bacterial species numbers in August, whereas fungal decline appeared in September.

Discussion

Methodology

The homogeneity of the Breuil study site provided similar mesoclimatic and geological conditions within involved forest plots. Biases in results were thus eliminated and all differences were assumed to be caused by the changed vegetation. The PCR-TGGE method gives a rapid, repeatable, and relatively confident opportunity to analyze the distribution of microbial species in environmental samples without any cultivation step. In addition, it has been shown that DGGE/TGGE profiles can be used for semiquantitative comparison [11, 23]. PCA confirmed higher resemblance within the replicates from a tree species than to the samples from other tree species.

Time and Tree Species Effect

Incubation time effect was more important in microbial community development than tree species. The most evident shift in species structure within time was found in the native forest, followed by spruce and beech. Time effect was not significant for the fungal community in beech because of an important species shift in summer samplings. The pronounced temporal shift in microbial communities because of changing composition of the decomposing plant material was accompanied by a significant seasonal influence.

Each tree species supported its own unique communities for both kinds of organisms; however, the incubation time and environmental conditions were the main determining factor for the community structure. Certain microbial species were more rare in a tree plot compared to other plots. The overall composition was closer for the young plantations of spruce and beech. The native forest community was the most different for both types of organisms (Fig. 2a, b). Such similarity between beech and spruce communities may indicate that the different plantation environments selected similar communities. Alternatively, these two tree species have not strongly affected the microorganism communities because the plantation. In our opinion, the effect of clear-cutting and replanting disturbances might still be influential for microbial communities. Higher soil temperatures, easier drying, more sunlight, or development of spontaneous vegetation at the clearing stage could cause an important species shift. The cutting influence might be still evident, being not completely overlapped by planted tree species effect yet. The plantations also varied in stand structure from the native forest. They were more dense, even-aged, monospecific, and still in their young stage of development. It can be hypothesized that all species present in plantations originate from the native forest, where they could be present in less active or dormant stages and where they become favored or dominant after tree species change. Several authors [11, 16, 28] have mentioned the potential importance of biodiversity for the stability of an ecosystem after disturbances by providing new species that may ensure the continuation of key functions.

Several recent studies screened the microbial diversity among forest stands, e.g., Hackl *et al.* [14] described the differences of bacterial communities present in 12 different types of native forest in Austria. The shift of communities' structure along the fertility gradient in boreal forests was detected by Pennanen *et al.* [24] and variant spatial distribution of microorganisms in mixed spruce-birch forest was found by Saetre and Baath [27]. Virzo de Santo *et al.* [29] defined differences of litter colonizing fungi among tree coniferous litter species, whereas Wilkinson *et al.* [30] showed the sensitivity of microbial populations' structure associated with decomposing conifer litter to changes in environmental conditions. Grayston and Prescott [13] showed floor layer and site effect to be more important than tree species role in a study of four forests in British Columbia. According to the results of our study, the incubation time effect was more important than the site effect during the decay of beech leaves in Japan [23]. Aneja *et al.* [3] noticed the shift in microbial community during the course of degradation of spruce and beech litter and a unique community profile for each litter sample. In spite that many investigations have demonstrated the influence of

tree species on soil processes, these studies are difficult to compare because they have considered different forest ecosystems located at different sites and have often been affected by factors other than tree cover, such as variation in pedoclimatic conditions [18]. The studies also used different approaches, whether molecular or PFLA, and different scales to answer different questions, which further complicates comparison, generalization, and extrapolation of the results. Thus, further studies are still needed to improve the knowledge of forest ecosystem processes.

Fungal vs Bacterial Communities

The highest fungal richness was found in the spruce forest and the lowest in the beech forest whereas it was opposite for bacteria. This might be indicative of the antagonistic relationship between fungi and bacteria documented, e.g., by Boer *et al.* [8] or Moller *et al.* [21]. SR of fungi and bacteria developed comparably in spruce, which was not the case of the beech and native forest. But generally, bacterial SR decreased already in August, a month earlier than the fungal one. Both fungal and bacterial communities differed in dependence mainly on ecological conditions, incubation time, or tree species. Similarly, Lejon *et al.* [19] documented a significant discrimination between fungal and bacterial communities indigenous to the tree species, using the RISA approach in the same study site. The bacterial communities were found to be more discriminating than fungal ones in general and the tree species effect in individual sampling dates was more often significant for bacteria as well. Agnelli *et al.* [2] also pointed out higher bacterial than fungal diversity between soil profiles in an 80-year-old *Picea alba* plantation in Apennines. On the contrary, Lejon, *et al.* [19] showed that fungal diversity was more discriminated compared to the bacterial community in the upper soil layers in the same study site, and Leckie *et al.* [18] demonstrated the same in forests of the Vancouver Island. In the present study, fungal communities were conversely found to be more dynamic with faster species turnover because of actual ecological conditions than bacterial ones. Both communities were clearly unaffected by tree species in the first sampling after 3 months of decay in early stages of colonization. Fungal community was also highly unaffected by tree species in September, when the role of environmental conditions dominated.

Environmental Condition Effect

Environmental conditions were found to have a major importance for both species numbers and community structure. Their effect overlapped the incubation duration and tree species role for both fungal and bacterial communities. Few species were found at all dates, although the majority was

found at least randomly present in all tree stands. SR increased with the beginning of summer but decreased later because of dry conditions in the site before September sampling. Next, SR increase followed moisture increase in October. The next decline was observed later because of the influence of the temperature decline in late autumn. During the summer, many species present in colder and more humid months were replaced by others that prefer the more extreme hot and dry summer conditions. Another hypothesis could be that the fade-away of otherwise common species in the summer allowed feeble competitors to develop even in unfavorable conditions. Analyses confirmed that many species were present both in early and later months whereas different species were detected only in summer samplings and some were rare or present just randomly. These “summer” species, although less numerous, produced high biomass, especially in spruce and beech. In our opinion, summer months still had higher amounts of nutrients released from litter because of higher microbial activity, which could by feedback effect sustain the higher activity and/or biomass production of drought-surviving species. Such seasonal changes in community composition show the importance of studying the whole pool of species present in the soil and forest floor and not only the species present at a unique period. Berg and McLaugherty [6] also found that the abundance of bacteria and fungi in a Scots pine forest in The Netherlands was influenced by soil water content, and that of fungi also by temperature, and showed clear seasonal pattern. Hyphae length was positively related to moisture and negatively to temperature in the same study. Inorganic forms of nitrogen concentration did not seem to be widely important either for fungal or bacterial communities, which is in agreement with our results.

Conclusions

The TGGE was used as a powerful fingerprinting technique to analyze fungal and bacterial communities present from the 3rd to the 10th month of the decay of cellulose strips, covering the period from spring to autumn. SR was not highly affected by tree species change. It did not increase with time, mainly because nonbounded cellulose is an easily accessible material for microbial ingrowths. The microbial communities were distinct in the individual tree stands and developed over the period of decomposition. Many species were detected in all three tree stands, but highly differed in patterns. Conversely, no species were typical for only one tree stand. The actual community composition was highly related to environmental conditions, which seemed to be more important for the presence or absence of microbial species than incubation time or tree species. An interesting situation was found in the summer, when the fungal biomass reached its highest value,

although the SR was low after the elimination of commonly abundant species, probably by drought combined with high temperatures. At the same time, rare or infrequent species were detected, disappearing later with the reappearance of the abundant species. Such species were for the most part present both in the spring and in the autumn, being replaced only in the summer. The correlation coefficients between studied variables highly differed between the tree stands, which could be caused partly by the environmental conditions and partly by different decomposer community responses in a given tree stand. However, all variables seemed to be very complex and inseparable. To link the decomposer diversity to ecosystem functioning, we also studied several parameters of the strip decomposition, such as the decay rate, cellulase activity, or nitrogen accumulation. The results and the aggregate discussion are given in the second part of this study.

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Microbial diversity during cellulose decomposition in different forest stands:

II. Functioning and role of diversity

Ariana Kubartová ^{1*}, Judicaël Moukoui ^{2,3}, Colette Munier-Lamy ³, Jacques Ranger ², Jacques Berthelin ³, Thierry Beguiristain ³

(1) Department of Botany, Faculty of Sciences, Charles University, Benátská 2, 128 01, Praha 2, Czech Republic

(2) INRA, Centre de Nancy, Unité Biogéochimie des Ecosystèmes Forestiers, 54 280, Champenoux, France

(3) LIMOS, CNRS UHP UMR 7137, Université Henri Poincaré, BP 239, 54 506, Vandoeuvre-les-Nancy, France

Correspondence to: Ariana Kubartová; Department of Botany, Faculty of Sciences, Charles University, Benátská 2, 128 01, Praha 2, Czech Republic

Tel: +(420) 221 951 646, E-mail: arikul@centrum.cz

Abstract

The objective of this study was to relate microbial diversity and environmental conditions to decomposition activity of the microbial communities. The experiment was performed in two monospecific plantations and in a native forest as a reference stand. Cellulose strips, as a model substrate, were incubated into the forest floor over ten months to assess for decomposition rate, microbial diversity and activity. The highest mass loss at the end of the experiment was found in beech and the lowest in the native forest. Beech stand also showed the highest N accumulation and the lowest C/N ratio. The decomposition rate of cellulose and the concentration of accumulated nitrogen were increasing over the year. The cellulase activity was mainly related to fungal biomass and was the highest in summer. Decomposition activity was not significantly influenced by fungal or bacterial species richness. The studied variables were highly affected by seasonal changes, mainly during summer, which overlapped both time and tree stand effects. Many microbial species were suppressed in summer and replaced by other infrequent species. However, such changed communities were able to produce the highest cellulase activity, decomposition rate or biomass values. Important differences in correlations of diversity, activity and environmental variables occurred among tree species. Tree substitution did not cause any significant

decrease in biodiversity, which would limit decay. Moreover, high functional redundancy and competition were probable, when decomposing simple cellulose material, not bound to lignocellulolytic complexes. The process of decomposition seems to be very complex and dependent on many factors, therefore a simple generalization cannot easily be made.

Keywords: Cellulose; Decomposition; Microorganisms; Forests; Biodiversity; Activity.

1. Introduction

Most literature on the relationship between biodiversity and ecosystem functioning has focused on aboveground parts of the systems. Advanced techniques such as molecular approaches have opened new possibilities to investigate the diversity of soil microorganisms and functions (Anderson 2004, Leckie 2005). Changes of aboveground species diversity also have implications for belowground ecosystem functioning, which may, in turn, affect the decomposition of organic material and, thus, the mineralization of nutrients and cause feedback effect on the plant community and whole ecosystem (Wardle et van den Putten 2003). Over 95% of the biomass of most decomposer food webs is composed of microbes, *i.e.*, fungi and bacteria (Setälä 2002) and soil communities are among the most species-rich components of terrestrial ecosystems (Jones et Bradford 2001). Despite that, the linkages between microbial biodiversity, its functions and soil processes have not yet been clarified. It is of overriding importance, whether species diversity is the main driver of ecosystem functions (Schwartz et al. 2000, Srivastava 2002) or if its effects are rather unimportant in comparison to the more direct effect of habitat changes. The extent to which the complex relationship between plants and micro-organisms influence organic matter dynamics is also critical to our understanding of global carbon cycles, sources and sinks, in the view of the global change. Related questions include how management practices affect ecological processes to set goals of sustainable management (Bengtsson et al. 2000, Griffiths et al. 2001).

Increasing tree diversity in forests should lead to an increase in microbial biomass and activity by higher niche variability (e.g. diversity of carbon sources in more variable litter types and root exudates, higher primary production, variable microclimate, presence of wood debris or spatial and age heterogeneity). Further, it is expected that greater microbial diversity ensures faster organic matter decomposition at a higher rate than a system with a few taxa, because the reduction in complexity may result in reduced activity. Nevertheless, the species richness is not the only issue; the gene-bank, functional characteristics and resource use

efficiency of individual species are important as well. Positive or negative interactions between co-occurring microbial taxa may also be relevant, e.g. in the acquisition of nutrients, in the sequential attack of substrate or in competition for space (Setälä et al. 2004, Deacon et al. 2006). The effect of diversity on ecosystem function could be negative, due to over-saturation and enhanced competition, or weak because of saturation at low species richness or functional redundancy interactions, as reviewed by Ekschmitt et al. (2001), Mikola et al. (2003), Wardle et al. van der Putten (2003). However, even if soil biodiversity is unimportant under steady-state conditions, it may significantly affect the ecosystem stability in response to disturbances. A diverse community would be more likely to contain species able to tolerate a disturbance than a poor one (Wardle et al. van der Putten 2003, Setälä et al. 2004).

This study aims to assess the relationship between microbial species richness, community composition and decomposing activity to identify the interest of biodiversity to forest ecosystem processes. The questions to answer include: i) how does tree species and time affect the dynamics of cellulose decay, ii) which variable is the most relevant to explain the relationship between ecosystem functioning and species richness, community composition or specific activity of species iii) what is the role of individual species and their frequency of occurrence.

2. Materials and Methods

2.1. In situ experiment

The information about decay and microbial colonization of a model material, such as cellulose, in monospecific and even-aged forest sites can improve our understanding of the factors regulating complex ecosystem processes. The study site of Breuil, situated in French Burgundy in the west part of Massif Central, includes stands of different tree species in 30-year-old plantations and a semi-native forest as a control stand, 1000m² each of them. The native forest (*Fagus sylvatica* L., *Quercus petraea* Liebl. in the high forest strata, *Betula pendula* Roth., and *Corylus avellana* L.) and two plantations (Norway spruce, *Picea abies* Karst., and European beech, *Fagus sylvatica* L.) were chosen for the incubation of cellulose strips. Vegetation should have caused all environmental differences between these three forests due to the homogeneity of the study site. Five round cellulose strips (Whitman, ø 47mm, total weight ±0.9g) were enclosed in a litter bag with mesh size 5µm and placed into the terrain in late February 2004. Seven bags were collected in 4 week intervals, i.e. once a month, in each of the stands over 10 months until early December 2004.

2.2. Biotic variables

The molecular analyses of microbial communities were described in detail in a previous paper (Kubartova et al. 2007); a brief overview is provided here. Bags from the 3rd to 10th samplings (May-December) were used to analyze the diversity of fungal and bacterial species. DNA was extracted from all samples using the Dneasy plant mini kit (Qiagen) following the manufacturer's protocol. DNA was amplified by PCR using ITS1F and CGITS2 primer pairs for fungi (50 µl of PCR assays contained 2 µl of template, 5 µl of PCR buffer (Sigma, 100mM Tris-HCl, pH 8.3, 500mM KCl, 15mM MgCl₂), 1 µl of dNTP (10mM), 1 µl of each primer (20 µM), 0,5 µl of Taq-polymerase (5 units/µl, Sigma), 1 µl of GC-rich solution (Sigma); the amplification regime was as follows: initial cycle of denaturation at 95° C for 3 min, followed by 35 cycles of the following: denaturation at 94° C for 45 s, annealing at 55° C for 45 s and extension at 72° C for 1min 15 s, ending with the final elongation at 72° C for 8 min. 1401r and CG968f primer pairs were used for bacteria (similar reaction mixture with 2 µl of BSA (3%) added, the PCR regime differed slightly: denaturation at 94° C for 5 min, 35 cycles of the following: denaturation at 94° C for 40 s, annealing at 56° C for 30 s and extension at 72° C for 1 min, and the final elongation step at 72° C for 5 min). All PCR reactions were performed with iCycler Thermal cycler (Biorad).

PCR products were then separated on acrylamide gel by temperature gradient gel electrophoresis (TGGE) and visualized under UV light after staining with ethidium bromide. TGGE was performed with a Dcode Universal Mutation Detection system (Biorad). For fungi, the polyacrylamide gels were prepared with 8% of acrylamide (wt/vol), 8M of urea, 1.25x TAE, 0.2% of glycerol (vol/vol), and with 300 µl of ammonium persulphate (10%) and 30 µl of TEMED as polymerization agents. Amplified DNA samples (10 µl plus 10 µl loading buffer) were separated by electrophoresis in 1.25x TAE at a constant voltage 145V with a temperature gradient from 50° C to 55° C with a temperature increment of 1 degree per hour and stained with ethidium bromide. For bacteria, TGGE differed in that we used 7% acrylamide gels (wt/vol) and a temperature gradient from 58° C to 63° C with the same temperature increment. TGGE optimization enabled the detection of 50 bands, differing in $T_m=0.1^\circ\text{C}$. Species distribution and relative abundance matrices were created both for fungi and bacterial communities. Then i) total species number ii) average species richness iii) Shannon-Wiever diversity index (SW) and iv) community composition were recorded and used for statistical analyses. Fungal biomass on cellulose strips was determined measuring ergosterol content in fungal membranes (Ruzicka et al. 2000, Zhao et al. 2005) and described

in a previous paper (Kubartova et al. 2007). Twenty of the most important fungal bands were sequenced previously (Kulhankova et al. 2006). However, the species are presented only as numbers, since their identification to genus level was not possible.

In this study, fungal and bacterial community composition data were related to each of the activity variables using RDA in order to detect the most important species (data not shown, for summarizing RDA plot (fungi) see Fig. 5). Five fungal species, the most relevant for the activity and/or diversity, were detected. Their abundances and life-strategies are described more closely below.

2.3. Environmental variables and microbial activity

Soil temperature (Campbell data logger CR10 and probes 107, Shepshed England) and moisture (TDR Trase system BE, Soil Moisture equipment Corp. Santa Barbara, California US) were continuously measured every 4 hours in the studied plots (except the native forest due to technical reasons) and their average values during the four week period between samplings were used for analyses. Ammonium and nitrate concentrations were continuously monitored in soil solutions collected under the forest floor in the three stands using zero tension thin plate lysimeters (Ranger et al. 2004, Kubartova et al. 2007).

In this study, microbial activity was targeted. Mass of the strips was determined after 24 hours of drying at 65°C. Decomposition rate was expressed in mg per day with 4 weeks of interval between the sampling dates. Olson's exponential model $k = -1/t \cdot \ln(X_t/X_0)$ was used for the whole period of decomposition, where X_0 = initial mass loss, X_t = final mass loss and t = time of the decomposition (Olson 1963). Cellulase activity was investigated using colorimetric determination of reducing sugars (Deng et Tabatabai 1994, Criquet 2002). Reducing sugars were extracted from an aliquot of powdered cellulose with deionised water. After mechanical shaking (60 min) and centrifuging (20 min at 3500 rpm), extracts were filtered through 0.2µm pore size membranes. The filtrate was treated with Somogyi-Nelson reagent and the absorbance measured at 710 nm with a spectrophotometer Beckman DU-70. The calibration curve was established with glucose, and cellulase activity was expressed as µg glucose/mg cellulose. Each sample was finely ground, dried at 65°C and treated using CHNS-O 1108 Carlo Erba analyzer (combustion at 1020°C) in order to assess carbon and nitrogen concentrations.

The activity data (mass loss, decomposition rate, cellulase activity, N accumulation) were tested by computing ANOVA using Statistica software (StatSoft). The correlations between the activity data and environmental variables or microbial parameters (Kubartova

2007) were described by Pearson's correlation coefficient (correlation $\rho_{X, Y}$ between the variables X and Y with respected values μ_X and μ_Y and standart deviations σ_X and σ_Y was defined as: $\rho_{X, Y} = \text{cov}(X, Y) / \sigma_X \sigma_Y$, where $\text{cov}(X, Y) = E((X - \mu_X)(Y - \mu_Y))$, in which E is the expected value of the variable and cov means covariance). Principal component analysis, PCA, and redundancy discriminate analysis, RDA, were used to visualize the relations among more variables and fungal species. These were computed by Canoco (Microcomputer Power).

3. Results

3.1. Microbial activity during decomposition

The mass loss of cellulose strips was measured along ten months, from February to December (Fig. 1). The common trend for all tree species was an increase in decomposition rate until September (Fig. 2a). The mass loss was significantly lower in beech until May (Fig 1), but the highest of all tree species in June. The remaining mass was 13.9% in the last sampling. The mass loss was the lowest in spruce and intermediate in the native forest between June and October. The decomposition slowed down in the native forest after September and thus the highest remaining mass was observed there at the end of the experiment (39.5%, compared with 16.0% in spruce). Decomposition constant k was the highest in beech, middle in spruce and the lowest in the native forest (2.51, 2.3 and 1.44*year⁻¹ respectively). Mass loss values differed significantly among tree stands.

Likewise, the cellulase activity increased until September, mainly in beech (Fig. 2b). It was intermediate in spruce and lowest in native forest until September, but with similar values in December for all (Fig. 2b). The average cellulase activity values were 0.68, 0.43 and 0.34 μg glucose/mg cellulose in beech, spruce or the native forest, respectively. Cellulase activity differed significantly among tree stands and over time in an individual tree stand.

Nitrogen concentration on the cellulose strips increased over time (Fig. 2c), as nitrogen was immobilized by the microorganisms. It attained up to 0.5% in the native forest, 2.7% in the spruce and 3.8% in the beech at the end of incubation. Nitrogen concentration significantly differed among tree stands.

The carbon/nitrogen ratio (Fig. 2d) decreased over time, probably because nitrogen accumulated in the remaining cellulose and the carbon concentration increased very slightly (beech) or maintained almost the same value throughout the period (spruce and the native forest, data not shown). At the end of the experiment, the lowest C/N ratio was found in beech and the highest in the native forest, but the differences were not significant among tree stands.

The colonization of the strips, represented by an increase in biomass, species richness, decomposition rate and N concentration, was significant in the early stages of the experiment. It was overbalanced by effect of season in summer.

3.2. Links between microbial diversity, activity and environmental conditions

Microbial activity variables, targeted in this study, were correlated to fungal and bacterial diversity as well as to environmental conditions, described previously (Kubartova 2007). The Pearson's correlation coefficients were shown in Table 1. Decomposition rate of the four week intervals between samplings was the most positively correlated to time of decomposition, slightly positively correlated to temperature and fungal biomass. It was not significantly related to fungal or bacterial species richness. Cellulase activity showed the highest positive relationship with fungal biomass; however it was often negative with fungal species richness. It was moderately negatively correlated to time and there was no link to bacterial species richness. Soil temperature influenced the cellulase activity value rather positively, moisture rather negatively. The nitrogen accumulation was highly correlated to decomposition rate (Table 1). The lowest C/N ratio coincided with the fastest decay in the beech stand.

Significant differences in correlations of diversity, activity and environmental variables occurred between tree species. In the native forest individually, decomposition rate was more positively correlated to both bacterial and fungal species richness, to moisture and temperature than in other tree species. It was more negatively related to fungal biomass (Table 1, Fig. 3). In spruce, decomposition rate was more positively related to bacterial species richness than in the other stands and less influenced by moisture and temperature. In beech, the neutral relationship of decomposition rate to fungal species richness and negative relationship to bacterial species richness was found. It was more positively correlated to fungal biomass than in other tree stands.

PCA plots (Fig. 3) showed correlations of studied variables in the individual stands, as well as the position of sampling dates in the ordination space. The RDA diagram in Fig. 4 showed the same characteristics, when tested within time and tree stands. The increase in decomposition rate over time was evident, contrary to cellulase activity. It also documented only a weak relationship between cellulase activity and species richness. Time, joined to the x-axis, was a major parameter in comparison to tree stands, spread along the y-axis. Spruce was well separated from both broadleaved stands. The next RDA plot (Fig. 5) showed relation

of the fungal species to the variables. There were no species highly correlated with time. The summer-abundant species were grouped in the lower left part of the plot.

3.3. Abundance and distribution of selected fungal species

We showed in a previous paper (Kubartova 2007), that the most distinct community structure was found in the native forest (tested by RDA), both in bacterial and fungal communities. Many of both fungal and bacterial species were present in all three tree stands, but with different patterns. Distribution and abundances of five fungal species, the most relevant for decomposition and/or diversity (tested by RDA, not shown), within time and tree stands are shown in Fig. 6. The closest sequences from the database (Genbank) are indicated in the graph headlines. All the related species are known as woodland saprophytes.

Species number 7, closely related to *Megacollybia platyphylla*, was the third common species, detected in 41 TGGE profiles from 72. Macrocarps of the species had been found in the study site previously (Ranger et al. 2004). Its abundance increased with time, mainly in the native forest and spruce. Its dominant appearance was detected in the native forest, where it was present in all three replicates during the last four months. It appeared more randomly in beech than in spruce, but as often (11 and 12 occurrences, respectively). It showed a neutral relationship to soil moisture, temperature and cellulase activity.

Species number 46, most resembling to the sequence of *Chaetosphaeria pulviscula*, was detected 19 times, mostly in spruce. In beech and the native forest, it was detected in all replicates in November and almost no other. In spruce, it was typical both in spring (June, July) and in autumn (October, November) and avoided summer months, which were possibly too warm or dry. Thus, it preferred a combination of high moisture and temperature. It was slightly negatively correlated to cellulase activity.

The next species, number 23, close to *Phlebia albida*, was detected 26 times, most frequently in the native forest. It also represented a species decreasing over time there, even though it was detected again in December. It was also common in spruce, mainly during the summer months. It could support its preferences for moderate moisture and temperature values. In beech, it appeared rather randomly. It was only slightly correlated to cellulase activity.

Species number 16 has not been sequenced. It appeared mainly in summer and thus was well correlated to the cellulase activity (tested by RDA, not shown). It was moderately frequent with 17 occurrences, mainly in spruce. It seemed to prefer higher temperatures at sufficient moisture levels. The Ascomycete number 37, with a sequence resembling to

Coniosporium apollinis sequence, was an example of a rare, randomly present species, detected only seven times, mostly in beech. Still, it appeared in periods of high cellulase activity and was positively correlated to it.

4. Discussion

4.1. Microbial activity

The use of cellulose strips was supposed to eliminate the effect of differences in initial chemical composition of the substrate and, thus, effects of tree stand or season should be more obvious. However, quite an important accumulation of nitrogen from the surrounding environment was found in the later months of the incubation (Fig. 2c). That might be relevant for micro-organism abundances and activities in later stages. The increasing importance of biotic interactions throughout time could also be influential (Dilly et al. 2001), especially in the case of an easily accessible substrate, such as cellulose. All the tree stands in the Breuil research site had originally the same abiotic conditions. Thus, variations in physical and chemical properties were expected to be related to tree species change. For the same reasons, changes in microbial diversity and activity are also supposed to be driven by the tree species.

Decomposition rates and nitrogen accumulation have been studied and discussed in many experiments since ecosystem studies have become common (Berg et McClaugherty 2003, Prescott 2005). Similarly, cellulase activity of forest soils has been described in several studies, e.g. in dependence to nitrogen supply (Saiya-Cork et al. 2002, Waldrop et al. 2004), among native and exotic species (Kourtev et al. 2002) or across European beech, spruce and pine forest gradients (Andersson 2004). The latter noted the burst of cellulase activity in the litter layer in July, which coincided with the summer increase of cellulase activity in the present study. Some common phenomena, such as acceleration of the decomposition rate or nitrogen accumulation can be found in all of the studies. As shown by Carreiro et al. (2003), the nitrogen stimulates cellulolytic enzymes such as β -glucosidase, cellobiohydrolase and endocellulase. This could explain the high decomposition rate observed under the beech plantation, where nitrogen incorporation was significantly highest. The slowing of decomposition and the decrease in cellulase activity in the native forest during the later stages was hard to explain. It could coincide with the strong retention of organic forms of nitrate and phosphorus and low nitrification rate, detected in the native forest (Ranger et al. 2004).

It is generally assumed that fungi are the main decomposers of organic matter in forests (Ruttimann et al. 1991, Leckie et al. 2004). Bacteria are considered not to degrade tree litter as efficiently due to their restricted ability to penetrate solid substrates and to be

confined to easily accessible, i.e. non-bound, cellulose. They are mostly dependent on substrate released by fungal activity, mainly when considering very recalcitrant material such as a lignocellulose complex. A microcosm experiment showed that cellulase activity on beech leaves was mainly of fungal origin (Moller et al. 1999). However, in the case of semi-degradable organic matter, such as cellulose, both competitive and mutualistic strategies have been noted, and bacteria and fungi are seen to compete most likely for carbon substrate (Moller et al. 1999, Boer et al. 2005). That was most probably the case in the present study, because non-bound cellulose from strips should be easily accessible for both and thus the role of bacteria could be more important.

4.2. Links between microbial diversity and activity

Important differences in correlations were found among tree stands. Hence, there may not be a universal and clearly predictable relationship between diversity and function in forest soils.

The contribution of fungal species richness to decomposition rate was both positive (the native forest and spruce) and negative (beech). The effect of fungal species richness to the cellulase activity was either neutral or negative (the native forest, beech). Similarly, the effect of bacterial species richness was neutral or negative (beech). Comparing the tree stands together, beech showed the lowest fungal- and the highest bacterial species richness, the highest cellulase activity and decomposition rate. Conversely, spruce had high fungal- and low bacterial species richness, the cellulase activity was only moderate and mass loss was the lowest until September. That signified importance of other variables than the species richness alone. Functional efficiency of fungal communities could theoretically increase with the number of taxa, but it was previously found to be significant at the species-poor end of the gradient only (Cox et al. 2001, Setälä et McLean 2004). The biomass had a positive effect on decomposition rate as well, except for the native forest, and was independent on fungal species richness (Kubartová et al. 2007). Fungal biomass and cellulase activity were positively correlated in all tree stands.

Community composition is likely to be more important than species richness when related to ecosystem functions (Jones et Bradford 2001, Setälä et McLean 2004). In this study, the community composition differed among tree stands, as well as the activity variables. However, spruce and beech monocultures displayed similar microbial communities within all samplings together. In spite of that, the decomposition variables differed significantly between them. This study also indicated that a diagnostic species with respect to

diversity may not necessarily be a key species for the functions. In addition, there may be no relationship between taxonomic and functional diversity for fungal species, because intra-specific variation in function can be high (van der Heijden 2004). No key species seemed to be lost in this study. The non-bound cellulose was supposed to be easily accessible for the microorganisms. In richer communities, high functional redundancy and niche overlap could be expected and the diversity effects could be grossly buffered (Ekschmitt et Griffiths 1998, Cox et al. 2001). Thus, the decomposition would be influenced by competition among species rather than by necessity of presence of highly specialized, and thus keystone species. The differences in the decomposing activity among studied tree stands were caused by diverse microclimatic conditions, nutrient status and organic matter quality in the stands (Ranger et al. 2004) rather than the microbial diversity decline.

4.3. Links between microbial activity and environmental conditions

The results showed that the biodiversity was not connected just to the number of species present at a given time, but to a pool of species present during different periods of the year. Microbial activities were previously documented to be regulated by seasonality and environmental changes (Aerts 1997, Berg et al. 1998, Zhang et Zak 1998, Wilkinson et al. 2002). In this study, temperature had a significant positive effect on both decomposition rate and cellulase activity in all tree stands. Conversely, the effect of soil moisture was always negative due to low moisture in warm months and vice versa (Kubartova et al. 2007). Community composition changed during the dry summer months. Several abundant fungal species were not detected, while infrequent species, normally undetectable, were present, probably because of the low competition.

The highest fungal biomass and cellulase activity of the entire study period were reached simultaneously. It was found that several species that correlated to high cellulase activity and biomass were not very frequent even in the high activity periods. It was the case of e.g. species nr. 16 (Fig. 5, 6). Such infrequent species might possibly be the bad competitors, but stress-tolerant and capable of high activity when a disturbance eliminated the abundant species. Accordingly, Osono and Takeda (1999) found that occasional and infrequent fungi play important roles in decomposition and were potentially more active than the frequent taxa. In their study, several infrequent species having a higher overall activity were able to use a wider range of substrates than the abundant species. Even the remaining abundant species might show higher than normal activity in summer, coinciding e.g. with favorable temperatures and higher amounts of nutrients released from the surrounding litter.

The spruce stand differed in environmental and microbial activity characteristics from both the young plantation and the native forests dominated by beech (Fig. 4). However, the microbial communities resembled more closely in the young plantations comparing to the native forest (Kubartova et al. 2007). The resemblance could possibly be explained by the importance of the development stage of the forests to the microbial communities. Further, community changes during the clear-cut, re-plantation and clearing stage might still have been in effect.

4.4. Conclusions

Decomposing activity during cellulose decomposition was studied and correlated with microbial diversity and environmental variables in order to describe the role of biodiversity to forest functions. The highest values of decomposition rate, cellulase activity, nitrogen accumulation and the lowest C/N ratio were predominantly found in the beech stand. Microbial species richness or community composition did not seem to be the main driver of the decomposing activity. Both diversity and activity appeared to be affected rather by the actual environmental conditions. Comparing the differences in correlations of the activity, diversity and environmental variables even between three studied neighboring forests we can suggest the variables to be highly interrelated and the processes to be very complex.

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Tables and figures

Table 1. Pearson's correlation coefficients between decomposition rate or cellulase activity and species variables, biomass and soil moisture, temperature, time of incubation, N accumulation and C/N ratio in individual tree species and the general trend for all. F – fungi, B – bacteria

Variable 1	Variable 2	Native f.	Spruce	Beech	all
Dec. rate	species rich. F	0.45	0.20	-0.08	0.47
	SW diversity F	0.14	0.05	-0.09	-0.04
	total sp. F	0.51	0.07	-0.05	0.30
	biomass F	-0.25	0.29	0.40	0.33
	species rich. B	0.35	0.40	-0.56	0.17
	SW diversity B	0.31	0.37	-0.70	-0.21
	total sp. B	0.16	0.43	-0.38	0.29
	moisture	-0.37	-0.14	-0.27	-0.18
	temperature	0.71	0.22	0.47	0.45
	time	0.67	0.97	0.94	0.94
	nitrogen (%)	0.96	0.92	0.91	0.92
	C/N ratio	-0.58	-0.69	-0.82	-0.71
	cellulase act.	-0.21	-0.61	-0.32	-0.31
	Cellulase act.	species rich. F	-0.54	0.08	-0.38
SW diversity F		0.04	-0.07	-0.52	-0.62
total sp. F		-0.65	0.48	-0.31	-0.17
biomass F		0.43	0.46	0.63	0.75
species rich. B		0.06	0.11	-0.56	0.07
SW diversity B		-0.32	-0.19	-0.53	-0.08
total sp. B		-0.03	0.04	-0.29	0.00
moisture		-0.46	-0.51	-0.27	-0.41
temperature		0.40	0.45	0.41	0.47
time		-0.78	-0.58	-0.41	-0.53
nitrogen (%)		-0.24	-0.81	-0.61	-0.64
C/N ratio		0.05	0.14	0.19	0.00

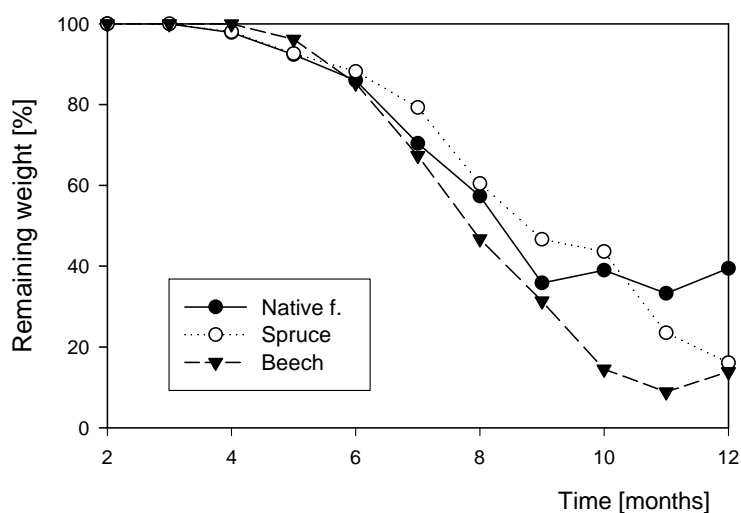


Figure 1. Mass loss of the cellulose strips in the three tree species stands studied from installation (February, 100%, number 2 on x-axis) to the 10th month (December, 13.9%-39.5%, number 12) of the decomposition.

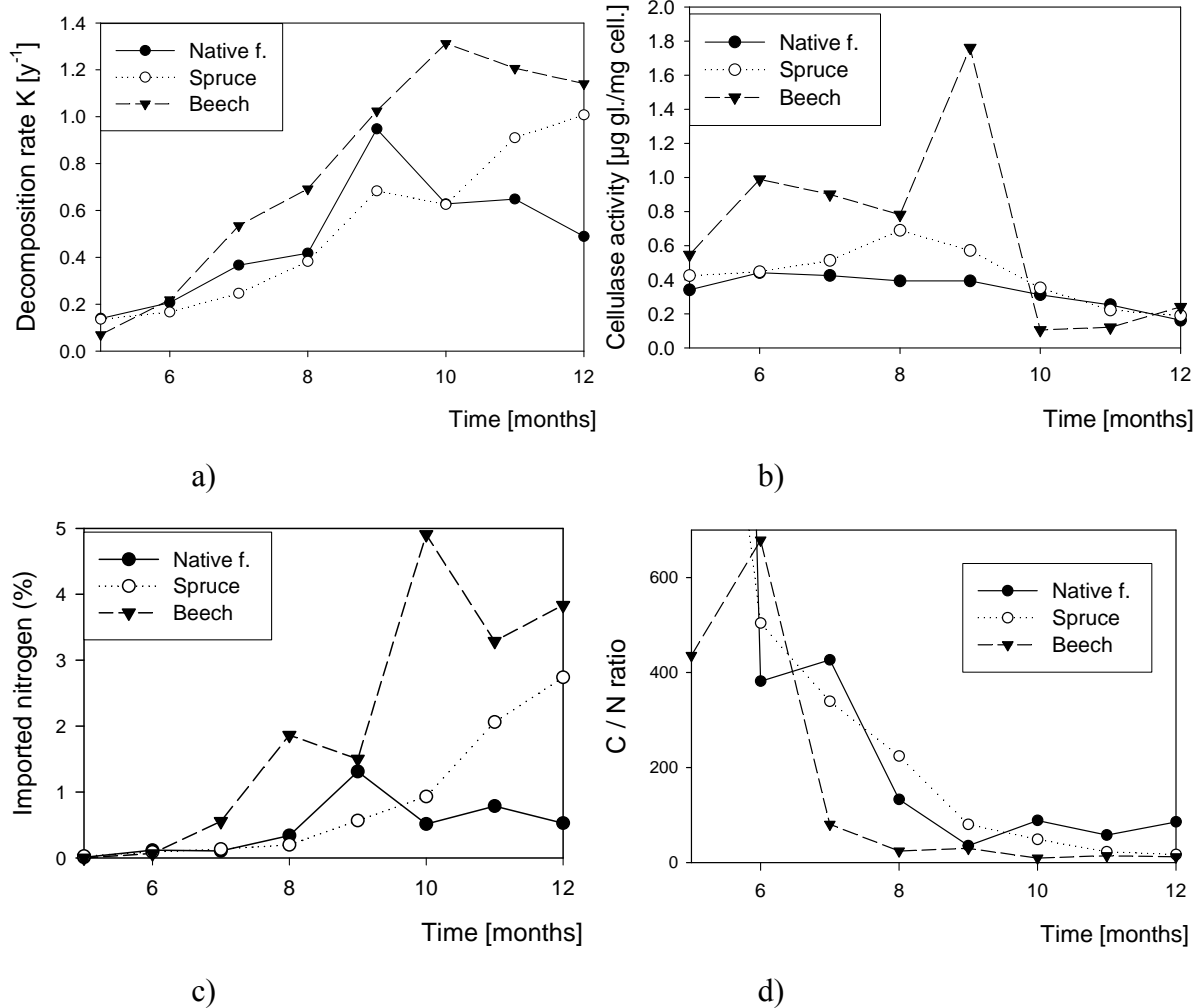
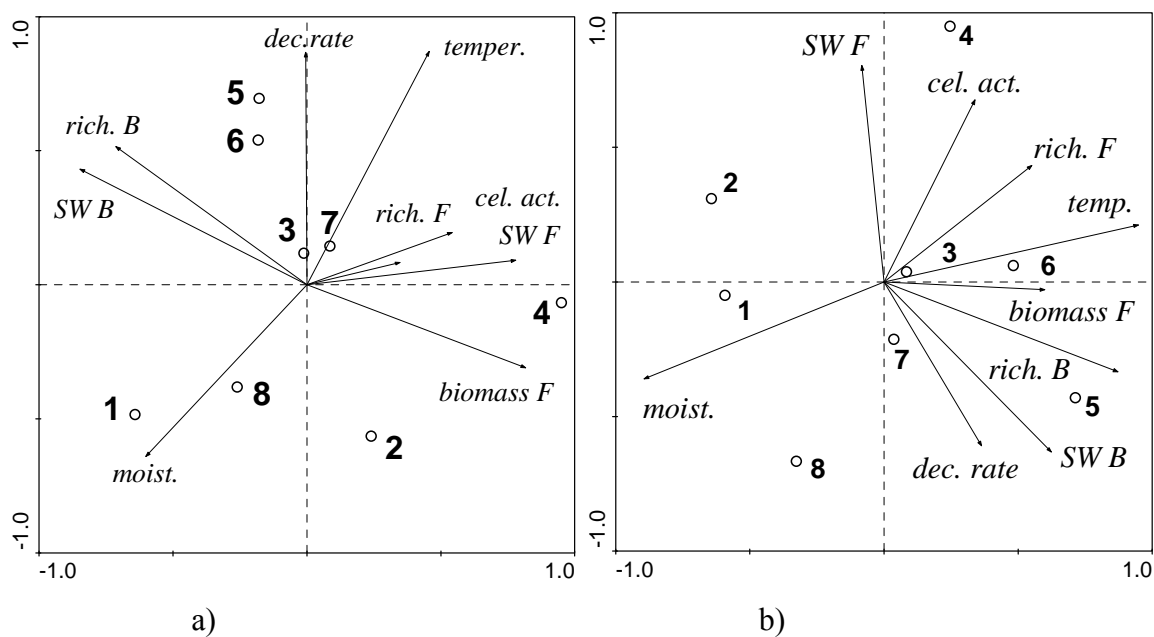
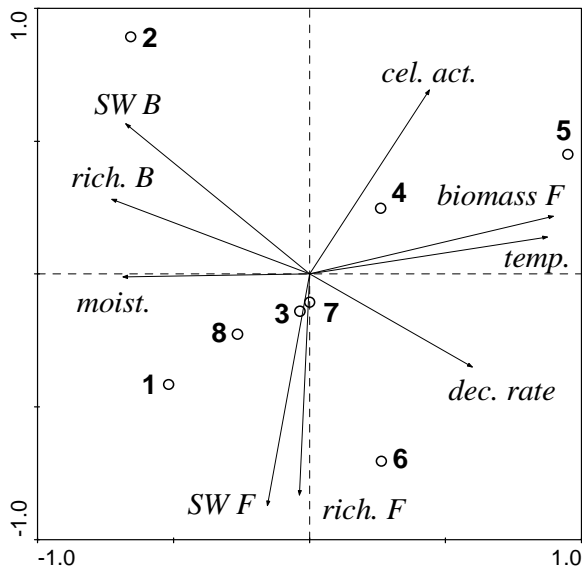


Figure 2. Curves of variables along time, from the third sampling (May 2004, 3 months of incubation,) to the last sampling (December 2004, 10 months of incubation). Numbers on x-axis correspond to months (6-June, 12-December). Decomposition rate (K /year) of the cellulose in four week periods between the samplings, expressed according to Olson's exponential model (a); cellulase activity ($\mu\text{g glucose/mg cellulose}$) (b); accumulation of nitrogen (%) (c); carbon/nitrogen ratio (d).





c)

Figure 3. PCA ordination plot showing relationships among studied variables and positions of particular sampling dates (1 - after 3 months of decay, in May; 8 - after 10 months of decay, December). Upper left-beech, upper right-spruce, lower left-native f., lower right-general trends based on average values from all tree species were plotted. The divergent position of September values (n.5, lower right) from the general trend, caused by high biomass and cellulase activity values (mainly in beech), is evident. Arrows point in the direction in which the scores would move if the value of the variable increased. Length of arrow indicates the relative importance of the variable (or species, Fig. 5) in explaining the variability. The angle between arrows indicates the degree to which they are correlated.

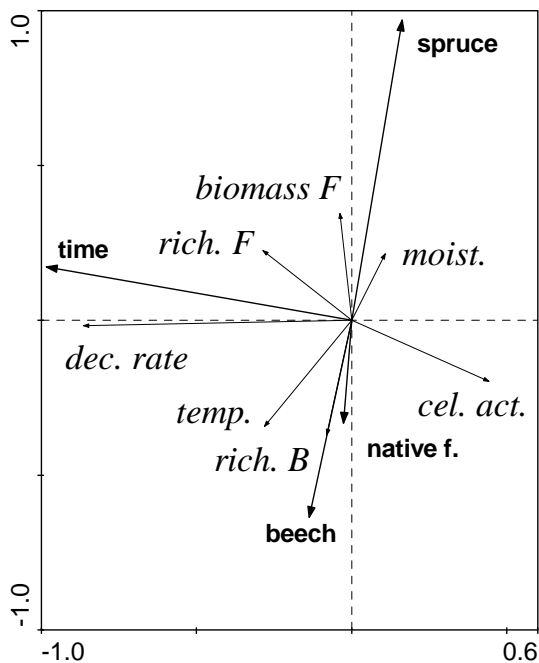


Figure 4. RDA plot illustrating the tree species position along the two first canonical axes according to studied variables throughout time. Time was a more determining variable than tree species and thus is closer to the x-axis; native forest and beech, with more resembling characteristics, would be separated beyond on the z-axis. For a better understanding of the plot see Fig. 3 legend.

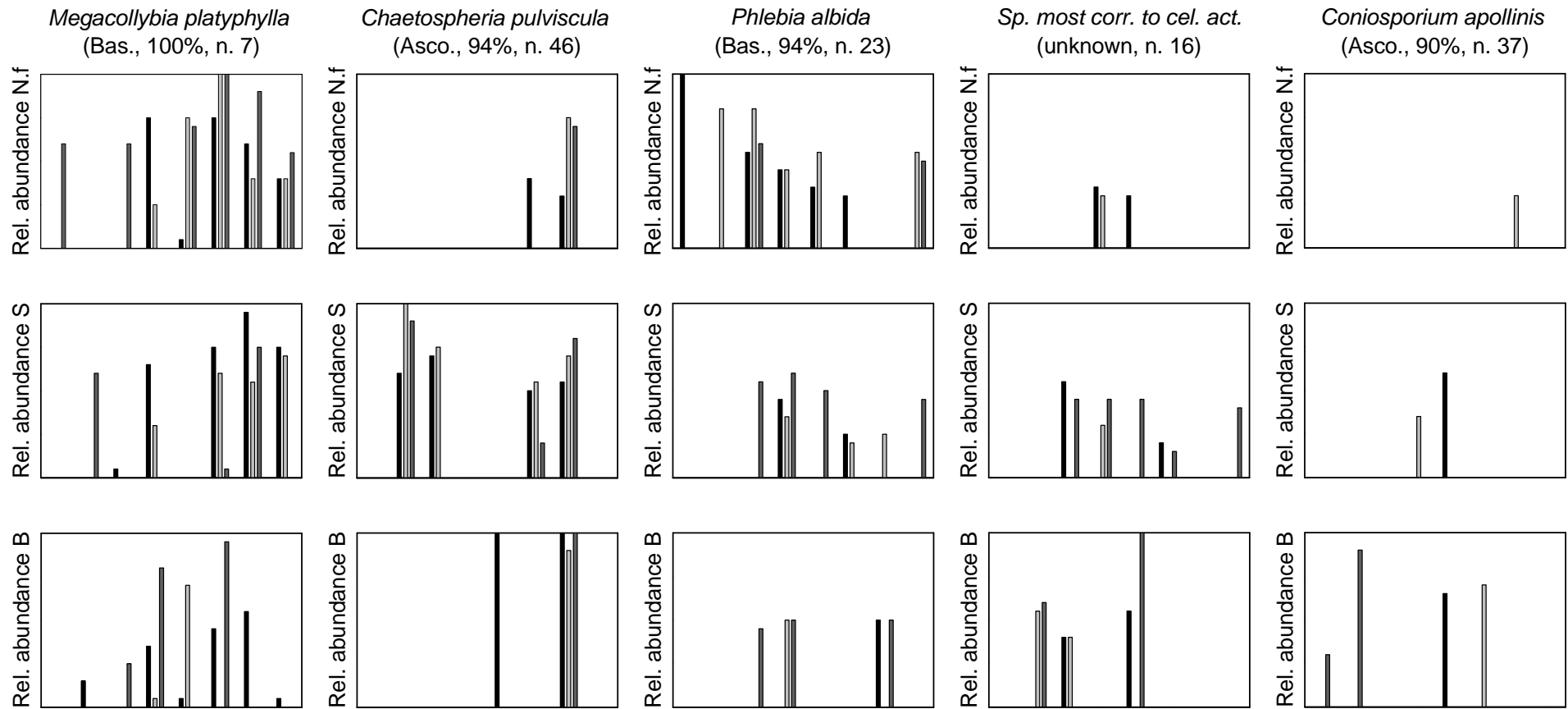


Figure 6. Detailed visualisation of abundances of five selected fungal species in particular tree species stands along time (3rd to 10th months, May to December, x-axis). 1-3 connected columns of different tints express the species presence or absence in the three replicates of each sampling date. Height of column indicates the relative abundance of a given species in the sample (max. shown=25%, y-axis). The headlines describe fungus name, number code used in ordination plots and percentage of similarity with DNA sequence available in the Genebank. Species 16 was not sequenced, but was chosen as the species most correlated with high fungal biomass and cellulase activity. Clear differences of fungal species presences within tree species can be seen as well as different life-strategies between fungal species.

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Spatial and temporal diversity of wood decomposer communities in different forest stands, determined by ITS rDNA targeted TGGE

Ariana KULHÁNKOVÁ^{a,b*}, Thierry BÉGUIRISTAIN^b, Judicaël MOUKOUMI^{a,b},
Jacques BERTHELIN^b, Jacques RANGER^a

^a INRA, Centre de Nancy, Unité Biogéochimie des Écosystèmes Forestiers, 54280 Champenoux, France

^b LIMOS, CNRS UHP UMR 7137, Université Henri Poincaré, BP 239, 54506 Vandœuvre-les-Nancy, France

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Abstract – The aim of this study was to determine the dynamics of colonisation and composition of the wood-decomposer community in a native forest and four monocultures over time. A fingerprinting method of TGGE (temperature gradient gel electrophoresis) with rDNA amplified by ITS1F and ITS2 primer pairs was optimized and used as a culture-independent approach to determine the dominant fungal species and biodiversity over a two-year period of decomposition of beech-wood samples. The bacterial community after two years was also investigated. Data showed that each tree species, as well as sampling date, displayed the characteristic community structure. There was no strong decrease in microbial species richness or Shannon-Wiener diversity index caused by a change of tree species. Nevertheless, a strong shift in decomposer community structure was evident among the tree species both for fungi and bacteria. The effect of environmental conditions was also significant.

wood / decomposers / forest / ITS / TGGE

Résumé – Étude par TGGE de la diversité spatiale et temporelle des communautés de décomposeurs du bois en fonction des essences forestières. L'objectif de cette étude était de caractériser la dynamique de colonisation d'un matériau modèle (du bois de hêtre) par les lignivores au cours de la décomposition, dans une forêt native et sous quatre essences de substitution à la forêt native feuillue. La technique d'empreinte génétique de TGGE (électrophorèse sur gel en gradient de température) ciblée sur l'ADN ribosomique, amplifié par les amorces ITS1F et ITS2, a été optimisée et utilisée pour déterminer les espèces fongiques dominantes ainsi que la diversité totale au cours des deux années d'incubation in situ des échantillons. La diversité bactérienne a également été étudiée sur les échantillons prélevés après deux années d'incubation. Les données indiquent que le peuplement et la durée d'incubation modifient la structure des communautés fongiques. Il n'y a cependant pas de forte diminution du nombre d'espèces ni de l'index de diversité de Shannon-Wiener associée au changement d'essence forestière. Toutefois, il y a une forte évolution dans la structure des communautés de décomposeurs entre les peuplements, à la fois pour les champignons et les bactéries. Les effets des paramètres environnementaux sont discutés.

bois / lignivores / forêt / ITS / TGGE

1. INTRODUCTION

A significant part of European forests has been replaced by even-aged plantations of native or alien tree species, especially during the last century. Such large changes modify soil conditions such as microclimate or organic and mineral chemistry of the litter. This can significantly influence the abundance, structure and activity of the soil microbial community regarding its and, thus, the functional integrity of the forest soil.

Plant litter decomposition is an important factor controlling organic matter decay, nutrient turnover and humus formation in forest ecosystems. It is also relevant at the biosphere level, because decomposition releases significant amounts of greenhouse gases. Thus, soil organic matter represents a major sink for carbon [6]. Therefore, research of belowground species diversity, with respect to the environment and microbial activity, is required to achieve a full understanding of forest ecosystem functioning. It is an important goal in regards to sustainable

forestry as well as having a potential role in global climate change.

The decay activity is expected to be positively related to the number of decomposer taxa. Even recalcitrant substrates, such as lignin, should degrade faster in diverse systems relative to simple ones, probably due to the complementary roles of different fungal species [26]. Fungal succession during litter decomposition has been recently studied for several forest floors under different tree species [22, 27, 31].

However, the dynamics of complex systems are not easy to describe. The behaviour of a single, isolated species or a simple community in a micro- or mesocosm study might not reflect its behaviour in a more complex natural environment. The use of model material decomposing in a tree monoculture could be a possible way to control variability by decreasing the effects of total natural complexity.

Suitable molecular techniques to study microbial species composition are based on direct extraction of DNA from environmental samples, thereby circumventing the culturing step

* Corresponding author: arikul@centrum.cz

and giving a more realistic view of total diversity [1]. The use of internal transcribed spacer (ITS) primers to amplify fungal ITS of rDNA was originally described by White et al. [32] and pioneered by Gardes et Bruns [9]. Studies based on the ITS region showed that intraspecific variation was very small when studying fungal isolates from the same geographical area, while interspecific variation was large enough to distinguish different species [16]. Moreover, analysis of the ITS region was more specific and taxonomically more informative than other genomic regions (e.g. 18S) [19]. Molecular methods to describe microbial succession were used in different studies, e.g. for investigating the decomposition of spruce and pine litter across a moisture gradient [33], beech and spruce litters [3] or to study wood-inhabiting fungi [14, 15, 29].

The present study deals with microbial colonisation of wood samples incubated for two years below five different tree species under the same ecological conditions. The first objective was to improve the method of ITS1F and ITS2 targeted TGGE. It was hypothesized that the samples would show differences in decomposer patterns when compared (i) between native forest and corresponding broadleaved plantations, (ii) among forest species and (iii) within the duration of the decay process. We also expected to identify the key species which characterise the stands, as well as the temporal succession.

2. MATERIALS AND METHODS

2.1. Site description

The experimental site of Breuil-Chenue forest is situated in the Morvan Mountains, Burgundy, France. Its altitude is 640 m, latitude 47° 18' 10" and longitude 4° 4' 44". Mean annual temperature is 9 °C, with 1280 mm precipitation and evapotranspiration of 640 mm. The substrate is granite with desaturated acid soil (pH 4–4.5, 60% of sand, 20% of clays). The natural potential vegetation would be a mixed broadleaf forest. It evolved with management towards a semi-natural coppice with a forest dominated by *Fagus sylvatica* L. and *Quercus sessiliflora* Smith, associated with several other dominant species like *Betula verrucosa* Ehrh. and *Corylus avellana* L. This native forest was partially cut down in 1976 and replaced by various monocultures planted in 1000 m² plots each. Five different stands were selected for the treatment: four young plantations, namely beech (*Fagus sylvatica* L.), oak (*Quercus sessiliflora* Smith.), Norway spruce (*Picea abies* Karst.) and Douglas fir (*Pseudotsuga menziesii* Franco.) and the native forest as a reference plot. All of these stands had the same environmental conditions. Thus, all variations in physical and chemical properties are expected to be caused by the forest tree species. For the same reasons, changes in the biological characteristics, such as the structure, of the decomposer community were supposed to be driven by the tree species.

2.2. Field experiment

Beech-wood pieces (4.5 × 3.5 × 0.5 cm) were incubated in the five different forest stands using the classical litter-bag method. Mesh size of the bags was 5 µm and the inside area 5 × 5 cm. The distance

between two replicates was 0.5 m and 1.5 m between two blocks. The bags were placed in the stands in late November 2002. They were collected after either 3, 9, 12 or 24 months (February, September and November 2003, and early December 2004). Three replicates were sampled and stored in a deep freezer until analysis.

2.3. Molecular analyses

The surface layer, including mostly fungal mycelia and a small amount of degraded wood, was scraped from each sample. Total genomic DNA was isolated from these prepared samples using the Dneasy plant mini kit (Qiagen) following the manufacturer's protocol without any modification.

Fungal communities were analysed using ITS1F and ITS2 primer pairs to amplify by PCR the 300bp fragment of the fungal ITS rDNA. The primer ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') [13] is higher fungi ITS specific, while ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3') is a universal primer amplifying the ITS region from Eucaryotes, including both Ascomycetes and Basidiomycetes [32]. A 40 bp GC-clamp was attached to the 5' end of the primer ITS2 to avoid a complete separation of DNA strands during denaturing electrophoresis. The reaction medium consisted of 5 µL of PCR buffer (Sigma, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂), 1 µL of dNTP (10 mM), 1 µL of each primer (20 µM), 0.5 µL of Taq-polymerase (5 units/µL, Sigma), 1 µL of GC-rich solution (Sigma) and 2 µL of genomic DNA brought to a final volume of 50 µL. The amplification regime consisted of an initial cycle of denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min 15 s. The amplification concluded with a final elongation step at 72 °C for 8 min.

Bacterial communities were analysed using the eubacterial primer set, 968f and 1401r, to amplify by PCR a 475-bp fragment of 16S rDNA from the same DNA extract as above [13]. A 40-bp GC clamp was attached to the 5' end of the primer 968f. A 50 µL PCR mixture containing 5 µL of PCR buffer (Sigma, 100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1 µL of dNTP (10 mM), 1 µL of each primer (100 µM), 0.5 µL of Taq-polymerase (Fastart, Roche Diagnostic), 2 µL of MgCl₂, 1 µL of GC-rich solution (Sigma), 2 µL of BSA (3%) and 2 µL of genomic DNA was used. The amplification regime consisted on an initial cycle of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min concluded with an elongation step at 72 °C for 5 min.

All PCR reactions were performed using an iCycler Thermal cycler (Biorad). PCR products (7.5 µL, 1.5 µL of loading buffer) were loaded onto 1.5% (wt/vol) agarose gels and electrophoresed (1× TAE buffer, 110 V, 45 min). Then the gels were stained with ethidium bromide (0.5 mg/L, Biorad) and the DNA bands visually inspected under UV light to verify the size and quality of the generated amplicons.

TGGE was performed with a Dcode Universal Mutation Detection system (Biorad). In the case of fungi, polyacrylamide gels (8% acrylamide (wt/vol), 8 M urea, 1.25 × TAE and 0.2% glycerol (vol/vol), 300 µL of ammonium persulphate (10%) (wt/vol) and 30 µL of TEMED) were allowed to polymerize over 1.5 h. Amplified DNA samples with the same volume of loading buffer (10 µL each) were separated by electrophoresis in 1.25 × TAE at a constant voltage (145 V) at a temperature gradient from 50 °C to 55 °C with a temperature increment of 1 degree per hour. For bacteria, TGGE conditions

differed by using 7% acrylamide gels (wt/vol) and a temperature gradient from 58 °C to 63 °C with the same temperature increment. Gels were stained with ethidium bromide after electrophoresis and evaluated under UV light. Twenty of the most important fungal bands were excised from the gel and re-amplified with the previous primers set (non-clamped), purified (Quiagen purification kit) and sequenced (MWG Biotech). The DNA sequences obtained were compared to sequences available in the Genbank database of the National Centre Biotechnology Informatics (NCBI) using the Blast software.

2.4. Statistics

Species distribution and relative abundance were calculated based on data analyses using the Quantity One software (Biorad) with a position tolerance of 2 mm, giving a maximum of 50 detectable species. Bands with intensities less than 5% of the intensity of the dominant band were excluded from the analysis. For each profile, the total number of species (species richness) and the Shannon-Wiener diversity index (referred to as SW hereafter) were calculated based on a natural logarithm transformation of the abundance data [10].

The significance of time and tree species on microbial species richness and SW diversity was tested by ANOVA using the Statistica software. The banding patterns of TGGE profiles were analyzed using multivariate techniques, reviewed in Fromin et al. [8]. These methods facilitate finding the variation in a multidimensional data set in a reduced space and assessing the association between species present and selected environmental factors. An initial DCA analysis was used to assess the trend of the data along the gradient. Because the structure was mostly linear, PCA and RDA were calculated using the square-root transformed relative abundances for each species in a profile. DCA and CCA were used in two cases of unimodal distribution. In the ordination plots presented, arrows indicate the direction in which the scores would move if the value of the environmental variable increased. The length of the arrows indicates the relative importance of the species/variable in explaining the variability between profiles. The angle between the arrows indicates the degree to which they are correlated. Monte Carlo permutation tests calculated the significance of a given factor. Forward selection was used to rank environmental variables in importance for determining the species data. All ordinations were computed using the Canoco software package. The significance level was set at $p < 0.05$ for both the ANOVA and multivariable analyses.

3. RESULTS

3.1. Fungal species richness and diversity

All fifty potential fungal species were detected in the whole stands-time matrix. The number of species per TGGE profile ranged from 5 to 22. Generally, the total number of species present in all stands increased with time from 36 species at the first sampling date to 45 after 2 years of incubation (Tab. I). The average number of species per sample exhibited the same trend (from 10.5 to 16.5 species detected) as did the average SW diversity index (0.866–1.083 in 4 months and 2 years, respectively). The only exception was the 8 month sampling, which had the lowest values of all of these characteristics. The

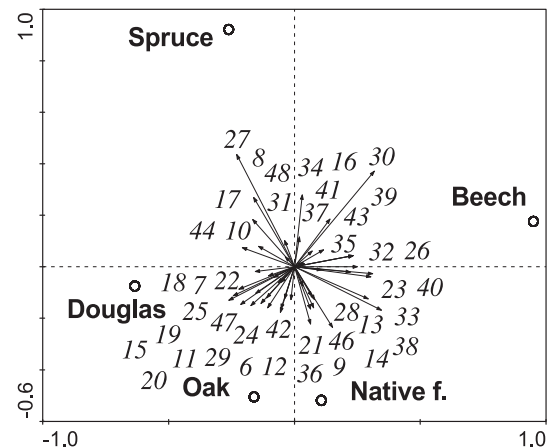


Figure 1. Fungal species distribution (arrows with numbers) and tree species position (circles and tree names) in ordination space according to the fungal community composition, based on the whole tree species-time matrix (5 tree species, 4 samplings together). Each arrow points in the direction of the steepest increase of abundance for the corresponding fungal species. The angles between arrows indicate correlations between them. The numbers correspond to the fungal species codes used in the text and Table II.

effect of time was found to be significant both for species richness and SW diversity ($p < 0.001$).

Tree species had a negligible influence on the total number of species detected, which varied from 40 to 43 within all the samplings (Tab. I). There were no significant differences either in average species richness or SW index for individual stands, although the native forest showed the highest measures (13.5 species in each sample, SW = 0.993) and the spruce site the lowest (only 11 species and SW = 0.904). Intermediate values were found in the oak, beech and Douglas-fir stands (12.6 and 0.937; 12.3 and 0.920; 12.2 and 0.934, respectively).

Twenty of the selected bands of the more common species were sequenced. The most similar species in the Genbank database are listed in Table II, together with the percent similarity to the reference sequences.

3.2. Fungal community structure over time and among stands

The first PCA component expressed 13.5% of the fungal species variability within all tree species and sampling dates. In RDA, the community composition was shown to be significantly affected by both time and tree species, together explaining 16.3% of total variation for the whole data set. A slightly higher weight could be noted for the tree species effect (8.8%) than for the time effect (7.5%). These values matched 9.5 and 8.2%, respectively when analysed with the second factor as a covariable. Random tests confirmed the influence of both variables with p -values of 0.001. Beech was the most discriminating tree species given by forward selection, followed by spruce, native forest, oak and Douglas-fir (Fig. 1).

Sampling dates were analysed separately (five tree stands together for each sampling date) in order to obtain a clearer

Table I. Results of the multivariable analyses, together with the total number of species present, average species per sample (“richness”) and Shannon-Wiener (SW) diversity index values. Scores for fungal communities in the sampling dates and tree species, for all stands and dates together, and bacterial diversity after 2 years are given. Variability explained by the 1st axis of indirect analyses (mostly PCA), all canonical axes (mostly RDA) and results of Monte Carlo permutation tests of stand or time effect (bold where significant) can be found.

Data set	Tested effect	Species number	Undirect 1st axe	Direct anal. can. axes	<i>p</i> value	F ratio	Species richness	SW index of diversity
F All matrix	Time*	50	13.5	8.2	0.001	4.807	12.3	0.937
F All matrix	Stand*	50	13.5	9.5	0.001	1.797	6–22**	0.52–1.28**
F 4 months	Stand	36	22.5	39.5	0.010	1.629	10.5	0.866
8 months		32	26.5	30.4	0.350	1.088	8.6	0.806
12 months		42	19.1	42.3	0.002	1.829	13.6	0.995
24 months		45	17.6	30.0	0.300	1.071	16.5	1.083
F Native f.	Time	43	22.9	17.7	0.002	2.144	13.5	0.993
Spruce	***	40	21.5	14.7	0.018	1.700	11.0	0.904
Beech	***	41	22.0	12.0	0.076	1.340	12.3	0.920
Oak		40	24.2	15.9	0.016	1.188	12.6	0.937
Douglas		43	19.6	14.0	0.016	1.623	12.2	0.934
B 24 months	Stand	41	23.5	37.5	0.020	1.507	12.4	0.931

* Unimodal analysis used (DCA and CCA), ** second effect (time or stand) as covariable, *** total range of values. F: Fungi; B: Bacteria.

Table II. Blast comparison of the sequenced bands. The most particular species (identity), their database match and percentage of similarity are given (sequenced fragments of 210–220 pb were compared).

Species n.	N. of samples	Identity	Blast match	Similarity
6	27	Leaf litter ascomycete strain its408 isolate	A AF502889	96
7	37	<i>Megacollybia platyphylla</i>	B AF498289	100
10	19	Unpublished species	–	–
12	32	<i>Epacris microphylla</i> root associated fungus	AY268197	98
14	16	Uncultured fungus clone B1c from forest	AY324159	98
17	23	Leaf litter ascomycete strain its356	A AF502859	97
19	17	<i>Trametes versicolor</i>	B AY673076	93
21	27	<i>Gerronema strombodes</i>	B U66433	92
23	16	<i>Phlebia albida</i>	B AY219368	94
25	22	<i>Dactylaria</i> sp. P24	A AY265332	88
26	35	<i>Phanerochaete sordida</i>	B AY219383	96
28	26	Fungal endophyte WMS13	A AY063309	94
30	25	<i>Cryphonectria</i> sp. CMW 11302	A AY214324	98
33	10	<i>Epacrid</i> root endophyte E4-5-5	A AF148952	91
35	9	<i>Menispora tortuosa</i>	A AF178558	92
37	10	<i>Coniosporium apollinis</i>	A AJ244271	90
39	17	<i>Helotiales</i> sp. sd2aN4b(A)	A AY465452	95
41	16	Ascomycete sp. olrim349	A AY354279	95
42	23	<i>Woollisia</i> root associated fungus XVIII	A AY230788	90
46	11	<i>Chaetosphaeria pulviscula</i>	A AF178544	94

Species n. is the number used in the text and images, N. of samples implies in how many samples the species was detected. A: Ascomycetes, B: Basidiomycetes.

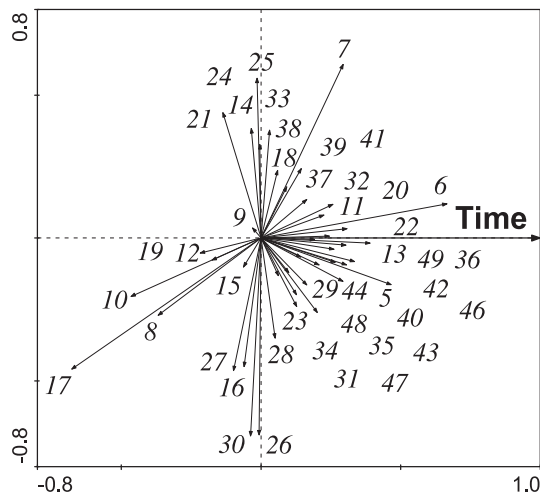


Figure 2. RDA bi-plot showing fungal species (arrows with numbers) responses and succession over time in all tree species together. The five tree species and four samplings were analysed together, with time as the independent variable (arrow connected to x-axis).

picture of the relationship between fungal community composition and tree species effect. The tree species effect was detected as significant in the first and third samplings (4 and 12 months) (Tab. I). In those cases, the variability of community structure explained by RDA canonical axes also achieved higher values (39.5 and 42.3%, respectively), the expressed portion was weaker (30.4 and 30.0%, respectively) for non significant sampling times (8 and 24 months). A slight decrease in the importance of the first PCA axis appeared with time (from 22.5 to 17.6%) with the exception of the 8th month sampling in late summer 2003 (26.5%).

Simultaneously, the time effect on species succession was tested in individual tree stands (four sampling dates together in each tree stand) (Tab. I). Its significant influence was detected for all tree species except beech, where a trend could still be seen ($p = 0.076$). Beech, together with spruce, also displayed a non-linear species distribution along the time gradient so that unimodal analyses (DCA) had to be used. The first component of the indirect analyses explained from 19.6 to 22.9% of the variability, whereas it ranged between 12.0 and 17.7% for linear techniques. The largest portion of the temporal community evolution was expressed for oak and native forest by both analyses.

There was a relationship between fungal species numbers and occurrence with particular tree species (Fig. 1). Fungal species numbers also changed over time (Fig. 2). Fungal community composition differed clearly among tree stands both in the first (Fig. 3a, 4 months) and the last (Fig. 3b, 2 years) sampling time. A distinct temporal shift can be detected. Coniferous and deciduous stands were separated along the y-axis in the first sampling period. This trend was less obvious in the last sampling, with the native forest situated above the x-axis. Coniferous stands tended to occupy the upper left portion of the ordination plane in both cases even when the Douglas-fir site displayed only low species specificity later. There were

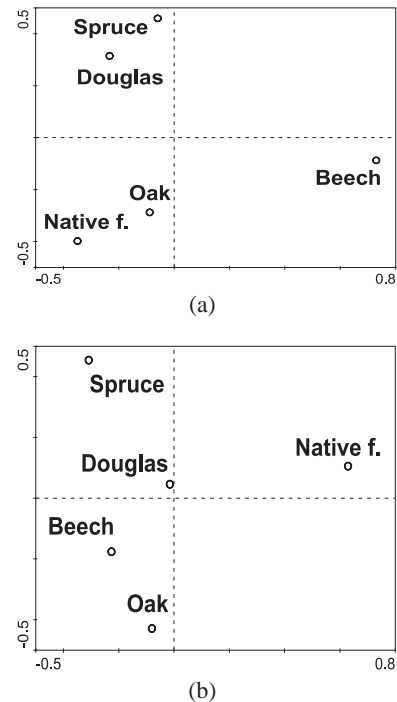


Figure 3. RDA ordination plots illustrating the tree species position at (a) the first sampling time (4 months) and (b) the last sampling time (24 months) according to the fungal species distribution.

noticeable shifts in the young beech and native forest stands. At the beginning of the study, a specific fungal community was found in the beech stand while its structure in the native forest was similar to that in the oak stand. By the end, the native forest had a separate species structure and the young broadleaf monocultures were grouped together.

3.3. Stand condition effect on fungal communities

There were definite relationships between tree species and their environment, as shown by the PCA of the important characteristics of forest floor, upper soil layer and soil solution (Fig. 4). The native forest, young broadleaf monocultures and coniferous stands were well separated along the x-axis. The oak site was positioned separately along the y-axis in the upper right quarter.

The importance of soil temperature and moisture were tested separately, based on average values for the month before sampling. The native forest was not included in the analyses because of missing data. Both factors had significant impacts on the species composition ($p = 0.002$ for both) and explained 5.7 and 5.8% of the total data set variability, respectively. As expected, temperature and moisture were correlated.

3.4. Bacterial communities

Forty-one different bacterial species from the 50 potentially detectable ones were found under the different tree species in

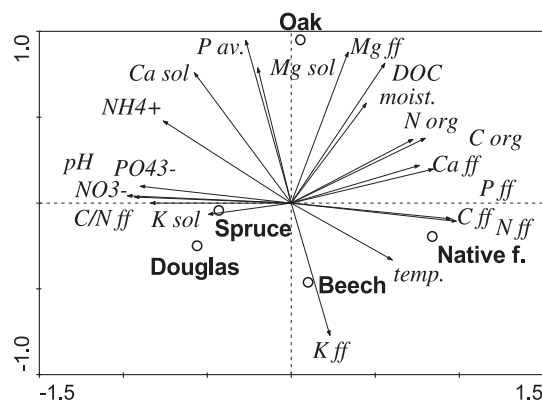


Figure 4. PCA ordination plot displaying relations of the environmental variables (arrows) in the studied tree species. ff means nutrient concentration in forest floor (C, N, C/N, P, K, Ca, Mg); sol means concentration in soil solution in the top 5 cm (K, Ca, Mg). Other variables in solution are also shown (pH, PO_4^{3-} , NO_3^- , NH_4^+ , DOC); concentrations of available P, organic C and N were also measured in the top 5 cm of the soil profile. The length of arrow indicates the relative importance of the variable in explaining the position of the tree species in the ordination space. The angle between the arrows indicates the degree to which they are correlated.

the last sampling time (24 months). There were 12.4 species on average per sample. The highest species richness was detected in the native and oak forests (24 different species), followed by beech (23), Douglas-fir (22) and spruce (20 species). The native forest also had the highest average number of species present per sample (14), followed by oak (12.7), spruce (12.3), Douglas-fir (11.7) and lastly beech (11.3). The highest diversity index value was also associated with the native forest ($\text{SW} = 0.998$), followed by the conifers and oak (approximately 0.95), with the lowest rate in beech (0.745). However, the influence of tree species on bacterial species richness and diversity was not significant.

The first PCA component explained 23.5% of the bacterial data set variability. The canonical axes of RDA, with tree species as independent variables, expressed 37.6% of the variability. Permutation tests showed a significant influence of tree species on the bacterial community composition ($p = 0.02$, Tab. I). The distribution of the tree species, as a function of bacterial species distribution, showed a clear separation along the visualized axes (Fig. 5). The native forest appeared to have a similar bacterial composition as the spruce stand. Young deciduous monocultures were grouped in the lower left part of the plot while the Douglas-fir stand differed clearly from the others, being located in the upper left quarter. The Douglas-fir stand was the most discriminating, based on the forward selection of environmental variables, followed by oak and then the three other stands.

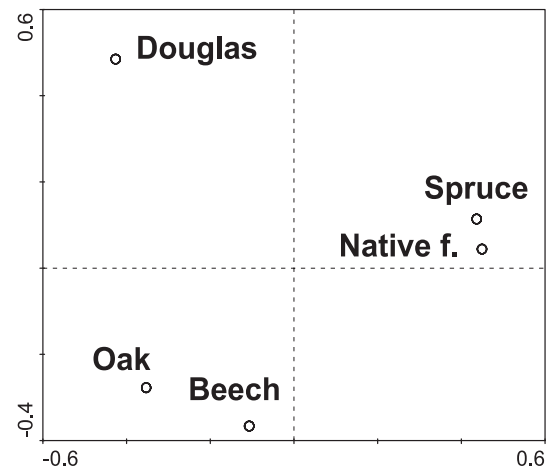


Figure 5. RDA ordination plot showing tree species position according to bacterial species distribution after 2 years of samples colonisation. The native forest and spruce did not differ either by position on the x or y-axis and thus they had similar bacterial communities.

4. DISCUSSION

4.1. Molecular approach

The fungal ITS region was targeted because it appeared to be taxonomically more informative than other genomic regions [19]. We also tried the 18S amplification with the same DNA samples. The number of visible bands after electrophoresis was lower than in the case of ITS. In addition Anderson et al. [2] noted that the ITS1F and ITS2 primers gave the sharpest and most consistent electrophoresis profiles in their fungal diversity study in organic soils. The primer pair efficiently amplified both asco- and basidiomycetes species in the present study as well as in Gardes and Bruns' study [9]. TGGE has been shown to be a sensitive and robust method for investigating soil fungal communities, particularly where the aim was to investigate shifts or changes in community composition [1, 30]. The ordination methods allowed comparison of both the position and the relative intensity of different bands within gels, as well as the interpretation of ecological significance [8].

In total, 20 fungal bands were sequenced and compared to similar sequences present in the GenBank database. A certain limitation imposed by existing databases must be noted when characterizing natural communities. Therefore, blasting did not completely provide determination at the species level. The majority of sequences had a high similarity to the fungal ITS sequences of the database. However, four of them matched uncultured fungi generated from previous molecular studies, but were not named to species, while one sequence had no similarity to any in the database. Despite only approximate identification, it was assumed that all sequences belonged to fungal species that occur in forest stands and are mostly saprophytic. It is also possible that there are un-cultivable species present, which are not currently represented in databases.

It was also realized that single bands did not necessarily originate from one species and that one band could contain

different sequences [20]. We tried to sequence several bands at the same position, as well as to have more replicates of the same bands. The results obtained by the sequencing were always similar. Thus, it was possible to assume that TGGE provided a quick and high resolution in the separation of microbial DNA fragments.

It is likely that the decomposer community present in the forest soil, as well as the site conditions, were more important for the resulting diversity than the origin of the incubated material (beech-wood). Thus, both non specific and specific fungi should be present in all of the stands. An advantage of this is that it gives the ability to compare situations using only a single probe. Nevertheless, it is a disadvantage to use a model material which is one of the set of species being investigated. It would be interesting to make a comparison with a wood probe from a different species. It has been documented that fungal mycelial ingrowths and the rates of mass loss and respiration could differ with litter material when incubated in the same site [31]. The sample size used in the study should be adequate to ensure high diversity, since small diameter debris has a relatively larger surface area per volume than larger ones [11]. Accordingly, Krøys and Jonsson [17] found smaller spruce logs to host more cryptogams than logs with diameters over 10 cm. Moreover, spatial heterogeneity within the soil is high and can be linked to the distribution of micro-organisms; there is a close relationship between sampling strategy and the observed species richness [28]. However, the PCA analyses (not shown) confirmed high variability of diversity between stands but a relatively low variability between sample replicates. Therefore, our design recorded well the species distribution patterns.

4.2. Tree species and time effect

Each tree species, as well as sampling time, displayed a characteristic fungal community structure. The effect of both factors was highly significant. Oak and native forest had quite similar species structure over the whole observation period. There were also other species that are present in other stands, except for spruce whose species composition was highly specific. Douglas-fir had some species in common with Norway spruce but tended to converged more towards the oak site. There was very little species overlap between Douglas-fir and beech at the same time. The species composition of the young beech stand was rather similar to the community observed in the native forest. Nevertheless, both beech and spruce stands had fewer species that were common in the three other stands.

The fungal communities in some particular spruce and Douglas-fir samples seemed to be less variable than over the whole period of incubation. This might be caused by the presence of some species typical for coniferous sites at a given time (e.g. species 10 or 17). There were clear shifts over time in the fungal communities of the native forest and beech stands. At the beginning of the incubation, the fungal species composition of the native forest resembled that of oak, while the beech fungal communities were distinct from these stands. The situation was reversed in the last sampling. Similar fungi

were present in the young deciduous monocultures whereas the native forest had a unique species composition. This might not be so surprising, because the native stand was composed of both oak and beech trees. Rather, it could reflect the presence of several species typical for broadleaf stands but differing in certain ecological demands (e.g. species 42, present in the native forest at first, but in the oak and beech monocultures at the end) or to the greater importance of ecological conditions in earlier rather than later stages of decay. This could be determined by the analyzing the stands distribution in relation to environmental variables (Fig. 4) and within the first sampling time (Fig. 3a). Interestingly, the time effect was highly significant in the oak and native stands whereas the beech was the only one where its role was not evident. Moreover, several characteristic of the native forest, such as a greater amount of dead wood on the forest floor, age differentiation of the trees, species mixture and more energy reaching the soil surface, would support greater niche complementarity for both specialist and generalist fungal species.

Both species richness and SW diversity clearly increased over time. No substantial changes between the stands were found in overall quantity of species present during the whole period. Nevertheless, the average species number per sample was slightly higher in the native forest and lowest in spruce. Even though no significant decrease was observed in the wood-colonizing species number caused by a change in tree species, other ecological effects could be expected. For example, microbial biomass could be reduced in response to less favourable litter quality or certain functional changes might occur. Previous studies in the same research site [23] showed the highest amount of both saprophytic and ectomycorrhizal fungal species occurred in the native forest (104 species together), while the lowest was in the oak and beech stands (26 species only). Lejon et al. [18], using the ARISA molecular approach, also showed a strong discrimination of fungal communities under the studied tree species. They showed by comparing that the microbial biomass in the upper layer was highest in the native forest, followed by oak, spruce and Douglas-fir (beech was not included) [18]. Plant species richness was also highly affected [23], being highest in the oak stand followed by beech, spruce and Douglas, with 16, 10, 7 and only 2 species present in the under-storey respectively (no data was available for native forest).

Several recent studies have screened macro-fungal species richness on decaying wood. Bader et al. [5] found the greatest number of species at intermediate stages of wood decay and a negative correlation between species number and logging activities in a Swedish boreal forest. Accordingly, Heilmann-Clausen and Christensen [12] detected a decrease in fungal species number with forest age in beech sites in Denmark. This could be caused by a loss of weakly competitive non-specialized species, to the benefit of highly competitive or narrow niche specialists, as the fungal community matures, whereas wood that has started to soften may contain a mixture of early, intermediate and late successional species. A meta-analysis of diversity studies showed high macro-fungal diversity associated with high tree species richness [25]. Rodriguez et al. [24] found no significant difference in the total

population of lignin and lignocellulose degrading fungi imperfecti from laurel and pine forests on Tenerife. Species of *Penicillium* and *Fusarium* were identified as the most efficient lignin degraders. Cox et al. [7] illustrated the importance of the effects and interactions of specific fungi during lignin decomposition in a Scots pine forest.

Bacterial communities were also specific for each forest stand. The greatest numbers of species were detected in native and oak stands, followed by beech and conifers. Accordingly, the highest average species number per sample was in the native forest whereas the beech site had the lowest value. Similarly as for fungi, ARISA profiles showed that tree species induced a specific genetic structure of the bacterial communities [18].

4.3. Fungal species patterns

Ascomycetes comprised the highest portion of identified fungi rather than basidiomycetes. Nordén et al. [21] found more basidiomycetes than ascomycetes species in their study of wood-inhabiting fungi in temperate broadleaved forests. However, their study was based on recording stroma and fruit-body diameters of 1 mm or more. The molecular approach could offer a more reliable detection of fungal mycelium in belowground wood debris than the distribution of fruit bodies [15].

Five of the fifty defined fungal species occurred only once (these were not shown in the ordination plots), while twelve species occurred in more than one third of all samples. The most abundant were species 7, 26 and 12, appearing in 37, 35 and 32 TGGE profiles, respectively. The most common species (number 7) was identified as *Megacollybia platyphylla*, the order Agaricales. This was the only one with a 100% similarity with the Genebank database. It was also the only species detected in the same forest site where carpophore diversity was studied [23]. Twenty-eight species were found in all sampling times and 31 under all tree species. The 23 species representing the intersection of these categories were present in all stands in all sampling times, but varying in the number of occurrences. Some species were associated with coniferous species (e.g. 7, 8, 10, 17, 27 or 44), young deciduous trees (14, 26, 28, 33, 38, 40, 45) or with the native forest (9, 23, 29, 36 and 49). Others seemed to prefer sites dominated by beech, i.e. native forest and young monoculture (13, 23, 39). Species (8, 10, 17 or 19) were identified mostly in the early stages of colonization (Fig. 2). Species (6, 13, 36, 40, 42, 46) were positively correlated with the later stages. Species appearing largely in intermediate periods were distributed along the positive part of the y-axis (14, 21, 24, 25, 33). Species mostly absent during the second sampling (26, 28, 30) were in the negative range. Species which showed no time response are presented only by short arrows (9, 15, 23). Only a few fungi species displayed no or only weak tree stand or sampling time preferences (e.g. 11, 12, 15, 16, 18 and 31).

For bacteria, nine of forty-one species occurred at least once in each tree species after 2 years of incubation. Five species were present in more than two-thirds of the TGGE

profiles. Conversely, 12 species appeared in only one profile. Also, characteristic bacteria species for different stands could be found.

4.4. Relation with environmental conditions

All differences in the studied forest site conditions were assumed to be caused by the dominant tree species, because geology and meso-climate conditions were very homogenous and site histories were the same (plantations were made after clear-cutting the broadleaf semi-native forest). Coniferous stands appeared to have certain specific characteristics when compared to broadleaved stands three decades after plantation establishment. They had low concentrations of total organic carbon and nitrogen in the forest floor and higher C/N ratios. Coniferous stands also had higher pH of the soil solution and phosphate and potassium concentrations. There was more inorganic nitrogen in the spruce and Douglas-fir stands. Douglas-fir stands displayed extremely high concentrations of nitrates. On the contrary, both nitrate and ammonium concentrations were very low in the native forest, as was phosphate content. Low pH was also typical of the native forest.

Conifers had the lowest phosphorus and potassium amounts in the forest floor. Oak had a nutrient-rich forest floor, which could lead to higher concentrations of organic carbon and nitrogen in the humus, together with high amounts of DOC, ammonium, available phosphorus, calcium and magnesium. Beech seemed to have intermediate values for most of these characteristics.

Native and beech forests differed in stand age and certain ecological characteristics, but had similar dominant species. The native forest could be assumed to be a climax-like ecosystem with well balanced processes unlike in the young monocultures, as shown by the high values of mineral nitrogen and phosphorus in conifers, compared to the strong retention of organic forms in the native forest.

Physical conditions that significantly changed according to season, such as temperature and moisture, might control both species composition and their decaying activity. Meso and pedo climatic conditions (air temperature and relative humidity, soil temperature and moisture) were monitored continuously since 2001 [23]. Beech was the warmest stand in all samplings, spruce the coldest one, while oak and Douglas-fir were intermediate (no data for the native forest). The highest soil moisture content was observed in the oak site, followed by spruce and beech, with lower soil moistures observed in the Douglas-fir [4, 34]. Thus, the oak and spruce stands were relatively cold and humid, while the beech and Douglas fir stands were warmer and drier [23]. The soil conditions at the first sampling time in late February 2003 were the coldest and most humid of all. Conditions were slightly drier and warmer in the late November samplings. The second sampling in September 2003 occurred during extremely warm and dry conditions. The effect of both soil temperature and moisture content was significant, even though these factors were less discriminating for community composition than tree species and time.

Cold or dry months could cause a reduction in species richness or fungal biomass. Such conditions would also favour less competitive species allowing them to be detected in profiles. This was noted in the second sampling time in September 2003, which followed an extremely warm and dry summer period. For that sampling time, species number was lower and the structure was different from the general trend with some characteristic species present only there. Such a seasonal variation, if present at all, was dependent more on the site conditions than on the tree species effect. The influence of environmental conditions could also change with the decay phase, being greatest in the early stages [6]. On the contrary, the stand effect was not significant even in the last sampling.

5. CONCLUSION

Each tree stand and sampling time had the characteristic wood decomposer species community structure. There was no strong decrease of microbial species number caused by change of tree dominant but strong shift in community structure was evident. In order to better understand the complex effect of tree species change it is necessary to consider also other factors, such as functional diversity, biodiversity of other organisms or fungal groups, soil development or ecosystem function at the landscape level. Thus more research is still needed in this field.

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Link of fungal diversity and wood decay in temperate forests

Kubartová Ariana ^{1*}, Székely Anna ², Beguiristain Thierry ⁴, Moukoui Judicaël ^{3,4}, Ranger Jacques ³, Berthelin Jacques ⁴

¹ Department of Forest Mycology and Pathology, SLU, Box 7026, 750 07, Uppsala, Sweden

² Department of Microbiology, Faculty of Sciences, Eötvös Oránd University, Pázmány Péter stny 1/c, Budapest, 1117, Hungary

³ INRA, Centre de Nancy, Unité Biogéochimie des Ecosystèmes Forestiers, Champenoux, 54 280, France

⁴ LIMOS, CNRS UHP UMR 7137, Université Henri Poincaré, BP 239, Vandoeuvre-les-Nancy, 54 506, France

* Corresponding author: tel: +(420) 221 951 646, e-mail: arikul@centrum.cz

Abstract

The purpose of the present work was to identify the relations between saprophytic fungal diversity and decomposition rate of wood in a native forest and four planted stands. We assessed the fungal community structures by T-RFLP and compared the results to results obtained by TGGE previously. Further, we targeted *in situ* decomposition rate of the wood samples after 3, 9, 12, 24 and 30 months in order to determine the possible links between fungal diversity and decomposition rate of wood, as well as to describe consequences of tree species substitution in forests.

Even though there were certain differences between results obtained by the two fingerprinting techniques, the same main trends were identified by both approaches. There was no significant decrease of species richness caused by a change of dominant tree species. The species richness was affected rather by stage of decomposition. The fungal community composition differed within tree species and changed over the time of decay. Mass loss values differed significantly among the tree species. Most wood was decomposed in young broadleaved plantations and least in spruce stand in the end of the study, i.e. after 30 months of decomposition.

Neither species richness nor community composition seemed to have a direct implication to decomposition rate of the wood samples. The changes of diversity did not mean loss of

functional abilities which could detriment the decomposing functions provided by the species assemblage. The interactions between fungal species and environmental conditions were possibly more important to decomposition rate than the species richness and diversity. However, the diversity effect might become more important in the very late stages of wood-decay.

Keywords: saprophytic fungi, diversity, wood decomposition, molecular methods

1 Introduction

Silvicultural interventions, including planting of particular tree species, may have considerable influence on soil microbial communities and the processes they mediate. One of the main processes is decomposition of plant litter. Its full understanding is required not only for sustainable forest management, but nowadays also for identifying potential carbon sources and sinks as a consequence of the global change. The role of saprophytic fungi is crucial in the decomposition process, since they are able to degrade the lignocellulose matrix of litter material, while other organisms cannot (Dix & Webster 1995; Adl 2003; Heilmann-Clausen & Christensen 2003).

Theory predicts positive effects of microbial diversity to decomposition of litter and the hypotheses were widely reviewed, e.g. in Ekschmitt & Griffiths (1998), Jones & Bradford (2001), Emmerling (2002), Mikola *et al.* (2003) or Wardle & van der Putten (2003), Hättenschwiler *et al.* (2005). However, few existing experiments with litter provide conflicting results (Griffiths *et al.* 2001; Setälä 2002; Setälä & McLean 2004; Deacon *et al.* 2006). The conclusions were that the increase of the fungal species richness should not necessarily lead to higher litter decomposition rates. That could be caused by high functional redundancy of many fungal species and by a saturation process at low species level relative to the richness observed in natural ecosystems. Further, the decomposing ability depended not only on the genetic disposition of the species, but also on environmental conditions and interactions with other fungi within the fungal community. Thus, the question how the ecological processes are related to biodiversity has not yet received satisfactory answers. Further, the issue of factors driving diversity of wood-decaying fungi and whether their diversity affects wood decomposition rate has been seldom addressed (Lonsdale *et al.* 2008).

During the last decades, the common use of molecular fingerprinting techniques, such as

TGGE or T-RFLP, simplified the soil microbial diversity estimation and species pattern detection (Anderson & Cairney 2004; Kirk *et al.* 2004; Leckie 2005) and hence it allowed studies of functioning of the complex microbial communities from environmental samples.

The main purpose of this study was to determine the link between decomposition rate of wood samples and diversity of saprophytic fungal community, colonising the samples, in five different forest stands. The specific aims were to answer following questions i) is species richness positively correlated with decomposition rate of wood or is it already saturated at low species numbers ii) does the community composition matter more than the species richness alone iii) are the results of different fingerprinting methods comparable iv) lastly, what are the consequences of tree species substitution for the studied variables. In the precedent work, we described the ecological conditions of the forest stands, fungal community structure in the stands and their succession over time, studied by TGGE (Kulhankova *et al.* 2006). In this work, we applied the T-RFLP approach to compare the two fingerprinting methods. Then, we assessed the mass losses of the wood samples to answer the highlighted questions.

2 Materials and methods

Site description and field experiment

The study underwent in Breuil forest research site, situated in the Morvan highlands, NE of Central Massif, France (altitude 640m, latitude 47° 18' 10", longitude 4° 4' 44", mean annual temperature 9°C, precipitation 1280mm, evapotranspiration 640mm). The substrate is granite with desaturated acid soil (pH 4 - 4.5). The previous coppice with standard forest dominated by European beech (*Fagus sylvatica* L.) and secondarily by sessile oak (*Quercus petraea* Liebl.) in the high forest strata and by birch (*Betula pendula* Roth) and hazel (*Corylus avellana* L.). It was replaced by various mono-specific plantations in 1000m² stands thirty years ago (Ranger *et al.*, 2004). Breuil study site is homogenous, the tree species plots should not differ in substrate or meso-climatic conditions. However, changing the tree species lead to modifications in physical, chemical and biological conditions (Ranger *et al.* 2004). More detailed description was given previously (Kulhankova *et al.* 2006).

The beech wood samples of the size 4.5x3.5x0.5cm were exposed in the field for 2.5 years (November 2002 – May 2005) in litter bags of mesh size 5µm. The composition of wood was: 5.1% of soluble substances, 23.2% of hemicelluloses, 59% of cellulose, 12.4% of lignin and

0.2% of ash content. The sampling of five replicates was realised after 3, 9, 12, 24 and 30 months of the exposition (February, September and November 2003, early December 2004, May 2005) in four 30-year-old monospecific stands of European beech, sessile oak, Norway spruce and Douglas fir (*Fagus sylvatica* L., *Quercus petraea* Smith, *Picea abies* Karst., *Pseudotsuga menziesii* Franco) and in a reference stand of the native forest.

Laboratory analyses

The fungal community structures in 3, 9, 12, and 24 months were assessed by T-RFLP of ITS1F and ITS4. The HEX labeled ITS1F was combined with universal ITS4 primer (White *et al.* 1990). The reaction medium consisted of 5µl of PCR buffer (Sigma, 100mM Tris-HCl, pH 8.3, 500mM KCl, 15mM MgCl₂), 1µl of dNTP (10mM), 1µl of each primer (20 µM), 0,5µl of Taq-polymerase (5 units/ µl, Sigma) and 2µl of genomic DNA brought to a final volume of 50µl. The amplification regime consisted of an initial cycle of denaturation at 95° C for 3min followed by 35 cycles of denaturation at 94° C for 45s, annealing at 55° C for 45s and extension at 72° C for 45s. The amplification concluded with a final elongation step at 72° C for 8min. PCR product were purified using a commercial kit (Qiagen). Hydrolysis of the fluorescently labeled PCR products were performed with *AluI* [AG'CT] (MBI Fermentas) endonuclease enzyme. Digestion was done in accordance to manufacturer's instructions for 3 hours (incubation temperature of 37°C). After ethanol precipitation, aliquots (2 µl) of each digest were mixed with 12 µl deionized formamide and 0.65 µl of internal size standard (GeneScan-500 TAMRA, Applied Biosystems). After denaturing of the DNA at 98°C for 5 min and immediate chilling on ice, samples were injected electro-kinetically at 15 kV for 8s on an ABI Prims™ 310 Genetic Analyser (Perkin Elmer). Electrophoresis was run for 30min at 15kV and 60°C. After electrophoresis, the size of each TRF was determined in comparison to the internal size standard using GeneScan analysis software (Applied Biosystems). The relative abundance of TRFs was determined by calculating the ratio between the peak area of each peak and the total peak area of all peaks within a sample. Analyses of the chromatograms was conducted using GeneMapper 3.7 software and the data was analyzed with T-Align web based tool (Smith *et al.* 2005). Total number of different ribotypes per a data set (a tree stand, a sampling date, whole matrice) was detected as well as average ribotype number, i.e. species richness (SR), per a data set.

The results obtained by T-RFLP were compared to results obtained by TGGE of ITS1F

and ITS2 amplified rDNA fragments, described previously (Kulhankova *et al.* 2006). The samples decomposed for 30 months were analysed by TGGE, using same protocol as previously (Kulhankova *et al.* 2006). The mass loss of the wood samples was determined after 3 days drying at 65°C. Decomposition rate was expressed in % decomposed per months during the periods between samplings.

The significant of tree stand and decomposition stage to studied dependent variables (species richness, mass loss) were tested by ANOVA using the Statistica software (StatSoft). Multivariable analyses (DCA, RDA) of community composition were performed by Canoco software packages (Microcomputer Power) to give the proportion of variation explained by tree stand and decomposition stage (for description of the analyses see Ramette 2007). Monte Carlo permutation test was used to calculate their significance to community composition. The significance level was set at $p < 0.05$ for both kind of analyses.

3 Results

T-RFLP

The total number of 125 different ribotypes were identified within all tree species and sampling dates using the T-RFLP technique. The average number of ribotypes per sample, *i.e.* ‘species richness’ (SR below), was of 12 within whole dataset. Nevertheless, there was a wide variation between samples, from the only one ribotype per sample to a maximum of 36. The total number of ribotypes identified in individual sampling dates within all five tree species showed an increasing trend from 71 to 88 different ribotypes after 3 and 24 months of exposition, respectively. The exception was found after 9 months of decomposition, *i.e.* sampling after summer, with only 44 ribotypes identified. The SR of the whole dataset was the highest after 3 and 24 months, the lowest again after 9 months, *i.e.* 14 or 8 ribotypes per sample, respectively. In individual tree species, the highest total number of different ribotypes during the whole period was detected in oak and spruce stands (77 and 76), followed by the native forest and Douglas-fir (66 and 65), while only 42 ribotypes were found in beech. The similar order of tree species was found also for SR (14.6, 14.7, 11.7, 11.3 and 6.8, respectively). Both decomposition stage and tree stand effect to SR were significant within whole data set. The trends of SR evolution in the stands are shown in Fig. 1a. Oak and both conifers had the highest SR after 3 months of

decomposition. It decreased sharply during the summer to low values in 9 months sampling and increased again during the autumn. Then, it still increased in spruce, while decreased in oak and Douglas. Only the native forest showed a slight increase in SR after the summer months. It reached its highest SR value after 2 years of decomposition. It was also the case of beech, which had the lowest values from all tree species during whole study period, except for Douglas in the 2-years sampling.

Both decomposition stage and tree stand effect were found to be significant to community composition, explaining 5.6 and 10.7% of the variability within all data set. Beech showed the most discriminated community composition within all exposition period, followed by spruce, oak, the native forest and Douglas-fir (Fig. 2). No ribotypes were strictly related either to early or later stages of the decomposition, though some of them showed certain tendency (Fig. 3).

Sampling dates and individual stands were analyzed also separately in order to detect their effects more accurately. The tree stand role was found to be the most important after short period of decomposition, explaining 47.6% of the fungal variability. It was less determining in the longer decomposition periods (37.3 and 34.5% after 1 and 2 years, respectively) and the least in nine months after the summer, only 27.5% of the community composition diversity. The tree stand effect was significant after 3 and 12 months, non-significant after 9 and 24 months. Decomposition stage effect was the most important in spruce stand, explaining 23.7% of fungal variability, followed by oak stand (19.2%), and was significant in these stands. It explained 14.4, 13.7 or 11.5% in Douglas, beech and the native forest, respectively, but its effect was not significant.

Fingerprinting method comparison

The TGGE results were described previously by Kulhankova *et al.* (2006). The total number of detected ribotypes was higher in case of T-RFLP than in TGGE (125 and 50, respectively). T-RFLP also showed higher differences of ribotypes numbers among the tree species. Nevertheless, SR was found to be similar between the two techniques, when compared both tree species or sampling dates. Hence, the T-RFLP ribotypes were less frequent in the samples and many of them were rather rare, comparing to TGGE species. The redundancy analysis (RDA) thus showed lower discrimination of tree species effect (Fig. 2) than TGGE did (Kulhankova *et al.* 2006).

There were certain differences between SR of particular tree species determined by the two methods. However, both indicated that there was no strong reduction in number of fungal ribotypes after the change of dominant tree species thirty years ago. The evolution of SR along time also partly differed (Fig. 1a, b). However, the general trend of SR decrease after the hot and dry summer 2003 was documented in both cases. Similarly, the total number of detected ribotypes increased with the decomposition time in both approaches. Both approaches also indicated substantial differences in fungal community compositions within the tree species or between decomposition stages.

In the whole data set, both decomposition stage and tree stand were founded to have significant effects to SR as well as to community composition, using both techniques. Separately, the tree stand effect to SR was significant after 3 and 12 months of the decomposition and highly insignificant after 9 months, independently on approach. Similarly, the influence of tree stand to community composition decreased with the length of decomposition. The exception was the nine months sampling, when the summer conditions overlapped the tree species effect. In individual tree species, the decomposition stage was not significant in the native forest, beech and Douglas-fir, when based on T-RFLP data, while was not significant only in beech in TGGE approach. Beech had the best discriminated community composition from all tree species within all sampling dates; spruce was the second and Douglas the last, which classification resulted from both methods. However the tree species distribution in RDA plot of the whole dataset partly differed using the methods (Fig. 2, (Kulhankova *et al.* 2006)). However, beech was separated on x-axis in both approaches and even spruce was clearly discriminated from other tree species. The native forest, oak and Douglas were rather grouped based on T-RFLP as well as TGGE data. The distributions of tree species in the second sampling after the hot dry summer of 2003 were plotted in Fig. 4a, b. This date was specific by high importance of environmental conditions, reducing the species richness and significantly shifting the community composition by favouring less frequent, low competitive species able to survive the drought disturbance. The discrimination of fungal communities in individual tree species can be seen in both graphs, however the distances and positions differed.

Decomposition rule

The wood mass loss values significantly differed between the tree species (Fig. 5a),

ranging from 67% mass loss in oak to 30% in spruce in 30 months of decomposition. Both beech and oak stand values significantly differed from spruce and Douglas-fir; another significant difference was detected between the native forest and beech stand. Tree species effect was significant within all dataset as well as in all sampling dates except the first one after 3 months of decomposition. After 9 months, in early September, by far most (40%) was decomposed in beech. Three months later, after 12 months, beech still showed the highest mass loss value, even though the decay slowed down there, compared to oak, where it started to accelerate (Fig. 5b). After the second year of decomposition, mass loss was the highest in oak, followed by beech. The native forest and Douglas-fir plantation displayed similar mass loss values during entire study period and more wood was decomposed there then in the spruce stand with the slowest decay. The decomposition rate was not correlated to time (Fig. 5b). It was relatively high in the beginning of the experiment (3 months), differed significantly among tree stands during hot dry summer (9 months), and it started to decrease in the later stages (30 months).

Thirty months of decomposition

The native forest displayed significantly highest SR in the end of the experiment (13), followed by spruce (11), Douglas-fir, beech and oak stand (8.5, 8 and 7.5, Fig. 1b). This meant a significant decrease of the species richness in all tree species, when comparing with the sampling at 24 months. Tree species explained 36.4% of the community variability and its effect was not significant. All three sequenced species from the seven most common species in this sampling belonged to Basidiomycetes, which might confirm the expected trend of increasing abundance of white-rot fungi in the later stages of wood decay.

Distribution of 4 selected species

Abundances and distribution of 4 selected fungal species (based on TGGE results) are shown in Fig. 6. Majority of the species was detected in several of the tree species, but many of them showed significant preferences to some of them or to the earlier or later stages of decomposition (Kulhankova et al. 2006). Two Ascomycetes and two Basidiomycetes were chosen to etch in this more closely. Species n. 7 was determined as *Megacollybia platyphylla*, detected in the studied stands even in fruit-body form and widely known as a saprophytic species both from hardwood and coniferous forests. It was also the most common species in our study,

relatively equally distributed within sampling and tree species. Species n. 17 was highly positively correlated to early stages of the colonisation, when it appeared in all tree stands. The sequence in GenBank came from beech leaf-litter from Germany. *Phlebia albida* (n. 23), known as lignin degrading species from decaying logs, was moderately common in our samples. It was present rather in the earlier stages in the beech-dominated stands and later in oak, being less common in conifers. *Chaetosphaeria pulviscula* (n. 46) was relatively uncommon and related to later stages of the decay in all tree species. It is a saprobic pyrenomycete known from dead, often rotten wood, usually in hardwood forests.

3 Discussion

Methodology used in the study

T-RFLP was initially developed as a community profiling technique by Liu *et al.* (1997). It has been used to study a range of fungal communities in different environments, as reviewed by Avis *et al.* (2006). The study also documented, that the choice of the restriction enzyme and the presence of multiple peaks may influence the accuracy of species identification and estimates of species diversity. However, TRF peak counts may be valid for use to compare samples processed with similar methods (Avis *et al.* 2006). It is also to be noted, that in T-RFLP, a ribotype was identified with one base accuracy. In TGGE, the melting temperature of DNA fragments differed about 0.1°C to be identified as a different species, which interval could include more species with very close AT-CG ratio. Previously, Brodie *et al.* (2003) also documented higher ribotypes number detected by T-RFLP than by DGGE in grassland soil measured by 18S TRFs and DGGE bands. Though, they found T-RFLP approach to show the lower similarity values between transect positions compared to DGGE (Brodie *et al.* 2003), unlike results of this study.

The model wood material was of beech origin. It was found to decompose the fastest in the young beech forest. However, the native forest was dominated by beech as well and the wood mass loss was lower there. Similarly, pure cellulose strips also decomposed the fastest in the beech, comparing to the spruce plantation and to the native forest (Kubartova *et al.* xxxx). That could mean that the highest decomposing potential generally occurred in beech. The environmental conditions of the stands differed, as described previously (Kulhankova *et al.* 2006).

Effect of species richness to mass loss

It did not seem that the fungal species richness would be important for the decomposition rate values when studied by TGGE. The Pearson's correlation coefficient equalled to 0.08 (tested without beech in 9 months value) for the whole period of decay. The relationship was the most negative in 3 months, when spruce, beech and oak stands displayed low SR, but high decomposition rate. The most positive relationship was found in all broadleaved stands in 12 months.

Based on T-RFLP results, the correlation coefficient between SR and DR was higher (0.49). Oak and spruce stands displayed high values of SR and DR after 3 months and decrease of both variables after 9 months. Oak showed generally high values of both SR and DR. In spruce, DR was related to SR. Beech displayed very high DR after 9 months despite low SR (not included in correlation). Later, after 12 and 24 months, both SR and DR were low in beech, probably due to depletion of easily decomposing substrate during fast decay in early stages. In the native forest, DR was related to SR during the first year, but not after the second year.

Similarly to our results, Deacon *et al.* (2006) observed even a negative effect of SR increase on lignin degradation in grassland soil. Cox *et al.* (2001) provided further example of inhibition of pine litter decomposition when colonised by more complex fungal community than by a single species. A surprisingly simple community appeared to be effective in maintaining respiration and it saturated in low species richness in study with six diversity treatments (Setälä & McLean 2004). Explanation of such insignificant or even rather negative effect of SR it could be higher competition among species in rich community together with high functional redundancy between the decomposing taxa (Schwartz *et al.* 2000; Setälä & McLean 2004; Deacon *et al.* 2006).

Effect of community composition

Each tree species supported its own fungal community with specific fungal species, as shown using both molecular methods. The community composition seemed to be more important for the decomposition process than SR. Based on T-RFLP results, beech and oak stands after 9 months had similar community composition (Fig. 4a), but the DR values significantly differed between them. Conversely, spruce displayed similar DR to oak though diverse community

composition in 9 months. Nevertheless, the native forest and Douglas-fir showed similar DR and resembling community composition within all sampling dates (Fig. 2). Their communities were more distant only after 9 months (Fig. 4a). RDA results showed that no ribotypes were either positively or negatively related to DR values (data not shown).

Based on TGGE data (Kulhankova *et al.* 2006), beech stand with the highest decay rate during early 9 months had highly distinct community composition (Fig. 3b). Later, at 12 and 24 months, oak showed fast decomposition and the community composition resembling to the beech one, with species common for both. Certain of that species present mainly in beech and oak were correlated to higher DR (tested by RDA, data not shown). These were e.g. species 28, 33 and 41 (the closest Blast matches were AY063309, AF148952 and AY354279 respectively) (Kulhankova *et al.* 2006). Certain other species were correlated with DR, e.g. 6, 42 and 46 (AF502889, AY230788 and AF178544), even though they were not characteristic for beech or oak stand. They were correlated rather to time of exposition. All of these species belonged to Ascomycetes. Basidiomycetes made up the minority of the sequenced species and seemed to be less frequent, at least in the early stages of the decomposition. Being rather rare, they might be both not so clearly related to fast decay as abundant species or have the bands not intensive enough to be sequenced.

It is to be noticed that the community composition in all tree species highly differed in 9 months after dry summer, using both approaches. It distinguished even from the general rule of the species succession observed. The infrequent species partly replaced the common species and SR decreased. Despite that, decomposition ran very fast in beech, slightly faster than usually in spruce and the native forest, while it was suppressed in oak and Douglas-fir (Fig. 5). This could document the role of the rare species and the importance of the whole pool of the species present in a stand, not only species active in a single sampling time. Accordingly, in the study of Deacon *et al.* (2006) infrequent taxa played important role in decomposition and were potentially more active than common ones. Indeed, the decomposing ability of particular fungi can highly differ, as documented by Osono and Takeda (2002) when testing beech litter decay and Osono *et al.* (2003) testing Japanese larch litter decomposed by many fungal isolates under laboratory conditions. However, the inter-species interaction in natural condition might decrease the genetic decomposing ability effect. Cox *et al.* (2001) illustrated the importance of interactions of specific fungi rather than the functional competence of diverse communities, when studied the process of

Scots pine litter decomposition. Actual environmental conditions and nutrient status sustaining or inhibiting microbial activity also involve decomposition process, co-acting with other variables (Adl 2003).

No really key-species in terms of decomposing activity were detected. However, they might appear in very late stages, when only highly recalcitrant substrate will be available for decomposers, as certain trends prompted in 30 months sampling. Some fungal species were observed only in later stages of the experiment and the number of Basidiomycetes in samples seemed to increase. The SR dropped significantly in 30 months compared to 24 months and DR started to slightly decrease as well. The effect of tree species was insignificant in both 24 and 30 months, indicating decreasing importance of microclimatic variables, which were highly affected by tree species. Even after the 30 months of decomposition, high amount of the wood remained to be decomposed (33-70%) and presence of highly specialised species might be needed to decompose such recalcitrant substrate more completely.

Tree substitution effect

The humus layer physical conditions and chemical composition changed during the thirty years from the native forest cut and the monoculture plantations (Ranger *et al.* 2004). However, this period might not be enough to exhaust the soil environment completely to cause a radical change of biodiversity and eventually a loss of key-stone species. However, this could happen after a few generations of monoculture plantation. In the present case, the fungal community composition differs between the tree species, but the species pool seems to rest still sufficient to cover the function of organic matter decompositions. Moreover, it is complicated to separate particular variables easily. Species typical in the broadleaved stands were replaced by other species characteristic for coniferous stands. Such species could maintain the decomposition processes in these stands. Species present both in broadleaved and conifers could be either tree-species generalists and rather resource-specific, or preferring the broadleaved, but still surviving in conifer stands, despite of an activity inhibition there. Decomposition run relatively slower in the coniferous stands, but it is not easily explainable by the species lack and/or the community change. It is more likely that the lower decomposition rate in conifers was the cause of less favourable conditions, e.g. lower temperature, higher moisture, higher concentration of inhibitors, such as phenols and tannins or by lower concentration of nutrients (Ranger *et al.* 2004,

Kulhankova *et al.* 2006). Further, functional redundancy and interactions among species might also influence decomposition in the studied stands (Cox *et al.* 2001).

The native forest contained more dead wood in different stages of decay than the young monocultures. The coarse woody debris was found to benefit the saprophytic fungal diversity in European beech forests (Heilmann-Clausen & Christensen 2004; Lindhe *et al.* 2004; Odor *et al.* 2006). Even fine woody debris was documented to support species rich fungal communities (Kruys & Jonsson 1999; Norden *et al.* 2004). SR in the native forest was higher both during the first year as well as in 30 months in this study. That might indicate higher diversity of highly specialised species in 30 months, comparing to plantations. A meta-analysis study (Schmit *et al.* 2005) confirmed tree species diversity to be a significant predictor of macrofungi diversity as a whole, but mainly for ectomycorrhizal and soil/leaf species, while the significance was lower for wood inhabiting macrofungal richness. It could be due to lower specialisation of the wood-invaders to the tree species and higher to substrate, i.e. wood.

5 Conclusions

Tree dominant change affected the fungal community composition in the studied stands, as documented by both T-RFLP and TGGE approach. T-RFLP results showed more positive relationship between species richness and decomposition rate, while TGGE results displayed higher importance of particular fungal species for the decay. However, neither species richness nor community composition seemed to have direct impact on the process of wood decomposition. It might be for several reasons, such as primary control by abiotic factors, or strong overlap of resource use by different species. Important fall of species richness and change of community composition was detected after dry hot summer, though decomposition rate slowed only in two of the five tree species. It documented similar functional potential of different fungal community, importance of environmental conditions as well as the necessity to maintain the diversity of the decomposers to sustain disturbances. The results also indicated that presence of specialised species should have increasing importance in later stages of the wood decay.

Figures

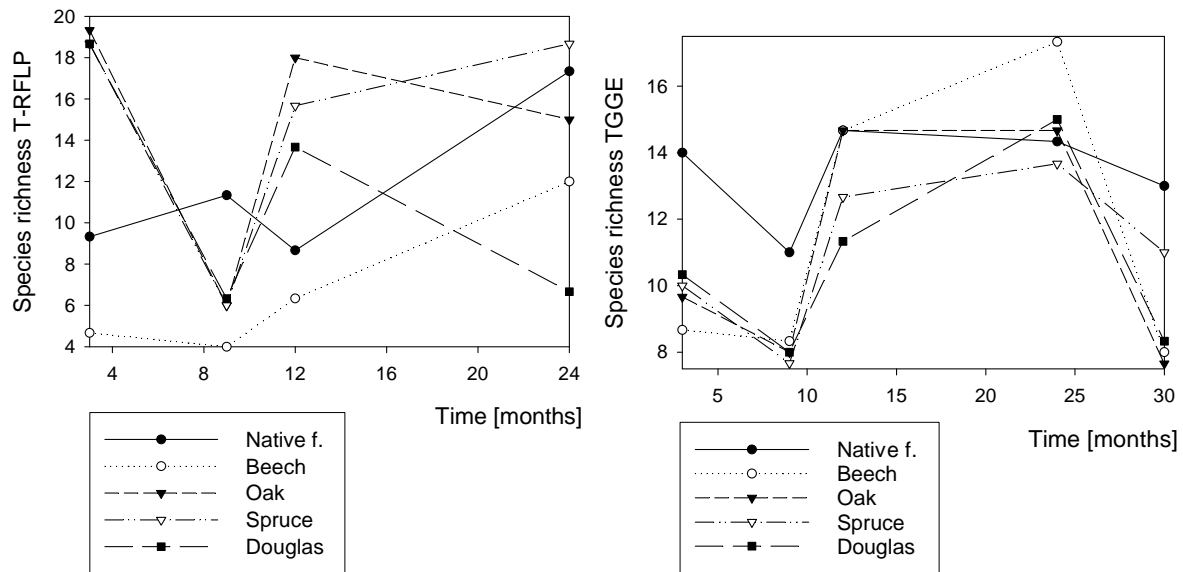


Fig. 1 Species richness detected by two fingerprinting methods; a) by T-RFLP as the number of TRF peaks, b) by TGGE as the number of bands.

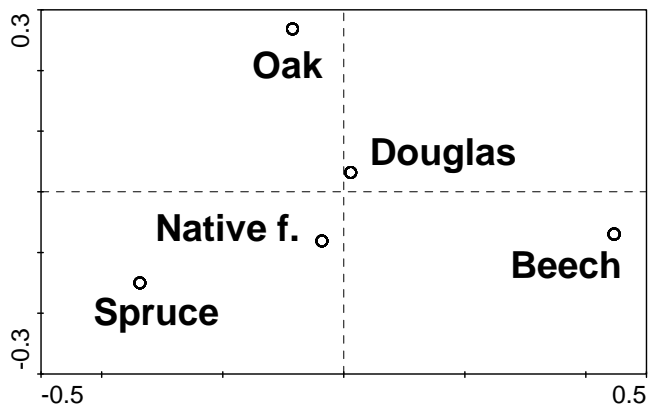


Fig. 2 Tree species positions in ordination space according to whole T-RFLP data set, i.e. the ribotype matrix of all five tree species and four sampling dates tested together (analyzed by RDA).

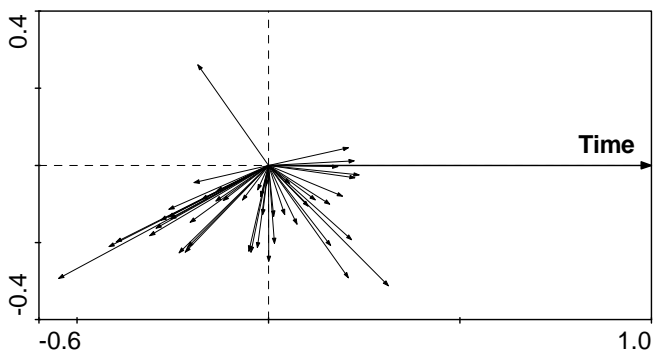


Fig. 3 RDA biplot documenting T-RFLP ribotypes (represented by arrows) correlation to time of decomposition in all tree species together (ribotypes detected less than 5x not shown). No ribotypes were strictly related to early or later stages of the experiment.

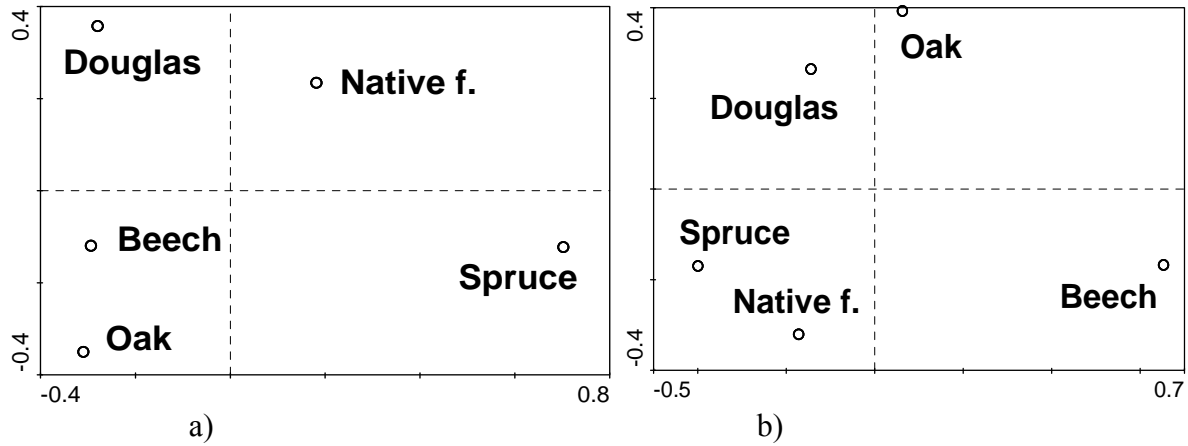


Fig. 4 Distribution of tree species in RDA plots, according to fungal community composition in the second sampling after nine months of the decomposition. Hot and dry conditions during the summer preceding the sampling reduced the species richness and suppressed certain species, given the ability to weak competitors to develop; a) based on TRF peaks, b) based on TGGE bands.

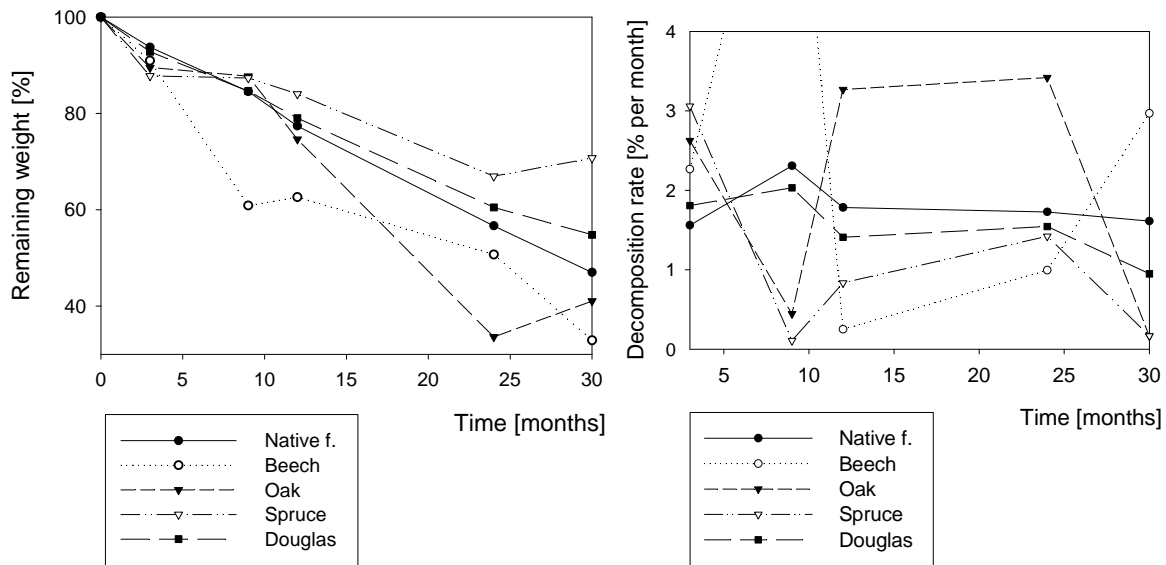


Fig. 5 Decomposition of wood samples during 30 months of the experiment in the five studied tree species, a) remaining weight of the samples, b) decomposition rate per months.

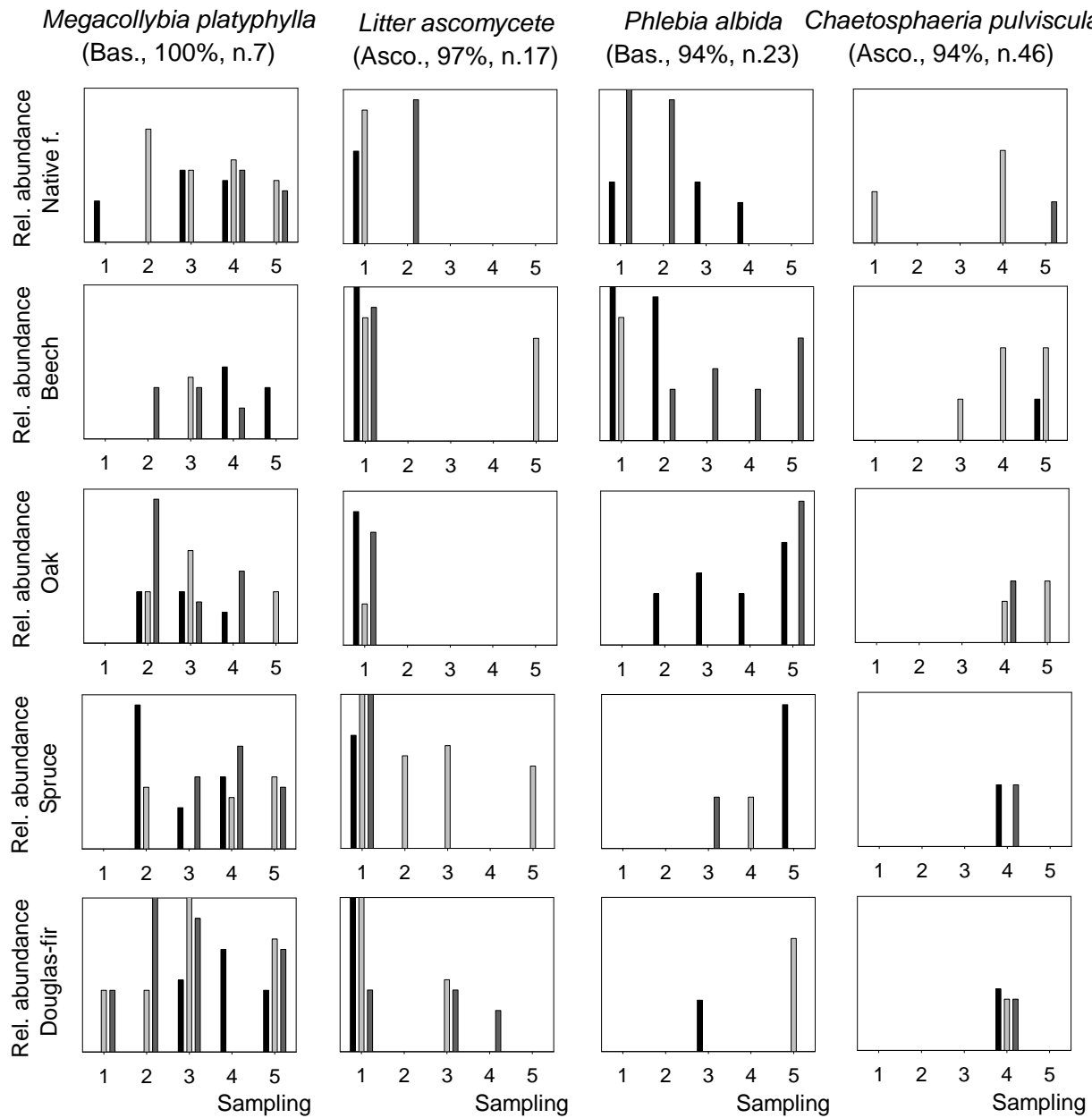


Fig. 6. Detailed visualization of abundances of four selected fungal species in particular tree species stands over time (3, 9, 12, 24 and 30 months of decomposition, x-axis). 1-3 connected columns of different tints express the species presence or absence in the three replicates of each sampling date. Height of column indicates the relative abundance of a given species in the sample (max. shown=20%, y-axis). The headlines describe fungus name, number code used in ordination plots and percentage of similarity with DNA sequence available in the Genebank database.

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Diversity and decomposing ability of saprophytic fungi from temperate forest litter

Kubartová Ariana ^{1*}, Moukoumi Judicaël ^{2,3}, Ranger Jacques ², Berthelin Jacques ³, Thierry Beguiristain ²

¹ Department of Forest Mycology and Pathology, SLU, Box 7026, 750 07, Uppsala, Sweden

² INRA, Centre de Nancy, Unité Biogéochimie des Ecosystèmes Forestiers, Champenoux, 54 280, France

³ LIMOS, CNRS UHP UMR 7137, Université Henri Poincaré, BP 239, Vandoeuvre-les-Nancy, 54 506, France

* Corresponding author: tel: +(46) (0)18 671 804, e-mail: Ariana.Kubartova@mykopat.slu.se

Abstract

This study was designed to examine saprophytic fungi diversity under different tree species situated in the same ecological context. Further, the link between the diversity and decomposition rate of two broadleaved, two coniferous and two mixed broadleaved/coniferous litter types was targeted. Litter material was decomposed in litter bags for 4 and 24 months to target both early and later stages of the decomposition. Fungal diversity of L and F layer was also investigated as a parallel to the litter bag method. Temperature gradient gel electrophoresis (TGGE) fingerprinting method was used to assess fungal diversity in the samples. Mass loss values, organic and nutrient composition of the litter were also measured. The results showed that the species richness was not strongly affected by the change of the tree species. Nevertheless, the community compositions differed within tree species and decomposition stages. The most important shift was found in the mixed litters from the litter bag treatment for both variables. Both mixed litters displayed the significantly highest species richness and the most distant community composition comparing to pure litters after 24 months. Even the mass loss after 24 months was higher in the mixed litter than in both original pure litter types. This was probably due to higher niche variability and to synergistic effect of nutrient transfer between litter types. Concerning pure litter, mass loss values were the highest in oak and beech litter. That was probably caused by more favourable microclimate and litter composition in broadleaved than in coniferous plantations. These variables also seemed to be more important to pure litter decomposition rates than fungal species richness or community structure.

Keywords: fungal diversity, litter decomposition, mixed litter, forests, TGGE

1 Introduction

It is widely viewed that changes in forest above-ground diversity and structure indirectly affect the soil microbial community and its functions. Plant litter decomposition is a key process in nutrient recycling and humus formation in forest ecosystems (Berg 2000; Berg & McClaugherty 2003; Prescott 2005). Saprophytic fungi play an important role in decomposition because they can attack lignocellulose matrix in litter that other organisms are not able to assimilate (Dix & Webster 1995; Adl 2003). The change in the litter quality during decomposition induces a succession of microbial communities: the r-strategists (opportunists) dominate during the early stages and are replaced later by K-strategists (persisters) due to growth of limiting substrate concentrations (Dilly *et al.* 2001). The process typically begins with litter colonisation by bacteria, Ascomycetes, and imperfect fungi (Deuteromycetes), which consume the less recalcitrant components. The cellulose present in non-lignified tissues is then attacked by some of these organisms. Subsequently, the remaining lignified litter is colonised mainly by brown-rot and white-rot Basidiomycetes that degrade it further. The remaining highly recalcitrant compounds become a part of the soil organic matter.

An increase of tree species diversity in forests should leave to an increase in microbial biomass, diversity and activity by higher niches variability (e.g. diversity of carbon sources in more variable litters and root exudates, higher primary production, variable microclimate, presence of dead wood or spatial and age variability of trees) (Setälä 2002). Deciduous litter should be more favourable for microbial decomposers than the coniferous one and its greater quality may have a positive influence on the diversity and decomposition processes. Several recent studies focussed on decomposition rate, nutrient dynamics and/or decomposer activity of both pure and mixed plant litters, as reviewed by Gartner & Cardon (2004). Similarly, certain studies assessed microbial diversity within forest ecosystems using molecular or phospholipid fatty acid analyses, as reviewed by Leckie (2005). However, studies investigating decomposition rates *in situ* together with the fungal species richness and community composition are still scanty.

Higher microbial species richness is generally expected to increase the average rates of relating ecological processes by higher enzyme diversity and niche complementarity (Ekschmitt *et al.* 2001). Nevertheless, decomposing ability of each species vary depending on environmental conditions and also on interactions with other fungi. Many fungi are supposed to be functionally redundant and potential capacity for inter-specific competition can be large. In such case the effect of diversity could be also weak caused by saturation at low species

richness and/or by enhanced competition (Cox et al. 2001; Wardle & van der Putten 2003; Setälä and McLean 2004, Hättenschwiler et al. 2005).

The main purpose of this study was to assess *in situ* fungal colonization of forest tree litter and to investigate the role of the fungal diversity on the decomposition rate. The targeted tasks were: i) how does a tree species affect fungal species richness and community structure during the litter decay ii) how are they affected by stage of decomposition iii) how do the litter mass loss rates coincide with the fungal species richness and diversity.

2 Materials and methods

Experiments were conducted in the Breuil forest experimental site, Burgundy, France. This site represents an opportunity to specifically evaluate the tree species influence, being composed of control semi-native forest and mono-specific even-aged plantation stands in homogeneous area. Hence, the impact of the vegetation cover results only from different stand structure, litter quality and/or changed microclimate. The site of Breuil is located in the Morvan Mountains, France. Its altitude is 640m, latitude 47° 18' 10" and longitude 4° 4' 44". Mean annual temperature is 9° C, with 1280mm precipitation and evapotranspiration of 640mm. The previous forest was an old coppice with standards, dominated by *Fagus sylvatica* L. and *Quercus petraea* Smith, associated with several other dominant species like *Betula verrucosa* Ehrh. and *Corylus avellana* L. It evolved slowly towards a high forest structure, being not harvested for more than 50 years. This native forest was partially cut down in 1976 and replaced by various monocultures planted in 1000m² stands each. Five different stands were selected for the litter bag experiment: four young plantations, namely European beech (*Fagus sylvatica* L.), sessile oak (*Quercus petraea* Smith.), Norway spruce (*Picea abies* Karst.) and Douglas-fir (*Pseudotsuga menziesii* Franco.) and the native forest as a reference stand.

Leaf and needle litters of the four plantations were decomposed in 5x5cm litter bags of mesh size 5µm in the plantation where they originated. Two leaf-needle litter mixtures were decomposed on the border of the adjoining involved stands using the same bags. These included spruce-beech mixture and Douglas-fir-oak mixture, both 50% of each litter type. The distance between two replicates was 0.5m. The bags were placed in the stands in late November 2002. They were collected after four months (March 2003) and twenty-four months (November 2004) of the incubation. Five replicates were sampled at each tree species, three of them were later used for molecular analyses. Mass loss of the decomposed litter was

calculated for the five replicates as a difference between initial and final weight of litter dried at 65°C for 24 hours.

Forest floor sampling was carried out in late June 2004 in the four plantations and in the native forest. Three replicates of L and F layer were sampled in each stand. They were distant 10cm from each other. All the samples were immediately deeply frozen for the storage in the lab. Organic and nutrient composition of the falling litter and nutrient composition of the forest floor were analyzed.

The structure of fungal communities was assessed by TGGE, which has been shown to be a sensitive and robust technique for investigating soil microbial communities (Muyzer 1999; Anderson & Cairney 2004). Total genomic DNA was isolated from the samples using the Dneasy plant mini kit (Qiagen) following the manufacturer's protocol without any modification. Fungal communities were analysed using ITS1F and ITS2 primer pairs to amplify by PCR the 280bp fragment of the fungal ITS rDNA. The primer ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes & Bruns 1993) is higher fungi ITS specific, while ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3') is a universal primer amplifying the ITS region from Eucaryotes, including both Ascomycetes and Basidiomycetes (White *et al.* 1990). A 40 bp GC-clamp was attached to the 5' end of the primer ITS2 to avoid a complete separation of DNA strands during the denaturing electrophoresis. The reaction medium consisted of 5µl of PCR buffer (Sigma, 100mM Tris-HCl, pH 8.3, 500mM KCl, 15mM MgCl₂), 1µl of dNTP (10mM), 1µl of each primer (20µM), 0,5µl of Taq-polymerase (5 units/µl, Sigma), 1µl of GC-rich solution (Sigma), 2.5µl of BSA (3%) and 2µl of genomic DNA in a final volume of 50µl. The amplification regime consisted of an initial cycle of denaturation at 95° C for 3min followed by 35 cycles of denaturation at 94° C for 45s, annealing at 55° C for 45s and extension at 72° C for 1min 15s. The amplification concluded with a final elongation step at 72° C for 8min. The PCR reactions were performed using an iCycler Thermal cycler (Bio-rad). PCR products were checked on 1.5% (wt/vol) agarose gels followed by ethidium bromide (0.5 mg/l, Bio-rad) staining. TGGE was performed with a Dcode Universal Mutation Detection system (Bio-rad). Polyacrylamide gels ((8% acrylamide (wt/vol), 8M urea, 1.25x TAE and 0.2% glycerol (vol/vol), 300µl of ammonium persulphate (10%) (wt/vol) and 30µl of TEMED) were used. Amplified DNA samples with the same volume of loading buffer (10µl each) were separated by electrophoresis in 1.25x TAE at a constant voltage (145V) at a temperature gradient from 50° C to 55° C with a temperature increment of 1 degree per hour. Gels were stained with silver nitrate after the electrophoresis.

A matrix of species distribution was calculated based on image analyses using the Quantity One software (Bio-Rad) with a band position tolerance of 2mm, giving a maximum of 50 detectable species. Presence/absence matrix was created. Species richness, i.e. species number per sample, was calculated (referred SR below). ANOVA tests of SR and mass loss were counted using the Statistica software (StatSoft). Multivariable analyses of the litter bags and forest floor fungal community composition were done using CANOCO (Microcomputer Power). Principal component analysis (PCA) was used first, corresponding to the successive dimensions of maximum variance of the scatter of samples. Redundancy analysis (RDA) then ordinated fungal communities and tree stands as environmental variables, such that the relative position of the communities reflect their similarity and/or dissimilarity. The relative significance of the fungal species vectors was indicated by their length and direction from the axes origin. Monte Carlo permutation tests calculated the significance of the environmental factors (Ramette 2007).

3 Results

3.1. Decomposition rate and litter properties

The values of cumulative mass loss in the litter bags were given in Fig. 1. The major part of the litter material was decomposed in both mixed litter experiments after four months of incubation. Concerning the pure litter material, the mass lost decreased from oak, Douglas-fir, spruce, and beech. The effect of litter type was significant. Beech and coniferous mass loss values significantly differed from oak and both mixed litter values. The tree species order changed after 24 months of incubation, when most of the material was decomposed in oak-Douglas-fir mixed litter. The classification was then: oak > beech-spruce mixed litter > beech > Douglas-fir and spruce. The litter-type effect was not significant in this case: only spruce significantly differed from oak and mixed litters.

The litter types differed in their mineral and organic composition (Tab. 1). Litter from beech, oak and the native forest had higher nitrogen content than spruce and Douglas-fir. This was both falling litter and forest floor case. The broadleaved trees also had lower C/N ratio, higher potassium and calcium concentration in falling litter. The native forest had the highest concentration of N, P and Ca from all tree species and second highest of K in the forest floor. Beech litter was the richest on soluble compounds, while Douglas was the poorest. However, it had the highest concentrations of both cellulose and hemicelluloses. The lowest concentrations had oak and beech, respectively. Beech showed the lowest amount of lignin, oak the highest. Oak had also high lignin/N ratio, followed by spruce, and high ash content.

3.2. Fungal diversity in litter decomposed in litter bags

All of the fifty potentially detectable fungal species were identified from the incubated samples. The species richness (SR, i.e. number of species per sample), ranged from five to twenty. The average SR per sample within all tree stands was 11.8 after four months of incubation and 10.6 for the twenty-four months sampling. Hence, it did not significantly differ between samplings. Beech stand showed the highest and significantly different SR in the first sampling, followed by conifers, mixed litter samples and oak. Mixed litter samples presented the highest SR in the second sampling, followed by spruce, oak, beech and Douglas-fir. The Douglas-fir value was significantly different from all the others and beech value significantly differed from both mixed litters. Comparing the two sampling dates, higher SR was found in oak and mixed litters in the later sampling than in the former, but lower in the others (Fig. 2a). Tree species effect on SR was found to be significant in both samplings.

Each tree species was characterised by a specific fungal species pattern in the litter bag samples. In RDA, 49.3% and 47.7% of the variability of the community composition was explained by the forest tree species effect in the first and the second sampling, respectively. Decomposition stage effect explained 11.5% of the fungi species shift between former and later sampling within all tree species together. Random test confirmed the significant influence of both forest tree species and the decomposition stage. In the RDA plot of the first sampling (Fig. 3a), beech was clearly discriminated from other tree species on the x-axis with presence of certain characteristic species. Spruce stand was the most distant on the y-axis, also having different species composition. The mixed litter samples were positioned in the central part of the graph. No typical species were associated with them in the former sampling. Nevertheless, they were highly separated from pure litter types in the second sampling, tending to occupy the right part of the RDA plot (Fig. 3b). However, they were well discriminated on the y-axis from each other. Lot of species were present only in these mixed litter samples in the later sampling. Spruce showed the most distinct community structure from the pure litter samples, similarly to the first sampling. It was positioned separately along the y-axis, but kept the same position as other pure litter types along the x-axis. These pure litter types, i.e. beech, oak and Douglas-fir, were clustered together, indicating relatively similar fungal communities involved in their decomposition process. Beech stand showed the most pronounced shift in community composition between the former and later sampling dates. The shift of the mixed litter communities was also significant. Oak and beech communities changed less and Douglas-fir community remained almost similar.

Thirty three of the fifty species were identified in both former and later sampling. However, there was a clear abundance difference in case of the majority of species between the sampling dates. Five species were detected in all tree litter types. Five other species were detected only in one sample. Two species were specific to only one tree stand. Certain species detected in mixed litter samples were not identified in any of pure litter samples. Lower number of species was identified in pure litter types, but not in mixed litter samples. Thus, species occurring in mixed litter samples were not always present in either of the original pure litter samples and conversely.

3.3. Fungal diversity in forest floor layers

Forty-five different fungal taxa were detected in L and F forest floor layers together. From eight to eighteen species were identified per sample. The average SR was 13.7 species per sample in L layer and 12.1 species in F layer, being not significantly different. Beech stand expressed the highest SR in L layer, followed by conifers, the native forest and oak, but only beech and oak values differed significantly. In F layer, Douglas-fir was the species richest and the only significantly different stand, followed by the native forest, spruce, beech and last oak again (Fig. 2b). Comparing the layers together, Douglas-fir and the native forest had higher SR in F layer than in L layer, the other tree species in L layer. The tree species effect to SR was not significant in L layer, but it was in F layer.

When testing tree species effect to community composition by RDA, it explained 43.6% of L layer variability and 46.1% of F layer variability. L or F layer effect (i.e. decomposition stage) explained 10.7% of the variability within all five tree species together. Random test showed both the tree species effect in both L or F layer and the L or F layer effect to be significant to fungal species distribution. In the RDA plot of L layer diversity (Fig. 4a), the fungal communities associated with the native forest and spruce were discriminated from the others along the x-axis. Beech and oak stands occupied similar position on the x-axis. However, beech was discriminated on the y-axis, while oak was positioned in the central part of the plot. Douglas-fir position was distant from the other tree species on both axes, being the closest to oak. The native forest and spruce had similar position on the first axis even in F layer RDA graph (Fig. 4b). Similarly, oak and beech did. Douglas-fir was discriminated from other tree species again. The native forest displayed high SR value but had many species common with the plantations. The most pronounced community shift between L and F layer were found in Douglas-fir. The communities changed in other tree species as well, the least in oak and beech stands.

Forty of forty five species occurred in both forest floor layers, often showing preferences to one or the other. Sixteen species were present in all tree stands, at least in one of the layers. Only one species was identified only once. All the samples both from litter bags and forest floor layers were also analysed together (Fig. 5). Canonical axes expressed 16.2% of the whole data set variability. Samples from 24 months sampling were positioned between L and F layer on both axes, clustered in the upper left part of the RDA plot. Four months sampling, representing the early stages of decomposition, was clearly separated from the others on the x-axis. F layer, representing the later stages, was well discriminated on the y-axis. This corresponded well to expected decomposition stages of the samples (4 months > L layer > 24 months > F layer).

4 Discussion

4.1. Decomposition rate

The differences in decomposition rate among forest tree species are associated not only with different organic composition of litters (Osono & Takeda 2002), the heterogeneous distribution of carbon and nitrogen resources (Osono & Takeda 2001a), but also with diverse SR and community composition and/or different microclimate of the stands (Berg & McClaugherty 2003). Early decomposition rates, i.e. 4 months here, are supposed to be strongly related to microclimate and litter chemistry of water soluble nutrients and structural carbohydrates. Later decomposition rates (24 months) are more influenced by lignin concentrations in the litter material (Berg 2000). Oak, as the warmest and moistest stand (Ranger *et al.* 2004) with the nutrient richest litter displayed the highest mass loss from pure litter samples both in four and twenty four months of decomposition, despite high lignin concentration and high lignin/N values. In the beech stand, the decomposition was the slowest after 4 months of decomposition, despite the highest N amount. It might be caused by high concentration of soluble compounds, which included inhibiting phenolic substances (Wardle 2003). However, it had the second highest mass loss in 24 months, coinciding with low lignin concentration and lignin/N values. Conifers had less favourable litter composition and microclimatic conditions and displayed lower mass loss values than broadleaved in the 24 months. Douglas needles decomposed more rapidly than spruce litter, being richer in N and having lower soluble compounds and lignin concentration. Mixed litter mass loss values were higher than values of both original pure litter types both in formal and later sampling time and are closer discussed below (4.5).

4. 2. *Fungal diversity in litter decomposed in litter bags*

The study supported the hypothesis of tree species influence on decomposing fungi communities. As a matter of fact, majority of species should originate in the native forest, being suppressed or favoured by the conditions in the young plantations. The high SR in beech at the beginning stage of litter colonisation coincided with its lowest lignin/N value. Oak showed similar N concentration as beech, but the highest lignin/N value of all stands. The high SR values in both mixed litter types in the later sampling could be explained by higher niche variability for decomposers and related decreased competition. Low SR in Douglas-fir might coincide with insufficient moisture during preceding months, since the Douglas-fir stand was the driest of all (Kulhankova 2006). In the 4 months sampling in March, fungi could be inhibited by unfavourable conditions during winter, mainly by low temperature values. Conversely, later sampling in November could have high fungal activity due to favourable moisture and temperature conditions and to input of nutrients leached from newly fallen litter.

Lejon *et al.* (2005) also documented significant discrimination of fungal communities within 5-10cm of the soil profile in the same study site, using ARISA technique. Certain other studies documented the differences in microbial community composition in diverse forest ecosystems (Pennanen *et al.* 1999; Saetre & Baath 2000; Priha *et al.* 2001; Myers *et al.* 2001; Wilkinson *et al.* 2002; Leckie *et al.* 2004; Hackl *et al.* 2004; Grayston & Prescott 2005; White *et al.* 2005), seasonal changes in community composition (Myers *et al.* 2001) or succession of fungi during decomposition (Osono 2002), reviewed by Osono & Takeda (2001b) or Virzo De Santo *et al.* (2002). Microbial activity also differed within forest ecosystems, as documented e.g. by Ohtonen & Vare (1998), Emmerling (2002) or Fisk *et al.* (2003). However, all the studies are hard to be compared, since using different scales and techniques in various climatic conditions.

4.3. *Links between biodiversity and decomposition rate*

No general positive effect of SR on the mass loss increase was detected (Fig. 1, 2), the variables were not necessarily correlated. Certain positive effect was found for 24 months for species-rich, well decomposed mixed litters. Rather negative relations were also found, e.g. in the oak stand in the 4 months sampling, where low SR was detected together with relatively fast decay. Conversely, the beech litter with significantly higher SR decomposed slowly. After 24 months, litter bags from Douglas-fir, relatively poor in fungal species, showed higher mass loss than spruce with rather higher SR, comparing to other pure litter types.

The tree species studied seemed to retain sufficient fungal diversity to compensate for species suppressed by the change of the dominant tree. There was no clear diversity decrease observed to influence the decomposition rate negatively by elimination of key-species. Community composition seemed to have only minor effect to decomposition rate, probably due to a high degree of functional redundancy of decomposing fungi, as documented by Deacon *et al.* (2006) in assemblages of cultivable fungi from grassland soil. Similarly, Setälä & McLean (2004) reported that functional efficiency of fungal communities increased with the number of taxa only at the species poor end of the gradient, when studying a gradient of SR in mixtures of saprophytic fungi from pine-spruce forest. Favourable microhabitats might be preferentially colonised, but less favourable are also exploited as total abundance and competition rise, largely independently of the number of species involved (Ekschmitt *et al.* 2001). Unexploited gaps after a species loss can thus be eventually closed by the remaining species (Jones & Bradford 2001). Biotic interaction between the fungal species in the community might be more important than its composition, e.g. in the beech stand in 4 months, where enhanced competition in rich community might slow the decay process. Conversely, the competition might be decreased by higher niche availability in mixed litter samples in 24 months and thus not slowing the decay. According to the results of the present study, not either certain decrease in functional diversity of microorganisms in plant debris did result in decline of decomposition rate (Degens 1998). Similarly, the experimental reduction of the microbial communities had no direct effects on soil functions in arable soils (Griffiths *et al.* 2001). (Cox *et al.* 2001) demonstrated similar mass loss values of Scots pine needles inoculated by a cellulolytic species and a species able degrade lignocellulose, and higher mass loss values compared to control litter colonised by a more complex fungal community. However, the two species affected the overall dynamics of decomposition at a biochemical level and illustrated importance of interactions of specific fungi. Conversely, short term decomposition in pasture soil decreased with decreasing biodiversity after fumigation (Griffiths *et al.* 2000).

Nutrient status of the litter and its organic properties, together with actual activity of species according to environmental conditions (Aerts 1997) seemed to be more important for mass loss values than SR or community composition. Accordingly, Wilkinson *et al.* (2002) documented decomposer community composition in pine and spruce forests to be largely determined by climatic conditions and litter quality while differences in community composition had no apparent functional consequences for litter decomposition.

4.4. Fungal diversity in forest floor layers

Forest floor was sampled at the end of June 2004, thus during a rather warm and humid period, supposed to support high fungal activity. L layer was mainly composed from well fungi-colonised litter material fallen down the preceding autumn or slightly older, while F layer represented later stages of litter decomposition. Slight decrease or increase of SR was detected between L and F layer, according to the tree species, but the differences were mostly insignificant. In previous studies, total and living fungal hyphal length was found to be higher in L layer than in F and H layers (Berg *et al.* 1998; Osono *et al.* 2003). Lindahl *et al.* (2007) found a clear shift in fungal community composition between the L and F horizon in a Scots-pine boreal forest. Comparing the forest floor layer and litter bag results, samples of 24 months showed species structure intermediary between L and F layer (Fig. 5), even when analysing the results for the four plantations together. The samples of the very early stages of colonisation, i.e. four months, displayed clearly different community composition than the older ones. That agreed with the assumed colonisation time since the litter shed. However, fungal community in the litter bags was expected to develop under partly different conditions, mainly modified moisture and exclusion of soil fauna, compared to surrounding litter layers (Prescott 2005).

4.5. Mixed litters

From numerous previous experiments using mixed litter material, it is clear that decomposition patterns are not always predictable from simple-species dynamics (King *et al.* 2002; Gartner & Cardon 2004). Also in the present study, the mixed litter showed different dynamics than corresponding pure litter types. Litter mixture is expected to support a greater number of microhabitats and chemical diversity and can also influence overall decomposition rate and microbial activity through the transfer of nutrients and secondary chemicals. Nutrients released from rapidly decaying, higher quality litter can stimulate decay in adjacent, more recalcitrant litter or conversely, decay can be slowed by release of inhibitory compounds such as phenolics and tannins (Gartner & Cardon 2004, Hättenschwiler *et al.* 2005). This study results documented litter variability to enhance fungal diversity and to increase the decomposition rate in temperate forests.

The decomposition rate change after mixing of broadleaved litter with coniferous litter was documented previously (Prescott *et al.* 2000; Wardle *et al.* 2003; Gartner & Cardon 2004). Additionally, this study brings data on the fungal succession changes in the litter. The mixed litter samples were colonised by similar species as the pure litters in the former

sampling and were not outstanding in SR values. However, mixed litter mass loss was higher than the pure litter values. In the later sampling, the highest SR and well discriminated community structure was typical for both mixed litter types. Both of them decomposed faster than corresponding pure litter samples. Indeed, it seemed that higher niche variability and resource diversity allowed more fungal species to coexist in mixed litter without increasing the competition and inhibiting their activity.

5 Conclusions

The study showed that saprophytic fungal communities under the studied tree species differed in species composition. Succession of species according to decomposition stage was observed as well. The mixed litter samples were found to sustain fungal diversity, inducing the most distant communities composed of characteristic species in the later decomposition stages. Decomposition rate was also increased comparing to corresponding pure litter. The species richness was not found to be generally positively correlated to the decomposition rate. Higher decomposition rate values in species-rich mixed litter might be explained rather by higher niche and resource variability. On the contrary, enhanced competition in the species-richest communities might possibly slow the decay (e.g. beech litter in 4 months).

Tables and figures

Tab. 1 Properties of the studied substrates; A) organic composition of the litters decomposed in the litter bags and in the native forest [%], B) concentrations of nutrients in the litters decomposed in the litter bags and in the native forest [g/kg], C) concentrations of nutrients in the forest floor [g/kg]

A	soluble	hemicellul.	cellulose	lignin	lignin/N	ash
Native f.	32.2	16.7	23.5	24.1	1.5	3.5
Beech	44.2	17.6	18.0	16.2	1.2	4.1
Oak	30.2	13.3	22.1	29.9	2.2	4.5
Spruce	37.1	16.5	19.4	23.6	1.8	3.5
Douglas	19.6	19.0	42.5	17.1	1.4	1.9

B	N	C/N	P	K	Ca	Mg
Native f.	23.2	22.3	1.03	9.51	2.28	0.84
Beech	25.2	21.0	1.00	8.24	2.33	0.50
Oak	23.9	21.3	1.16	8.52	2.75	1.20
Spruce	15.1	34.4	1.11	7.34	1.44	0.71
Douglas	18.0	29.1	0.99	6.77	2.26	1.08

C	N	C/N	P	K	Ca	Mg
Native f.	16.1	32.1	0.81	1.19	2.72	0.61
Beech	13.9	37.3	0.63	1.22	2.46	0.56
Oak	13.6	38.1	0.70	1.12	2.64	0.69
Spruce	12.9	36.9	0.67	1.12	1.27	0.55
Douglas	12.6	44.6	0.63	1.2	1.98	0.58

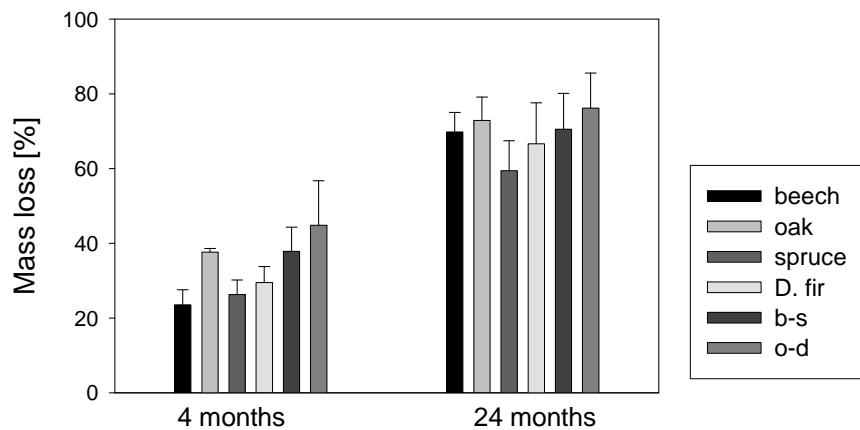
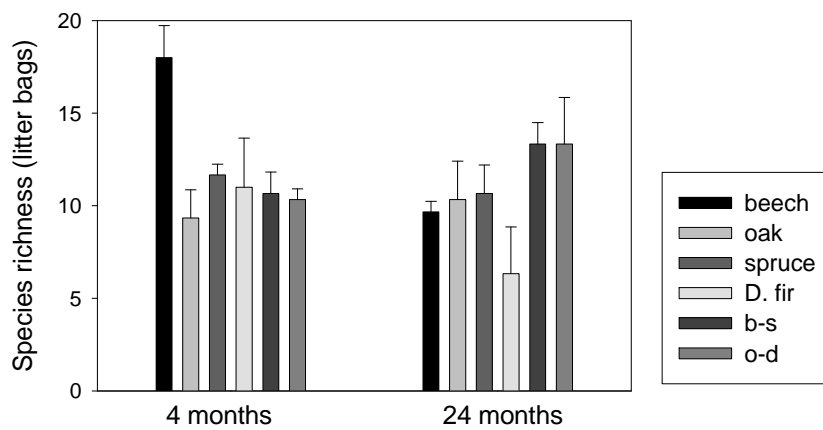
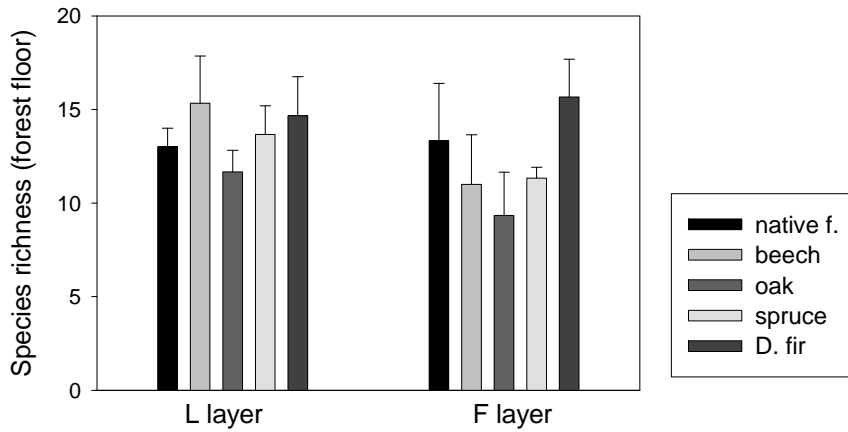


Fig. 1 Mass loss of the litters decomposed in the litter bags, sampled after 4 and 24 months of decomposition (b-s = beech-spruce mixed litter, o-d = oak-Douglas mixed litter). 4 months: beech and coniferous litter values significantly differed from oak and both mixed litter values; 24 months: only spruce differed significantly from oak and mixed litters.



a)



b)

Fig. 2 Fungal species richness (SR), i.e. average number of species per sample, detected:
 a) in litter decomposed in litter bags, sampled after 4 and 24 months of *in situ* decomposition (b-s = beech-spruce mixed litter, o-d = oak-Douglas mixed litter); beech significantly differed in 4 months, Douglas significantly differed from all the others and beech differed from both mixed litters in 24 months; b) in L and F layer of forest floor; in L, only beech and oak values differed significantly, in F, Douglas was the only significantly different stand.

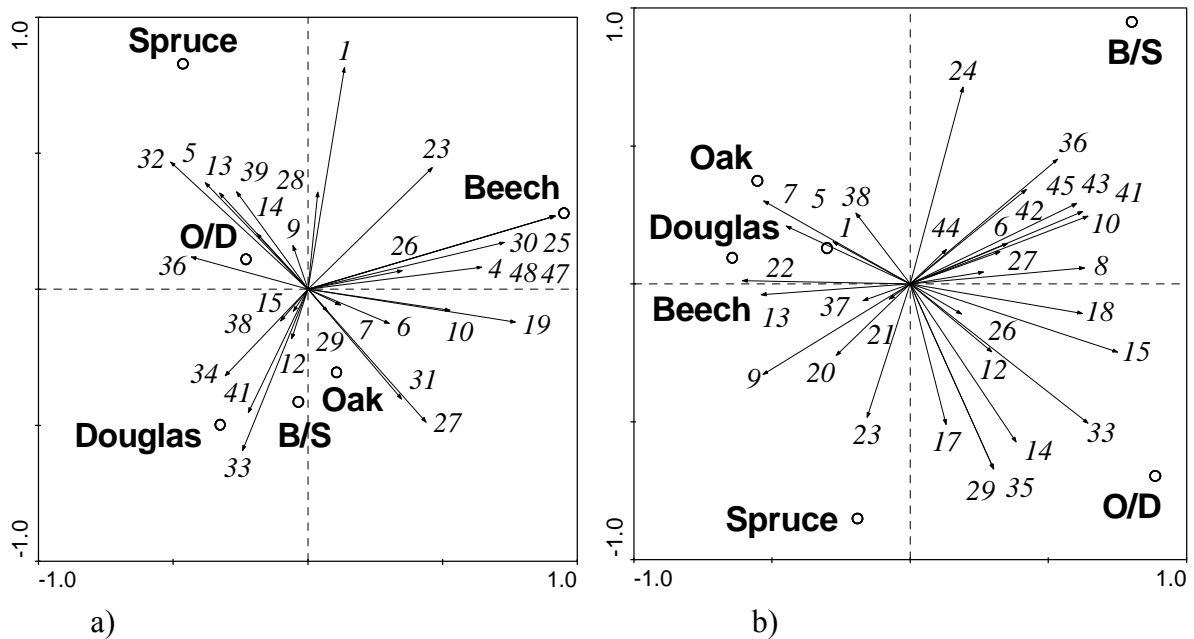


Fig. 3 RDA plot showing fungal community composition in litters decomposed in litter bags, after a) four months, b) 24 months of the decomposition in two broadleaves, two coniferous plantations and in two mixed litters (B/S – beech, spruce; O/D – oak, Douglas), decomposed on the border of adjoining plantations. Length of arrow indicates the relative importance of the fungal species, while the angle between arrows indicates the degree to which they are correlated.

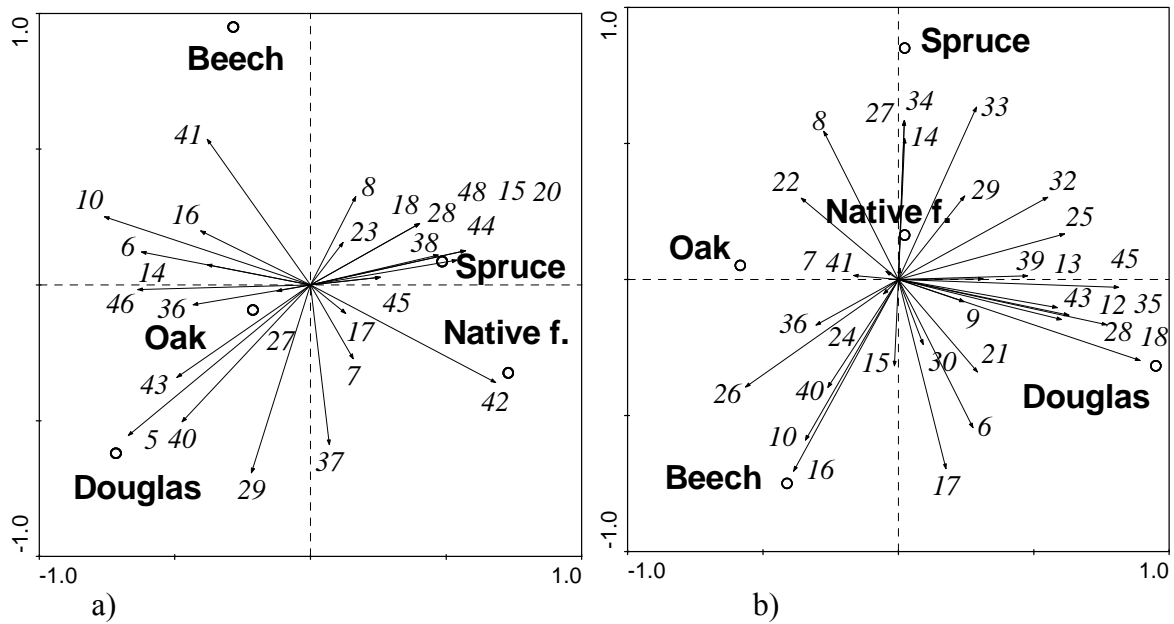


Fig. 4 Fungal community composition in L and F layer of the forest floor, in the four plantations and in the native forest as the control stand a) L layer, b) F layer

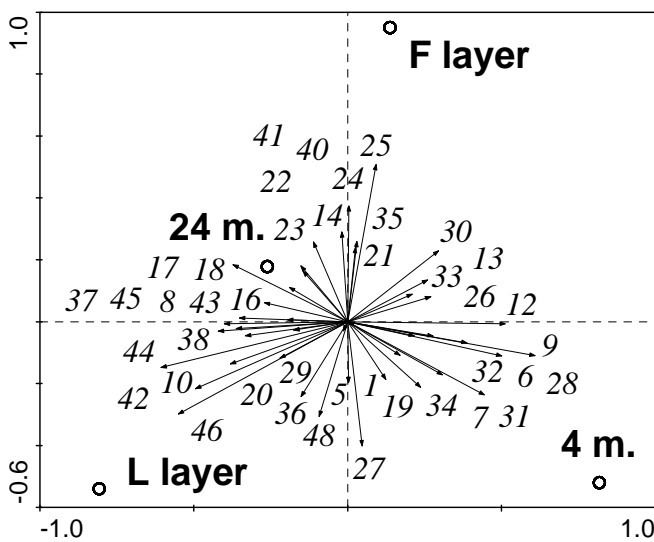


Fig. 5 Fungal community compositions of all samples analysed together in order to show the resemblance/divergence between the data sets (L and F layers of the forest floor, 4m. = four months sampling of litters decomposed in the litter bags, 24m. = twenty-four months sampling)

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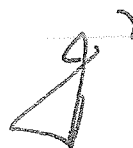
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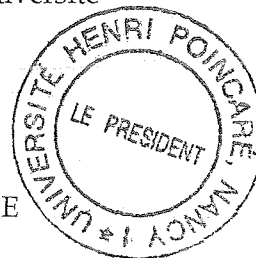
VU, APPROUVÉ ET PERMIS D'IMPRIMER

Nancy, le 17 décembre 2002

Le Président de l'Université



J.P. FINANCE



Effect of forest tree species change on decomposer diversity: A case study of the Breuil research site (Burgundy, France)

Microbial diversity and its relation to decomposition activity were studied in forest soils. An optimized fingerprinting method of TGGE (temperature gradient gel electrophoresis) was used to determine the patterns mainly of fungal but also of bacterial communities during decomposition of different substrates (cellulose, wood, tree litters) in a native forest and four monocultures. The diversity data were compared to measurements of decomposition rate in the samples (plus cellulase activity, fungal biomass and C/N). Further, effect of environmental variables (soil chemistry, moisture and temperature) to diversity was also targeted. Substitution of tree species caused changes in the composition of fungal and bacterial communities. Similarly, the communities were changing during the incubation period. However, any significant decrease of species richness was detected for any substrate or tree species. The differences in the decomposition rate did not seem to be influenced by a species loss or community change, but rather by a change in the microclimatic and nutrient conditions, influencing biotic interactions and/or species activity. Leaf-needle mixed litters sustained fungal diversity and increased the decomposition rate. During summer, species richness tended to decrease and common species were replaced by infrequent ones, able to tolerate dry conditions and to be very active.

Keywords: decomposers, biodiversity, temperate forests, litter, molecular techniques, decomposition rate

Effet des essences forestières sur de la diversité des décomposeurs: étude du site atelier de Breuil (Bourgogne, France)

La diversité microbienne et sa relation à l'activité de décomposition ont été étudiées dans des sols forestiers. La méthode moléculaire de TGGE (électrophorèse sur gel à gradient de température) a été optimisée et utilisée pour établir des diagrammes des communautés fongiques et aussi bactériennes pendant la décomposition de divers matériaux (cellulose, bois, litière) en forêt native et dans quatre monocultures. La diversité a été comparée aux mesures de vitesse de décomposition des échantillons ainsi qu'à l'activité cellulolytique, la biomasse fongique et le C/N. L'effet de variables environnementales (propriétés chimiques, humidité et température) ont aussi été étudiées. La substitution d'espèces cause des changements de composition des communautés fongiques ou bactériennes. Les communautés changent aussi durant la période de décomposition. Pourtant, aucune diminution de la richesse en espèces n'est observée pour les substrats ou essences étudiés. Les différences de vitesse de décomposition ne sont pas liées à la disparition d'espèces ou à des changements de communautés, mais plutôt au changement de conditions microclimatiques et nutritionnelles, ayant des conséquences sur les interactions biotiques et/ou sur l'activité des espèces. Les litières mixtes feuillus-résineux augmentent la diversité fongique ainsi que la vitesse de décomposition. Pendant l'été, la richesse des espèces diminue et les espèces communes sont remplacées par des espèces moins fréquentes, capables de tolérer des conditions sèches et restant très actives.

Mots clés: décomposeurs, biodiversité, forêts tempérées, litières, techniques moléculaires, vitesse de décomposition