Fungal Communities in Branch Litter of Norway Spruce: Dead Wood Dynamics, Species Detection and Substrate Preferences.

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2005

Acta Universitatis Agriculturae Sueciae
2005: 125

ISSN 1652-6880
ISBN 91-576-6924-4

Tryck: SLU Service/Repro, Uppsala 2005

Abstract

Allmér, J. *Fungal communities in branch litter of norway spruce: dead wood dynamics, species detection and substrate preferences*. Doctor's dissertation
ISSN 1652-6880, ISBN 91-576-6924-4

The aim of this thesis was to study the fungal communities on fine woody debris (FWD) of Norway spruce (*Picea abies* [L.] Karst.) and the importance of FWD as a substrate for these fungi. Three different methods for detecting fungi were used, sporocarp survey, mycelial isolation and ITS T-RFLP.

FWD has been suggested to be an important wood component in managed forest where the amount of coarse woody debris (CWD) is scarce. Therefore FWD may act as a substitute substrate for species negatively affected by the decrease in CWD in managed forests. However, slash, i.e. branches and tops, is being removed from about 50 % of the clear-cut sites in southern Sweden and about 70 % of the slash is removed from the clear-cut. So far no studies has been undertaken to investigate the possible effects of slash removal on wood-inhabiting fungal communities.

This thesis indicate that FWD host numerous fungal species, but essentially all of them are also found on other fractions like CWD and that species primary confined to CWD are essentially missing on FWD, except for some species that occurred on tree tops.

Different types of dead plant matter in the litter layer is largely colonised by the same fungal species. The strategy of the species utilising plant litter with little or even without discrimination to the type of substrate seems successful as they represent at least a third of the fungal community in the forest floor.

It is apparent that litter from needle and fine roots form the greater part of the decomposed plant matter during a forest generation, while wood, and particularly CWD constitute a minor part, 20-30%.

No differences in species composition due to slash removal could be detected at site level, nor could any differences be detected when comparing substrate types separately. Thus, the results suggest that slash removal after clear-cutting have a negligible long-term impact on the saprotrophic fungal community, at least under the experimental conditions used.

Keywords: Picea abies, fungi, woody debris, FWD, slash, T-RFLP, sporocarps, mycelia, Old growth, clear cut.

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Appendix

Papers I – V

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

I. Allmér, J., Vasiliauskas, R., Ihrmark, K., Stenlid, J., and Dahlberg, A. (2005) Wood-inhabiting fungal communities in woody debris of Norway spruce (*Picea abies* (L.) Karst.): as reflected by sporocarps, mycelial isolations and T-RFLP-identification. *FEMS Microbiology and Ecology*. In press.

II. Allmér, J., Stenlid, J., and Dahlberg, A. ITS T-RFLP identification of fungi colonising wood and needle baits show negligible effects of slash removal in in Norway spruce stands after 25 years. Submitted manuscript

III. Allmér, J., Stenlid, J., and Dahlberg, A. Fungal communities on fine woody debris of norway spruce (*Picea abies* [L.] Karst.) in clear-cuts, thinned and old-growth forests in east-central Sweden. Manuscript.

IV. Dahlberg, A. Allmér, J., Kruys, N., Nyström, K., Hyvönen R., Ågren, G. & Majdi, H. Carbon availability in litter for saprotrophic fungi in Norway spruce forests: a modelling approach of mass and flux of dead plant matter from the tree-, field- and bottom-layer. Manuscript.

V. Menkis, A., Allmér, J., Vasiliauskas, R., Lygis, V., Stenlid, J & Finlay, R. (2002) Ecology and molecular characterization of dark septate fungi from roots, stems, coarse and fine woody debris. *Mycological Research* 108, 965-973.

Introduction

This thesis focuses on wood-inhabiting fungi on fine woody debris (FWD) of Norway spruce (*Picea abies* [L.] Karst.) and the importance of FWD as a substrate for these fungi. Dead wood forms the substrate for about 30 % of the 25 000 - 30 000 multicellular forest organisms in Fennoscandia and is thus a key component for biodiversity in these forests (Dahlberg & Stokland, 2004; Jonsson, Kruys & Ranius, 2005). The term dead wood includes a wide and heterogeneous variety of types, such as dead standing trees, logs, stumps and roots from different woody bushes and trees of varying diameter and degree of decay. Species that at some stage of their lifecycle are associated with dead wood are in this thesis referred to as wood-inhabiting. The most species rich groups of wood-inhabiting organisms in Fennoscandia are insects and fungi, represented by at least 3 000 and 2 500 taxa, respectively (Dahlberg & Stokland, 2004). From an ecological view, wood-inhabiting fungi are important for decomposition processes in forest ecosystems as they are able to degrade wood, thereby releasing carbon and nutrients into the ecosystem (Dix & Webster, 1995).

Background

Modern forest management practices have dramatically decreased the amount of dead wood in the forest landscape: The amount of coarse woody debris (CWD) in managed forests is only ca. 2 – 30 % of that in old growth forests (Fridman & Walheim, 2000; Siitonen, 2001). A number of studies undertaken in Fennoscanidia during the last decade have indicated that CWD of Norway spruce can host a great number of fungal species (Renvall, 1995; Niemelä, Renvall & Penttilä, 1995; Bendiksen & Høiland, 1997; Lindblad, 1998; Vasiliauskas *et al*., 2005a). However, wood-inhabiting fungi are negatively affected by the reduced availability of CWD in managed forests (Siitonen, 2001) and that fungal species richness on CWD declines with increased intensity of forest management (Bader *et al*., 1995; Lindblad, 1998; Sippola *et al*., 2001). The species primarily affected are those that are rare to less common, often species that are red-listed (Bader *et al*., 1995; Sippola *et al*., 2001). Of the nearly 1000 red-listed organisms in Sweden many are associated with CWD (Dahlberg & Stokland, 2004; Gärdenfors, 2005). Coarse woody debris is now being monitored and used as a biodiversity indicator in the Swedish National Forest Inventory and the Swedish government has set a goal to increase the amount of CWD in forests by 40 % by 2010 (Anon., 2001).

In addition to CWD, another important component of dead wood for wood-inhabiting organisms in managed forests may be FWD, which mainly comprises branches and tree tops left after clear-cuttings and thinnings (Kruys & Jonsson, 1999). In view of the shortage of CWD in managed forests, the comparatively high amounts of FWD may act as a substitute substrate for species negatively affected by the decrease in CWD in managed forests. The FWD would effectively act as a reservoir of fungal species more commonly associated with CWD. It is therefore important to determine the importance of FWD as a substrate for wood-inhabiting

fungi, particularly the degree to which FWD and CWD host the same species pools and how species composition can be affected by forest management. This is of great interest at present because increased awareness for rising CO_2 levels and energy prices has led to an increased removal of FWD for use as a bio-fuel. In southern Sweden, clear-cut slash is already being removed from 50 % of the clear-cut areas and used for energy production (Eckerberg, 2004). Currently, about 70% of the slash is being removed from the clear-cuttings in pine, spruce and birch forests (Egnell, *et al*., 1998). Prior to this thesis, only a single study (Kruys & Jonsson, 1999) had examined wood-inhabiting fungi on FWD of Norway spruce. Kruys and Jonsson (1999) concluded that FWD was important for the species richness in managed forests.

Ecology of wood-inhabiting fungi

Wood degrading fungi

Saprotrophic fungi is an essential group of organisms in forest ecosystems as they are the main decomposers of litter and wood and are responsible for a large part of the heterotrophic respiration in forest ecosystems (Rayner & Boddy, 1988). Wood degrading fungi have a range of catabolic enzymes that enable them to utilise wood as an energy and nutrient source (Rayner & Boddy, 1988). There are three basic types of wood decay; white-, brown- and soft-rot (Cooke & Rayner, 1984). By having both lignolytic and cellulytic enzymes, white-rot fungi are capable of degrading lignin, cellulose and hemicellulose (Cooke & Rayner, 1984). Fungi responsible for this type of decay are mainly basidiomycetes (e.g. *Resinicium bicolor* (Albertini & Schwein.:Fr.) Parmasto (**Paper I, II & III**), but some xylariaceous ascomycete species also have this capability (Cooke & Rayner, 1984). Brown-rot fungi, which appear to be exclusively basidiomycetes, degrade cellulose and hemicellulose, leaving the lignin only slightly modified (Cooke & Rayner, 1984). Soft-rot is caused by a wide range of ascomycetes and, as with brown-rot, it is only the cellulose and hemicellulose that are utilised (Cooke & Rayner, 1984). Soft-rot differs from brown rot mainly in that the decay process is much slower (Cooke & Rayner, 1984).

Endophytic fungi

Numerous of fungal species live as endophytes inside living trees (Redlin & Carris, 1996). Endophytic fungi are defined as fungi causing symptomless infections in plant tissues (Carlile *et al.,* 2001). Fungi with diverse ecological roles may occur endophytically as latent pathogens, dormant saprobes or as mutualists (Espinosa-García, *et al*. 1996). Needles and leaves are reported to host numerous endophytes but a great number has also been reported from branches and twigs (Petrini & Müller, 1979; Petrini & Fisher, 1988; Barklund & Kowalski, 1996). Living Norway spruce seems to host a great number of endophytic fungi. In a recent study on fungal diversity in a healthy Norway spruce, as many as ninety-nine operational chemotaxonomic units (OCTUs) were detected (Müller & Hallaksela, 2000). The OCTUs are based on fatty acid and sterol profiles derived from the fungal isolates

(Müller & Hallaksela, 2000). Even though it is difficult to say to what degree these OCTUs correspond to taxa at species level, it is a clear indication that living Norway spruce trees may host a considerably diversity of taxa. Müller & Hallaksela (2000) estimated that the above ground part of a healthy, undamaged Norway spruce tree can harbour ca. 200 fungal species.

Other wood-inhabiting fungi

The trophic status is not known for all wood-inhabiting fungi. For example, not all degrade wood, certain species, e.g. some resupinate ectomycorrhizal species like *Amphinema byssoides* (Fr.) John Erikss., *Piloderma* spp. and *Tomentella* spp. appear only to use the wood as a support for their sporocarps (**Paper I, II & III**). There are also many fungi that rely on supplies of relatively assimilable substrates such as simple sugars, starch and proteins (Rayner & Boddy, 1988). Many of these non-decay fungi are micro-fungi that do not form large sporocarps (Rayner & Boddy, 1988). Furthermore, some fungi may be mycoparasitic on the mycelia on wood degrading fungi (Holmer, *et al.* 1997; Boddy, 1999).

Resource capture

Wood degrading fungi may be divided into those specialised in either primary or secondary resource capture (Cooke & Rayner, 1984). Primary resource capture refers to establishment on undecayed wood and is carried out by species with stress-tolerant strategy like pathogens and other primary wood degraders. Secondary resource capture is mainly by species that have a more competitive strategy, which are able to colonise wood that is occupied (Holmer, 1995). Undecayed wood in living trees is a stressful hostile environment for a fungus as it contains high levels of phenols, tannins and other antifungal aromatics (Dix & Webster, 1995). Species living under these conditions are considered to be poor competitors while species establishing in a later phase have to be stronger competitors in order to overcome existing mycelia. Secondary resource capture can be divided into selective and non-selective replacement (Cooke & Rayner, 1984). In selective replacement, a secondary decay fungal species is almost always found in close physical association with a particular primary decay fungal taxon. Whereas in non-selective replacement, there is no obvious association between the succeeding species and preceding species.

Colonisation of wood

Fungal dispersal and colonisation to new substrates can take place in several ways. The common mode for long distance dispersal is by propagules, e.g. sexual and asexual spores or hyphal fragments. Dispersal within forest stands can take place either through dissemination of propagules or by mycelial growth (Boddy, 1999). Wood-inhabiting fungi can be classified as unit-restricted, i.e. dependent on propagules as a mode of arrival and establishment on a new substrate, or as non-unit-restricted, being able to colonise substrates through extended mycelial growth such as hyphal-cords, rhizomorphs or fairy-rings (Rayner & Boddy, 1988). The mode of establishment is critical for the amount of resource captured, where colonisation via mycelial growth usually is more effective than propagules in

colonising resources (Holmer & Stenlid, 1996). Dead plant litter like woody substrates is distributed heterogeneously and often discontinuously in space and time (Boddy, 1999). Non-unit-restricted species are able to overcome this heterogeneity as they can extend from one substrate to others, enabling individual mycelia to actively search for new substrates in the neighbouring area and to translocate energy and nutrients to newly colonised substrates (Boddy, 2001). This gives non-unit-restricted species a considerable advantage over unit-restricted species in terms of initial inoculum potential when colonising new substrates as unit-restricted species are limited to the energy and nutrient content in the dispersed propagule (Rayner & Boddy, 1988). The classical view is that most primary wood degraders are unit-restricted species, which rely on spore dispersal for colonisation on substrates that have little or no ground contact. However, as shown in **Paper II** there are some species like *Armillaria* spp. that commonly spread in-between substrates through rhizomorphs even though they are primary degraders (Wahlström, 1992). Additionally, non-unit-restricted species are also often secondary wood degraders like *Resinicium bicolor* and *Phanerochaete* spp. (Kirby et al., 1990; Boddy, 1999; **Paper I, II & III**).

Determining the diversity of fungal communities on woody debris

Current ecological knowledge of the wood-inhabiting fungi relies almost exclusively upon observations of sporocarps (e.g. Renvall, 1995; Lindblad, 1998; Nordén, *et al*., 2004). However, such investigations do not reveal the entire species richness present as mycelia nor the relative activity of the fruiting species. For example, many fungal species were present as mycelia inside Norway spruce logs without producing sporocarps (Käärik & Rennerfelt, 1957; Johannesson & Stenlid, 1999; Gustafsson, 2002). Wood-inhabiting fungi may also be detected by culturing mycelia from wood on selective media, and by biochemical, chemical, and immunological analyses (Käärik & Rennerfelt, 1957; Stalpers, 1978). However, these methods are time consuming and some wood-inhabiting fungi are unculturable (e.g. Rayner & Boddy, 1988). Recently, PCR-RFLP methods have been employed not only to identify fungi from mycelial cultures, but also to discover and identify the presence of fungal directly in wood (e.g. Johannesson & Stenlid, 1999; Jasalavich, Ostrofsky & Jellison, 2000). One obstacle has been to develop efficient methods for extracting amplifiable fungal DNA from wood (e.g. Johannesson & Stenlid, 1999). A promising and efficient method to analyse samples with multiple species is terminal restriction fragment length polymorphism, T-RFLP (Lord, *et al*., 2002). T-RFLP targeting the ITS region of rRNA genes has been used to effectively characterize the presence of ectomycorrhizal hyphae in soil (e.g. Dickie, *et al*. 2002; Edel-Hermann, *et al*., 2004), and on wood-inhabiting fungi from wood of Norway spruce (**paper I & III**).

Wood-inhabiting fungal communities

Wood-inhabiting fungal communities are dynamic, constantly changing in time and space, following a diverse array of pathways rather than occurring in simple stages and following an ordered pattern (Rayner & Boddy, 1988; Boddy, 2001). There

are a number of factors influencing the changes in the community e.g. physical and chemical properties of the host trees, the microclimate of the growth site and biological interactions taking place in the substrates (Rayner & Boddy, 1988; Renvall, 1995; Holmer & Stenlid, 1996). However, there are some general patterns that can be seen during the decomposition of wood substrates. The environmental stress imposed on the fungi in the beginning of the community development can range from very high in living trees to relatively low in dead, un-colonised wood (Rayner & Webber, 1984). Typically, species attacking living trees through their roots or sapwood are stress-tolerant, whereas species colonising dead, un-colonised wood or wood in the vicinity of wounds of living trees have predominantly ruderal characteristics (Holmer, 1995; Boddy, 2001). When the primary resource capture is completed i.e. there is no un-colonised part of the substrate left, the community will shift to a more combative phase (Rayner & Webber, 1984; Holmer, 1995; Boddy, 2001). Finally, when the nutrients in the substrate are almost depleted the community will once again shift to a more stress tolerant phase (Rayner & Webber, 1984; Holmer, 1995; Boddy, 2001). Field observations on Norway spruce logs indicate that the fungal species richness is highest at the intermediate stages of the decomposition process, i.e. at the combative phase, and decreases as stress increase (Niemelä, Renvall & Penttilä, 1995; Renvall, 1995; Lindblad, 1998).

Objectives of the thesis

The overall aims of this thesis were to characterize the fungal communities occurring on fine woody debris (FWD) of Norway spruce and to analyse if slash removal from clear-cuts affects the communities of wood-inhabiting fungi. A number of methodological questions were also addressed in relation to the detection of fungi in woody substrates.

More specifically the objectives were:

- To study how different identification methods influence the detection of fungal communities in the wood (**Paper I**).
- To describe the fungal communities on branches and tops in slash (**Paper I** & **III**).
- To determine if needle litter and dead wood are colonised by the same saprotrophic fungi (**Paper II**).
- To study the differences in species composition and richness of fungi growing on branches and tops in old-growth, thinned and clear-cut stands (**Paper III**)
- To model the yearly production of different litter fractions from the tree-, field- and bottom-layers in managed forests and determine how the proportions between these fractions varies over one forest generation (**Paper IV**).
- To test if slash removal affects the presence and frequency of the dominant saprotrophic fungi 25 years after the removal (**Paper II**).
- To specifically describe the contribution of dark septate mycelia to the fungal diversity (**Paper V**)

Material and methods

Study sites and sampling regimes

The study in **paper I** was conducted in a 50-year-old plantation of *Picea abies*, located in east central Sweden, about 150 km north-west of Stockholm (Figure 1). It was thinned 7 years prior to the study. Slash, branches and tops, representing individual trees, from the thinning was left in piles. Slash piles from twenty individual trees were randomly selected within an area of 0.5 ha. From each pile,

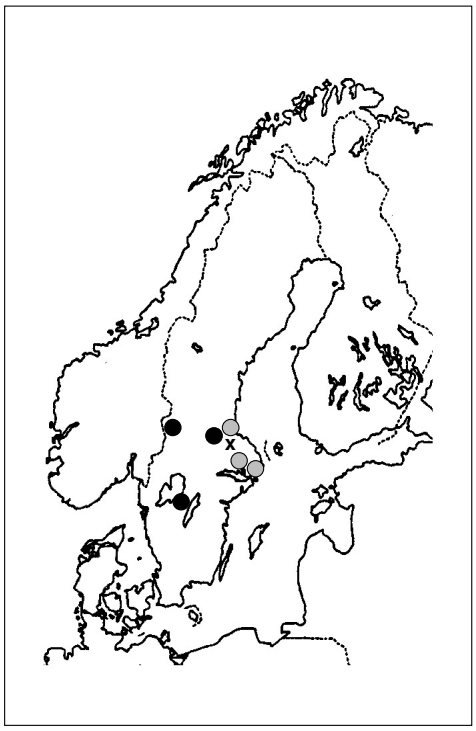

Fig. 1. Map showing the locations for the different localities in this thesis. **X** refers to the locality used in **paper I**, black dots refer to localities in **paper II** and grey dots refer to the localities used in **paper III.**

five branches and the top were sampled. Wood discs were sliced from the branches and tops and subsamples were taken for mycelial cultures and T-RFLP identification. Wood samples from the branches were taken from the centre of the discs whereas the wood samples from the tops were taken from where different decay zones were visible. All sporocarps on the sampled branches and tops were collected and identified to the species level. In **Paper I,** the combined approach of mycelial isolations, ITS T-RFLP and sporocarp surveys were used for detection of fungi in the wood samples.

In **Paper II,** we used three unique sets of experimental sites in southern Sweden that had been forested with Norway spruce forest prior to clear cutting (Figure 1). The sites were established in the late 1970s and set up to evaluate long-term effects

of slash and stump removal on the field-layer vegetation and the production of berries and mushrooms (Kardell, 1981). Each site consisted of two replicated blocks, each with four quadratic 1600 m^2 experimental plots; 1) one control plot where slash and stumps from the clear cut were left, 2) one plot where all the slash was removed, 3) one plot where all the stumps was removed, and 4) one plot where both all the slash and stumps were removed. In this study, we used two treatments, the control and slash removal plots in both blocks. The slash, i.e. all left branches and tops, was removed manually. In principle this means 100 % were removed compared to operational slash removal for bio-fuel where commonly only ca. 70 % is removed (Egnell *et al*., 1998). We placed standardized wood sticks and needle-litter-bags as bait substrates at pre-defined locations on all studied plots. After 2 ½ year, the litter bags and wood sticks were collected from each site, in total of 60 litter bags and 119 wood sticks. Samples were taken at 22 points from each wood sticks by drilling 2 cm into the wood with a drill bit, sterilized in 70% ethanol between the drillings. All of the drill dust obtained from each stick was mixed and frozen at -20 ºC in a plastic bag. Five randomly collected needles from each litterbag was put in a 2 ml microcentrifuge tube and freeze-dried. In **Paper II,** ITS T-RFLP was used for detection of fungi in the wood and needle samples.

The study in paper **III** was conducted in three different regions in east-central Sweden (Figure 1). At each region, three different forest stands of Norway spruce were selected, one clear-cut site about 3 – 5 years old, one mature thinned site about 3 – 5 years since last thinning, and one old-growth site. All of the sites were mesic spruce forests of *Vaccinium myrtillus* – type. At clear-cut and thinned sites, slash piles from twenty trees were randomly selected within an area of 0.5 ha. From each pile, three branches were selected for sporocarp survey and molecular identification of fungi. In addition to the branches, five tops were selected from each site for sporocarp survey and molecular identification of fungi. In old growth sites branches were selected from natural "piles" of branch-litter from living trees within an area of 1.5 ha, otherwise the same sampling regime were as for clear-cuts and thinnings. Since natural tree tops were scarce in the old growth stands, five natural logs per site with a maximum diameter of 10 cm and a maximum length of 5 m were selected for molecular identification and sporocarp inventory. All woody debris had an intermediate stage of decay, i.e. they were neither hard nor so well decayed that they would fall apart when lifted / handled. After sporocarps had been recorded and collected from the branches and tops, wood discs were sliced from these and brought back to the laboratory. For molecular work, drill-samples were taken from the wood discs and DNA was extracted from the drill dust. In **Paper III,** ITS T-RFLP and sporocarp surveys were used for detection of fungi in the wood samples.

In **Paper IV,** the yearly litter production from the tree-, field- and bottom-layers was modelled. The yearly availability of carbon for saprotrophic fungi in different litter and wood types was modelled based on data and estimations of the annual production of dead wood and other plant litter types in Norway spruce, bilberries (*Vaccinium myrtillus*) and feather-mosses (*Pleurozium schreberii* and *Hylocomium splendens*). We selected to model ideal forest stands with 100 % Norway spruce during one forest generation with estimates for the coverage for *V. myrtillus* and

feather-mosses. We analysed three site quality indexes, G18, G 22 and G30 at latitude 57°N in Sweden. Two levels of forest management scenarios were modelled; 1) all thinning and harvesting residues are left at the site, 2) Slash is removed after clear-cutting and after all thinnings. The amount of respired carbon from each litter fraction was estimated by modelling. Standing crop biomass of tree-layer at different forest ages was modelled by using information from conventional forest yield research. Field- and bottom-layers was modelled by using information from National Forest Inventory. The annual inflow of dead plant litter was modelled as a fraction of the standing crop biomass. An overview of the simulation of the stand dynamics, biomass, dead plant matter flux and heterotrophic respiration in this study is illustrated in Figure 2. Occurrences of saprotrophic fungi on different plant litter fractions were compiled based on published data and **Papers I**, **II** and **III**. An attempt to analyse the relative importance of different plant litter types were performed.

A total of 127 strains of DS fungi were used in **paper V**. The strains were collected and isolated during previous work; stem bases of living and dead Silver birch (*Betula pendula* Roth) and Scots pine (*Pinus sylvestris* L.) (Lygis, Vasiliauskas & Stenlid 2004, Lygis *et al.* 2004), stumps, trunks, branches and tops of Norway spruce (Vasiliauskas *et al*., 2005a; **paper I**), root tips and decayed roots (Menkis *et al*., 2005a; Menkis *et al*., 2005b). All our isolates were subcultured by excising hyphal tips from the front of uniformly growing mycelium. All strains studied in this work are deposited in the culture collection of the Dept. Forest Mycology & Pathology, Swedish University of Agricultural Sciences (R. Vasiliauskas). The extraction of DNA, PCR amplification and DNA sequencing procedures followed the methods of Kåren *et al*. (1997). Phylogenetic analyses and neighbour-joining analyses were performed using PAUP* 4.0b10 (Swofford, 2002).

Methods used for detection of species

ITS T-RFLP

In **Paper I,** the same sampling regime was used for both mycelial cultures and ITS T-RFLP. Whereas in **Paper II** and **III,** a modified sampling regime was used, making greater use of the potential of T-RFLP. Among the different fragment profiling methods available, T-RFLP seems to be particularly useful for fungi, especially when used on the ITS region (Lord *et al*, 2002). With this method it is possible to detect multiple species present in an individual environmental sample with a high precession as to the fragment sizes of the individual species (Dickie *et al.*, 2002). This method is rather new and there are few studies on fungi published where T-RFLP has been used (Dickie *et al.*, 2002; Lord *et al*., 2002; Anderson & Cairney, 2004; Paper **I**, **II** & **III**). For this reason the protocols used in this thesis will be given below.

In **Paper I,** wood samples (small wood blocks) were freeze-dried and ground with a ball mill. In **Paper II** and **III,** the drill dust was transferred to 2ml

microcentrifuges tubes to a volume of 1 ml lightly packed dust. In **Paper II,** the needles were freeze-dried and ground in a FastPrep® preparation shaker.

800 µl 2% CTAB (2 % hexadecyltrimethylamonium bromide, 0.1M Tris-HCl, 1.4M NaCl (pH 8), 0.02M EDTA) was then added to both needle samples and wood samples. The samples were vortexed for 20 seconds and put in a heating-block at 65 ºC for 2 h. The samples were then centrifuged for 20 minutes and the supernatant transferred to a new 1.5 microcentrifuge tube and precipitated with isopropanol. The DNA was dissolved in 50 µl autoclaved H_2O, thereafter cleaned with GENCLEAN III kit (**Paper I**, **II** & **III**).

The internal transcribed spacer (ITS) 1 and 2, including the 5.8S region of nuclear rDNA, was amplified using the primers ITS1F and ITS4 (Gardes & Bruns, 1993) labelled with WellRED dyes D4-PA and D3-PA, respectively. PCR amplification was performed using an Applied Biosystems GeneAmp PCR System 2700 Gradient Thermal Cycler (Applied Biosystems, Foster City, CA). Initial denaturation at 95 °C for 5 minutes then 35 cycles with denaturation at 95 °C for 30 seconds, primer annealing at 55°C for 30 seconds, and primer extension at 72 °C for 30 seconds, thereafter a final primer extension at 72°C for 7 minutes. In the PCR-reactions, final concentrations of 2mM $MgCl_2$, 200µM dNTP, 0.2µM of each primer, 0.3 units/µl Redtaq (Sigma, St. Louis, MO), and corresponding reaction buffer was used (**Paper I,II** & **III**).

The PCR products were digested with *Taq*I, *Cfo*I (**Paper I**, **II** & **III**) and *Alu*I (**Paper II** & **III**) separately. The T-RFLP patterns were analysed with a Beckman Coulter CEQ™ 8000 Genetic Analysis System using CEQ™ DNA Size Standard Kit-600.

Reference library of T-RFLP patterns and T-RFLP identification

A selection of samples containing $\geq$ 3 taxa was cloned so that each of the different taxa in one sample could be sequenced. Samples containing unique T-RFLP patterns were also cloned to allow for sequencing. The cloning was performed with Invitrogen's TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). The cloning was performed according to the recommendation of the manufacturer. The sequences obtained from the clones were compared with the reference database at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden, and the GenBank database (Altschul et al., 1997).

The fragment sizes from the unknown T-RFLP patterns were compared to the reference database using TRAMP (Dickie et al., 2002). To account for base calling during analysis in the Beckman Coulter CEQ™ 8000 Genetic Analysis System and for within taxa variation of the ITS region, a threshold level for fragment size in TRAMP was set to $\pm$ 2 base pairs.

Mycelial isolations

The procedures for fungal isolation from wood samples and sub-culturing of fungal strains used in **Paper I** were similar to those performed in earlier studies by Vasiliauskas and Stenlid (1998) and Vasiliauskas *et al*. (2005a). The fungal strains were grouped according to mycelial morphology and two to seven randomly selected mycelia from each group was further identified using sequencing of the ITS of the nuclear rDNA. The sequences were compared with the reference database at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden, and the GenBank database (Altschul et al., 1997). Culturing of fungal mycelia on nutrient agar plates is a well known method and has been used in many studies of wood-inhabiting fungi (Käärik & Rennerfelt, 1957; Stalpers, 1978; Rayner & Boddy, 1986; Vasiliauskas & Stenlid, 1998). The method is simple to use and involves placing surface sterilised pieces of wood on nutrient agar plates and allowing the mycelia to grow out from the wood onto the nutrient agar. Since more than one species is likely to be present in the wood piece, it is necessary to check the plates frequently and subculture when necessary to prevent slow growing species to be overgrown by faster growing ones. Identification of mycelial cultures it is not easy. It requires a lot of experience to base the identification on morphological characters. In general, there is a low resolution as to the taxonomic level that specimens can be assigned to by this visual method compared to identification based on molecular methods like DNA sequencing.

Sporocarps

All specimens visible on a substrate was either identified and noted in the field or brought back to the laboratory for identification using microscopical examination (**Paper I & III**). Sporocarp surveys require some knowledge on how to identify species, but when that knowledge in obtained it is a relative time efficient method compared to the other methods used in this study.

Results and Discussion

Fungal identification from wood by mycelial isolations, ITS T-RFLP and sporocarps (paper I)

There were clear differences in the species composition and richness detected by the three methods used to examine the fungal communities on branches and tops. Out of a total of 58 species, 27 were identified by sequencing of mycelial cultures, 31 using sporocarps survey, whereas only five species were detected with ITS T-RFLP (**Paper I,** fig. 2). One species, *Hypochnicium lundellii* (Bourd.) John Erikss., was detected by all methods and four out of the five species detected by ITS T-RFLP were also recorded in mycelial cultures. One species, *Phlebia* sp. JA1., was only recorded by ITS T-RFLP (**Paper I**). There were only a few species found as both mycelia in the wood and as sporocarps: Only ca. 10 – 14 % of the species recorded by sporocarps were recovered as mycelia in the wood (**Paper I**).

The proportion between number of detected basidiomycete and ascomycete species differed between the methods: 30:1 in the sporocarp survey, 1:3 with mycelial cultures and 2:3 with ITS T-RFLP (**paper I**). Possible reasons for these discrepancies are discussed below.

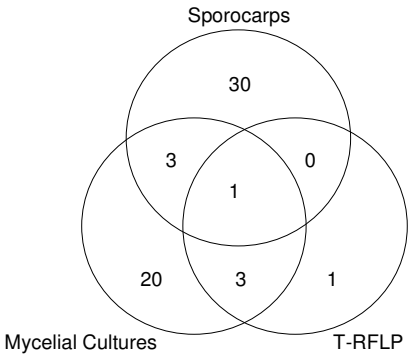

Fig 2. Comparison of the number of fungal species recorded as sporocarps, cultured mycelia and from direct T-RFLP. The numbers show the number of species documented with either method, individual or in combination with two or three methods

In **Paper I,** the sampling strategy for mycelial cultures and ITS T-RFLP was not optimal; wood-samples from the exterior of the wood-discs should also have been included to get a better representation the fungi present in the wood. In **Paper III,** drill-samples were taken by drilling through the wood surface into the centre of the wood from different directions along a circle corresponding to a wood-section, resulting in a larger volume sampled and both the exterior and the interior represented. This increased the number of species detected from the branches and tops.

In **Paper I,** mycelial cultures revealed five times more species than were found by ITS T-RFLP from wood samples. The most frequently recorded species from the mycelial cultures, *Trichoderma polysporum* (Link:Fr.) Rifai, a non-fruiting ascomycete, was not detected by ITS T-RFLP. It was probably present as conidia or minute mycelial units in the wood which successfully grow on the agar plates but were too insignificant to be detected by the ITS T-RFLP. All detection methods have inherent problems favouring certain species. For mycelial isolations, the nutrient media used would most likely favour fast growing species, enabling them to out-compete slower growing species even though the latter might have a larger inoculum to begin with. From this point of view molecular methods seem to

be more reliable in terms of reflecting the species present in wood. However, during PCR with mixed samples the primers used may have a higher affinity to the DNA of certain species resulting in a skewed representation of species abundance in the samples in e.g. T-RFLP profiles. Even though there is no evidence for this, I would recommend to base estimates of fungal abundances on other criteria than the height of the T-RFLP peeks. It would be better to base the abundance on species frequencies from the number of samples that contains a species. The PCR reaction may also bias the outcome of multiple samples as one or both of the primers may be unable to bind to the DNA of some species due to introns in the binding site. This would result in the exclusion of some taxa. This is known to occur in e.g. *Cantharellus* species when using certain ITS primers.

Fungal fruiting is environmentally controlled and may vary between years and among different species. Thus, sporocarp inventories are recommended to be repeated during a season and over years to gain a more comprehensive picture on the fungal community (Huhndorf *et al*., 2004). When comparing fungal species detected from mycelial isolations (**Paper I**) or ITS T-RFLP (**Paper I**, **III**) and sporocarp surveys, sporocarps has a strong bias towards basidiomycetes whereas mycelial isolations mainly detected ascomycetes. ITS T-RFLP seems to be less biased towards ascomycetes compared to mycelial cultures (**Paper I**). The number of basidiomycete species detected by all three methods was fairy low (**Paper I**, **III**). In both **Paper I** and **III,** a number of basidiomycetes that commonly produce sporocarps was recorded only as mycelia in the wood. Sporocarp surveys may miss mycelia in wood if not conducted at the appropriate fruiting time (Gustafsson, 2002; Rayner & Boddy, 1988). Different species may also allocate varying amount of resources for reproduction and dispersal in relation to mycelial growth, as has been discussed in ectomycorrhizal fungi (Gardes & Bruns, 1996). Some ascomycete taxa may also be overlooked as they entirely or predominantly rely upon asexual reproduction by microscopic conidia.

Fungal communities in FWD, branches and tops, in managed forests (Paper I & III)

Sixty-four species were detected in **Paper I,** whereas in **Paper III,** 65 and 94 species were detected by ITS T-RFLP and sporocarps, respectively. It was not possible to give a total species number in **Paper III** due to a large number of unidentified species, but since no ascomycetes were found as sporocarps and at least 40 species are ascomycetes as detected by the ITS T-RFLP, a minimum total estimate would be 134 species.

Even though the studies in **Paper I** & **III** were not primary set up to test for differences between branches and tops, some comments can be made. Most fungal species occurred on both branches and tops, but there were some species that seemed to have a preference for one or the other. In both **Paper I** and **III,** the number of branches sampled was five to six times greater than tops. Despite this, a number of species seemed to occur either only on tops (**Paper I** & **III**) or had a significant preference for the tops (**Paper III**). Due to the over representation of

the branches, it is only possible to say something about the species preferences on the branches for the species that were frequent enough to test statistically (**Paper III**). From detection with ITS T-RFLP, two species were found to prefer branches and as detected by sporocarps, six species were found to prefer the branches.

Stump data from Vasiliauskas *et al*. (2005a) was included in **paper I** to have as a comparison with the branches and tops, where the stumps represented CWD. The stump data comprised 68 species and the stumps derived from the same area as was used in **paper I.** When comparing the number of species identified from branches, tops and stumps many more species were uniquely occurring on the stumps than on the other two fractions (**Paper I**). The difference between the stumps and the branches and tops indicates that CWD has a capacity to host many more species than FWD. This is notable, as the tops had a greater volume than the stumps.

It is possible that some fungal species utilize both FWD and CWD, but only fruit on CWD as the amount of energy from FWD is not sufficient for sporocarp production. Polypore species with conspicuous sporocarps e.g. *Phellinus viticola* (Schwein. in Fr.) Donk, *Postia* spp. and *Skeletocutis biguttulata* (Romell) Niemelä fruited on tops (**Paper I** & **III**) and stumps (**Paper I**), but were not detected as sporocarps or as mycelia in the wood of the branches. This group of fungi are probably adapted to CWD and are thus only rarely reported from branches. Some of the fruiting species found in **Paper I** and **III**, like *Athelia bombacina* Pers., *Hypochnicium lundellii* and *Merulicium fusisporum* (Romell) Johan Erikss. & Ryv. on the other hand, are known to prefer branch litter and other small debris of Norway spruce (Eriksson & Ryvarden, 1973 & 1976; Johan Allmér, personal observation). However, all the recorded fruiting species in **Paper I** and **III** have been reported as sporocarps from both FWD and CWD, though with different frequencies (Dahlberg & Stokland, 2004; Johan Allmér, personal observation).

Saprotrophic fungi colonising needle and wood baits (Paper II).

This project suggests that different types of dead plant matter in the litter layer is largely colonised by the same fungal species. At three sites studied, at least 35 % of the species and more than 40 % of the fungal frequencies of the frequent species occurred on both needles and wood buried in the litter layer. The strategy of the species utilising plant litter with little or even without discrimination to the type of substrate seems successful as they represent at least a third of the fungal community in the forest floor. Fungal dynamics in the litter layer results most likely mainly from interactions of established mycelia, whereas dynamics in undecayed wood probably to a large extent depend on establishment from propagules (Rayner & Boddy, 1988). Being more of a generalist in terms of substrate utilisation may be favourable in the sense that the fungus has a wider range of substrates available than more specialised species has.

The most frequent colonisers of the baits, dominating the community were non-unit restricted species. All these species were rhizomorphic and hyphal-cord formers allowing them to spread between substrates (Rayner & Boddy, 1988). This

enables them to efficiently allocate nutrients to newly colonised substrates from already established ones, giving them an upper hand compared to species that relay upon colonisation via propagules (Rayner & Boddy, 1988). This strategy may be of advantage for species that are adapted to utilise patchy distributed substrates in the litter layer like wood. Species primary adapted to colonising needle litter on the other hand might not have the same needs to be mobile since needle litter is much more evenly distributed on the forest floor.

Species composition and species richness in old-growth, thinned and clear-cut stands (Paper III).

There were clear differences in species composition and species richness between the three forest management classes. The old-growth sites supported 90% of the species found from the three management classes, whereas the thinned sites supported 70% and clear-cut sites only 45%. The most frequent species were present in all of the management classes. The species overlap between old-growth sites and thinned sites were greater than between old-growth and clear-cut or clear-cut and thinned sites. There are several possible causes to explain this pattern. The old-growth sites were more shaded than the other two management classes and may therefore favour more species by more even moist conditions. Furthermore, there is a difference in the way FWD is produced in managed and old-growth forests. In clear-cuts and thinnings, healthy branches and tops are cut and fall to the ground, usually with no primary decay, and essentially all dead wood is produced at the same time. This most likely gives the fungi a narrow window of opportunity for colonisation as has been shown in baiting experiments of wood discs (Vasiliauskas *et al*. 2005). Whereas in old-growth forests, a large portion of the branches and tops might already have started to decay by the time they fall to the ground. Also, and maybe more importantly, there is a more continuous supply of these substrates, giving rise to a much greater window of opportunity for colonisation.

Availability of different plant litter fractions in managed forests (Paper IV)

According to the estimates of substrate availability from different fractions during forest management with no slash removal, branches represent some 15 – 17 % of the relative production of litter during one forest generation, depending on the forest site index (Paper **IV**). Stems make up about 4 – 5 % of the relative litter production, whereas stumps and coarse-roots represent 16 – 19 % and the litter produced from the bottom and field-layers represents 6 -13 %. About 50 % of the relative litter production is represented by needles and fine roots. The main inflow of woody substrates in managed forests is regulated by clear-cutting and thinnings. In stands with slash removal, the inflow of branches is reduced by at least 40 %, whereas stumps and coarse-roots will not change.

The estimates of the relative share of annually heterotrophic respired carbon give an indication as to what litter fractions saprotrophic fungi are mainly utilising

during a forest generation. According to the amount of carbon respired from different fractions after clear-cutting and no slash removal, branches, stumps and coarse-roots, together with needle and fine-root litter, represent the dominant part during the first 30 years due to the accumulated slash from the clear-cut. As the canopy closes, the respiration of needle and fine-root litter dominates. In stands with slash removal, the respiration of the needle and fine-root litter is reduced about 50 % after the clear-cutting (**paper IV**).

Slash removal and its effects on FWD- and litter fungal communities (paper II)

No differences in species composition due to slash removal could be detected at site level, nor could any differences be detected when comparing substrate types separately (**Paper II**). Thus, the results suggest that slash removal after clear-cut have a negligible long-term impact on the saprotrophic fungal community, at least under the experimental conditions used. However, there are some issues that need to be considered. We used experimental plots where all of the slash was removed. For practical reasons about 70 % of the slash is removed in normal forest practise (Egnell *et al*., 1998). Thus, in practise more slash will be available for saprotrophic fungi, further supporting our conclusions that the impact of slash removal will be negligible in practical forestry. However, as the practice is to remove slash also at thinnings, the amount of slash over the rotation time will decrease by ca. 40 % (**Paper IV**).

It also has to be considered that stumps left after slash removal represent a substantial amount of dead wood as they are estimated to represent about 20 % of the relative litter mass at the time of clear-cutting (**Paper IV**). Species like *Armillaria* spp., *Hypholoma capnoides* (Fr.) Kumm., *Resinicium bicolor* and *Sistotrema brinkmannii*, all frequent on the wood baits in **Paper II**, are reported from CWD in many studies (Wahlstöm, 1992; Vasiliauskas *et al*., 2005a,b; **Paper I**) and Vasiliauskas *et al*. (2005a) reported a high fungal diversity from stumps of Norway spruce. Thus, stumps might presumably function as a food base for species lost from logging residues during the first 30 years. Furthermore, as indicated by the carbon respiration of different litter fractions, the dominant litter fractions utilised are needle and fine-root litter, whereas branches and tops are marginal contributions.

However, we can not over-look the possibility that the high conformity in the community between the control plots and the plots with slash removal may partly be an edge effect as the experimental plots were only 900 m^2 and located on large clear-cuts where the slash was left in the remaining area. Some of the species may thus have originated from the surrounding where no slash removal had occurred.

Dark septate endophytic fungi in FWD of Norway spruce (Paper V)

A dark septate (DS) fungus was frequently isolated from branches and tops; being the second most common species form mycelial isolations (**Paper I**). Since a number of ongoing studies encountered these DS fungi, a taxonomic investigation was set up to assess their identity and ecological range (**Paper V**). The DS fungi derived from a broad range of ecological niches including CWD (logs, snags, stumps and roots), FWD (branches and tops), living healthy-looking stems, and healthy root tips. Sequencing of the ITS region of 127 DS strains revealed a close affinity to the genus *Phialocephala* W.B. Kendr., as all of the strains had a 95 – 100% homology to identified *Phialocephala* species they were all assigned to that genus.

Five distinct clusters were identified in a neighbour-joining analysis, most likely representing distinct taxa. Representatives from each cluster were analysed together with known *Phialocephala* spp. and other related species in a maximum parsimony analysis in order to assess the identity of the five clusters. The parsimony analysis revealed *P. fortinii* Wang & Wilcox and *P. dimorphospora* Kendrick as belonging to two of the clusters whereas the remaining three did not group with any of the known species. As a result, they were defined as *Phialocephala* sp. 6, *Phialocephala* sp. 18 and *Phialocephala* sp. 35, thus three new fungal taxa were revealed.

The DS fungal strains so frequently isolated from branches and tops in **paper I** turned out to consist of both *Phialocephala* sp. 18 and *Phialocephala* sp. 35 were the latter was the most frequent. Both species were also detected with ITS T-RFLP from branches and tops (**paper III**), but also from wood baits buried in the litter layer (**paper II**). Both *Phialocephala* sp. 18 and *Phialocephala* sp. 35 were also detected from living stems of Silver birch, the latter were also detected form CWD (**paperV**).

Phialocephala fortinii is commonly isolated from both healthy and decayed roots (Wilcox & Wang, 1987; Holdenrieder & Sieber, 1992; Menkis *et al*., 2005a; Menkis *et al*., 2005b). However, this study revealed the presence on several new substrates, like living stems of Scots pine (*Pinus sylvestris* L.), dead stems and stumps of Silver birch (*Betula pendula* Roth) and stumps of Norway spruce (**paper V**). *Phialocephala dimorphospora* has been found in healthy roots and conifer branches (Holdenrieder & Sieber, 1992; Kowalski & Kehr, 1992). It has also been isolated from stained sapwood of declining oaks and decayed CWD (Kowalski, 1991; Kehr & Wulf, 1993; Harney, Rogers & Wang, 1997). *Phialocephala* sp. 6 was associated with healthy conifer seedling roots (Menkis *et al*., 2005a).

General conclusions

The results from this thesis demonstrate that fungal communities detected from wood are strongly dependent on the detection and identification method used (**Paper I** & **III**). Therefore, it is highly advantageous to combine methods such as sporocarp surveys with methods that detect taxa occurring in the wood as mycelia when studying fungal communities. Choice of methods will be influenced by the aims of the study. If the aim is to study the occurrence of red-listed species, a sporocarp survey is the appropriate method since the classification of the red-list for fungi is based on the occurrences of sporocarps. On the other hand, if the focus is on the occurrence of fungal mycelia in wood, then it would be preferable to use methods like mycelial isolations or some molecular detection method.

It is usually easier to detect a higher number of rare species with sporocarp surveys than using molecular detection methods e.g. T-RFLP (**Paper I** & **III**). The molecular based detection methods are of course also able to pick up rare species, but not to the same degree relative to the total amount of species detected by respective method. Molecular detection methods will on the other hand pick up species that are active in the wood whereas sporocarp surveys also detect species that only uses the substrate as a support for fruiting. Mycelial isolations will also pick up species that are active in the wood but this method may favour species only present as propagules in the samples and not as active mycelia (**Paper I**). One advantage with culturing based methods is that it is much easier to do laboratory experiments on individual species, for example to asses the trophic status of a species since each species is cultured on individual plates. Mycelial cultures and sporocarp surveys are old and well used methods where it is easy to find good reference literature. T-RFLP based methods are, on the other hand, quite new and the number of publications referring to that method is still fairly low. Regardless of the methods used, it is important to be aware of its limitations.

Of the three methods used, sporocarp surveys still present the best reference data about the occurrences on different wood fractions, particularly on CWD. This has made it possible for us to compare what we have found on FWD (**Paper I, II & III**) with what is known from CWD. It has also been possible to compare some of the species, especially basidiomycete species, detected by mycelial isolations and ITS T-RFLP. Also, for some of the micro-fungi detected by mycelial cultures or ITS T-RFLP it has been possible to assess their occurrences on different wood fractions in relation to our findings. However, many of these fungi are only found on a few times and there are so few published studies in this area, it is really not possible to draw any conclusions about possible substrate preferences.

Fungi inhabiting FWD seem to be present also in CWD; but it seems that some species might have a preference for FWD (**Paper I & III**). All of the fruiting species found in this project has been reported from CWD, even the ones that seem to prefer FWD. For a number of species, the occurrences on branches seem to be restricted to mycelial growth as they seem not to produce sporocarps on these fractions (**Paper I & III**). Furthermore, many species e.g. *Antrodia* spp., *Phellinus*

spp. and *Postia* spp. seem to be restricted to coarser wood fractions than branches, e.g. tree tops. Thus, it is unlikely that FWD, and especially branches, is an important refuge for species mainly occurring on CWD. An interesting observation was that a number of species known as primary wood degraders were frequently found in fine-diameter wood-baits in the litter layer, with some even found on needle baits, e.g. *Resinicium bicolor* and *Sistotrema brinkmannii* (**Paper II**).

Many saprotrophic fungi appear to be able to utilise different plant litter fractions e.g. needle and wood litter (**Paper II**). Considering the dynamics of these litter fractions in managed forests (**paper IV**), this strategy must be advantageous as it may allow these species to alternate between these fractions as they become available. Thus, they are less likely to be affected by slash removal.

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Acknowledgements

I would like to thank my main supervisor Anders Dahlberg for giving me the opportunity to work with wood-inhabiting fungi and their ecology and for his support and willingness to discuss things even though you've been a busy man. I have enjoyed working with you. Thanks to my second supervisor Professor Jan Stenlid for help and support along the way. I'm grateful to Rimvis Vasiliauskas, who has functioned as a second supervisor for a large part of this thesis, for help and support all the way. I'm also grateful to Karin Backström for all practical help, Katarina Ihrmark, for endless help in the in the PCR lab and Andy Taylor for coming with valuable comments on the manuscript for this thesis. You to, Jonas, who spent a great deal of time on it even though you had tons of other things to do. A special thanks to Riitta Hyvönen for all the work you put in to get the modelling parts of the manuscript finished. Audrius, thanks for the help in the end when I prepared the thesis, hope I haven't disturbed you to much. Thanks to all of you who came with suggestions for improvements on the manuscripts.

There are of course many more people involved that has supported and helped me in different ways, both at the department and outside. Thanks to all of you for doing so!!

To my closer friends both at the department and outside, thanks for support and especially a great time together. My family is of course not forgotten, thanks for everything.

I am forever grateful to Dr. Åke Strid for introducing me to the field of mycology and for all the help and support .

This project was financed by STEM.