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Impacts of clear-cutting on soil fungal communities and their activities in boreal forests

A metatranscriptomic approach

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Abstract

Large-scale forestry has reduced and fragmented the area of primary forest and greatly impacted communities of organisms, above and below ground. Fungal communities, which are pivotal in boreal forest soil functions, are vulnerable to tree harvesting. Changes in their community composition may be followed by loss of key functions and ultimately affect carbon and nutrient cycling. By using various molecular approaches, such as metabarcoding, transcriptomics and metatranscriptomics, this thesis aims to investigate how clear-cut forestry affects the composition and traits of soil fungal communities.

In a shorter time perspective, clear-cutting eliminate ectomycorrhizal fungi but stimulates growth of saprotrophic fungi. Clear-cutting also enhanced cellulose and lignin decomposition, which may reduce soil carbon stocks in the short-term and potentially cause eutrophication in the mid-term. After 35 years, the ectomycorrhizal fungal community composition in re-established secondary forest was still not restored to the same composition as in forest with longer continuity, although its biomass had recovered. Particularly *Cortinarius*, a genus with a key functional role in lignin decomposition, was largely missing in secondary forest. Ectomycorrhizal Cortinarius species accounted for a large fraction of gene transcription of ligninolytic peroxidases in forests with long continuity, and loss of this function could impair long-term nitrogen cycling and soil fertility.

Overall, this thesis presents evidence that clear-cutting forestry has extensive effects on fungal biodiversity, with major short-term consequences for soil fungi and their facilitation of decomposition and nutrient cycling, but also long-term effects on ectomycorrhizal communities that should be considered in further evaluation of management practices.

Keywords: forestry, soil fungi, metabarcoding, transcriptomics, metatranscriptomics, decomposition, carbon-use efficiency, ectomycorrhiza, *Cortinarius*

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Effekter av trakthyggesskogsbruk på marksvampar och deras funktioner i boreal skogsmark

Sammanfattning

Storskaligt skogsbruk har minskat och fragmenterat ursprungliga skogsområden med stor påverkan på organismsamhällen, såväl ovan som under markytan. Svampsamhällen spelar en avgörande roll i den boreala skogsmarkens funktion och är känsliga för avverkning. Förändringar i artsammansättning kan leda till att viktiga funktioner går förlorade med långsiktiga konsekvenser för kol- och näringsomsättning. Genom att använda olika molekylära metoder, såsom DNA-baserade samhällsanalyser, transkriptomik och metatranskriptomik, syftar denna avhandling till att undersöka hur trakthyggesskogsbruk inverkar på svampsamhällens artsammansättning och funktionella egenskaper i boreal skogsmark.

I ett kortare tidsperspektiv eliminerar kalavverkning ektomykorrhizasvampar men ökar tillväxten av saprotrofiska nedbrytarsvampar. Avverkning stimulerade också cellulosa- och ligninnedbrytning, vilket på kort sikt kan leda till kolförluster, och på lite längre sikt till ökad näringstillgängligheten i marken (eutrofiering). Efter 35 år avvek det ektomykorrhizabildande svampsamhället fortfarande tydligt i artsammansättning från skogar med längre kontinuitet, även om den totala biomassan hade återhämtat sig. Särskilt *Cortinarius* (spindlingar), ett släkte med en central roll i ligninnedbrytning, saknades i stort sett i återplanterade skogar. Ektomykorrhiza-bildande *Cortinarius*-arter låg bakom en stor del av genuttrycket av ligninnedbrytande peroxidaser i skogar med lång kontinuitet, och förlust av detta viktiga släkte kan på längre sikt ha negativa effekter på kväveomsättning och markbördighet.

Sammanfattningsvis visar avhandlingen att trakthyggesskogsbruk har tydliga effekter på svampbiodiversitet, med stora kortsiktiga konsekvenser för nedbrytning och näringsomsättning, men också långsiktiga effekter på mykorrhizasvampar som bör beaktas när skogsbruksmetoder utvärderas.

Nyckelord: skogsbruk, marksvampar, DNA-baserad samhällsanalys, transkriptomik, metatranskriptomik, nedbrytning, metabolisk effektivitet, ektomykorrhiza, Cortinarius

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Dedication

To my parents

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Hasby, F.A., Barbi, F., Manzoni, S. & Lindahl, B.D. (2021). Transcriptomic markers of fungal growth, respiration and carbon-use efficiency. FEMS Microbiology Letters vol. 368 (15). DOI: https://doi.org/10.1093/femsle/fnab100

II. Hasby, F.A., Clemmensen, K.E. & Lindahl, B.D. (2021). Impact of rotation forestry on soil fungal communities and associated enzymatic activities. (manuscript)

III. Hasby, F.A., Barbi, F., Clemmensen, K.E., Manzoni, S. & Lindahl, B.D. (2021). Metatranscriptomic analyses of fungal decomposition in managed boreal pine forest. (manuscript)

The contribution of Fahri Hasby to the papers included in this thesis was as follows:

I. Planned the study together with the co-authors. Performed all laboratory work and data analysis. Interpreted the results and wrote the manuscript together with the co-authors.

II. Participated in planning together with the co-authors. Conducted the field sampling and performed all laboratory work and data analysis. Interpreted the results and wrote the manuscript together with the co-authors.

III. Participated in planning together with the co-authors. Conducted the field sampling and performed all laboratory work. Contributed to development of the bioinformatic pipeline and conducted all data analysis. Interpreted the results and wrote the manuscript together with the co-authors.

Abbreviations

AA2	Class II peroxidase encoding gene family
CUE	Carbon-use efficiency
ECM	Ectomycorrhizal fungi
ERM	Ericoid mycorrhizae
GT48	1,3-β-glucan synthase encoding gene family
GTR	Green tree retention
GH6	Cellobiohydrolase encoding gene family
ITS	Internal transcribed spacer
KGD	2-Oxoglutarate dehydrogenase encoding gene family
mRNA	Messenger RNA
NMDS	Non-metric multidimensional scaling
PCR	Polymerase chain reaction
rRNA	Ribosomal RNA
TCA	Tri-carboxylic acid

1. Introduction

1.1 Ecology of boreal forest

Boreal forest, also known as the Taiga, is a biome that spans across the circumpolar belt, including Finland, Scandinavia, Russia, Canada and Alaska (Figure 1) and covers roughly 11% of the global land surface. The climate in the boreal forest is characterised by short, moderately warm and moist summers and long, cold and dry winters. However, in Fennoscandian boreal forest winters are not as severe, as a strong influence of coastal climate makes the intra-annual temperature differences smaller. As a result of the seasonal variation, boreal plants experience a short growing season (Bonan & Shugart 1989). The average annual temperature in Fennoscandian boreal forest is around 1-4˚C (Ruiz-Pérez & Vico 2020).

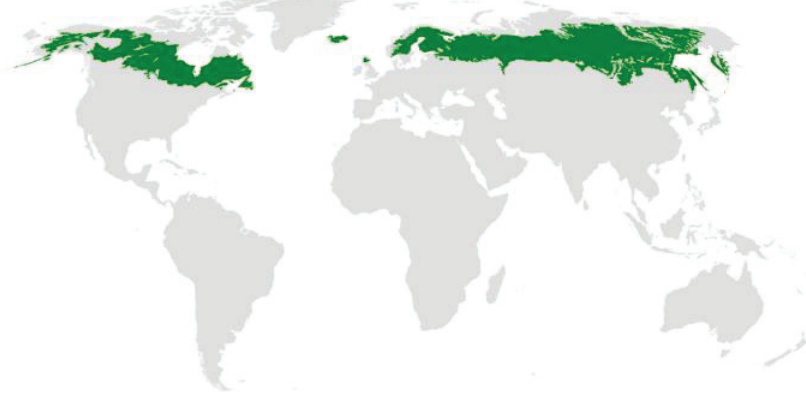

Figure 1. Global distribution of the boreal forest biome (green; image by MBS; reproduced under CC BY-SA 3.0 licence).

In these conditions, only plant species characterised by tolerance to large variations in air temperature and low nutrient availability have come to dominate, resulting in low plant diversity. The Fennoscandian boreal forest is mostly dominated by evergreen coniferous trees, particularly Scots pine (*Pinus sylvestris*) and spruce (*Picea abies*). Dwarf shrubs (*Calluna vulgaris*, *Empetrum*, *Vaccinium etc*. of the Ericaceae family) form the major part of the understory vegetation, while larger shrubs (*Salix*, *Juniperus etc*.) comprise a minor part (Esseen *et al.* 1997). Boreal forests are adapted to regular wildfires that regenerate the vegetation in successional cycles. This succession may shift dominance from spruce to pine, which is more tolerant to fire than spruce (Engelmark *et al.* 1994). Lichens, such as *Cladonia*, and mosses, such as *Pleurozium*, *Hylocomium* and *Dicranium,* are also common in boreal forests (Esseen *et al.* 1997).

1.2 Soil characteristics in boreal forest

Boreal forest soils are typically classified as Podzols, characterised by an organic layer (O-horizon) residing on top of mineral soil, commonly with a sandy texture and illuvial mineral layer (Bonan & Shugart 1989). In the O-horizon, organic matter accumulation combined with the absence of mixing by soil fauna, leads to vertical stratification, where the age of the organic matter increases with depth (Trumbore & Harden 1997). The O-horizon can be further divided into a litter (L), fermentation (F), and humus (H) layers, which differ in the degree of litter decomposition (Lindahl *et al.* 2007). The dominating evergreen conifers and ericoid dwarf shrubs, together with their associated fungi, play an important role in shaping the soil characteristics of boreal forest (Clemmensen *et al.* 2013).

Tree growth and microbial decomposition in boreal forest soils are characterised by severe nitrogen limitation. The needle litter in boreal forest is enriched in recalcitrant compounds, such as tannins, lignin and phenols (Prescott & Vesterdal 2021). Efficient mycorrhizal nutrient recycling, competition between functional guilds and tannin-organic matter interactions further restrict decomposition. The low rates of decomposition and scarcity of nitrogen imply that nitrogen is either immobilised in microbial (mainly fungal) biomass or more or less stabilized in dead organic matter (Lindahl &

Clemmensen 2016). The low nitrogen availability makes it even more difficult for other types of plants, apart from evergreen conifers, to grow in boreal forest (Cornwell *et al.* 2008).

1.3 The functional role of fungi in boreal forest

1.3.1 Saprotrophic fungi

Fungi occupy a central role in decomposition of soil organic matter in the boreal forest biome (Lindahl & Clemmensen 2016). Saprotrophic fungi are free-living decomposers that usually dominate in freshly deposited litter layers (Schneider *et al.* 2012) and are the main producers of extracellular hydrolytic enzymes (Baldrian *et al.* 2011). Saprotrophic fungi depolymerise organic material in leaf litter, dead roots and dead mycelium into assimilable compounds using extracellular enzymes, in order to obtain the nutrients and carbon (C) they need for energy and growth (Jennings, 1995).

The saprotrophic community in boreal forest soil are dominated by basidiomycetes and ascomycetes. Basidiomycetes, mainly agaricomycetes, encompasses potent white-rot and brown-rot lignin decomposers. Ascomycetes, mainly leotiomycetes and the order Pezizales, are also dominant (Sterkenburg *et al.* 2015; Kyaschenko *et al.* 2017a). During the early phase of litter decomposition, ascomycetes, which are generally less efficient decomposers compared to basidiomycetes (Boberg *et al.* 2011a), selectively decompose cellulose over lignin (Lindahl *et al.* 2010). Over time, approximately within a year, they are gradually replaced by basidiomycetes (Kohout *et al.* 2018) that are able to produce a wider variety of extracellular enzymes (Baldrian & Valášková 2008; Osono *et al.* 2021) and capable of degrading more chemically resistant organic matter.

The dominant part of the litter is in the form of fine roots and associated fungal mycelium (Prescott & Vesterdal 2021). Recent meta-analysis indicated that 48% of litter input comes from fine roots (Freschet *et al.* 2013; Xia *et al.* 2015), but numbers as high as 80% have been reported from boreal forests (Kyaschenko *et al.* 2019). Fine roots decompose at a slower rate compared to leaf litter (Guo *et al.* 2021), possibly due to higher tannin content, and may lead to lower rates of carbon and nitrogen mineralisation. Fine root

decomposition has been proposed to be an important mechanism in soil organic matter stabilisation in boreal forest (Adamczyk *et al.* 2019b). Therefore, it is important to understand which fungi decompose fine roots.

1.3.2 Mycorrhizal fungi

Over the course of evolution, plants have formed a close partnership with fungi, and this symbiosis has been proposed to be crucial for plants to be able to colonise terrestrial ecosystems (Redecker *et al.*, 2000; Brundrett, 2002). In general, fungi play a pivotal role in helping plants to explore soils, by accessing nutrients and offering protection from pathogens (Courty *et al.*, 2010). This mutualistic partnership, also known as *mycorrhiza*, occurs in several forms. In the boreal forest biome, three types of mycorrhizal association are present: arbuscular mycorrhizae (AM), ectomycorrhizae (ECM) and ericoid mycorrhizae (ERM), where the latter two play a more important role in ecosystem functioning and nutrient cycling.

The AM fungi first appeared about 460 million years ago and probably enabled plants to colonise the terrestrial environment during the Devonian period (Redecker *et al.*, 2000; Brundrett, 2002). Globally, arbuscular mycorrhiza is the most abundant type of plant–fungi interaction and is formed by obligate symbiotic fungi in the phylum Glomeromycota that penetrate the cortical cell of vascular plant roots (Figure 2). Arbuscular mycorrhiza is characterised by fungal structures called *arbuscules*—hence the name 'arbuscular', and sometimes also vesicles within the plant cells (Smith & Read, 2008). Today, about 80% of terrestrial plants form arbuscular endomycorrhizae with glomeromycetes (Brundrett, 2002).

The ERM association is a type of symbiosis between ascomycetes (and certain basidiomycetes) and plants from the Ericaceae family that first appeared around 100 million years ago (Strullu-Derrien *et al.* 2018). Ericoid mycorrhiza is usually formed in harsh environmental conditions, such as in highly acidic and nutrient-poor soils, and are characterised by fungal coils that form within the epidermal cells of the fine hair roots of Ericaceae plants (Figure 2). Ericoid mycorrhizal fungi form loose hyphal networks around the root hairs, from which they penetrate the walls of cortical cells (Smith & Read, 2008).

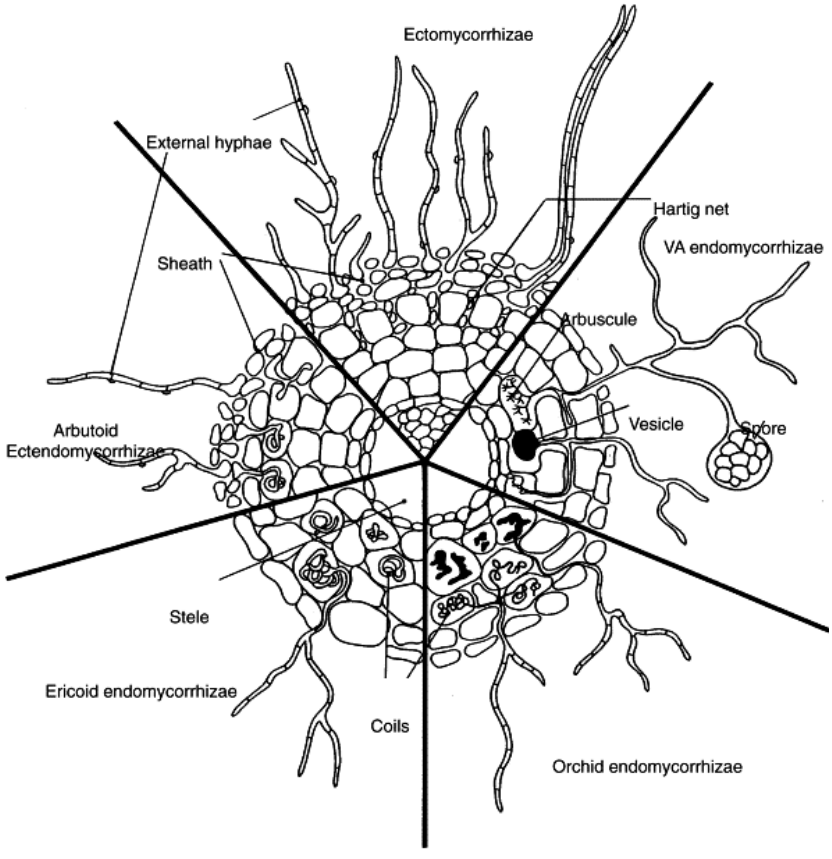

Figure 2. The different types of mycorrhizal association between root tip and arbuscular mycorrhizae (AM), ectomycorrhizae (ECM), vesicular-arbuscular (VA) endomycorrhizae, orchid mycorrhizae, and ericoid mycorrhizae (ERM) fungi (Selosse & Le Tacon, 1998; reproduced with permission from the publisher).

ECM forming fungi have existed at least since the Tertiary period, *i.e*. around 60 million years ago (LePage *et al.*, 1997). This type of symbiosis forms between fungi and around 2% of plant species in the world, mostly woody plants (Tedersoo *et al.*, 2010). However, it involves more than 6000 fungal species belonging to the basidiomycetes (and certain ascomycetes), as well as many of the temperate tree species and practically all boreal trees (Smith & Read, 2008). Unlike AM fungi, ECM fungi do not penetrate the host cell, but rather form highly branched hyphal networks called 'Hartig nets' that extend into the spaces between epidermis and cortical cells of the root, as

well as dense mycelial mantles around the root tips (Figure 2). Most ECM are also able to form extra-radical mycelium, which extends from roots into the soil matrix (Smith & Read, 2008). The ECM symbiosis is regarded as mutualistic and biotrophic, as the fungi obtain metabolic carbon from their plant hosts in the form of hexoses. In exchange for the carbon, the fungi share nutrients, such as nitrogen and phosphorus (P), obtained via their decomposition and scavenging activities (Harvey *et al.*, 1986; Abuzinadah & Read, 1989; Lindahl *et al.*, 2002; Smith & Read, 2008).

The harsh climate of boreal forests in combination with high soil acidity and low decomposition rates of organic matter, leads to strong nitrogen limitation and provides a strong selection force against AM fungi in favour of ECM fungi (Steidinger *et al.* 2019). As a result of that, coniferous trees with ECM and dwarf shrubs with ERM dominate in boreal forest. Besides nutrient uptake, ECM fungi also offer transport of water in many species, increasing the functional water access area (Duddridge *et al.*, 1980). In addition, the hyphal sheath enveloping the root tips acts as a physical barrier, shielding plant tissues from pathogens, predators and toxic substances (Kennedy & de Luna, 2005).

Dead mycorrhizal mycelium (*i.e.* necromass) plays an important role in carbon accumulation in boreal forests. In the past, aboveground plant litter dynamics were believed to be the principal predictor of carbon accumulation in soils (Adamczyk, 2021). However, Clemmensen *et al*. (2013, 2015) showed that the dynamics of roots and root-associated fungi are an important additional factor explaining variations in carbon sequestration. For example, on one hand, carbon sequestration may be decreased by the rapid mycelial turnover and well-developed decomposer capacity of certain ECM species. On the other hand, carbon sequestration might be increased by the highly melanised ERM mycelium. The ECM fungi seem to play a significant role in decomposition of organic matter in the humus layer via the activity of manganese (Mn) peroxidases (a potent oxidative enzyme; Bödeker *et al.* 2014), iron (Fe) peroxidases or via the Fenton reaction (op de Beeck *et al.* 2020; Wang *et al.* 2020, 2021b). These mechanisms of oxidative decomposition have been proposed to benefit the fungus by increasing its access to organic nitrogen, rather than by providing a source of metabolic carbon (Lindahl & Tunlid 2015). Mn-peroxidases are energetically expensive and an external input of carbon might be needed to drive their production (Bödeker *et al.*, 2014). In a trenching experiment, Sterkenburg *et al.* (2018)

found that after removal of ECM, decomposition of surface litter increased by 11% due to easing of nitrogen limitation. However, trenching also caused a 91% decrease in Mn-peroxidase activity in the deeper organic layer, with supposed negative effects on decomposition. These findings imply that Mn-peroxidases are very effective, yet costly, enzymes, and their activity needs to be supported by a large amount of external carbon supply to the fungal community. A similar effect of ECM elimination has been suggested to occur after clear-cutting (Kyaschenko *et al.*, 2017a) when the supply of host C to the mycorrhizal community is disrupted at harvest.

Under severe nitrogen limitation, the balance of bidirectional transfer of resources between ECM fungi and its host may shift. Plants will deliver more carbon to their fungal partner to stimulate nitrogen mineralisation. However, more carbon allocation by the plant will cause ECM fungi to increase extra-radical mycelial growth and retain nitrogen in their own biomass, ultimately putting further constraints on plant growth (Näsholm *et al.* 2013; Högberg *et al.* 2017). Nitrogen limitation may indirectly restrict decomposition, as it favours stress-tolerant plants and ERM fungi. Yet, nitrogen limitation may also directly constrain the performance of fungal decomposers, basidiomycetes in particular (Boberg *et al.* 2011b). The highly melanised biomass of ERM fungi has been proposed to be more resistant to enzymatic decomposition and to make the nitrogen cycle even slower, restricting decomposition even more, which may lead to 'ecosystem retrogression', *i.e*. a substantial decline in ecosystem processes over a long time period (Lindahl & Clemmensen, 2016). This feedback mechanism may be re-set by disturbances, such as fire or forestry (Clemmensen *et al.*, 2015; Kyaschenko *et al.*, 2017a).

1.4 A glimpse into forestry in Sweden

In Sweden, forest currently occupies around 58% of the total land area, and around 84% of the Swedish forest is productive forest—forest areas where production is at least 1 m^3/ha/year (NFI, 2021). Forestry plays a crucial role in the Swedish economy. It also accounts for 9-12% of total employment, sales, exports and added value of Swedish industry. Around 90% of the annual forest biomass increment (~130 million m^3) is exported (valued around 130 billion SEK), making Sweden the third largest forest product exporter in the world (KSLA, 2015). Forestry in Sweden underwent a very rapid and radical change in the mid-20th century. Throughout the 20th century, forests

were managed into even-aged stands, which were harvested by clear-cutting. To enhance production, rationalisation in agriculture to limit grazing disturbance in forest also took place. Even though tree harvesting involved clear-cutting, active regeneration after harvest, together with extensive reforestation of agricultural land and pasture land, led to an increase in the total forested area in Sweden (KSLA, 2015).

In the early 1970s, large-scale clear-cutting began to draw criticism, but mainly based on aesthetic rather than ecological concerns. Ecological considerations only began to be included in the debate as recognition of the impact of forestry on rare and threatened species increased. It has become evident that replacing old forested landscape with even-aged monospecific stands of spruce or pine is detrimental to the presence and abundance of rare and threatened birds, mammals and insects. In the 1990s, ecological efforts were supported by the compilation of a Red List of rare and threatened species (Simonsson *et al.*, 2015).

The clear-cutting strategy has long been argued to have a detrimental effect on the forest. Although clear-cutting is usually followed by large-scale replanting, the efficacy of replanting in ensuring that the forest diversity and structure will return to its original complexity and long-term productivity has not been thoroughly evaluated, due to the long-term perspectives involved (Gamfeldt *et al.* 2013). Various studies have been conducted to better understand the impact of clear-cutting on other forest communities, apart from plants (Paillet *et al.* 2010).

In order to ameliorate the negative effect of clear-cutting, alternative forestry methods, called 'retention forestry', have been adopted in various countries. Much similar to continuous-cover forestry, a large number of trees are intentionally left behind during harvest. In Swedish forestry, the method implemented as 'green tree retention' (GTR), involves leaving 1-20% stand coverage and tree species with high conservational value from the old forest during tree harvesting (Figure 3) (Rosenvald & Lõhmus, 2008; Simonsson *et al.*, 2015). The main objectives of GTR are to: (1) 'life-boat' species and processes over the regeneration phase, (2) enrich re-established forest stands with older trees, and (3) enhance landscape connectivity (Franklin *et al.*, 1997). Its implementation is a result of a compromise between critics of the old large-scale clear-cutting method and the economic rationale.

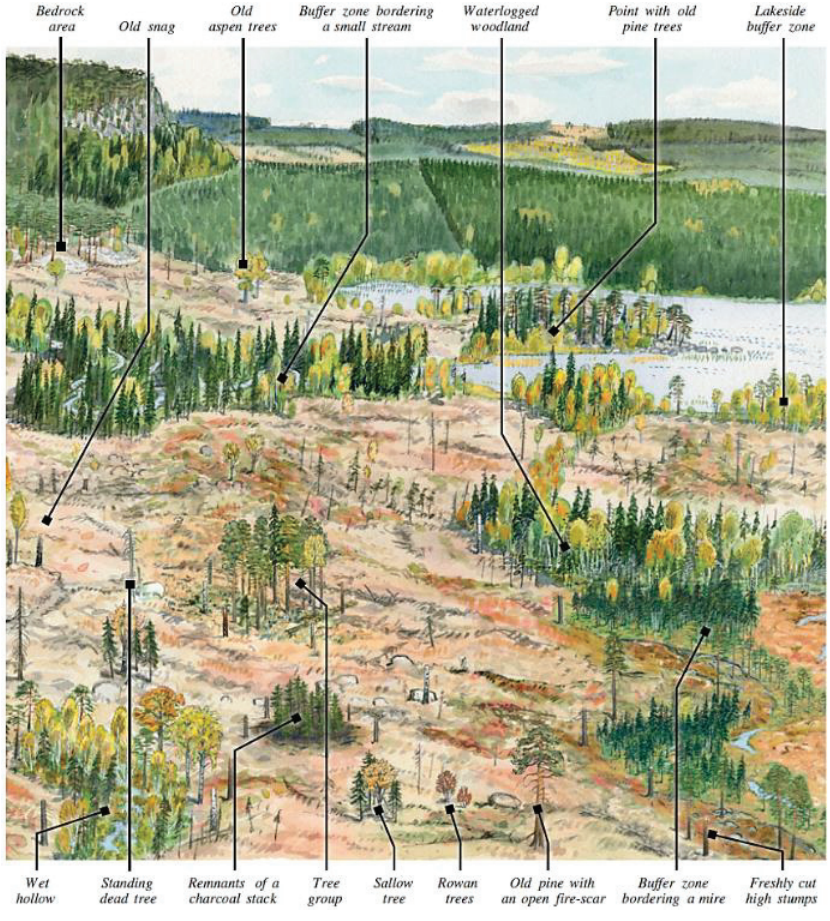

Figure 3. Example of a retention forestry plan implemented by a Swedish forest company (Simonsson *et al*., 2015).

In a meta-analysis of studies examining the effects of GTR implementation, Rosenvald & Lõhmus (2008) found that GTR lowered the harvest-related loss of species richness by 72% of the studies analysed. However, Varenius *et al*. (2017) claimed that the current model of GTR implemented in Sweden has not succeeded in mitigating the effect of clear-cutting on ECM fungal communities, as tree retention only succeeds in maintaining the local ECM fungi communities immediately around trees that are permanently retained. A recent study showed that the level of tree retention is critical for the effectiveness of GTR, as a positive effect on ECM fungi can be seen only when

at least 30% of the trees are retained (Sterkenburg *et al.* 2019). These findings have put GTR implementation into question, creating a need for evaluations of GTR and for development of new forestry strategies to ensure conservation of belowground fungal taxa that are important for ecosystem functionality.

1.5 Effect of forestry on soil fungal communities

At present, less than 1% of European forests are still primary forest. This is considerably less than in Canada and USA, where 40-52% and 13%, respectively, of natural forest is estimated to remain (Heywood & Watson, 1995; Parviainen *et al.*, 2000). In Sweden, primary forests currently comprise about 37% of total forest area (NFI, 2021), but these are highly fragmented (Sabatini *et al.*, 2018; Peter *et al.*, 2021). A major part of forested land in Sweden consists of planted secondary forest (52%) and recent clear-cuts (10%). Forestry has altered the community structure of vegetation and other taxa, such as insects, birds and mammals. However, taxa that live belowground are also greatly affected and are beginning to receive more attention regarding their important role in the ecosystem (Rosenvald & Lõhmus, 2008).

Recently, belowground fungal communities have garnered more interest, as many fungi lead a symbiotic lifestyle with plants and play an important role in soil carbon and nutrient cycling. Fungal communities undergo several major changes after tree harvesting, including: a) a decrease in C input to the ECM fungi as their hosts are removed (Last *et al.* 1979); b) changes in age distribution and community structure of the host trees; c) displacement or removal of ECM from the organic soil horizons caused by soil preparation following harvest (Harvey *et al.* 1986); d) changes in temperature and moisture due to loss of large over-story trees; e) changes in the fungal community structure, as well as changes in processes in which these organisms are involved, such as organic-matter decomposition and N cycling (Perry *et al.*, 1984; Forge & Simard, 2000; Prescott *et al.*, 2000; Bradley, 2001).

Ectomycorrhizal fungi are eliminated shortly after clear-cutting and take about 10-20 years to recover to their original rate of mycelial production (Wallander *et al.*, 2010). The relatively fast recovery of ECM fungal biomass is usually not paralleled by recovery of fungal biodiversity, for which negative effects may persist for several decades. Based on a meta-analysis of 12

studies in boreal forest, Paillet *et al.* (2010) concluded that fungal species richness is significantly lower in forest that undergone clear-cutting and re-forestation compared to primary forest. Varenius *et al*. (2016) found that tree harvesting in boreal Scots pine forest had altered the species composition of ECM fungi in the long-term (50-60 years), in particular for rare species. This finding was corroborated by Kyaschenko *et al.* (2017a) in a chrono-sequence study, comparing managed boreal Scots pine forest with older forests. They found that after clear-cutting, the ECM fungi community changed from dominance by the genera *Cortinarius* and *Russula* in old forests to genera in the family Atheliaceae (*Piloderma, Tylospora, Amphinema*) in young, planted forests.

In the harsh environment of boreal forests, saprotrophic fungi and mycorrhizal fungi may compete for space and resources, such as nitrogen. Such competitive interactions contribute to partitioning of the fungal community into two vertically separated and functionally distinct sub-communities, where saprotrophic fungi dominate the litter layer and mycorrhizal fungi dominate the humus layer (Lindahl *et al.* 2007; McGuire *et al.* 2013). Following tree harvest, saprotrophic fungi have been proposed to be released from competition with ECM fungi and proliferate (Chatterjee *et al.*, 2008; Kohout *et al.*, 2018). As a result of this and in combination with a sudden supply of dead roots and mycelium, decomposition may increase. This phenomenon is known as the 'Gadgil effect' first described by Gadgil & Gadgil (1971). As a result of increased decomposition of mycorrhizal fungal necromass, nitrogen availability may increase in clear-cuts and further stimulate proliferation and decomposition by saprotrophs, creating a positive feedback where mineralised nitrogen from decomposition promotes growth of saprotrophic fungi and even further decomposition (Kyaschenko *et al.* 2017a; b, 2019).

1.6 Functional traits of fungi

1.6.1 Linking fungal traits, diversity and ecosystem functions

Traits are expressed characteristics or attributes of an organism that are genetically determined and/or influenced by the environment. The term 'response traits' denotes phenotypic traits that determine how organisms respond to changes in the ecosystem, while 'effect traits' describes alterations

in the phenotype that, in turn, affect the ecosystem, *e.g*. by changing ecosystem processes or multi-trophic interactions (van der Putten *et al.*, 2001; van der Putten *et al.*, 2013; Bardgett, 2018). When response traits and effect traits are connected in fungal species, fungal communities become pivotal links between environmental changes and ecosystem responses (Crowther *et al.*, 2014; Koide *et al.*, 2014). Fungal traits may have a major influence on soil carbon and nitrogen cycle. For example, symbiotic vs. saprotrophic lifestyle and enzymatic capacities might influence organic matter stabilisation, as recalcitrant organic matter requires certain enzymes that are unique to particular taxa. (Schmidt *et al.* 2011). Dead mycelium is part of soil organic matter, and the abundance of fungal mycelial components, such as melanin, might determine its decomposability (Fernandez & Koide 2014; Fernandez *et al.* 2016).

In trait-based modelling, affiliation of these functional traits to specific taxa is needed, and assays that provide taxonomic resolution are urgently required (Allison & Martiny, 2009; Allison, 2012, 2014; Manzoni, 2017; Zhang *et al.*, 2018). Expression of effect traits by individual species may be concealed at the community level, if there is a high degree of functional redundancy among species, which might undermine the influence of microbial diversity and community composition on ecosystem processes (Andrén & Balandreau 1999; Nannipieri *et al.* 2003). However, presence of microbial keystone taxa may exert a major influence on community effect traits and ultimately influence ecosystem processes (Banerjee *et al.* 2018). Recently, Lindahl *et al*. (2021) found that the presence of a specific group of closely related fungal species in the ectomycorrhizal genus *Cortinarius* exerted a major influence of belowground carbon storage across Swedish forests with long continuity, demonstrating a low degree of functional redundancy among boreal forest soil fungi.

1.6.2 Fungal enzymatic decomposition

Crystalline cellulose and lignin are the most abundant components of plant litter (Talbot & Treseder 2012). Fungi utilise a plethora of extracellular enzymes that polymerise and depolymerise complex carbohydrates (CAZymes; Lombard *et al.* 2014). Cellulose, the most abundant polymer in nature, is degraded in several steps. After initial cutting by endocellulases, shorter cellulose chains are further degraded by cellobiohydrolases into cel-

lobiose – a disaccharide. Cellobiose is further degraded into assimilable glucose by β-glucosidases (Payne *et al.* 2015; Yue *et al.* 2016). This process releases metabolically useful carbon, and expression of cellobiohydrolases is characteristic of saprotrophic litter decomposers, which use dead plant matter as their principal source of metabolic carbon (Žifčáková *et al.* 2017). Biotrophic organisms, including mycorrhizal fungi, obtain glucose directly from their living hosts, and are thereby independent of cellulose decomposition (Kohler *et al.* 2015; Lindahl & Tunlid 2015).

Due to a lack of regularly occurring C–O linkages, lignin is resistant to hydrolytic enzymes and requires oxidative reactions for its degradation (Sinsabaugh 2010). Lignin and similar phenolic macromolecules are commonly not used for metabolic purposes, but hinder access to hydrolysable compounds and have to be degraded for efficient mobilisation of carbohydrates and other resources (Baskaran *et al.* 2019; Barbi *et al.* 2020). Class II peroxidases are enzymes that are able to indirectly decompose lignin by using H_2O_2 as electron acceptor and oxidize Mn^{2+} into Mn^{3+}. The Mn^{3+}, in turn, may oxidize and break up lignin crosslinks and make hydrolysable substrates, such as cellulose and proteins, accessible for further degradation (Hofrichter *et al.* 2010; Rahman *et al.* 2013; Yue *et al.* 2016). In lignin-rich boreal forest litter, removal of hampering lignin by fungal peroxidases regulates overall decomposition rates (Barbi *et al.* 2020), with increasing importance of lignin degradation as decomposition progresses (Craine *et al.* 2007). Oxidative lignin decomposition is characteristic of saprotrophic "white-rot" basidiomycetes and uncommon among mycorrhizal fungi (Zak *et al.* 2019), but some ectomycorrhizal genera like *Cortinarius* have retained efficient oxidative capacity (Lindahl & Tunlid, 2015; Lindahl *et al.* 2021).

1.6.3 Carbon-use efficiency

To represent the partitioning of carbon in fungal metabolism, carbon-use efficiency (CUE) is defined as the amount of carbon incorporated in growing biomass relative to the total amount of carbon taken up, normally assessed as the sum of growth and respiration (Geyer *et al.*, 2016; Manzoni *et al.*, 2018). Microorganisms play a central role in formation, transformation and release of soil organic carbon (Lehmann & Kleber, 2015; Liang *et al.*, 2017; Lehmann *et al.*, 2020). Through decomposition of plant litter, or via biotrophic interactions with symbiotic plants, fungi obtain carbon that is either transformed into biomass, and eventually into soil organic matter, or released

to the atmosphere as carbon dioxide (CO_2) (Schmidt *et al.*, 2011). High CUE of decomposers means lower proportional carbon losses during decomposition and higher accumulation of microbial biomass, which could enhance storage of fungus-derived carbon in soils (Cotrufo *et al.*, 2013). On the other hand, high CUE can instigate rapid proliferation of fungal decomposers, leading to more efficient exploitation of plant litter, with associated organic matter losses and lower storage of plant-derived carbon (Allison *et al.*, 2010; Manzoni *et al.*, 2018). Meanwhile, Hagenbo *et al*. (2019) observed that CUE of ectomycorrhizal fungi was relatively low (0.03-0.15) compared to saprotrophic decomposer (0.1-0.4), and decrease significantly with increasing forest age. This decrease might be explained by shift of fungal community towards ectomycorrhizal dominance and general lower N availability in older forest.

However, empirical assessment of CUE is challenging, because this parameter integrates processes occurring at different scales in time and space, carried out by diverse communities of organisms with contrasting properties (Geyer *et al.*, 2016). As a consequence, estimates of CUE are sensitive to the chosen methodology. In laboratory incubations, carbon-labelled substrates (Dijkstra *et al.*, 2011, 2015; Rousk & Bååth, 2011; Frey *et al.*, 2013; Sinsabaugh *et al.*, 2013), oxygen-labelled water (Schwartz, 2007; Blazewicz & Schwartz, 2011; Spohn *et al.*, 2016; Geyer *et al.*, 2019) or calorespirometry (Hansen *et al.*, 2004; Barros *et al.*, 2016; Geyer *et al.*2019) have been used to measure microbial growth in soils. Respiration is more frequently measured directly in the field, but with major problems in isolating the microbial component from plant roots and soil fauna (Sapronov & Kuzyakov, 2007; Vargas *et al.*, 2011). In addition, the current focus on separating 'autotrophic' and 'heterotrophic' respiration is not very informative in regards to fungal communities, as fungal carbon sources vary from old organic matter to recently photosynthesised carbon provided by symbiotic hosts (Ryhti *et al.*, 2021).

1.7 Molecular methods in soil microbial ecology

A large part of the work described in this thesis centred on the development and implementation of metatranscriptomics in the study of soil microbiota. Thus, it can be useful to summarise the developmental history and compare

existing molecular methods. It is estimated that only about 1% of all microbial taxa can be easily cultured (Harwani, 2012; Epstein, 2013). This so-called 'great plate anomaly' (Staley & Konopka, 1985) makes capturing the full diversity of soil microbes into culture virtually impossible. Due to that severe limitation, new methods have been developed based on 'molecular markers', *i.e*. different molecules produced in cells, such as lipids, DNA and RNA, to analyse the abundance, the taxonomic and functional diversity, as well as activities of soil microbial communities (Zuckerkandl & Pauling, 1965).

1.7.1 Lipids as markers

Lipid membranes are ubiquitous in cellular organisms and mainly composed of phospholipids and sterols. Phospholipid-derived fatty acid (PLFA) analysis is a method that uses phospholipids to estimate microbial biomass, and also to some extent as taxonomic markers (Morris *et al.*, 2008). Phospholipids are major components of cellular membranes and different groups of microorganisms have a different variety of di-glyceride tails. PLFA analysis does not require cultivation (Kaur *et al.*, 2005; Buyer & Sasser, 2012) but has a low level of taxonomic resolution (Frostegård *et al.*, 2011) and has mainly been used to partition biomass at the level of entire kingdoms (*e.g.* fungi:bacteria ratios). Ergosterol, a main sterol component in fungal cell membranes, is normally used as a marker of living fungal biomass (Nylund & Wallander 1992), as it is rapidly degraded in dead mycelium. Ergosterol concentrations may be used to estimate the total fungal biomass through a conversion factor (Salmanowicz & Nylund 1988).

1.7.2 DNA as a marker

The use of DNA as a molecular marker depended on the development of the polymerase chain reaction (PCR) technique, which enables a gene fragment of interest to be amplified *in vitro*. PCR uses the unique properties of the Taq enzyme, a polymerase isolated from the thermophilic aquatic bacterial species *Thermus aquaticus*, and short nucleic acid strands called 'primers' (Saiki *et al.*, 1988). This was a great improvement over the previously used cloning methods, which were time-consuming and laborious (Cohen *et al.*, 1973).

The development of molecular methods in microbial ecology saw another great improvement with recent advances in sequencing technology. The ar-

rival of massive parallel sequencing platforms has offered unprecedented sequencing yield and possibilities to quantify the composition of complex communities. It also means that sequencing is more economically feasible, lifting constraints on large-scale microbial ecology studies. In the past decade, many sequencing platforms have become available, such as 454 pyrosequencing (Roche), flow cell technology (Illumina Inc.), single molecule real-time (SMRT) sequencing (Pacific Biosciences), ion torrent sequencing (ThermoFisher Scientific) and, most recently, a table-top sequencing from Oxford Nanopore Technologies. All these have their own advantages and disadvantages (Liu *et al.*, 2012; Shokralla *et al.*, 2012; van Dijk *et al.*, 2014; Goodwin *et al.*, 2016). These new technologies, combined with collective global efforts to build more efficient bioinformatics tools and reference databases, have propelled the study of microbial diversity into a new age.

Metabarcoding

Metabarcoding, also known as targeted metagenomics, is a robust technique that directly sequences PCR-amplified gene markers (a so called 'barcode') from environmental samples. With high sequencing yield, it is possible to obtain detailed analyses of large numbers of samples in one sequencing run, making this a high-throughput method. Both Ribosome coding genes and functional genes can be used as barcodes. Ribosome coding genes, such as 16S rDNA (prokaryote), 18S rDNA (eukaryote) and the internal transcribed spacer (ITS) region (fungi), are the most commonly used taxonomic markers (Medlin *et al.* 1988; Meyer *et al.* 2010; Lindahl *et al.* 2013; Öpik *et al.* 2014; Nilsson *et al.* 2019). The presence and diversity of genes coding for enzymes that play central roles in various biological processes, such as the *nifH* and *nirK*/S genes of the nitrogen cycle, may also be used to study functional diversity (Penton *et al.*, 2013). These functional genes can be amplified from isolated total DNA or from cDNA (see Section 1.7.3) produced from mRNA templates (Baldrian *et al.*, 2012). However, one disadvantage with metabarcoding is that only the relative abundance of a taxon can be determined, and not its absolute abundance in terms of biomass, *i.e.* the method is semi-quantitative. Primer bias and sub-optimal amplification during the PCR step can also skew the community, as some primers have higher affinity to some species, leading to overrepresentation of particular taxa, and blind spots for others (Tedersoo & Lindahl, 2016). Taxonomic binning is also dependent on availability of reference sequences in the database.

Metagenomics

Metagenomics aims to capture all the genetic potential and species diversity of a community. Using a 'whole-genome shotgun' sequencing approach, DNA extracted directly from environmental samples is randomly sequenced (Venter *et al.*, 2004) and yields large amounts of raw sequence reads. These overlapping small fragments of sequences (usually 50-150 bp) might be assembled to longer sequences, thereby reconstructing the original DNA sequence (gene contigs) using a *de novo* assembly approach. The major advantage of this method is that it does not depend on PCR, therefore avoiding bias mentioned above. Another advantage is that it also provides information about the genetic potentials of communities, in addition to taxonomic composition. However, since metagenomics (and metabarcoding) only captures the genetic potential of the environment, it does not represent the 'active' community, but rather indicates what the community is able to do, *i.e*. which genes are present (Nilsson *et al.* 2019). Yet presence of genes does not necessarily imply that they are expressed. On top of that, the process of assembling genomes into biologically meaningful information is challenging and requires a lot of computational resources.

1.7.3 RNA as a marker

Interestingly, RNA, or more specifically ribosomal RNA (rRNA), was the first molecule considered to be a taxonomic marker, since it is present in all organisms and has conserved regions that may be compared between organisms. Direct sequencing of rRNA precludes PCR bias mentioned above and makes the method potentially quantitative. However, it was deemed too time-consuming and technically demanding at the time of its conception (Woese & Fox, 1977). Other than rRNA, messenger-RNA (mRNA) or 'transcript', may be used to study the activity of organisms and their responses to environmental stimuli. The study of transcriptomes intensified with the development of reverse transcriptase-PCR (RT-PCR), a method that enables mRNA to be reversely transcribed to complementary DNA fragments (cDNA) (Bustin, 2000; Weber & Kuske, 2011).

Transcriptomics

With the recent advances in sequencing technology, enormous number of fragments can now be sequenced simultaneously and at significantly lower

cost (Liu *et al.,* 2012; Shokralla *et al.*, 2012; van Dijk *et al.*, 2014; Goodwin *et al.*, 2016). This enables comprehensive studies of all genes expressed by an organism by so-called transcriptomics or RNA-seq (Figure 4). The mRNA isolated from cell or tissue samples is first reversed to cDNA and then fragmented. Data analysis usually requires a combination of different bioinformatics software tools (a so called "pipeline") and can generally be broken down into four stages: i) quality control, ii) alignment to a reference genome, iii) quantification, and iv) estimation of differential gene expression (Van Verk *et al.*, 2013).

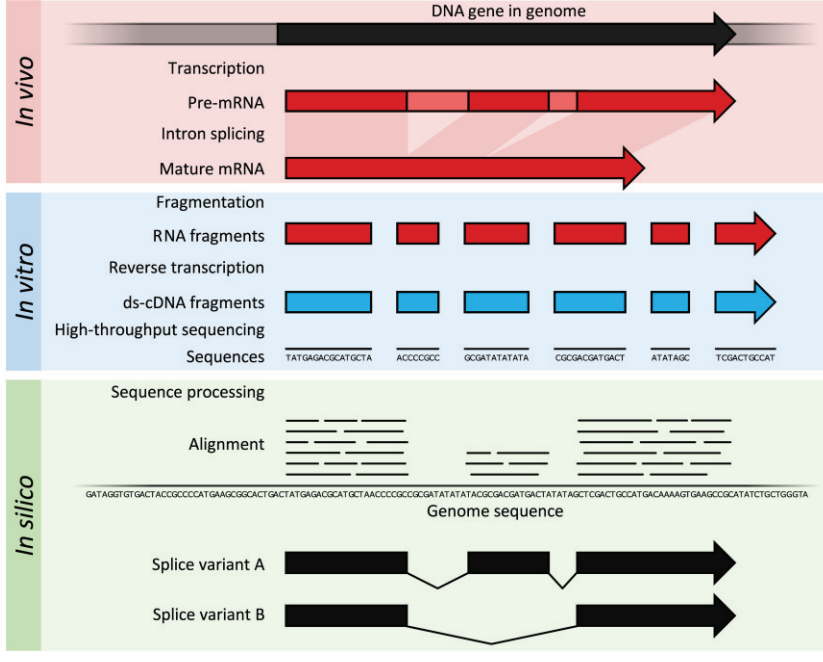

Figure 4. Workflow diagram of transcriptomic analysis from transcription in cells (*in vivo*), library preparation and sequencing (*in vitro*) and bioinformatic analyses (*in silico*) (Lowe *et al*., 2017).

1.8 Metatranscriptomics

When applied to environmental samples, the transcriptomics approach allows sequencing of all expressed genes from all organisms in a community at a given time and condition, reflecting its collective expressed traits and processes, an application known as metatranscriptomics. Prior to fragmentation and sequencing, the total RNA can be further selected to capture only

the type of RNA of interest (*e.g.* poly-A selection or rRNA removal to specifically isolate mRNA). Metatranscriptomics generates copious amounts of data, which can be assembled into the full metatranscriptome (*de novo* assembly) or sieved through a reference-based assembly (Kuske *et al.*, 2015).

Metatranscriptomic analyses can provide information about relationships between the composition of fungal communities and their roles in ecosystems, as mRNA sequences can be linked to specific processes, metabolic conversions, biogeochemical transformations or interactions, while at the same time carrying a phylogenetic signature of the fungal species responsible (Treseder & Lennon, 2015). Patterns of gene expression may provide important mechanistic clues to explain the link between response traits and effect traits. Metatranscriptomics has important advantages over other -omics approaches, such as metabarcoding and metagenomics, which suffer from the limitation that they are not capable of discerning the active component of communities.

Particularly for fungi and other eukaryotes with large and complex genomes, phenotypic traits are likely to depend more on which genes are expressed (and how much) than on genomic composition (Barbi *et al.* 2020), making metatranscriptomics more informative than metagenomics. Metatranscriptomics can provide holistic information about broad patterns in traits at community level, but is still not used regularly in fungal ecology. The limited use may be explained partly by technical challenges, but also by the failure of holistic (inductionistic whole transcriptome analyses) metatranscriptome analyses to answer specific questions. However, metatranscriptomics can also be used to address targeted questions about traits and functions by focusing on specific gene families, signals and metabolic pathways. Targeted assessment of expression of specific enzyme-encoding genes that correlate with important traits and processes may be more informative than holistic transcriptome-wide patterns to decipher roles of fungal communities in ecosystems (Treseder & Lennon, 2015). Although such targeted analyses make use only of a minor portion of metatranscriptomic data, problems with primer generality, or with specificity of PCR-based mRNA assessments of functional genes (Kellner & Vandenbol, 2010; Bödeker *et al.*, 2014), are avoided and openly accessible metatranscriptome data may be used repeatedly to target different questions. However, this method (and also transcriptomics) has some drawbacks. For example, RNA is difficult to isolate from environmental samples due to its instability and

requires handling in frozen condition. On top of that, mRNA only make up a small fraction (less than 10%) of the isolated total RNA. Therefore, large samples are required to obtain the material needed for sequencing, rendering this method low-throughput, methodologically challenging and computationally demanding.

Previously, metatranscriptomics in a fungal context has focused primarily on traits of resource acquisition. For example, genes that code for enzymes involved in depolymerisation of organic compounds (*e.g*. CAZymes) can be directly related to organic matter decomposition (Bailly *et al.*, 2007; Damon *et al.*, 2012; Žifčáková *et al.*, 2016, 2017; Hori *et al.*, 2018; Romero-Olivares *et al.*, 2019). For example, Žifčáková *et al*. (2016) used metatranscriptomics to study changes in the dynamics of soil and litter microbial communities between summer and winter in *Picea abies*-dominated coniferous forest. They found that fungi dominated the microbial transcripts, especially in litter. The transcript profiles of fungi, archaea and most bacterial phyla were significantly different between seasons. Further investigation of the transcriptome revealed that genes encoding for enzymes involved in turnover of reserve compounds, such as starch or trehalose, were highly expressed in the soil during winter, while during summer ligninolytic and cellulolytic enzymes, which indicate exploitation of external resources, were highly expressed (Žifčáková *et al.*, 2017). In another metatranscriptomic analysis, Hori *et al*. (2018) found high expression of ligninolytic enzymes from white rot and brown rot Basidiomycetes during the decay process of pine (*Pinus contorta*) logs. Hesse *et al*. (2015) used metatranscriptomics to study the effect of atmospheric nitrogen deposition on soil microbial communities in two maple (*Acer saccharum*) forests. With this approach, they were able to simultaneously study the total metabolism of bacteria, archaea and fungi and its shifts in response to disturbances. They also explored the expression of bacterial and fungal CAZymes in each metatranscriptome. They found that, although the two ambient forests were similar in community biomass, taxonomic structure and active CAZyme profiles, the shifts in active CAZyme profiles in response to nitrogen deposition differed between the sites. They also identified a need for higher-resolution time course studies with more field replicates, and improvement of databases to annotate the entire expressed mRNA collection.

By targeting more general metabolic markers, metatranscriptomics also has the potential to provide information about resource utilisation and other,

broader ecophysiological traits of communities and component taxa. Genes coding for enzymes that facilitate polymerisation of cell wall components (transferases) are potential markers for growth. Synthesis of complex carbohydrates by glycosyl transferases (GT families in the CAZyme classification) (Lombard *et al.*, 2014) is a particularly interesting target, as these enzymes are involved in the synthesis of major cell wall components and may be linked directly to fungal growth (Kelly *et al.*, 1996; Sreenivasaprasad *et al.*, 2000) The citric acid cycle, which is central to aerobic metabolism and a hub of several different catabolic and anabolic pathways, contains many different potential gene markers linked to respiration. If useful transcriptional markers for growth and respiration can be identified, the expression ratio of these markers should reflect CUE.

Metatranscriptomics has potential as a means to assess collective growth rates, respiration and CUE of fungal communities, and to analyse differences between community members. While admittedly being an indirect approach, metatranscriptomics has the advantage of estimating dynamic processes on environmental samples, maintaining realism and being unaffected by laboratory incubation, while also providing taxon-specific data (Bailly *et al.*, 2007; Kuske *et al.*, 2015). However, in contrast to genes coding for extracellular enzymes, for which the relationship between gene expression and the observed process may seem relatively straightforward (Lindahl & Kuske, 2013), relationships between gene markers and growth and respiration need to be verified under controlled conditions. In order to qualify as suitable markers in metatranscriptomic analysis, relationships between gene expression and phenotype have to be consistent across a variety of fungal species, and in various environments.

2. Description of the work in this thesis

2.1 Laboratory study (Paper I)

In a liquid-culture-based study, variations in growth rates, respiration and CUE between four fungal isolates and between contrasting growing conditions (high and low nitrogen availability) were related to gene expression data. This study focused on the expression of glycosyl transferases encoding genes and genes that codes for enzymes active in tri-carboxylic (TCA) cycle that produces CO_2.

2.1.1 Objectives

By relating gene expression to empirical measurements of growth, respiration and CUE, the aim was to identify specific gene markers that could be used to assess inter- and intraspecific phenotypic variation in fungal communities. Specific objectives of the study were to:

i) Find a genetic marker for fungal growth.
ii) Find a genetic marker for fungal respiration.
iii) Test the extent to which expression ratios of these markers may be used to indirectly predict fungal CUE using the gene markers identified.

2.1.2 Hypotheses

The hypotheses tested were that:

1) Transcription of genes coding for glycosyl transferases active in polymerisation of fungal cell wall components is correlated with relative mycelial growth rate.

2) Transcription of genes coding for enzymes in the TCA cycle is correlated with fungal respiration.
3) The expression ratio of selected glycosyl transferase genes over TCA cycle genes is correlated with CUE.
4) The above correlations are valid both between and within fungal isolates under different environmental conditions.

2.1.3 Methods

Experimental design

Four genome-sequenced fungal strains were selected to represent various taxonomic classes and ecological strategies. The fungal strains used were:

i) *Chalara longipes* (Preuss) Cooke, an ubiquitous coloniser of needle litter (Koukol, 2011)
ii) *Laccaria bicolor* (Maire) P. D. Orton, an ECM fungus (Martin *et al.*, 2008)
iii) *Serpula lacrymans* (Wulfen) J. Schröt., a brown-rot wood decomposer (Kauserud *et al.*, 2007)
iv) *Trichoderma harzianum* Rifai, an opportunistic mycoparasite (Weindling, 1932)

The fungi were precultured to produce agar-free inoculum. To establish variations in growth, respiration rate, and CUE, the isolates were cultivated under two different levels of nitrogen availability.

Glucose concentration in the medium was measured at regular intervals using a GM-100 glucose monitoring system (BioReactor Sciences, USA). Immediately before harvest, respiration was measured using an EGM-4 portable infra-red gas analyser (PP Systems, USA). Mycelium was harvested by filtration and immediately shock-frozen in liquid nitrogen, freeze-dried, weighed and stored at -80 °C. Relative growth rates (μ, day^{-1}) at the time of harvest were estimated by the following equation under the assumption that growth was exponential,

$$\mu = \frac{\ln\left(\frac{B_t}{B_0}\right)}{t}$$

where B_t is biomass at harvest, B_0 is the amount of added inoculum, and t is number of days in culture.

The metabolic quotient (qCO_2, day^{-1}) was calculated by dividing the respiration rate measured immediately before harvest with the dry mass of the harvested mycelium. The mycelium was harvested through filtration and dry weight was measured through gravimetric method. The CUE was calculated as the ratio between relative growth rate and the sum of relative growth rate and the metabolic quotient. A gene index, CUE_{gene}, was calculated from coefficients and intercepts of linear models relating the selected gene markers to μ and qCO_2.

RNA isolation and sequencing

Total RNA was extracted from the harvested mycelium using the RNA mini kit (Qiagen, Hilden, Germany) and cleaned of remaining DNA using the DNAse I kit (Sigma-Aldrich, St. Louis, USA). Poly-A selection was used to isolate mRNA from other RNAs, and library preparation was conducted using the TruSeq library preparation kit (Illumina, San Diego, USA). Libraries were sequenced on the Illumina NovaSeq 6000 SP platform. Poly-A selection of the mRNA, library preparation and sequencing were performed by the SNP&SEQ Technology Platform of SciLifeLab, Uppsala, Sweden.

Bioinformatic analyses

Raw paired-end reads were subjected to quality control using FastQC (Andrews *et al.*, 2010). Removal of sequencing adapters and low-quality bases were performed in the program 'Trimmomatic' (Bolger *et al.*, 2014), using the default settings. Reference genomes and gene annotations of *T. harzianum* (Druzhinina *et al.*, 2018), *C. longipes* (Barbi *et al.*, 2020), *L. bicolor* (Martin *et al.*, 2008), and *S. lacrymans* (Eastwood *et al.*, 2011) were downloaded from the JGI-Mycocosm database (https://mycocosm.jgi.doe.gov/mycocosm/home). Filtered mRNA sequences were aligned to respective genomes using 'bowtie2' (Langmead & Salzberg, 2012). Data were sorted, indexed and converted to gene expression count tables using 'SAMtools' (Li *et al.*, 2009; Li, 2011). Gene expression data were normalised for gene length and sequencing effort according to the reads per kilobase per million mapped reads (RPKM) method (Mortazavi *et al.*, 2008). To enable analysis of expression of enzyme-encoding genes across fungal isolates, the data were aggregated according to Enzyme Commission (EC) numbers, which denote a numerical classification of enzymes (Kanehisa, 2017).

2.2 Field study (Papers II and III)

To evaluate the generality of functional responses linked to fungal community shifts after clear-cutting, suggested by a previous chronosequence study (Kyaschenko *et al.* 2017a; Hagenbo *et al.* 2019), an extensive field study was conducted in which organic topsoil samples were collected from 18 sites distributed over three distinct types of forest land: recent clear-cuts, secondary forests established after clear-cutting and older forests that might have been subjected to forestry but have never been clear-cut (longer continuity).

The community composition of soil fungi was analysed by sequencing of internal transcribed spacer (ITS2) amplicons (metabarcoding). The decomposition capacity was assessed by enzyme activity assays. In addition, metatranscriptomics was used to investigate expression of selected extracellular enzyme encoding genes and general metabolic markers. The targets of the analysis were the GH6 family of genes that code for cellobiohydrolases (Baldrian *et al.*, 2012; Žifčáková *et al.*, 2017; Barbi *et al.*, 2020) and the AA2 family of genes that code for Class II peroxidases (Kellner & Vandenbol, 2010; Bödeker *et al.*, 2014; Bonner *et al.*, 2019). These two families of genes were selected as markers for cellulose and oxidative decomposition, respectively. Other targets were the GT48 family of genes that code for 1,3-β-glucan synthase, an enzyme that synthesises β-glucan (a dominant fungal cell wall component), and KGD genes that code for 2-oxoglutarate dehydrogenase, which is part of the TCA cycle.

2.2.1 Objectives

The effect of clear-cutting on fungal communities and fungal traits at the community level, as well as between taxonomic groups and functional guilds, was investigated using metatranscriptomics. Specific objectives of the study were to:

i) Investigate the effect of clear-cutting on fungal abundance and community composition at the levels of genera and functional guilds.
ii) Investigate the effect of clear-cutting on fungal decomposition by extracellular enzymes, using assays of enzymatic activities and gene expression analyses (metatranscriptomics).
iii) Assess differences in CUE of the fungal community at different stages of forest management based on transcriptional markers, established in the lab study.

2.2.2 Hypotheses

The hypotheses tested were that:

1) The biomass of saprotrophic fungi, as well as the activity of cellobiohydrolases and the GH6 gene expression, would be higher on recently clear-cut sites than in grown-up forests. This would be expected because disruption of ECM symbiosis will release saprotrophic fungi from competition.
2) ECM fungi would occur abundantly in both secondary and continuity forest but with different community composition. Specifically, the genera *Cortinarius* and *Russula* would be less abundant in secondary forest along with a lower Mn-peroxidase activity and AA2 expression based on previous observations by (Kyaschenko *et al.* 2017a).
3) The GT48 over KGD gene expression ratio would be highest on the clear-cut, intermediate in secondary forest, and lowest in forest with long continuity based on previous observations by Hagenbo *et al.* (2019).

2.2.3 Plot design and sampling

Eighteen managed hemi-boreal forest sites around Uppsala, Sweden, were selected by filtering geographic information system data (provided by Bergvik Skog AB). The selection criteria were as follows: 1) tree species composition of at least 80% *Pinus sylvestris* L.; 2) productivity site index (Geyer & Lynch, 1987) in the range 26-27; 3) soil moisture class ‘mesic; 5) understory vegetation dominated by *Vaccinium myrtillus* L.; 6) within 50 km north of Uppsala. Areas subjected to fertilisation and recent thinning operations were avoided. The filtering was conducted in ArcGIS Desktop version 10.7.1 (ESRI 2019). Within these criteria, six sites were chosen from each of three different age classes (Figure 5A-C): a) clear-cut less than three years old; b) forest established 30-35 years ago by planting after clear-cutting (hereafter ‘secondary forest’); and c) long-continuity forests as indicated by presence of trees older than 90 years (hereafter ‘continuity forest’).

Twenty-five soil cores (3 cm diameter) from the O-horizon (organic horizon) were sampled at 5 m intervals in a regular grid pattern within 20×20 m plots at each site (Figure 5D). Mineral soil and green plant parts were discarded, and the O-horizon material was immediately frozen on dry ice within

one minute and then pooled within sites. The sample pools were stored at -80°C until they were freeze-dried and used for metabarcoding of fungal communities, metatranscriptomics, enzyme analyses and fungal biomass estimation by ergosterol analysis. Five additional cores were sampled at random locations within each plot and pooled for measurements of soil pH, organic matter content and extractable ammonium (NH_4) and nitrate (NO_3).

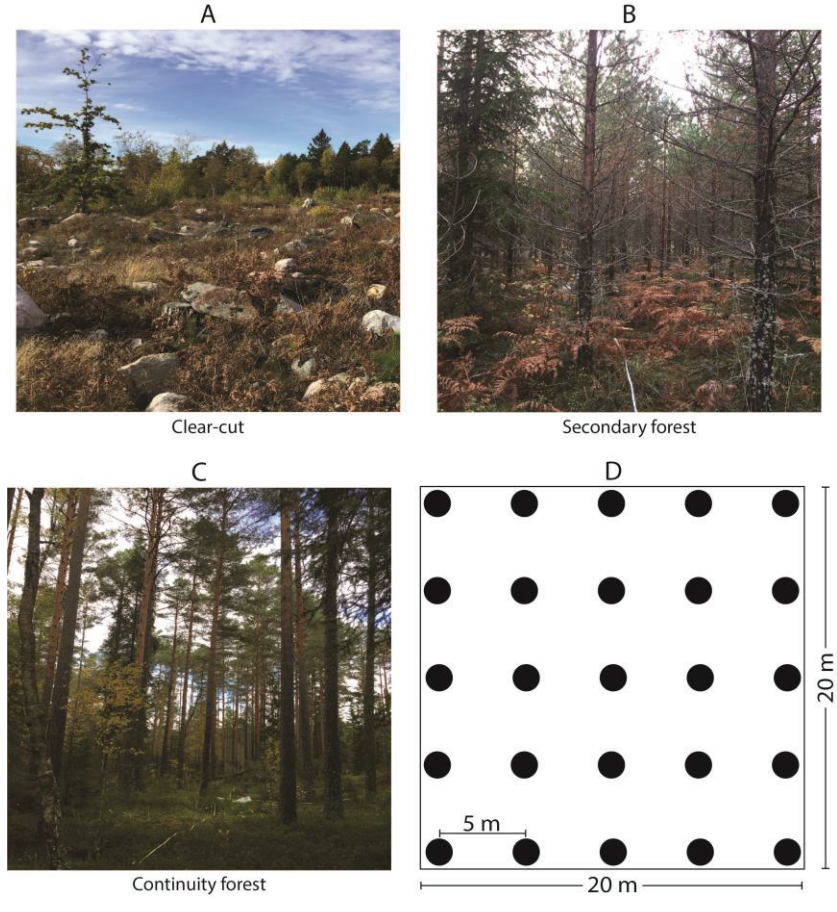

Figure 5. View of a representative (A) a clear-cut site, (B) secondary forest and (C) continuity forest, and (D) schematic diagram of the soil sampling strategy (photos by K.E. Clemmensen).

2.2.4 Analyses

Ergosterol analysis

Ergosterol was extracted as described by Nylund and Wallander (1992) and analysed by high-performance liquid chromatography (HPLC). The ergosterol peak was detected at 280 nm and fungal biomass concentration was calculated based on an assumed mycelial ergosterol content of 3 mg g^{-1} tissue (Salmanowicz & Nylund, 1988) and expressed in per gram of organic matter.

Enzyme activity analyses

Activities of selected extracellular enzymes involved in organic matter decomposition were measured in soil samples following the protocols of Saiya-Cork *et al*. (2002) and Sinsabaugh *et al.* (2005) with modifications. Activities of cellobiohydrolase, β-glucosidase, acid phosphatase and N-acetyl-β-D-glucosaminidase were measured fluorometrically using umbelliferone substrates. Measurements of Mn-dependent peroxidase activity were based on colorimetric oxidative coupling of 3-dimethylaminobenzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) in the presence of Mn^{2+} and H_2O_2 (Daniel *et al.*, 1994).

Metabarcoding

DNA was extracted according to a customised protocol (Kyaschenko *et al.*, 2017a) and further cleaned with the Macherey-Nagel NucleoSpin soil kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. The ITS2 markers were PCR-amplified using the forward primer gITS7 (Ihrmark *et al.*, 2012) and the reverse primer ITS4 (White *et al.*, 1990) mixed with ITS4a (Urbina *et al.*, 2016). Both forward and reverse primers were attached to short sample identification nucleotide tags. Pacific Biosciences (PacBio) PSII sequencing were performed by SciLifeLab (NGI, Uppsala, Sweden). The PacBio platform was chosen as it is superior in reflecting the original composition of ITS amplicons (Castaño *et al.*, 2020).

Sequences were subjected to quality control and clustering using the SCATA bioinformatic pipeline (https://scata.mykopat.slu.se) (Ihrmark *et al.*, 2012) using single-linkage clustering with the minimum similarity set at 99%. The resulting 'species hypotheses' (hereafter 'species'; Kõljalg *et al.*, 2013) were identified and assigned to ecological functions based on sequence similarity to references in the UNITE database (Abarenkov *et al.*, 2010). The following functional groups were distinguished: ECM fungi,

root-associated ascomycetes (excluding known ECM taxa, but including ericoid mycorrhizal fungi), litter-associated ascomycetes, litter-associated basidiomycetes, moulds and yeasts (Mortierellales, Eurotiales, Pucciniales, Filobasidiales, Tremellales, Trichosporonales and Chytridiomycetes). The biomass of each functional group was estimated by multiplying total fungal biomass, as assessed by ergosterol analysis, by relative abundances based on ITS2 sequencing.

Metatranscriptomics

RNA was extracted from freeze-dried soil. Considering the probable fragmentation of extracted mRNA and to avoid biased selection of 3'-end mRNA fragments, mRNA isolation was conducted by rRNA removal instead of poly-A selection. Ribosome removal was performed serially using hybridisation to biotinylated bacteria and yeast probes (Ribominus Transcriptome Isolation Kit, Thermo Fisher Scientific, USA), followed by biotinylated pan-eukaryote probes (Ribominus Eukaryote Kit v2, Thermo Fisher Scientific, USA) and removal by streptavidin magnetic beads according to the manufacturer's instructions. Libraries were prepared using the TruSeq stranded mRNA library preparation kit (Illumina, San Diego, USA) and sequenced on the Illumina NovaSeq S4 platform by the SNP&SEQ Technology platform of SciLifeLab, Uppsala, Sweden.

Based on information from sequenced fungal genomes available in the Mycocosm database, one to three Hidden Markov Models (HMMs) per assessed gene family were constructed to extract matching sequence homologues from the filtered reads (see Quantizyme bioinformatic pipeline for more details). From the resulting pools of targeted sequences, open reading frame sequences were extracted using TransDecoder (https://www.github.com/TransDecoder) and used to build gene contigs in SPAdes (Bankevich *et al.*, 2012). For each target gene family, the most highly expressed fungal contigs, which together accounted for 80% of the total mapping sequences, were subjected to BLASTx searches against the Mycocosm database. The best match for each contig was selected as reference. All references and assembled contig sequences were translated to amino acid sequences, aligned using the Clustalw algorithm and reverse-translated into cDNA sequence in MEGA X (Kumar *et al.*, 2018). Approximately-maximum-likelihood phylogenetic trees were constructed using FastTree (Price *et al.*, 2009, 2010). Taxonomic identification of assembled contigs was based on inclusion of contigs in well-supported clades (Shimodaira-Hasegawa test

> 80%; Shimodaira & Hasegawa, 1999) that also contained one or several reference sequences. Contigs were assigned to genera, if possible, or to higher taxonomic levels depending on the phylogenetic distribution of reference sequences. The functional groups of saprotrophic basidiomycetes and ECM fungi were distinguished among identified contigs.

Quantizyme bioinformatic pipeline

Extraction of mRNA sequences affiliated to specific gene families was conducted using a new in-house bioinformatic pipeline 'quantizyme' (https://github.com/domenico-simone/quantizyme_snakemake_pipeline).
The pipeline was designed to construct HMMs from a large number of reference sequences. Since certain taxonomic groups might be over-represented in the gene databases, due to ease of sequencing and longer history of study, the pipeline was designed to maximise the number sequence extracted in an unbiased manner by phylogenetically informed subsampling of reference genes. Reference sequences were downloaded from the Mycocosm database, using Enzyme Commission (EC) numbers (Kanehisa, 2017) and PFAM family number (Mistry *et al.*, 2021) as search queries. The multiple sequence alignment was converted into a maximum likelihood phylogenetic tree using the 'PhyML' program (Guindon *et al.*, 2010). The resulting tree was split into sub-trees (one to three) at basal branches and each sub-tree was further subdivided into a number of sub-groups that were equal to 30% of the total number of reference sequences in the sub-tree. To obtain a phylogenetically balanced selection of references, a single sequence was picked randomly from each sub-group and this step was repeated 10 times to ensure consistency. Using the references picked in each repetition, HMMs were constructed using the 'hmmbuild' command in HMMER (http://hmmer.org). The 10 HMMs from each sub-tree were then used to extract homolog sequences from the filtered metatranscriptome reads, using the 'nhmmer' program within HMMER. The results of these 10 replications were merged to obtain the number of reads extracted from each sub-tree. Finally, the results of the different sub-trees were compared using a Venn diagram, to ensure that the model was unbiased and to choose the optimal number of sub-trees used in the analyses.

3. Results and Discussion

3.1 Evaluation of gene markers for growth and respiration

In the laboratory study (paper I), I aimed to identify transcriptional markers for growth and respiration and chose the gene families of GT48 and KGD as the most suitable markers. GT48 codes for 1,3-β-glucan synthase, an enzyme involved in the biosynthesis of 1,3-β-D-glucan, which is a major component of fungal cell walls. GT48 was found to be the best (highest coefficient of determination) gene marker (R^2=0.64) for relative growth, and explained a significant ($p < 0.001$) part of the variation across isolates and media (Figure 6A; see Table 2 in Paper I for comparisons with other gene marker candidates). At the mycelial level, the abundance of GT48 mRNA in the transcriptome may reflect the proportion of growing hyphal tips in the mycelium as well as the rate of growth of these cells. At this scale, hyphal branching frequency might be an important determinant of relative growth of mycelial biomass. The significant negative intercept in the linear model of growth predicted by GT48 expression implies basal expression (Figure 6A; x-axis intercept at x = 6.89 RPKM), possibly linked to cell wall maintenance in non-growing cells. It is also possible that growth responds to gene transcription in a logarithmic trend, as hinted by the dashed line in Figure 6A. The linearity of the correlation between GT48 and fungal growth under more natural conditions remains to be confirmed.

KGD is a gene family that codes for 2-oxoglutarate dehydrogenase. This enzyme is part of the TCA cycle and catalyzes the decarboxylation of alpha-ketoglutarate, producing succinyl-CoA and CO_2. Thus, the enzyme is directly linked to the respiratory release of CO_2. KGD gene expression explained a significant ($p < 0.001$) part of the variation in qCO_2 (respiration per existing biomass) across fungal isolates and growth media, with R^2 = 0.48

(Figure 6B; see Table 2 in Paper I for comparisons with other gene marker candidates). Similarly to GT48, a significant negative intercept was observed in the linear model of expression, indicating baseline expression (Figure 6B; x-axis intercept at x = 6.32; see Table 2 in Paper I for comparisons with other gene marker candidates). Constitutive expression of this gene may not result in CO_2 production if the TCA cycle is constrained by other regulatory bottlenecks, such as substrate availability.

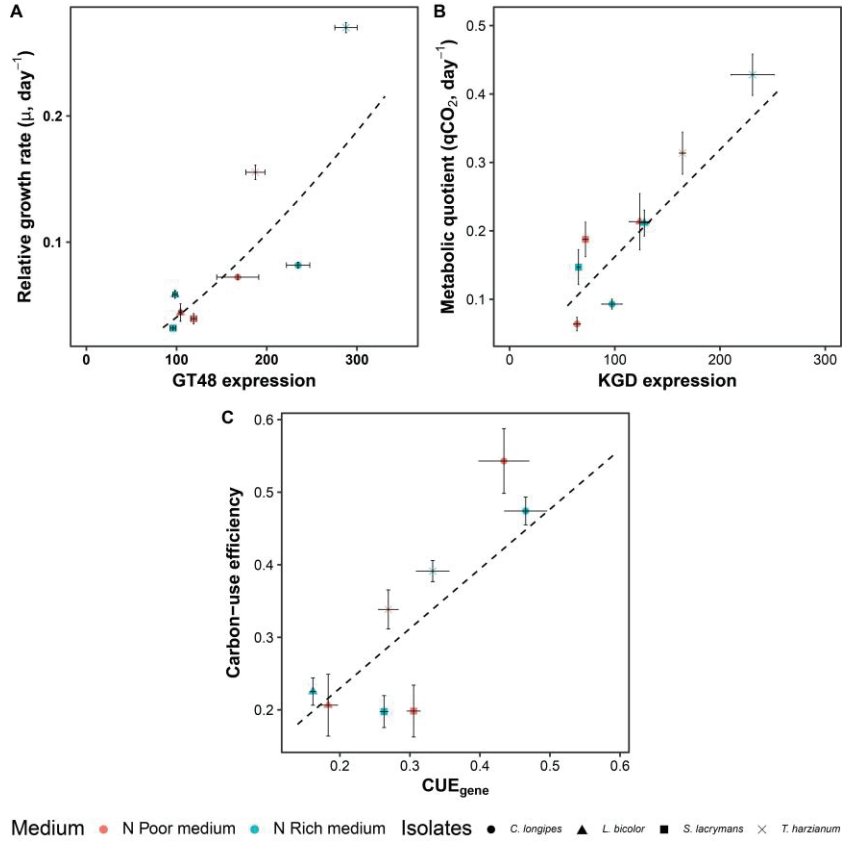

Figure 6. Relationships between (A) growth rate and 1,3-β-glucan synthase (GT48) expression, (B) qCO_2 and 2-oxoglutarate dehydrogenase (KGD) expression, and (C) measured carbon-use efficiency (CUE) and gene index (CUE gene) of four different fungal isolates grown in two different media. Symbols represent mean ± SE and gene expression levels are RPKM-normalised. Dashed lines represent models presented in Table 1 in Paper I.

CUE_{gene}, a gene expression index of CUE, was calculated based on the predictive models of relative growth and qCO_2. The CUE_{gene} was significantly correlated with measured CUE, with $R^2 = 0.42$ (Figure 6C, see Table 2 in Paper I for detail). Although this index is probably not directly transferable to other studies (due to different bases for data normalisation), the simple expression ratio of GT48 over KGD was also a useful indicator of CUE (See paper I supplementary Figure S2).

3.2 Soil chemistry

Soil pH was significantly lower ($p < 0.01$) in the O-horizons of continuity forest (3.8 ± 0.07) than in clear-cut and secondary forest (both 4.2 ± 0.1). There was no significant difference ($p = 0.11$) between ammonium concentration (Figure 7) in clear-cut (0.98 ± 0.06 µg g organic matter (OM) $^{-1}$), secondary forest (0.06 ± 0.05 µg g OM^{-1}) and continuity forest (0.005 ± 0.001 µg g OM^{-1}). There was no difference between nitrate concentration in clear-cut (1.6 x 10^{-4} ± 0. 7 x 10^{-4} µg g OM^{-1}), secondary forest (0. 3 x 10^{-4} ± 1.2 x 10^{-5} µg g OM^{-1}) and continuity forest (0.3 x 10^{-4} ± 0. 7 x 10^{-5} µg g OM^{-1}).

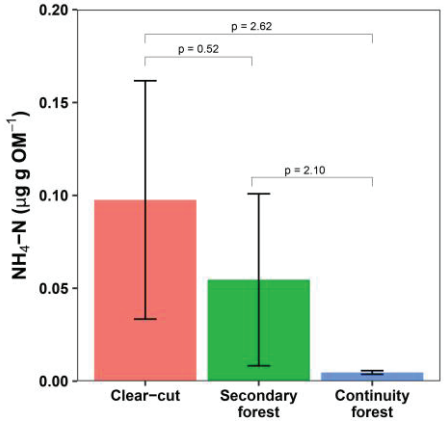

Figure 7. Ammonium (NH_4-N) in clear-cut (less than 3 years old), young secondary forest (30-35 years old) and continuity forest (with trees older than 90 years). Bars and whiskers indicate mean ± SE. Significance values based on Tukey's pairwise comparison.

3.3 Effect of forestry on fungal community composition

In the field study I investigated the effect of clear-cutting on fungal community composition at functional guilds level (paper II). One sample from a secondary forest was excluded from the fungal community data, since it strongly diverged in ITS amplicon composition, with strong (78%) dominance by sequences from *Archaeorhizomyces*, which in other samples contributed between 4% and 35% of the sequences with a median of 10%.
Fungal community composition in the clear-cut was significantly different (Figure 8A) from that in grown-up forests, and the total estimated fungal biomass was significantly lower than in the forests. Fungal communities in the clear-cut was dominated by litter-associated basidiomycetes, accounting for 47% of the ITS markers, with *Mycena*, *Gymnopus, Luellia* and *Trechispora* as particularly abundant genera. A sizeable amount of litter- and root-associated ascomycetes, accounting for 36% of the ITS markers (Figure 9), mainly in the classes Archaeorhizomycetes, Eurotiomycetes and Leotiomycetes, was also found in clear-cuts. *Plectania* and *Pseudoplectania* from the order Pezizales were prominent genera. The abundance of moulds and yeasts was comparably low but significantly higher in clear-cuts compared with the two forest types.

Decomposition of dead roots and mycorrhizal mycelium in clear-cuts may lead to the proliferation of saprotrophic fungi and opportunistic moulds and yeasts (Chatterjee *et al.*, 2008; Kohout *et al.*, 2018). The higher abundance of saprotrophs in the clear-cuts may also be related to the elevated levels of inorganic N (Figure 7). In the absence of ECM fungi, saprotrophs are released from competition and proliferate deeper into the organic layer, further increasing the rate of organic matter decomposition (Bödeker *et al.* 2016; Kyaschenko *et al.* 2017a; b). In addition, tree harvesting opens up the canopy, raises soil temperature, increases light penetration and often increases soil water content, due to disrupted root uptake and tree transpiration (Chen *et al.* 1993; Keenan & Kimmins 1993; Kovács *et al.* 2018). These changes alter understory vegetation and increase litter quality, with potential effects on microbial communities (Chatterjee *et al.*, 2008).

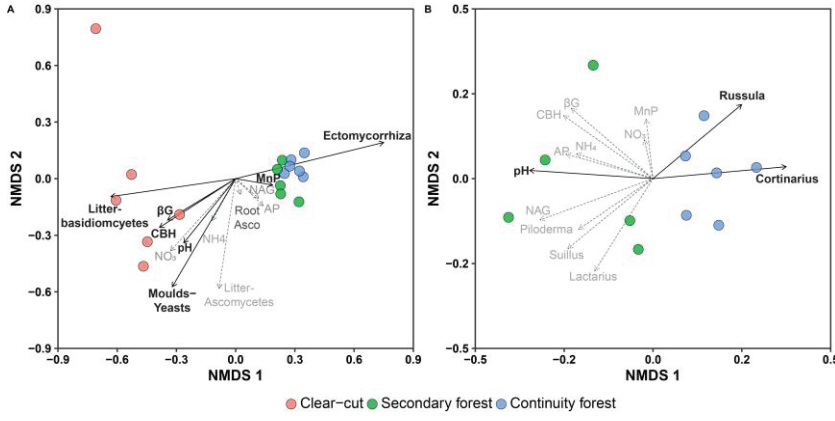

Figure 8. Variation in fungal community composition in (A) all age classes, *i.e.* clear-cut (less than 3 years old), young secondary forest (30-35 years old) and older forest with long continuity (with trees older than 90 years); and (B) in long-continuity, as visualised by non-metric multidimensional scaling (NMDS) of abundances of fungal genera based on PacBio sequencing of amplified ITS2 markers. Solid-line vectors represent parameters that were significantly different between forest types (hatched vectors represent non-significant parameters).

Even though fungal communities in both secondary forest and continuity forest were dominated by ECM fungi (on average 47% and 52% of ITS marker respectively; Figure 9), community composition was significantly different between these two forest types (Figure 8B). This was primarily attributed to differences within the ECM fungal communities, with dominance of *Piloderma* species and relatively low abundance of *Cortinarius* and *Russula* species in secondary forest. The abundance of litter-associated basidiomycetes was similar in both forest types, with *Mycena* and *Luellia* species dominating, but significantly lower than in clear-cuts. The biomass of litter- and root-associated ascomycetes was similar for secondary forests and continuity forests, and the dominant taxa remain dominated by fungi from the class Archaeorhizomycetes, Eurotiomycetes and Leotiomycetes. Moulds and yeasts accounted for a tiny proportion of the estimated fungal biomass and did not differ significantly between the two forest types.

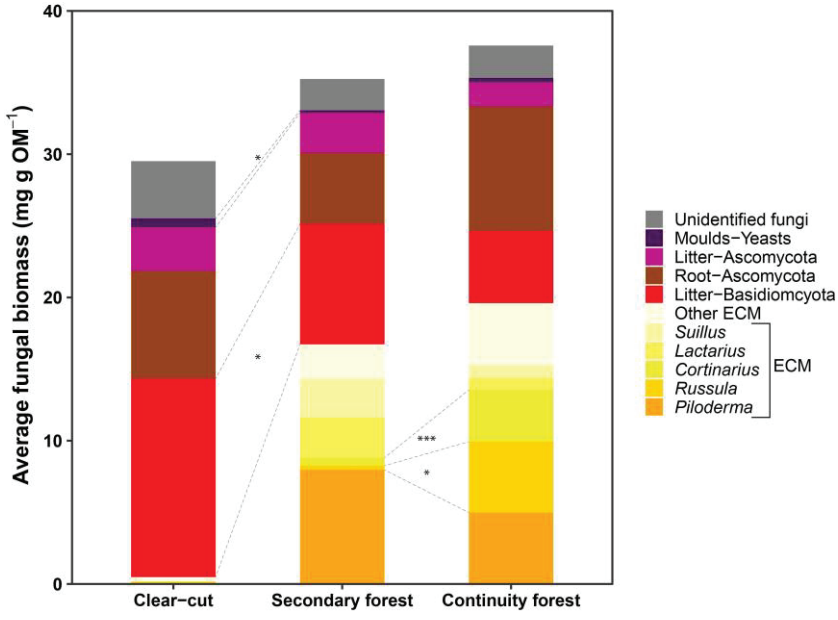

Figure 9. Estimated fungal biomass of fungal guilds and ectomycorrhizal (ECM) fungal genera in clear-cut (less than 3 years old), secondary forest (30-35 years old) and continuity forest (with trees older than 90 years). Biomass estimates were based on ergosterol concentrations multiplied by relative abundances established by sequencing of amplified ITS2 markers. Data represent averages of 5-6 sites per forest type. Asterisks indicate statistically significant difference in the biomass of guilds or genera. One asterisk indicate $p < 0.05$ and three asterisk indicate $p < 0.001$. Other genera within ECM functional guild was tested only between secondary and continuity forest by Student's T-test due to negligible biomass in the clear-cut.

This observation confirms previous findings by Kyaschenko *et al.* (2017) that the ECM fungal genera *Cortinarius* and *Russula*, which are predominant in forests with long continuity, are largely missing in re-established secondary forest, where *Piloderma* species were instead strongly dominant. It is also in agreement with previous observations that fungi of the Atheliaceae family are present in high abundance in young forest stands (Twieg *et al.* 2007; Wallander *et al.* 2010). *Piloderma* species are generally regarded as nitrophobic (van der Linde *et al.*, 2018), but less negatively affected by nitrogen fertilisation than *Cortinarius* and *Russula* species (Jörgensen *et al.*, 2021). This means that *Piloderma* species might have a competitive advantage when forest re-establishes after clear-cutting and the availability of inorganic nitrogen is higher. *Piloderma* species also have the ability to form

a spore bank (Glassman *et al.*, 2015), which may enable rapid re-colonisation after clear-cutting. In later stages of stand development, predominance of *Piloderma* species may prevent re-colonisation by other ECM fungi, due to a strong priority effect—an advantage that a particular species have during succession due to its prior arrival on a particular site (Kennedy *et al.*, 2009).

The actively growing fungal community, as assessed based on GT48 expression, largely corresponded to the ITS2 amplicon-based data. Saprotrophs dominated the GT48 transcripts in clear-cuts, with the basidiomycete genera *Mycena* and *Gymnopus* being prominent (Figure 10). *Gymnopus* accounted for a significantly larger share of GT48 transcripts in clear-cuts than in both forest types. ECM fungi accounted for a tiny fraction of GT48 expression in clear-cuts, but accounted for more than 50% of it in both forest types. *Piloderma* species accounted for a significantly higher share of GT48 transcripts in secondary forests compared with continuity forests, while *Cortinarius* and *Russula* accounted for significantly higher shares in continuity forests.

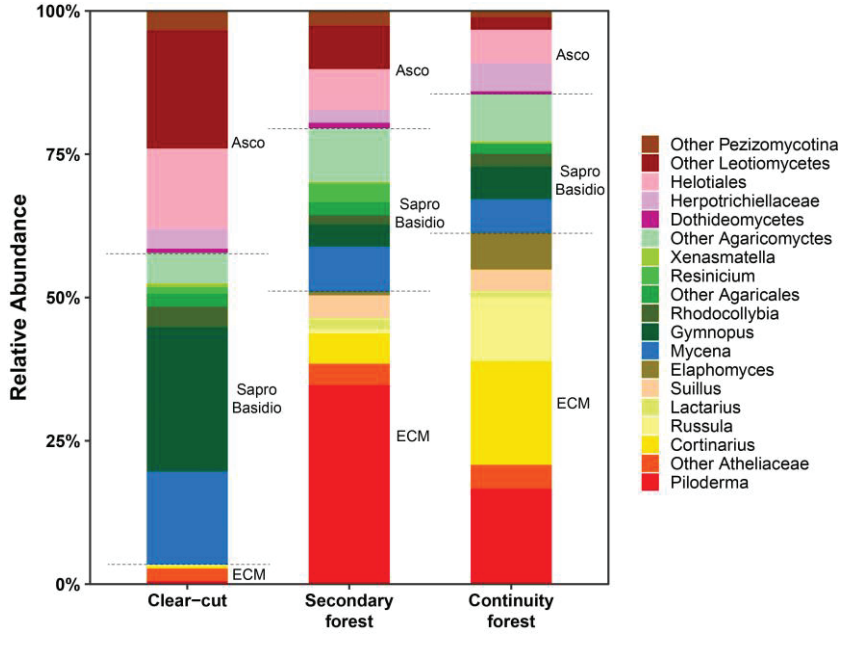

Figure 10. Relative abundance of GT48 transcripts (proxy of growth) in clear-cut (less than 3 years old), secondary forest (30-35 years old) and continuity forest (with trees older than 90 years). Data represent averages of six sites per age class. 'Asco' = ascomycetes; 'Sapro-Basidio' = saprotrophic basidiomycetes and ECM = Ectomycorrhizal fungi.

3.4 Effect of forestry on fungal decomposition traits

There were no significant differences in any enzymatic activity between the secondary and continuity forest types (Figure 11), even though cellulolytic enzyme activities in secondary forests were intermediate to those in clear-cuts and continuity forests. Cellobiohydrolase and β-glucosidase activities were significantly higher in clear-cuts than in continuity forests, with intermediate levels in secondary forests (Figure 11A, 11B). The higher activity of these cellulolytic enzymes was congruent with a higher abundance of saprotrophic fungi (litter-associated basidiomycetes, moulds and yeasts) in the clear-cuts (see Figure 9, Voříšková *et al.*, 2011), particularly the genera *Mycena* (Steffen *et al.*, 2007; Boberg *et al.*, 2011a; Kyaschenko *et al.*, 2017a) and *Gymnopus* (Větrovský *et al.*, 2013). No significant differences in N-acetyl-β-D-glucosamine, acid phosphatase and Mn-peroxidase activities were seen between the clear-cuts and the forest sites (Figure 11C-11E).

Although *Cortinarius* species have been linked to production of Mn-peroxidases (Bödeker *et al*., 2014; Kyaschenko *et al*., 2017; Perez-Izquierdo *et al*., 2021), and were largely missing in the secondary forest, Mn-peroxidases activities in secondary forests and continuity forests were similar. The lack of *Cortinarius* species in secondary forests may have been offset by higher abundance of ligninolytic saprotrophic basidiomycetes compared to long-continuity forest, in line with the slightly higher relative ITS abundance and higher cellulolytic activities. However, there was a large variation between individual forests in Mn-peroxidase activity (Figure 11C), potentially caused by high spatial heterogeneity, with localised ‘hotspots’ of activity, making representative sampling difficult (Kyaschenko *et al.* 2017a; Jörgensen *et al.* 2021).

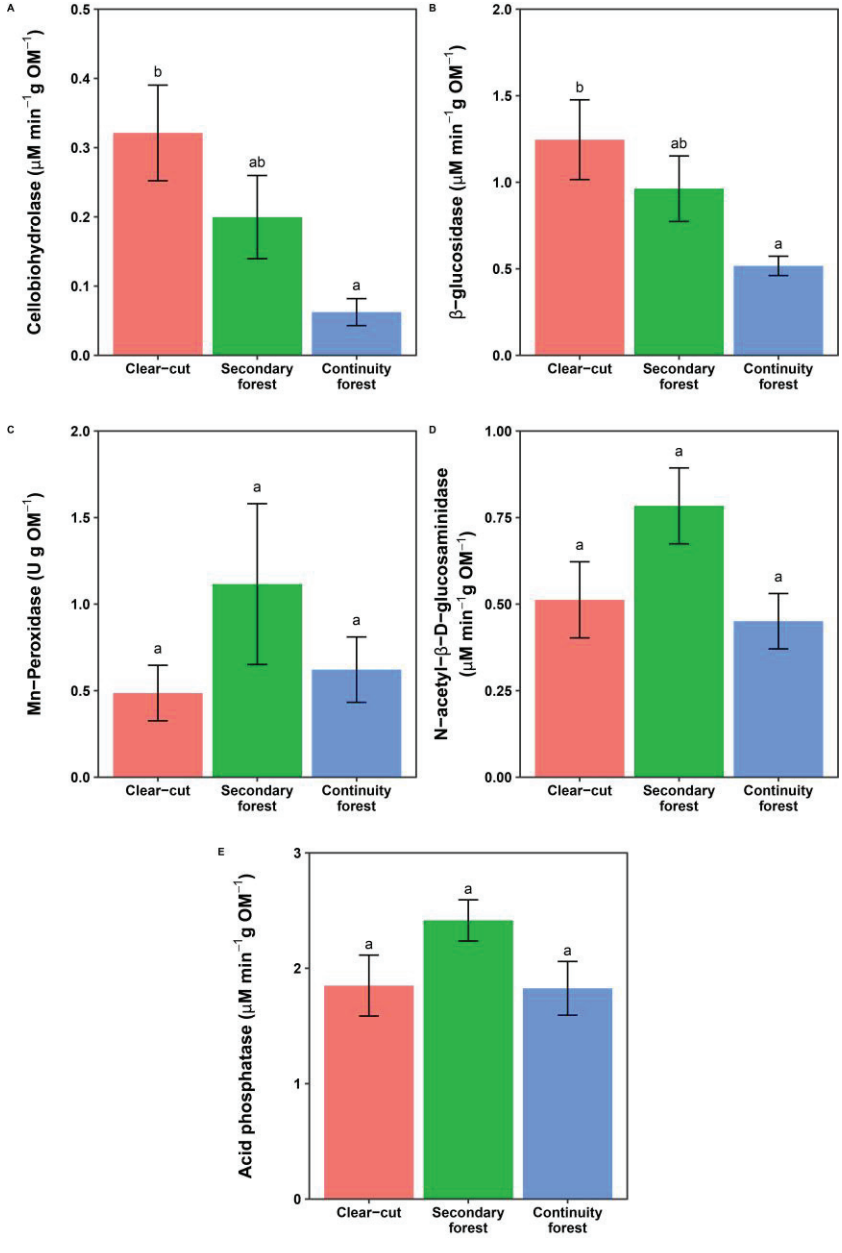

Figure 11. Enzymatic activity of (A) cellobiohydrolases, (B) β-glucosidases, (C) manganese-peroxidases, (D) N-acetyl-β-D-glucosaminidases and (E) acid phosphatases in clear-cut (less than 3 years old), young secondary forest (30-35 years old) and continuity forest (with trees older than 90 years). Bars and whiskers indicate mean ± SE. Different letters indicate statistically different values for the different forest types.

To enable comparison of decomposition traits between individual forests, expression of extracellular encoding genes was presented relative to GT48 expression (*i.e*. normalised to fungal growth). Relative expression of GH6 (cellobiohydrolase) was significantly higher in clear-cuts than in secondary and continuity forests, with no significant difference between the two forest types (Figure 12A). Overall the relative AA2 expression was significantly lower in the secondary forests than in both clear-cuts and continuity forests. (Figure 12B). Thus, both GH6 and AA2 were highly expressed in the clear-cuts (Figure 12C), indicating a fungal community that is active in cellulose and lignin decomposition. Higher decomposition in the clear-cuts, as indicated by enzyme activities and gene expression data, supports the presence of a Gadgil effect, *i.e.* increased decomposition when saprotrophs are released from competition with ECM fungi (Fernandez & Kennedy, 2016). There could be a positive feedback between decomposition, nutrient release and saprotrophic growth, which may increase nitrogen availability, but have a negative effect on short- to medium-term belowground carbon storage (Magnani *et al.*, 2007; Kyaschenko *et al.*, 2017b; Mayer *et al.*, 2021).

The community GH6-expressing fungi was taxonomically similar in the clear-cuts and both forest types (Figure 13). It was mainly comprised of saprotrophic basidiomycetes (*Mycena* and *Gymnopus*), ascomycetes (dominated by Helotiales, Rhytismatales and the genus *Plectania* (Peziales)); and chytridiomycetes (Figure 13). The observation of GH6 expression (albeit relatively low) by *Hyaloscypha* and *Oidiodendron* species, main genera of ERM fungi, supports the suggestion that certain root-associated ascomycetes, including ericoid mycorrhizal species, act as saprotrophic decomposers in the initial stages of root decomposition (Müller *et al.*, 2001; Korkama-Rajala *et al.*, 2007; Kohout *et al.*, 2018; Martino *et al.*, 2018). Chytridiomycetes were previously regarded as having little importance or an uncertain role in soils (Eric *et al.*, 1999; Freeman *et al.*, 2009), but the results in this thesis identify the family as an important component of the cellulolytic fungal community. The lifestyle of Chytridiomycetes were principally thought to be biotrophic-pathogenic (van de Vossenberg *et al.*, 2019). However, a species isolated from an aquatic environment has been described as free-living and saprotrophic (Longcore *et al.*, 2016). The observation of a higher proportion of GH6 transcript assigned to Chytridiomycetes in continuity forests supports the idea that chytridiomycetes might be litter decomposers that are tolerant to acidic and nutrient poor environments.

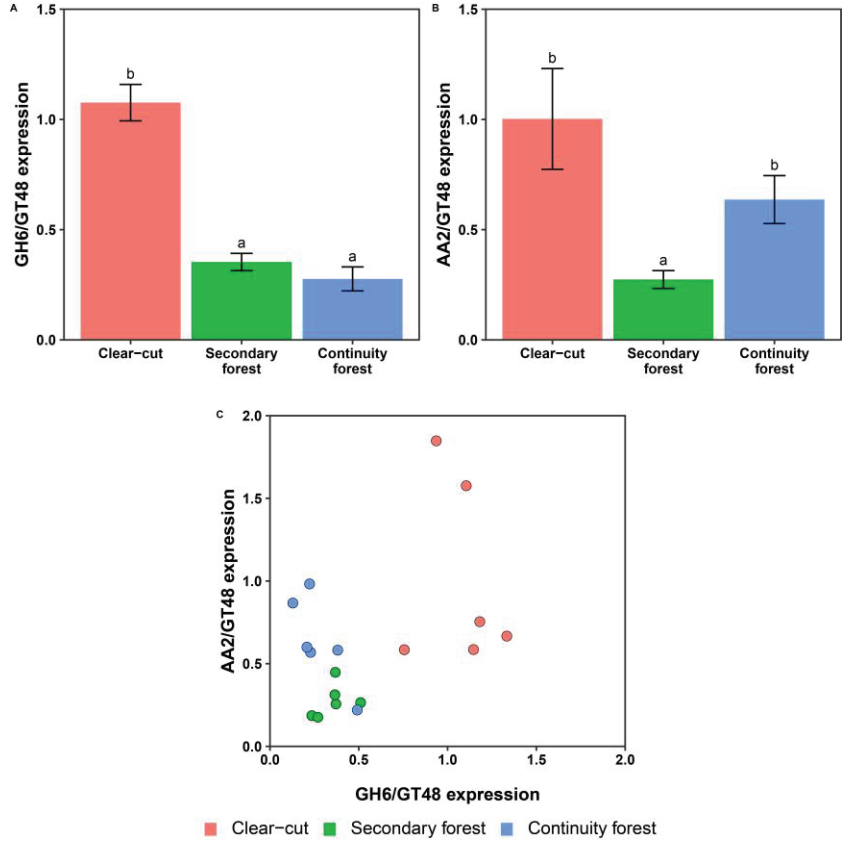

Figure 12. Expression ratios of (A) GH6 (cellulolytic enzyme encoding gene) and (B) AA2 (ligninolytic enzyme encoding gene) relative to expression of GT48 (growth-related gene). (C) Scatter plot based on expression of both enzymes in clear-cut (less than 3 years old), young secondary forest (30-35 years old) and older forest with long continuity (90 years old). Bars and whiskers indicate mean ± SE. Different letters indicate statistically significant differences between forest types.

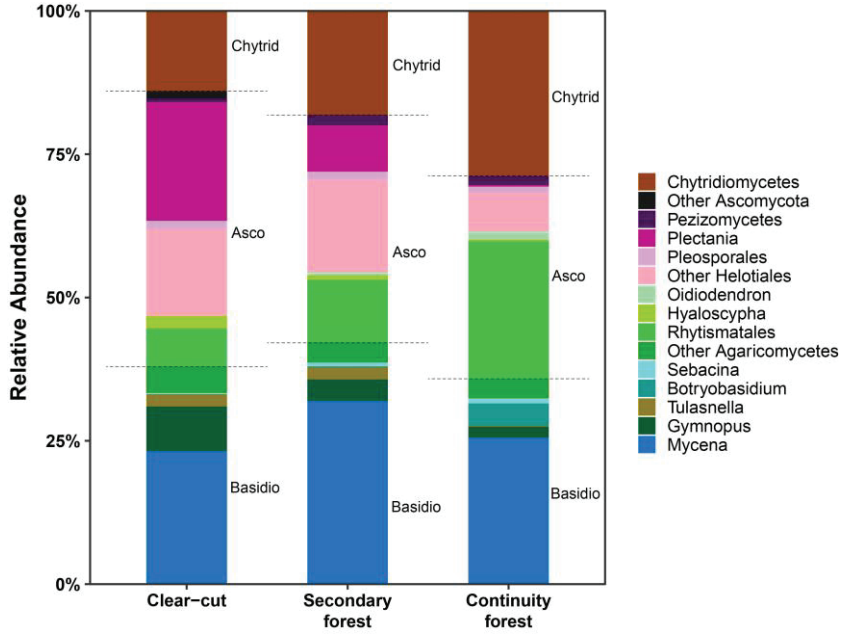

Figure 13. Relative abundance of GH6 transcripts in clear-cut (less than 3 years old), secondary forest (30-35 years old) and continuity forest (with trees older than 90 years). Data represent averages of six sites per age class. 'Chytrid' = Chytridiomycetes; 'Asco' = ascomycetes and 'Basidio' = saprotrophic basidiomycetes.

The composition of the fungal AA2 expressing community in forests with long continuity was significantly different from that in clear-cuts and secondary forests. Extracellular peroxidases are only expressed by basidiomycetes, both saprotrophic and ECM (Floudas *et al.* 2012). The AA2 expression of saprotrophs was dominated by *Mycena*, *Gymnopus* and *Rhodocollybia* species as well as members of the order Trechisporales (Figure 14). *Mycena*, *Gymnopus* and *Rhodocollybia* species have been found previously to exert ligninolytic activity through production of oxidative enzymes under both artificial and field conditions (Ghosh *et al.*, 2003; Boberg *et al.*, 2011b; Osono *et al.*, 2021). Basidiomycetes in the order Trechisporales, which contains poorly studied fungi with inconspicuous, crust-like fruit bodies (Ordynets *et al.*, 2018; Spirin *et al.*, 2021), were here identified as potential ligninolytic white-rot fungi, supporting findings in a previous comparative genomic study (Nagy *et al.*, 2016).

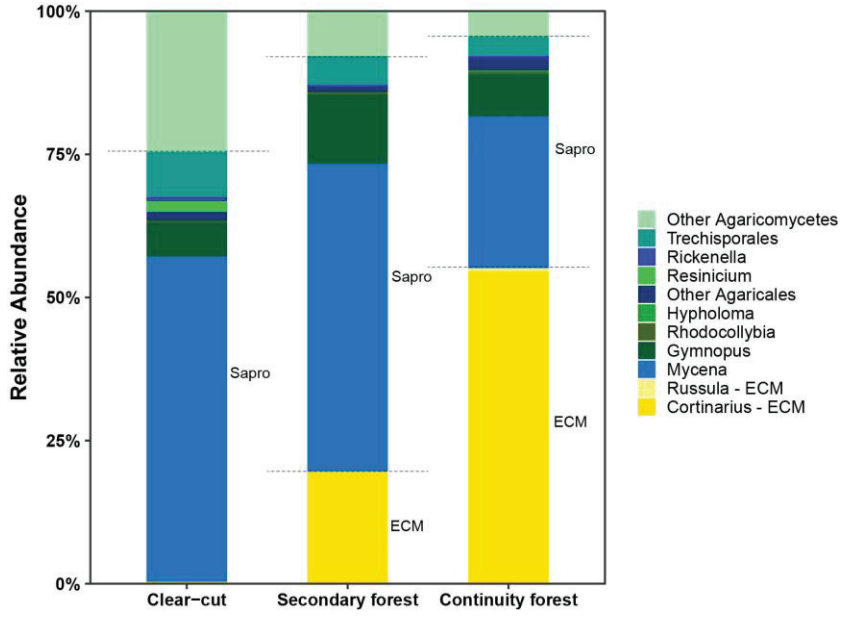

Figure 14. Relative abundance of AA2 transcripts in clear-cut (less than 3 years old), secondary forest (30-35 years old) and continuity forest (with trees older than 90 years). Data represent averages of six sites per age class. 'Sapro' = saprotrophic fungi, ECM = Ectomycorrhizal fungi.

Peroxidase expression by ECM fungi was almost exclusive to the genus *Cortinarius* (minuscule amounts of *Russula* sequences were also found in both forest types), and the *Cortinarius* share of AA2 expression was significantly higher in continuity forests compared with secondary forests. The share of class II peroxidase transcripts by *Russula* species was much lower than the share of GT48 transcripts (Figure 10), indicating lower oxidative capacity compared with *Cortinarius* species. There was no AA2 transcripts were detected for *Piloderma* species, which dominated the ECM community in secondary forest. These observations provide transcription-based evidence for the claim by Kyaschenko *et al*. (2017b) and Lindahl *et al*. (2021) that *Cortinarius* species are vital in regulating soil soil carbon and nitrogen stocks and dynamics in old boreal forests .

Peroxidases produced and utilised by ECM fungi have been proposed to increase nitrogen availability (Lindahl & Tunlid, 2015). This was supported by reports of down-regulation of peroxidase activity in response to increased nitrogen availability (Bödeker *et al.*, 2014; Argiroff *et al.*, 2019; Bonner *et*

al., 2019). Oxidative decomposition by ECM fungi might be a costly adaptation, which may trade off against growth and competitive strength under nitrogen-rich conditions (Lindahl & Tunlid, 2015; Argiroff *et al.*, 2021; Jörgensen *et al.*, 2021). In a theoretical model, Baskaran *et al.* (2016) showed that ample allocation of carbon from host to ECM fungi without significant decomposer capacity will hamper tree growth under nitrogen limitation. This is because a significant fraction of acquired nutrients will be retained in mycorrhizal fungal mycelium, rather than transferred to the host trees, and fungi will compete with the trees for a limited mineralised nitrogen pool (Näsholm *et al.*, 2013; Kyaschenko *et al.*, 2019). In the theoretical model, a similar carbon investment in mycorrhizal fungi with significant decomposer capacity (*e.g. Cortinarius* species) stimulated tree growth, as trees and fungi in symbiosis gain access to a pool of organically bound nutrients that would otherwise be inaccessible to either of the partners (Lindahl & Tunlid, 2015), bypassing saprotrophic mineralisation (Read, 1992; Lindahl *et al*., 2002).

Re-establishment of ECM fungi in secondary forest requires large amounts of carbon and nitrogen (Wallander *et al*., 2010) to sustain mycelial growth. This demand potentially leads to nitrogen immobilisation in ECM fungi mycelium and intensified soil nitrogen limitation (Näsholm *et al*., 2013). In this situation, ECM fungi that possess ligninolytic capacity, such as many *Cortinarius* species (Bödeker *et al*., 2014), may be important to maintain nitrogen availability and benefit both microbial and plant communities. In boreal forest, where the C:N ratio of litter is relatively high, nitrogen mineralisation is low (Vitousek, 1981). This phenomenon, exacerbated by N immobilisation in the mycelia of ECM fungi, might further hamper decomposition due to competition between saprotrophic and ECM fungi (Fernandez & Kennedy, 2016). The combination of low mineralisation and restricted decomposition may lead to nitrogen shortage when the benefits of the initial increase in nitrogen after clear-cutting have expired. This nitrogen limitation may lead to declining nutrient cycling and, potentially, reduced tree growth, *i.e*. retrogression (Wardle *et al.*, 2004; Clemmensen *et al.*, 2015).

In a short-term perspective, clear-cutting may affect the soil carbon pool negatively due to increased decomposition by saprotrophs via the Gadgil effect. Substantial net losses of carbon from soil organic matter have been observed in the first decade after clear-cutting, followed by increases in carbon sequestration along with stand development (Peltoniemi *et al.*, 2004;

Magnani *et al.*, 2007). In line with this observation, suppression of ECM decomposers in the medium term may contribute to a strong belowground carbon sink in secondary boreal forests, but in a longer-term perspective reduced nitrogen cycling may potentially reduce tree growth and hamper aboveground carbon sequestration.

Presence of ECM fungi that are able to do oxidative decomposition might play a pivotal role as drivers of carbon and nitrogen cycling, as boreal forest litter is rich in lignin and tannin (Sterkenburg *et al.* 2018; Adamczyk *et al.* 2019a; Pérez-Izquierdo *et al.* 2021). Failure of ECM decomposers to re-establish, when the eutrophication effects of clear-cutting declines and nitrogen limitation intensifies, may promote accumulation of larger soil carbon stocks and a strong below-ground C sink in secondary forests (Magnani *et al.*, 2007). However, with nitrogen cycling in boreal forests being relatively closed, there is a clear risk that accumulation of undecomposed organic matter in soils decreases soil fertility and leads to ecosystem retrogression (Wardle *et al.*, 2004; Janzen, 2006; Clemmensen *et al.*, 2013, 2015).

3.5 Effect of forestry on fungal carbon-use efficiency

The expression ratio of GT48 over KGD (Figure 15) was used as a proxy for CUE. The expression ratio was significantly higher in clear-cuts compared with both forest types, with no significant difference between the two forest types. This suggests that the death of roots and ECM mycelium after tree harvest may increase nitrogen availability and release saprotrophs from nitrogen limitation (Manzoni, 2017) and stimulate their growth (Sterkenburg *et al*., 2018). The proliferation of saprotrophic fungi in the clear-cut could be sustained both by decomposition of fresh litter and by more decomposed litter in deeper layers that becomes accessible after elimination of ECM fungi (Boberg *et al*., 2014). The increased decomposition eases nitrogen limitation, increases CUE and promotes growth (Boberg *et al.* 2008; Maynard *et al.* 2017; Silva-Sánchez *et al.* 2019). The higher biomass of saprotrophic fungi observed in the clear-cuts (Figure 9) might be a direct effect of higher CUE and lead to higher activity of extracellular enzymes. Higher cellulolytic and ligninolytic activity might lead to nitrogen mineralisation. Based on previous observations we expected lower CUE in old continuity forest than in younger secondary forest, linked to a larger proportion of fungi with empirically low

CUE (Hagenbo *et al*., 2019), potentially due to high metabolic costs of organic matter oxidation (Shimizu *et al.* 2005). However, the GT48/KGD ratio was similar for the two forest types.

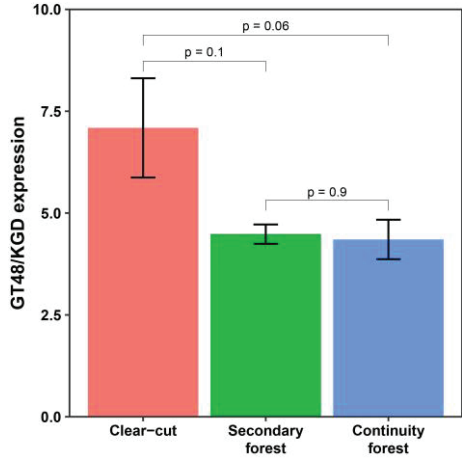

Figure 15. Expression ratio of GT48 over KGD as an estimate of carbon-use efficiency in clear-cut (less than 3 years old), young secondary forest (30-35 years old) and older forest with long continuity (90 years old). Bars and whiskers indicate mean ± SE. Significance values based on Tukey's pairwise comparison.

4. Concluding remarks and future outlook

4.1 Considerations on metatranscriptomics

Metatranscriptomics data on fungal traits need to be interpreted with caution, as gene expression does not quantitatively measure morphological features, processes and fluxes, but rather the momentarily regulatory investment (*i.e.* 'intention') of the fungi to alter its traits or facilitate a process. There are multiple steps involved in the manifestation of mRNA abundances as eco-physiological traits and processes, such as translation, post-translational modification, mRNA turnover, substrate availability and enzyme turnover. For some gene families such as GH6, the relationship between gene expression and process might be more straightforward. In contrast, class II peroxidases act indirectly on organic substrates, have a wide range of substrates and depend on H_2O_2 production and the availability of manganese in the soil. Clearly, gene expression data are best utilised to assess and compare traits, whether between different groups of organisms or at the community level between different environmental conditions, rather than to quantify processes or fluxes in absolute terms.

Use of GT48 as a taxon-specific marker of fungal growth theoretically only captures the 'active' part of the fungal community, as glucans are synthesised primarily in growing hyphal tip cell (Paper I). This can be seen as an advantage over DNA-based methods, such as ITS-metabarcoding and qPCR, which have been criticised for including taxa with low activity (Amend *et al.*, 2010; Nilsson *et al.*, 2019). Despite this, the GT48-based community was highly correlated and quantitatively similar to the ITS-based community (see Procrustes analyses in Paper III), supporting the validity of the more affordable and straightforward ITS-sequencing for community analyses where trait information is not required.

In this thesis, a targeted approach was used to analyse gene expression, as the specific objective was to investigate some potential gene markers, rather than analysing a larger set of genes or conducting a global analysis of the entire metatranscriptomes. An alternative approach would be to analyse everything and let the data 'speak' for themselves, particularly since the *a priori* marker candidates might not be the ones that are statistically best correlated with empirical values. However, such an approach would not be hypothetico-deductive, but rather inductionistic, *i.e*. involving interpretation of observations *post hoc* and posing the risk of adjusting the results to conform to presumptions and arriving at spurious conclusions. Gene markers found through such screening analyses might be un-intuitively correlated with traits or processes. A targeted approach, on the other hand, is based on hypothesis testing and arguably more efficient, as it requires less computing resources than *e.g.* attempting *de novo* assembly of the whole transcriptome or applying blind machine learning to screen for patterns. The focus in this thesis was on investigating gene families that are well understood in both functional and evolutionary terms. The decision to avoid combinatorial analyses, such as gene set enrichment (Subramanian *et al.*, 2005) or the use of broad gene ontology (GO) terms (Carbon *et al.*, 2009) allowed for more detailed analyses and provided the possibility to obtain valuable phylotaxonomic information.

The work in this thesis only targeted a small selection of fungal traits that are important in the regulation of soil functions. The following potential gene markers might be interesting to investigate in future analyses:

1. Genes that code for ergosterol (fungal cell membrane component) synthesis, such as ERG4 and ERG5 (Jordá & Puig, 2020), which might be suitable markers for standing biomass or biomass turnover, as ergosterol is rapidly degraded and needs to be replenished.
2. Genes that code for transporters, such as AMT2 (ammonium transporters) and PHO4 (phosphate transporters), which can be potentially useful markers for uptake of inorganic resources (Damon *et al*., 2012; Treseder & Lennon, 2015).
3. Glutamine synthetase encoding (Gln) genes (Teichert *et al.*, 2004), which can be potential markers for nitrogen deficiency.
4. Glutaminase encoding (gls) genes, as an indicator of nitrogen surplus or mineralisation (Durá *et al.*, 2002; Ito *et al.*, 2012).

5. Acid phosphatase encoding (ACP) genes (Hidayat *et al.*, 2006), as potential markers for exploitation of organic P resources.
6. Superoxide dismutase encoding (Sod) genes (Wang *et al.*, 2021), which might be useful as markers of reactive oxygen species (ROS) production and as indicators of oxidative stress.

By measuring and comparing expression of these gene markers in different conditions, more knowledge on growth and turnover, resources dynamics and stress response of soil fungal community in boreal forest might be gained.

4.2 Future outlook

Overall, the results presented in this thesis indicate that clear-cutting has persistent (> 30 years) effects on the community composition of soil fungi. The long-term alteration of ectomycorrhizal fungal communities is linked to clear alteration in functional traits,and is likely to have important implications for organic matter decomposition, a critical process in the biogeochemical cycle of forest ecosystems. Clear-cutting favours saprotrophic fungi that possess high cellulose and lignin decomposition capabilities, which might have a short-term negative effect on soil carbon storage and lead to increased nitrogen mineralisation after harvest. Persistent effects of tree harvesting are evident, as successional re-establishment of ECM fungal communities in secondary forest excludes genera with key functions, such as *Cortinarius*. Their absence might hamper oxidative decomposition and increase soil carbon stocks. However, in the long run, organic matter accumulation is linked to nutrient retention belowground, which could decrease soil fertility (Wardle *et al.* 2004a; Janzen 2006; Kyaschenko *et al.* 2019) and, consequently, overall forest productivity. It is uncertain whether these key genera will return as secondary forest develops further. Continuous cover forestry or harvest strategies with higher levels of tree retention might be considered to conserve fungal biodiversity and functional properties in forest soils. It is clear that more attention should be devoted to fungal diversity when trying to understand and predict the human footprint in the carbon cycle of forests.

In the future, metatranscriptomics and other omics-based analyses may evolve into to a “data-mining” approach (Figure 16). The transcriptomic profile obtained from environmental samples can serve as a database of infor-

mation that can be applied to different research questions. This could potentially lead to a change in the way ecological studies are conducted, with more *in silico* coupled with *in situ* studies to increase understanding of various ecological phenomena, such as interactions between taxa, nutrient cycling and adaptation strategies. The analysis could even be extended into a multitrophic approach, exploring the roles of several groups of organisms (*e.g.* roots, fungi, bacteria and soil fauna) at the same time. It is also likely that combining metatranscriptomics with other types of -omics (such as metagenomics and metabolomics) will strengthen the analysis and open up new perspectives in the understanding of ecological processes.

It could be very interesting to combine a metatranscriptomic approach with metapopulation theory, which posits that a population in a landscape at a given time is a 'population of populations' that disperse from and into interconnected habitats (Keymer *et al.* 2006). Such theory might be applicable also to gene expressions, *i.e.* populations of functional genes. In the context of Swedish forestry, there are likely spatially interconnected patches of forests that have never been subjected to clear-cutting, which act as a gene 'source', and productive forest, which act as a gene 'sink'. There might be dispersal of fungi between these patches, accompanied by migration of functional genes from one patch to another. The gene expression at these source and sink can be quantified *in situ* by metatranscriptomics and the dispersal of fungi might be measured by using a 'spore trap' method (Peay & Bruns 2014). By applying metapopulation theory, it might be possible to assess the likelihood that a function will become extinct in a forest patch. In addition, when key functions can be linked to particular species, the approach could possibly bolster conservation efforts regarding that particular species.

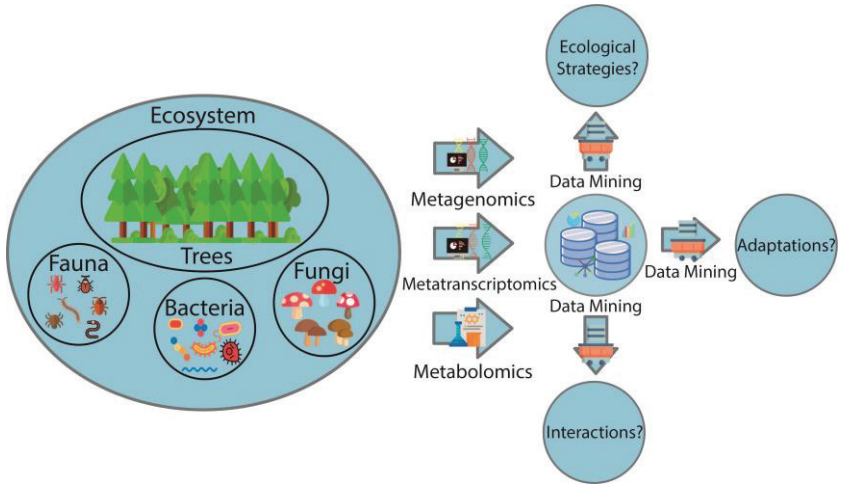

Figure 16. A multi-taxa and multi-omics approach could help extend the study of ecological processes and strategies into a 'data-mining' form of analysis.

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Populärvetenskaplig sammanfattning

Världen skulle inte vara densamma utan svampar, en grupp mångsidiga organismer som innefattar både mikroskopiskt små mögel- och jästsvampar samt stora fruktkroppsbildande svampar. De finns i alla typer av miljöer och spelar en central roll i många ekosystem, till exempel som symbiotiska partners till växter eller som nedbrytare av organiskt material i marken. Den boreala skogen, en skogstyp som karaktäriseras av en hög förekomst av barrträd, är en viktig global kolsänka och den domineras av ektomykorrhiza – en symbios mellan träd och svampar. I detta ekosystem drivs kol- och kväveomsättningen i marken till stor del av svampar.

Mänsklig aktivitet, som till exempel skogsbruk, påverkar skogsekosystem genom att fragmentera naturliga skogar och därmed påverka flora och fauna. Mikroorganismer i marken, däribland svampar, har inte undkommit denna förödande påverkan. Denna avhandling visar på att kalavverkning på kort sikt har en stor påverkan på mängden svamp, samt artsammansättningen av svampsamhället genom att gynna tillväxten av svampar som livnär sig på döda löv och rötter. Detta leder till en ökad omsättning av kol och näringsämnen i marken.

Kalavverkning minskar mängden ektomykorrhizasvampar genom att deras värdväxt dödas. Efter återplantering av skogen återvänder ektomykorrhizasvamparna, men artsammansättningen blir inte densamma som innan kalavverkningen. Cortinarius (spindelskivlingar), ett svampsläkte som har förmågan att både kunna bryta ned komplext organiskt material och bilda ektomykorrhiza, saknas i markens svampsamhälle i skogar som tidigare har kalavverkats. Förlusten av Cortinarius kan få stora oönskade konsekvenser. Till exempel kan näringsomsättningen i marken minska, vilket kan leda till lägre markbördighet och trädproduktivitet. För att göra skötseln av skogsekosystem mer hållbar, och för att motverka negativa

effekter på biodiversitet och ekosystemfunktion, bör alternativa brukningsmetoder såsom att lämna en högre andel levande träd vid avverkning eller kontinuitetsskogsbruk övervägas när man utvärderar effekterna av rotationsskogsbruk.

Popular science summary

The world as we know it would not be the same without fungi, cellular organism popularly known as moulds, yeasts and mushroom. Fungi are present in various ways and occupy a pivotal role in ecosystems, for example as symbionts of plants or as decomposers in soil. Boreal forest, a forest ecosystem that characterized by coniferous trees, is known to act as a global carbon sink and dominated by ectomycorrhizal symbiosis. In this ecosystem the carbon and nutrient cycling in boreal forest soils are largely driven by fungi.

Human activities, such as forestry, have altered forest ecosystems by fragmenting natural forests, affecting flora and fauna alike. Soil microbes, including fungi, have not escaped the devastating effects. Results obtained in this thesis showed that clear-cutting severely alters soil fungal abundance and species composition in the short term, promoting growth of fungi that feeds on dead leaves and roots and accelerating carbon and nutrient release from soil.

Clear-cutting eliminate ectomycorrhizal fungi by killing its tree hosts. After reforestation, the ectomycorrhizal fungi return, but the species that make up the fungal community are not the same. *Cortinarius*, a key fungal species with a combination of decomposer and symbiosis capabilities, is absent from forest soils that have been subjected to clear-cutting. This loss of *Cortinarius* may have some major consequences, such as impaired nutrient cycling, which will ultimately reduce soil fertility and tree productivity. To ensure sustainability and counteract the negative effect of forestry on biodiversity, ecosystems management, such as higher tree retention or continuous cover forestry, should be taken into account when evaluating rotational forestry and efforts to mitigate impacts on biodiversity and ecosystem function.

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I

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RESEARCH LETTER – Environmental Microbiology & Microbial Ecology

Transcriptomic markers of fungal growth, respiration and carbon-use efficiency

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One sentence summary: Development of genetic markers for fungal growth, respiration and carbon-use efficiency assessment.

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ABSTRACT

Fungal metabolic carbon acquisition and its subsequent partitioning between biomass production and respiration, i.e. the carbon-use efficiency (CUE), are central parameters in biogeochemical modeling. However, current available techniques for estimating these parameters are all associated with practical and theoretical shortcomings, making assessments unreliable. Gene expression analyses hold the prospect of phenotype prediction by indirect means, providing new opportunities to obtain information about metabolic priorities. We cultured four different fungal isolates (*Chalara longipes*, *Laccaria bicolor*, *Serpula lacrymans* and *Trichoderma harzianum*) in liquid media with contrasting nitrogen availability and measured growth rates and respiration to calculate CUE. By relating gene expression markers to measured carbon fluxes, we identified genes coding for 1,3-β-glucan synthase and 2-oxoglutarate dehydrogenase as suitable markers for growth and respiration, respectively, capturing both intraspecific variation as well as within-strain variation dependent on growth medium. A transcript index based on these markers correlated significantly with differences in CUE between the fungal isolates. Our study paves the way for the use of these markers to assess differences in growth, respiration and CUE in natural fungal communities, using metatranscriptomic or the RT-qPCR approach.

Keywords: growth; respiration; carbon-use efficiency; metatranscriptomics; gene markers; fungi

INTRODUCTION

Upon uptake, metabolic carbon is partitioned between biomass growth and respiration, with carbon-use efficiency (CUE) defined as the share of acquired carbon incorporated into growing biomass (Geyer *et al.* 2016; Manzoni *et al.* 2018). High CUE of microbial (in particular fungal) decomposers means lower proportional carbon losses during decomposition and higher accumulation of microbial biomass, which could enhance microbial-derived carbon storage in soils (Cotrufo *et al.* 2013; Wang *et al.* 2021). On the other hand, a high CUE can promote microbial proliferation, leading to more efficient plant litter exploitation with associated organic matter losses and lower plant-derived carbon storage (Allison, Wallenstein and Bradford 2010). Information about growth rates, respiration and CUE of microbial decomposers is, thus, essential in trait-based microbial modelling (Allison 2012; Manzoni *et al.* 2017; Zhang *et al.* 2018). However, direct empirical assessment of growth is challenging, since it depends on repeated measurements and intrusive disturbance. As a consequence, estimates of CUE are

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sensitive to the chosen methodology, and indirect short-term assessments may prove useful. For example, isotope-labeled substrates or calorespirometry have been used to assess growth and CUE in laboratory incubations (Geyer *et al.* 2019). CUE data on fungal decomposers is particularly limited, motivating our focus on fungi in this contribution.

'Omics' approaches, made possible by recent advances in sequencing technology, are becoming more common and are transforming research on fungal ecology (Nilsson *et al.* 2019). Variations in growth, respiration and CUE have a genetic basis, but are more likely related to differences in gene expression rather than gene presence and diversity (Barbi *et al.* 2020). Therefore, gene expression data may provide information about fungal metabolic traits, as mRNA sequences can be linked to specific metabolic conversions (Treseder and Lennon 2015). Genetic markers might even be used to assess differences in fungal traits in complex, natural fungal communities, using metatranscriptomic or RT-qPCR approaches (Kuske *et al.* 2015).

Genes coding for enzymes that facilitate polymerization of cell wall components such as glycosyl transferases (GT families in the CAZyme classification, Lombard *et al.* 2014) are particularly interesting targets for markers. Chitin synthase (GT2) and 1,3-β-glucan synthase (GT48) expression can be expected to be directly linked to fungal growth, since these enzymes are involved in the synthesis of chitin and β-glucan (Kelly *et al.* 1996; Sreenivasaprasad, Burton and Wood 2000), which are main components of fungal cell walls (Bowman and Free 2006). The tricarboxylic acid (TCA) cycle, which is central to aerobic metabolism and a hub of several catabolic and anabolic pathways, contains many different potential gene markers for respiration. Both NAD-dependent and NADP-dependent isocitrate dehydrogenase catalyse oxidative decarboxylation of isocitrate to 2-oxoglutarate and release CO_2 (Haselbeck and McAlister-Henn 1993; Gálvez and Gadal 1995). Consecutively, the 2-oxoglutarate dehydrogenase complex catalyses oxidative decarboxylation of 2-oxoglutarate to succinyl-coenzyme A and CO_2 (Repetto and Tzagoloff 1989). If useful transcriptional markers for growth and respiration may be identified, the expression ratio of these markers should reflect CUE. However, in contrast to genes coding for extracellular enzymes, for which the relationship between gene expression and observed process may seem relatively straightforward (Lindahl and Kuske 2013), relationships between gene expression and growth and respiration need to be verified under controlled conditions. In order to qualify as suitable, general markers, relationships between gene expression and phenotype have to be consistent across a variety of fungal species and in various environments.

Here, we devised a laboratory study where differences in growth rates, respiration and CUE among four fungal isolates and between contrasting growing conditions (high and low nitrogen availability) were related to gene expression data, with the aim to identify transcriptional markers that may be used to assess inter and intraspecific phenotypic variation. We specifically targeted a set of markers linked to growth and respiration, and evaluated these markers in relation to other potential marker genes. We hypothesized that:

1. Transcription of genes coding for glycosyl transferases that are active in polymerization of fungal cell wall components would correlate with relative mycelial growth rate.
2. Transcription of genes coding for enzymes in the TCA cycle would correlate with fungal respiration.
3. The transcription ratio of selected glycosyl transferase genes over TCA cycle genes would correlate with CUE.
4. That the above correlations would be valid both among fungal isolates as well as within isolates under different environmental conditions.

METHODS

Culture experiment

A total of four fungal isolates with sequenced genomes were selected to represent various fungal classes and life strategies: *Trichoderma harzianum* Rifai—an opportunistic mycoparasite (Weindling 1932) in the class Sordariomycetes, *Chalara longipes* (Preuss) Cooke—an ubiquitous colonizer of needle litter (Koukol 2011) in the class Leotiomycetes, *Lacaria bicolor* (Maire) P. D. Orton—an ectomycorrhizal fungus (Martin *et al.* 2008) in the order Agaricales (Agaricomycetes) and *Serpula lacrymans* (Wulfen)—a brown-rot wood decomposer (Kauserud *et al.* 2007) in the order Boletales (Agaricomycetes).

Fungal stock cultures were kept on Modified Melin-Norkrans (MMN) agar (Marx 1969) at room temperature in darkness. To produce agar free inoculum, colonized agar plugs were floated on the surface of liquid MMN medium, and extending mycelium was separated from the agar, further cultivated in liquid MMN for 1 week and macerated in the growth medium using an Ultra-Turrax (IKA, Germany). The concentration of mycelium in the inoculum was established by weighing the mycelial content of 2 mL of inoculum after drying at 40°C for 24 h ($N = 10$ for each isolate), and ranged 2–10 mg/mL.

To establish phenotypic variation in growth, respiration rate and CUE, the isolates were cultivated at two different conditions of nitrogen availability: liquid MMN medium with only glucose as the carbon source was modified to C:N ratios of 22 (N rich) or 221 (N poor) by altering the content of $(NH_4)_2HPO_4$. The experimental system consisted of 50 mL of medium autoclaved in 250 mL Schott Duran® Bottles (DWK Life Sciences, Germany). Two connectors in the lid were attached to sterile syringe filters (0.2 µm; VWR, Radnor, Pennsylvania), to allow gas exchange. Systems were inoculated by adding 0.5 mL of mycelial macerate (1-5 mg of mycelium). Each treatment included nine replicates and one negative control (without inoculation). In total, 72 systems (four isolates × two growth media × nine replicates) were set up, and due to logistic constraints, the four isolates were assessed sequentially.

Glucose concentration in the medium was measured at regular intervals using a GM-100 glucose monitoring system (BioReactor Sciences, Lawrenceville, Georgia). The systems were harvested when roughly 30% of the glucose in the medium had been consumed (Figure S1 and Table S1, Supporting Information). Immediately before harvest, respiration was measured using an EGM-4 portable infra-red gas analyser (PP Systems, Amesbury, Massachusetts) with a closed sampling loop. Prior to measurement, the systems were flushed with filtered air for 1 h to allow dissolved CO_2 to equilibrate with the atmosphere. CO_2 accumulation was measured during 2.5 min for each system.

Mycelium was harvested by filtration through Whatman filter paper; ø 55m, pore size 12 mm (ThermoFisher Scientific, Waltham, Massachusetts) and immediately shock-frozen with liquid nitrogen, freeze dried, weighted using an ES120A analytical balance (Precisa Gravimetrics, Switzerland) and stored at –80°C. Total RNA was extracted from the harvested mycelium using the RNA mini kit (Qiagen, Germany) and cleaned from remaining DNA by the DNAse I kit (Sigma-Aldrich®, St. Louis, Missouri). Poly-A selection and mRNA library preparation was conducted using the TruSeq library preparation kit (Illumina,

San Diego, California). Libraries were sequenced on the Illumina NovaSeq 6000 SP platform, yielding 50 bp paired-end sequences. Poly-A selection, library preparation and sequencing were performed by the SNP&SEQ Technology Platform of SciLifeLab, Uppsala, Sweden.

Calculations

Relative growth rates (μ, day^{-1}) at the time of harvest were estimated by the following equation under the assumption that growth was exponential,

$$\mu = \frac{\ln\left(\frac{B_t}{B_0}\right)}{t}, \quad (1)$$

where B_t is biomass at harvest, B_0 is the amount of added inoculum, and t is number of days in culture.

Measured increases in CO_2 concentration (ppm) over 2.5 min was converted to respiration rates (mmol carbon/day) using the ideal gas law with 1 atm air pressure, 20°C temperature and a 274 cm^3 sampling loop volume. The C content of harvested mycelium was calculated based on assumed mycelial carbon content of 0.43 g carbon/g dry mass and then converted to mmol carbon (Zhang and Elser 2017). The metabolic quotient (qCO_2, day^{-1}) was calculated by dividing respiration rate measured immediately before harvest with the mass of the harvested mycelium and expressed in units of day^{-1}. The CUE was calculated as the ratio between relative growth rate and the sum of relative growth rate and metabolic quotient,

$$CUE = \frac{\mu}{\mu + qCO_2}. \quad (2)$$

Bioinformatic analyses

Raw paired-end reads were subjected to quality control using FastQC (Andrews *et al.* 2010). Sequencing adapter trimming and removal of low quality bases were performed in the program 'Trimmomatic' (Bolger, Lohse and Usadel 2014) with default settings. Reference genomes and gene annotations of *T. harzianum* (Druzhinina *et al.* 2018), *C. longipes* (Barbi *et al.* 2020), *L. bicolor* (Martin *et al.* 2008) and *S. lacrymans* (Eastwood *et al.* 2011) were retrieved from the JGI—Mycocosm database. Filtered mRNA sequences were mapped against respective genomes using 'bowtie2' (Langmead and Salzberg 2012) with default settings. Data was sorted, indexed, and converted to transcript count tables using 'SAMtools' (Li *et al.* 2009; Li 2011). Transcript data were normalized for gene lengths and sequencing effort according to the RPKM method (reads per kilo base per million mapped reads; Mortazavi *et al.* 2008). To enable analysis of expression of enzyme-encoding genes across fungal isolates, data was aggregated according to Enzyme Commission (EC) numbers, which denote a numerical classification of enzymes (Kanehisa 2017). The complete sequence was uploaded to NCBI-Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA721630.

Statistical analyses

All statistical analyses were performed in R version 3.6.2 (R Core Team 2019). Effects of isolates, medium and their interaction on relative growth rate, qCO_2 and CUE, were evaluated by two-way ANOVA, with a post hoc Tukey test to evaluate differences between isolates. Due to large differences in variance between isolates, effects of growth medium were evaluated post hoc by t-tests for each isolate separately.

In order to evaluate gene markers for growth, we specifically targeted expression of 1,3-β-glucan synthase (EC 2.4.1.34) and chitin synthase (EC 2.4.1.16) encoding genes (Table S2, Supporting Information). We established a linear model with relative growth rate as response variable and expression of 1,3-β-glucan synthase or chitin synthase encoding genes (paralogs aggregated) as explaining variables, using the 'lm' function in the 'stats' package of R. Both response and explaining variables were log transformed. The same approach was applied to evaluate gene markers for respiration. We specifically targeted the expression of NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41), NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) and 2-oxoglutarate dehydrogenase (EC 1.2.4.2) encoding genes (Table S2, Supporting Information), and established linear models with qCO_2 as response variable and gene expression levels of either of the three enzymes classes as explaining variables.

A gene index (CUE_{gene}) was calculated from coefficients and intercepts of linear models relating the selected gene markers to μ and qCO_2. The linear models were defined as,

$$\log \mu = \alpha_1 + \beta_1 \log GT48 + \varepsilon \quad (3)$$

$$\log qCO_2 = \alpha_2 + \beta_2 \log KGD + \varepsilon, \quad (4)$$

where α is the intercept, β is the slope coefficient for the explaining variable and ε represent the residuals; GT48 is the expression of 1,3-β-glucan synthase, and KGD is the expression of 2-oxoglutarate dehydrogenase. After rearranging and assuming $\varepsilon = 0$, Eqs. (3) and (4) can be expressed as,

$$\mu = e^{\alpha_1} GT48^{\beta_1} \quad (5)$$

$$qCO_2 = e^{\alpha_2} KGD^{\beta_2}. \quad (6)$$

Thus, CUE_{gene} was defined as,

$$CUE_{gene} = \frac{e^{\alpha_1} GT48^{\beta_1}}{e^{\alpha_1} GT48^{\beta_1} + e^{\alpha_2} KGD^{\beta_2}}. \quad (7)$$

The gene index was evaluated as a predictor of measured CUE by linear regression. The *a priori* selected markers for growth and respiration were evaluated against other potential gene markers. All EC categories with an aggregated gene expression level of at least 10 RPKM in all samples were included, resulting in a list of 431 enzyme classes. Pearson's correlation coefficients between log-transformed expression levels of individual enzyme classes and log-transformed relative growth rate or qCO_2 were calculated with the 'cor' function of the 'stats' R package.

To verify that fungi were nitrogen limited in the nitrogen poor medium, we investigated the expression of glutamine synthetase (EC 6.3.1.2), which is a central enzyme in ammonia assimilation (Montanini *et al.* 2003), as a marker. Two-way ANOVA was conducted to assess effects of isolates, medium and their interaction on expression of selected gene markers (log transformed) as well as on the CUE_{gene} index with post hoc Tukey tests to evaluate differences between isolates. Post hoc t-tests of effects of growth medium were conducted for each isolates separately, due to large differences in variance between isolates. The complete dataset used in the analyses was uploaded to Dryad reository (doi:10.5061/dryad.pvmcvdnkm).

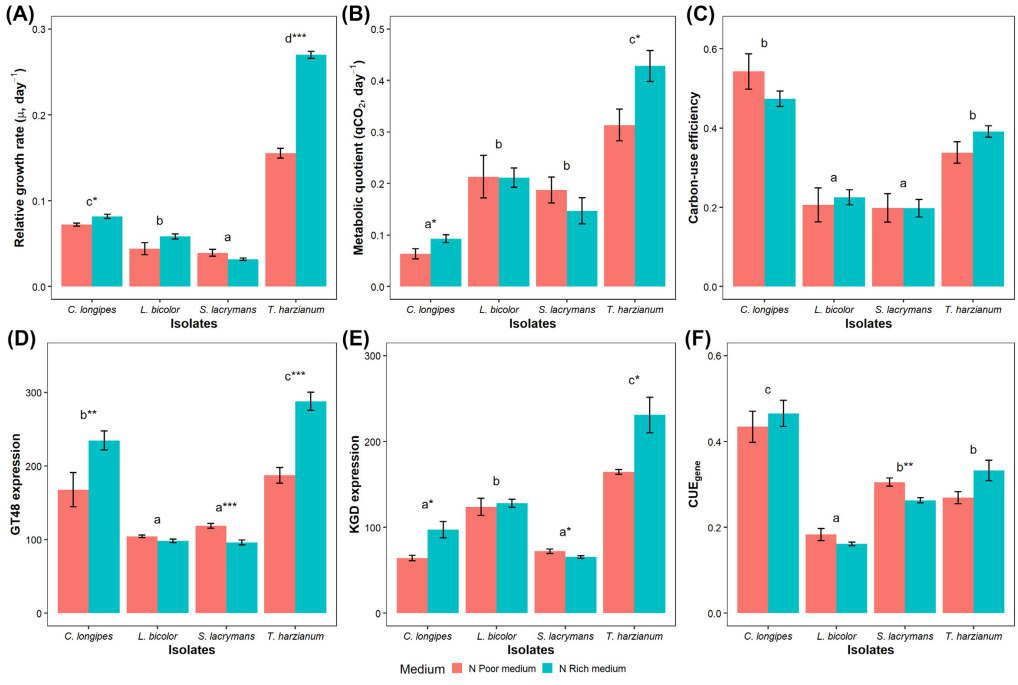

Figure 1. Effect of poor (red) vs. rich (blue) medium on: **(A)** relative growth rate, **(B)** metabolic quotient (qCO_2), **(C)** carbon-use efficiency, **(D)** 1,3-β-glucan synthase (GT48) expression, **(E)** 2-oxoglutarate dehydrogenase (KGD) expression and **(F)** gene expression index of carbon-use efficiency (CUE_{gene}) for different fungal isolates. Bars and whiskers indicate means ± SE. Different letters indicate statistically significant differences between isolates and asterisks (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$) indicate statistically significant differences between medium (within isolates). Gene expression levels were RPKM normalized.

RESULTS AND DISCUSSION

Sequencing and alignment

Transcriptome sequencing was successful for 65 of the 72 samples. One replicate of *C. longipes* was removed as an outlier due to very low expression of 1,3-β-glucan synthase and one replicate each of *L. bicolor* and *S. lacrymans* were removed due to negative relative growth rates. Thus, all statistical analyses were based on 62 observations. In total, 408 million Illumina sequences (96%) of *C. longipes*, 477 million sequences (95%) of *L. bicolor*, 487 million sequences (93%) of *S. lacrymans* and 378 million sequences (97%) of *T. harzianum* passed quality control. Of these, 316 million sequences (77%) mapped to the *C. longipes* genome, 374 million sequences (78%) mapped to the *L. bicolor* genome, 344 million sequences (71%) mapped to the *S. lacrymans* genome and 212 million sequences (56%) mapped to the *T. harzianum* genome.

Growth markers

For *C. longipes* and *L. bicolor* the rates of glucose consumption were increasing, as indicated by downward concavity in the time trajectories, supporting our assumption of exponential growth. This was also the case for *T. harzianum* in rich medium, whereas in poor medium growth was retarded towards the end of the culture period, presumably due to N limitation. Glucose consumption by *S. lacrymans* was more irregular and with a linear trend (Figure S1, Supporting Information). Biomass at harvest ranged from 1.3 to 54 mg, with *T. harzianum* having the highest relative growth rate, followed by *C. longipes*, *L. bicolor* and *S. lacrymans* (Fig. 1A). There was a significant interaction effect of isolate and medium on growth rate (Table 1) with *T. harzianum* and *C. longipes* growing significantly faster in the rich medium.

Gene expression of both 1,3-β-glucan synthase and chitin synthase explained a significant fraction of the total variation in relative growth rate. However, 1,3-β-glucan synthase expression was a much better predictor of relative growth across isolates and medium ($R^2 = 0.64$) than chitin synthase expression ($R^2 = 0.09$), and was chosen as the best marker (Table 2 and Fig. 2A). For comparison, an ANOVA in which relative growth rate was predicted by isolate, medium and their interaction had an R^2 of 0.90 (Table 1). 1,3-β-glucan synthase expression was significantly different between isolates ($P < 0.001$) with higher expression in the faster growing *T. harzianum* and *C. longipes* than in the slow-growing *L. bicolor* and *S. lacrymans* (Fig. 1D). There was a significant interaction between the effects of isolates and medium on 1,3-β-glucan synthase expression (Table 1) with higher 1,3-β-glucan synthase expression in rich medium for *T. harzianum* and *C. longipes* but higher expression in poor medium for *S. lacrymans*. When comparing correlation with growth across all enzyme-encoding genes, 1,3-β-glucan synthase ranked 14th out of 431 enzyme classes (Fig. 4A). The 13 enzyme classes that exhibited stronger correlations with growth rate than1,3-β-glucan synthase are listed in Table S3 (Supporting Information), and were mainly related to glycolysis, cell membrane synthesis and post-translational modification of proteins.

Table 1. Two-way ANOVA table of C dynamics.

	Log growth rate (mmol C/day)					Log metabolic quotient (qCO_2, day^{-1})					Log carbon-use efficiency				
	Sum Sq	Df	*F* value	Pr(>F)	R^2	SumSq	Df	*F* value	Pr(>F)	R^2	Sum Sq	Df	*F* value	Pr(>F)	R^2
Isolates	27.38	3	143.43	0***	0.90	17.39	3	45.64	0***	0.73	0.90	3	46.44	0***	0.73
Medium	0.66	1	10.44	0.0021**		0.21	1	1.61	0.21		9×10^{-5}	1	0.014	0.91	
Isolates : Medium	1.17	3	6.12	0.0012**		0.96	3	2.52	0.067		0.026	3	1.36	0.26	
Residuals	3.44	54				6.86	54				0.35	54			
	Log GT48					Log KGD					CUE_{gene}				
	Sum Sq	Df	Fvalue	Pr(>F)	R^2	Sum Sq	Df	Fvalue	Pr(>F)	R^2	Sum Sq	Df	Fvalue	Pr(>F)	R^2
Isolates	8.55	3	171.10	0***	0.92	9.83	3	89.92	0***	0.85	7.37	3	104.29	0***	0.86
Medium	0.14	1	8.38	0.0055**		0.30	1	8.22	0.0059**		0.0027	1	0.11	0.74	
Isolates : Medium	1.11	3	22.21	0***		0.55	3	5.06	0.0037**		0.29	3	4.13	0.011*	
Residuals	0.90	54				1.97	54				1.27	54			
	Log GS														
	Sum Sq	Df	Fvalue	Pr(>F)	R^2										
Isolates	8.32	3	36.19	0***	0.73										
Medium	4.47	1	58.36	0***											
Isolates : Medium	0.64	3	2.77	0.051+											
Residuals	4.14	54													

Notes: + $P < 0.10$; *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.

Table 2. Linear model output of different genes and gene index.

Dependent variable	Explaining variable	Intercept	β	R^2	Residual SE (df = 60)	F statistic (df = 1; 60)
Log relative growth rate (μ, day^{-1})	Log GT48	–9.64***	1.40***	0.64	0.45	107.46***
	Log GT2	–6.37***	0.63*	0.09	0.71	6.02*
Log metabolic quotient (qCO_2, day^{-1})	Log KGD	–6.32***	0.98***	0.48	0.47	55.69***
	Log IDH_{NAD}	0.20	–0.15	0.02	0.64	1.12
	Log IDH_{NADP}	0.22***	0.12	0.01	0.65	0.50
Carbon-use efficiency	CUE_{gene}	0.07	0.82***	0.42	0.11	43.24***

Notes: + $P < 0.10$; *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.

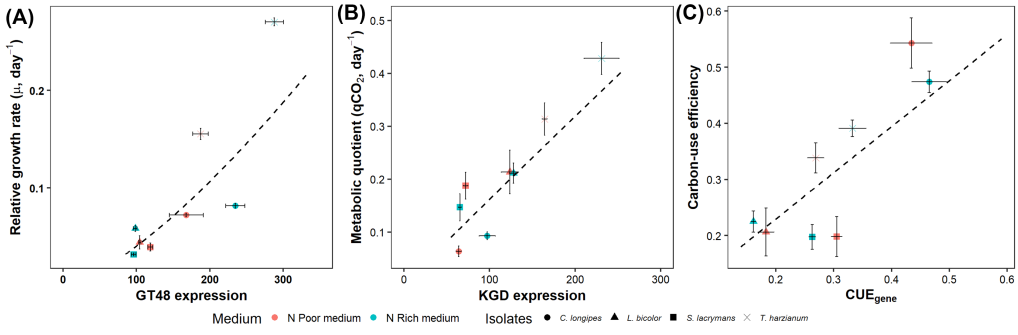

Figure 2. Relationships between **(A)** growth rate and 1,3-β-glucan synthase (GT48) expression, **(B)** qCO_2 and 2-oxoglutarate dehydrogenase (KGD) expression and **(C)** measured carbon-use efficiency and gene index (CUE_{gene}) of four different fungal isolates grown in two different media. Symbols represent means ± SE and gene expression levels were RPKM normalized. Dashed line represents linear model presented in Table 1.

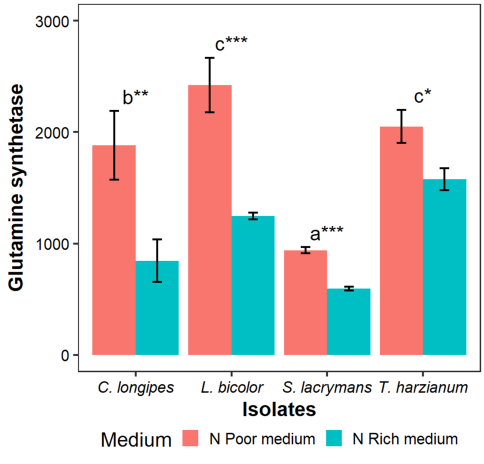

Figure 3. Effect of poor (red) vs. rich (blue) medium on glutamine synthetase expression of different fungal isolates. Bars and whiskers indicate means ± SE. Different letters indicate statistically significant differences between isolates and asterisks (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$) indicate statistically significant differences between media (within isolates). Gene expression levels were RPKM normalized.

The relative growth rates of *C. longipes* and *T. harzianum* (ascomycetes) were higher than those of *L. bicolor* and *S. lacrymans* (basidiomycetes), and in spite of the very high C:N ratio of the poor medium (Fig. 1A), growth was supressed by nitrogen limitation only for the two most rapidly growing isolates. Nitrogen limitation was, however, apparent for all isolates in the consistent upregulation of glutamine synthetase (EC: 6.3.1.2) in poor medium (Fig. 3), which is required to maintain ammonium transport across the cell membrane when external ammonium concentrations are low (Kershaw and Stewart 1989). Our results show that 1,3-β-glucan synthase expression reflects the variation of growth rate between media (across environments) and among isolates (across taxa). At the mycelial level, the relative proportion of 1,3-β-glucan synthase mRNA in the transcriptome may reflect the proportion of growing tip cells as well as the rate of growth of these cells. At this scale, morphological differences, such as hyphal branching frequency might be a more important determinant of relative growth rates than metabolic differences at the cellular level.

The significant negative intercept in the linear model of growth predicted by 1,3-β-glucan synthase expression (Table 2) implies a basal expression of 1,3-β-glucan synthase (Fig. 2A; x-axis intercept at x = 6.89), possibly linked to cell walls maintenance in non-growing cells, balanced by continuous turnover of cell wall material. In addition, the rate of cell wall assembly (growth) could be hampered by restricted allocation of sugars for growth (i.e. substrate limitation rather than enzyme limitation). However, it is also possible that growth responds to gene transcription in a logarithmic manner. Although our results represent a wide range of growth rates, linearity along the full range of variation present under natural conditions remains to be confirmed.

In this study, the expression of 1,3-β-glucan synthase reflected short-term relative investment in growth at harvest (i.e. the 'intention' of the fungi to grow). For this reason, we

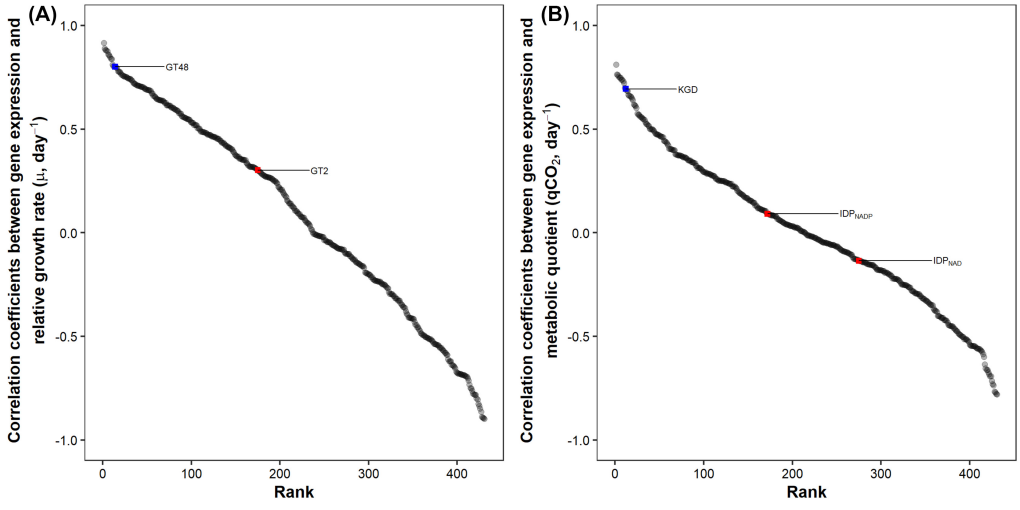

Figure 4. Ranked Pearson's correlation coefficients of all log-transformed enzyme encoding genes with log-transformed growth rate **(A)** and qCO_2 **(B)**. The blue square represents the marker selected to be used in CUE_{gene} and the red square represents marker(s) not selected.

expected that expression of this gene would correlate with the relative growth rate at the time of harvest, as approximated by Eq. (3). In future studies, isotope labeled substrate added shortly before harvest might help to elucidate the relation between carbon uptake and short-term realized growth. We also acknowledge that there growth rate might be underestimated due to uncertainties in the amount of added inoculum, since the added macerate might have contained both active cells and dead tissues. A lag phase before growth might have commenced after inoculation, contributing additional uncertainty, particularly for *S. lacrymans*, which took a long time to start growing after inoculation and exhibited low estimated growth relative to 1,3-β-glucan synthase expression. However, exponential growth models do account for a possible initial slow growth rate (they assume time invariant *specific* growth rate), thus reducing the negative consequences of a lag phase.

Apart from cell proliferation and elongation, 1,3-β-glucan synthase has been proposed as a marker for stress response, since the 1,3-β-glucan production is involved in cell fortification to protect from environmental stressors (Treseder and Lennon 2015). However, this might also be considered as part of growth, since thicker cell walls have higher mass.

Respiration markers

The qCO_2 was significantly different between isolates ($P < 0.001$), with highest values in *T. harzianum*, intermediate values in *L. bicolor* and *S. lacrymans* and lowest values in *C. longipes* (Fig. 1B), and a marginally significant isolates x medium interaction effect ($P = 0.07$), with *C. longipes* and *T. harzianum* having significantly higher qCO_2 in rich medium (Table 1). This is contrary to previous observations of a negative relationship between qCO_2 and nitrogen availability in complex microbial communities in soils (Riggs *et al.* 2015; Spohn 2015; Spohn *et al.* 2016). The discrepancy may be ascribed to the artificial conditions of the pure culture system, for which it has been speculated that fast growing isolates may suffer higher metabolic costs of growth and cell maintenance (Lipson 2015).

Among gene candidates in the TCA cycle, only the 2-oxoglutarate dehydrogenase gene expression explained significant ($P < 0.001$) variation in qCO_2 across isolated and medium composition, achieving $R^2 = 0.48$ (Fig. 2B and Table 2). For comparison, a two-way ANOVA in which qCO_2 was predicted by isolate, medium and their interaction had $R^2 = 0.73$ (Table 1). 2-oxoglutarate dehydrogenase expression was significantly different between isolates with higher expression in *T. harzianum* and *L. bicolor* and lower in *S. lacrymans* and *C. longipes* (Fig. 1E). There was a significant isolates x medium interaction effect with higher 2-oxoglutarate dehydrogenase expression for *T. harzianum* and *C. longipes* in rich medium but slightly lower expression in poor medium for *S. lacrymans* (Table 2). Similar to 1,3-β-glucan synthase, we observed a significant negative intercept in the linear model of expression (Table 2), indicating baseline expression (Fig. 2B; x-axis intercept at $x = 6.32$). Constitutive expression of this genes may not result in CO_2 production, if the TCA cycle is constrained by other regulatory bottlenecks. When comparing correlation with qCO_2 across all enzyme encoding genes, 2-oxoglutarate dehydrogenase ranked 12th out of 431 enzyme classes (Fig. 4B). The 11 enzyme classes that exhibited stronger correlations with growth rate than 2-oxoglutarate dehydrogenase are listed in Table S4 (Supporting Information) and were mainly related to glycolysis, pentose-phosphate pathway and post-translational modification of proteins.

Gene index of CUE

Measured CUE was significantly different between isolates ($P < 0.001$; Table 1), with highest efficiency for *C. longipes*, followed by *T. harzianum* and lowest efficiency in *L. bicolor* and *S. lacrymans*, without any significant effect of culturing medium (Fig. 1C and Table 1). Based on the predictive models of relative growth and

qCO_2, a gene expression index for CUE was calculated as:

$$CUE_{gene} = \frac{e^{-9.64}GT48^{1.4}}{e^{-9.64}GT48^{1.4} + e^{-6.37}KGD^{0.98}}. \quad (8)$$

After rearranging, Eq. (8) can be expressed in a more compact form as,

$$CUE_{gene} = \left(1 + 26.3\frac{KGD^{0.98}}{GT48^{1.4}}\right)^{-1}. \quad (9)$$

CUE_{gene} was significantly correlated with CUE, with $R^2 = 0.42$ (Fig. 2C and Table 2). For comparison, an ANOVA in which CUE was predicted by isolate, medium and their interaction had $R^2 = 0.80$ (Table 1). The higher fraction of explained variance in the latter statistical model is explained by its higher number of parameters (six parameters) compared to our simpler linear regression between CUE_{gene} and CUE (two parameters). CUE_{gene} was significantly different between isolates, with highest values in *C. longipes*, intermediate values in *T. harzianum* and *S. lacrymans* and lowest values in *L. bicolor*. There was a significant isolates x medium interaction effects on CUE_{gene} with a negative effect on *S. lacrymans* in rich medium (Fig. 1F).

We observed no significant effect of N availability on measured CUE. This finding is surprising given the high C:N ratio (220:1) of the nutrient poor medium—typically CUE decreases at high C:N ratio as microorganisms become nitrogen limited and excess carbon is eliminated or invested in non-growth processes (Manzoni *et al.* 2017). The lack of CUE response could be due to allocation adjustments in the face of nitrogen shortage that did not involve waste respiration (Camenzind *et al.* 2021). As carbon and nitrogen were supplied in uncoupled form in liquid media, the isolates could adjust the relative acquisition of different resources, as indicated by upregulation of glutamine synthetase gene expression. In contrast, CUE varied across isolates, and in particular, the two ascomycetes had significantly higher CUE than the two basidiomycetes. A similar pattern was also observed in our gene expression based assessment (CUE_{gene}). The CUE_{gene} of *S. lacrymans* was intermediate and similar to *T. harzianum* with higher expression of 1,3-β-glucan synthase than expected from direct measurements of growth.

Although this index is probably not directly transferable to other studies (due to different bases for data normalization), the simple expression ratio of 1,3-β-glucan synthase over 2-oxoglutarate dehydrogenase was also a useful indicator of CUE (Figure S2, Supporting Information).

CONCLUSIONS

Although we identify many potential genetic markers for growth and respiration, we see pedagogic values for the use of our *a priori* selected markers. 1,3-β-glucan synthase is directly active in polymerization of the main constituent of the fungal cell wall and its interpretation as a growth marker is intuitive. Similarly, 2-oxoglutarate dehydrogenase is directly involved in the generation of CO_2 and, thus, an attractive marker for respiration. This study presents a basis for theoretical linkages of transcripts, growth rates, respiration and CUE under idealized conditions, but we see a potential utility of these markers in future assessment of differences in growth, respiration and CUE in natural fungal communities, using metatranscriptomics or RT-qPCR approaches.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflicts of interest. None declared.

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Supplementary information

Table S1. Total culture period and average glucose consumption of all isolates in different media

Isolates	Medium	Culture period	Glucose consumption (Means ± SE)
C. *longipes*	N poor media	26 days	43.0 ± 1.8 %
	N rich media		58.2 ± 3.2 %
L. *bicolor*	N poor media	23 days	34.3 ± 2.8 %
	N rich media		35.2 ± 2.8 %
S. *lacrymans*	N poor media	25 days	16.9 ± 1.2 %
	N rich media		24.4 ±1.4 %
T. *harzianum*	N poor media	7 days	32.2 ± 1.5 %
	N rich media		80.5 ± 2.2 %

Table S2. Gene marker candidates of different isolates

Isolates	Markers	Enzyme family	E.C. number	JGI transcript ID	JGI protein ID
C. *longipes*	Growth	GT48	2.4.1.34	346190, 455219	346138, 455167
		GT2	2.4.1.16	327914, 328554, 376611, 377305, 378237, 426211, 539183, 546778	327862, 328502, 376559, 377253, 378185, 426159 539131, 546726
	Respiration	KGD	1.2.4.2	352321	352269
		IDP_{NAD}	1.1.1.41	539034, 550863	538982, 550811
		IDP_{NADP}	1.1.1.42	435426	435374
L. *bicolor*	Growth	GT48	2.4.1.34	187667, 317105	187667, 317105
		GT2	2.4.1.16	181144, 186476, 187522, 188623, 239741, 246643, 247128, 380073, 676353, 689201	181144, 186476, 187522, 188623, 239741, 246643, 247128, 380073, 676353, 689201
	Respiration	KGD	1.2.4.2	245492, 691668	245492, 691668
		IDP_{NAD}	1.1.1.41	311842, 311861	311842, 311861
		IDP_{NADP}	1.1.1.42	317084	317084
S. *lacrymans*	Growth	GT48	2.4.1.34	87516, 172886	87516, 172886
		GT2	2.4.1.16	55348, 64710, 106295, 107038, 113672, 115801, 151765, 168131, 172317	55348, 64710, 106295, 107038, 113672, 115801, 151765, 168131, 172317
	Respiration	KGD	1.2.4.2	123478	123478
		IDP_{NAD}	1.1.1.41	177461, 177525	177461, 177525
		IDP_{NADP}	1.1.1.42	51285	51285
T. *harzianum*	Growth	GT48	2.4.1.34	3225, 83485	3225, 83485
		GT2	2.4.1.16	1177, 1178, 2190, 84529, 85658, 95405, 131849	1177, 1178, 2190, 84529, 85658, 95405, 131849
	Respiration	KGD	1.2.4.2	510673	510673
		IDP_{NAD}	1.1.1.41	71098, 508954	71098, 508954
		IDP_{NADP}	1.1.1.42	86285	86285

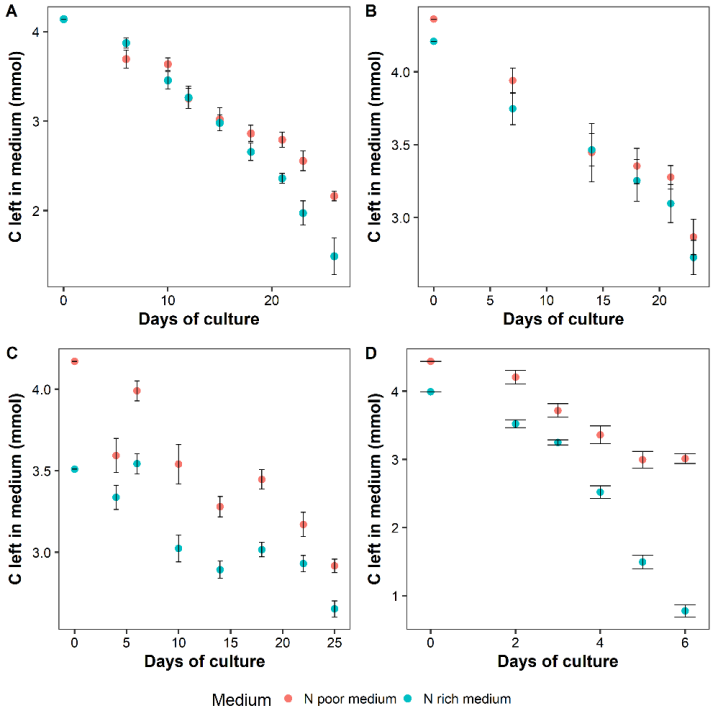

Figure S1. Glucose consumption of (A) *C. longipes*, (B) *L. bicolor*, (C) *S. lacrymans* and (D) *T. harzianum* in two different media. Symbols represent means ± SE.

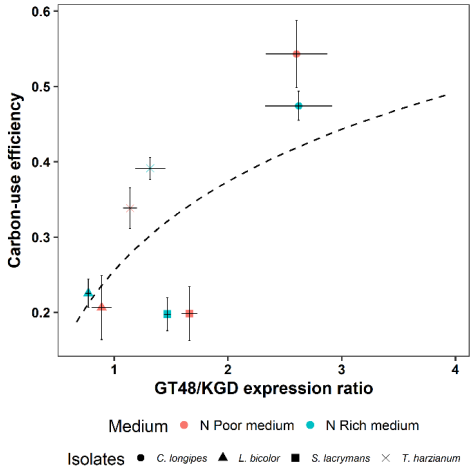

Figure S2. Relationships between measured carbon-use efficiency and expression ratio of 1,3-β-glucan synthase (GT48) over 2-oxoglutarate dehydrogenase (KGD) of four different fungal isolates grown in two different media. Symbols represent means ± SE and gene expression levels were RPKM normalized. Dashed line represents regression line on log-transformed expression ratio.

Table S3. Genes that ranked higher than 1,3-beta-glucan synthase in correlation to relative growth rate

Rank	Enzyme	Pearson's correlation coefficient (r)	E.C. Number
1	Peptide alpha-N-acetyltransferase	0.92	2.3.1.88
2	Methionyl aminopeptidase	0.89	3.4.11.18
3	Phosphorylase	0.88	2.4.1.1
4	Ubiquitin thiolesterase	0.88	3.1.2.15
5	Glutamate--tRNA ligase	0.88	6.1.1.17
6	NAD(+) kinase	0.86	2.7.1.23
7	Cytochrome-c oxidase	0.86	1.9.3.1
8	Choline kinase	0.85	2.7.1.32
9	Di-trans-poly-cis-decaprenylcistransferase	0.84	2.5.1.31
10	3-oxo-5-alpha-steroid 4-dehydrogenase	0.84	1.3.99.5
11	Phosphoglycerate kinase	0.81	2.7.2.3
12	Phosphotransferases (nitrogenous group acceptor)	0.80	2.7.3.-
13	Serine--tRNA ligase	0.80	6.1.1.11
14	1,3-beta-glucan synthase (GT48)	0.80	2.4.1.34

Table S4. Genes that ranked higher than 2-oxoglutarate dehydrogenase in correlation to qCO_2

Rank	Enzyme	Pearson's correlation coefficient (r)	E.C. Number
1	Leucine--tRNA ligase	0.81	6.1.1.4
2	Cyclopropane-fatty-acyl-phospholipid synthase	0.76	2.1.1.79
3	Fructose-bisphosphate aldolase	0.76	4.1.2.13
4	Chitin synthase	0.75	2.4.1.16
5	Saccharopine dehydrogenase	0.75	1.5.1.7
6	Transaldolase	0.75	2.2.1.2
7	Licheninase	0.74	3.2.1.73
8	Guanosine-diphosphatase	0.74	3.6.1.42
9	Argininosuccinate synthase	0.73	6.3.4.5
10	CDP-diacylglycerol--serine O-phosphatidyltransferase	0.72	2.7.8.8
11	Exoribonuclease II	0.70	3.1.13.1
12	2-oxoglutarate dehydrogenase (KGD)	0.69	1.2.4.2

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Rotation forestry has a large impact on soil fungal community. By using advanced molecular techniques, this thesis investigates how forestry changes soil fungal community composition and traits, and how it might affect short- and long- term nutrient cycling. This study offers an in-depth analysis on how the current practice needs to be evaluated for sustainability.

Fahri Hasby received his post-graduate degree at the Department of Soil and Environment, SLU, Uppsala. He holds a M.Sc. in biotechnology from School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, Indonesia.

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