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PERTURBANCE AND STIMULATION

Using Nitrogen Addition and High-Throughput Sequencing to Study Fungal Communities in Boreal Forests

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"The generative principle of the mushroom is in the slime and the fermenting juices of the damp earth, or of the roots of most of the glandiferous trees. It appears at first in the shape of a sort of viscous foam, and then assumes a more substantial but membranous form, after which, as already stated, the young mushroom appears."

Pliny the Elder, the Natural History (AD 77). Translation by John Rostock and Henry T. Riley (1855).

Abstract

Fungal communities are major players in globally important nutrient cycling processes, and form symbioses with most terrestrial plants. In the nitrogen (N) limited Swedish boreal forest, ectomycorrhizal (EcM) fungi colonize most roots of the economically important and stand dominating conifer species, Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), with significant implications for tree nutrition and decomposition processes. Long-term sustainable forestry practices require a deeper understanding of biotic and abiotic factors influencing forest health and tree growth. While high-throughput sequencing technologies such as DNA amplicon sequencing or RNA-based metatranscriptomics have furthered our understanding of fungal communities, there are still many details of EcM symbiosis and decomposition processes that we do not understand. In this thesis, I have used these sequencing methods to further our understanding of fungal communities in the boreal forest, and how they are influenced by forest management and N addition.

In the first part of this thesis, I investigated how early fertilization of seeded or planted seedlings affects seedling growth and survival, and the fungal communities associated with the growing seedlings, assessed by amplicon sequencing. In two manuscripts I show that seeds or seedlings planted into scarified clearcut soil are rapidly colonized by site indigenous fungi, including many EcM species. I show that small doses of added N increase survival of sown seeds and that organic N (in the form of arginine) can increase early root growth of planted seedlings. This light fertilization did not perturb early fungal community succession.

In the second part, I co-developed a workflow for *de novo* assembly as well as functional and taxonomic annotation of complex fungal community RNA sequencing data, in order to advance our ability to utilize metatranscriptomics (not only) as an alternative to DNA amplicon approaches. I assessed the outcome of this workflow by comparison to the currently most widely employed method of DNA amplicon sequencing, finding that both methods provide highly congruent insights into among-sample relationships and alpha and beta diversity. I then demonstrated use of the functional annotation of the metatranscriptomic data to provide biological insight into fungal community responses to high levels of N addition. It is known that N addition to boreal forests, apart from stimulating tree growth, perturbs the natural, N-limited

status and leads to significant changes in fungal community composition and soil chemistry. Using metatranscriptomic data and the newly designed workflow enabled us to test the hypothesis that N addition can inhibit decomposition in cold climates, at least in part, by rendering the oxidative enzymes used for so-called “white rot” ligninolytic decay energetically uncompetitive. Moreover, in a study using transcriptomic data from Norway spruce roots and the associated EcM fungi, we show that N addition leads to a reprogramming of the mycorrhizal symbiosis controlled by the tree, thus favoring fungal species that have previously been described as N tolerant.

In general, high-throughput sequencing methods have furthered our understanding of fungal community dynamics, and this thesis provides a powerful new part of the toolbox for studying these highly complex systems and contributes new perspectives to our knowledge of how fungal communities respond to N addition and forest management, from the perspective of soil biochemical processes and the EcM symbiosis.

Abbreviations

AM	Arbuscular mycorrhiza(l)
ASV	Amplicon sequence variant
C	Carbon
CAZymes	Carbohydrate-active enzymes
COG	Clusters of Orthologous Groups of proteins
DA	Differentially abundant
DNA	Deoxyribonucleic acid
EcM	Ectomycorrhiza(l)
eggNOG	evolutionary genealogy of genes: Non-supervised Orthologous Groups
EMBL	European Molecular Biology Laboratory
ErM	Ericoid mycorrhiza(l)
eCO_2	elevated CO_2
FACE	free-air CO_2 enrichment
GO	Gene ontology
HTS	High-throughput sequencing
IQR	Inter-quartile range
ITS	Internal Transcribed Spacer
JGI	Joint Genome Institute

KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Ortholog
mRNA	messenger RNA
N	Nitrogen
NE	Nutrient enriched
NL	Nutrient limited
ORF	Open reading frame
OTU	Operational taxonomic unit
P	Phosphorous
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PCWDE	Plant cell wall degrading enzyme
PLFA	Phospholipid fatty acid
polyA	poly-adenylated
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
rRNA	ribosomal RNA
SMRT	PacBio single-molecule real-time sequencing
SOTU	Swarm operational taxonomic unit
SSP	Small secreted protein

SWEET	Sugars Will Eventually be Exported Transporter

Papers in this thesis

Paper I: Castro, D.*, Schneider, A.N.*, Holmlund, M., Näsholm, T., Street, N.R., & Hurry, V. (2021). Effects of Early, Small-Scale Nitrogen Addition on Germination and Early Growth of Scots Pine (Pinus sylvestris) Seedlings and on the Recruitment of the Root-Associated Fungal Community. *Forests, 12(11), 1589*. (https://doi.org/10.3390/f12111589)

Paper II: Schneider, A.N.*, Castro, D.*, Holmlund, M., Näsholm, T., Hurry, V., & Street, N.R. (2022). Organic N addition improves root growth without changing fungal communities in outplanted conifer seedlings (In preparation)

Paper III: Bonner, M.T., Castro, D., Schneider, A.N., Sundström, G., Hurry, V., Street, N.R., & Näsholm, T. (2019). Why does nitrogen addition to forest soils inhibit decomposition? *Soil Biology and Biochemistry, 137, 107570*. (https://doi.org/10.1016/j.soilbio.2019.107570)

Paper IV: Schneider, A.N., Sundh, J., Sundström, G., Richau, K., Delhomme, N., Grabherr, M., Hurry, V. & Street, N.R. (2021). Comparative Fungal Community Analyses Using Metatranscriptomics and Internal Transcribed Spacer Amplicon Sequencing from Norway Spruce. *Msystems, 6(1), e00884-20*. (https://doi.org/10.1128/mSystems.00884-20)

Paper V: Law, S.R., Serrano, A.R., Daguerre, Y., Sundh, J., Schneider, A.N., Stangl, Z.R., Castro, D., Grabherr, M., Näsholm, T., Street, N.R., & Hurry, V. (2022) Metatranscriptomics captures dynamic shifts in mycorrhizal coordination in boreal forests (Accepted)

* these authors contributed equally to this work

Authorship contributions

1. Castro *et al.* [1]: I collected samples, contributed to laboratory procedures, library preparation and statistical analyses of the C/N and sequencing data, significantly contributed to writing the manuscript.
2. Schneider *et al.* [In preparation]: Sample collection, contributed to laboratory procedures, library preparation and statistical analyses of the C/N and sequencing data, writing and editing of manuscript.
3. Bonner *et al.* [2]: I contributed to field work and data interpretation.
4. Schneider *et al.* [3]: I co-designed the metatranscriptomic workflow, ran all of the analyses except random forest and pipeline diagnostics, and wrote the manuscript.
5. Law *et al.* [Accepted]: I ran analyses connected to alpha diversity and ITS data comparisons and contributed to discussion of results, and writing and revisions of manuscript.

Aims and goals of this thesis

The main aim of this thesis was to both develop and use high-throughput sequencing tools to study fungal communities in the boreal forest, in the context of forest management and N addition experiments. I studied early seedling establishment to determine if we can use organic nitrogen (N) addition to maximize early seedling survival and growth, while at the same time accounting for the fungal community succession on seedlings in early forest regeneration. Here the methodological aim was to establish a reproducible DNA amplicon sequencing workflow to study fungal communities under the influence of small-scale seedling fertilization. Furthermore, I have made use of long-term nutrient enrichment and N addition experiments to study fungal communities in the natural, N-poor state in comparison to a perturbed and N rich forest. To further our understanding of fungal dynamics in this comparison, I have developed and used metatranscriptomic methods, which allow for in-depth study of fungal (and host tree) functional activity. My hope is that the approaches demonstrated in this thesis can contribute to further our knowledge and understanding of how fungal communities respond to N addition and forest management.

Sammanfattning

Svampsamhällen har global betydelse som drivande krafter i viktiga näringskretslopp, och ingår symbioser med de flesta landväxter. I de kvävefattiga svenska boreala skogarna koloniserar ektomykorrhizasvampar majoriteten av rötter på de ekonomiskt viktiga och dominerande trädarterna gran (*Picea abies*) och tall (*Pinus sylvestris*). Detta har signifikanta implikationer för både trädnäring och nedbrytningsprocesser i marken. För att säkerställa hållbart skogsbruk på lång sikt är det nödvändigt att vi får en djupare förståelse för biotiska och abiotiska faktorer som kan påverka skogens hälsa och tillväxt. Moderna sekvenseringsmetoder som DNA amplikon-sekvensering och RNA-baserad metatranskriptomik har hittills lett till en förbättrad förståelse för svampsamhällen, men det finns fortfarande oklarheter i många av processerna de är viktiga för. I denna doktorsavhandling har jag använt dessa sekvenseringsmetoder för att fördjupa vår förståelse av svampsamhällen i boreal skog, och hur de påverkas av skogsbruk och kvävetillförsel.

I avhandlingens första del har jag undersökt hur gödsling vid plantering och sådd påverkar plantornas överlevnad och tillväxt, och dessutom de associerade svampsamhällen som undersöktes med hjälp av amplikon sekvensering. I två manuskript visar jag, hur efter plantering på markberedda kalhyggen, plantorna fort blir koloniserade av olika svampar från marken, bland annat mykorrhizasvampar. Jag visar att små doser av kvävegödsel höjer överlevnad av sådda frön, och att organiskt kväve (i form av arginin) kan förbättra tidig rottillväxt vid plantering. Svampsamhällen blev inte påtagligt förändrade av denna lätta gödsling.

I andra delen av denna avhandling har jag varit involverad i utvecklingen av en bioinformatisk pipeline för *de novo*-assemblering, såsom taxonomisk och funktionell annotering av komplex RNA sekvenseringsdata, för att göra framsteg i vår förmåga att använda metatranskriptomik (inte bara, men också) som ett alternativ till DNA amplikon sekvenseringsmetoder. Jag utvärderade resultatet av denna pipeline i en jämförelse med den vanligaste amplikon sekvenseringsmetoden, och kom fram till att båda metoder ger jämförbara insikter om förhållanden mellan prover och jämförbara resultat från diversitetsberäkningar. Jag visar sedan hur den funktionella annoteringen från metatranskriptomik data kan användas för att ge biologiska insikter om svampsamhällens reaktion till höga kvävegivor. Vi

vet att gödsling av boreal skog, bortsett från stimulation av trädens tillväxt, leder till en perturbation av det naturliga, kvävelimiterade tillståndet och orsakar betydande förändringar i svampsamhällen och markens kemiska sammansättning. Genom att använda metatranskriptomisk data i samband med den tidigare nämnda metoden testade vi hypotesen att kvävegödsling leder till en hämmad nedbrytning av organiskt material i marken, i alla fall delvis, genom att göra så kallad ”vitröta” mindre konkurrenskraftig. Dessutom, i en annan studie med RNA sekvenseringsdata från granrötter och dess associerade svampsamhällen, visar vi att kvävegödsling leder till att träden inleder en omprogrammering av mykorrhizasymbiosen, vilket gynnar svamparter som tidigare har beskrivits som kvävetåliga.

Allmänt sett har moderna sekvenseringsmetoder gett oss en bättre förståelse av dynamikerna i svampsamhällen, och denna avhandling tillhandahåller en kraftfull ny del i verktygslådan för att studera dessa högst komplexa system och bidrar med nya perspektiv till vår vetskap om hur svampsamhällen reagerar på kvävetillförsel och skogsbruk, från perspektivet av biokemiska processer i marken och ektomykorrhiza symbiosen.

1 Swedish Forest and Forestry

Around 70% of Sweden is covered in forest, and around 75% of these forests are used for production. Of this productive forest, around 50% is owned by private owners, with the other half being split between forestry companies (~30%) and state and public owners [4]. The forestry industry is an important part of the Swedish economy, with roughly 2.5% of the working population employed in the forestry or directly related sectors, and Sweden is the fifth largest export nation of pulp, paper and timber in the world. Around 80% of Swedish forestry products are exported, with a total export value of around 145 billion Swedish crowns [5]. Apart from these purely economic considerations, the forest has an immense recreational value, with millions of Swedes choosing to spend their free time in some sort of forest related activities. Moreover, forests in Sweden are home to a fascinating array of biodiversity and habitats, above and belowground.

One of the principles of forestry in Sweden is the aim of achieving a balance between environmental and economic goals [6], and long-term sustainable forestry has to take into account both economic sustainability and sustainability from the perspective of the forest as an ecosystem of not only trees, but all kingdoms of life. In this thesis I have studied fungal communities in the Swedish forest, in the context of early forest regeneration, and forest fertilization through the addition of nitrogen (N).

1.1 The Boreal Forest

A large part of forests in Sweden are in the conifer dominated boreal forest zone, with only the area south of (roughly) Stockholm falling into the transition zone to the nemoral forests of central Europe, which are more characterized by broadleaf trees such as oaks (*Quercus* spp.) and beech (*Fagus sylvatica*). The boreal forest covers about 11% of the global terrestrial land surface [7], stretching as a belt between the temperate broadleaf forests to the south and the barren tundra to the north, and is estimated to contain about one third of global terrestrial carbon (C) stocks [8]. It is characterized by long cold winters and a short growing season.

In the Swedish boreal forest, Norway spruce (*Picea abies* (L.) H. Karst.) and Scots pine (*Pinus sylvestris* L.) are the ecologically and economically

most important forest tree species and comprise the majority of standing wood. Norway spruce is often found on more nutrient rich sites with higher water availability, and is widely spread throughout the whole European boreal forest, as well as montane areas in central and eastern Europe [9]. Scots pine is considered a pioneer species, and is often found dominating on relatively dry and nutrient poor sites, growing even in the poorest and most acid soils [10], with very high morphological variability. It is very common in the boreal forest but tolerates a variety of climatic conditions and can also be found in many areas of central Asia and southern Europe. Other common tree species in the Fennoscandian boreal forest are European aspen (*Populus tremula*), silver birch (*Betula pendula*), white birch (*Betula pubescens*), grey alder (*Alnus incana*), different willow species (*Salix* spp.) and rowan (*Sorbus aucuparia*). The understory can be, depending on site characteristics, dominated by ericaceous shrubs (e.g. *Vaccinium* spp., *Rhododendron tomentosum*, *Calluna vulgaris*), juniper (*Juniperus communis*) and a variety of herbaceous plants, mosses and lichens.

Soils in the boreal forest are generally acidic and nitrogen (N) poor, with tree growth in Sweden being limited by this low N availability, and in southern Sweden additionally limited by water availability [11, 12]. Despite this general low N availability, there are local differences in N availability that can be recognized by the vegetation. N poor sites are dominated by dwarf shrubs, while forests with higher N availability are rich in herbaceous plants [12]. These shifts in understory vegetation are correlated with measures such as N content, soil pH, exchangeable Ca^{2+}, and tree growth [13]. Often these big variations can be found on small spatial scales, for example close to groundwater discharge areas, which are characterized by relatively high N contents and differences in plant community composition [14]. Another important characteristic of boreal forest soils is the vertical stratification, with a clearly separated organic layer (also called mor-layer) and mineral (inorganic) layer below. The organic layer can be further divided into litter, fermentation, and humus layers [15] that represent organic matter in different stages of decomposition, each colonized by distinct communities of microorganisms [16]. The soils, and especially the mor-layer, are inhabited by highly diverse communities of these microorganisms, and fungi take an especially important role, in particular ectomycorrhizal (EcM) fungi that form mutualistic interactions with trees to supply N in exchange for C [17]. These EcM fungi are a focus of this thesis and will be discussed in greater detail below.

1.2 Clearcutting and genetic selection

Sweden has a long history of land use and human influence impacting forests, with evidence of this happening for at least the past 6,000 years. Starting roughly from the Middle Ages, larger areas of forests were turned into pastures, agricultural areas through slash-and-burn, or logged for warfare and iron production, which reached its peak in the eighteenth century. During this period timber forestry was, in most cases, performed as semi-continuous cover forestry, where the largest stems within an area were selectively harvested, leaving only smaller, weaker trees [6]. This changed around the time of the second world war, when large-scale rotation-based forestry was introduced. This type of forestry, where patches of forest are cleared almost completely and replanted, has been practiced in the large majority of Swedish forests ever since. According to a study published in 2012, annually 50,000-70,000 sites are clear-cut in Sweden with a total area of 150,000-300,000 ha [18], which corresponds to about 1% of the total forested area. Until the introduction of rotation-based forestry, forestry practice mostly relied on natural regeneration from seed present at a clear-cut site for reestablishment.

Another important factor in modern Swedish forestry are the breeding programs, which have the goal of increasing forest productivity by selecting for trees with preferred traits. This could, for example, entail faster growth, different wood properties for different purposes, or an increased resistance to diseases or environmental stresses such as drought [19]. The breeding programs were initially based on the selection of so called “plus trees”, which were the largest, healthiest looking trees identified in forest stands. Seeds from these plus trees are cultivated in seed orchards and subsequently used to produce seeds for planting, or, more commonly, for production of seedlings in nurseries. Planted Norway spruce has been genetically improved in this way since the 1940s [20], and Sweden is among the countries with the biggest breeding program for this species. For Scots pine, the first provenance trials were already established at the end of the nineteenth century, followed by numerous breeding programs in several countries in the twentieth century [21]. In Sweden, around 75% of productive Scots pine stands are now regenerated by planting using nursery seedlings grown from seed orchard seeds. For both Norway spruce and Scots pine, the genetic gain for volume growth was estimated to be around 10% after the first generation of breeding, and could reach up to a maximum of 25% from the third generation of seed orchards [19]. However, there seems to be a tradeoff between growth and wood quality traits [22], and

considering climate change and uncertain changes in future economy, there are a number of different traits that need to be balanced against each other for a long-term sustainable silvicultural practice [23].

1.2.1 Site preparation

Seedlings planted in a freshly harvested, but unscarified site often do not survive or grow well. Experiments in the early twentieth century by the Swedish botanist and forester Henrik Hesselman found that "poor" boreal forest sites had all the nutrients needed for plant growth stored in the soils, but that the N in the humus layer seemed to be inaccessible to plants for growth until it was mixed with the mineral soil below. A range of experiments were conducted, finally showing that plowing the soil, and mixing in some additional peat for fertilization, improved growth of naturally regenerating seedlings [24]. These seedlings were observed to otherwise often stagnate and slowly decline when establishing in unprepared sites. The observed improvement was hypothesized to largely result from the scarification process, which would kill many of the plants and fungi remaining in the ground after tree removal, and these would then decompose and supply the growing seedlings with easily accessible nutrients. An additional factor inhibiting seedling development in unprepared soils was believed to be both aboveground (for light) and belowground (for nutrients) competition with nearby trees and other vegetation. These factors have been confirmed to inhibit seedling regeneration in more recent studies [25, 26].

Consequently, soil in fresh clear-cuts is nowadays almost always prepared by scarification, which aims to invert the organic soil layer in order to expose the mineral soil (Fig 1.1C). Many people complain that this is not particularly pleasing to look at, but several newer studies have also shown that Norway spruce and Scots pine seedlings planted on scarified sites survive better, grow better, and even get a slightly higher degree of root colonization by EcM fungi, when compared to unprepared sites [27–30], concordant with the early experiments discussed above. Natural regeneration, that is the growing of trees from seeds from the surrounding areas, is also not affected negatively by soil scarification [31]. Another positive effect of site preparation is that the exposed mineral soil acts as a deterrent for the feared pine weevil (*Hylobius abietis*), which avoids open mineral soil and only attacks seedlings growing in organic soil. Pine weevil attacks are a major cause of seedling death, especially in the south of Sweden [29].

Regardless of these clear advantages for forest regeneration, the other side of the coin is that clear-cutting and subsequent soil scarification disrupts the organic layer of the soil and the residing fungal communities, leading to a shift from mostly EcM fungi to a dominance of saprotrophic (that decompose organic material and release N) and opportunistic fungi [32, 33]. This is primarily due to the cutting of the trees, which eliminates the C source for EcM fungi. The scarification further disrupts the mycelial networks and speeds up this community shift. Furthermore, water retention capabilities of the soil are reduced, and the higher nutrient accessibility can also lead to increased nutrient leaching [34, 35].

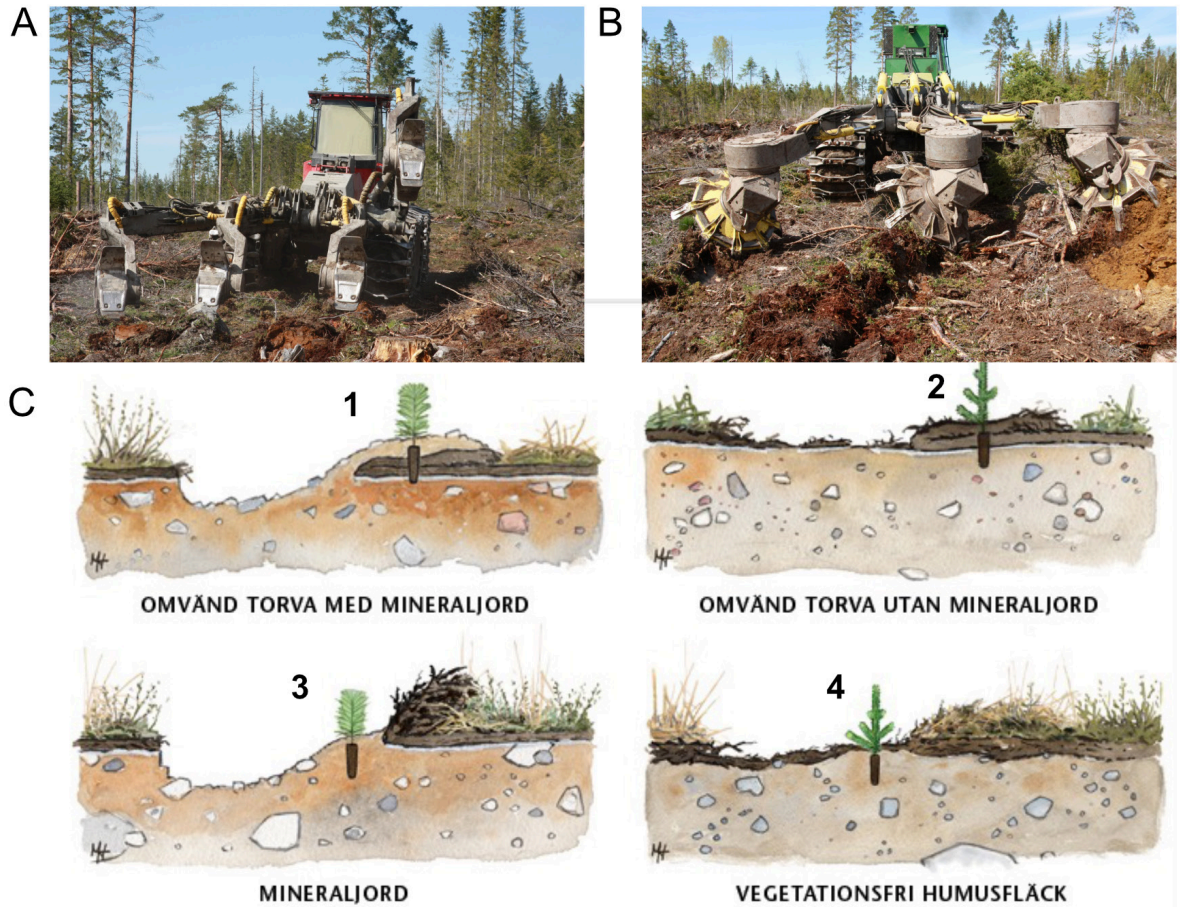

Figure 1.1. *Soil scarification techniques and resulting seedling planting positions.* ***A.*** *Attached mounders, with each of the triangular attachments forming regular, interspersed capped mounds and spots of bare mineral soil. Image used with permission from Mats Hannerz/skogskunskap.se* ***B.*** *Attached disc trencher, resulting in continuous trenches of bare mineral soil, next to continuous capped mounds. Image used with permission from Mats Hannerz/skogskunskap.se* ***C.*** *The four most commonly seen planting positions on a prepared site. Following numbered seedlings: 1) The ideal planting position, the seedling is surrounded by mineral soil, and the roots reach into the double inverted organic layer. 2) The seedling is planted into the double layer of inverted organic soil, but there is no protective cap of mineral soil around the base of the plant. 3) The seedling is planted in the top portion of the bare mineral soil, granting good water availability but a lower amount of nutrients. 4) The scarification did not reach all the way down into the mineral soil, so the seedling is planted into a position of bare organic soil. Images reproduced with permission from Leif Johansson/SCA.*

There are different techniques for soil scarification, with the two most common being mounding and disc trenching. During mounding (Fig 1.1A), a triangular attached mounder creates regularly interspersed positions of exposed mineral soil, with a so-called capped mound next to it. When using a disc trencher (Fig 1.1B), continuous trenches of exposed mineral soil are created, with a continuous capped mound next to them. In both cases, this capped mound ideally consists of a double inverted

layer of organic soil, covered by a 'cap' of mineral soil (Fig 1.1C, planting position 1) [36]. This is the ideal planting position, providing the seedlings with access to the nutrient rich organic layer, while at the same time having a top layer of protective mineral soil on the surface around the seedling. In addition, there is a lower risk for frost damage due to the elevated position. However, the main disadvantage with this planting position is lower water availability, which is why it is recommended to plant seedlings higher up in the bare mineral soil of the trench (Fig 1.1C, planting position 3) in the south of Sweden, or in very dry conditions [37]. In agreement with this recommendation, a recent study on several sites in the boreal zone in Sweden found that planted Scots pine seedling survival depended to a significant part on the amount of precipitation in the first month after planting, and that survival in dry conditions was higher in bare mineral soil than capped mound planting positions [36].

1.2.2 Seeding vs planting

In most cases, seeds from seed orchards are grown in nurseries for one to three years before being planted on clear-cut sites, usually around three years after harvest. In 2020, more than 400 million plants (of which 81% were grown from seed orchard seeds) were produced in Swedish nurseries, with Scots pine overtaking Norway spruce in production numbers for the first time [38]. Direct seeding is used less commonly, with Scots pine being directly seeded at only around 1-2% of regenerated sites in Sweden [21]. In the north of Sweden this proportion can be higher in certain areas, and direct seeding can be a viable option for forest regeneration [39, 40], but in Sweden it is usually only an option on sites that fulfill the following conditions: i) low nutrient availability that limits competing vegetation, ii) adequate moisture that ensures survival in the most critical early stages, and iii) minimal abundances of *Hylobius abietis*. Direct seeding comes with the advantage of not having to pre-grow seedlings in nurseries, but due to the lower survival a much higher number of seeds are needed, which is especially critical for Norway spruce for which orchard seeds are in short supply due to the erratic flowering of this species [41]. One proposed method of improving survival rates for direct seeding is the use of a protective micro-environment for the germinating seed. Two examples are the LandPuck™, a peat-briquette enveloping the seed, and so-called SeedPads™, consisting of a vermiculite disk and a carbohydrate envelope. If these systems can be manufactured at a lower cost than the production of seedlings, they might become a part of Swedish forestry policy in the future [42, 43].

1.3 Nitrogen addition

1.3.1 Nitrogen deposition

In the global N cycle, atmospheric N is fixed through an energy intensive fixation reaction, mostly by diazotrophic bacteria. In boreal forests, a portion of natural N fixation is performed by cyanobacteria associated with feather mosses [44]. Since the discovery of the Haber-Bosch process in the early twentieth century, humans have artificially fixed massive amounts of N, mainly to use for fertilization of crops. The positive side of this is a lower area of land needed to feed humanity, while on the negative side are the massive use of energy and increasing amounts of this artificially fixed N being released into the environment. This has led to eutrophication of water bodies [45], large scale effects on plant communities in some areas [46], as well as large scale effects on fungal communities, as determined based on fruiting body counts [47]. A large-scale study in North America showed that N deposition correlates with a decline of EcM trees in favor of arbuscular mycorrhizal (AM) trees, which was additionally correlated with a decrease in soil C stocks [48]. However, in boreal Sweden the levels of N deposition are still not substantial and only exceed background levels of 2-5 kg ha^{-1} $year^{-1}$ in a few areas, where N deposition levels have declined in recent years [12, 49]. Nevertheless, it has been shown that conifer-associated EcM fungi that are adapted to N-limited soils (and which are discussed in detail in this work) may already be negatively affected by N deposition levels above 5-6 kg ha^{-1} $year^{-1}$ [50], making it all the more relevant to study how the boreal forest overall, and fungal communities specifically, react to different levels of N addition.

1.3.2 Fertilization in forestry

Tree growth in Fennoscandian boreal forests is largely limited by the low N availability, with fertilization to increase growth often being discussed and experimented with. In Swedish forestry practice, fertilization can be used to increase stand volume in the last years of the rotation, about 10 years before harvest. This was found to be profitable on specific sites in an assessment study of different forestry measure from 2010 [51]. In forestry nurseries it is common practice to fertilize seedlings to improve growth. Moreover, a process called ‘nutrient loading’ is used in nurseries, which entails carefully fertilizing the seedlings after growth cessation in autumn to induce N storage without promoting further growth. This has been shown to improve field performance after outplanting in the spring [52, 53]. Early experiments with fertilization of seedlings (naturally

regenerating at the time, since outplanting was not practiced yet [24]) observed that fertilized seedlings initially showed growth improvement, but that their development then slowed compared to their unfertilized counterparts a few years later. This was attributed to decreased root:shoot ratios caused by increased aboveground growth without corresponding root growth. Low root:shoot ratios are correlated with a higher susceptibility to drought and other stresses in different types of herbaceous plants [54–56]. It is also a well characterized phenomenon that lower plant nutrient availability leads to an increase in root:shoot ratio [57]. A relevant example study on forestry seedlings was performed in North America using Douglas fir, showing that fertilization with inorganic N at outplanting led to poorer root development and decreased drought tolerance compared to control seedlings [58]. As a result of such reported negative impacts, seedlings in Sweden have generally not been fertilized at outplanting, but further studies are needed to determine the conditions in which fertilization of planted seedlings might improve survival and growth.

1.3.2.1 Organic fertilizer

Previous experiments on seedling fertilization were performed using inorganic N sources, but we have since learned that a significant portion of N in forest soils is available in the form of amino acids [59]. In this thesis (**Papers I** & **II**) I have worked with the fertilizer arGrow (Arevo, Umeå, Sweden) that is based on arginine, the amino acid with the highest N content, having four N atoms per molecule. It has been demonstrated that conifers can take up amino acids directly and use arginine for N storage [60–64]. In *Arabidopsis thaliana*, arginine supplementation has been shown to rescue seedling root growth after knock-out of a crucial enzyme in the nitric oxide pathway, an important plant signaling molecule associated with a wide range of physiological processes [65], and other studies indicate a connection between arginine supply and root growth [66]. More relevant to forestry, arginine used as a nursery fertilizer has been shown to increase root growth and the proportion of mycorrhizal root tips, as well as overall field performance after outplanting [67, 68], which is why several Swedish nurseries are now using arginine phosphate to fertilize seedlings despite its currently higher cost. Initial root growth is considered one of the most important factors in seedling establishment [56, 69]. A recent large scale study on several sites across boreal Sweden found that Scots pine seedlings are more likely to survive with a small dose of arginine phosphate at outplanting [36]. A study in Spain, also on Scots pine, showed that seedlings fertilized with an amino acid mix had improved P uptake

capabilities and higher chlorophyll concentrations [70]. An additional advantage of arginine phosphate specifically is that it reduces leaching of N into the environment [71] due to the positively charged molecules adhering to soil particles [72]. An increasing number of outplanted seedlings in Sweden is supplied with a small dose of arginine phosphate at outplanting due to these first positive results.

1.3.3 Perturbation experiments

Perturbation experiments are commonly used in biological sciences to "infer and quantify interactions between components of a biological system" by deducing these interactions from the system's response to a targeted perturbation [73]. In the N-limited boreal forest, some of the earliest forestry experiments described previously [24] used the addition of peat and N salts to test whether this fertilization increased natural regeneration. In the last 50 years, there have been a number of experimental sites in Sweden established for the purpose of adding controlled amounts of N to N-limited boreal forest sites, with the aim of observing the changes, with different questions and experimental designs. Large parts of northern Sweden do still only receive pre-industrial levels of N deposition [12], making them ideal to study N limitation, as well as the artificial perturbation of this natural state.

Experimental sites in Lisselbo and Norrliden (Fig 1.2) were set up to supply Scots pine with an amount of N (and other nutrients) that maximizes tree growth. These experiments confirmed that N is the limiting factor for tree growth, but that N addition by itself leads to deficiencies in other nutrients (notably phosphorous (P)), and also made inferences regarding N pollution and soil acidification from their results [74]. The Rosinedal site (Fig 1.2) is a mature Scots pine forest on a very nutrient poor and sandy site that has been exposed to high levels of yearly N addition, mainly to study CO_2 flux components in the presence and absence of N addition using specialized equipment [75]. The site has also been used in several other studies exploring the effects of N addition on tree and soil microbiome, and soil chemistry levels [76–78]. The Flakaliden experimental site (Fig 1.2) was designed to supply Norway spruce trees with an optimized, balanced nutrient solution to further test whether nutrient limitation is the main factor that limits tree growth in boreal Fennoscandia [11]. The full nutrient solution was additionally compared to the effect of irrigation, to determine the extent to which water limitation is also a factor. Other experiments from the Flakaliden site included soil warming and elevated CO_2 climate chamber experiments [79], with the result that the nutrient limitation was found

to be the strongest factor limiting tree growth. This experimental design allows the effect of increased N to be studied without induced limitations of other nutrients. Finally, it also has to be noted that natural N-gradients do exist in the boreal forest, for instance at groundwater discharge areas [12], and studying them can also reveal a lot about boreal forest dynamics, while perturbing a site that starts from an identical state as a control site has its own scientific merits.

A benefit of these experimental sites is that they can be used for many different types of studies once they have been established. In this thesis I have made use of the Rosinedal site (Fig 1.2, **Paper III**) and the Flakaliden site (Fig 1.2, **Paper III, IV & V**) to study belowground changes after N addition, more specifically we have used metatranscriptomic data from tree root samples to study the fungal communities under N limited and N enriched conditions.

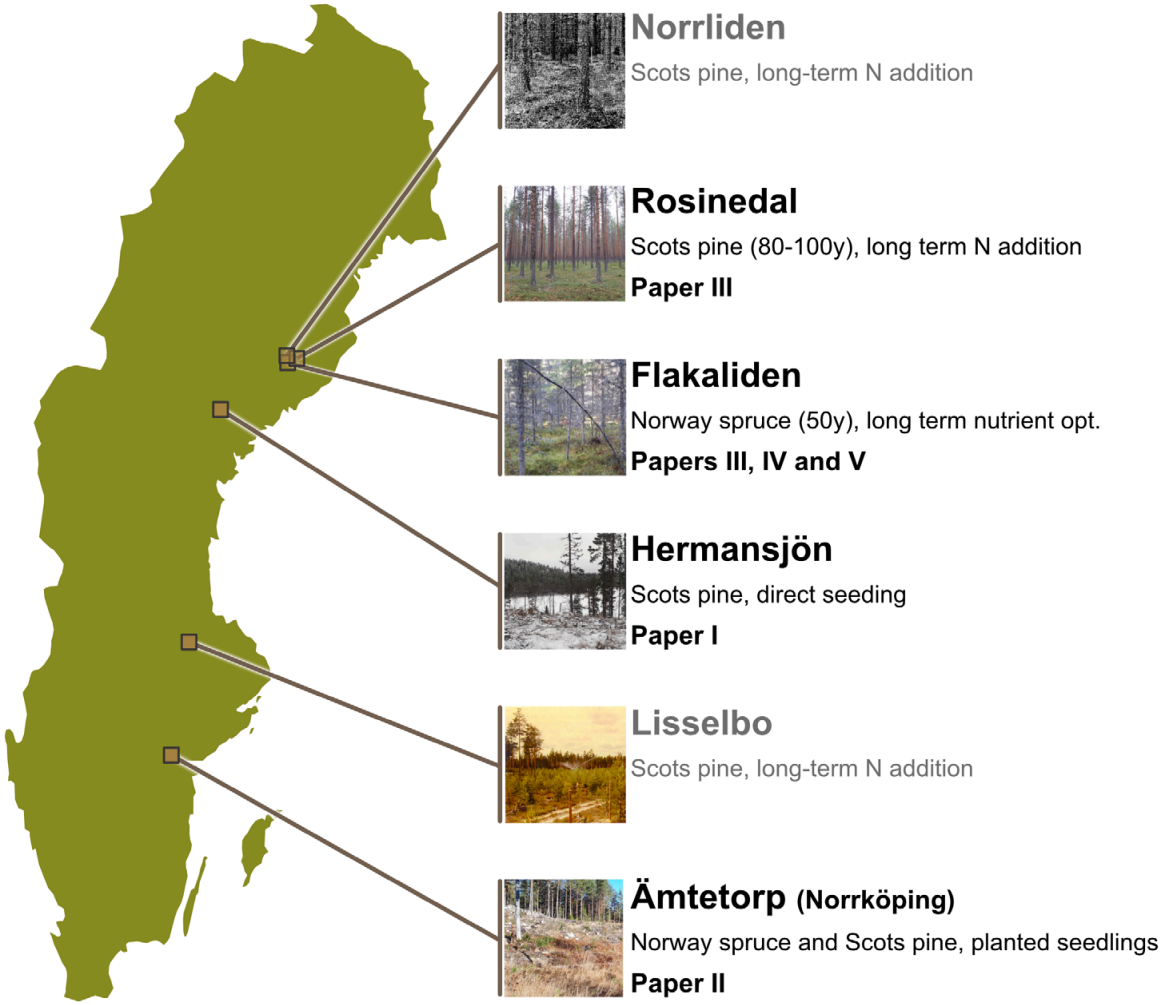

***Figure 1.2**. Map of Sweden showing experimental sites used in this thesis (dark text) and mentioned in this section (greyed text).*

2 What are fungi and why are they important?

Fungi are one of the eukaryotic kingdoms and contain a vast diversity of species with an array of different survival strategies. Most fungi grow as filamentous, finely branched structures called hyphae (which in multitude comprise what is called mycelium) [80], enabling an explorative and adaptive lifestyle. Several fungal lineages have (independently) lost this mode of action and are mostly unicellular, known collectively as yeasts [81]. Fungi can be found in all environments [82–84], and have a global importance as nutrient cyclers and ecosystem keystone species. They are, with bacteria, the most important part of the global soil microbiomes, which consist of a plethora of different types of microorganisms, and comprise a significant portion of global carbon stocks in living and dead biomass (reviewed in [85]). The boreal forest has some of the largest fungal and bacterial carbon stocks per area in the world [86]. Arguably one of the most important clades in the fungal kingdom (especially in the boreal forest) is the subkingdom Dikarya, which contains the phyla Ascomycota and Basidiomycota [87]. Ascomycota and Basidiomycota are distinguished by, and named after, their reproductive structures (asci and basidia). All macroscopic mushrooms we know and the large majority of saprotrophs and EcM fungi in the boreal forest are in these phyla, making them especially important for the work in this thesis.

2.1 Trophic modes

Fungi are often classified by their trophic mode, that is their mode of obtaining nutrition in the form of carbon, more specifically complex carbohydrates. Like animals, all fungi are obligate heterotrophs, which means they must rely on pre-existing organic molecules for nutrition [88]. This can be achieved in several ways with the most important fungal strategies being pathotrophy (feeding by parasitism and/or causing disease on other organisms), saprotrophy (feeding on dead organic matter), and symbiotrophy (feeding by entering a symbiosis with primary producers).

Pathotrophic fungi are pathogens and parasites that attack other organisms and parasitize or even kill them to obtain nutrients. Notable examples for this are fungal diseases affecting hundreds of millions of

humans [89, 90], different types of fungal plant pathogens causing massive losses in agriculture and forestry [91], and invasive fungi decimating wild plant [92] or animal populations [93]. Swedish forestry is affected by several economically significant pathogenic fungi. The most important example is *Heterobasidion* spp., which cause root and butt rot on Norway spruce and Scots pine in a necrotrophic (causing the death of the host) manner [94, 95], causing annual losses for forest owners up to a billion Swedish crowns.

Saprotrophic fungi feed on dead organic matter, thereby depolymerizing organic compounds, and are major drivers of decomposition. In boreal forest soils, they dominate fungal communities in the litter and upper organic soil layers [16], and mainly decompose leaf litter, dead roots and dead mycelium. Decomposition by saprotrophs can be split into two phases: the earlier phase occurs in the uppermost litter layer, where more easily degradable polymers like cellulose and chitin are degraded primarily by Ascomycetes [96, 97], which generally are “weaker” decomposers [98]; the lower phase (in the lower litter and upper mor-layer) is dominated by Basidiomycete saprotrophs, which generally have a larger range of enzymes and higher lignin degrading capabilities [99] and comprise the majority of fungi in the next step of decomposition succession. However, it should be noted that this separation is not absolute, since there are many ascomycetes that are efficient lignin decomposers [100, 101], and basidiomycetes lacking such capabilities. Indeed, this is one of the challenges faced when attempting to assign ecological roles or functions on the basis of taxonomic annotations represented in a community.

Symbiotrophic fungi obtain all or most of their carbohydrates through symbiosis with a primary producer. Lichens fall into this category, consisting of a fungal thallus hosting photosynthetic organisms like algae and/or cyanobacteria, as well as a whole array of different microorganisms [102]. Another notable example are endophytic fungi, living an invisible life inside most plants. In many cases these endophytic fungi can be beneficial for the host [103], but some are opportunistic pathogens that wait for the host to be weakened before they cause disease [104]. In tree leaves many of the endophytes are part of the first successional stage of decomposers after the leaves have senesced [97]. Arguably the most impactful symbiotrophic fungi are mycorrhizal fungi, forming associations with over 90% of described plants, with the general definition of a mycorrhiza (from Greek mykes, “fungus”, and rhiza, “root”) being that there is a specific organ for mutual nutrient transfer between certain phylogenetic groups of plants and fungi [105]. Broadly,

the mycorrhizal symbiosis entails a trading relationship, with the fungus trading nutrients like N or P for carbohydrates from the plant, with this exchange happening in specialized structures in plant roots, such as the Hartig net for EcM fungi and the arbuscules for arbuscular mycorrhizal (AM) fungi. The dominating tree species in the Swedish boreal forest are all ectomycorrhizal, and, as such, this group of mycorrhizae are the primary focus of this thesis. Interestingly, symbioses with different types of microorganisms seem to have a common genetic basis in plants, with, for example, the gene *SymRK* being involved in root symbioses both with AM fungi and rhizobial bacteria in different plant lineages [106].

In the context of symbiosis and symbiotrophy, the term "holobiont" must be mentioned. The concept has been developed by several researchers independently since the 1940s [107], and has gained popularity in recent years. The definition is somewhat fuzzy and is used differently by different people, but it can be defined as the whole "anatomical, physiological, immunological or evolutionary units" [107] of a host organism and associated microorganisms. The concept is important as it influences many aspects of biological research, such as studies of environmental response, adaptation, and evolution. Perhaps most importantly, it conceptualizes that an individual is the sum of its parts. This has been highly influential in many biological fields, most notably humans where the importance of the many microbial communities we host has increasingly been appreciated and understood to the extent that terms such as pro- and pre-biotics are now commonplace. It is only more recently that the same emphasis has been placed on these kinds of concepts in plants.

While symbiotrophy and saprotrophy may seem like clearly distinct trophic modes, the reality is more complicated. Probably owing to the fact that all hitherto known EcM fungi are descendants of saprotrophic ancestors [108], there are a surprising number of saprotrophs that are able to colonize roots to some degree, with some even being able to initiate Hartig net formation [109]. Moreover, EcM fungi have retained plant cell wall degrading enzymes (PCWDEs) to different degrees [110], and some have been shown to be facultative saprotrophs [111]. Nevertheless, in most cases the transition from saprotroph to EcM seems to be correlated with a significant loss of PCWDEs, but further studies are needed to explore why exactly this seems to be the rule [110]. Generally, one could say that the lines between trophic modes are rather blurred, and there are many examples of species that are seen as being able to sustain different trophic modes in different situations [111–113].

2.1.1 Mycorrhizal fungi

Mycorrhiza is in fact an umbrella term for different types of fungal symbioses with plants, with mycorrhizal associations having evolved independently numerous times in almost all plant, and many fungal lineages. Mycorrhizae were traditionally broadly divided into endo- and ecto-mycorrhiza, depending on whether the fungal hyphae penetrate the host root cells or not. Intracellular mycorrhizal associations include AM, ericoid mycorrhizal (ErM) and orchid mycorrhizal symbiosis. AM is defined by the presence of so-called arbuscules inside host cells, and can form associations with almost 80% of described plant species [105]. They are all from the fungal phyla Glomeromycota and Mucoromycota. AM fungi are almost everywhere but are especially important in tropical and grassland ecosystems. ErM are limited to members of the Ericaceae (excluding some subfamilies) and Diapensiaceae. ErM is usually formed by fungi within the groups Helotiales, Chaetothyriales, Serendipitaceae and *Pekloderma*. Orchid mycorrhizae, as the name implies, only occur in the Orchidaceae. Remarkable in orchid mycorrhizal symbiosis is that in many cases the fungi do not seem to obtain any nutritional benefit from the association with both C, N and P being given by the fungi to the plant [105].

2.1.2 The ectomycorrhizal symbiosis

EcM symbiosis has evolved repeatedly in about 30 plant and 80 fungal lineages [114] since its first known emergence about 200 million years ago. Only around 2% of plant species form EcM, but it is of great importance, especially in temperate and boreal forests. Ectomycorrhizae are named after the fact that the fungal hyphae do not enter the host cells, instead forming a structure called the Hartig net (Fig 2.1B) by growing in between epidermal and cortical root cells, and additionally forming what is called a mantle around the mycorrhizal root tips. This mantle constitutes a significant portion of root tip weight and can be used as a fungal storage organ [17]. However, not all EcM fungi form these structures, with, for example, some strains of the common species *Paxillus involutus* forming a nutrient-exchanging mycorrhizal symbiosis without it, while other strains exhibit normal ectomycorrhizal behavior with Hartig net and mantle formation [115]. The Hartig net is the root-mycelium interface, where the exchange of nutrients is realized, but also where crosstalk between trees and fungi occurs. In order to form the ectomycorrhizal roots both plant and fungus undergo significant developmental changes [116], the coordination of which is achieved through the exchange of a large number of signaling molecules and metabolites [117, 118]. This results in the formation of mycorrhizal root

tips, which are often clearly recognizable by their phenotype (Fig 2.1A). On the plant side, this bilateral process requires lowering of defense responses [119] and loosening of cell-to-cell adhesion to enable the fungus to enter the apoplastic space. This is achieved through secretion of various specific signaling molecules and effectors by the fungus [120]. An important group of such signal molecules are the small secreted proteins (SSPs), which are exuded by fungi and play a crucial role in mycorrhiza formation [121].

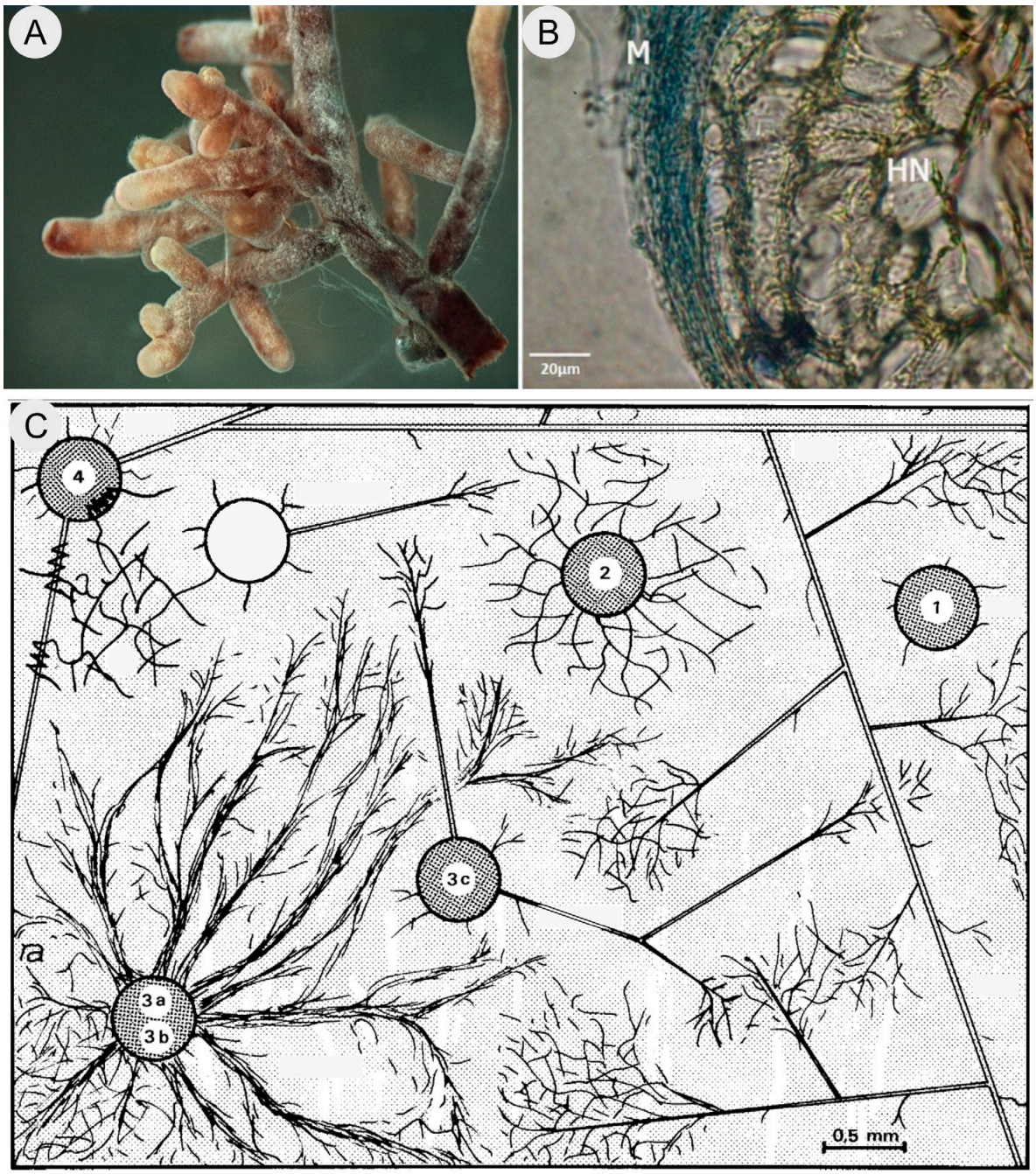

Figure 2.1. *Microscopy photos of ectomycorrhizal roots, and drawings of ectomycorrhizal exploration types.* ***A.*** *Ectomycorrhizal root tips, colonized by* Amanita *sp. Modified from [122].* ***B.*** *Cross section of ectomycorrhizal root between* Pinus pinea *and* Pisolithus *sp., with clearly visible mantle (M) and Hartig net (HN) structures. Modified from [123].* ***C*** *Ectomycorrhizal exploration types, big circles indicate mycorrhizal roots. 1 Contact exploration. 2 Short-distance exploration. 3a Medium-distance fringe exploration. 3b Medium-distance mat exploration. 3c Medium-distance smooth exploration. 4 Long-distance exploration. Modified from [124].*

The ectomycorrhizal symbiosis has been shown to provide host trees with a number of benefits, including improved drought tolerance and nutrient uptake [125–128]. Large public attention has been given to the concept of a common mycorrhizal network that transports nutrients between trees since it was found that C can indeed be transported between plants through fungal mycelium [129]. From another angle, the mycorrhizal symbiosis has often been described as an economic relationship, with both symbiotic partners having higher access to a resource that is highly desirable to the other party. In the N-limited Fennoscandian boreal forest soils EcM fungi control tree N supply, but studies have shown that in these N-limited conditions additional N mostly ends up being immobilized (at least from the plant's perspective) in fungal mycelium and not taken up by the trees. This leads to the hypothesis that EcM fungi might be in a competitive relationship with the trees and that they potentially might even end up aggravating tree N limitation [130]. Fitting within this conceptual framework, a later study showed that an increase in C export from boreal forest trees leads to reduced N uptake, at least when soil N is limiting [77]. This can be explained by the EcM fungi using this additional C to increase their own biomass (and thus also using up N) instead of returning equal value to the trees in the form of N. This could lead to the hypothesis that the conifer-EcM holobiont has a competitive advantage in the boreal forest through the large C investments of the trees contributing to the strong N limitation [130]. This could then reduce competition from other trees, a theory that has been supported by another modelling study [131]. In a larger context, this could be part of what has been called "plant-soil feedback", which in essence is the modification of soil properties by plants and the resulting change in performance of conspecific or heterospecific seedlings [132]. Plant-soil feedback has been confirmed to be positive (i.e. favoring conspecific trees) in EcM forests [133]. A recent meta-analysis came to the conclusion that EcM trees invest more C into fungal mycelium, while AM trees invest more into feeder roots, which is also in line with these findings [114]. Taken together, this shows that the relationship between trees and fungi is not straightforward or as simple in all cases as a purely mutually beneficial relationship where both partners always get the best possible outcome [134], even though it certainly can be. An additional level of complexity in the forest is that trees and EcM fungi are interconnected via a web of fungal hyphae, or network, i.e. one tree is connected to a number of fungal individuals, and one fungal individual can likewise be connected to several trees. This can lead to interesting trading dynamics, and while we still do not understand the full extent or implications of this, there have been several interesting studies in recent years. For instance, a Swedish greenhouse

study found that competition between Scots pine seedlings connected to the same *Suillus variegatus* isolate leads to higher immobilization of N in fungal biomass through an increase in collective C export [135]. An Australian study using mesocosms with *Eucalyptus grandis* seedlings and different *Pisolithus* isolates found that competition (for plant C) between different fungal strains was controlled by the plant by an upregulation of defense responses when the fungus did not reciprocate the C investment with appropriate amounts of N compared to the other isolates [136]. In the field, there is likely to be competition and interaction in all directions (tree-tree, fungus-fungus and fungus-tree) and moreover very likely significant individual behavioral differences between species and interspecific genotypes on both the tree and the fungal side that are influenced by both genetic and environmental factors.

While only around 2% of all plant species form ectomycorrhizal symbioses [105], in many temperate ecosystems, and especially N-limited ecosystems like the boreal forest, it is the dominating form of tree mycorrhiza [82]. In a typical boreal Norway spruce forest, around 95% of tree root tips are colonized by EcM fungi [137], and looking at the global distribution of mycorrhizal types, the majority of aboveground plant biomass in boreal forests is of ectomycorrhizal plants [138]. Connected to aboveground C storage and sequestration, a recent meta-analysis showed that EcM fungal abundance patterns and functional capacities are at least strongly correlated with tree growth levels on a larger scale, even if causality or directionality could not be determined [139]. With that said, aboveground C is actually the smaller portion of total boreal forest C stocks, with the majority believed to be stored belowground [8, 140]. Overall, it has become increasingly clear in the last decades that understanding how EcM fungi are involved in important processes such as decomposition and nutrient cycling, tree growth and nutrition, and ecosystem services is going to be an important piece of the puzzle for ensuring a sustainable future.

2.1.3 Carbon cycling

At its core, the C cycle is what happens between the fixing of CO_2 by photosynthetic organisms and the eventual release of the same C into the atmosphere as CO_2, which can happen after very short times (plant respiration) or after millions of years (e.g. the burning of fossil fuels). In between, plants can incorporate this C into their own biomass, either above or belowground, export it to mycorrhizal symbionts that then incorporate it into their own biomass or use it to “mine” for more N by

investing it into extracellular enzymes. If a system emits more C than it stores it is called a C source, while it is called C sink if it stores more C than it emits. C is emitted through respiration (including as a part of decomposition), and stored through e.g. accumulation of organic matter [8].

As discussed before, boreal forest soils are usually vertically stratified, both by decomposition stage and by functional fungal guilds, with the litter and upper mor-layer being dominated by saprotrophic fungi and with EcM fungi dominating below [16]. Large-scale comparative analyses have shown that EcM fungi are especially good at taking up nutrients through their extensive mycelia and efficient means of transportation, which could be why EcM fungi may be able to exclude other fungi in "their" soil layer, while AM fungi seem to be more "cooperative" and take advantage of bacteria and saprotrophs surrounding them to gain access to nutrients [114]. Another finding from the same meta study was that EcM fungi are a more costly investment for trees than AM fungi, using up more C due to their more extensive mycelium and higher enzyme expression levels [114], which again could be part of the explanation for why EcM trees have a competitive advantage only in N-limited soils. This corresponds to real-life observations revealing a correlation between high levels of N deposition and a shift from EcM to AM tree dominance [50], and generally higher soil C storage per unit of N in ecosystems dominated by EcM and ErM plants [141].

The localized "exclusion" of other fungi by EcM fungi has been hypothesized to originate from a competition between EcM fungi and saprotrophs. This was first described by Gadgil and Gadgil in 1975 [142], who found that removal of mycorrhizal *Pinus radiata* roots increased decomposition. In support of this 'Gadgil effect', EcM fungi have also been found to decrease soil respiration [143]. However, it has been widely acknowledged that strength and directionality of this effect are variable and need to be studied further [144]. This ties in well with the observation (described in 1.2.1) that EcM fungi drastically reduce in abundance after clearcutting and soil preparation. The felling of trees cuts off C supply for the EcM fungi, and the soil scarification disrupts the fungal mycelia and plant roots and mixes the soil layers, thus eliminating competitiveness of EcM fungi and providing ideal conditions for saprotrophic and opportunistic fungi.

2.1.3.1 Decomposition

Beside respiration, decomposition is the other major biological process that releases CO_2 back into the atmosphere. Due to the sheer magnitude of these carbon fluxes on a global scale [145], it is important that we understand how decomposition works in different environments and conditions, and how it might change in the future. One of the most important organic molecules is lignin, which is a major structural polymer of wood and the second most abundant plant-based biopolymer on earth after cellulose, thus comprising a major portion of global carbon stocks in living and dead plant biomass [146].

There are two important groups of fungi called “brown rot” and “white rot” that account for degradation of dead plant material. Brown rot fungi generally lack the oxidative enzymes needed to completely break down lignin, and instead rely on generating reactive oxygen species through the reaction of Fe^{2+} and H_2O_2 [147]. This is also called non-enzymatic chelator-mediated Fenton chemistry [148], and temporarily depolymerizes the lignified plant cell walls sufficiently for the fungi to access more easily degradable polymers that can be processed using carbohydrate-active enzymes (CAZymes) closer to the mycelium. In contrast, white rot fungi rely directly on enzymatic reactions by extracellular oxidative enzymes, such as lignin peroxidases and manganese peroxidases [147], which enables them to comprehensively break down lignin. Depolymerizing lignin in this way is very energy intensive, and until it was shown that there are indeed fungi that can live off lignin alone [149] it was debated whether a white rot lifestyle could exist as a stand-alone nutritional acquisition mode. Consequently, only a small group of filamentous basidiomycetes is able to achieve this feat, even though comparative genomic analyses have led to the likely hypothesis that a very early lineage of current Agaricomycetes (the class of fungi that contains all basidiomycete mushrooms) was a white rot fungus, and that lineages with EcM fungi and brown rot fungi within the class have secondarily lost the enzymatic capacity required for white rot decomposition [150].

Different studies in the boreal forest have shown that fertilization with high doses of inorganic N inhibits decomposition and increases organic matter accumulation [15]. On a broader scale, it has been found that this organic matter accumulation occurs through inhibition of substrate mineralization [75], and reduction of soil respiration [151]. These changes in microbial activity have been hypothesized to partly be caused by soil acidification [152], which is commonly observed after N addition

[12]. Other effects are that overall microbial biomass decreases [153], and trees produce more fine roots, resulting in a higher ration of fine root:fungal biomass [154]. However, it is worth noting that these effects only occur at high N addition rates that far exceed current N deposition levels in boreal Sweden, while at lower N deposition levels decomposition may actually be stimulated instead [155]. To delve a little deeper into these N addition effects, we must look at things from the tree side first, as well as explore the functional differences between nitrophobic and nitrophilic fungi, i.e. fungi with a low or high tolerance to high N availability. When large amounts of inorganic N are added to a previously N-limited boreal forest site, over time the trees undergo significant morphological changes compared to controls. If we take the Flakaliden site (used in this thesis in **Papers III**, **IV** and **V**) as an example, in response to the nutrient enrichment Norway spruce trees have been shown to increase production and turnover of roots [156], as well as significantly increase their above and belowground biomass [157]. A more immediate effect of the fertilization treatment is that the trees decrease their C export to fungi [158, 159], which has been observed to lead to the disappearance of fruiting bodies in fertilized forests [160]. Over time, the composition of the fungal communities also changes belowground, from a community dominated by nitrophobic fungi that are adapted to the soils with low N availability such as *Cortinarius* (Fig 2.2) and *Tricholoma*, to more nitrophilic taxa such as *Lactarius* and *Tylospora* [161]. Some of these EcM fungi that seem to be more tolerant to N addition belong to a group of fungi that can incorporate high amounts of melanin into their cell walls, which is believed to grant them protection against environmental stress such as drought [162]. Melanized hyphae (e.g. in *Cenococcum geophilum* or *Meliniomyces bicolor*) can lead to slower decomposition [163] and consequently contribute to additional C accumulation [164]. This ties in well with the concept of microbial necromass, which has gained importance in recent years as a major component of soil C stocks at least as quantitatively significant as dead plant biomass [165, 166].

2.1.3.2 Known properties of "nitrophobic" and "nitrophilic" EcM fungi

Fungi have often been classified as nitrophobic or nitrophilic, depending on their ability to grow and sustain themselves in nutrient rich soils. While this can be a useful way to classify fungal taxa, it is not informative as to the reasons for this preference for high or low nutrient availability. A review from 2011 summarized different traits of nitrophilic and nitrophobic EcM fungi [161], finding that nitrophobic taxa are mostly able to use complex organic N sources like proteins, and have a medium-

distance fringe exploration type with hydrophobic rhizomorphs. Rhizomorphs are long, root-like mycelial structures, often used for nutrient and water transport. Ectomycorrhizal exploration types were defined by Agerer in 2001 [124] to create a classification for morphological differences between the mycelia of different ectomycorrhizal species. The different exploration types defined were contact exploration, short-distance exploration, medium-distance fringe exploration, medium-distance mat exploration, medium-distance smooth exploration, and long-distance exploration (Fig 2.1C). These terms signify the distance that the hyphae will extend from the host root (short-medium-long), and the physical characteristics, where fringed and smooth describe an abundance and a lack of emanating hyphae from the mantle and the rhizomorphs. As said above, many of the generally strongly nitrophobic genera such as *Cortinarius* and *Tricholoma* fall within the medium-fringe exploration type (Fig 2.2C) [161]. Furthermore, they use oxidative enzymes (like white rot wood decomposing fungi) to break down organic matter and mobilize N from the soil. Especially *Cortinarius* has been found to both express oxidative enzymes and to participate in decomposition in boreal forest soils [167], and the sequenced species *C. glaucopus* has retained an unusually high number of PCWDEs in its genome, when compared to other EcM fungi [110, 168]. This has led to the proposition that EcM fungi can be powerful decomposers, but without being saprotrophs (since they decompose mainly to access N, and not C like “true” obligate saprotrophs) [169]. Moreover, numerous studies have shown that *Cortinarius* generally responds very negatively to high soil N content in different contexts [167, 170–173]. One could argue the combination of an exploration type with extensive mycelial growth (medium fringe, Fig 2.2C), and the “expensive” N mining strategy of using oxidative enzymes entails a lifestyle with a very high C demand, which the trees will only be able (or willing) to fulfill when conditions are strongly N limited (as discussed in section 2.1.2). A recent study showed that a species complex of *Cortinarius* (*C. acutus* s. l.) was restricted to older forests, and correlated with lowered soil C stocks [174], further suggesting that these EcM fungi can be powerful decomposers and are important for larger nutrient cycling processes. Taking a broader view, plant-microbe symbioses in general often have this additional cost of N mining. This could mean that many current climate models potentially underestimate climate change and overestimate C sequestration, especially for boreal forests, where N limitation is a strong factor and requires plants to invest more C to get enough N to grow [175].

Figure 2.2. *Fruiting bodies of the common nitrophobic genus* Cortinarius *from the Flakaliden and Rosinedal experimental sites, and* Cortinarius *ectomycorrhizal (EcM) root tips with extramatrical mycelium from the literature, showing medium-fringe exploration phenotype.* ***A***. Cortinarius semisanguineus, *Flakaliden*. ***B***. C. sanguineus, *Flakaliden*. ***C***. C. traganus, *Rosinedal*. ***D.*** Cortinarius *EcM root tips and extramatrical mycelium. From left to right:* Cortinarius *sp. and* Pseudotsuga menziesii *roots (wikimedia commons).* Cortinarius *sp. and* Castanea dentata [176]. Cortinarius torvus [177]. Cortinarius *sp. on Scots pine root, eh = emanating hyphae, rh = rhizomorph* [178].

In contrast, nitrophilic fungi are characterized by hydrophilic exploration types with mostly contact, short-distance and medium-distance smooth types [161]. These types require a lower C investment due to less extensive transport structures, and many of them also lack proteolytic capabilities in culture, which would indicate a specialization for more easily accessible N forms. As with everything in nature, there are exceptions to these patterns, for instance chantarelles (*Cantharellus cibarius*), which have medium-distance smooth exploration type, but do possess proteolytic capabilities [179] and have been reported to decline in areas with high levels of N deposition [180].

2.1.3.3 Nitrophilic fungi in forestry nurseries

Several studies have been performed on the fungal communities in Fennoscandian forestry nurseries. A typical forestry nursery can be considered a heavily disturbed, and nutrient rich environment. It is thus not surprising that nursery seedlings have been found to be colonized by a mix of nitrophilic and stress tolerant EcM fungi including *Thelephora terrestris*, *Amphinema byssoides*, and opportunists such as *Penicillium* spp. [181–184]. After outplanting, *Thelephora terrestris* has been shown to persist on inoculated Scots pine seedlings after the first and the second growing season in a Polish study [185]. A Finnish study also found nursery EcM fungi to persist on outplanted seedlings, especially in scarified soil [30]. An early Swedish study found that while the majority of outplanted Scots pine and lodgepole pine (*Pinus contorta*) seedlings they examined had to some degree been colonized by site indigenous EcM fungi after one growing season, the majority of newly formed EcM root tips were still colonized by fungi that came from the nursery [186], with a very similar result reported in an additional study [187]. A Canadian study also found that new ectomycorrhizal roots on establishing seedlings were to a large part colonized by the fungi already present in the nursery [188]. This pre-colonization is believed to provide the seedlings with higher drought tolerance and nutrient uptake capabilities [30, 189]. The continued persistence of these nursery fungi is likely due to priority effects, where EcM fungi that are already present on the root system of a tree have a higher chance of colonizing new roots [190].

2.2 What is known about the effect of forestry on fungal communities?

There are concerns that modern, rotation-based forestry has negative effects on species that are adapted to the N-limited boreal forests, which naturally experience only rare disturbances from events such as natural fires or severe storms. There have been a number of studies showing that while overall EcM fungal mycelial abundances only take about 10-20 years to recover after clearcutting and replanting [191, 192], *Cortinarius* spp. take at least several decades more to establish at pre-harvest abundances [174], which is also true for many other fungal species [193, 194]. While it is not entirely clear whether this is a clearcutting or a stand-age effect, the concern is that in the long term this could lead to a threatening of such species of not only fungi, especially if harvest cycles are shortened, but also to plants and animals that evolved in these

conditions [195, 196]. In fact, a large number of forest-inhabiting species are already threatened in Sweden [197], with the exact role of forestry being heavily debated in the Swedish public and academic circles in recent years [198]. It is currently too early to say if the measures taken by the forestry industry to prevent biodiversity loss, such as leaving retention trees and dead wood and favoring broadleaf trees, are going to be sufficient or what the long-term and accumulated impacts of changes to fungal species composition may be more specifically. What can be said on the positive side is that the area of older forests (>160 years), and the amount of dead wood in Swedish forests have both increased significantly since the 1990s, when these protective measures were introduced [198]. Well-designed large-scale experiments and meta studies will be needed to answer these questions with certainty. However, time might be limiting if threatened species are to survive, especially with climate change further impacting ecosystems beyond the effect of forest management. We know from post-agricultural land that plant communities and soil properties can remain changed for extended periods of time after cessation of agricultural use [199–201], and this has also been shown for microbial communities [202]. The number of studies on EcM fungi specifically is not as comprehensive, but secondary forest stands may take a very long time to be colonized by EcM fungal species characteristic for older forests [203], depending on the distance to the nearest old forest. Apart from an adaptation of forestry practices, a potential solution for this issue could be the targeted reintroduction of threatened species into secondary forest stands that have reached a certain age, however this requires an in-depth understanding of which species are threatened across all kingdoms and what the consequences of such reintroductions are exactly.

2.3 The rhizosphere

The rhizosphere is defined as a zone around plant roots that is affected by root exudates, and often colonized by specific microorganisms. Mycorrhizal fungi are an obvious example, but there is usually a distinct rhizosphere microbiome different from the surrounding soil. Moreover, rhizosphere processes are important components of terrestrial C and nutrient cycles [204]. While fungi are important members of the plant, rhizosphere and soil microbiomes, other microorganisms deserve a brief mention. Bacteria are, depending on the environment, at least as important as fungi in global C and N cycles [205]. Moreover bacteria are important for mycorrhizal formation, and there is a large amount of published research on so-called “mycorrhizal helper bacteria” [206]. In

the boreal forest, important bacterial groups include Alphaproteobacteria, Acidobacteria, Actinobacteria and Gammaproteobacteria [171, 207]. It has been shown that N addition increases the bacterial:fungal biomass ratio and favors autotrophic nitrifying bacteria, which further on leads to leaching of nitrate and denitrification processes [12]. Other notable soil microbiome members are protists, archaea, viruses and microfauna such as nematodes and Collembola [205]. An especially understudied part of the boreal forest soil microbiome are viruses, which can be present in both bacteria and fungi, and that currently represent something of an unknown 'dark matter' of forest ecosystems [208]. First results have shown that viruses might represent overlooked components of C cycling in soil [209], thus warranting further research. While I have focused on fungi in this thesis, the methodologies I applied can also be used to study the other kingdoms in the earth's microbiomes.

3 Methods to study fungi

When studying fungi, we are faced with different challenges. Even fungi developing macroscopic structures (macrofungi) spend large portions of their lifecycles in microscopic or filamentous stages, which naturally makes them hard to observe and study. Furthermore, fungi are mostly active belowground or inside a host species, hindering easy access and visibility. For this reason, many of the methods employed to study fungi today are indirect, for example inferring species abundances by extracting DNA from a sample and then assigning species to the resulting sequences, or by incubating a soil extract with a substrate expected to be activated by a specific enzyme. Other, more traditional methods use direct observation of fungi, either through their macroscopic structures or microscopy.

3.1 Methods for direct observation

The earliest method used to study fungi, apart from macroscopic observations of fruiting bodies (also called sporocarps), was microscopy. The first person to look at fungi with a microscope was Robert Hooke in 1667 [80], and around 60 years later Pier Antonio Micheli created the first mycological work using microscopic characteristics of around 900 different fungi. However, it wasn't until the 19th century that more people became interested in studying fungi, mostly connected to plant diseases, but also the mycorrhizal symbiosis, which was first described in 1885. With the technical possibilities today, fungal microscopic structures can be visualized in different ways, for example staining different compounds in fungal cell walls using different dyes [210], fluorescent in situ hybridization [211], or expression of fluorescent proteins [212].

Another commonly used method for macrofungi is to count fruiting bodies. This can tell us which fungi are developing reproductive structures in an area and has been used as a proxy for fungal populations. It is a good method because many important saprotrophs and EcM fungi form fruiting bodies that can be identified and counted and does not require any specific equipment or technologies. In my view, however, making localized statements about fungal communities based on fruiting body counts is problematic due to i) erratic fruiting of many species, meaning that they might be present in the area of interest but not fruit in that particular year due to suboptimal environmental conditions, and ii) temporal limitation of sporocarp emergence, meaning

that many fungi only develop fruiting bodies at specific times of the year. So, to comprehensively cover all macrofungi in any area, one would have to undertake weekly sampling over the whole growing season, ideally over several years. Even then it has been shown that sporocarp surveys only correlate well with belowground data for some species [213]. Despite these drawbacks, fruiting body counts are a useful method to determine fungal reproductive output in an area, especially for large-scale studies. This has the advantage that citizen science projects can be included and that global species occurrence databases from museums and other institutions can be utilized in order to collate millions of observations into big datasets. These datasets in turn can be used to answer questions about long-term population developments in response to environmental changes [47, 214, 215].

EcM fungi were also commonly studied using so-called mycorrhizal morphotyping, which assumes that every EcM fungal species forms morphologically distinct mycorrhizal roots. Identification keys were developed in the 80s and 90s [216], and used widely in ecological studies of EcM fungi. However, since the widespread use of sequencing technologies several studies have shown that mismatching between sequencing and morphotyping results is common, because the same fungal species can form different morphotypes, and the same morphotypes can be formed by different species [181].

Another, more important method of studying fungi is culturing, which means cultivating the fungus of interest in a compatible growth medium, usually in a petri dish or flask. Culturing is a very straightforward way of studying microbes and has been used for a long time. We have gained understanding of many phenomena and processes by using cultured isolates, and it has led to the discovery of many new taxa [217, 218]. Culturing has the advantage of having the organism of interest available *in vitro*, which allows for endless possibilities for experimentation, observation, and manipulation. However, many microorganisms are notoriously difficult (if not impossible) to culture [219], although improvements in culturing media and techniques are starting to overcome this challenge in part [219–224], at least for bacteria and free-living fungi. Obligate symbiotrophs, which includes many EcM fungi and most AM fungi, can be more difficult to establish and maintain in culture over a longer time, depending on species. Generally, AM species cannot be cultivated without presence of the plant host [225].

3.2 Indirect methods to assess fungal biomass, enzyme activity and nutrient flows

Indirect methods must be used to determine fungal biomass, for which it is best to rely on ubiquitous markers that can be readily extracted from a soil sample. Components of the lipid cell membranes are commonly used, since they represent a proxy for the number of microbial cells in the sample. Ergosterol is very commonly used as a fungal marker [226], and has the advantage that it degrades relatively rapidly in dead mycelium [227]. Another commonly used microbial marker are phospholipid fatty acids (PLFAs), which can provide information of microbial biomass of different microbial groups, and to some degree even allows for division by taxonomy on a very high level [228]. In this way, one assay can be used to obtain an estimate of bacterial and fungal biomass, for instance the commonly used fungal PLFA marker 18:2ω6,9, which is considered a good indicator for EcM fungi and that has a high correlation with ergosterol abundances [229].

The activity of fungal enzymes can be assessed using enzyme assays. Enzyme assays to study biogeochemical reactions in soil samples have been utilized for almost 100 years [230]. The basic approach is to make an extract from the soil, and to then incubate said extract with potential substrates for enzymes of interest, with fluorophores used as indicators of a conversion that can be fluorometrically quantified. Enzyme assays provide the potential enzyme activity of a soil sample, not necessarily the actual *in situ* activity [230].

When it comes to nutrient fluxes between plants and fungi, stable isotopes like ^{15}N and ^{13}C can be used to determine strength and directionality of such nutrient transport processes [231]. The heavier isotope ^{15}N can be used for short-term labelling studies, where ^{15}N is added to a system and subsequently N-flow can be traced. Natural occurrences of stable isotopes can also be used to answer biological questions, because there is a tendency to discriminate against heavier isotopes in biological systems [232]. This makes it possible to infer previous nutrient fluxes from abundances of ^{15}N (expressed as $\delta^{15}N$ which expresses the ratio of ^{15}N and ^{14}N compared to a standard) in a sample, for instance ^{15}N abundances in plant tissues can inform on mycorrhizal presence and mycorrhizal type [232].

3.3 High-throughput sequencing

One of the most important methodological revolutions for the study of microbial communities has been the development of next generation sequencing (NGS), also called high-throughput sequencing (HTS), which enables the parallel sequencing of millions of DNA sequences in relatively short amounts of time and at ever-decreasing cost. Soon after its development, the first studies using HTS were performed on microbial communities [233, 234], coming to the conclusion that microbial diversity was much larger than previously thought. Nowadays, HTS is one of the most used methods for the study of microbial ecology, and we have seen a shift from HTS mainly being used for more explorative studies to more and more studies using HTS to answer specific hypotheses in combination with other methods. The most used sequencing methods today are Illumina, which yields paired-end, short (100-300bp) reads, and PacBio, which is a long-read technology yielding average read lengths in the tens of kilobases.

3.3.1 Amplicon sequencing

The most used sequencing method to study microbial communities is DNA amplicon sequencing (also called marker gene sequencing or metabarcoding), where a specific marker gene is PCR amplified from a sample and then sequenced. This marker gene is required to have variable regions that allow for taxonomic identification, while at the same time needing to have flanking conserved (for the group of interest) regions, where the PCR primers can anneal. For fungi, the internal transcribed spacer (ITS) region, located between the small and the large ribosomal subunits, is most often used for taxonomic identification [235]. The ITS region consists of the variable ITS1 and ITS2 sequences, interspersed with the highly conserved 5.8S subunit. When using Illumina short read sequencing, it is only possible to cover one of the two subregions if full length coverage is desired. It has been found that the ITS2 region is the better alternative for taxonomic resolution and accuracy [236], although other studies reported the difference to be marginal [237]. Even though amplicon sequencing has proven to be very useful for taxonomic profiling, it generally suffers from methodological biases such as not accounting for multiple rRNA copies per cell and preferential primer binding, leading to bias for or against certain taxa

[238, 239]. Moreover, amplicon sequencing is only semi-quantitative, meaning that we only obtain relative abundances of taxa and cannot infer absolute abundances from the results (i.e. the data is compositional).

After sequencing, there are several ways to process the data in order to obtain an approximation of species counts. Commonly used approaches include operational taxonomic units (OTUs), which are considered a species level approximation. For this purpose, quality filtered and processed sequences are clustered at a hard similarity threshold, for example 97% or 95% [240]. However, this approach has been criticized due to varying degrees of interspecific differences, leading to a loss in detection of some species [241]. For this reason, I have used a type of single linkage clustering in this thesis [242], which is expected to yield more biologically meaningful clusters than a hard threshold [243]. Another option is to not cluster the reads at all, and use processed and quality filtered amplicon sequence variants (AVSs) [244]. Recent studies, however, suggest that it is better to do some level of clustering to avoid inflation of species numbers due to intraspecific variation [245].

3.3.2 Metagenomics and metatranscriptomics

While amplicon sequencing only considers one marker gene, and is thus limited to taxonomic community composition profiles, there is also the possibility of sequencing all DNA (or at least a representative subsample of the total DNA) in a sample. Metagenomics aims to capture both genetic and taxonomic diversity of a microbial community. This approach is also called “shotgun sequencing”, since it is an untargeted sequencing of randomly fractured DNA from a sample [246]. Shotgun metagenomics has been vital for research on complex microbiomes and has led to many spectacular discoveries and new ways to approach microbiological research [247, 248]. The major disadvantage of metagenomics is that it can only tell us the genetic potential of a microbial community, that is the genes that are present in genomes, but not whether these genes are actually “used”, that is expressed and translated into functional proteins. Moreover, all DNA-based methods are unable to distinguish living from dead sources of DNA, and studies have shown that “relic DNA” makes up a substantial amount of DNA in environmental samples [249].

This is where metatranscriptomics shines. In contrast to DNA-based metagenomics, it is based on RNA sequencing (RNA-Seq), which captures actively expressed genes and the relative abundances at which

they are expressed. One major quality of RNA-Seq is that it covers the whole transcriptome, which in the case of an environmental sample (such as soil) would entail all the collective transcriptomes of all microorganisms in the sample. In plant samples, the majority of reads will originate from the plant host but we can also extract microbial reads to describe the functional activity of the endophytic and epiphytic microbial community [250]. RNA-Seq data from root samples has successfully been used to study both plant and microbial community response to abiotic stress in *Salix purpurea* [251]. Several studies have been performed on systems where the transcriptomes of one plant species and one specific fungal species (often termed dual RNA-Seq) in the roots have been investigated under different conditions [252–254], but studies looking at both host and the total fungal transcriptome in field studies are scarce. Nevertheless, there have been several successful studies on complex environmental metatranscriptomes [255–259]. Within the context of a holobiont system, the availability of functional information from both components (the collective transcriptomes of the plant host and the hosted microbial community) could be transformative in advancing our understanding of the development, dynamics, interactions, and effects of these two components [258, 260, 261]. Furthermore, RNA-Seq applied to a holobiont system would, in principle, enable taxonomic profiling of all represented species, offering taxonomic information in addition to covering the biological processes actively represented in the metatranscriptome. It is important to consider that the protocol details for RNA extraction and sequencing library preparation will determine which microbial species can be assayed. In particular, it is always necessary to deplete ribosomal RNA (rRNA) as this represents the vast majority of RNA but is not informative. The most common method for achieving this is to perform a selection for poly adenylated (polyA) RNAs but, importantly, while this removes rRNAs it also removes bacterial transcripts as neither are poly adenylated.

Despite all the benefits and potential of metatranscriptomics, there are some disadvantages that it is good to be aware of: Firstly, it cannot show actual biological activity, since mRNA is just a precursor and there are several post-transcriptional and post-translational processing steps that could change relative levels of the products of expressed genes beyond the abundance levels of mRNA [262, 263]; secondly, metatranscriptomic mRNA data is not able to differentiate between abundance and expression, which means higher counts of one gene could be due to higher gene expression, but also because of a higher overall cell

abundance of said organism. Adding to this second complication, it must be remembered that RNA-Seq data is compositional.

While DNA amplicon methods currently remain the most widely utilized approach of profiling microbial metacommunities, each of the above introduced methods have their strengths and weaknesses and the method or combination of methods employed within a study must be selected to ensure that the data generated enable answering the biological question or specific hypothesis. I have summarized the relative advantages and limitations of the methods used in this thesis in Table 3.1.

Table 3.1. *Summary of methods to study fungi that were used in this thesis, with possibilities, advantages, and disadvantages of each method.*

	What does it tell us?	**(+)**	**(-)**
PLFAs ***Paper II***	Fungal and bacterial biomass	- Quantitative method - Bacterial and fungal biomass can be distinguished	- Low taxonomic resolution - Only works in certain sample types
Enzyme assays ***Paper III***	Potential enzyme activity	- Shows ”realized” gene expression	- Noisy data - Only shows potential activity
ITS Amplicon sequencing ***Papers I, II, IV***	Relative abundances of fungal taxa	- Relatively cheap - High taxonomic resolution - Comprehensive databases	- Primer biases - Relic DNA - Multiple ITS copies
Metatranscriptomics ***Papers III, IV, V***	Fungal community gene expression	- Only shows active genes and species - Taxonomy and function	- Post-transcriptional regulation not captured - Abundance vs expression - Lack of reference databases

3.3.2.1 Metatranscriptomics – the “dry lab” part

When it comes to metatranscriptomics, generating the data is the relatively easier part, as far as RNA extraction from environmental samples and subsequent library preparation can be considered “easy”. When this data comes back from the sequencing facility, we are faced with millions of sequences from tens of thousands of genes, from thousands of different organisms. While the first steps after any sequencing run are always quality assessment and filtering, these reads can then either directly be aligned to reference databases, or they can be assembled *de novo* into contigs, of which as many as possible should represent full-length transcripts. Due to the complexity of such data, assembly is difficult and many of the available public tools and pipelines do not offer assembly with the reasoning that it is not worth the effort,

especially with short read technologies [264–266]. Full pipelines including assembly are rarer, but do exist [267]. The major advantages with an assembly-based workflow are i) it increases the proportion of the data that is used and ii) the on average bigger query length increases the probability of a correct taxonomic or functional assignment [268]. Several assembly algorithms for mixed species transcriptomic datasets exist, such as megaHIT [269], VICUNA [270], or MetaCRAM [271].

The next challenge after assembly is annotation. Functional annotation is relatively easier, since there are large databases available with many orthologous genes, allowing for at least an approximate functional assignment for significant parts of the data. Commonly used databases are the Kyoto Encyclopedia of Genes and Genomes (KEGG) [272], the database of Clusters of Orthologous Groups of proteins (COG) [273], the gene ontology resource (GO) [274], or the Carbohydrate-Active Enzymes database (CAZy) [275]. Conveniently, there is a tool called eggNOG mapper that combines orthology results from all of these databases into one result [276].

The biggest challenge for taxonomic annotation of metagenomic data has always been the low number of sequenced genomes of microbes in environmental samples [277]. Luckily, we are currently in the exponential phase of genome sequencing, so the situation is improved constantly. In this thesis, I have used the JGI Mycocosm resource [278] in **Papers IV** and **V**, which has gone from 1 681 to 2 181 published and annotated genomes in the last 12 months. Despite these promising numbers this is still only a tiny fraction of the true diversity of fungi out there, and thus we will have to live with uncertain taxonomic annotations for large portions of our metatranscriptomic data for the foreseeable future.

As other -omics methods, metatranscriptomic data can be used either to study the whole system at once, and to try to draw conclusions from the whole dataset (**Papers IV and V**), or we can use it to interrogate for example specific enzyme groups, in order to answer specific hypotheses (**Paper III**). Both approaches have their strengths and weaknesses. A more hypothesis-driven approach allows for the formulation of *a priori* questions and hypotheses, that the data then can be interrogated for, and follows the scientific method of deduction and testing of falsifiable hypotheses [279]. In contrast, explorative or descriptive methods can be used to interrogate a whole dataset and can also be seen as a part of hypothesis generation. A criticism of this approach is that it does not

follow rigorous scientific theory and can lead to biases, but it has also long since been acknowledged to be a necessary part of science [280].

3.3.3 Long read sequencing – the future (?) for both amplicon sequencing and metagenomics/transcriptomics

In the 2010s, sequencing technologies allowing for the sequencing of long, contiguous reads were first introduced. PacBio single-molecule real-time (SMRT) sequencing and nanopore sequencing are the two most established technologies [281]. In the beginning, these technologies could not really compete with short read HTS technologies, due to low output and high error rates of 10-20%. This has changed in a positive direction in the last years, and long-read technologies have been successfully applied to study microbial communities using marker genes [282, 283]. They currently still suffer from needing large amounts of high-quality input material, but the continued development of these methods will make it possible to improve both amplicon sequencing and metatranscriptomic approaches. In amplicon sequencing it will be possible to easily cover the entire ITS region, but also longer markers like, for example, the entire 16S region for bacteria, which enables much better taxonomic resolution and annotation [284, 285]. For metatranscriptomics, it will be possible to sequence entire transcripts, for example using the PacBio IsoSeq protocol [286], which relieves assembly difficulties and will enable higher taxonomic and functional resolution.

4 Results & Discussion

The main aim of this thesis was to further our understanding of how fungal communities in the boreal forest respond to N addition, which generally stimulates tree growth and, at larger scales, leads to accumulation of organic matter in forest soils. I have used HTS methods in combination with biochemical assays to elucidate fungal community response to both small doses of fertilizer to stimulate seedling survival and growth during early establishment (**Papers I** and **II**), and to high dose N addition in mature forests used to perturb the nutrient poor status and to study tree N limitation in the boreal forest (**Papers III**, **IV**, and **V**).

4.1 The importance of giving seedlings a "good start"

Forestry in Sweden is based on clear-felling followed by replanting the harvested area with genetically selected seedlings. This involves substantial investments in breeding programs, nurseries and other infrastructure to produce these seeds and grow the seedlings [287], and places high importance on maximizing seedling survival after outplanting, or seeding. It is therefore critical to provide the best possible initial growth conditions, taking into consideration as many factors as possible that can influence seedling survival and early growth, not only for economic reasons but also for the increased aboveground C sequestration that will result from higher survival and improved establishment of genetically selected seedlings. EcM fungi have been shown to provide growing seedlings with a number of benefits including improved nutrient uptake [189] and drought tolerance [126], and research in the last decades has shown that EcM are also crucial for nutrient cycling processes in the forest (discussed in section 2.1). In **Paper I** and **Paper II** we investigated the extent to which the addition of small doses of inorganic and organic N would support early seedling survival and growth, and at the same time whether the fungal communities associating with the growing seedlings would be affected by this treatment (positive or negative). For this purpose, we used ITS2 DNA amplicon sequencing to characterize the belowground fungal communities in early seedling establishment in two separate experiments.

4.1.1 Improvement of direct seeding using seedPAD in combination with fertilization

In **Paper I**, we used a clearcut site in the boreal zone of Sweden to investigate whether Scots pine seeding establishment using seedPADs, which consist of a protective envelope that increases survival after germination (Fig 4.1A) [43], was improved through the addition of small amounts of organic and inorganic N, and how this affected the fungal communities in the soil and on seedling roots. Arginine phosphate has been shown to improve seedling root growth and EcM colonization under nursery conditions [67], thus we hypothesized that there would be an increase in seedling root:shoot ratio and differences in fungal community composition on seedlings grown from fertilized seeds. We set out seedPADs with 10 mg, representing a highly localized low dose, of slow-release arginine phosphate or ammonium nitrate on a scarified clearcut site in northern Sweden.

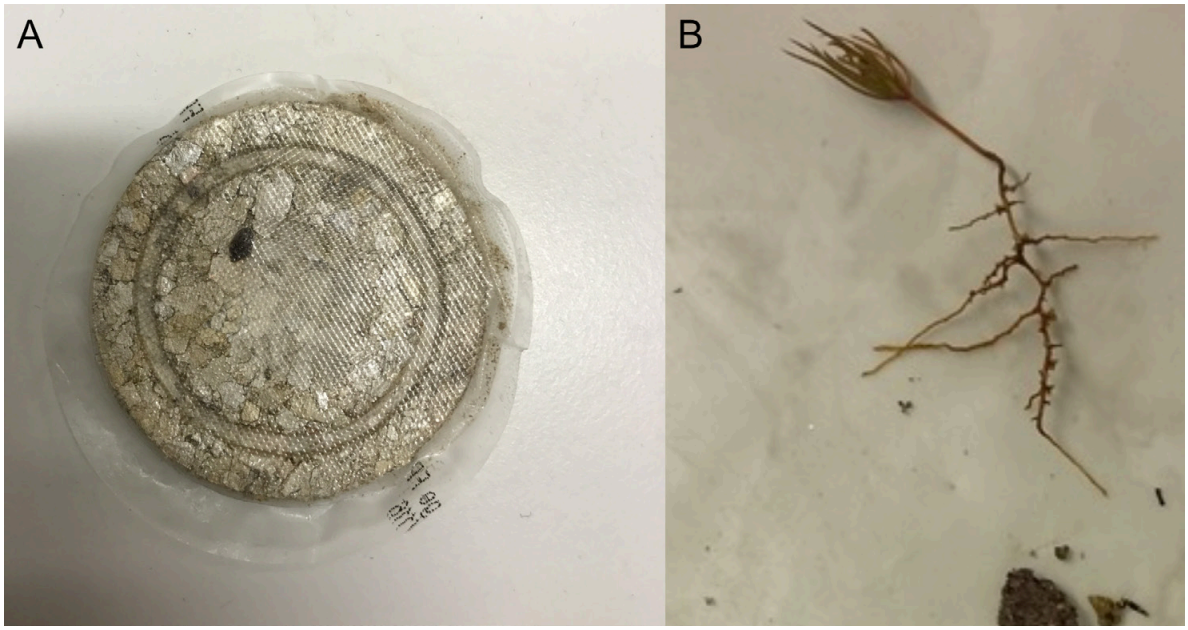

Figure 4.1. A*. SeedPAD with Scots pine seed. Slow-release fertilizer pellets can be added into the circular groove.* ***B****. Scots pine seedling after one growing season on a clearcut in northern Sweden, with clearly visible mycorrhizal root tips on main and secondary roots.*

One growing season after planting the seedPADs we assessed survival rates, collected the surviving seedlings (Fig 4.1B) for DNA extraction and collected soil samples. We found that the small amount of fertilizer (as expected) did not have a marked influence on soil N or C contents in the seedling rhizosphere (**Paper I**, **Fig 1**). Seedling survival and needle C content were increased significantly by both fertilization treatments, but

the treatments did not have an influence on other measured parameters such as root or shoot mass or needle N content (**Paper I**, **Fig 2**). We then used ITS2 sequencing on extracted DNA to characterize the fungal communities on seedling roots and surrounding rhizosphere and block soil. In control samples, seedling roots had lower alpha diversity values than the rhizosphere and scarified bare mineral soil samples (**Paper I**, **Fig 3**). In agreement with previous studies [32], we found the scarified clearcut soil to be dominated by saprotrophic and opportunistic taxa. The rhizosphere was dominated by taxa such as *Penicillium*, *Umbelopsis* and *Mortierella*, but we observed an enrichment of several symbiotrophic taxa in root samples when compared to rhizosphere and soil (**Paper I**, **Fig 4**), indicating a rapid recruitment on the growing seedling roots. In terms of EcM fungi specifically, several taxa were significantly enriched in root samples (*Russula*, *Piloderma*, *Suillus, Tylospora, Amphinema*), while others (identified as *Russula densifolia* and *Cortinarius* sp.) were not identified on seedling roots but only in the bulk soil samples. Most *Cortinarius* and some *Russula* species (*Russula* is a large genus, containing both nitrophobic and nitrophilic species) are known to be sensitive to disturbance and only increase in abundance in older forest stands [191], which might indicate that we sequenced remaining propagules, such as spores, mycelium or sclerotia from the previous stand [288], that however did not colonize the seedlings due to these preferences and adaptations [289].

Fertilization treatments did not modify colonization patterns significantly, and we found that there was a rich community of fungi on seedling roots including several EcM fungi such as *Tylospora*, *Russula*, *Piloderma* and *Suillus*, regardless of treatment (**Paper I**, **Fig 5**). We found arginine phosphate addition increased fungal richness, but without alpha or beta diversity effects, indicating a higher number of species with low abundance. Despite this lack of a general-scale significant fertilization effect on the fungal communities in roots and rhizosphere, we did find significant effects of fertilization treatments on individual fungal ASVs. For instance *Suillus*, which has been reported to be nitrophobic previously [161], had lower abundance in both fertilization treatments while the overall generally nitrophobic *Piloderma* showed a positive response to arginine phosphate addition. The dearth of any stronger fertilization effect on the fungal communities is probably best explained both by the small dose and the short timeframe of this study.

4.1.2 Fertilization of planted seedlings

SeedPADs (in combination with fertilization) may have the potential to increase survival of sown seeds and thus make direct seeding a viable alternative on more sites, but Scots pine is currently only seeded on about 1-2% of regenerated sites in Sweden (with close to 0% for Norway spruce) [21]. The majority of sites are planted with seedlings grown in nurseries, with both Norway spruce and Scots pine seedlings being produced and planted in large numbers (see section 1.2). While there have been studies on the effect of fertilizer on seedlings, and on the EcM communities of planted seedlings, the combination of both is not well understood, especially in combination with organic instead of inorganic N. In **Paper II**, we used a site in the nemo-boreal zone close to Norrköping in southern Sweden to investigate how planted seedlings and their fungal communities respond to planting position (capped mound or bare mineral soil, Fig 1.1C) in combination with small-scale fertilization with either inorganic or organic N. The sampling site is characterized by relatively high nutrient availability (for Sweden), with comparatively low C:N ratios measured in the soil and has a history of logging for cattle grazing up until the 19th century. Seedlings were planted in spring 2017, with 40 mg of N in the form of either arginine phosphate or ammonium nitrate slow-release fertilizer added into the planting hole. Seedlings and soil samples were sampled after one and two growing seasons to determine survival, growth, and fungal community composition in response to planting position (capped mound and bare mineral soil, Fig 1.1) and fertilization treatment. To assess fungal communities, we used ITS2 sequencing and resulting ASVs were clustered into swarm operational taxonomic units (SOTUs) using single linkage clustering.

Before planting, Norway spruce and Scots pine nursery seedlings were colonized to a large degree by typical nursery EcM taxa such as *Thelephora* and *Amphinema*, and a number of saprotrophic and opportunistic fungi [181, 182]. Norway spruce and Scots pine had similar alpha diversity and a large overlap in which taxa they were colonized by, but significantly different beta diversity, indicating differences in community composition (**Paper II, Fig S5**) stemming from consistent differences in relative abundances of these taxa. Previous studies found that differences between nurseries were bigger than differences between Norway spruce and Scots pine within the same nursery [182]. In our case it is hard to judge the scale of the observed differences between the two species as we have results from only one nursery.

After the first growing season, both Norway spruce and Scots pine control seedlings survived better in bare mineral soil. This difference can be connected to the 45% below-average precipitation in the first month after planting, since the bare mineral soil planting position provides better water availability to seedlings [36, 290]. Interestingly, Scots pine had consistently higher mortality in both planting positions (**Paper II, Fig 2A**). This was surprising as Scots pine often grows on dryer sites than Norway spruce and is described as having a greater drought tolerance [21]. However, some newer studies indicate that Scots pine seedlings might have a higher physiological drought susceptibility compared to Norway spruce [291, 292]. This could explain the contrasting results to those reported for drought tolerance, which is likely due to the deeper roots or lower surface transpiration of mature Scots pine trees [19]. However, we also have information from the nursery that some of the Scots pine seedlings were afflicted with grey mold (*Botrytis* sp.). While they were still considered healthy enough for sale and planting, this infection may have contributed to the lower observed survival and could possibly indicate that greater consideration should be placed on this as a factor influencing establishment and survival after outplanting. While we did not identify any *Botrytis* in our sequencing data, we found high abundances of several unidentified Helotiales SOTUs (that we could not identify beyond order level) on Scots pine seedlings, potentially supporting this prior infection. Norway spruce also grew better in bare mineral soil, while Scots pine showed no growth differences between planting positions (**Paper II, Fig 3**). After the second growing season we measured plant height and stem diameter and found no remaining significant aboveground growth differences between Norway spruce and Scots pine control seedlings.

Root samples of control seedlings, expectedly, changed significantly in both alpha and beta diversity after the first growing season after outplanting. Despite the obvious effect of novel colonization by species not present in the nursery, the majority of nursery fungi were still present after one season in the field (Fig 4.2). This persistence of nursery species has been reported by several previous studies [30, 185–188] and might be due to so-called priority effects, meaning that fungi that are already present have a better chance of colonizing new roots than site indigenous fungi that have to colonize roots *de novo* [190]. Norway spruce roots had higher richness after one year, but Scots pine had more significantly differentially abundant (DA) novel colonizers, indicating a more consistent colonization by fewer species in Scots pine. This might be attributable to the site being populated mostly with Scots pine before harvest, which would explain the higher number of DA colonizers, but

not the higher richness in Norway spruce. We compared fungal communities between planting position for both Norway spruce and Scots pine, finding significant differences only for Norway spruce, and only after the first growing season. This may be related to the increased root growth, which was only observed in Norway spruce growing in bare mineral soil and that could have led to a higher degree of novel colonization. While there were no large differences between the first and the second growing season in terms of fungal community composition, we observed a higher differentiation between first and second growing season for Norway spruce (**Paper II, Fig 3A**) than for Scots pine (**Paper II, Fig 3B**), which again could be connected to the better growth in the first year for Norway spruce. After the second growing season, seedling roots were still, to a large part, dominated by nursery species, and novel colonizers were often from closely related or different phylotypes of the same species (Fig 4.2). Overall, we observed that many (more than half) of the SOTUs present on nursery seedlings were also present at the site, which would be consistent with the site being relatively nutrient rich (C:N ratios lower than on other, comparable sites in Sweden [16, 171]) and having a history of disturbance (logging for pasture in the past).

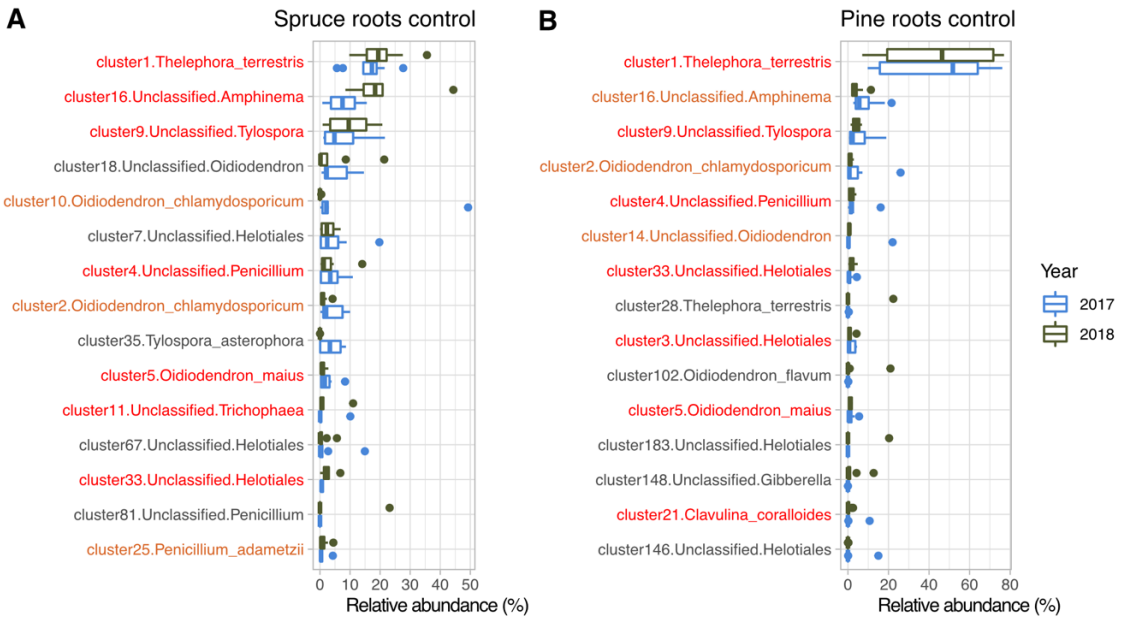

Figure 4.2. *The 15 most abundant swarm operational taxonomic units (SOTUs) in Norway spruce (**A**) and Scots pine (**B**) seedling root samples after planting. Boxplots show relative abundances split by year. Colors indicate nursery presence: SOTUs present in nursery root samples (>0.01% average rel. ab.) are colored orange, and SOTUs that were among the 15 most abundant in nursery root samples are colored red.*

Due to their important role in tree nutrition, we were interested in EcM fungi specifically and how they were affected by planting position and tree species. The site was surprisingly poor in EcM fungi, even in the surrounding forest, and instead strongly dominated by saprotrophs and putative ErM fungi such as *Oidiodendron*, *Trichophaea*, *Meliniomyces*, and *Penicillium*. Many of the EcM fungi we identified at the site were taxa that are known to tolerate high N and disturbance, e.g. *Thelephora terrestris*, as well as several members of the Atheliaceae such as *Tylospora* and *Amphinema*, but also several *Suillus* species, which are considered to be more nitrophobic [161]. The bulk of DA EcM fungi were novel colonizers (i.e., significantly higher abundance compared to nursery to field roots), confirming that most of the nursery fungi persisted on seedling roots after outplanting. Most novel colonizers were already found after the first growing season, and there were few novel EcM colonizers after the second growing season (**Paper II, Fig 6**).

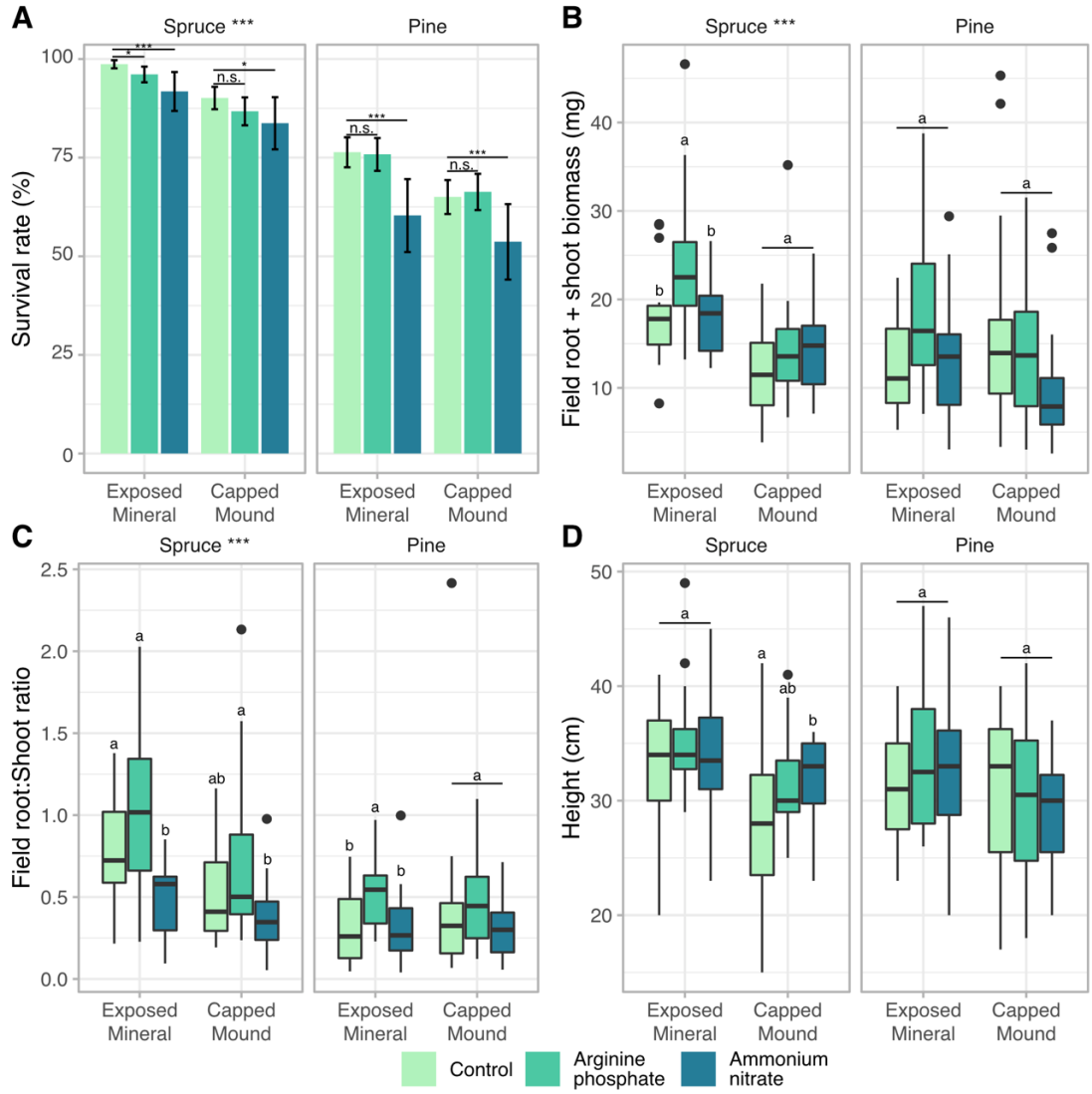

Figure 4.3. *Survival and growth statistics of Norway spruce and Scots pine control seedlings colored by treatment, growing in exposed mineral or capped mound soils after one growing season. Number of stars indicate p-value: *: p<0.05; **: p<0.01; ***: p<0.001. n.s.:p>=0.05. Letters above boxplots (**B**, **C**, **D**) represent statistical differences resulting from Kruskal-Wallis Rank Sum test, and whiskers represent 1.5 × inter-quartile range (IQR). **A**. Barplot of survival rates. Stars represent statistical differences of Cox proportional hazard regression. Error bars represent 95% confidence intervals calculated after survival curve. **B**. Boxplots of combined biomass of shoot and field roots. **C**. Boxplots of field root:shoot ratio. **D**. Boxplots of seedling height.*

After the first growing season, the addition of ammonium nitrate at the time of panting had significant negative effects on survival on both tree species in both planting positions (Fig 4.3A), with no consistent effects on field root growth or root:shoot ratio. We can only speculate on the reasons for this, but for one we know that uptake of inorganic and organic N are regulated differently in seedlings [293]. These seedlings

were acclimatized to an amino acid fertilization regime in the nursery, and the change to inorganic N around the roots, in combination with drought stress, could have led to a higher mortality. The control seedlings were exposed to the clearcut soil, which we would expect to contain a high proportion of organic N sources [59]. Moreover, inorganic N availability is known to increase plant transpiration through aquaporin activity [294], which may also have added to the drought sensitivity [295]. Arginine phosphate did not have major effects on survival after the first growing season, but increased seedling root:shoot ratios, especially in bare mineral soil (Fig 4.3C), which is in agreement with the previous nursery studies [67]. Fungal communities were largely unaffected by fertilization, with no significant alpha or beta diversity effects (**Paper II, Fig 8**), or effects on fungal biomass (**Paper II, Fig S12**). However, similar to **Paper I**, there were a number of SOTUs that were DA in response to the fertilization treatments (**Paper II, Fig S11**). The overall limited fungal response to fertilization does fit within existing literature, and we know from large-scale studies that relatively large amounts of N and long time frames are needed to significantly change fungal community composition [160, 171, 296].

4.1.3 Conclusions

Both **Paper I** and **Paper II** investigated the fungal communities of regenerating seedlings, either seeded or planted, in scarified clearcut soil. Previous studies have described how the fungal community changes from EcM dominated to a community dominated by saprotrophs and opportunists after clearcutting and soil preparation. Clearcutting removes the trees and thus eliminates C flow to EcM fungi [297] and reduces their competitiveness against saprotrophs [33, 142]. Over a timeframe of about 20 months this results in a fungal community dominated by saprotrophs [32], and initiates large-scale decomposition processes breaking down dead roots, plant material and microbial necromass. The additional process of soil scarification disrupts the mycelial networks in the upper soil layers and is likely to speed up this process. In both these studies we have shown that seeds or seedlings planted into this disturbed environment are rapidly colonized by site indigenous fungi, including many EcM. This can be seen as the start of a new EcM succession cycle, where the growing trees over the next decades will be progressively colonized by species that are more associated with older forests [191]. We have shown that small doses of N increases survival of seedPAD seeds and that organic N can increase root growth, which in the long term might lead to better seedling establishment. We found the effects of this small-scale N addition on the

fungal communities to be negligible, which can be seen as positive as it does not add an additional disruption to the regeneration dynamics. The long-term effects of this fertilization on both seedling growth and fungal community succession remains to be tested in further experiments.

4.2 Large scale N addition and the effects on fungal communities

As shown in the previous results section, targeted small-scale addition of organic N can improve early seedling survival and (root) growth, while overall not having large effects on the fungal communities establishing around the seedling roots. This can be contrasted with the large-scale N addition experiments at the Flakaliden and Rosinedal experimental sites (Fig 1.2), which were used for the second part of this thesis (**Papers III, IV, and V**), and where the fungal communities underwent significant changes after long-term addition of ammonium nitrate (Rosinedal) [160] or a full nutrient solution for optimized Norway spruce growth (Flakaliden). At the Flakaliden site, long-term nutrient enrichment (NE) has led to a fourfold increase in tree aboveground biomass [157], while decreasing the relative flux of photosynthates belowground [298] and decreasing soil respiration [151]. A first study on fungal community response at the site used mycorrhizal morphotypes [299], and found that fertilization had a significant influence on community composition. A later study using PLFAs found an overall decline in microbial biomass after NE, and a decreased fungi:bacterial ratio [300]. The first sequencing-based study [171] used ITS1 amplicon sequencing to investigate the fungal community composition under the influence of seasonal and short term (5 years) and long term (25 years) nutrient enrichment. The main findings were that after initiation of NE, the fungal community slowly shifts from a community strongly dominated by nitrophobic genera, such as *Cortinarius* and *Piloderma*, to a community with higher alpha diversity, and a more even community composition, with a marked increase in abundance of many nitrophilic taxa such as *Tylospora* or *Hyaloscypha*.

4.2.1 Why does nitrogen addition to forest soils inhibit decomposition?

When large amounts of N are added to boreal forest sites, decomposition is inhibited and organic matter accumulates [15], which is associated with an inhibition of soil respiration and substrate mineralization [75, 151]. Oxidative enzymes are crucial for decomposition of organic matter

[301], which led us to the hypothesis in **Paper III**, that the inhibition of decomposition after N addition is caused by a relative shift in efficiency of different enzyme classes. Logically, one could think that N addition should ease N limitation at least for saprotrophic microorganisms and thus stimulate decomposition. However, representatives of both EcM fungi and saprotrophs are inhibited in their overall mycelial growth after N addition [169, 302]. For a long time, it has been observed that this disproportionally affects fungi with "white rot" ligninolytic capabilities [303]. It was previously thought that this is due to the formation of recalcitrant compounds by chemical reactions called "browning". Browning, in short, entails a side reaction of lignin breakdown that was thought to increase with higher amounts of amino compounds, which are a consequence of added N [304]. However, further studies showed that these compounds were not actually present in the soil at high abundances and that these findings were an artefact of the soil extraction methods used at the time [305].

For this study we explored the hypothesis that these browning reactions still occur when oxidative enzymes are used in high N conditions, but that they do not end up creating large amounts of recalcitrant compounds. Instead, several additional steps must be catalyzed to break down these additional compounds that result from browning reactions. These additional reactions by themselves are not very energy intensive but create just enough of an extra energy cost to render the already "expensive" strategy of white rot decomposition not worthwhile and competitive anymore (**Paper III, Fig 2**). To test this hypothesis, we used a combination of metatranscriptomic data and enzyme assays to test whether the ratio of white rot to brown rot activity would decrease after long term N addition. Both transcriptomic data (**Paper III, Fig 3**) and enzyme assays (**Paper III, Fig 5**) showed that oxidative enzyme activity was reduced, while enzyme activity associated with brown rot was not affected, thus supporting our hypothesis. These results are corroborated by other studies [303], and further evidence for this hypothesis has come from an additional study using spectroscopy methods to discern the chemical composition of mor-layer soil along a fertilization gradient (at the Rosinedal site, Fig 1.2), and showing an increase of lignin derivatives in fertilized soil [78]. We believe this mechanism to be of higher importance in colder ecosystems, where lower soil temperatures often limit enzymatic reactions. In tropical ecosystems, fertilization should thus not lead to accumulation of organic matter, which is concordant with a meta-study looking at soil respiration in response to N addition [306]. In this study, the majority of oxidative enzyme metatranscriptomics sequencing reads were determined to

originate from saprotrophic fungi. However, EcM fungi such as *Cortinarius* (discussed in section 2.1.3, Fig 2.2), which also rely on oxidative enzymes and that have been shown to participate in decomposition [169], are likely to also be an important part of this story.

The implications of this are that N addition hampers decomposition in cold climates, at least in part by rendering the oxidative enzymes used for "white rot" ligninolytic decay energetically uncompetitive. This further confirms that N deposition may result in higher C sequestration, but also means that this balance might shift when temperatures rise.

4.2.2 Designing a metatranscriptomic workflow to study fungal communities

The current *de facto* standard of studying microbial communities is DNA amplicon sequencing, which is used to infer taxonomic profiles that, for example, can be used for alpha and beta diversity analyses. Metatranscriptomic data can be used to study microbial activity, providing functional information and insights. However, whether it also captures the same taxonomic information as amplicon data and can be used to perform the same alpha and beta diversity analyses has received little attention to date, with few systematic comparisons. One example using total RNA sequencing on human stool samples concluded that metatranscriptomic data have higher sensitivity and reproducibility than both ITS and 16S amplicon sequencing data [307]. Currently, what is seen more commonly is that amplicon sequencing (or DNA based metagenomics) is used in combination with metatranscriptomics in a complementary way, in what has been called "multi-omics" integration [308, 309]. In **Paper IV**, I made such a comparison using ITS1 amplicon sequencing data and mRNA sequencing data from the same samples; needle and root samples from Norway spruce trees growing at the Flakaliden site. I used samples from NE trees that had been fertilized with an optimized nutrient solution for 5 years and 25 years at the time of sampling, in comparison to the nutrient limited (NL) control plots. Samples were taken at four different seasonal timepoints in the growing season, and then used for RNA-seq and ITS1 amplicon sequencing. The aims of this study were: i) to implement a bioinformatic pipeline for metatranscriptomic data that filters fungal reads, assembles them into transcripts and assigns taxonomic and functional profiles ii) to compare the taxonomic profiles to those of ITS1 amplicon sequencing from the same samples and iii) to demonstrate some of the additional possibilities we have using the resulting functional profiles.

The first aim was to create a metatranscriptomic pipeline, and we wanted this workflow to separate host and fungal reads and to perform a *de novo* assembly of fungal transcripts. This necessitated writing a new pipeline as there was no suitable, existing option. First, fungal reads were selected by mapping to the taxmapper (which has a number of representative genomes spread across the tree of life) [310] and JGI Mycocosm [278] databases. Selected reads were cross-checked against the Norway spruce reference genome [311]. We compared different assemblers, determining that megaHIT [269] produced the most suitable transcript assemblies due to better length statistics and a higher number of reads assigned to assembled transcripts with open reading frames (ORFs). The resulting assembly comprised 547 305 ORFs. The eggNOG mapper [312] was used to obtain functional profiles from different databases (see method section). For the comparison between taxonomic profiles in the two datasets, it was important to assign taxonomy as accurately as possible, for which we used a tool based on DIAMOND (a fast equivalent to blastp) and taxon-specific thresholds [277, 313], called contigtax [314], combined with the Mycocosm and taxmapper databases. The majority of ORFs received at least some level of taxonomic or functional annotation (**Paper IV, Fig 1A**; Fig 4.4A). ITS1 data was preprocessed using dada2 [244] and then single-linkage clustered using swarm [242] (**Paper IV, Fig 1B**; Fig 4.4B). Taxonomy was assigned using the UNITE database [315]. In the RNA data, root samples had consistently much higher number of fungal reads than needle samples (**Paper IV, Fig 1A, inset 4**).

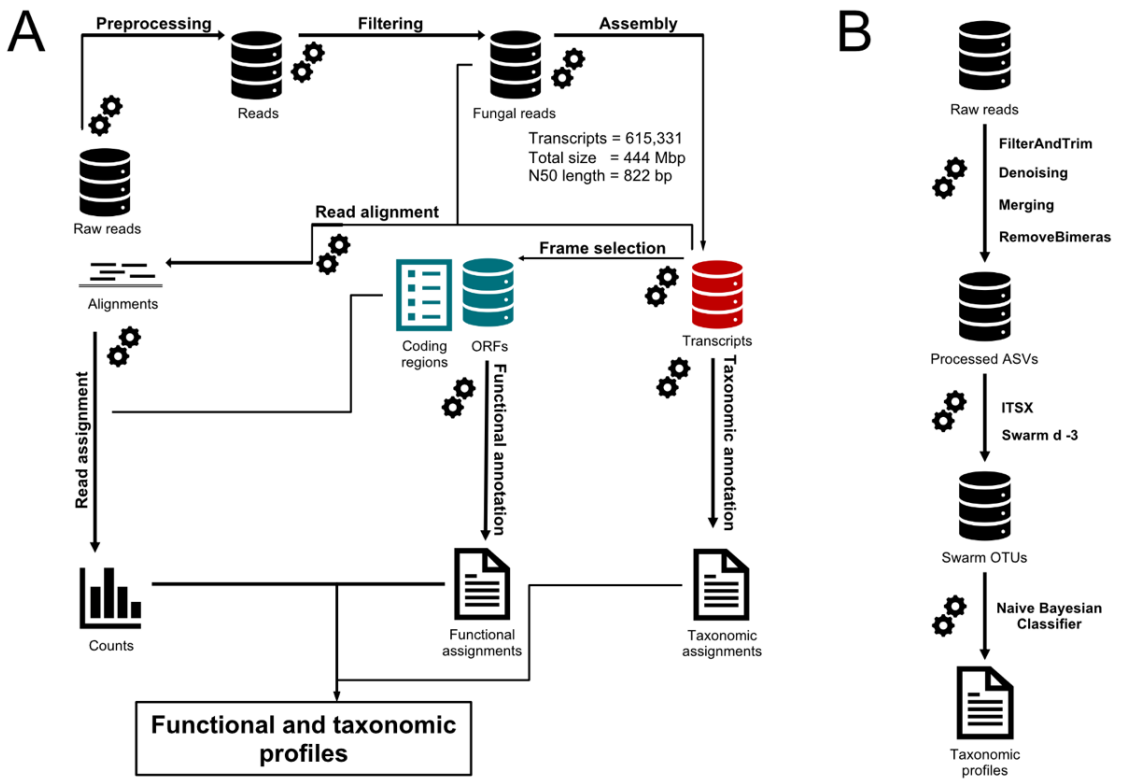

Figure 4.4. *Paper IV bioinformatic pipelines overview. A more detailed version of this figure can be found in Paper IV (Fig 1) [3].* ***A.*** *Metatranscriptomic pipeline. After preprocessing and filtering using fungal genomes, fungal reads were assembled using megaHIT* [269]. *Assembled transcripts were used for taxonomic annotation. Open reading frames (ORFs) were used for functional annotation using eggNOG mapper* [276]*, and for calculating read counts. Functional and taxonomic profiles were used for analysis and comparison to internal transcribed spacer (ITS) sequencing data.* ***B.*** *ITS amplicon sequencing pipeline. Raw reads were filtered, trimmed, and denoised before merging of forward and reverse sequences and chimera removal. Processed ASVs were filtered using ITSx* [316] *and clustered using swarm* [242].

As a first comparison between the two datasets, I looked at NL control samples and compared ITS and transcript data from needle and root samples. The sample clustering pattern was highly congruent using ITS and transcript data, with a clear difference between sample types and with the needle samples showing much smaller variation, despite higher richness observed in needle samples in the ITS data and a much smaller number of transcripts in needle samples in the RNA data (**Paper IV, Fig 2**). This low number of transcripts, only about 2 000 transcripts remained after filtering to remove low abundance transcripts, led us to decide not to consider needle samples for the later analyses in this study. In phyllospheric tissues, the relative fungal load (ratio of fungal to host nucleic acid in extract) is significantly lower than in roots [317, 318],

which, without amplification steps, yields less comprehensive data. This data can still be analyzed and inform about the activity of the phyllospheric fungal community [250], but I reasoned that focusing on root samples would ensure a fairer and more comprehensive methodological comparison.

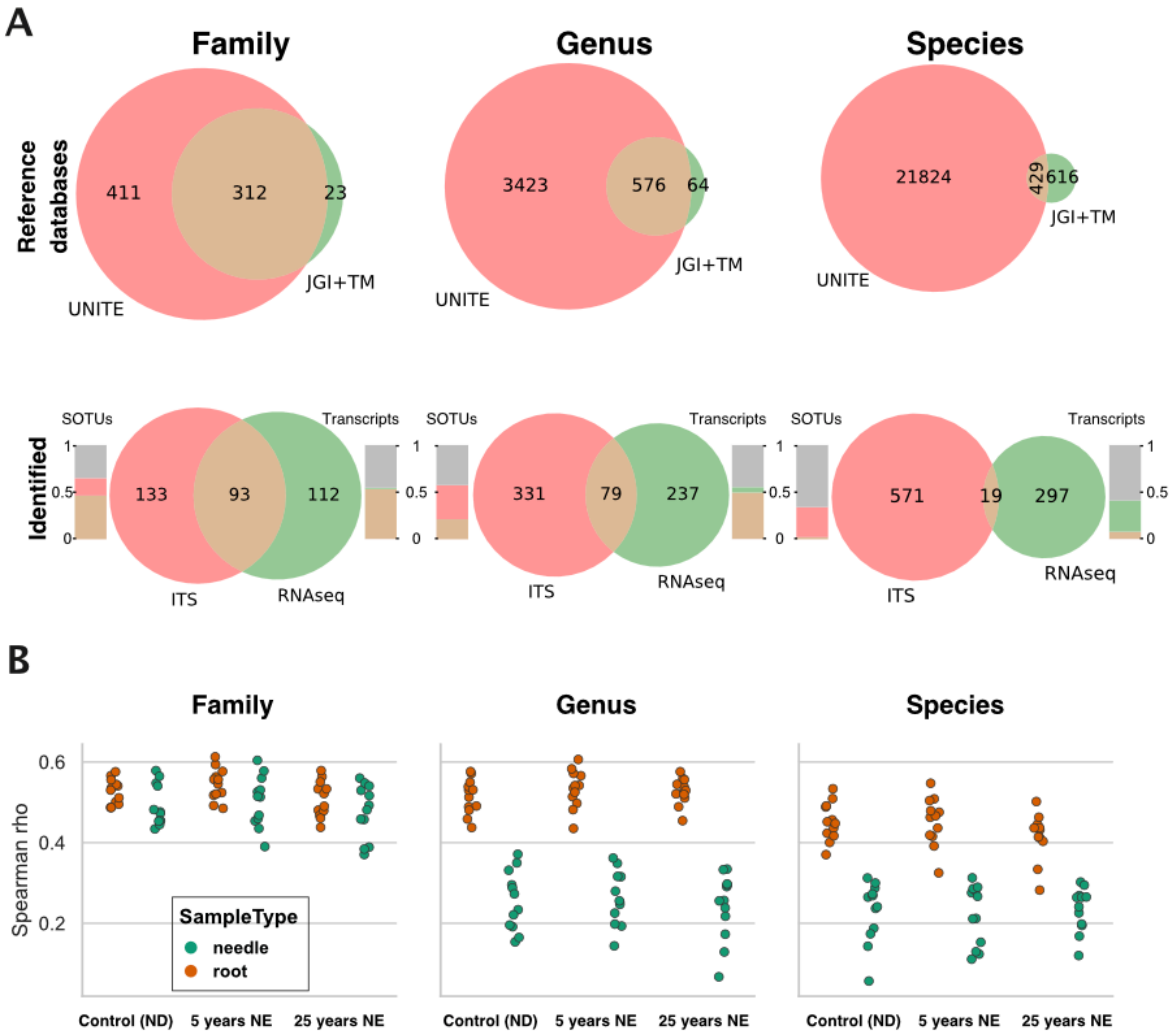

Figure 4.5. *Taxonomic congruence between RNA-Seq and ITS amplicon sequencing data sets.* ***A.*** *(Top) Venn diagrams showing taxonomic unit overlap between the UNITE database and the JGI MycoCosm and TaxMapper (JGI+TM) genome databases at the family, genus, and species levels. (Bottom) Taxonomic unit overlap of identified taxa in the RNA and the ITS sequencing data sets. Bars indicate the proportion of transcripts and Swarm operational taxonomic units (SOTUs) belonging to the unique and common portions of the Venn diagrams (colors correspond; gray indicates unidentified transcripts/SOTUs on the corresponding taxonomic level).* ***B.*** *Spearman rank correlations of taxonomic abundance (family, genus, and species levels) between RNA-Seq and ITS amplicon sequencing samples. Colors indicate samples of root (brown) or needle (green) origin.*

To be able to interpret the taxonomic annotations between the datasets I decided to first perform a comparison of the taxonomic annotations between the databases used to annotate the data (JGI Mycocosm and UNITE), since those are the basis for, and limit, the annotations obtained (Fig 4.5A). I found progressively lower overlap at lower taxonomic levels, especially in the datasets, but also in the databases. This low overlap, apparent in the databases and compounded in our data, clearly highlights that the current main limitation with metatranscriptomic data is the number of available reference genomes. This is likely to improve in the future as more genomes are sequenced and more annotated ITS sequences are added to the UNITE database. The Spearman's rank correlations between taxonomic abundances in the two datasets, expectedly, also decreased with lower taxonomic levels (Fig 4.5B), and more so in needle than in root samples (further validating the decision to focus on root samples). Comparing treatment effect on root samples in both datasets, I found that a principal coordinate analysis (PCoA) on ITS1 samples and principal component analysis (PCA) of RNA samples clustered samples in a highly congruent manner (Fig 4.6A+B). Similarly, Shannon diversity index values on genus level had high correlation between the two datasets (Fig 4.6C). As such, the two methods provide similar insights into the among-sample relationship. Next, I compared the most highly abundant taxonomic annotations on family level between the two datasets, finding that some families correlated well between the two datasets (e.g. Cortinariaceae), while others exhibited lower to low correlation (**Paper IV, Fig 5A**). These differences might stem from methodological biases known from amplicon sequencing [239], but it has also been found that DNA and RNA abundances do not necessarily correlate well in general [319, 320]. There was a lower proportion of transcripts that had any assigned taxonomic annotation at the family level, and, importantly, RNA-seq metatranscriptomics cannot separate abundance and expression. Considering these limitations and confounding factors it would almost have been surprising to have identified a higher congruence than we observed.

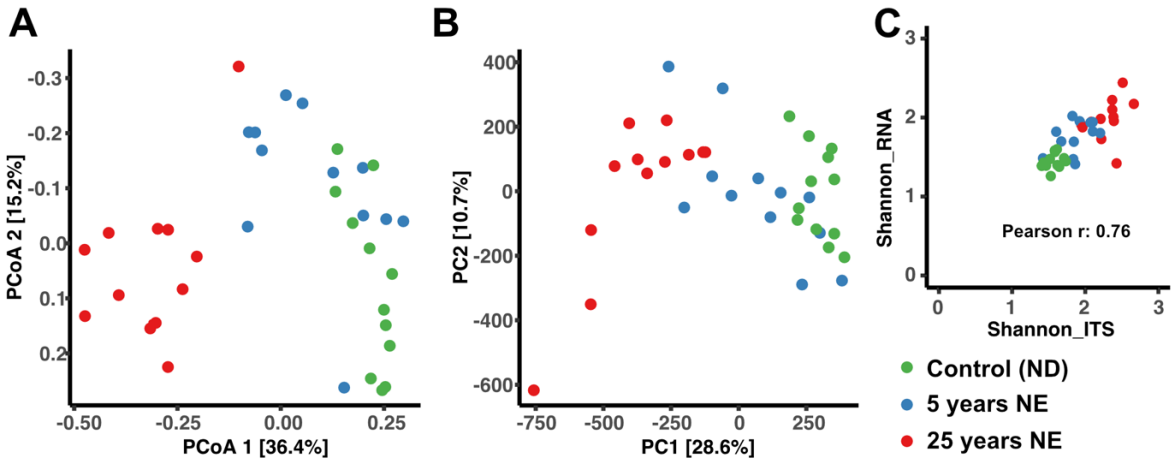

Figure 4.6. *Ordination and alpha diversity index comparison of root samples.* ***A.*** *Principal-coordinate analysis of rarefied Swarm operational taxonomic unit (SOTU) counts, colored by treatment.* ***B.*** *Principal-component analysis of variance stabilization transformed transcript counts, colored by treatment. (C) Sample-wise relationship between Shannon diversity index values (genus level) using ITS amplicon sequencing (x axis) and RNA-Seq (y axis), colored by treatment.*

As a final comparison, we ran a random forest classifier (on species level) to classify samples by treatment to determine whether we observed higher biological signal in either data type, but found similar accuracies were achieved using both datasets (**Paper IV, Fig 5B**). Interestingly, in the ITS data the same accuracy was achieved using fewer, and more highly abundant, species than in the RNA data. In bacterial communities it has repeatedly been shown that low abundance community members can have significant functional importance [321–323], which is potentially a part of the explanation here as well.

In conclusion, we compared the taxonomic profiles between ITS and RNA data and found that while the taxonomic annotations on lower levels did not overlap greatly, we could still draw similar conclusions about the fungal communities in the samples and the relationship among samples based on ecological diversity analyses and random forest classification.

Further analyses were performed on the metatranscriptomic data to explore the functional annotations deeper in addition to the taxonomic information. We ran random forest analysis on KEGG ortholog (KO) level, to see if a similar classification accuracy could be achieved as by species annotations (**Paper IV, Fig 6**). Classification accuracy was indeed similar for treatment and higher for seasonal timepoint, and by

using feature importance we could identify KEGG pathways that were of high importance in distinguishing between NL and 25 years NE treatments. In the carbohydrate metabolism pathway category, KOs related to carbohydrate metabolism decreased in abundance, while KOs more involved in amino acid metabolism increased in abundance. Summing by KOs has the advantage that the data becomes less sparse (i.e., suffers less from zero inflation), which might have allowed for better classification compared to random forest on species level. We also observed a seasonal signal that was obscured on species level and that probably has a functional explanation, although we did not explore this further.

As an alternative to the random forest analysis, I conducted a differential abundance (DA) analysis on KO level and tested for DA KOs between 25 years NE and NL at the four seasonal timepoints (**Paper IV, Fig 7A**). This identified seasonal differences in functional differentiation between NL and NE treatments, and demonstrated that we can perform KEGG pathway enrichment analysis (**Paper IV, Fig 7B**) and taxonomic profiling of these KOs (**Paper IV, Fig 7C**) to elucidate functional and taxonomic level responses to treatment. Finally, I extracted all genes from one taxon (the genus *Cortinarius*, Fig 2.2) and examined transcript abundance dynamics in the NL and the 25 years treatments (Fig 4.7). *Cortinarius* was selected as an interesting example as it shows a widely reported negative response to N addition [161], and relies on oxidative enzymes [167]. A first notable observation was that even after 25 years of NE, *Cortinarius* was still present and transcriptionally active in low abundances. *Cortinarius* transcripts were divided into three top level clusters based on expression patterns, of which only one (albeit by far the biggest cluster) showed consistent strong decrease in abundance after 25 years of NE. GO enrichment in this strongly decreasing cluster showed several GO terms associated with the metabolism of aromatic compounds (Fig 4.7B), which fits well with the results of **Paper III**, and is in line with previous literature, indicating that *Cortinarius* are nitrophobic, rely on oxidative enzymes and participate in decomposition [110, 174].

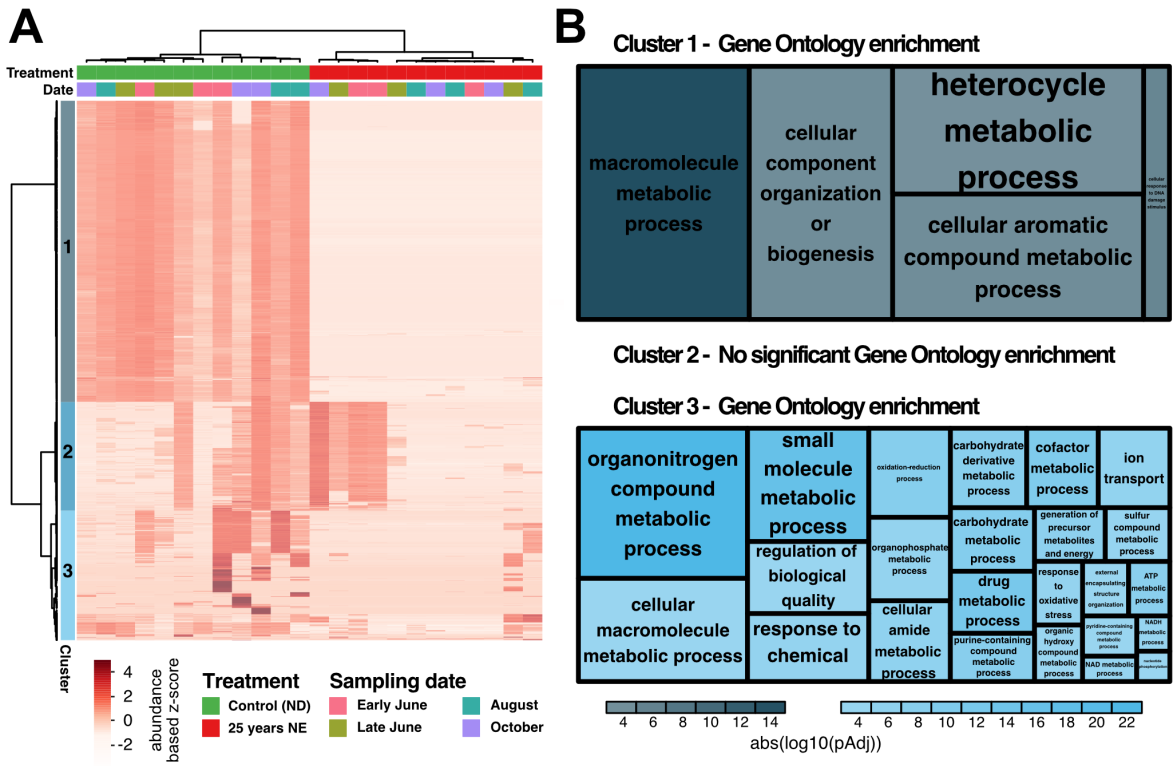

Figure 4.7. *Hierarchical clustering of transcripts assigned to the genus* Cortinarius *and Gene Ontology (GO) enrichment.* ***A.*** *Heat map of all transcripts assigned to the genus* Cortinarius. *Normalized variance stabilization transformed expression values were transformed to z-scores per row to highlight differences between samples. Hierarchical clustering of samples and transcripts was performed using Ward's minimum variance method. Colors on top indicate treatment and sampling date, while colors to the left indicate the three highest-level clusters. A color legend is provided below the heat map. The Venn diagram below the heat map shows KEGG orthologs (KOs) derived from the three clusters and the respective overlap between them.* ***B.*** *Tree maps showing GO enrichment of all transcripts identified as belonging to the three highest level clusters. The upper tree map summarizes significant GO enrichments for cluster 1 (4,047 transcripts), and the lower tree map shows the enrichment for cluster 3 (1,741 transcripts). There were no significant enrichments for cluster 2 (1,465 transcripts). Color intensity is determined by adjusted P value of the enrichment, while rectangle size is proportional to the number of transcripts mapping to the respective GO term. Table S3 shows all terms and corresponding statistics.*

In conclusion, we demonstrated that RNA-seq metatranscriptomics, under the condition of a sufficient microbial load, can provide similar information on alpha and beta diversity of fungal communities as ITS amplicon sequencing. With improved technologies and larger numbers of reference genomes, taxonomic and functional accuracy of metatranscriptomics will be improved. In this study, we did not make use of RNA-seq reads from the host tree, which comprised the majority of the RNA-Seq data from these samples. Combining these two sources of transcriptome data would enable many additional analyses, providing insight into the interaction between host and associated community. In

Paper V, we performed such a combined analysis using this metatranscriptomic workflow combined with host tree transcriptomics to gain a deeper understanding of the EcM symbiosis and how it is influenced by N addition.

4.2.3 Metatranscriptomics captures dynamic shifts in mycorrhizal coordination in boreal forests

Soil microbes in general are important mediators of C and N cycling globally (see section 2.1.3), and form mycorrhizal symbioses with the majority of plants [105]. The tree-EcM holobiont has crucial importance for the boreal forest ecosystem, with global implications [86, 324]. In **Paper V**, we used the metatranscriptomic workflow designed in **Paper IV** in combination with host tree transcriptomics to study transcriptomic responses (to long-term NE) of Norway spruce and hundreds of associated fungal species, obtained from root samples. As in **Paper IV**, the Flakaliden site was used, but the main dataset for this study was a seasonal sampling profile with 19 timepoints, covering the whole growing season. This was essential to be able to undertake such a fine-grained and in-depth coordination analysis of host and mycobiome transcriptomics. One challenge with such highly temporally resolved data is replication, which is why the higher replicated 2012 dataset (used in **Paper IV**) was used to cross-validate the 2011 dataset (**Paper V**), which was generated from only one replicate block per treatment (with only within-block replicates).

In roots from NE samples, the proportion of fungal reads was significantly lower than in NL samples (**Paper V, Fig 1A**), supporting previous results showing a decrease in fungal biomass after NE [300]. NE had the largest effect on the overall fungal transcriptome summed by KOs (**Paper V, Fig 1B**), while seasonal timepoints did not have a clearly discernible influence. On a taxonomic level, the fungal metatranscriptome was dominated by the basidiomycete families Cortinariaceae, Atheliaceae and the ascomycete families Gloniaceae and Hyaloscyphaceae. Within the first three of these families, the majority of reads were assigned to one species each: *Cortinarius glaucopus*, *Piloderma olivaceum*, *Cenococcum geophilum*, while in Hyaloscyphaceae most reads were assigned to the two species *Meliniomyces bicolor* and *M. variabilis*. From comparison to the published ITS data (**Paper IV**, [171]), we believe that the true number of species is higher and that each of these species level assignments are likely to include at least several species from these genera. However, we decided to use these species level annotations for further analyses as

available reference genomes did not allow for greater taxonomic resolution. As in the amplicon sequencing results, *Cortinarius* (Fig 2.2) and *Piloderma* decreased significantly in relative abundance in NE, while *Cenococcum* and *Meliniomyces* increased, concordant with previous descriptions of these taxa as nitrophobic and nitrophilic, respectively.

A co-expression network of fungal expression data was constructed, showing a separation into two "hemispheres" (**Paper V, Fig 2A**) of which each was dominated by these species with different responses to NE, respectively. *Cortinarius* and *Piloderma* showed high functional modifications in expression profiles in response to NE, with many processes linked to fungal growth having lower abundance in NE conditions. *Cenococcum* remained more stable between the treatments (**Paper V, Fig 2C**) and consistently expressed many genes related to melanin biosynthesis and competitive interactions with other microorganisms, which suggests a combination of competitiveness and resilience, enabling it to persist in NE conditions.

In contrast to the fungal community, Norway spruce gene expression was influenced to a larger degree by seasonal timepoint, which was common to both treatments (**Paper V, Fig 3A**). Functional enrichments tests showed that these seasonal changes were to a large part connected to growth processes, as would be expected. NE did not significantly modify these seasonal patterns, but instead changed the magnitude of expression dynamics. Processes with higher expression magnitude were largely found to be connected to defense mechanisms and stress responses. In contrast, one group of transcripts with lower magnitude after NE were sugar efflux transporters, such as the family of Sugars Will Eventually be Exported Transporters (SWEET), which play an important role in plant C export to the rhizosphere [325]. Both upregulation of defense responses and reduced sugar efflux indicate a remodeling of the EcM symbiosis in response to NE.

Global fungal community analysis did not indicate a seasonal response in coordination with the strong tree responses, which could be attributed to the Simpson's paradox. We therefore decided to continue with directed coordination analyses between fungal and gene tree expression focusing on the three most abundant fungal species (*Cortinarius*, *Piloderma*, *Cenococcum*) separately. To calculate these inter-kingdom correlations, we used eigengenes of network modules of co-expressed genes. For *Cortinarius* and *Piloderma*, all fungal gene expression modules that were strongly correlated with tree modules in NL

conditions lost coordination after NE. Fungal modules that lost coordination were enriched in growth and metabolism processes, while disassociated tree modules were enriched in several transporter categories. Notably, the Norway spruce module most strongly connected to *Cortinarius* in NL became coordinated with *Piloderma* in NE. This might explain why *Piloderma* was still abundantly present after NE but may also indicate shifts in colonization between different *Piloderma* species. For *Cenococcum*, the majority (almost 90%) of expression modules connected to Norway spruce in NL remained connected in NE, and a large number of new coordinated modules were gained by this species (**Paper V, Fig 4**). These comprised Norway spruce modules that were previously coordinated with *Cortinarius* and *Piloderma* and included some plant genes that are thought to be essential for mycorrhizal establishment [326].

Finally, a more targeted approach was used to search the fungal transcripts for effector proteins, and to map coordination of fungal effectors with Norway spruce gene expression modules, since effectors such as SSPs are known for their crucial role in EcM formation [119]. These results were congruent with the global analyses, showing a decrease in coordination of *Piloderma* and *Cortinarius* effectors with Norway spruce gene expression modules (a loss in coordination for over 5 000 Norway spruce genes in total), and high remaining coordination of *Cenococcum* effectors. This targeted approach exemplifies how the data can be explored for identification of candidate genes for experimental validation and to provide molecular understanding to observed changes.

In conclusion, these findings provide one of the first examples of an *in situ* analysis of metatranscriptomics in the EcM symbiosis, and how these expression dynamics change after long term NE. While **Paper III** demonstrated how soil chemistry processes might contribute to the decline of certain fungi after NE, **Paper V** provides a more "symbiotic" picture, with Norway spruce upregulating defense responses and downregulating important sugar transporters to exclude symbiotic EcM partners with higher C costs. The persistence of *C. geophilum* might be an additional factor contributing to the buildup of organic matter after N addition, since its melanized hyphae slow decomposition [162]. Studies in recent years have stressed how important microbial necromass is for soil organic matter buildup [166], consequently any changes in the formation of more recalcitrant necromass may have large scale consequences.

5 Conclusions and future perspectives

It has been known since at least the early 1990s that N addition profoundly changes fungal communities, with studies in the 90s determining that N deposition was the cause of the decline (to a large part) of many EcM species in central Europe [215]. Thirty years later we have gained a lot more knowledge in this regard, and we now know which species are positively and negatively affected by increased N deposition [161], and what some of the biological differences between nitrophobic and nitrophilic species are. In this thesis, I have used HTS in combination with other molecular methods to further our understanding of the EcM symbiosis in the context of forest management and N addition. In **Paper I** and **Paper II**, we used ITS amplicon sequencing to study fungal communities on establishing seedlings on scarified clearcuts, showing that we can provide seedlings with small amounts of fertilizer without perturbing early colonization by EcM fungi. Future experiments need to assess these effects in the longer term, with the ideal outcome being that the positive effects we identified on seedling survival (seeding) and root growth (outplanting) perpetuate and lead to more successful future growth with no long-term negative effects. Moreover, we need to study additional sites in different geographic locations, as different microclimates, soil types and weather conditions are likely to especially influence seedling performance [36, 327]. When it comes to the widely criticized practice of soil scarification, studies are needed to show why seedlings establish better in scarified soil in the Swedish boreal forest. We know from other studies that belowground competition is a part of the answer [25, 26], but the how and why are less clear. Further experiments using isotopic labelling to determine directionality and strength of transport processes between EcM fungi and establishing seedlings are needed to understand more about this phenomenon. Resolving these kinds of questions is also important because the need for distance to adult trees and soil scarification for seedling establishment contradicts results from other (non-Swedish) studies where it has been reported to be positive when seedlings are connected to established mycorrhizal networks (for example [328]).

In **Papers III**, **IV** and **V** we used metatranscriptomic data to further study how decomposition processes and EcM symbiosis are affected by N addition. While it is already possible to perform in-depth functional analyses based on known candidate genes or orthology databases, the current limitation for metatranscriptomics is the low number of available sequenced reference genomes. Luckily there is hope on the

horizon, with the global collaborative effort “Earth Biogenome Project”, launched in 2018, aiming to sequence all known eukaryotic species within the next 10 years [329, 330]. In the context of the studies presented in this thesis, we have collected sporocarps from the Flakaliden and Rosinedal (Fig 1.2) sites to perform genome sequencing on some of the most abundant local species that currently lack reference genomes. It goes without saying that these major new sequencing efforts will not only facilitate more in-depth metatranscriptomic analyses but will also enable us to gain fundamental new knowledge on different aspects of biodiversity on earth. A major advantage of many -omics datasets is that their usefulness does not necessarily end after one study has been conducted, but can be used for “data mining”, to answer other questions that arise with new knowledge, or in the case of metatranscriptomics, new reference genomes. There are already numerous online tools for interactive data mining, for example the PlantGenIE.org web resource to explore genomic and expression data for different plant species [311], or fungi.guru for visualization of fungal expression data and networks [331]. Metatranscriptomics can be nicely combined with many other methods to study microbial communities in different contexts and their combined inclusion in public resources with intuitive visualization and exploration tools will be a powerful resource for the community to develop and test hypotheses. In the context of rhizosphere processes, it is now possible to mimic root exudates with microdialysis probes that were originally designed to sample brain metabolites, but that can be used in a reverse approach to exude compounds on very small spatial scales [332]. Metatranscriptomics could be a powerful tool to study how microorganisms in an artificial rhizosphere react to different exuded compounds, enabling exact control over C economics, for example.

A recent initiative by the EMBL is called “planetary biology” and aims to “understand ecosystems at the molecular level” by using molecular biological techniques to study *in situ* ecosystem processes [333]. Ecosystems are complex and can only be fully understood by identifying how the biotic and abiotic components interact to shape processes such as nutrient and energy flows. Metatranscriptomics can be one very powerful tool in the toolbox of planetary biology to generate and validate hypotheses in this context, allowing both for more global approaches or more targeted methods, as I have shown in this thesis. A further example for targeted metatranscriptomics can be the use of certain biomarkers for e.g. growth or carbon use efficiency of fungi [334]. One aspect that we have not considered in **Paper IV** and **V** are bacteria, viruses, and other components of soil microbiomes, which also play major roles in

symbiosis and decomposition processes. Future studies can include other parts of soil and root microbiomes to further our understanding.

While I have not directly worked with climate change questions in this thesis, we know that fungal communities are major players in global C cycling, and that both rising temperatures and rising CO_2 concentrations (through effects on photosynthesis and C allocation) are likely to impact boreal forests and their fungal communities. A large-scale study from the Baltic region, using long read sequencing to characterize fungal communities at thousands of sites found that soil pH and tree species (especially Scots pine and Norway spruce) had the biggest effect on fungal communities, with surprisingly low effects of microclimates. The authors concluded that climate change will probably not have a strong direct effect on fungal communities, however it will have an effect on trees, which indirectly will also effect fungal community composition [335]. In terms of elevated CO_2 (eCO_2), a metastudy based on free-air CO_2 enrichment (FACE) experiments found that eCO_2 will lead to increased plant growth in forest ecosystems (compared to grasslands), but this increase might happen at the cost of soil organic carbon storage [324], possibly leading to a net loss of stored C. These outcomes contradict some previous studies and models, and we can only conclude that a better understanding of all aspects of C cycling is needed, not only in the boreal forest.

It has been suggested that overall C storage in the boreal forest could be increased by adding N on larger scales, which is also a topic I have (indirectly) worked on within this thesis. While N addition increases C sequestration, there is also evidence from a large-scale study that increased N substantially shifts microbial communities and might lead to higher abundance of pathogenic and opportunistic fungi [336], which in turn may have unforeseen consequences. The significant shifts in the dynamics of the EcM symbiosis that we elucidated in **Paper V** in this thesis also suggest that long-term fertilization leads to shifts in coordination processes between trees and their fungal communities. An interesting follow-up question from our thus far gathered knowledge that N fertilization massively changes fungal communities would be whether those communities can recover after fertilization ceases. In particular, it is important to know how long it takes for fungal community composition and soil chemistry to return to “normal”; Can traces of the fertilization still be detected several decades after fertilization ceases? If so, what are the implications? The Rosinedal site, where fertilization has now ceased, is being sampled to study this process and there are numerous older fertilization experiments that can

be used for further comparisons. Previous studies on some of these sites have reported lingering effects (based on sporocarp counts, ITS data and plant communities) decades later [160, 337, 338], and it will be interesting to elucidate this further. Until we have more certainty on these long-term effects, I would be cautious in suggesting fertilization of large forest areas. I think there has to be a balance, but we should not prioritize C sequestration over everything else, an approach that has already been widely criticized, nicely summarized as "bio-perversity - negative biodiversity and environmental outcomes arising from a narrow policy and management focus on single environmental problems without consideration of the broader ecological context" [339].

In this thesis, a focus has been on EcM fungi, which have received increasing attention in recent decades, with EcM fungi now being included in many global modelling studies on, for example, C sequestration or large-scale tree growth [139, 324]. In a review from 2019, Lilleskov et al. [50] discussed how effects of N deposition on fungal communities can arise both directly (fungal or soil-mediated) and indirectly (tree-mediated). In this thesis I have contributed to elucidating both processes: in **Paper III** I provide an example for a hypothesis showing how changes in soil chemistry after N addition can influence fungal communities; in **Paper V** I illustrate how trees upregulate defense responses and downregulate sugar transporters when N is abundant, and how N addition results in an overall loss of coordination between tree and fungi. *In situ*, both processes probably play a role, and we need to gain a deeper understanding of both sides of the symbiosis, not only for basic knowledge but also to develop and enhance management practices to ensure future sustainable forestry.

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