# Master Thesis

## How does the ectomycorrhizal community change along a chronosequence of spruce and oak on an afforested area

Submitted by

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in the framework of the Master programme

Environmental Sciences – Soil, Water, Biodiversity (ENVEURO)

in partial fulfilment of the requirements for the academic degree

Master of Science, MSc

Skamstrup, January 2022

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# Affidavit

I hereby declare that I have authored this master thesis independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included.

I further declare that this master thesis has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Skamstrup, 3.1.2022                          Robin Mikaela KOTSIA *(manu propria)*

# Acknowledgements

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# Abstract

Afforestation is an attempt to restore a land by planting trees, aiming to both improve biodiversity and as a powerful tool to sequester carbon, therefore applied for climate change mitigation. Although there is a growing understanding among ecologists that above and belowground communities are strongly connected, the majority of studies are focused on the above ground contribution to carbon storage. Ectomycorrhizal (ECM) fungi form associations with 80-90% of tree species in temperate forest ecosystems and constitute the connecting link of the trees with the soil by transferring photosynthetically fixated carbon into the soil ecosystem. It is therefore an important group of fungi that deserves attention with regard to their re-establishment after afforestation, as well as their potential for carbon storage of both of living as well as dead tissue. In this report I investigated the effect of afforestation with Norway spruce and oak monocultures on ECM species composition and their decomposability, through two chronosequences available in Denmark. The aims of the study were to compare the effects of a native and an introduced tree species on ECM fungi composition and decomposability, over time. The external investigated parameters that affect ECM decomposability included soil pH, C/N ratio and litter cover. The sampled ECM were additionally molecular identified to study the species composition and fungal succession. Melanization of the fungi was taken into consideration as a "decomposition trait". Results showed that the spruce stands provided conditions that retard decomposability and had lower fungal biodiversity compared to the oak stands. These results suggest that native tree species may be better for promoting fungal biodiversity through afforestation, but might not be the most efficient choice for carbon storage in the soil.

# 1. Introduction

## 1.1. Afforestation, succession, and soil organic matter

Land-use change is one of the main factors causing environmental changes globally, so the knowledge concerning the current and past land-use can therefore serve as an indicator for environmental change (Gerard et al. 2010). It is especially interesting to investigate these land-use changes that affect the ecosystems through altering C fluxes and stocks, as these can additionally provide insights to other environmental changes (Lal 2005; Bárcena et al. 2014b). Additionally, the decision of the Kyoto protocol to investigate the effects of land-use and land-use change on the global C budget adds even more incentives for examining the shifts in the soil C storage in areas that have undergone changes such as afforestation, reforestation, and deforestation (IPCC 2015).

One type of land-use change that has been getting a lot of attention due to its potential for contributing to mitigation of climate change effects, is afforestation. Afforestation is the establishment of a forest either by planting trees or by allowing natural succession in previously treeless areas such as grasslands, shrublands or croplands. The act of afforestation would historically also involve the legal aspect of defining the area of subject as an area bound to the forest laws (Allaby 2019). Afforestation naturally builds up carbon above ground but is also expected to contribute with a net increase in the forest soil C in Europe, counteracting the negative effect that the projected warming has on the C fluxes (Lorenz and Lal 2010). It is therefore no surprise that afforestation, along with reforestation, is seen as one of the most effective practices for sequestering C and is applied as part of climate change mitigation strategies (Sauer et al. 2012). The potential of afforestation as a biological atmospheric $CO_2$ removal strategy that aims at storing C in forest biomass may have many co-benefits, but also negative ecological effects which also deserve further analysis and attention (Smith and Torn 2013). There are several reasons, along with C sequestration, for converting arable land into forests, either environmental or economic, but in countries like Denmark with an intense agricultural sector, the reduction of croplands equals reduction of food production (Fissore et al. 2010). So, understanding whether this conversion is beneficial and to what extent, is rather interesting.

Afforestation is an attempt to restore a land by planting trees and aims to improve biodiversity faster than it would have if it was left to the natural process of secondary succession which is a long and complicated process of creating forests (Krawczyk 2015). Afforestation on former croplands, where soil degradation and ecosystem imbalances are severe, may improve soil quality faster compared to abandoning the cropland and leaving it to secondary succession (Zethof et al. 2019). Either pure or mixed species forests are used for afforestation of previously agricultural lands, but we know little about the effects of different tree species on altering soil function through e.g., improving C and nitrogen (N) storage or supporting nutrient cycling, as we also know little about the effects of different tree species on soil microbial communities (Gunina et al. 2017). Secondary vegetation and soil microorganisms that colonize because of the initial tree planting can also be seen as natural succession following the afforestation initiative. Different tree species, soil conditions and climate will affect the subsequent succession and the development of various understory plant species, soil microorganisms and fungal communities. Investigating the colonization and development of secondary species that find their way into the afforested ecosystems depending on the different tree species that are planted through afforestation, would provide useful knowledge for supporting management decisions to benefit both above and below-ground diversity.

Belowground processes and C storage are often neglected when estimating C budgets or fluxes, and there is need for better understanding them. The effect of afforestation on C sequestration in the growing biomass is the most obvious contribution to the net sink for atmospheric $CO_2$. However, C is

also sequestered in the soil because of afforestation, even though this process is significantly slower compared to C fixation in forest biomass. Since C stored in soil is less susceptible to changes in forest management than C stored in forest biomass, the effect of afforestation on C storage in the soil is necessary to be evaluated (Vesterdal et al. 2002).

The use of afforestation on former croplands for restoring lost SOC stocks is a slow and complicated process that is not well understood. Several factors seem to be affecting the restoration of SOC after afforestation, with the major ones including previous land-use, tree species planted, soil clay content, pre-planting disturbance and, to a smaller degree, climate zone. Additionally, there is evidence showing that broadleaf tree species have a higher potential for increasing SOC than coniferous species (Laganiãre et al. 2010). Organic C will mainly increase in the mineral topsoil layer (Kukuļs et al. 2019), so soil samples taken from the top layers can be examined for determining the effects of afforestation on SOC the subsequent years.

Forests contain a great part of the global terrestrial C stocks; approximately 80% of all global aboveground C and 70% of all global belowground C (Laganiãre et al. 2010). Soil can store great amounts of C; more than what can be found in living plant biomass and atmospheric $CO_2$ combined (Jobbágy and Jackson 2000). SOC in stable fractions can reside in the soil for more than 1000 years (Lützow et al. 2006), which makes it a very important and stable sink compared to living plant biomass. Restoration of SOC appears therefore to be a promising way to mitigate climate change effects and for reducing $CO_2$ concentrations in the atmosphere. The contribution of afforestation to restoring SOC is, however, to be debated, since several studies seem to present conflicting results. SOC stocks may decrease, increase, or even remain unaffected because of afforestation. Despite the inconsistency in the various studies results, there seems to be a trend, since afforestation will repeatedly cause a loss in SOC during the first years and eventually C stocks will steadily reach levels that can be compared to those in the control agricultural soil, and then in some cases it will lead to net C gains, which, however, are usually lower than the values we would find in a comparable natural forest. Overall, it may be said that the scientific community does not seem to agree on the factors affecting the restoration of SOC after afforestation (Laganiãre et al. 2010).

## 1.2. Ectomycorrhizal fungi role in forests

Studying the effects and changes caused in species composition of plants, animals and fungi following afforestation, can provide valuable insights concerning C fluxes and storage in the ecosystems. This thesis project is specifically focused on the fungal group of ectomycorrhizae, for reasons that are elaborated in the following section.

Ectomycorrhizal (ECM) fungi form associations with plants, they dominate temperate and boreal forest soil ecosystems around the globe and they are involved in numerous fundamental ecosystem processes (Phillips et al. 2013). As high as 80-90% of tree species in temperate forest ecosystems form associations with ECM fungi, with many tree species relying on these fungi to fulfil their nutrient requirements (Allen 1992). The ECM symbiosis is an essential component to the forest ecosystems of the globe, in which host plants supply fungal partners with photosynthetically fixated C (Högberg et al. 2001), and in return they receive a variety of other services such as enhanced water and nutrient uptake, protection from soil pathogens, and heavy metal tolerance (Smith and Read 2008). ECM fungi assist tree nutrition through mineral weathering (Landeweert et al. 2001) or by mobilizing nutrients from organic complexes (Read and Perez-Moreno 2003). In addition, they play a significant role in transferring carbon into the soil and are responsible to a large extent for the forest-soil C fluxes (Read 1992; Högberg et al. 2001; Högberg and Högberg 2002; Godbold et al. 2006; Hobbie 2006; Clemmensen et al. 2013). ECM fungi are generally considered to be essential organisms in the forest nutrient cycles and are strong drivers of forest ecosystem processes (Read et al. 2004). ECM fungi are a relatively large and diverse group of fungi with numerous phylogenetic groups, with mostly

basidiomycetes and fewer ascomycetes, of which a great majority lives in symbiosis with trees (Smith and Read 2008). The morphology of the ECM root tips vary greatly due to the diversity of fungal taxa, and their macroscopic and microscopic observation and analysis is broadly applied for the identification of ECM fungi (Agerer 1987).

The role of ECM in the forest, however, is not restricted to assisting the nutrient uptake of the trees. The extensive mycelial networks formed by these fungi are an efficient way to distribute carbon and other nutrients, which provide an essential food source for numerous soil animals and saprotrophic microorganisms that feed on the fungal mycelia. ECM are therefore spreading energy derived from the photosynthesis of the trees into the soil ecosystem. Given that the great majority of root tips of ECM host trees are enclosed in ECM fungal sheaths, we could say that these fungi constitute the connecting link of the trees with the soil (Read et al. 2004). The fungus-plant interface represented by the Hartig net plays a crucial functional role in the symbiosis, as it comprises the platform through which nutrients and C are transferred between the partners (Smith and Read 2008).

The tree-fungi association involves the fungi receiving energy from their tree hosts in form of sugars from photosynthesis and, in return, providing essential nutrients for the tree, like nitrogen and phosphorus (Read et al. 2004). The ECM root consists of three structural components (fig. 1): a sheath or mantle of fungal tissue that surrounds the tree root, a network of hyphae which grows inwards and between the epidermal and cortical root cells, called the Hartig net, and a system of hyphae which grow outwards, called the extraradical or external mycelium; the latter has an important role, connecting the different fungal structures with the soil and the sporocarps. The colonized tree root tips and the extraradical mycelium are the key structures in the symbiosis, as they form the sites where the uptake and exchange of nutrients takes place between the partners (Smith and Read 2008). ECM mycelia, which expand from the mantle into the soil (fig. 1) are different among fungal taxa (Agerer 2001) and are functionally valuable for the symbioses as they are responsible for foraging and transferring nutrients and water (Anderson and Cairney 2007). Additionally, the mycelia colonize small lateral roots (Smith and Read 2008) and may be involved in forming common mycelial networks that allow C and/or nutrient translocation from one individual tree host to another (Simard and Durall 2004; Selosse et al. 2006). From a greater point of view, at the ecosystem level, the ECM mycelia are also important due to their contributions in mobilizing nutrients from organic and recalcitrant inorganic matter, either by collaborating or by competing with soil-dwelling saprotrophs, aiming at translocating nutrients within the soil-plant continuum and at releasing and spreading C belowground (Leake et al. 2003; Wallander 2006).

Mycorrhizal fungi control plant distribution and productivity and are therefore predicted to influence the mitigation of climate change drivers (Classen et al. 2015). Generally, it is believed that mycorrhizal fungi influence ecosystem responses through their direct effect on individual plant function and their indirect effect on other processes such as plant dispersal and community interactions (Bennett and Classen 2020). They are a very widespread group of fungi on the landscape and they form associations with more than 80% of plants in both managed and unmanaged ecosystems (Smith and Read 2008). Actually, there exist an astounding amount of observational and experimental evidence that mycorrhizal fungi control a great variety of community traits and ecosystem functions, plant productivity and community composition (van der Heijden et al. 1998; Jiang et al. 2017), decomposition, and soil nutrient cycling (Langley et al. 2006; Rosling et al. 2016), soil microbial community composition (Kyaschenko et al. 2017) and soil carbon stabilization (Clemmensen et al. 2013; Leifheit et al. 2014; Moore et al. 2015a; Moore et al. 2015b; Fisher et al. 2016; Jackson et al. 2017).
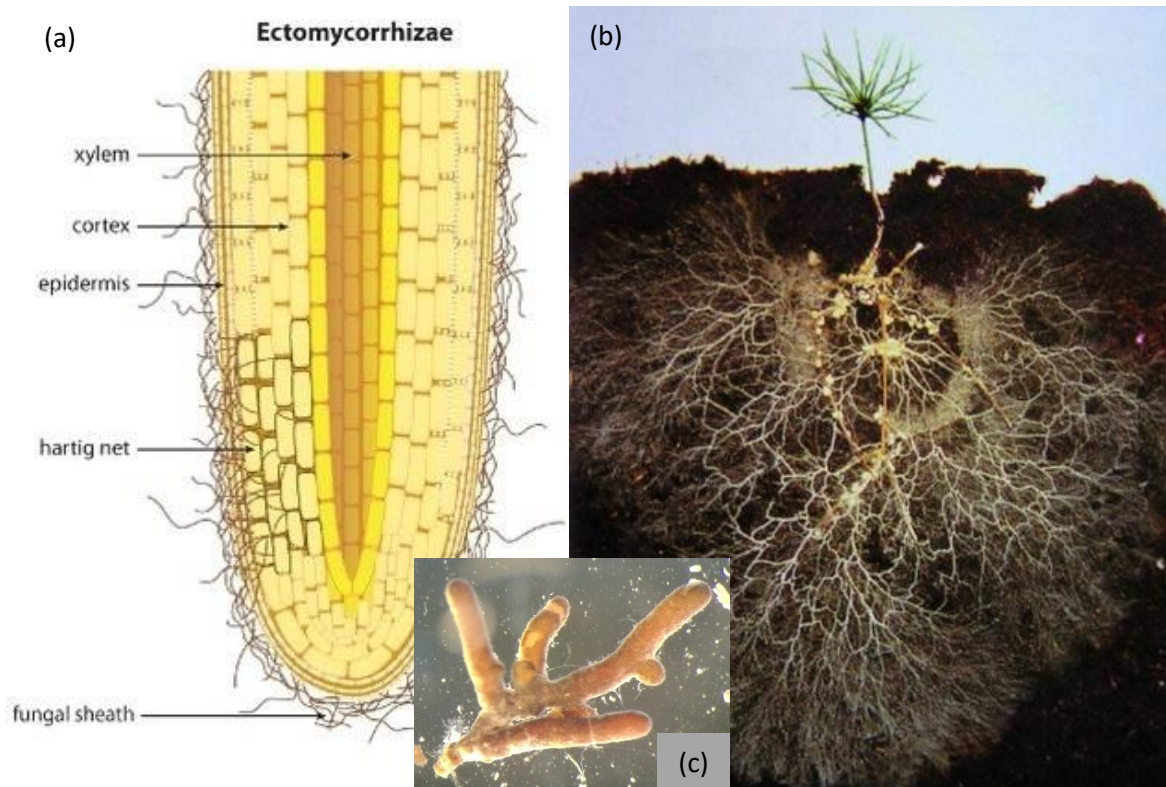
Figure 1. (a) Illustration of the ectomycorrhizal colonization of a tree root. Source: (Bonfante and Genre 2010). (b) Ectomycorrhizal mycelium surrounding the roots of a pine seedling and extending into the surrounding soil. Source: (Smith and Read 2008). (c) Ectomycorrhizae under stereomicroscope.

Many mycorrhizal fungal groups are equipped with exceptional traits which allow them to counteract the effects of climate change on mycorrhizal fungi (Treseder and Lennon 2015). Such traits include variations in hyphal exploration types (Agerer 2001; Helgason and Fitter 2009; Lilleskov et al. 2011; Peay et al. 2011), which is in turn related to root density (Bingham and Biondini 2009; Peay et al. 2011), disturbance and nitrogen availability (Lilleskov et al. 2011; Chen et al. 2018; Treseder et al. 2018) across mycorrhizal types and at species level (Bukovská et al. 2016). Additionally, many mycorrhizal fungal species show different variations in extra-radical hyphal density (Jakobsen et al. 1992; Duan et al. 2011) and turnover (Chagnon et al. 2013). The mentioned traits, along with a range of others, allow mycorrhiza to combat environmental changes (Fernandez et al. 2017).

The potential role of ECM fungi in climate change mitigation through their contribution to C storage in the soil, is the main motivation and driver for this thesis project. The importance of ECM for the soil ecosystems, but also for the reduction of atmospheric C, makes them extremely interesting to study.

## 1.3.  ECM fungi and succession

Even though there is a growing understanding among ecologists that above and belowground communities are strongly connected, the majority of studies on shifts in community composition over forest development are mostly conducted on aboveground biota (Wardle et al. 2004). In fact, we have limited knowledge concerning the ways soil microbial communities change after disturbance and with forest stand age, as well as the factors controlling possible changes in species composition (LeDuc et al. 2013). Regarding ECM fungi, due to their intimate symbiosis with plant roots, they may influence ecosystem structure and functioning (van der Heijden et al. 1998), and studies reveal that disturbances will cause changes in ECM species composition with forest age (Visser 1995; Twieg et al. 2007). The initial ECM species composition may be determined by the differences among the species ability to

disperse or persist in the soil and their efficiency in root tip colonization via spores or other propagules (Allen 1992; Taylor and Bruns 1999; Lilleskov and Bruns 2003). However, with the increase of stand age, these initial ECM fungi tend to get entirely replaced by other taxa or decline in abundance (Visser 1995; Twieg et al. 2007). These changes in species composition suggest a successional shift that is not yet well understood, as to what mechanisms or processes take place.

Studies on the mycorrhizal fungi in association with jack pine trees (*Pinus banksiana* Lamb.), which are heavily ectomycorrhizal with a wide range of fungi, show that the mycorrhizal associations with young jack pine are different from those found on roots of mature jack pine (Danielson 1984; Visser 1995). Additionally, it has been observed that throughout tree growth and stand development, the mycorrhizal species composition exhibit an orderly sequence as one species is replaced by another (Danielson 1991). Temporal designations of early-stage versus late-stage fungi following a successional sequence were observed for the first time in mycorrhizal communities forming associations in a mixed birch forest (*Betula pendula* Roth and *B. pubescens* Ehrh.) in Scotland (Mason et al. 1982; Mason et al. 1983). This concept of mycorrhizal fungal succession has been further observed and supported by several ECM studies, on different forest types with various tree species, such as *Pinus spp., Eucalyptus spp., Picea sitchensis* (Bong.) Carr. (Chu-Chou 1979; Chu-Chou and Grace 1982, 1988; Dighton et al. 1986; Natarajan et al. 1992; Richter and Bruhn 1993). These studies support the theory that ECM species richness increases until canopy closure and will then start decreasing due to changes in host physiology and forest floor organic matter quality (Moore 1985; Last et al. 1987). Although this concept of ECM succession is well supported by numerous studies, it is worth noting that the majority of them were carried out on afforested areas that have suffered anthropogenic disturbance, such as agriculture, plantation and clearcut systems, rather than natural disasters. For this reason, it is uncertain whether the succession concept is also applicable for the mycorrhizal communities appearing in stands reestablishing after an event of natural disturbance, such as wildfire (Visser 1995).

## 1.4. Decomposition and relevant ECM traits

ECM transfer a large proportion of the sequestered atmospheric C from the plants into the soil, and contribute to its long-term storage in the soil. In order to understand the extent of this contribution, it is relevant to consider the factors that affect the decomposition rates of ECM necromass. For the purposes of this thesis, the chosen investigated parameters include two direct decomposition traits, namely the fungal C/N ratio and melanin content, and two system factors that affect decomposition of organic matter, those being the soil C/N ratio and soil pH. The aim is to compare the potential for carbon storage in the soil, provided by the ECM in association among the two tree species found in the experimental site. I will not go into decomposition times and rates, as these findings would require much longer time in the field and lab, which the study program does not allow. A simple comparison of the mentioned parameters will be conducted to see whether it is possible to distinguish which tree species will result in more carbon storage in the soil when used for afforestation of former agricultural land in Denmark.

### 1.4.1. Fungal and soil C/N ratio

It is generally accepted that the availability of macronutrients, with nitrogen (N) being the most crucial one, can have a restraining effect on the storage and cycling of C in terrestrial ecosystems (Vitousek and Howarth 1991), and can limit soil microbial activity (Treseder 2008) and therefore also decomposition and C fluxes from the ecosystem (Schimel 2003; Mack et al. 2004). It is hard to understand the C/N dynamics that take place belowground, especially because of the apparently

contradictory role that N appears to be playing in microbial decomposition of organic matter (Averill and Waring 2018).

The past century, scientists have noticed that increased N availability favors the decomposition of organic matter (Aber and Melillo 1982; Richards and Norman 1931; Waksman and Tenney 1927). Plant litter tends to immobilize N during the first stages of decomposition (Parton et al. 2007), and litter with a low C/N ratio (higher N content) has a higher decomposition rate (Aber and Melillo 1982). In plant leaves, the main driver of decomposition rates is the N content, at all climatic zones, and in general, the decomposition of soil organic matter (SOM) appears to be positively correlated with N availability (Hu et al. 2001). Based on these observations it is safe to conclude that organic matter decay by soil microbiota is limited by N availability (Averill and Waring 2018). Decomposition of ECM necromass is also dependent on N availability, as these consist an important source of organic matter for the soil microorganisms to feed on.

Apart from the C/N ratio found in the litter or ECM biomass, which affects their decomposition rates, it is also important to consider the C/N ratio of the soil. Soil C/N ratio is a property with direct influence on the decomposing organisms living in the soil and will therefore have an effect on the SOM decomposition rates (Enríquez et al. 1993). A study based on data-assimilation analysis that tested the effect of different soil properties on the decomposition of soil organic carbon (Xu et al. 2016), reports that as the soil C/N ratio increases, the decomposition rates decrease considerably. Microbial activity and growth, and consequently the decay of organic matter, greatly depends on the availability of N. Soils with low N availability (high C/N ratio) do therefore retard the decomposition of organic matter (Xu et al. 2016).

## 1.4.2. Soil pH

Under natural conditions, soil pH directly affects the soil biogeochemical processes and is for that reason referred to as the "master soil variable". Soil pH is the driver of numerous biological, chemical and physical soil properties and processes that play a role in plant growth and biomass production (Minasny et al. 2016; Weil and Brady 2017). Soil pH is controlled by various processes taking place in the soil. Some examples include: the leaching of basic cations like Ca, Mg, K and Na well away from their release from weathered minerals, which results in the formation of $H^+$ and $Al^{3+}$ ions that dominate exchangeable cations; the mixing of $CO_2$ with soil water which leads to the production of carbonic acid, which in turn separates and forms $H^+$ ions; the humification of SOM that leaves humic residues which result in large amounts of carboxyl and phenolic groups that dissociate and release $H^+$ ions; the nitrification of $NH_4^+$ to $NO_3^-$ produces $H^+$ ions; N removal from animal and plant outputs; and acid rain absorption or N uptake by plants (White 2009). At the same time, soil pH regulates many biological processes in the soil. Therefore, the relationship between soil pH and the biogeochemical processes in terrestrial ecosystems, is bidirectional. This means that soil pH impacts numerous biogeochemical processes, and at the same time, some biogeochemical processes have an effect on soil pH, to some extent (Neina 2019). For this reason, pH is a chosen parameter to investigate when trying to understand the decomposability of ECM fungal necromass. The figure below (fig. 2) summarizes the relation of soil pH with some major biogeochemical processes.
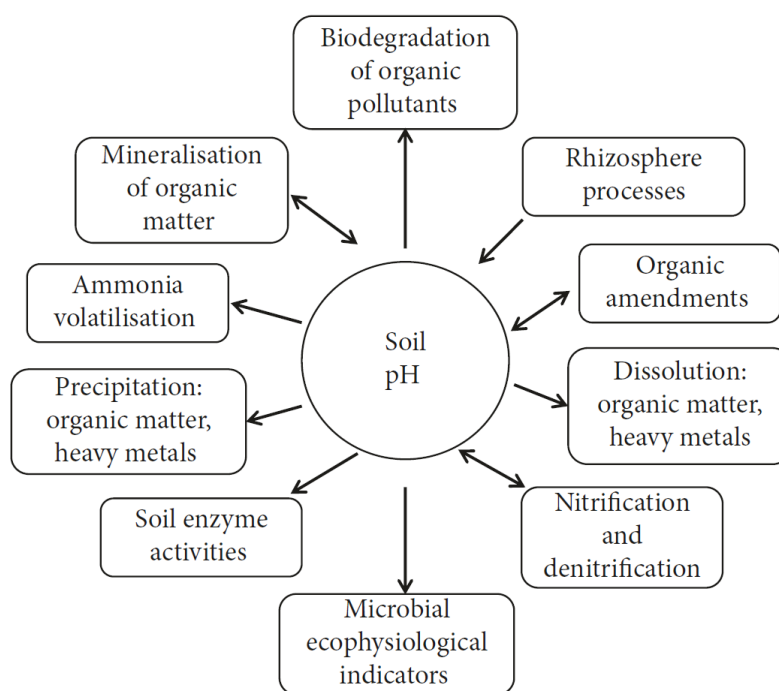
Figure 2. Soil pH and its relation to some major biogeochemical processes. Source: (Neina 2019)

Soil pH is a measure of the soil solution's acidity and alkalinity. By definition, pH is the 'negative logarithm of the hydrogen ion concentration [$H^+$]', meaning that pH = -log [$H^+$]. The pH values vary on a scale from roughly 0 to 14, with 7 being neutral (pure water), below 7 being acidic, and above 7 is alkaline (or basic). Depending on where on the scale a soil solution falls, it can be characterized accordingly. Since pH is a logarithmic function, this means that every unit of the pH scale is ten times more alkaline (less acidic) than its lower unit. As an example, a soil solution with a pH measure of 5 will have a $H^+$ concentration which is 10 times higher compared to a soil solution with a pH of 6, and 100 times higher compared to a pH 7 soil solution. Soil pH is affected by various ions in the soil, either acid- or base-forming. Frequent acid-forming cations include hydrogen ($H^+$), aluminium ($Al^{3+}$), and iron ($Fe^{2+}$ or $Fe^{3+}$), and common base-forming cations are calcium ($Ca^{2+}$) magnesium ($Mg^{2+}$) potassium ($K^+$) and sodium ($Na^+$). Precipitation can have a great influence on soil pH; low precipitation causes little leaching of base cations, which will saturate and the pH values will be higher than 7, whereas when precipitation is increased it will cause the leaching of base cations and the soil pH will drop. Other factors affecting the soil pH is the presence of elements like silica (rhyolite and granite) in the parent material, which tend to result in acidic soil, and highly sandy soils have low buffering capacities and may also be acidic (McCauley et al. 2009).

Over the last many years, extensive research has been focused on displaying the effect of soil pH on numerous biogeochemical processes. The significant role that the soil pH plays in the soil ecosystem processes is nowadays becoming more and more evident. It is a soil property which regulates the interaction of xenobiotics in all soil phases and determines their fate, translocation and transformation. For this reason, soil pH also determines the fate of substances in the soil ecosystem, which in turn means that it controls nutrient recycling and availability. Although soil pH has a functional role in soil biochemistry, in many studies it is only measured informally as a norm rather than giving it the attention and consideration its role in the soil deserves (Neina 2019).

Soil pH and C/N ratios of the soil environment, as well as the organic matter, seem to be two of the most central and determining factors that affect so many biogeochemical processes, including the decomposition of SOM. The efficiency of the decomposer community and the catalytic power of the exoenzymes they produce seem to be directly affected by soil pH or substrate C/N ratio (Leifeld and

Lützow 2014). For this reason, soil pH and C/N ratios are thoroughly investigated and analyzed in this report.

### 1.4.3. Melanin

Melanin is an ancient pigment with multiple functions that is found in all biological kingdoms (Cordero and Casadevall 2017). Historically, melanins have shown to be challenging to characterize and categorize because of their great variety and structural complexity. Melanins are unique among pigments and other biomolecules, as they carry very particular physicochemical and structural characteristics. Due to their natural complexity, it has proven to be hard to figure out their higher-order structure and consequently understand their functions. Only recently have studies been able to provide information concerning the multiple functions of melanin in eukaryotic systems (Abbas et al. 2009; d'Ischia et al. 2009; Meredith and Sarna 2006). It is interesting to comprehend the physicochemical properties of melanins, as this will allow to further exploring the distinct functions of melanin in fungal biology. Today, melanins are classified into eumelanins, pheomelanins, neuromelanins and allomelanins (Ambrico 2016). Below (fig. 3) can be seen illustrations showing the chemical structure of melanin precursors for each type of melanin (Solano 2014). All of them are heterogeneous polyphenols forming high-order structures with exceptional physicochemical properties, such as broadband optical absorption paramagnetism, charge transport and outstanding structural stability. As a result of these properties, melanins can achieve a great range of functions and melanization has come to symbolize a general adaptation mechanism to stress e.g. as imposed by climate changes. Since the responsible genes for melanin production provide protection from a number of factors connected to climate warming, this could potentially indicate that dark coloration may be indirectly selected by climate warming. Additionally, it has been observed that individuals with higher melanin concentrations are usually more combative compared to paler conspecifics, which gives them advantages during competitive interactions with invasive species that migrate to northern latitudes and higher altitudes (Roulin 2014). The extensive occurrence of melanins in biology indicates a functional significance for this class of biomolecules in the evolution of life on Earth (Cordero and Casadevall 2017).
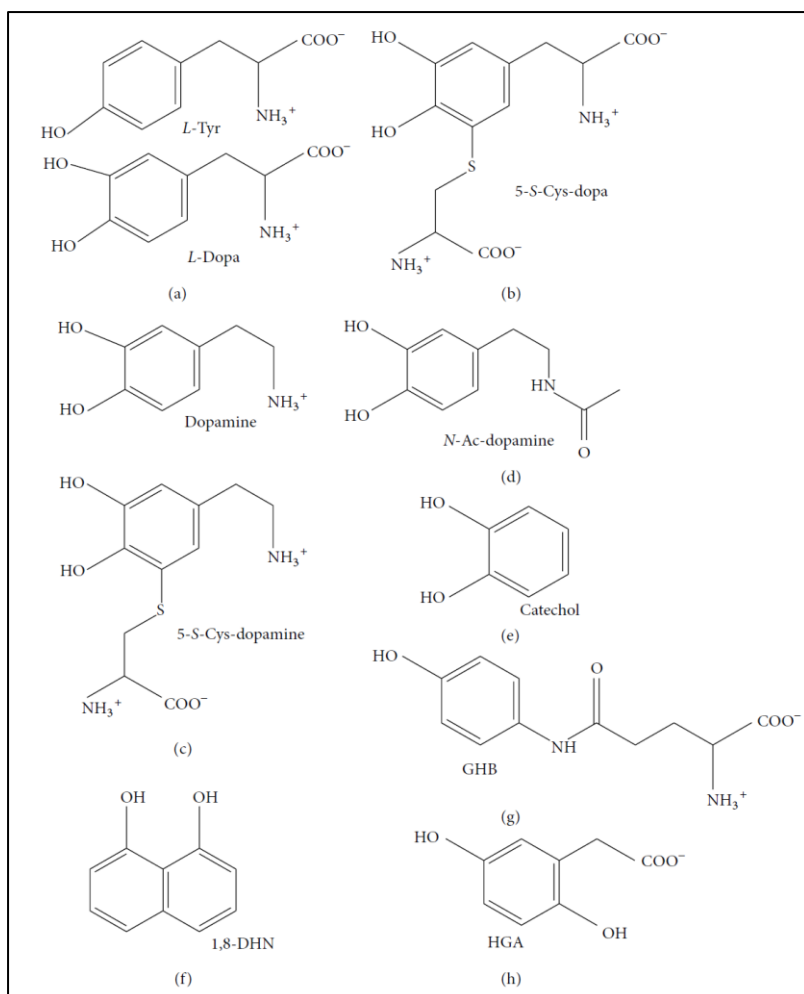
Figure 3. Illustration showing the chemical structure of melanin precursors for each type of melanin (in brackets). Only the most representative for each type is shown. (a) L-tyr and L-Dopa (eumelanin); (b) 5-cys-dopa (pheomelanin); (c) dopamine and 5-S-cys-dopamine (neuromelanin); (d)N-acetyldopamine (insect-melanin); (e) catechol (catechol-melanin, plants); (f) DHN, 1,8-dihydroxynaphthalene (DHN-melanin, fungi); (g) GHB, 4-glutaminyhydroxylbenzene (GHB-melanin, mushroom); (h) HGA, homogentisic acid (pyomelanin). Source: (Solano 2014)

The pigment melanin appears to be crucial in protecting fungal cells from various environmental stressors and serves essential survival functions in fungi (Butler and Day 1998). It is found at the cell surface or released into the extracellular space (Dong and Yao 2012; Doss et al. 2003; Gadd and Rome 1988; Jalmi et al. 2012), forming complexes with other cell wall components like proteins, β-glucans and chitin (Koide et al. 2014). In ECM fungi, the concentration of melanin in the cell walls differs considerably, as does the type of melanin they produce. Depending on their biochemical precursors, fungal melanins are divided into four classes: c-glutaminyl-3,4-dihydroxybenzene (GDHB) melanin, dihydroxyphenylalanine (DOPA) melanin, dihydroxynaphthalene (DHN) melanin and catechol melanin. The type of melanin found in fungi appears to be predominantly related to their phylogeny, with Basidiomycetes producing GDHB and DOPA melanins, whereas Ascomycetes produce mainly DHN melanin but may also produce DOPA and catechol melanins (Butler and Day 1998).

In the fungal kingdom, melanization is present in all phyla. There are fungal species which are always melanized, and others which melanize exclusively during certain developmental phases (i.e. conidia, yeast filamentous growth), in response to environmental shifts, and/or in the presence of phenolic melanin precursors (Bell and Wheeler 1986). Constantly melanized species of fungi are mentioned as melanotic, black, dematiaceous, microcolonial or meristematic fungi. Fungal species that melanize only under certain conditions are named "facultative melanotic" fungi (Cordero and Casadevall 2017). Melanotic fungi are phylogenetically diverse (Sterflinger et al. 1999) and can be found everywhere around the globe, usually colonizing rough environmental niches that cannot sustain most life forms. These habitats endure various conditions of low water activity and nutrient availability, extreme temperature fluctuations, high osmotic pressure, high radiation exposure and oxidative stresses. In such conditions, melanization allows fungi to tolerate the large range of physical and chemical

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intensity found in their surroundings, making them able to withstand extreme environmental conditions (Dadachova and Casadevall 2008; Gessler et al. 2014; Robinson 2001).

This thesis project does not focus on extremophiles, but they are interesting because they provide good examples to understand the properties of melanin. Melanin can be found in fungi from all biomes and this broad distribution implies that melanization comes with advantages in survival and/or adaptation abilities in harsh environmental conditions. These advantages may be related to a wide range of functions like photoprotection, free radical quenching, energy harvest, cell strength, metal chelation, protection against heat and cold stress, resistance to desiccation and cell development (Cordero and Casadevall 2017). Among ECM, the densely melanized and universal ascomycetous fungus *Cenococcum geophilum*, is able to tolerate high levels of water stress, compared to many other ECM fungi (Pigott 2006). This resistance to drought may be due to the heavy melanization of the fungus cell walls (Koide et al. 2014). Additionally, dry and seasonally water-deficient areas seem to be dominated by *C. geophilum* (Querejeta et al. 2009).

Finally, for this thesis, melanization of fungi is of particular interest as it appears to be affecting their decomposability. Again, using *C. geophilum* as an example, there is evidence that its melanized cell walls retards decomposition (Malik and Haider 1982). ECM fungal necromass comprises a large biomass input into forest soil ecosystems (Clemmensen et al. 2013). Therefore, it makes sense to assume that fungal communities which are dominated by *C. geophilum* or other melanized fungi may add surprisingly high concentrations of soil C stored as extremely recalcitrant fungal detritus or partial decomposed products. The idea that the different parts of ECM fungus *C. geophilim* necromass, or its sclerotia and other living fungal parts play an important role as stable C pools in forest soil ecosystems is supported by several studies (Fernandez et al. 2013; Fogel and Hunt 1983; Dahlberg et al. 1997; Watanabe et al. 2007; Scott et al. 2010).

Given the significant role of melanin in SOM decomposition, this thesis project included an attempt to develop a methodology for measuring the melanin content of small ECM fungal samples. More details about this follow in the methodology section.

## 1.5. Aim and Research Questions

The aim of this study is to get a better understanding of the effects of afforestation with native and introduced tree species on the ECM communities and the C storage in the soil.

Based on the above background information and theory, motivated to understand what methods and interventions are more efficient for carbon storage, and given the available study site (see Methods), I have formulated 5 research questions to guide my work.

- *Question 1:* Does biodiversity increase along the chronosequence? Are there more ECM morphotypes in the older stands?
- *Question 2:* As spruce is introduced and oak is naturally occurring in Denmark, I am interested to see whether oak will have associations with more morphotypes compared to spruce.
- *Question 3:* Do ECM from older and less nutrient rich stands decompose slower? Do we identify traits that indicate slower decomposition? (higher melanin and C:N ratio)
- *Question 4:* Does succession following afforestation have an effect on the soil parameters of pH and C/N ratio?
- *Question 5:* Is there a change in the ECM species along the chronosequence? What do the species indicate?

# 2. Materials and Methods

## 2.1. Site description

Vestskoven (55°70'N, 12°35'E) is an afforested area which lies 15 km west of the city of Copenhagen in the eastern part of Denmark. Prior to the afforestation, the land had historically been used for intensive crop production and nurseries for several centuries. The main purpose of the afforestation was to create a recreational area close to Copenhagen and the project was carried out by the Danish Nature Agency with the first afforestations starting in 1967 and continuing over the following decades. The afforested area of Vestskoven has been increasing successively over the years and today covers approximately 1400 ha (Danish Nature Agency 2021), with forest stands of approximately 1-10 ha in size and several clearings used as permanent pastures. Forest stands consist mainly of monocultures of oak, beech, pine and Norway spruce, with the dominant tree species mostly used for afforestation being Norway spruce (Picea abies (L.) Karst.) and pedunculate oak (Quercus robur L.). The soils found at Vestskoven are predominantly Mollic Hapludalfs (Soil Survey Staff 2014), derived from calcareous till deposits (Rahman et al. 2017). Soils are rich in nutrients and have a sandy-loam texture in the whole area, with no systematic differences in the nutrient contents or texture between the earliest and the latest afforested stands (Ritter et al. 2003). The topography is flat with an elevation ranging from 20-28 m, the climate is temperate with an average annual temperature of 9.8 °C and a mean annual precipitation of about 773 mm (DMI 2020).

Since afforestation was carried out successively at Vestskoven, it resulted in the creation of a range of forest stands with different ages, representing a chronosequence of two different tree species and covering a time span of 3 to 4 decades. Given that the area of Vestskoven is homogeneous with regards to soil type, climatic conditions and topography/elevation, the two established afforestation chronosequences provide an excellent experimental site for studying and comparing changes that develop over time. The two chronosequences allow us to investigate the influence of afforestation on ECM and their decomposition traits, when considering the effect of time and tree species. We commonly see the use of chronosequences in ecology, where sites of different ages are used in order to study and describe patterns which can be attributed to individual sites as they age (Yanai et al. 2000).

Apart from the chronosequences found in Vestskoven, a ~200-year-old forest named Ledøje Plantation within the same area was sampled. The patch is a mixed deciduous forest located approx. 1 km west of Vestskoven and covers an area of 5 ha. It is dominated by oak and sycamore maple (Acer pseudoplatanus L.), but also some ash (Fraxinus excelsior L.) and beech trees (Fagus sylvatica L.) are present in the plantation. These forest stands were also planted on former cropland and can provide data on the long-term effects of afforestation. However, the samples collected in Ledøje plantation are not considered as part of the chronosequence because the soil management prior to afforestation is probably largely different to that in the area of Vestskoven, which was afforested significantly later in time. The older stands are therefore providing information on ECM fungi that can be found in old forest areas with similar soil conditions to those in the chronosequence, and are used as a reference rather than being part of the strict afforestation chronosequence. It is important to consider that there may always be unexpected differences in soil conditions between the sampling stands and that the results might for that reason be affected to some degree.

Figure 4. The afforested area of Vestskoven presenting the investigated chronosequence, along with the year of plantation indicated adjacent to each plot or group of plots; Norway spruce (green dots), oak (orange) and Ledøje Plantation (blue).

## 2.2. Sampling

In 1998, three circular subplots with a radius of 10 m were established in each stand of the chronosequence (Vesterdal et al. 2002) for a study on the changes in SOC following afforestation. In 2010-2011, another study used the same plots for SOC monitoring and lost marking poles were replaced, as well as some additional plots were added in some stands (Bárcena et al. 2014a). For this thesis project, the same plots were used, limited to stands that were oak or spruce dominated. 6 stands of each tree species were available, 16 spruce plots and 18 oak plots. Some stands had been partially or entirely destroyed, so the current chronosequences covered an age range of 24-52 years for the spruce stands and 28-51 years for the oak stands. Most plots remained marked with poles since 2011, which made them easy to find, otherwise plots were relocated by using the GPS of a smartphone with the known coordinates of the plots (Google Maps 2021).

Soil and litter samples were collected in the first week of April 2021. Each plot sample consisted of three sample replicates that were collected and pooled in a composite sample. Forest floor litter was collected on an area basis by using a 15x15 cm wooden frame and soil samples were collected with a cylindrical soil sampler with a 5 cm inside diameter at a 10 cm depth, taken randomly within the plot but with a minimum of 2 m distance between samples. Litter samples were placed in paper bags and were dried at 60 °C for 48 hours to constant weight before weighing. Soil samples were kept in zip-lock bags and stored in 4 °C for up to 1 month until processed.

Root samples were collected in the last week of April 2021, as spring season that year was rather cold and an earlier attempt for sampling ECM root tips revealed that they were not developed enough for

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efficient observation. Each plot sample consisted of six sample replicates that were collected and pooled in a composite sample. Samples were collected using a cylindrical soil auger with a 2 cm inside diameter to 10 cm depth, taken randomly within the plot but with a minimum distance of 2 m between samples, as well as a minimum distance of 1 m from a tree, to ensure the collection of the relevant tree roots. A study focusing on the distribution of *Picea abies* in Europe from the boreal to temperate zones, showed that the bulk of the fine roots were mainly concentrated in the top 10 cm of the soil profile (Stober et al.), making the study of ECM root tips sufficient by sampling in that depth. Root samples with substrate were placed in zip-lock bags and stored in 4 °C for up to 3 weeks until processed.

Soil, litter and root samples from Ledøje Plantation were all collected in the last week of May 2021 as it was only decided later to include them for comparison. In total, 37 samples (soil, litter and roots) were collected from the available above-mentioned plots.



Figure 5. Demonstration of sampling procedure, from left to right: 15x15 cm frame for litter sampling, 5 cm metal core marked at 10 cm length for soil sampling, and 2 cm metal core marked at 10 cm length for root sampling.

## 2.3. Soil analysis

Soil samples were analyzed for water content, loss on ignition (LOI), pH and C/N ratio. Coarse and fine roots, stones and fragments of litter were removed by hand and the soil was passed through a 2 mm sieve as preparation for the analysis.

### 2.3.1. Water content

A minimum of 30 g of moist, sieved soil was placed in paper bags, was weighed and then dried for 48 hours at 80 °C to constant weight. The samples were then weighed again to determine the weight difference which is attributed to the loss of water. The soil was then ground in an IKA® A10 basic grinding machine for further analysis.

### 2.3.2. Loss on ignition

Dried and ground soil was placed in pre-weighed and labelled crucibles to about ⅓ to ½ of their volume. Crucibles filled with soil samples were then weighed again. Samples were ignited in a furnace at 550 °C for six hours. Crucibles were then cooled in a desiccator and weighed again.

### 2.3.3. Determination of pH

50 mL of distilled water were added to 10 g of moist soil (approximately a ratio of 2:1 v/v) and extracted for 1 hour on a rotary shaker. The soil solutions were then left to rest until settled for another hour, before proceeding to the pH measurement. A Mettler Toledo pH meter was used after calibration with buffer solutions and the glass electrode was rinsed between all measurements in distilled water.

### 2.3.4. Determination of C/N ratios

Determination N concentrations was done using the Dumas-method, through combustion at high temperature to $N_2$ and $NO_x$, followed by reduction of $NO_x$ to $N_2$ in preheated Cu-catalyst. The amount of $N_2$ was analysed by a thermal conductivity detector. Prior to the analysis, appropriate amounts of finely-ground soil (0.1-0.5 g) was packed in tin-capsules and weighed on a precision scale. The packed samples were combusted in a stream of $O_2$, resulting in the formation of $N_2$, $NO_x$, CO2 and $H_2O$. These gasses were then reduced to $N_2$ and analysed in the detector against a reference (EDTA) by thermal conductivity. At the same time, the soil C content was analysed through combustion at high temperature and determined as the amount of CO2 on an IRGA (infrared gas analyser).

### 2.3.5. Litter cover

Litter samples were dried in 70 °C for 48 hours and were then weighed for determination of the litter cover biomass per area.

## 2.4.  Mycorrhizal analysis

Root samples were soaked in tap water and left to soften overnight. Samples were then washed under high pressure flowing tap water on a 1 mm mesh standard sieve to capture roots with ECM root tips. The sieve was then emptied into a white photo-tray with water from which roots were collected for 10 minutes and placed in petri dishes with distilled water for observation under an Olympus SZX16 stereomicroscope with an SDF Plapo 1XPF lens at x10-60 magnification. Petri dishes were marked with grids to make observation easier and ensure that the entire area of the petri dish, and consequently all roots, were observed, for finding more than 98% of mycorrhizal tips in the sample. This was done in great detail in order to determine the fungal biomass per area to a 10 cm depth, both for the total ECM but also for each identified morphotype. ECM root tips were gently cut off the roots with the use of forceps and placed in salt trays for sorting into morphotypes according to their morphological and anatomic features. All morphotypes were given an ID number and were photographed under the microscope.

### 2.4.1. Morphological analysis

Criteria for sorting the ECM tips into morphotypes included surface color, presence of hyphae, presence of rhizomorphs, branching pattern and shimmering of surface color, which is an adapted and accepted methodology (Agerer 2001). In total, from the 37 plots, 169 morphotypes were examined by morphological, molecular and chemical analyses. Each morphotype group consisted of different numbers and sizes of mycorrhizal tips and were placed into separate Eppendorf tubes to be further

freeze-dried and weighed for determination of their biomass, before proceeding to the molecular and chemical analyses.

## 2.4.2. Molecular identification

From each identified morphotype group, one clean root piece was selected and transferred into 8-strip tubes with single lids. Total DNA was extracted from the identified morphotypes by using the Extract-N-Amp™ Plant PCR Kit by Sigma-Aldrich. In a cleaned laminar flow cabinet, the extraction solution was left to thaw on ice and, with the use of a pipette, 10 μl were put into each 8-strip tube containing the clean ECM tips. Samples were then incubated on 95 °C for 10 min and on 20 °C for another 10 min for DNA extraction. After the extraction, 10 μl of dilution solution was added into each 8-strip tube and samples were stored in the freezer until PCR.

PCR amplification was performed using 5x HOT FIREPol® Blend Master Mix with 10 mM MgCl2 (Cat. No. 04-25-00120), and the pair of forward primer ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990; Gardes and Bruns 1993), manufactured by Solis BioDyne (Estonia). The prepared PCR plates were subjected to an amplification program as follows: initial denaturation at 95 °C for 15 min followed by 35 cycles of 30 sec at 95 °C, 35 sec at 55 °C and 60 sec at 72 °C, and a final hold of 4 min at 72 °C. The quantity and quality of PCR products were checked by gel electrophoresis using the 100bp DNA Ladder by Solis BioDyne.

Two PCR plates containing all 169 samples were prepared and sent to Macrogen Europe B.V. in the Netherlands for Sanger sequencing by ordering the Standard-Seq service. Samples were additionally ordered to be purified with ExoSap-IT (product #78201, USB Corp., Clevand, OH, USA) prior to sequencing by enzymatic purification reaction at 37 °C for 15 min and at 80 °C for another 15 min. Cycle sequencing was performed using BigDye Terminator v3.1 and the primers ITS1F and ITS4 at a program starting with denaturation at 96 °C for 1 min followed by 25 cycles of 10 sec at 96 °C, 5 sec at 50 °C and 4 min at 60 °C. The sequence products were concentrated by ethanol precipitation.

The resulting sequences were trimmed using the R (R Core Team 2013) script "abifToFastq" from the CrispRVariants package by Helen Lindsay (Lindsay 2011), with a cutoff value of 0.01 corresponding to a phred score (quality score) above 20. The trimmed sequences were then subjected to BLAST through plutoF's massBLASTer (Abarenkov et al. 2010b) with standard settings. Below, figure 4 illustrates how the quality of each base varies throughout the sequence, with a usual drop towards the end. The result of each BLAST search was carefully examined and taxonomic affinity was only assigned if the sequences had ≥95 % similarity, 100% query cover and aligned over 400 base pairs.
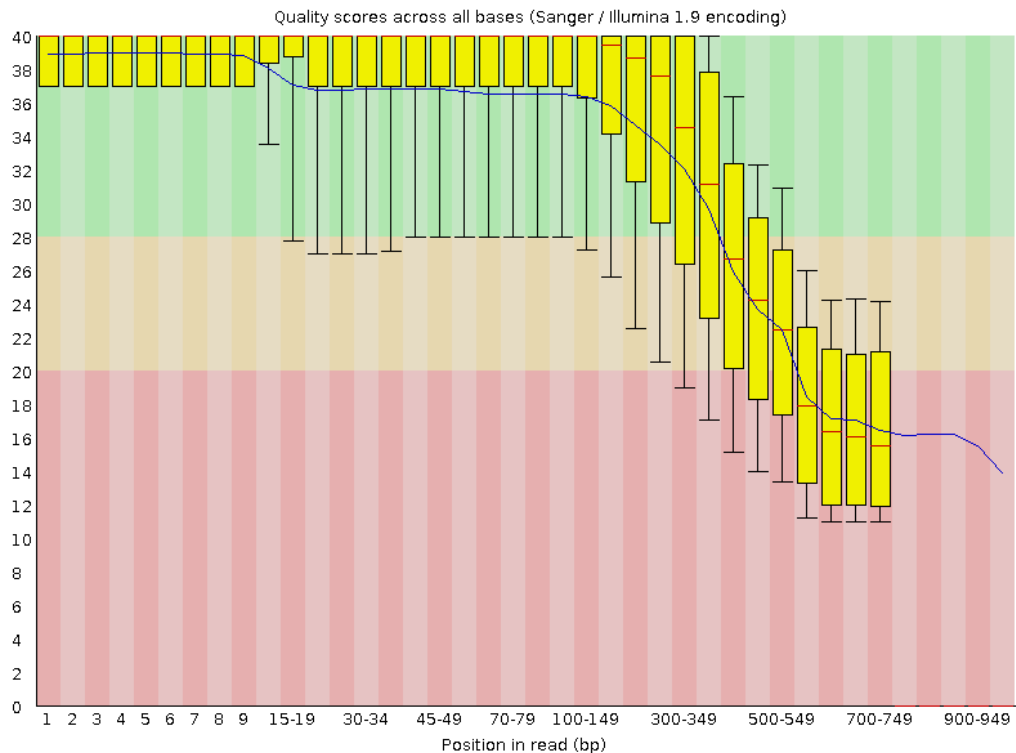
Figure 6. Illustrating how the quality score of each base (y-axis) usually falls towards the end of a sequence (x-axis).

A second round of BLAST was conducted for the sequences that had a similarity of ≥90% or down to 100 base pairs available for comparison. These sequences were analysed and edited with the use of MegaX software version 10.2.6 (Kumar et al. 2018), and were subjected to BLAST searches (http://blast.ncbi.nlm.nih.gov.ep.fjernadgang.kb.dk/) directly through the software, as well as with the UNITE (http://unite.ut.ee/) database (Abarenkov et al. 2010a), to determine similarities to sequences in the GenBank database (https://www-ncbi-nlm-nih-gov.ep.fjernadgang.kb.dk/). When results were differing, those provided by UNITE were preferred, as it is a database with focus on the eukaryotic nuclear ribosomal ITS region. The sequences were then carefully re-examined and were only used for identification if the available part of the sequences matched the start or end of a known species sequence. The results of the second BLAST round were marked as less reliable than the first one, and were included in the analysis. Based on BLAST results combined with the descriptions of the morphological characteristics of each morphotype, further identification was conducted for morphotypes with similar description, photograph and sequences.

## 2.4.3. Determination of C/N ratios

C and N concentrations were determined following the same method as described for soil samples.

## 2.4.4. Melanin concentration measurement

An attempt was made to establish a method for measuring melanin concentrations in ECM root tips by adapting the methodology used in other research projects for the measurement of melanin in cultured fungi (Butler and Lachance 1986; Frederick et al. 1999; Fernandez and Koide 2014). The adapted method is based on the ability of the dye Azure A chloride (Sigma-Aldrich® A6270) to bind strongly to melanin. Melanin concentrations are determined by measuring how the absorbance of the

dye solution changes before and after exposure to melanin. High melanin concentrations will result in significantly lower absorbance of the dye solution, and low melanin concentrations will result in small changes in the absorbance. The Azure A dye solution was prepared by dissolving the dye in 0.1 M HCl and then filtering the solution through a 0.45 µm syringe filter to remove any remaining undissolved dye. The dye solution was then further diluted with 0.1 M HCl until an absorbance of approximately 0.665 at 630 nm was accomplished.

In the cited projects (Butler and Lachance 1986; Frederick et al. 1999; Fernandez and Koide 2014), the fungi of interest were cultured, hyphae were isolated through filtration and further acidified with concentrated HCl. This was done in order to isolate the melanin from the fungal tissues which was achieved thanks to the fact that melanin is acid insoluble and will remain intact after hydrolysis of all other cell components (Butler and Lachance 1986). Next, the precipitated melanin was centrifuged (or filtered), washed with deionized water, and lyophilized at -20 °C to remove excess water. A standard curve was generated using melanin which was isolated from dark fungal species, namely the black yeast Phaeococcomyces (Butler and Lachance 1986), Gaeumannomyces graminis var. graminis (Frederick et al. 1999) and Cenococcum geophilum (Fernandez and Koide 2014). Known amounts of melanin ranging from 0.5 mg to 3 mg were added to 3 mL of Azure A stock solution and incubated for 90 minutes. The absorbance of the stock melanin and dye solutions was measured at 630 nm in a spectrophotometer, after filtering through a 0.45 µm syringe filter.

The fungi used in this thesis project were sampled from forests and were not cultured prior to chemical analysis. Additionally, ECM root tips were not separated from tree roots before the measurement of melanin, which means that they contained pieces of enclosed fine tree roots. The attempt was focused on developing an assay for measuring melanin concentrations in small sample sizes ranging from 0.1 to 15 mg of ECM root tip biomass. Not all steps described above were adapted in the method development. Instead of isolating melanin from cultured fungi, sampled and dried (at 70 °C) ECM root tips were directly placed in the dye stock solution and absorbance was measured before and after 90 minutes of incubation. Melanin was not isolated by acidification, washing and lyophilization. Additionally, a standard curve was prepared with the use of known amounts of synthetic melanin (Sigma-Aldrich® M8631) instead of isolated melanin from a dark fungal species.

The intention for this method was to develop a quick and easy way to measure melanin in fungi sampled directly in their natural habitat. This would hopefully provide values representing the melanin concentration as a result of the environment and conditions the fungi are found in. If fungi are cultured prior to the measurement, melanin concentrations may no longer correspond to the concentrations found in wild fungi, as growth conditions would vary significantly. Melanin concentration in fungi may be affected by a range of stress factors including UV radiation (Singaravelan et al. 2008), high concentrations of heavy metals (Gadd and Rome 1988) and reduced water potential (Kogej et al. 2006), and appears to play a crucial role in fungal necromass decomposition rates, which makes it interesting to measure directly in ECM found in their natural habitats. Using fungal tissues sampled from the forest, however, comes with a disadvantage. The collected ECM root tips are very small in size and biomass, resulting in extremely small sample sizes that are hard to work with. It was therefore clear that it would be too ambitious to extract melanin from these samples due to the small biomass collected. An attempt was therefore made to measure melanin concentrations in ECM root tip samples by adding them directly in Azure A dye solution to test whether the method can be adapted for small sample sizes without prior extraction of melanin.

A first test was made with samples collected from beech roots, sampled in a nearby park (Fælledparken). The standard curve was made with known amounts of synthetic melanin ranging from 0.7 to 3 mg that were placed in 3 mL of Azure A stock solution. In total 9 ECM samples weighing between 0.2 and 6.7 mg were dried and placed in Azure A stock solution. Samples weighing up to 3 mg were placed in 200 µL of stock solution and samples above that in 400 µL. After 90 minutes of incubation, everything was centrifuged twice and placed in 96-well UV-Transparent microplates for spectrophotometry. The generated standard curve did not seem to cover all the melanin

concentrations found in the small fungal samples. It was therefore decided to make a second test by generating a standard curve with a larger range of melanin concentrations. Additionally, some of the fungal samples changed the absorbance to the minimum and it was decided to place samples in larger volumes of stock solution in order to avoid extreme measurements that may imply saturated dye solutions where excess melanin has nothing to bind to and cannot be measured. Last, there was no significant difference in the change of absorbance, and subsequently in the calculated melanin content, between species with distinct visual difference in color.

A second test was then conducted by generating a standard curve with known amounts of synthetic melanin ranging between 0.2 and 3.4 mg that were placed in 1 mL of Azure A solution. 19 fungal samples ranging from 0.1 to 11.3 mg were placed in 1 mL of Azure A stock solution and were left to incubate for 90 minutes. This time the standard curve covered the whole range of resulting melanin contents in the fungal samples, and extreme values were avoided. However, once more, there seemed to be no significant difference in calculated melanin content between fungal groups with distinct visual color difference. Despite the obvious difference in color between the fungal groups, the absorbance appeared to change according to the biomass added to the stock solution rather than the color darkness, as seen in the scatter plot below. It was therefore decided that the method could not be used without extracting the melanin prior to addition to the stock solution. Possibly, apart from melanin, other molecules such as lignin may bind to the Azure A dye and cause additional change in the absorbance. No further investigation was carried out to determine the reasons why the absorbance changed according to biomass instead of color darkness. This method was not used for the samples collected in Vestskoven and no results for melanin content were generated.
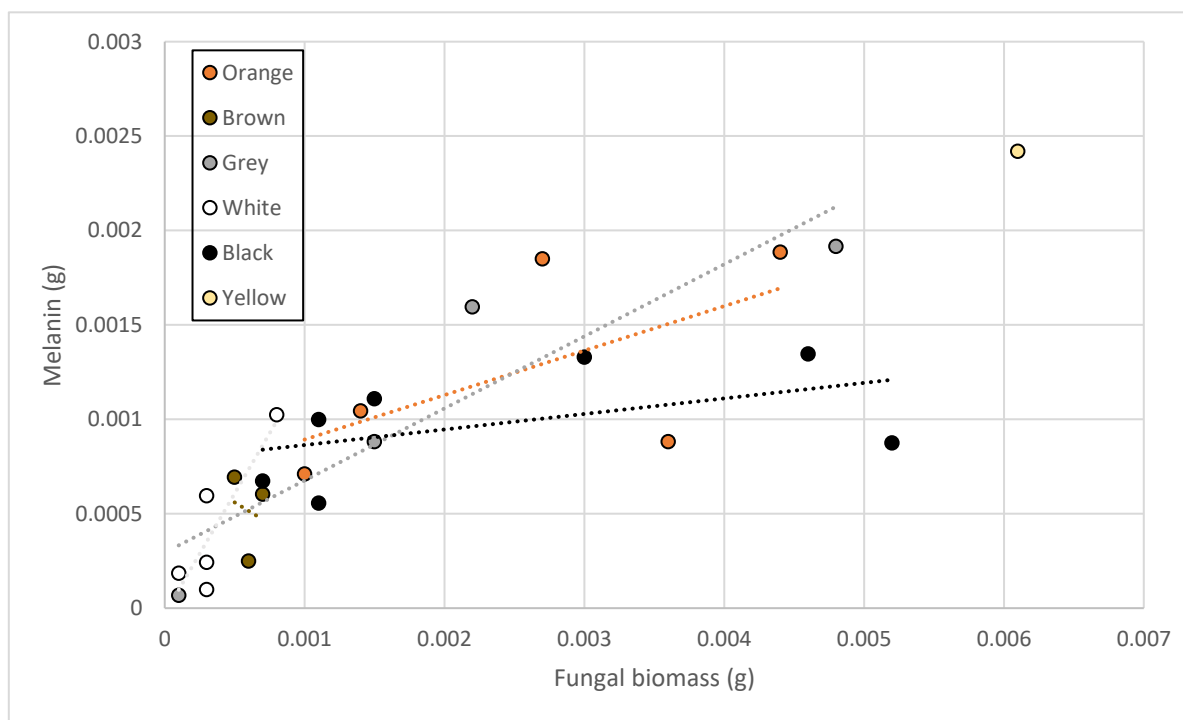


Figure 7. Scatter plot with lines illustrating the relation between the calculated melanin concentration and fungal biomass, among the different fungal groups divided based on color difference.

## 2.5. Data analysis

Soil data were statistically analyzed to investigate the potential effects of stand age and tree species on the measured variables: water content, LOI, pH, C/N ratios and litter cover. Simple linear regression analyses were performed for each dependent variable and for each tree species separately, with the

confidence level set at 95%, in order to understand whether stand age can predict the measured variables. Furthermore, single-factor ANOVA analyses were performed for each variable and tree host separately, with the a-value set at 0.05. When the result was significant, the single-factor ANOVA was supplemented with Tukey's test, to determine where the differences lie. Finally, to analyze the effect of different tree species on the measured soil variables, two-factor ANOVA analyses with replication were performed, with stand age and tree species as the independent variables and the alpha value set at 0.05. Since the spruce stand age 52 (afforested in 1969) had only one remaining subplot (since the other two were destroyed by windbreak), the measures of the remaining subplot were used three times, to represent three hypothetical subplots in order to be able to include this stand age in the single and two factor ANOVA analyses. Additionally, since the spruce and oak stand ages were not exactly the same, they were grouped into 6 categories as "youngest-young-low medium-high medium-old-oldest" in order to be able to perform the two-factor ANOVA analyses. All the soil statistical analyses were performed with the use of Microsoft Excel (Microsoft Corporation 2018). The soil measurements were also graphed as scatter plots with trendlines, to visualize their relation with stand age. Root data were statistically analyzed by following the same method used for the soil data.

ECM data were visually presented through column charts to see the relation of ECM fungal biomass and number of ECM species with stand age, scatter plots with trendlines to present the calculated biodiversity indices of each stand age, and box-and-whisker charts to see the relation of ECM C/N ratios with stand age. Additionally, sample-species matrices were generated for the ECM fungi found in association with each tree species separately, to detect and compare the dominant species and species richness, and to further calculate the diversity and evenness with the use of appropriate indices. Furthermore, an abundance-rank diagram was produced, to visualize the species richness and dominance found in the stands of the two tree species. All graphs and calculations were performed with the use of Microsoft Excel (Microsoft Corporation 2018).

# 3. Results

## 3.1. Soil data

### 3.1.1. Soil Water Content

There was no significant trend for soil water content with age for the two tree species in the samples (fig. 8) and neither did the 1-way ANOVA show significance, for either tree species. However, the two-factor ANOVA indicated that there was a statistic significant difference in soil water content by tree species ($p < 0.001$) but again not by stand age. The interaction between the two factors was also significant ($p < 0.05$).
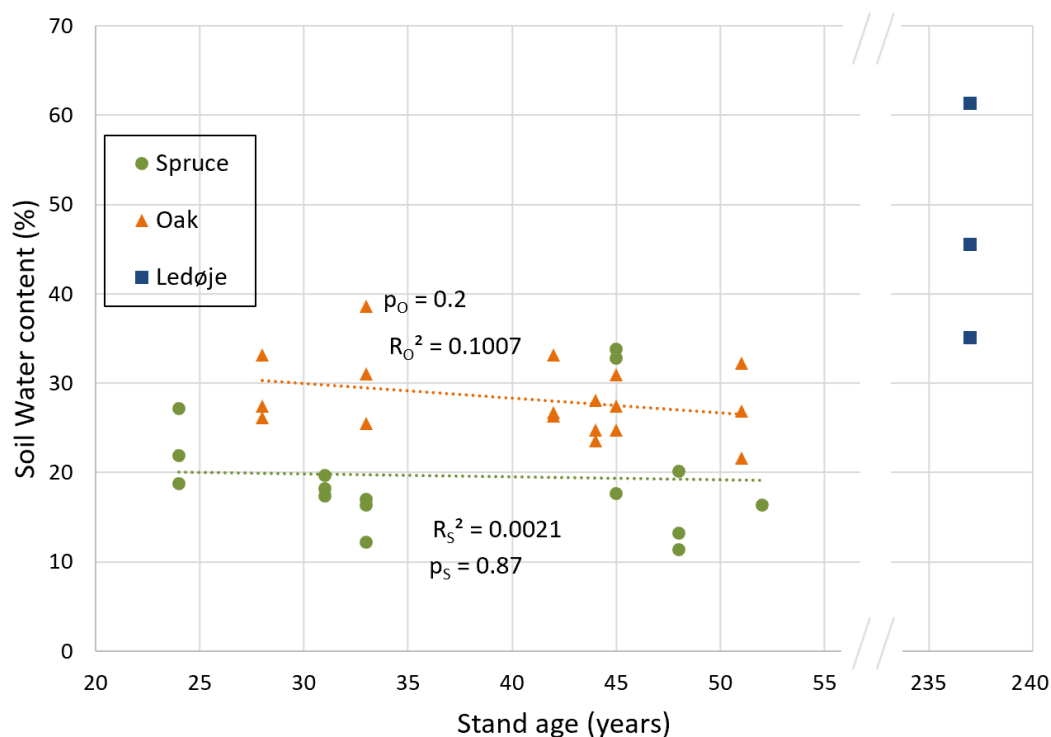


Figure 8. Scatter plot with trendlines showing the percentage of soil water content in samples taken from spruce and oak stands along the chronosequence in Vestskoven, as well as in Ledøje plantation (control) for comparison. Each plotted point in the figure represents a sample from one of the three subplots for each stand age and one tree species. Each sample consisted of three subsamples to cover the variation of the subplot that were pooled into one common subplot sample. R2 and significance (p-value) are shown for each regression.

### 3.1.2. Soil LOI

There was no significant trend for soil organic matter (LOI) with age for the two tree species (fig. 9). However, the single factor ANOVA analyses for spruce showed significance, which implies that there was a difference in LOI among the age groups of spruce stands, but not for oak. A complementary Tukey-Krammer test was performed to detect where the differences lie, and as seen also in figure 5, the stand with the age of 45 years (afforested in 1976) was significantly different from all other stand ages, with higher values of soil organic matter. Additionally, the results of the two-factor ANOVA indicated that both factors of tree species and stand age were significantly different (tree species $p <$

0.001 and stand age p < 0.01), suggesting that soil LOI was affected by both tested variables, as well as by the interaction of the two variables which was also significant ($p < 0.01$).
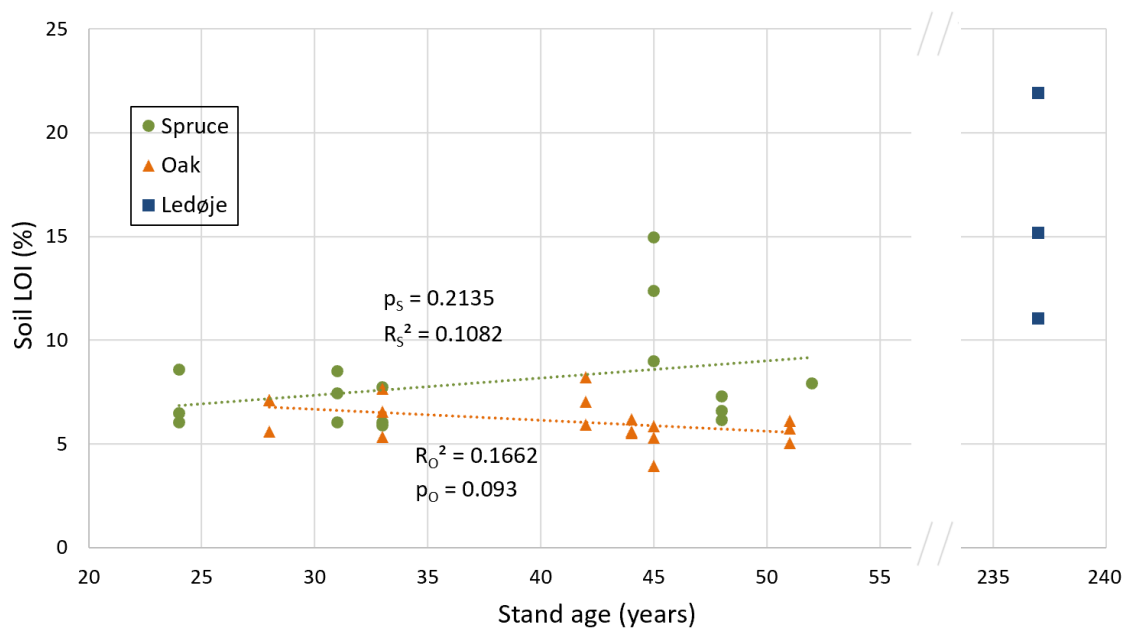


Figure 9. Scatter plot with trendlines illustrating the percentage of loss on ignition in samples taken from spruce and oak stands along the chronosequence in Vestskoven, as well as in Ledøje plantation (control) for comparison. Each plotted point in the figure represents a sample from one of the three subplots for each stand age and one tree species. Each sample consisted of three subsamples to cover the variation of the subplot that were pooled into one common subplot sample. $R^2$ and significance (p-value) are shown for each regression.

## 3.1.3. Soil pH

There was a significant negative trend for pH with age for both tree species (fig. 10). The greater the stand age the lower the pH. The single factor ANOVA results did not show significance for either of the tree species. However, the two-factor ANOVA analysis indicated that both stand age and tree species were statistically significant (stand age $p < 0.01$ and tree species $p < 0.01$), suggesting that soil pH is affected by both variables, stand age and tree species, but not necessarily by the two variables together, because the interaction was not significant ($p = 0.25$).
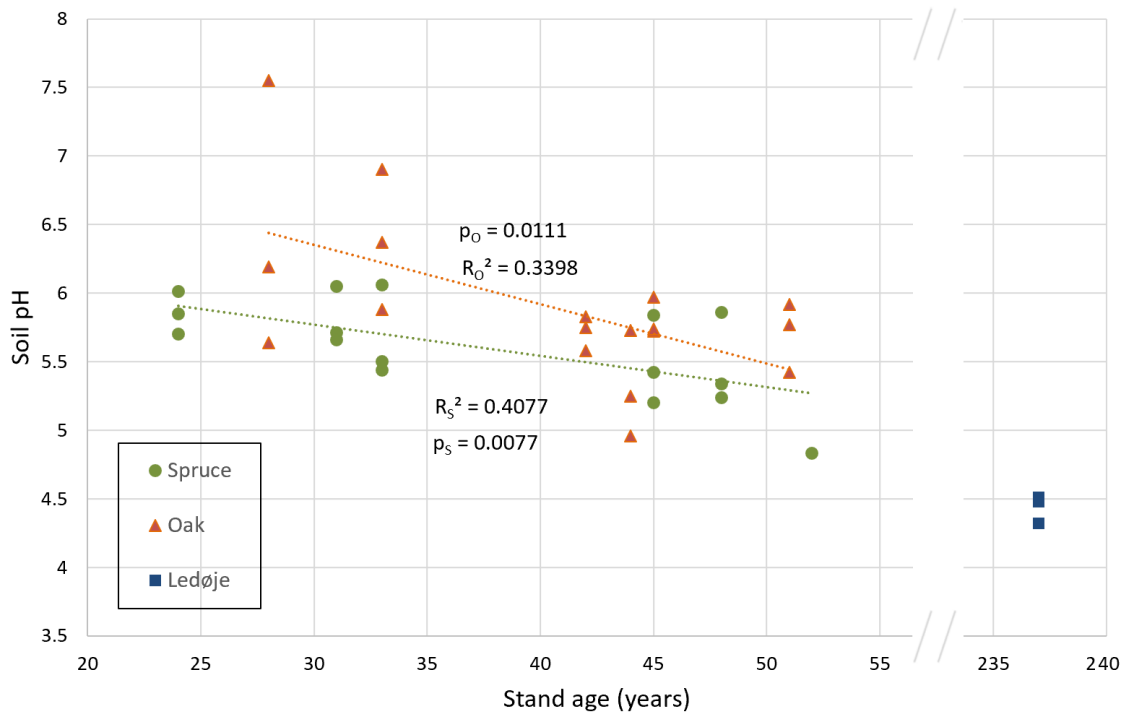
Figure 10. Scatter plot with trendlines showing the pH of soil in samples taken from spruce and oak stands along the chronosequence in Vestskoven, as well as in Ledøje plantation (control) for comparison. Each plotted point in the figure represents a sample from one of the three subplots for each stand age and one tree species. Each sample consisted of three subsamples to cover the variation of the subplot that were pooled into one common subplot sample. $R^2$ and significance (p-value) are shown for each regression.

### 3.1.4. Soil C/N ratios

There was no significant trend for C/N ratios with age for either tree species (fig. 11). However, the single factor ANOVA showed significance in the spruce stands ($p < 0.05$) but not in the oak stands, indicating that spruce trees will affect C/N ratios. A complementary Tukey-Krammer post hoc test was performed to confirm what can otherwise be seen in the scatter plot below (fig. 7), that the stand aged 45 years is significantly different from all others, with higher soil C/N ratios. Additionally, the two-factor ANOVA analysis indicated that both tree species and stand age were statistically significant (tree species $p < 0.001$ and stand age $p < 0.05$), suggesting that soil C/N ratio is affected by both variables, tree species and stand age, as well as by the two variables together because the interaction was also significant ($p < 0.001$).
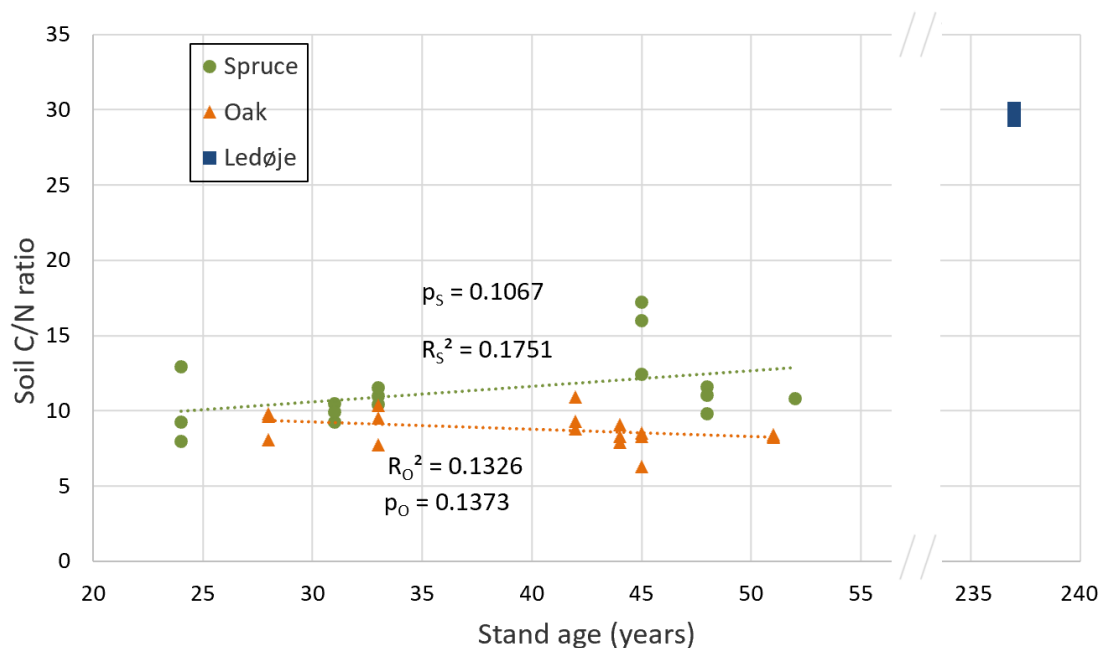
Figure 11. Scatter plot with trendlines illustrating the C/N ratio of soil in samples taken from spruce and oak stands along the chronosequence in Vestskoven, as well as in Ledøje plantation (control) for comparison. Each plotted point in the figure represents a sample from one of the three subplots for each stand age and one tree species. Each sample consisted of three subsamples to cover the variation of the subplot that were pooled into one common subplot sample. $R^2$ and significance (p-value) are shown for each regression.

### 3.1.5. Litter mass

There was no significant trend for litter cover with age for either tree species but a trend of more litter in spruce with age is indicated though the variation is large and one stand age falls out completely (fig. 12). However, the single factor ANOVA analyses showed significance for both tree species (spruce and oak $p < 0.01$), indicating that tree species affects C/N ratios. The complementary Tukey-Krammer test was performed to detect that the litter cover in the oldest spruce stand (52 years old) was significantly higher that the younger stands, and among the oak stands the one aged 33 (afforested in 1988) was significantly different from all others. Additionally, the two-factor ANOVA analysis indicated that both tree species and stand age were statistically significant ($p < 0.001$ for both), suggesting that soil C/N ratio is affected by both variables, tree species and stand age, as well as by the combination of the two variables because the interaction was also significant ($p < 0.001$).
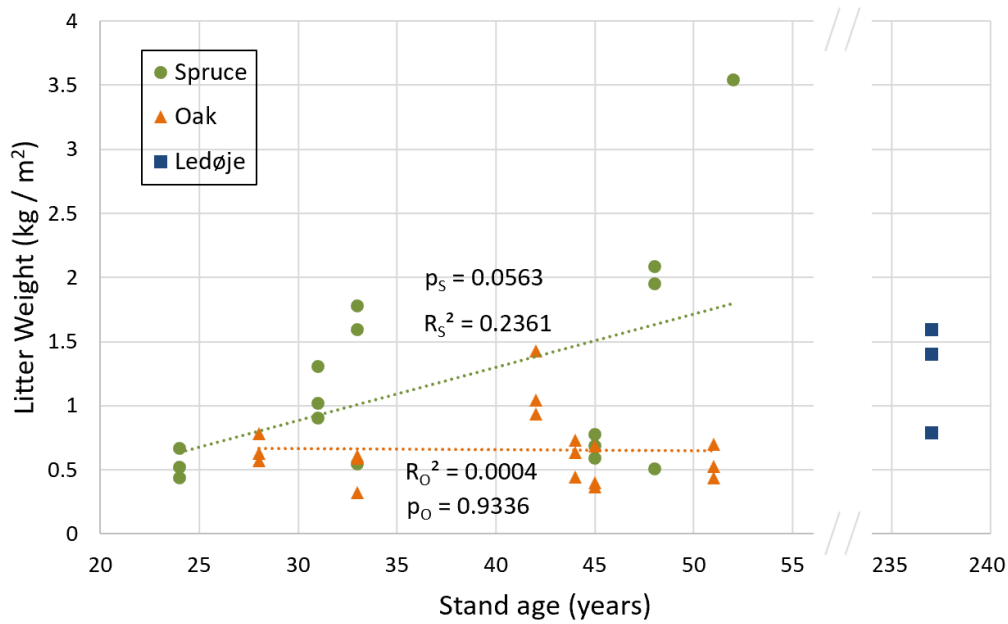
Figure 12. Scatter plot with trendlines showing the litter cover in kg per m$^2$ of soil in samples taken from spruce and oak stands along the chronosequence in Vestskoven, Denmark, as well as in Ledøje plantation (control) for comparison. Each plotted point in the figure represents a sample from one of the three subplots for each stand age and one tree species. Each sample consisted of three subsamples to cover the variation of the subplot that were pooled into one common subplot sample. R$^2$ and significance (p-value) are shown for each regression.

A summary of the soil statistics can be seen in the following table (fig. 13) that provides an overview of the p-values of all statistical tests that were conducted. Briefly, summing up the significant results given by the analyses of soil data; water content is affected by the interaction of the species and stand age variables, LOI is affected by the two variables as well as their interaction and the spruce stand aged 45 is higher than the rest, pH is affected by both variables but not by their interaction, C/N ratios are affected by the two variables as well as their interaction and the spruce stand aged 45 is higher than the rest, and last, litter mass is affected by the two variables as well as their interaction, the spruce stand aged 52 (the oldest stand in the chronosequence) had significantly more litter mass and the oak stand aged 33 was different than the rest.

| SOIL DATA STATISTICS OVERVIEW | | | | |
|---|---|---|---|---|
| | Regression | 1 -factor ANOVA | Tukey - Krammer | 2 - factor ANOVA |
| H2O % - Spruce | p > 0.05 | p > 0.05 | - | sp.: p < 0.001 |
| H20 % - Oak | p > 0.05 | p > 0.05 | - | age: p > 0.05 |
| | | | | inter.: p < 0.05 |
| LOI - Spruce | p > 0.05 | p < 0.05 | 45 yr | sp.: p < 0.001 |
| LOI - Oak | p > 0.05 | p > 0.05 | - | age: p < 0.01 |
| | | | | inter.: p < 0.001 |
| pH - Spruce | p < 0.01 | p > 0.05 | - | sp.: p < 0.01 |
| pH - Oak | p < 0.05 | p > 0.05 | - | age: p < 0.01 |
| | | | | inter.: p > 0.05 |
| C/N - Spruce | p > 0.05 | p < 0.05 | 45 yr | sp.: p < 0.001 |
| C/N - Oak | p > 0.05 | p > 0.05 | - | age: p < 0.05 |
| | | | | inter.: p < 0.01 |
| Litter - Spruce | p > 0.05 | p < 0.01 | 52 yr | sp.: p < 0.001 |
| Litter - Oak | p > 0.05 | p < 0.01 | 33 yr | age: p < 0.001 |
| | | | | inter.: p < 0.001 |

Figure 13. Overview of p-values for all statistical analyses conducted for the soil data. Significant results are highlighted with grey. In the Tukey-Krammer column is specified the stand age which showed significant difference from the others. In the 2-factor ANOVA column are given the three p-values of the test; Tree species effect (sp.), stand age effect (age) and the interaction of the two variables (inter.)

## 3.2. Fine root data

### 3.2.1. Fine root biomass

There was no significant trend for fine tree roots biomass with age for either tree species (fig. 14). The single factor ANOVA analyses showed significance for the spruce roots (p < 0.001) but not for the oak, indicating that spruce root biomass is greatly affected by the tree's age. The Tukey-Krammer test that was performed for the spruce data showed that the stand with age 31 years was significantly different from the others, with higher fine roots biomass. Additionally, the two-factor ANOVA analysis indicated that both tree species and stand age were statistically significant (p < 0.01 for spruce and p < 0.001 for oak), suggesting that root biomass is affected by both variables, tree species and stand age, but not necessarily by the two variables together, because the interaction was not significant.
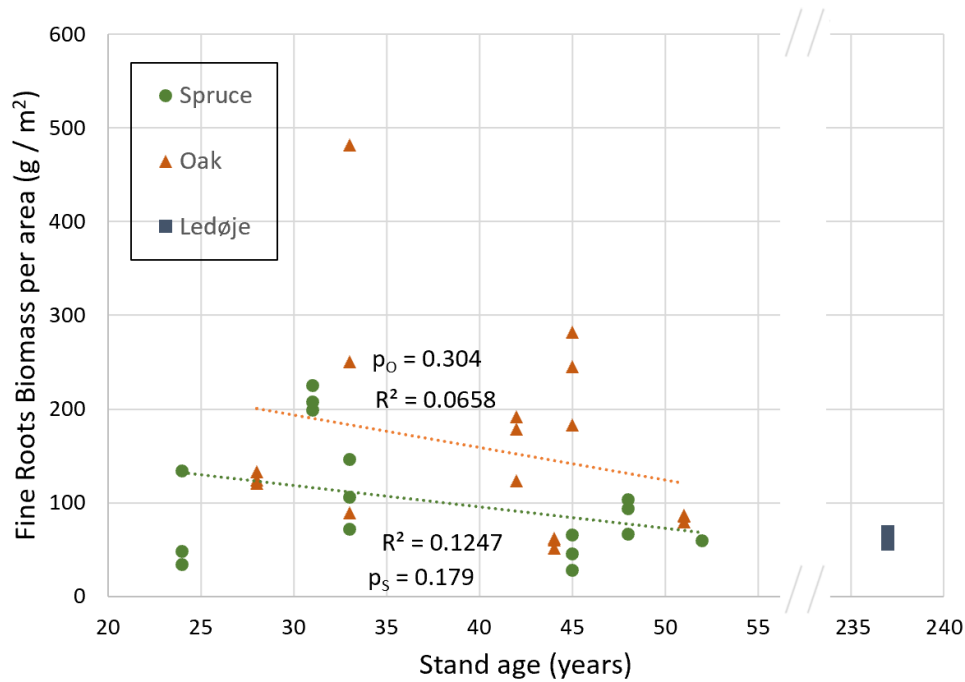
Figure 14. Scatter plot with trendlines illustrating the biomass of fine tree roots per m² in samples taken from spruce and oak stands along the chronosequence in Vestskoven, as well as in Ledøje plantation (control) for comparison. Each plotted point in the figure represents a sample from one of the three subplots for each stand age and one tree species. Each sample consisted of six subsamples to cover the variation of the subplot that were pooled into one common subplot sample. $R^2$ and significance (p-value) are shown for each regression.

## 3.2.2. Fine root C/N ratios

There was a significant negative trend for fine tree roots C/N ratio with age for spruce trees and not for oak (fig. 15). The single factor ANOVA analyses showed no significance for either tree species, indicating that the C and N concentrations of spruce and oak fine roots are not affected by the age within the examined ranges. Additionally, the two-factor ANOVA analyses indicated that neither the tree species or stand age were statistically significant, further confirming the 1-way ANOVA and showing that fine roots C/N ratios are not affected by either variable, tree species or stand age, neither by the two variables together, because the interaction was also insignificant.
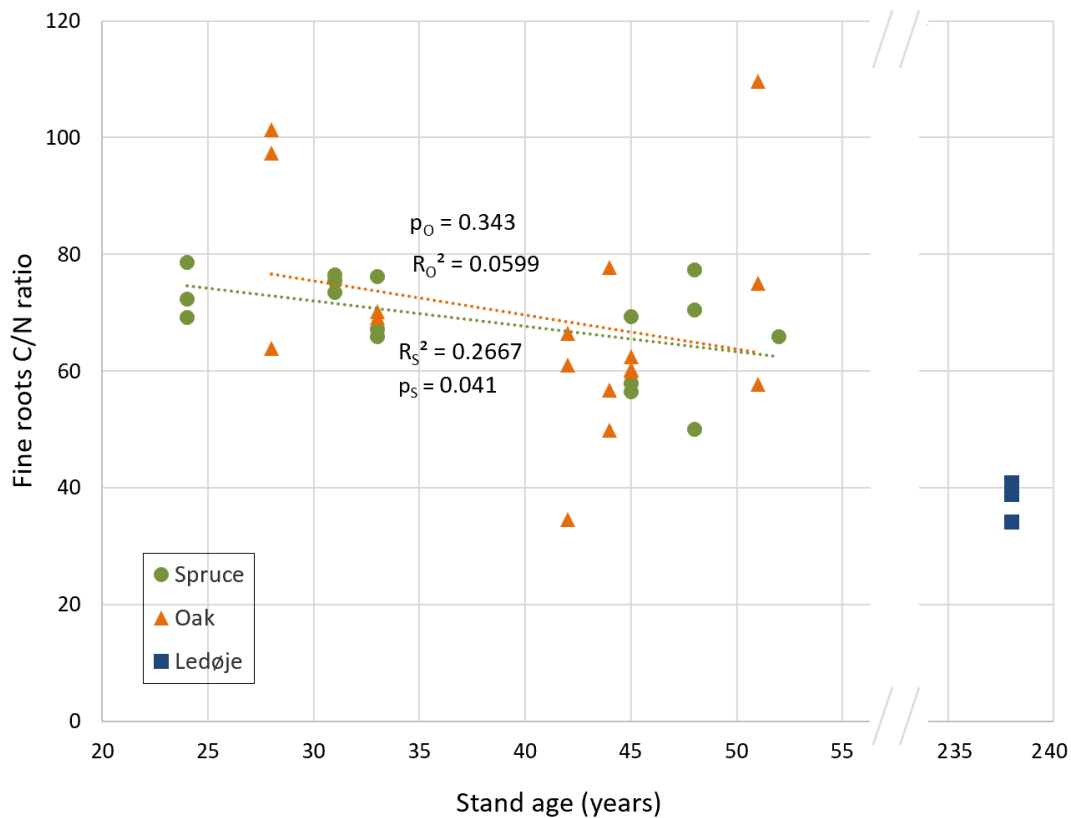
Figure 15. Scatter plot with trendlines showing the C/N ratio of fine tree roots in samples taken from spruce and oak stands along the chronosequence in Vestskoven, as well as in Ledøje plantation (control) for comparison. Each plotted point in the figure represents a sample from one of the three subplots for each stand age and one tree species. Each sample consisted of six subsamples to cover the variation of the subplot that were pooled into one common subplot sample. R2 and significance (p-value) are shown for each regression.

A summary of the fine root's statistics can be seen in the following table (fig. 16) that provides an overview of the significance of all statistical tests that were conducted. Briefly, summing up the significant results given by the analyses of the fine roots data; biomass is affected by both the species and stand age variables but not by their interaction and the spruce stand aged 31 is higher than the rest, and C/N ratios are not affected by either variable or their interaction.

FINE ROOTS DATA STATISTICS OVERVIEW

| | Regression | 1 -factor ANOVA | Tukey - Krammer | 2 - factor ANOVA |
|---|---|---|---|---|
| Biomass - Spruce | $p > 0.05$ | $p < 0.001$ | 31 yr | sp.: $p < 0.01$ |
| | | | | age: $p < 0.001$ |
| Biomass - Oak | $p > 0.05$ | $p > 0.05$ | - | inter.: $p > 0.05$ |
| C/N - Spruce | $p < 0.05$ | $p > 0.05$ | - | sp.: $p > 0.05$ |
| | | | | age: $p > 0.05$ |
| C/N - Oak | $p > 0.05$ | $p > 0.05$ | - | inter.: $p > 0.05$ |

Figure 16. Overview of p-values for all statistical analyses conducted for the fine roots data. Significant results are highlighted with grey. In the Tukey-Krammer column is specified the stand age which showed significant difference from the others. In the 2-factor ANOVA column are given the three p-values of the test; Tree species effect (sp.), stand age effect (age) and the interaction of the two variables (inter.)

## 3.3. ECM data

### 3.3.1. ECM C/N ratios

Both in spruce- and oak-associated ECM fungi, the C/N ratios showed very little fluctuations caused by the stand age (fig. 17 and 18). From the data it does not seem like stand age has a significant effect on the C/N ratios. By comparing the two tree species however, it becomes evident that ECM fungi found in spruce stands had higher C/N ratios ranging around 20-35, whereas ECM fungi found in association with oak trees had significantly lower C/N ratios ranging around 15-27, and those found in Ledøje plantation ranged between 12.5-19. The comparison of the oak-associated ECM fungi with the findings of Ledøje plantation indicate that there may be an effect of stand age on C/N ratios within oak stands, which is not evident in the young age ranges available in Vestskoven, and might only be noticeable in older stand ranges.



Figure 17. Box-and-whisker plot illustrating the C/N ratio in ECM fungi found in spruce stands along the chronosequence in Vestskoven, Denmark. The number of ECM species that were analyzed for C and N concentrations in each stand age differed ($n_{24}$ = 11, $n_{31}$ = 15, $n_{33}$ = 9, $n_{45}$ = 11, $n_{48}$ = 14, $n_{52}$ = 4). The boxes represent the values around the mean (marked with x) and whiskers show the minimum and maximum values range.
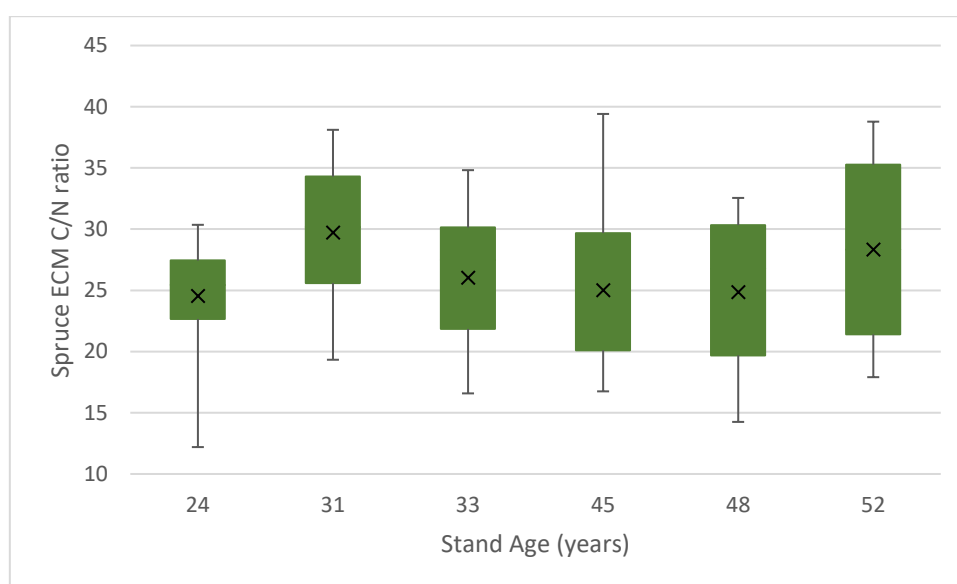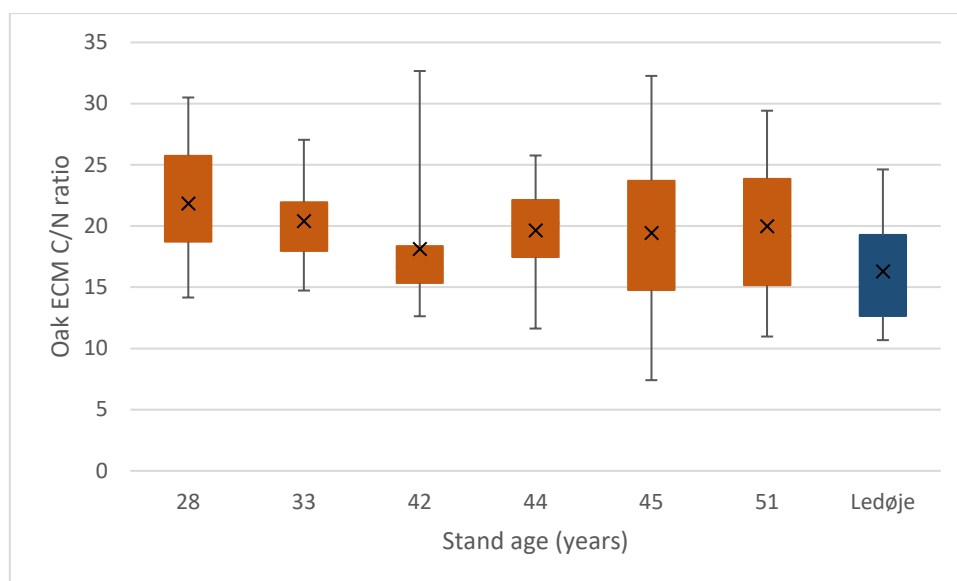
Figure 18. Box-and-whisker plot illustrating the C/N ratio in ECM fungi found in oak stands along the chronosequence in Vestskoven, Denmark, as well as Ledøje plantation. The number of ECM species that were analyzed for C and N concentrations in each stand age differed ($n_{28}$ = 11, $n_{33}$ = 15, $n_{42}$ = 18, $n_{44}$ = 12, $n_{45}$ = 14, $n_{51}$ = 15, $n_{Ledøje}$ = 10). The boxes represent the values around the mean (marked with x) and whiskers show the minimum and maximum values range.

### 3.3.2. Number of species and biodiversity

A total of 45 sequence verified ECM fungal species were found in the afforested area of Vestskoven. In the spruce stands, a total number of 45 morphotypes was found, of which 20 were sequence verified; 11 to the species level and 9 to the genus level only. Note that of the remaining unknown 25 morphotypes some may have been the same species and the total number of species would maybe not add up to 45 if the sequencing of all morphotypes had been successful. In the oak stands, a total number of 49 morphotypes was found, out of which 30 were sequence verified; 18 to the species level and 12 to the genus level only. Again here, the actual total number of species in the oak stands of Vestskoven is possibly between 30 and 49. Oak stands had therefore higher ECM fungal species richness than the spruce stands. The overlapping identified species between the spruce and oak stands were five, namely *Laccaria sp. 1*, *Lactarius rufus*, *Russula sp. 1*, *Russula sp. 2* and *Tomentella stuposa*.

As can be seen in the following figure (fig. 19), oak stands had higher species richness compared to spruce across all stand ages. Neither spruce- or oak- associated ECM communities showed a trend with stand age; at least for the age range available through the Vestskoven chronosequence.
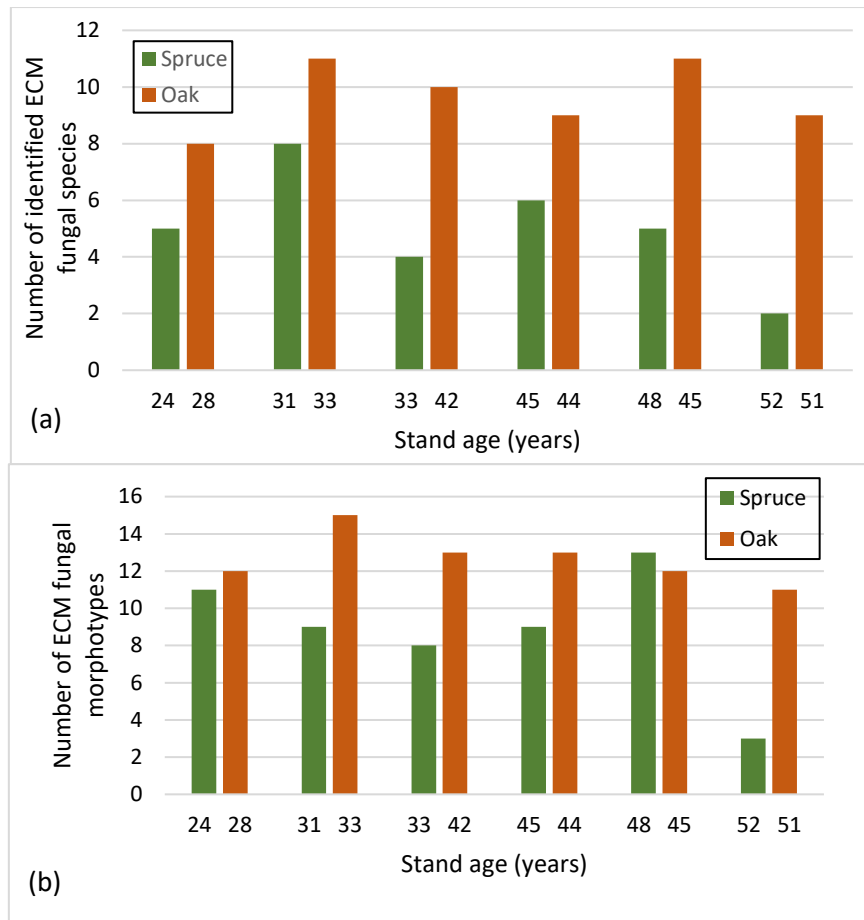
Figure 19. Column charts illustrating the number ECM fungal species (richness) found in association with the two tree species in the different stand ages. (a) presents the number of ECM fungal species that were successfully identified through the molecular analysis, and (b), the total number of ECM fungal species, including the unidentified morphotypes that could either be the same as one identified species or an additional unknown species, meaning that the real number of total species may be lower than indicated here. Note that spruce stand aged 52 only included one subplot available for sampling and the numbers are significantly lower than the rest probably because of the smaller sampling site size. All other stands consist of three subplots that were sampled and analyzed separately. Each sample consisted of six subsamples to cover the variation of the subplot that were pooled into one common subplot sample.

A rank-abundance diagram (fig. 20) was generated with the sequence verified ECM species to compare the fungal communities that were found in association with the two tree species in all age stands. The diagram allows us to observe that the spruce stands were less species rich but at the same time more dominated by few ECM fungal species, compared to the oak stands.

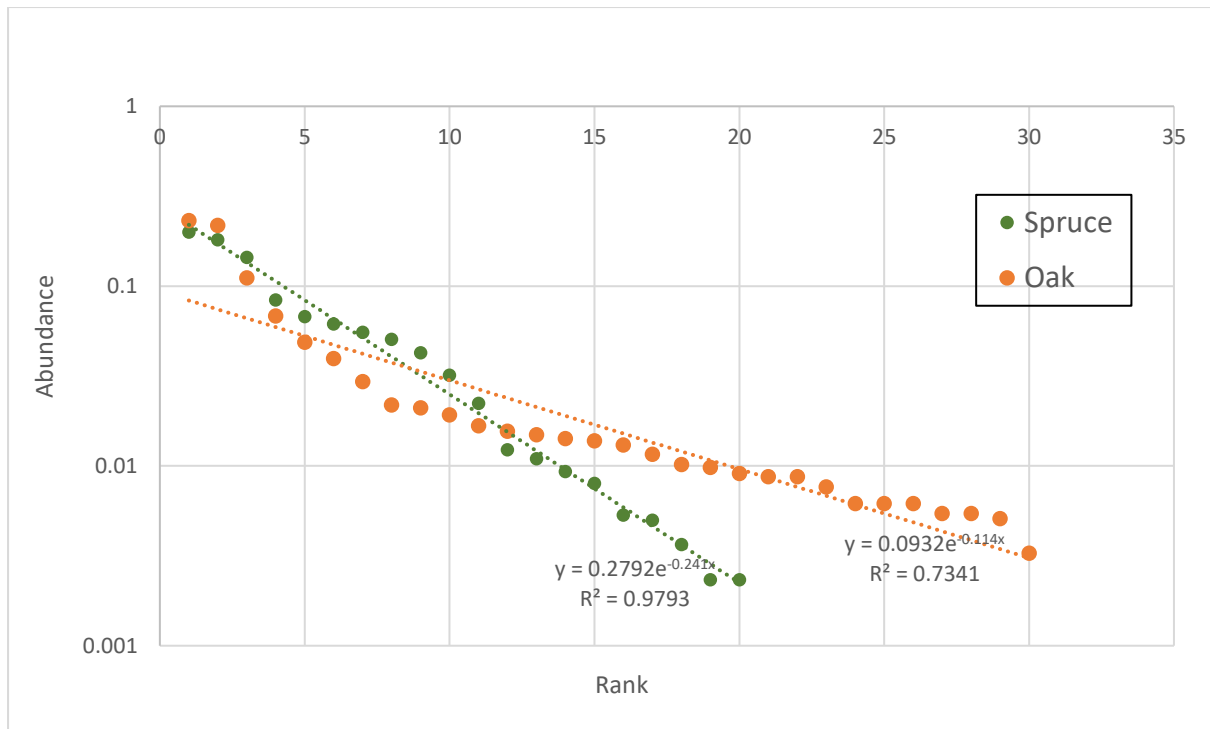Figure 20. Abundance - Rank diagram of sequence verified ECM fungal species in spruce and oak stands in logarithmic scale. Abundance is the relative abundance in biomass and each plotted dot represents the total biomass of a given species across all age stands per tree species. $R^2$ and equations are displayed on the graph.

To compare the biodiversity of the ECM fungi found in the stands of spruce and oak, the Shannon's Diversity Index (SDI) and the Pielou's Evenness Index (PEI) were used, as these allow us to make calculations based on the biomass rather than number of individuals, which applies more to the type of data used here. For the calculations, only the data of identified ECM species were included, as those were more reliable even though incomplete. Including the unidentified morphotypes measures in the calculations would give results based on an unreliable species richness, making all further results and comparisons untrustworthy. The SDI for the spruce- associated ECM community in all of Vestskoven (including all stand ages) was 2.4 and for the oak 2.6. The PEI for the spruce- associated ECM community of Vestskoven was 0.8 and for the oak 0.76. This means that the ECM community found in spruce stands had generally a lower diversity and a higher evenness than oak. Further, to understand how the biodiversity evolved over time, and to see the effect of succession on biodiversity, the SDI and PEI were also calculated for each stand age separately, and plotted into a graph to visualize it. As seen in the following figure (fig. 21a), the SDI of oak ECM species is slightly increasing whereas the diversity found in spruce stands appears to be decreasing over time. The PEI (fig. 21b) however, seems to be affected by succession in the same way for both the spruce and oak associated ECM communities, showing a positive trend over time.
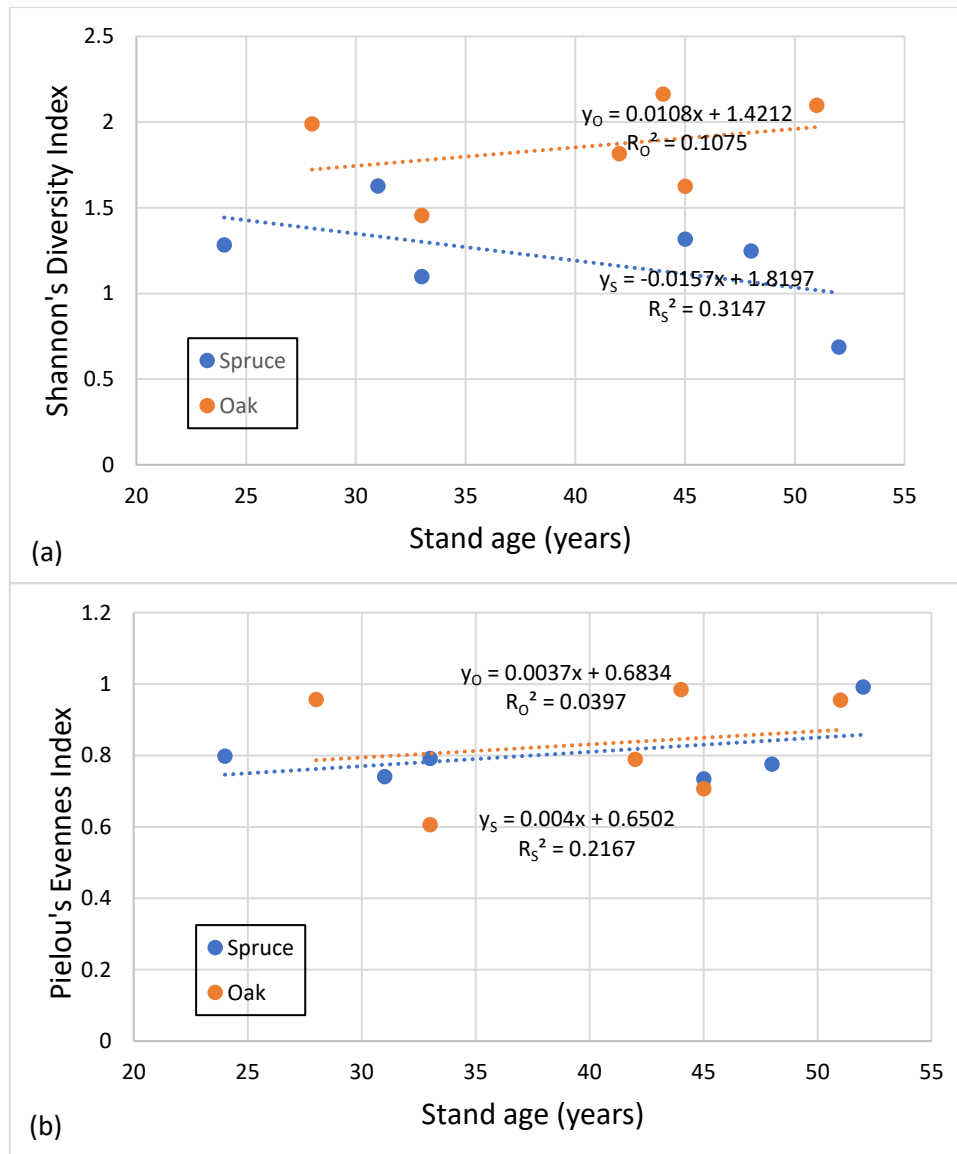
Figure 21. Scatter plots with trendlines showing the biodiversity of ECM fungi over the time range available through the chronosequence of Vestskoven, Denmark. Each dot represents the index of a stand age of spruce or oak and consists of 3 subplots that were sampled separately. $R^2$ and equations are displayed on the graphs. (a) Shannon's Diversity Index (b) Pielou's Evenness Index.

### 3.3.3. ECM root tip biomass

A total of 0.4613 g ECM root tip biomass was collected in all spruce stands and 0.407 g in all oak stands. This corresponds to 15.3 g of ECM root tip biomass per soil $m^2$ in the top 10 cm layer for the spruce stands and 12 g for the oak stands (153 and 120 kg per ha ECM root tip biomass in spruce and oak respectively). This biomass includes the small pieces of fine tree roots enclosed in the ECM fungal sheath (Smith and Read 2008). Although the species richness appeared to be significantly higher in the oak stands, the total fungal biomass was greater in the spruce stands. When looking at the following figure (fig. 22), which illustrates the total ECM fungal biomass per area found in each stand age of the two tree species, we see that there were fluctuations but no trend of biomass with stand age, for either tree species, at least for the age range found in the chronosequence of Vestskoven currently.

Figure 22. Column chart presenting the total ECM root tip biomass per area (g/m2) in the different stand ages of spruce and oak forest patches.

## 3.3.4. ECM species composition

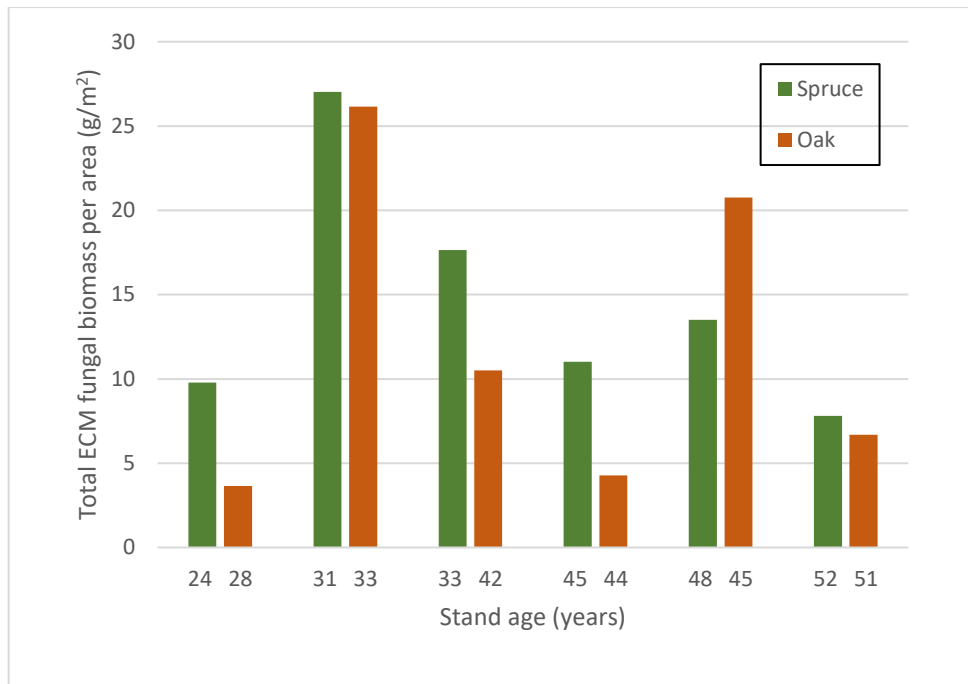The ECM species found in each age stand of the two tree species are listed in the table below (fig. 23). The changes in species composition are interesting as they may indicate tendencies or provide insights about the state of the ecosystem.

**OAK**

| 1970 | 1976 | 1977 | 1979 | 1988 | 1993 |
|---|---|---|---|---|---|
| Amanita phalloides | Cortinarius obtusus | Cortinarius diasemospermus | Amanita phalloides | Amanita phalloides | Cortinarius sp. 1 |
| Inocybe cincinnata | Cortinarius sp. 1 | Cortinarius obtusus | Cortinarius obtusus | Clavulina sp. 1 | Cortinarius sp. 3 |
| Inocybe sp. 1 | Inocybe cincinnata | Inocybe cincinnata | Elaphomyces sp. 1 | Cortinarius sp. 1 | Russula ochroleuca |
| Laccaria amethystina | Laccaria laccata | Laccaria sp. 1 | Inocybe cincinnata | Cortinarius sp.2 | Russula sp. 4 |
| Russula ochroleuca | Naucoria bohemica | Lactarius rufus | Laccaria sp. 1 | Inocybe cincinnata | Tomentella coerulea |
| Tomentella coerulea | Russula sp. 1 | Lactarius subdulcis | Lactarius quietus | Laccaria sp. 1 | Tomentella fuscocinerea |
| Tomentella fuscocinerea | Russula sp. 2 | Russula amoenolens | Russula amoenolens | Lactarius quietus | Tomentella stuposa |
| Tomentella sp. 2 | Russula sp. 3 | Russula sp. 3 | Russula fragilis | Lactarius subdulcis | Tricholoma scalpturatum |
| Tomentella sp.1 | Tomentella sp. 2 | Tomentella stuposa | Tomentella sp.1 | Russula amoenolens | |
| | Tomentella stuposa | | Tomentella stuposa | Tomentella sp.1 | |
| | | | | Tomentella stuposa | |

**SPRUCE**

| 1969 | 1973 | 1976 | 1988 | 1990 | 1997 |
|---|---|---|---|---|---|
| Lactarius rufus | Amphinema sp. 4 | Amphinema sp. 1 | Amphinema sp. 1 | Amphinema sp. 1 | Amphinema sp.2 |
| Tomentella stuposa | Hygrophorus pustulat | Amphinema sp. 3 | Amphinema sp.2 | Cortinarius flexipes | Laccaria sp. 1 |
| | Otidea bufonia | Hygrophorus pustulatus | Amphinema sp. 3 | Hebeloma leucosarx | Lactarius rufus |
| | Tomentella lateritia | Russula sp.1 | Hygrophorus pustula | Helvellosebacina helvelloides | Russula puellaris |
| | Tylospora asterophora | Russula sp.2 | | Hymenogaster sp. 1 | Tylospora asterophora |
| | | Tomentella lateritia | | Inocybe flocculosa | |
| | | | | Lactarius rufus | |
| | | | | Pseudotomentella sp.1 | |
| | | | | Tylospora asterophora | |

Figure 23. ECM species list. Species are listed as found per stand age, in association with the two tree species, oak and spruce. Each stand age list is sorted alphabetically.

# 4. Discussion

Through the results presented in the previous section, I aim at understanding the effect of afforestation on ECM and their decomposability, either directly by examining the changes in the ECM biomass and species, or indirectly by investigating the effect of afforestation on the different soil parameters that in turn may influence ECM. More specifically, I am interested in the effect of succession on ECM, following afforestation, and I therefore compare the findings of different age stands in the chronosequence. Additionally, I am interested in the differences between spruce and oak associated ECM and I further compare the findings among the two tree species. In this section, I also aim at answering the research questions I have formulated in my introduction. I repeat them here, before attempting to answer them.

- *Question 1:* Does succession following afforestation have an effect on the soil parameters of pH and C/N ratio?
- *Question 2:* Does biodiversity increase along the chronosequence? Are there more ECM morphotypes in the older stands?
- *Question 3:* As spruce is introduced and oak is naturally occurring in Denmark, I am interested to see whether oak will have associations with more morphotypes compared to spruce.
- *Question 4:* Do ECM from older and less nutrient rich stands decompose slower? Do we identify traits that indicate slower decomposition? (higher melanin and C:N ratio)
- *Question 5:* Is there a change in the ECM species along the chronosequence? What do the species indicate?

## 4.1. Soil pH

Investigating the effect of succession in the afforested area of Vestskoven, in spruce and oak patches, I found that the most affected soil parameter in 2021 was the soil pH.  Soil pH was the only parameter that clearly illustrated a significant negative trend with time. Both in spruce and oak stands, the soil pH decreased over time, dropping to 5.3 from 5.8 for spruce and 5.5 from 6.5 for oak. Oak stands had therefore higher overall soil pH compared to spruce stands.

Generally, many studies report that afforestation decreases soil pH, with many variations due to region differences (Parfitt and Ross 2011; Rigueiro-Rodríguez et al. 2012). However, a meta-analysis found that afforestation neutralizes soil pH (Hong et al. 2018), which disagrees with the results of this study, where soil pH decreased from 6 to 4.8 for spruce and from 7 to 5.5 for oak. Soil pH is the result of the production minus the consumption of soil hydrogen ions, which are often determined by the nutrient cycles, such as carbon, nitrogen, phosphorus, sulfur or calcium (Fisher and Binkley 2012; Paul et al. 2002; Laganiãre et al. 2010). Afforestation is a type of land-use change that may influence the nutrient cycles in different ways including the uptake of exchangeable cations by plants (Rhoades and Binkley 1996; Binkley et al. 1989), the capture of acid deposition (Fisher and Binkley 2012; Binkley and Richter 1987), or altering the quality and quantity of litter input and rhizosphere processes (Binkley and Richter 1987; Fisher and Binkley 2012), which are all factors that may change the generation and consumption of soil hydrogen ions and therefore the soil pH. There are several studies showing that soil pH is a strong driver shaping the ECM community (Ge et al. 2017; Hung and Trappe 1983; Kjøller and Clemmensen 2009; Glassman et al. 2017) as well as the total soil fungal communities (Tedersoo et al. 2014). This effect of pH on ECM could potentially be related to the fact that pH regulates soil nutrient availability; it directly affects the ability of negatively and positively charged materials in soil to hold charged ions, as well as the anion and cation exchange capacity (Sylvia et al. 2005). pH has also

a special effect on phosphorus availability which appears to be less biologically available at lower soil pH values (Kluber et al. 2012).

Returning to the first research question formulated above, it seems that soil pH was the most affected soil parameter, with a clear decrease over time for both tree species, which in turn can have an effect on the ECM and their decomposability. It therefore deserves further investigation combined with the ECM results.

## 4.2. Fine roots and ECM Biomass

Fine roots in the sampled upper 10 cm soil layer had the tendency to be decreasing in biomass over time, for both tree species. Fine root biomass found in oak stands decreased from 200 g/m$^2$ in the younger stands to 120 g/m$^2$ in the older ones, whereas in spruce stands the young stands had an average of 130 g/m$^2$ fine roots and the old stands as much as 80 g/m$^2$. ECM biomass had a slightly different trend which did not follow that of the root biomass precisely. In both spruce and oak stands, the youngest stands had very low ECM biomass, which then increased and peaked at the next oldest stand age, followed then by a decrease with time to the oldest stand.

Although as an individual, an old tree may produce more fine roots compared to a younger one, this is not necessarily true when comparing tree stands. Fine root biomass may vary among different forest stand ages due to a range of factors including canopy closure, stand tree density, aboveground standing biomass, local site conditions, soil depth and previous management practices (Finér et al. 2011). A study on the roots found in a Chinese fir chronosequence showed that the roots of understory plants decrease in biomass as stand age increases and the canopy becomes denser, allowing less light for the understory to develop (Pei et al. 2018). In my thesis project, during the selection of roots for analysis, roots were not separated into tree roots and understory roots. The samples were taken close to the trees to aim for tree roots and understory roots were likely also sampled during this process, but were not identified and excluded from the roots that were kept and were used for the biomass measurements. Therefore, a probable explanation for the trend observed in the fine root biomass over time, is that the trend of decreasing biomass is affected by the presence of understory plant roots which decrease as the tree canopy gets denser in older stands.

## 4.3. ECM biodiversity

By observing the ECM biodiversity data, there seems to be no increase of ECM fungal richness (fig. 12) as the stand age increases, in neither of the two studied tree species. Diversity (Shannon) had a tendency to increase in oak associated species and evenness was the same for both tree species with a slight increase following stand age increase (fig. 15). Returning to the second research question, there was not an increase in biodiversity as stand age increased.

One possible explanation for the stability observed in biodiversity over time could be the fact that all stands were well established and had reached an age where colonization of ECM is no longer dynamic and has taken place in earlier years. Regarding the stability of the fungal evenness over time, this is further confirmed by a study that observed the fungal colonization of the new artificial island Peberholm in Denmark (Nielsen et al. 2016). A study on the ECM fungal community in a spruce chronosequence planted on former agricultural lands in the Orlickè Hory Mountains in Czech Republic (Pešková et al. 2009), with stands aged 10, 50 and 80 years old, reports that the ECM symbionts were more species rich and had better quality in the youngest stands. Some of the less frequent fungal species that were found in the young stands tended to disappear in the older ones, which gradually reached a more adapted and fitting spectrum of fungi (Pešková et al. 2009). Changes in the fungal species along the chronosequence could also be explained by the fact that different fungi species have

different abilities of early- or late-stage colonization, following an ordered succession which can be influenced by factors like the accumulation of recalcitrant litter in the soil or the earlier occurence of wildfires (Last et al. 1987; Visser 1995).

In accordance with my expectancies, the ECM fungal richness and diversity was higher under oak than with spruce. As formulated in the third research question of this report, this was expected based on the fact that oak is an endemic species in Denmark, whereas spruce is introduced. The results support this assumption, though they don't prove it. The factors affecting the ECM fungal species richness may vary. A study in Poland (Trocha et al. 2012) attempted to examine whether the ECM community is affected by the plant being native or non-native, or whether the host plant phylogeny plays a more fundamental role. The study compared native and non-native species of oak and pine in a 35-year-old common garden. For the oak, the native species was significantly richer in ECM associations, whereas for the pine, the native species had lower ECM species richness than the non-native pine species. This leads to the assumption that although my findings support the idea that the endemic species will have a higher richness compared to the introduced tree species, this is still an open question and could be investigated further.

## 4.4. Decomposition traits

Regarding the decomposition of ECM fungi in Vestskoven, it can be partly evaluated based on certain ecosystem-level properties which influence decomposition, namely the C/N ratios and soil pH. According to the C/N ratio results, it appears that oak associated fungi had lower ratios compared to the spruce associated ones. It is generally accepted that N limitation can have a restraining effect on the storage and cycling of C in terrestrial ecosystems (Vitousek and Howarth 1991), and can also limit soil microbial activity (Treseder 2008) and therefore also decomposition and C fluxes from the ecosystem (Schimel 2003; Mack et al. 2004). Given that organic matter with higher C/N ratio will have decreased decomposition rates, it is only logical to assume that the ECM fungal necromass in the soil of the spruce stands will decompose slower than the oak associated ECM, which had a lower C/N ratio. Additionally, the soil C/N ratio was also higher in spruce stands compared to oak, which could potentially create a less favorable environment for decomposing microbiota to flourish. With regard to soil pH, in spruce stands a lower pH was observed compared to the oak stands. This is expected, since spruce litter is more acidic than broadleaf litter; however, it does not necessarily mean that coniferous forests will always have more acidic soil compared to broadleaf forests (Burgess-Conforti et al. 2019). Regardless what's causing the soil pH difference between the two tree species, it may have an effect on the microbial activity in the soil and therefore the decomposition of organic matter. As discussed earlier in this section, soil pH regulates the availability of many nutrients, including nitrogen and phosphorus (Sylvia et al. 2005; Kluber et al. 2012). Lower pH will decrease nutrient availability, which in turn will have a negative effect on decomposability in the soil. Since spruce stands have a lower soil pH compared to oak, the decomposition of ECM fungal necromass may be slower in the spruce soil.

Evaluating all the parameters available, it is hard to assess which forest stands provide the best conditions for carbon storage in the soil through the deposition of ECM fungal necromass. Low pH in spruce stands slows decomposition by not making nutrients readily bioavailable and at the same time they have higher C/N ratios, which is another indication for low decomposability. Further, the low C/N ratios found in the oak stands, combined with the higher – yet still acidic – pH values may be an indication that decomposition has been favored up to this point. Assuming that the soil has been relatively homogenous, we can imagine that both the spruce and oak stands had approximately the same nutrient concentrations at some earlier point in time. Findings of low soil C/N ratios at this point in time, may indicate that there has been a higher decomposition rate which favored the uptake and binding of nutrients into living biomass. Findings of low ECM fungal C/N ratios might be underlying

low nutrient availability in the soil which in turn could be a result of low pH and therefore low decomposability.

Another factor that could potentially be influencing the results and therefore also conclusions made in relation with the C storage in the soil through ECM, is the soil depth in which the samples were taken. I did not include several depths in the study, and can therefore not compare data across different depths. However, data from a former study in Vestskoven (Bárcena et al. 2014a), found that the C concentration was highest in the top 5 cm soil layer and decreased in the deeper layers. Soil was sampled down to a 25 cm depth and the C content decreased from an average of 35 mg/g in the top layer to 5 mg/g in the deepest layer, in both spruce and oak stands (Bárcena et al. 2014a). Based on this result, it is obvious that the major contribution and deposition of C to the soil is done in the top 5 cm layers. ECM fungi appear to be mainly distributed (>50%) in the top 5 cm soil layer, with the amount of ECM decreasing sharply below that depth (Hashimoto and Hyakumachi 1998). I can therefore safely assume that the C concentration is significantly higher in the top layer of Vestskoven due to increased ECM fungal activity in that soil layer.

Unfortunately, melanin could not be calculated from our samples due to technical limitations. Still, melanin is expected to be a strong decomposition trait and would provide essential information to understand and compare the decomposability of the different ECM species, between stands and over time. Melanin content affects stress tolerance in ECM and retards decomposability of their necromass, and could therefore change any decomposition trend predicted based on the conditions in the habitat. Lacking the information for this parameter is rather limiting the assumptions and conclusions regarding the comparison of the ECM decomposability in relation with the two tree species.

## 4.5. ECM species composition

Concerning the ECM species composition found in association with the two tree species, 10 genera were found in the oak stands and 14 in the spruce, with the majority being basidiomycetes and a few ascomycetes. There were some conifer specifics in the spruce stands, such as Amphinema, Tylospora and Hygrophorus species. However, a more thorough investigation would be necessary for the identification of most morphotypes collected, which would require more time than the framework of this thesis project could allow. Based on a complete list of identified morphotypes, this report could further go into the mycelial traits of each species, as reported by Agerer (Agerer 2001), which would provide information about the amount of mycelia produced in the soil. Additionally, with more time available, this could even be experimentally tested with the use of in-growth cores to better estimate the amount of mycelia and compare the carbon storage potential in the soil based on those estimates. This experiment could also include the observation of melanized fungi and measurement of decomposition rates in order to compare the conditions found in the different stands over time.

# 5. Conclusions

With regard to decomposability, I conclude that in Denmark, afforestation of former agricultural land with Norway spruce may result in conditions that retard the decomposition of soil organic matter, including the ECM necromass, compared to afforestation with pedunculate oak. This could be due to the lower soil pH, higher soil C/N ratios and significantly higher litter mass accumulation. However, when considering the biodiversity of ECM that arises due to afforestation with the two tree species investigated in this thesis project, it appears that oak will result in higher fungal biodiversity compared to spruce, as observed by the ECM species composition found in association with the two tree species. I can further conclude, that the introduced Norway spruce may be a better choice for afforestation in Denmark for its potential for carbon storage in the soil, due to the retarded decomposition conditions it offers; and the native pedunculate oak may be a better choice for afforestation in Denmark with regard to restoration of fungal biodiversity, as it appears to accommodate niches for a greater variety of ECM fungi compared to the introduced Norway spruce.

A deeper and more thorough comparison of the individual potential of the different ECM fungi with regard to their decomposability, would shed more light to the investigated questions of this report. The extensive mapping of the fungal species found in the area of Vestskoven and the establishment of a method that can effectively compare their melanization and resistance to decomposition would give a more accurate understanding of the potential for carbon storage in the soil through ECM necromass.

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