Biotic and abiotic effects on carbon storage in temperate forests on sandy soils

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List of abbreviations

C – Carbon

N – Nitrogen

Al – Aluminium

SOM – Soil Organic Matter

SOC – Soil Organic Carbon

CEC – Cation Exchange Capacity

BS – Base Saturation

AUR – Acid Unhydrolyzable Residue

CUE – Carbon Use Efficiency

LMM – Linear Mixed Model

GLMM – Generalized Linear Mixed Model

CI – Confidence Interval

Summary

Climate change is affecting the conditions on our planet and threatening our ecosystems along with the services they provide. One of those services, namely carbon sequestration, could be part of the answer to slow down the trend of rising atmospheric CO_2 concentrations. Where previously the focus was on aboveground systems, recently, soils are gaining more and more attention. However, in order to properly exploit this potential, it is important to understand the mechanisms behind the underlying processes. The biotic and abiotic conditions of the system and their interactions influence decomposition and stabilization of soil organic matter, ultimately determining the amount of C stored. The study area comprises temperate forests on sandy soils in Flanders and the Netherlands. The soils of these systems have known a long history of degradation: from litter and wood harvest for agricultural purposes, resulting in heathlands, to plantations of Scots pine accompanied by soil acidification. The remaining forests have consequently poor soil health and a low vitality and biodiversity. Introduction of rich litter species, with high nutrient and low lignin concentrations, could help restore these ecosystems through their positive influence on soil conditions. In this thesis, we tried to get a better understanding of the C stocks, their main drivers and the impact of rich litter species in these forests. Especially the role of soil biota, but also litter quality with a direct as well as intermediate effect, is still not well understood. A plot-wise sampling method is used consisting of a 'poor' plot, dominated by Scots pine and a 'rich' plot, with a large share of rich litter species. In two forests, an additional reference plot is sampled, characterized by a lower degradation status and natural climax vegetation. Soil characteristics such as pH, CEC and BS along with biotic factors such as soil respiration and the biomass of earthworms are measured, and the C stock is approximated based on organic C % and bulk density. All characteristics are described for the organic layer, top- (0-10 cm) and subsoil (10-20 cm). Also, descriptions of mesofauna and tree species and composition are used for analysis. Contrary to our hypothesis, the measured soil health indicators, both biotic and abiotic did not improve as a result of rich litter introduction for the rich and reference forest type. Likewise, the positive impact on C stocks is also not established for either treatment, contrary to our hypothesis. These phenomena could be attributed to a time-lag in the C sequestration process or the point-of-no-return status in terms of degradation. The largest C stocks are found in the Scots pine plots on the forest floor. Low pH-levels, found in these plots create hostile environments for earthworms and other soil biota, reducing decomposition and bioturbation. A larger productivity of poor litter species adds to these C stocks. The impact of the tree species and soil biota composition are less unambiguous, attributable to the complex synergy within and between both communities. C stocks in forest floors in monocultures are, however, unstable and vulnerable to climate change and other disturbances, endangering their existence in the future. Further research should sample deeper layers to provide a more complete understanding of the C stocks and a incorporate a temporal dimension to account for a possible time-lag.

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A. Introduction

1. Climate Change in temperate forests

1.1. Impacts

The climate is changing. Greenhouse gas (GHG) emissions resulting from combustion of fossil fuels, land use (change), and industrial processes are causing temperatures to rise (IPCC, 2022). In turn, these temperatures are causing climatic zones to geographically shift (Burrows et al., 2011; Cui et al., 2021) while also changing the global and local precipitation cycles. The frequency and magnitude of extreme rainfall but also drought events will increase throughout Europe (Lehner et al., 2006; Madsen et al., 2014). The changes in water, temperature and carbon fluxes are drastically altering the conditions in which our ecosystems naturally thrive. Hotter drought, a climate change induced phenomenon, predicted for temperate regions, is expected to cause high tree-mortality in forests (Allen et al., 2010; Breshears et al., 2005). Plants that experience drought-stress undergo physiological changes that can result in carbon starvation, which is exacerbated by elevated temperatures. Under these circumstances, their resistance to pests and pathogens is also reduced and both effects can cause severe die-back of trees and other species (McDowell et al., 2008). In turn, occurrences of large tree mortality can shift species distribution and ultimately forest ecosystems (Breshears et al., 2005; IPCC, 2022). Increasing frequency in temperate forest fires, due to drought and rising temperatures, could also lead to conversion to non-forest vegetation (Adams, 2013). Temperate forests provide many societal benefits, termed ecosystem services, such as timber, provisioning and regulation of water, carbon and nitrogen cycles, recreation, etc. (Rawat et al., 2022), which are thus also threatened by these climate change-induced events. Biodiversity plays a large role in both protection against climate-induced risks as well as support of the ecosystem services (Balvanera et al., 2006; I. Thompson et al., 2009). For example, Fargione & Tilman (2005) found that a more diverse forest plant community has a higher resistance against invasive species. Another study by Dale et al. (2010) indicated that a higher tree diversity results in a higher resilience against drought events. However, global biodiversity has experienced rapid deterioration in previous decades (Almond et al., 2022). Currently other anthropogenic disturbances such as eutrophication (Hautier et al., 2009) and habitat fragmentation (Krauss et al., 2005) are the main causes of biodiversity loss and degradation of forest ecosystems but it its predicted that climate change will become the dominant factor in the coming years

(Almond et al., 2022). This steep decline in biodiversity will lead to a lower delivery of ecosystem services (Balvanera et al., 2006).

1.2. Mitigation through carbon sequestration

Another important forest ecosystem service is the ability to sequester carbon (C) and thus mitigate climate change. Although for a long time the focus was on sequestration by aboveground vegetation, research shows that soils also play a key role as terrestrial carbon sinks (FAO, 2020; Jandl et al., 2007). Houghton (2007) reported that soils store two to three times more Soil Organic Matter (SOM) than vegetation. SOM is defined as "the non-living component of organic matter in the soil" and is difficult to analyse due to its variable nature in composition and turnover rate (Trumbore, 1997). It has become increasingly important to get a better understanding of the contributing factors in this dynamic ecosystem process to be able to properly address management and restoration measures and stimulate C uptake by the soil (Desie et al., 2021; IPCC, 2022; Jandl et al., 2007)

2. Carbon sequestration in forest soils

Atmospheric carbon is taken up by photosynthetic organisms and stored in above- and belowground biomass. Dead plant material, termed litter, comprising leaves, needles, twigs and bark but also root tissue, are frequently shed due to natural phenology or disturbances. This litter, combined with microbial necromass is the main carbon source of the soil and is either broken down and respired or stored (Liang et al., 2019; Mayer et al., 2020). The carbon sequestration capacity of a forest results thus from the balance between the productivity of the aboveground vegetation and its roots and the residence time of that carbon in the soil (Thompson et al., 1996). The latter depends on the decomposition of organic matter and the stabilization mechanisms (Mayer et al., 2020).

2.1. Decomposition
2.1.1. *Process*

Decomposition of litter is controlled by multiple factors, such as climatic conditions, soil properties, litter quality and the composition of the soil biota community and these factors are often interrelated (Krishna & Mohan, 2017; Swift et al., 1979) (Figure 1). The process can be separated into different phases in which different regulating factors, organic compounds and enzymatic processes dominate (Berg, 1986) (Figure 2). Throughout these phases, the composition of the litter changes and the decomposition rate decreases. Two main models, which are species-

specific, are suggested by Berg & McClaugherty (2020). In the first stage of Type I, unprotected (hemi)cellulose and water-soluble compounds are broken down. This is followed by the decomposition of Acid Unhydrolyzable Residue (AUR), consisting of cutin, tanin and lignin, which are more resistant to decomposition (Berg, 1986; Gholz et al., 2000; Preston et al., 2011). The first stage does not occur in decomposition according to Type II (Berg, 1986).
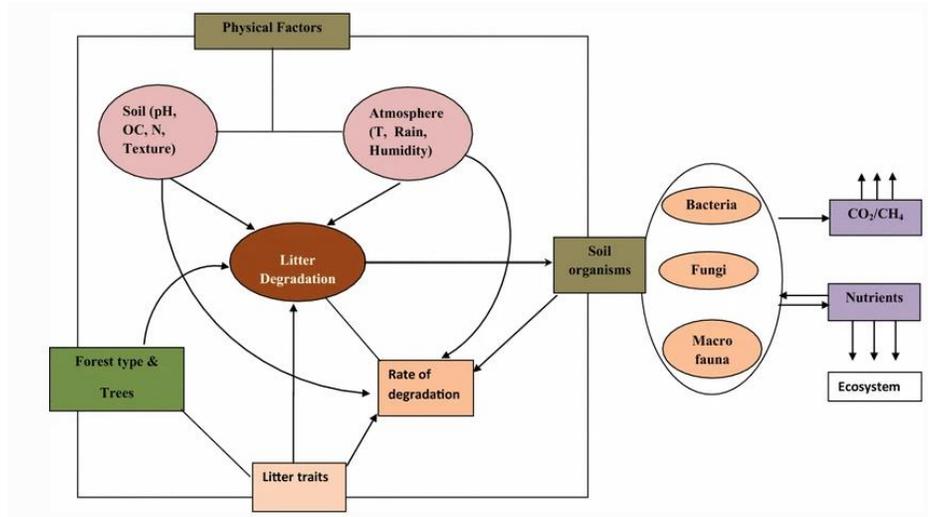
Figure 1: Schematic overview of drivers of litter degradation. Factors include physico-chemical soil properties, climatic variables and biological drivers such as different groups of soil organisms and the tree species composition and their litter traits. (Copied from Krishna & Mohan, 2017)
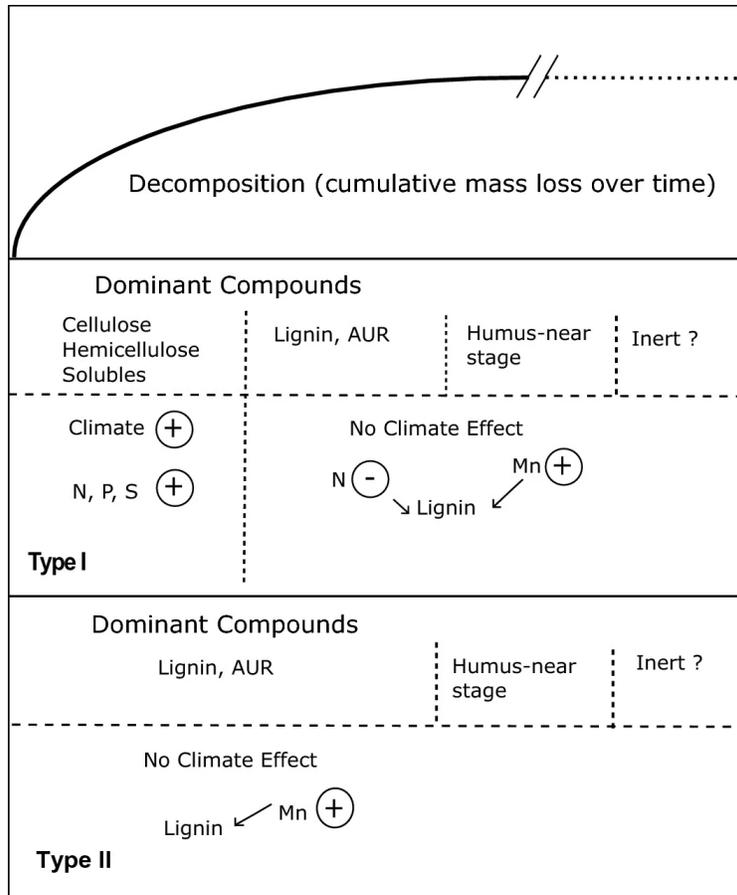
Figure 2: (Top) Decomposition rates are shown to slow down and ultimately become zero until the organic matter reaches a humus-near stage. (Middle) Decomposition model Type I, associated with pine species, is divided into two stages. In stage one, the break-down of (hemi)cellulose and solubles is stimulated by nutrients N, P & S and favorable climate conditions. This stage accounts for 25-30% of the accumulated mass loss. In the following stage, the remaining lignin-encrusted and AUR components determine the decomposition rate. (Bottom) Type II, associated with several species litter, does not include a first stage. In both types, Mangane (Mn) has an accelerated effect on AUR decomposition. (Copied from Berg, 1986)

2.1.2 Drivers of decomposition
i. Climate

Climatic conditions are summarised by temperature and moisture balance, which are often quantified by mean annual temperature and annual actual evapotranspiration rate. These directly affect the temperature and moisture conditions of the soil and show a positive correlation with the decomposition rate (Berg & McClaugherty, 2020; Gholz et al., 2000; Zhang et al., 2008). The soil biota's activity is stimulated by higher temperature and moisture availability but restricted by cold

temperatures and deficient or excess moisture, with thresholds at 10°C and 30% or 80%, respectively as proposed by Prescott (2010). These effects, however, were mostly significant during the early stages of decomposition and only in unfavourable conditions or aggregated on the biome level (Berg & McClaugherty, 2020; Djukic, 2018). An indirect effect is the influence of temperature and precipitation on the possible plant and decomposer community structure (Aerts, 1997; Prescott, 2010; Zhang et al., 2008).

ii. Tree species

The tree species community can either directly or indirectly affect decomposition. Trees directly influence decomposition rates through the composition of their litter and is often considered the key driver (Cornwell et al., 2008; Djukic et al., 2018; Prescott, 2010). The decomposability of plant litter depends on the partitioning into soluble compounds, AUR, nutrients and cellulose fractions and differs significantly between plant species (Parton et al., 1994). These species-based differences in plant traits are a result of ecological strategies and selection pressures (Cornwell et al., 2008; Reich et al., 1997) and evidence for global patterns was found by Reich (1997).

Multiple variables of litter quality can predict decomposition rate and AUR:N and C:N ratios, both with a negative correlation, are the most consistent in temperate regions (Prescott, 2010; Zhang et al., 2008). In conditions where N is not a limiting nutrient, the concentration of base cations (Ca, K, Mg and Na) becomes the main driver, as found by Desie, et al. (2020a). Most conifer species are considered "poor" or slow-decomposing litter trees with high lignin and low N content. Broad-leaved genera such as ahorn (*Acer*), ash (*Fraxinus*), linden (*Tilia*) and hazel (*Corylus*) species have fast-degrading, so-called "rich" litter with low lignin content and high base concentrations (Hommel et al., 2002; Krishna & Mohan, 2017; Cornwell et al., 2008). Oak (*Quercus*) and beech (*Fagus*) on the other hand are also labelled "poor" litter species (Hommel et al., 2002). But this litter quality is also a result of interaction with site characteristics such as soil properties, climatic conditions and species composition. (Desie et al., 2023; Hommel et al., 2002; Krishna & Mohan, 2017; Mayer et al., 2020).

Tree composition can also indirectly affect the decomposition potential through influence on the soil biota community. Hooper et al. (2000) describe linkages between above- and belowground biodiversity to be either positive, negative or neutral, depending on the mechanism and its strength. Conifer litter contributes to acidification of the soil with negative implications for soil biota, while

broadleaved species are associated with higher soil fauna activity (Mayer et al., 2020; Prescott, 2010). Carbon fixation, associated with pathways performed by soil macro- and microfauna, is thus more connected to broadleaved forests (Prescott, 2010). Another consequence is the difference in distribution of C stock throughout the soil depth. Carbon derived from broadleaved litter is incorporated in the mineral soil due to this elevated activity, termed secondary sequestration, while conifers accumulate more C in the upper soil layer (Mayer et al., 2020; Prescott, 2010; Berg & McClaugharty, 2020). Differences in root input could also explain the depth distribution of C stocks (Dawud et al., 2016). Dawud et al. (2016) found that tree species diversity can have a positive effect on carbon stocks, but the main driver is tree species identity (Desie et al., 2023; Mayer et al., 2020). This phenomenon can be ascribed to an increased productivity and litter input, and above- and belowground niche complementarity in certain compositions of functional groups (conifers vs. broadleaved or N-fixing species) (Dawud et al., 2016; Mayer et al., 2020).

iii. Soil type

Soil particle characteristics such as particle size, surface properties and chemical composition are responsible for other chemical and physical soil properties. On one hand, these properties, such as pH, porosity and thus gas and moisture exchange, etc. directly influence the decomposition rate. On the other hand, they determine both the possible plant and the decomposer community (Swift et al., 1979; Van Veen & Kuikman, 1990). Contradicting evidence regarding the relationship between soil type and C sequestration can be found in literature (Jandl, 2007). Van Veen & Kuikman (1990) found soil texture and structure to be the key driver of microbial decomposition. Finer, clayey texture is associated with a higher C retention capacity than more sandy soils due to formation of organo-mineral complexes and a higher fertility resulting in higher productivity. Other research reports that poorer sandy soils store more C, due to its slow decomposition rates (Vesterdal et al., 2007) This is confirmed by Vejre et al. (2003) who found that the clay content of the soil has a negative correlation with soil organic carbon (SOC). These C sinks are, however, mainly linked with the topsoil.

2.2. Stabilization mechanisms of SOC

Storage of organic carbon, and thus the lengthening of their residence time in the soil, can happen through several mechanisms, including physical stabilization and chemical reactions between OM particles and mineral surfaces or metal ions. These ensure protection from biological, aerobic and

enzymatic decomposition and thus release of CO_2. Selective preservation, which depends on the molecular structure of SOM (recalcitrance) and was often associated with the AUR-fraction (Sollins et al., 1996), was recently found not as significant as previously thought and happens mainly in the early stages of decomposition (Lützow et al., 2006). Spatial protection can occur through several pathways, such as aggregation (Elliott, 1986; Oades, 1984). Organic particles become entrapped in the centre of aggregates with a size < 0.2 µm diameter, which limits access to soil biota (Blanco-Canqui & Lal, 2004; Chenu & Stotsky, 2002). The number of pores with diameter < 0.2 µm are dependent on the pore system and thus on the soil texture (Van Veen & Kuikman, 1990). Chenu & Stotsky (2002) found that sandy soils have a considerably lower amount than clayey or loamy soils. Other mechanisms that limit spatial accessibility are intercalation within phyllosilicates, hydrophobicity, and encapsulation in organic macromolecules but these are difficult to verify due to unreliable measurement methods and lack of data (Lützow et al., 2006). Organo-mineral complexes, also protecting OM from decomposers, are formed through surface reactions of humic substances with mineral soil particles such as clay and sesquioxides (Carter & Stewart, 2022), although the mechanistic understanding is still lacking. Chenu & Stotsky (2002) suggested that irreversible adsorption of those substances makes them resistant to microbial consumption.

3. Temperate forests on sandy soils

3.1. History

Forests in the temperate sandy regions of Flanders and the Netherlands have a complex history of vegetation types (Figure 3). Tree compositions throughout the years have been reconstructed based on pollen analysis (Doorenbosch, 2013). Since the last Glacial Period, that ended around 11.700 years ago, forests have transformed from an open birch-pine (Betula-Pinus) species composition to a more species rich linden-oak (Tilia-Quercus) forest (van Geel et al., 1980; Doorenbosch, 2013). These were however quickly dominated by oak due to leaf harvesting for cattle and transformation into forest-meadows and arable lands. Further human interference, such as litter raking, sod cutting, wood harvesting and overgrazing has caused an even further degradation to heathland (Sauren et al., n.d.; Webb, 1998). At the end of the 19th century, the demand for wood grew exponentially to support the increasing mining activities and this was met by plantations of monoculture Scots pine (Pinus sylvestris) (Buis, 1985). Since WWII, a rise in awareness regarding environmental problems and the importance of biodiversity and ecosystem services has led to a

switch from focus on production to a multifunctional and more natural forest management (Sauren et al., n.d.).
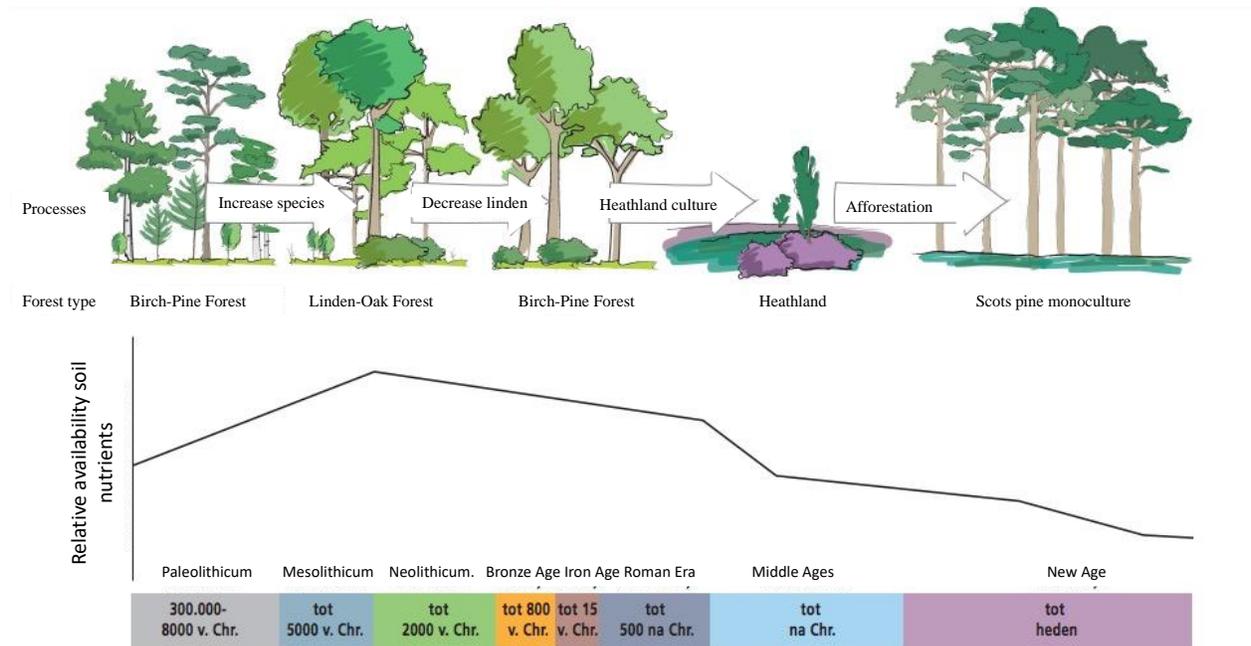
Figure 3: Chronological sequence, starting in the Paleolithicum and ending in the present, of the different ecosystems found in temperate regions on sandy soil and the processes that led to their transformations. The nutrient availability is shown below and characterized by a decline since the Neolithicum. An increase in anthropogenic deposition nullified recent soil rehabilitation efforts. (Copied from Sauren et al., n.d.)

3.2. Current state

Regardless of the mentality shift in forestry practices, a forest type where Scots pine dominates the stand, is still the most common in these areas. Lack of adequate management after the collapse of the mining industry has resulted in monocultures with low vitality, biodiversity and production potential (Sauren et al., n.d.). The general characteristics of a sandy soil texture, combined with the historical land use, have contributed to heavily degraded, acidic and nutrient-poor soils. First, the sandy soils in these areas have a large acidification potential due to lack of carbonate buffer and a low CEC with an inadequate buffer capacity. Nutrient-poor pine litter and decomposition dominated by fungi add to the natural acidification processes of plant respiration and rainwater (de Schrijver et al., 2010; den Ouden et al., 2010). These are amplified by previous agricultural activities and recent atmospheric deposition of N (de Keersmaeker et al., 1999). This has led to loss of nutrients, limited soil biota activity and Al-toxicity. Sandy soils are also characterized by

their low water holding capacity, so they are naturally drought sensitive (den Ouden et al., 2010). Scots pine is a light species and not demanding in terms of nutrients and is thus adept at these growing conditions. It can grow under both dry and wet conditions as long as the moisture regime is stable (de Vos et al., 2010).

3.3. Consequences for ecosystem functioning

The resulting low vitality and carrying capacity of these types of forest have led to an overall low biodiversity in flora and fauna (Sauren et al., n.d.). Decomposition in the soil is mostly characterised by fungi since other soil biota such as earthworms, mesofauna and bacteria cannot survive Al-toxicity and low pH-levels. This impedes the vertical nutrient cycle and results in an accumulation of the litter in the topsoil, termed mor and mormoder humic types (de Keersmaeker et al., 2010; Sauren et al., n.d.). As the acidification continues, the conditions will also become obstructive for fungi growth, which will have a negative cascading effect throughout the whole food web (Kuyper et al., 2010). Atmospheric deposition of N and therefore acidification has shown to lower the available food quality. Larger non-protein N quantities result in deficiencies in protein assimilation processes and a decline in insects and bird populations has been reported by van den Burg et al. (2014). Low biodiversity negatively affects their resilience to climate change, which is predicted to have drastic effects on the water table (IPCC, 2022). Increased drought stress during the summer will onset early leaf fall, while increased rain fall will decrease stem stability (Sauren et al., n.d.). Low vitality and poor nutrient and moisture conditions increase the large vulnerability to pathogens and diseases, which is already an occurring problem due to poor nutrient and moisture conditions and will worsen with climate change (Sauren et al., n.d.; van den Burg et al., 2014).

3.4. Restoration measures

The degraded and low-vitality state of our temperate forests on sandy soils requires adequate restoration measures. As stated by Hommel & de Waal (2003) and van den Burg et al. (2014) passive management will not suffice to achieve or protect a healthy forest ecosystem and limit acidification. The limitations of sandy soils can be overcome through addition of organic matter. Humic substances enhance CEC, moisture retention and improves the soil structure (de Vos et al., 2020). These improvements result in more nutrient and water availability, as was shown by Rode (1999). But the quality of this organic matter, characterized by C:N ratio, AUR content and base cation concentration, is an important factor to consider. It influences processes such as decomposition rates, pH-balance, soil biota activity and carbon sequestration, and thus overall soil

health (Hommel et al., 2002; Krishna & Mohan, 2017; Prescott, 2010; Reich et al., 2005). To restore the low-vitality, poor and acidic pine forests, a transformation to mixed stands with a rich-litter species such as linden, ash, ahorn and hazel has been suggested by Hommel et al. (2007). Their low C:N ratio stimulates the presence of soil organisms, and their high base cation concentration can upgrade the soil's buffer capacity and thus the pH. They have been found to also improve the humic types to moder or mull variants (Jabiol et al., 2000). The restorative impact of this transformation, however, needs nuance and is dependent on site characteristics. Rich-litter species are high-demanding in terms of nutrient-availability and pH, which is often deplorable in temperate forests on sandy soils (de Vos et al., 2020). The current status of CEC and buffer capacity of the soils determine the success of these restoration measures but the 'point-of-no-return' regarding degradation and acidification requires more research (Hommel & de Waal, 2003). Also, the clay content positively influences the extent of restoration (Desie et al., 2020b). So, while the proposed restoration measures could limit further degradation and improve soil biota activity, the effective 'regeneration or regime shift potential' in every situation is questionable (de Vos et al., 2020; Desie et al., 2020a). Moreover, it is unclear how this restoration measures impact carbon cycles in the belowground ecosystem compartment. Mixed stands could also increase C-stocks through complementary productivity above-and below ground. While plant tissues of "poor" species is considered more decay-resistant, AUR compounds do have a limited residence time in the soil. Physical and chemical protection of recalcitrant humic substances through microbial pathways would be the optimal strategy (Prescott, 2010). But these effects on carbon sequestration will also be a result of interaction between tree species composition and diversity, soil biota and abiotic conditions and remains unclear (Mayer et al., 2020).

4. The role of soil biota in carbon sequestration
4.1. Soil biota diversity

Soils are biodiversity hotspots, a phenomenon, which can be ascribed to the large heterogeneity of soil habitats, which is caused by ecosystem engineers and the variable nature of the soil matrix (Decaëns, 2010). The soil biota communities are influenced by both biotic (Figure 4) and abiotic factors. Biotic factors can range from interactions between and within trophic groups, such as predation and competition respectively, to plant species and community characteristics (Wardle, 2006). The latter entails differences in Net Primary Production and their influence on quality and availability of resources, such as root and leaf litter (Wardle, 2013). Abiotic factors determine

conditions that can inhibit or stimulate growth depending on the group soil biota. These include N content, pH, moisture, temperature, land use, texture, etc. (Birkhofer et al., 2012; Wardle, 2013).
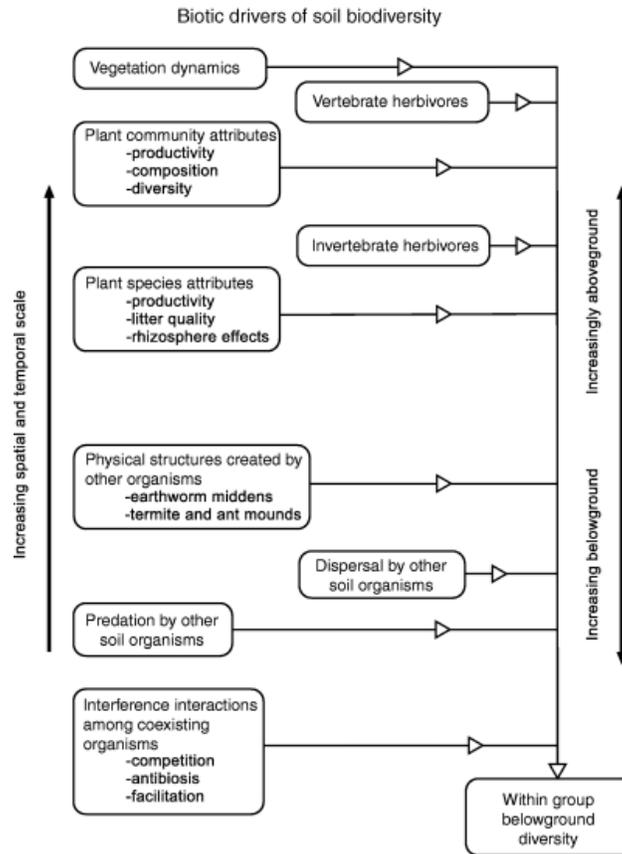
Figure 4: Schematic overview of biotic factors influencing soil biodiversity. The left axis represents the impact of the drivers on spatial and temporal level. The right axis indicates the height level at which the factors are at play starting above the soil going into deeper soil layers. The higher above the ground, the further the effect is in time and space. (Copied from Wardle, 2006)

However, these rich soil habitats are under pressure due to climate change and anthropogenic disturbances such as land use change and introduction of exotic species (Blankinship et al., 2011; Decaëns, 2010). This caused a decline in belowground biodiversity, which has been observed by the FAO (2020) and could negatively affect the delivery of the important ES service of sequestrating carbon. And while the complexity and responses of the species community belowground is much less understood, there is a consensus that a healthy soil is required for maintaining a high level of ES services (Briones, 2014).

Soil biota can be divided into four groups based on their size (Wall et al., 2012) (Figure 5). The microflora, which consists of the fungi and bacteria, have a body width between 0.3 and 20 μm. The microfauna, with a size smaller than 0.2 mm, include the protozoa and nematodes. The micro-arthropods and enchytraeidae form the mesofauna, with a size between 0.2 and 10 mm. The largest group in size, the macrofauna, consists of biota larger than 10 mm, such as earthworms, termites, ants…Earthworms can be further divided into three groups based on their feeding system and morphology (Bouché, 1972). Epigeic species reside and feed on the litter layer, endogeic earthworms live in the upper soil layers and move horizontally and anecic species burrow vertically but their diet consists mostly of litter.
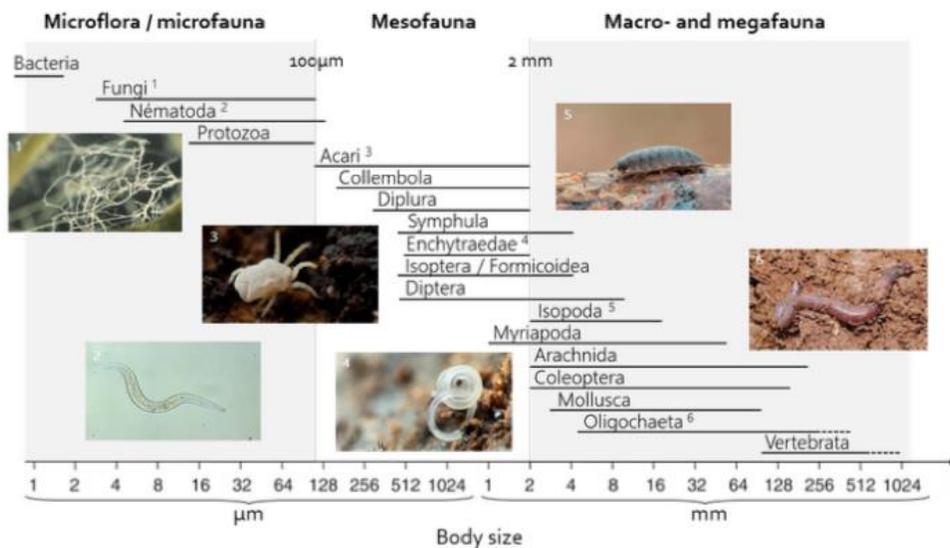
Figure 5*: *Representation of most important taxonomic groups of soil biota according to their body size. (Copied from Decaëns, 2010)

4.2 Soil biota in the carbon sequestration process

The carbon stock regulated by soil biota, is the net balance between their anabolic and catabolic activity. The former is the synthesis of simple compounds into complex ones which ultimately contributes to necromass and is gaining more attention recently. The latter is the breakdown of larger molecules into smaller units with the release of CO_2 and energy (Schimel & Schaeffer, 2012). The Carbon Use Efficiency (CUE) is used as an indicator for this and describes the substrate fraction consumed that is converted into microbial biomass (Winzler & Baumberger, 1938). Allison et al. (2010) found that this microbial efficiency determines the soil-carbon response to

rising temperatures. Two different mechanisms are proposed by Liang et al. (2017) in which the microbial community contributes to SOM: Ex-vivo modification and in-vivo turnover (**Figure 6**). Ex-vivo modification or transformation of plant-derived C, depends on specific plant traits (Cornwell et al., 2008) and the microbial composition (Waldrop & Firestone, 2004). In-vivo turnover, or anabolic activity, contributes to the SOC-pool through input of standing biomass, necromass and microbial-derived products. Microbial-derived C is often further stabilized chemically or physically (Schaeffer et al., 2015) and this process is referred to as "microbial entombing" (Liang et al., 2017). In combination with the high turnover-rates of microbial species, these two pathways can result in a large contribution to the C stock. However, the reverse process, called the "primer effect", stimulates the microbial decomposition or catabolism of stable SOM through the addition of new soil C (Liu et al., 2020). These processes are limited by the accessibility of the substrate, which is determined by their physical protection and the space and structure of the microbial landscape (Dungait et al., 2012; Liu et al., 2020)
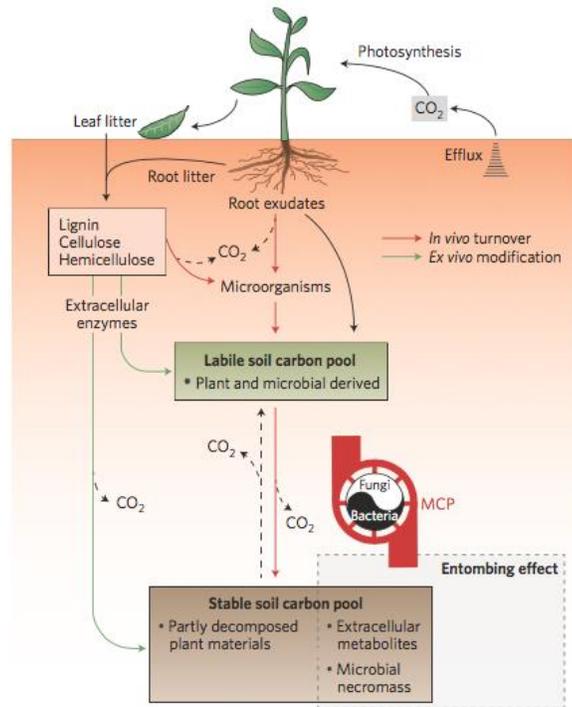
Figure 6: Schematic representation of two mechanisms involved in carbon sequestration by soil biota: in-vivo turnover (red) and ex vivo modification (green). Vegetation provides the soil with SOC through leaf litter and root litter and exudates. One part is decomposed and transformed into labile or stable soil carbon by ex-vivo modification. Another part is taken up by the microbial community and used for production of biomass, which becomes microbial necromass, and extracellular metabolites. After stabilization, it becomes part of the stable soil carbon pool and this process is referred to as the "entombing effect". (Copied from Liang et al., 2017)

Soil biota, in turn, influence this physical stabilization. Each group plays distinct roles in the assimilation process of soil aggregates, in which carbon is stored. Bacteria provide biopolymers as binding agents or mucilage (Deng et al., 2015; Oades, 1984), while fungi are capable of holding micro-aggregates together by intertwining soil particles through fungal hyphae (Degens, 1997; Oades, 1984). Macrofauna, such as earthworms, create macro-aggregates through bioturbation and soil ingestion and egestion (Blanchart et al., 2009). The soil community also affects chemical stability of C through the formation of organo-mineral complexes, since the type and amount of humic substances is dependent on the type of soil biota present (Brussaard & Juma, 1996).

5. Knowledge gaps

5.1. Forest characteristics

There is still a lack of understanding of how the plant species composition and thus their litter quality plays a role in the presence of soil biota and their decomposition activity (Prescott, 2010). Also, the context-dependency in terms of soil properties, and especially the non-linearity of pH and its depth distribution, of that litter quality and its impact on organic matter dynamics requires more research (Desie et al., 2021). The age of the stand at the time research on C stocks is conducted, might influence our perception on the C sequestration capacity of a forest system, since carbon sequestration in mineral soils often experience a time lag of three decades accompanied by redistributions in depth. Questions on whether all forest systems have a steady state are raised by Berg & McClaugherty (2020), since evidence of continuous C addition to forest soils, in the absence of disturbances, have been found.

5.2. Soil biota

The processes involving carbon sequestration, the part played by soil biota and the interaction between the different groups still leaves much to imagination and requires more research. It is also still unclear in what form the soil biota is related to the magnitude of carbon stocks. Are these processes linked to the biomass and species density (Cole et al., 2004) or to the numerical abundance as proposed by (Mulder, 2006). The reason for this large knowledge gap in soil biota and their functions can be ascribed to several factors. First, the taxonomic deficit of soil biota is more than 75% due to lack in taxonomic expertise and the challenges in identification at a small-scale level (Decaëns et al., 2008). Another explanation could be the disproportional interest in aboveground fauna, which has led to an underrepresentation of soil biota in scientific literature (Decaëns, 2010).

6. Aim, objectives & research questions

In this thesis, we will try to get insights on the current status of carbon stocks in temperate forests on acidified sandy soils. We will try to answer the question whether restoration measures, based on transformation to rich-litter tree communities, could affect C cycles. And whether soil biota play an integral role in the underlying processes of carbon sequestration. Is the magnitude of their effect depending on the amount of biota present in the soil or do some species or functional groups have more influence than others?

The goal of this thesis is to link belowground carbon stocks to abiotic and biotic characteristics of temperate forests on sandy soils in North-Brabant, The Netherlands and Limburg, Belgium. These factors include tree community, pH, texture, CEC and the presence of soil biota communities.

Hypothesis: A diverse "rich"-litter tree community results in a larger C-stock in the long term.

1. "Rich" species restore pH – balance, BS and overall soil health, when the acidity of the soil is within a certain buffer capacity.
2. A high tree diversity of rich-litter species will result in a larger aboveground productivity and more diverse soil communities, ultimately corresponding with a larger carbon stock.
3. There will be differences in the depth distribution of the C pool between "poor" and "rich" litter forests.
4. Certain compositions of soil communities will alter carbon stocks. The carbon sequestration will be most efficient when all soil biota groups (microflora, -fauna, meso- and macrofauna) are represented, which is the case for a healthy soil. Soil with both bacteria and earthworms present will have a higher sequestration capacity than one with only fungi. The more diverse the soil communities, the larger the carbon stock.

B. Methodology

1. Study area

The study area entails a region comprising North-Brabant, The Netherlands and the Northern part of Limburg, Belgium (Figure 7). Six sites were selected, of which two located in Belgium. The climate is classified as Cfb (temperate ocean climate) under the Koppen classification with an annual average temperature of 11°C and annual rainfall of 820 mm (Climate-Data, 2021). The area is characterized by Pleistocene cover sand fluvial deposits (Doorn et al., 1997).
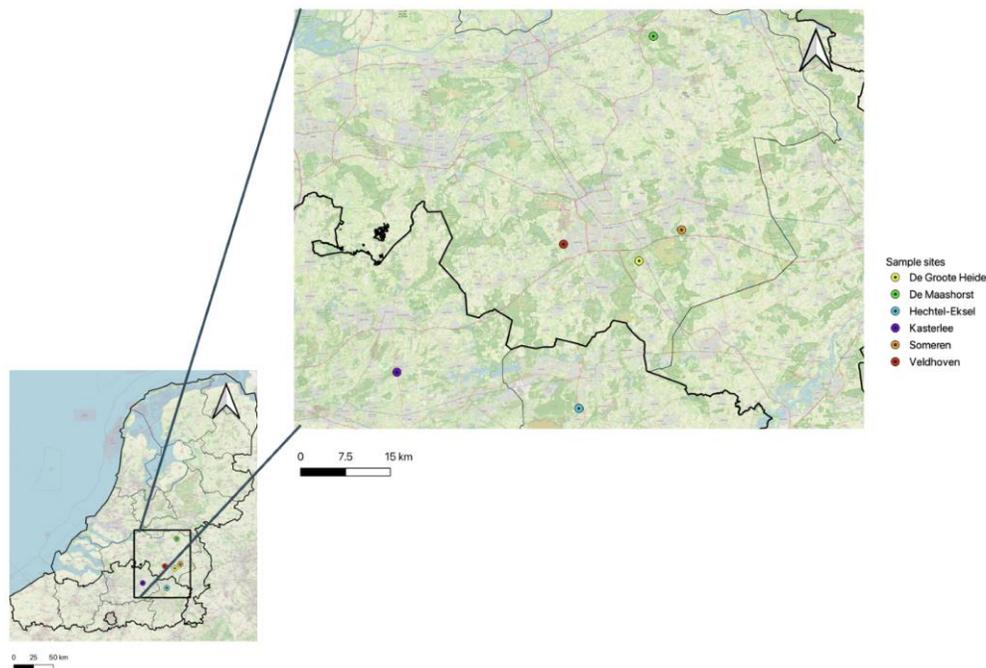
Figure 7: Study region located in North-Brabant, The Netherlands and the Northern part of Limburg, Belgium. The different sites are indicated by colour: De Groote Heide (yellow), Maashorst (green), Hechtel-Eksel (blue), Kasterlee (purple), Someren (orange), Veldhoven (red).

The forests in this region can be found along a revitalisation gradient (

Figure **8**). Monoculture Scots pine on heavily degraded sandy soils, combined with pedunculate oak (*Quercus robur L.),* silver birch (*Betula pendula*) and/or black cherry (*Prunus serotina*), characterize the poor sites (Table 1A). The rich sites comprise mixed forests where broadleaved species with rich litter were introduced or appeared due to spontaneous succession on these poor soils. Most occurring species include linden (*Tilia europeae*), maple (*Acer pseudoplatanus*) and

mountain ash (*Sorbus aucuparia*) and the invasive species black cherry (Table 1A). In all forests, two plots were sampled: one in a poor site and another in a rich site. This pair-wise sampling is needed to compare the effect of rich-litter species on the soil biota and consequently the C stock between sites where land use history and soil type are constant. In this region, also two additional reference sites are sampled. While these are not defined as "pure" reference forests, since they share the same degradation history as the rest of the study area, they do have reference characteristics of undisturbed, natural forests, such as more fertile soil (former agricultural land, higher loam percentage or Tertiar sand) and/or an optimal mix of broadleaved rich-litter tree species (Table 1A). In the plots in Someren, however, the soil types between rich and poor differ. The rich site is former agricultural land, while the poor site is characterized by a heavily degraded podzol. This site might thus not be able to indicate whether the revitalisation of the soil is due to its land use history or the introduction of rich litter species, which is why the rich plot is taken into account as reference site in further analysis.
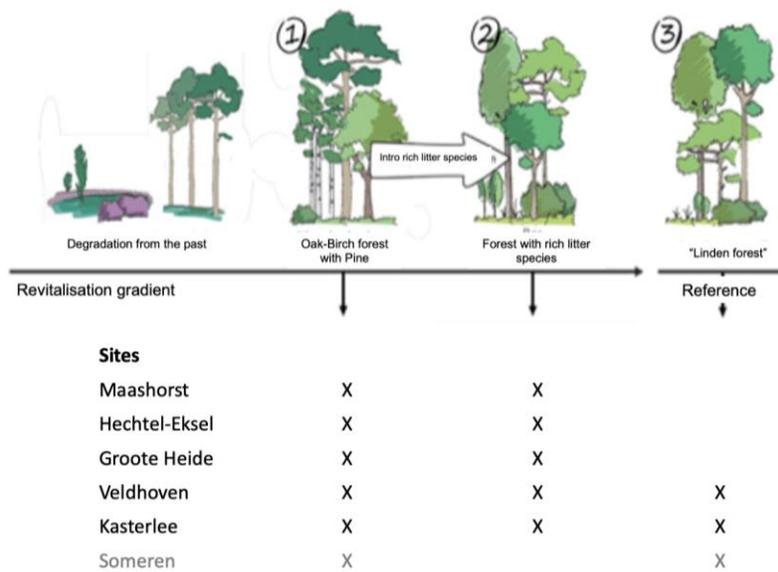
Figure 8: Study sites with plots found within depending on their recovery. The poor, degraded sites characterized by Oak-Birch forest with Pine start at the left of the gradient. In the middle there is, after introduction of rich litter species, a more restored forest type. The reference or Linden forest with a high vitality and without a history of degradation. Someren is shown in grey to indicate the exception to the pair-wise sampling of rich and poor due the use of the rich site as reference and might thus also influence the results. (Modified from Van Den Berg et al., n.d.)

2. Data collection

2.1 Field campaign

For each forest location, a rich and poor plot were pair-wise sampled. For the locations of Kasterlee and Veldhoven, a reference plot was also included. All plots were described by their location, tree composition, management history and canopy cover and samples were taken at the organic, top- and/or subsoil for biotic and abiotic characterization (Figure 9). The land use history and stand age was determined based on information from a local forest ranger or from maps available for that region (Geopunt, 2023; Topotijdreis, 2023). The tree species present were identified and the bitterlich method was used as a proxy for the basal area. Based on share of basal area, the forest type was classified. This descriptive information was summarized in Table 1.
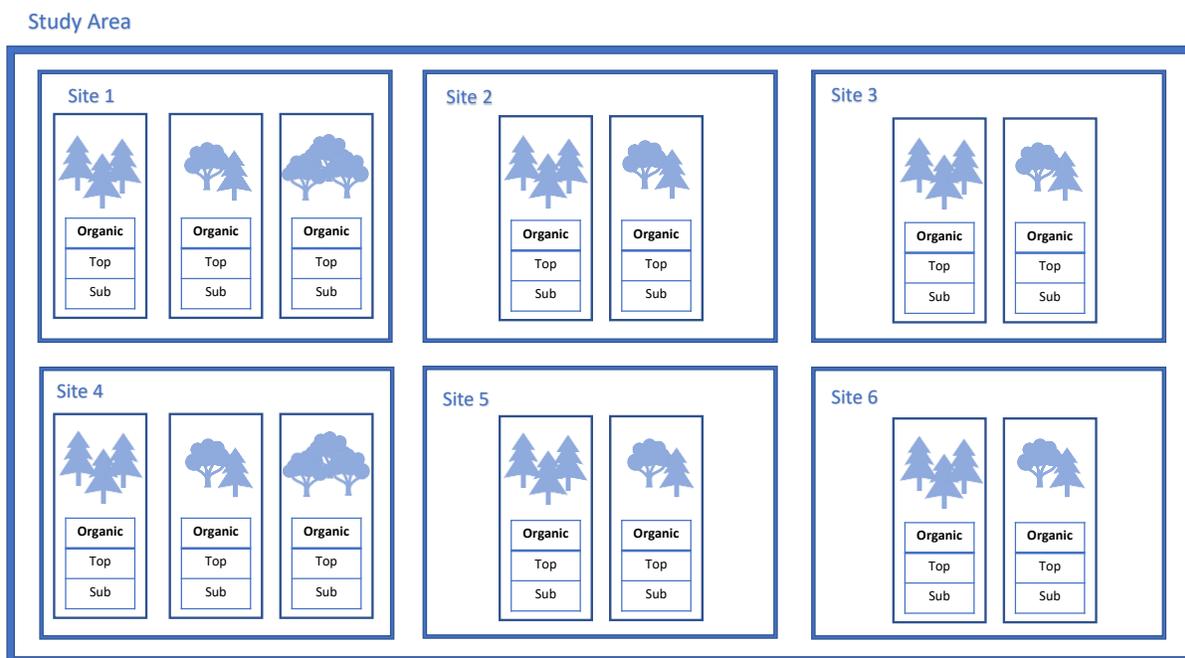
Figure 9: Sampling design for our study area, in which 6 different sites are incorporated consisting of either two (poor and rich) plots or an additional third reference plot. In each plot samples at three different soil depths are taken: Organic layer, topsoil (0-10 cm) and subsoil (10-20 cm).

First, samples that consist of a composite (ten points with a sterile gouge auger) were taken in the organic layer and the mineral soil layers between 0-10 cm (topsoil) and 10-20 cm (subsoil). These were used for analysis of chemical variables. Three samples were taken for the calculation of the bulk density: two with a Kopecky ring at a depth between 0-10 cm and 10-20 cm and one from a smaller plot of 25 cm by 25 cm of the organic layer. Next, earthworms were collected in three

separate sections of 71 cm by 71 cm within the plot. This was done by mustard extraction according to the FunDivEurope Protocol, where mustard powder was used as an expellant in combination with hand sorting from a smaller plot of 25 cm by 25 cm (Valckx et al., 2011). Three soil samples, consisting of the organic layer from a small plot of 20 cm by 20 cm and one auger point from the soil layer between 0-10 cm, were taken for the identification of other mesofauna present (Macfadyen, 1961). A description of the humus layer was executed according to the European Humus Reference Base (Zanella et al., 2011) in three different parts of the plot (Table 1A). Thickness of the humus layer was defined as the sum of the fragmented (OF) and humic (OH) layer. A soil description up to 1 m was also performed with an Edelman auger according to the FAO guidelines and used to determine the soil type according to WRB classification Table 1.

Table 1: Study site characteristics per site (Kasterlee, Veldhoven, Hechtel-Eksel, Someren, Maashorst and Grooteh Heide) for each treatment (poor, rich and reference). Characteristics include tree species identity, divided into rich and poor-litter species with a proxy of their basal area, measured with bitterlich, given between (). Also the landuse history and stand age is given up until the year of available information (NIA = No Information Available). Next, the forest type is determined based on share of basal area (gD = grove den or pinus sylvestris, zE = zomereik or Quercus robur L., avK = Amerikaanse vogelkers or Prunus serotina, rBe = ruwe berk or Betula pendula, Ha = Hazelaar or Corylus avellana, (w)Li = (winter)linde or Tilia (cordata), nEd = Noorse Esdoorn or Acer platanoides, wE = wintereik or sessile oak, gEd = gewone esdoorn or Acer pseudoplatanus L., bWi = boswilg or salix caprea, He = Hemlockspar or Tsuga heterophyla, zK = zoete kers or Prunus avium, Hb = haagbeuk or Carpinus betulus, wLij = wilde lijsterbes or sorbus aucuparia, / = left value > 50%, + = left value between 50-25%). The soil type is given according to the WRB classification and lastly a comment section is provided with additional information on the plots.

POOR	Kasterlee	Veldhoven	Hechtel-Eksel	Someren	Maashorst	De Groote Heide
Tree species identity	• Poor (26) • Rich (2.5)	• Poor (18) • Rich (8)	• Poor (11) • Rich (3.5)	• Poor (13) • Rich (7)	• Poor (11) • Rich (5)	• Poor (26) • Rich (3)
Land use history	1777: Heathland	NIA	1712: Heathland 1777: Heathland	1712: Heathland	Until 1955: Heathland 1978: Closed forest	1712: Heatland
Forest type	gD/zE	gD/zE, avK, rBe	gD/rBe	gD/avK + rBe	gD/avK + Ze	gD/rBe
Stand age	NIA	NIA	NIA	NIA	NIA	NIA
Soil type	Gleyic Cambisol	Cambisol	Podzol	Podzol	Cambisol	Gleyic Podzol
Comments					Big grazers such as wisent, Tauros and Exmoorpony	Eroded landscape, covered with mosses

RICH	Kasterlee	Veldhoven	Hechtel-Eksel	Maashorst	De Groote Heide
Tree species identity	• Poor (17) • Rich (20)	• Poor (2.5) • Rich (26)	• Poor (5) • Rich (7)	• Poor (8) • Rich (23)	• Poor (4) • Rich (25)
Land use history	1777: Heathland		1712: Heathland 1777: Heathland	Until 1955: Heathland 1978: Closed forest	1956: Larix forest (According to forest ranger, but not observed in the field)
Forest type	gD + Ha + Li, nED	avK	Li/wE + zE	avK/gD	gEd/bWi + He
Stand age	NIA	Prunus serotina : ± 32 years Quercus acer: ± 68 years	± 30 years	NIA	NIA
Soil type	Gleyic Cambisol	Cambisol	Plaggic Anthrosol	Anthric Umbrisol	Gleyic umbrisol
Comments				Big grazers such as wisent, Tauros and Exmoorpony	

REFERENCE	Kasterlee	Veldhoven	Someren
Tree species composition	• Poor (3) • Rich (30.5)	• Poor (5.5) • Rich (15)	• Poor (8) • Rich (13.5)
Land use history	Until 80s: Maize field 80s: Afforestation	NIA	NIA
Forest type	wLi + zK, Hb, zE	avK + Ha + zE, wLij	avK + zE, gEd
Stand age	± 38 years Quercus robur L. relics	NIA	Prunus serotina: ± 33 years Quercus: ± 40 years
Soil type	Plaggic Anthrosol	Cambisol	Plaggic Podzol
Comments	Experimental introduction of rich litter species		

2.2 Laboratory work

The soil samples for the chemical analysis were dried in an oven at 36°C and split to ensure a representative sample. The samples for the organic layer were grinded in a mill in advance for homogenization. Chemical variables, such as pH, texture, C:N ratio, base saturation (BS) and CEC were determined (Table 2A). The pH was measured with HI 991000 pH/Temperature meter by Hanna Instruments met HI 1292 electrodes from samples with 5 mg – 25 ml ratio soil to demi water. To prepare for texture analysis, HCl and H_2O_2 were added to the split samples to remove Ca and organic matter respectively. Next, texture was determined with a LS13 320 Laser Diffraction Particle Size Analyzer by Beckman Coulter. For determination of C:N ratio, the soil samples were grinded with a mortar and subsamples between 3-20 mg, depending on their organic matter content, were weighed and stored in tin casing. The analysis was performed with an elemental analyser by ThermoFisher Scientific, type Flash 2000. The bulk density was determined based on the dry weight. Based on the organic % C and bulk density, the total carbon stock was calculated in ton/ha, differing for organic and top-sub soil depending on the extraction method (Eq. 2.1 & Eq. 2.2) (Table 2A). The organic samples, however, were not completely dried upon weighing, which resulted in an overestimation of the bulk density and ultimately their carbon stock.

Organic: $C\ stock\left(\frac{ton}{ha}\right) =\ 0.25\ \frac{ton}{ha}\cdot m(g)\cdot C(\%)$ (Eq. 2.1)

Top-Sub: $C\ stock\left(\frac{ton}{ha}\right)\ =\ 10\,000\ m^2\ \cdot 0.1\ m\ \cdot bulk\ density\left(\frac{g}{cm^3}\right)\cdot C(\%)$ (Eq. 2.2)

Lastly, the CEC was determined with standardised protocol ISO 23470 or the cobalthexamine method. Due to the sandy texture of all the samples, 2000 mg was advised as sample extract to ensure an accurate analysis. The exchangeable cations (Ca, K, Mg and Na) remain in the supernatant solution, following a one-step centrifuge extraction with 0,0166 M cobalt (III) hexamine chloride solution (Cohex) $[Co[NH_3]_6]Cl_3$. The Co lost after extraction is an indicator for the CEC ($cmol_c$/kg). This exchange, however, was for the subsoil samples often lower than the ideal range of 15-33%, possibly underestimating their CEC. The CEC is used to calculate the BS (%). The micro- and mesofauna present in the samples were retrieved by means of multiple methods (incl. eDNA and PLFA analysis – not discussed in this study). The earthworms, collected in the field and preserved on 70% ethanol, were determined till species level and weighed (Table

3A). The remaining mesofauna is determined up to the taxonomic level of order with a Thulgrenn funnel (Simons, 2023). The respiration, or the production of CO_2 in the soil, is measured for the three soil layers with a portable gas dectector, the PBI Dansensor CheckPoint II, for a sample of 100 g and expressed in % (Table 3A) (Van Criekinge, 2023). This CO_2 percentage is converted to µg C / (day * g soil) (Eq 2.3).

$$Respiration\left(\frac{mass\ C}{day \cdot g\ soil}\right) = \frac{\left(0.007195 \cdot \frac{\frac{C(\%)}{100(\%)}}{m(sample) \cdot MM(C) \cdot \frac{10^6 \mu g}{g}}\right)}{\#hours \cdot 24} \qquad \text{(Eq. 2.3)}$$

3. Data analysis
3.1. Site characteristics

The sites are compared in terms of the characteristics described and measured, mentioned in section B.2.1 and B.2.2. Differences in the basal area for rich and poor-litter species between the treatments poor, rich and reference, were tested with a linear mixed model (LMM) (with site as random effect) with the lme4 package. An interaction with soil layer was added for the variables which were measured at organic, top- and subsoil level, namely pH, the thickness of the humus layer, C stock and respiration. CEC and BS were analysed for the top and subsoil layer. Differences in percentage of sand between sites was also tested. Lastly, diversity indices for mesofauna and tree species, calculated with the vegan package, were tested with generalized LMMs (GLMM) with the glmm package. For richness and evenness, the Poisson- and beta-distribution, respectively, were used, while Shannon and earthworm mass corresponds to a gamma-distribution. P-values were obtained with the lmerTest package and normality of the residuals were checked with histograms. The (G)LMM were used, even in case of no normal distribution, due to their robustness to these violations. The variables which showed a significant difference ($\alpha = 0.05$) between either treatment, soil layer or both were tested with a post-hoc Tukey test to determine which treatments or soil layers differed. Both tests were performed with the multcomp package. The predicted variables and raw data distribution were visualized with violin plots for each variable with the ggplot2, effects and gridExtra packages. All statistical analysis were performed in R (R Core Team, 2022).

3.2. Soil depth profile

The depth profile of the soil, obtained for each plot, was partitioned into horizons up to a depth of 1 m, depending on the type, characteristics and colour based on the Munsell colour chart. The depth distribution of the analysed soil characteristics, such as pH, texture and carbon were summarized using Excel for visual comparison in a graph (Microsoft Corporation, 2018).

3.3. Carbon stock analysis

To get an understanding of the drivers of the soil carbon stock in our study area, relationships with the other variables were tested, with a distinction between organic, top- and subsoil layers. Variables include pH, BS, CEC, litter type, thickness of humus layer, mass earthworms and respiration. To test the relationship with the mesofauna and tree species present, multiple diversity indices were also used as predictor variable. All data was standardized to zero mean and unit variance with the vegan package, to be able to compare effect size. LMMs with site as a nested random effect are fitted with the lme4 package. The distribution of the residuals was visually tested for normality with a histogram. To determine significance ($\alpha = 0.05$), the p-values were retrieved with the lmerTest package. The resulting relationships were visualized both with the fitted linear regression line and a confidence interval (CI = 0.95) based on the predicted values, determined with the ggeffects package, and the raw data presented by points. All statistical analysis were performed in R (R Core Team, 2022).

C. Results

1. Site characteristics

With the help of LMM and GLMM, biotic and abiotic variables are estimated and compared between treatments and soil layers, aiming to get better insight on the current conditions of the sites and the differences between treatments.

1.1 Abiotic variables

To understand the soil conditions and health status, soil variables, such as texture, pH, BS and CEC are analyzed. Kasterlee, Maashorst and Someren have a significantly higher percentage of sand, around 80%, in comparison to Veldhoven, Hechtel-Eksel and Groote Heide, whose texture consists of 60-70% sand ($p < 0.01$) (Figure 10).
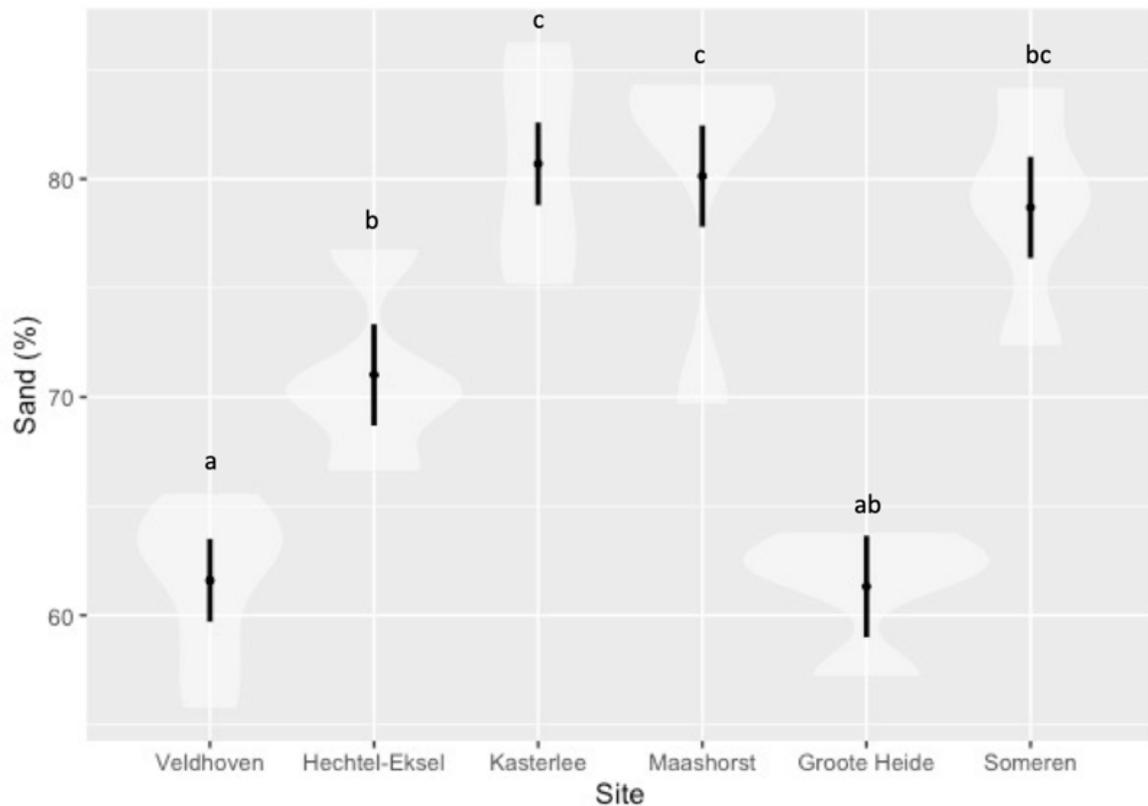
Figure 10: Comparison of texture, expressed in percentage of sand, between sites (Veldhoven - Hechtel-Eksel - Kasterlee - Maashorst - Groote Heide - Someren). The predicted value with the standard error of the linear model is displayed in black. The distribution of the raw data is represented by a violin plot for each site. The results of a post-hoc Tukey test are shown in small letters above each plot.

Both treatment and horizon have a significant effect on pH ($p < 0.05$). While there is a significant difference between the poor and the rich treatment in the organic layer (-1.64 ± 0.29, $p \ll 0.01$) for pH, this trend is not found for the other layers, nor between rich and reference (Figure 11).
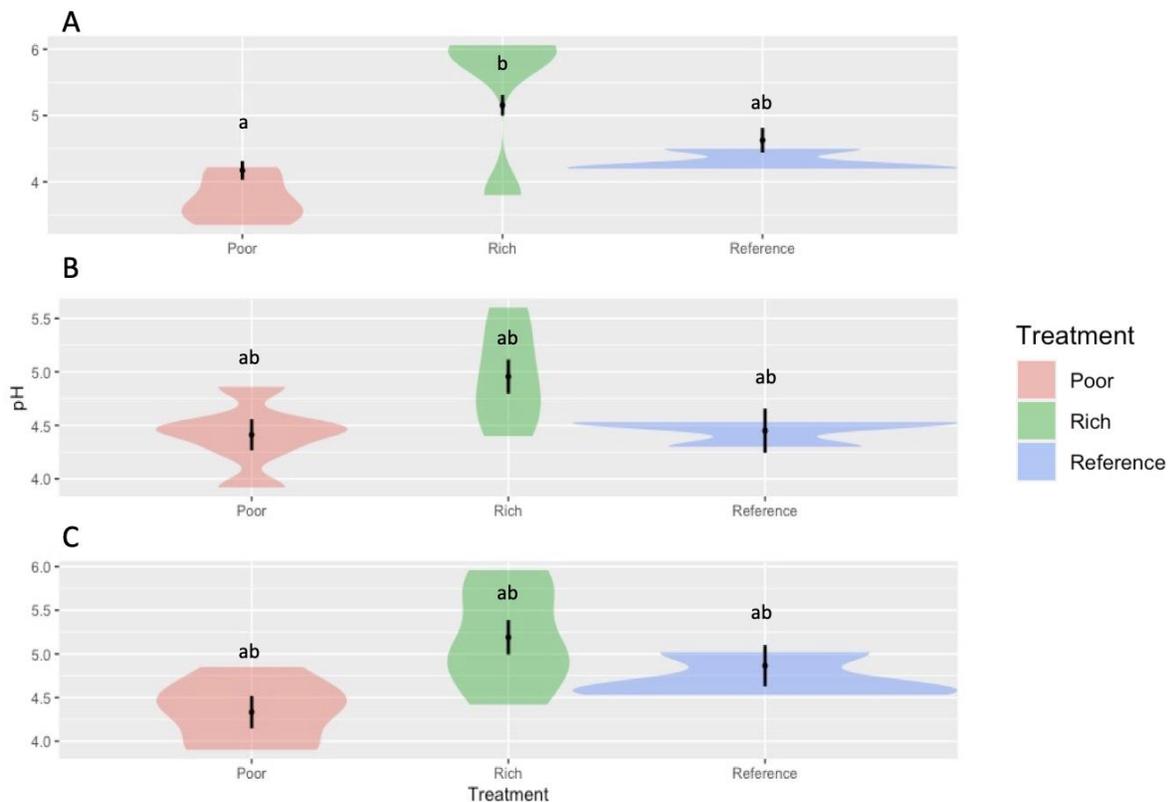
Figure 11: Comparison of pH between the treatments (poor – rich – reference) for the organic layer (A), 0-10 cm (B) and 10-20 cm (C). The predicted value with the standard error of the LMM, which takes the site into account as random variable, is displayed in black. The distribution of the raw data is represented by a violin plot for each treatment with poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test are shown in small letters above each plot.

Neither treatment, nor horizon have a significant effect on BS or CEC. The BS, however, is larger in the rich and reference treatments, while this is the opposite case for CEC (Figure 12).
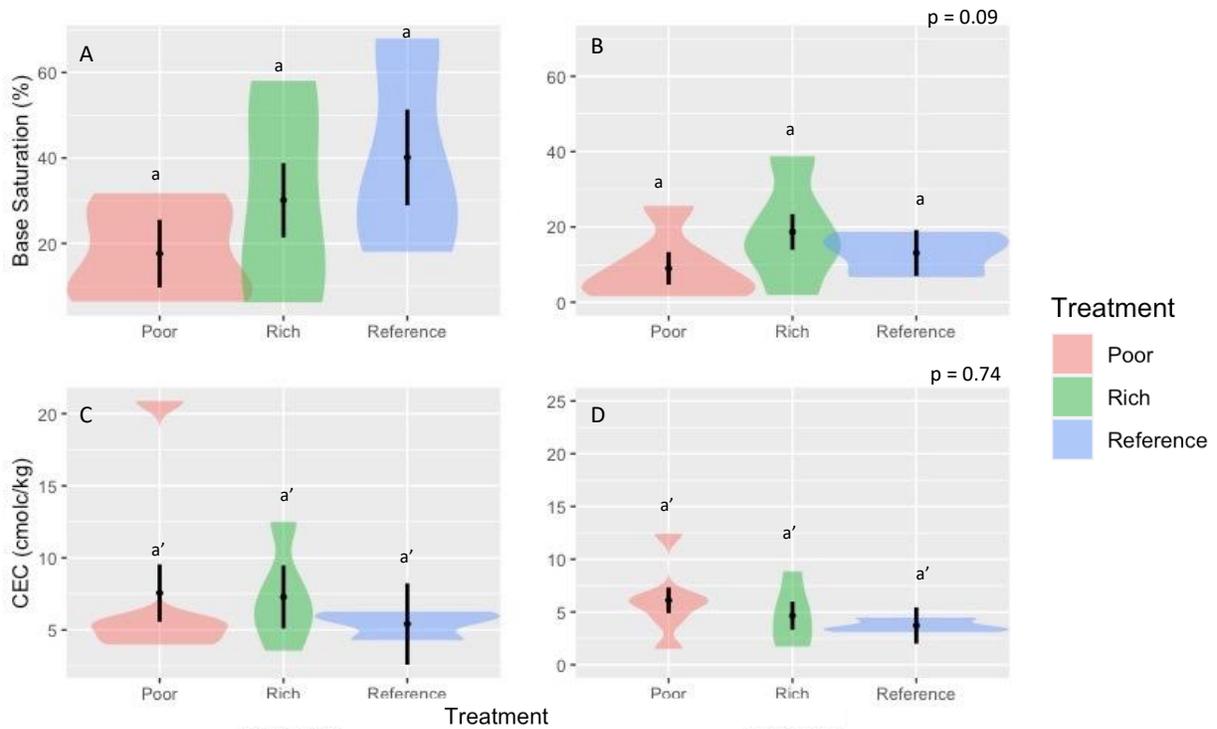
Figure 12: Comparison of BS (%) between the treatments (poor – rich – reference) at 0-10 cm (A) and 10-20 cm (B) and comparison of CEC at 0-10 cm (C) and 10-20 cm (D). The predicted value with the standard error of the LMM, which takes the site into account as random variable, is displayed in black. The distribution of the raw data is represented by a violin plot for each treatment with poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test, done separately for both variables, are shown in small letters above each plot.

1.2 Biotic variables

There are also several biotic variables characterizing our study sites, relating to tree species and their litter quality, and soil biota. Between treatments, there are no significant differences in richness and evenness of tree species (Figure 13). In the rich sites, there is a large variation for both variables, while this is limited for the reference treatment. The predicted values of the richness and evenness are also slightly higher for reference compared to poor and rich plots.
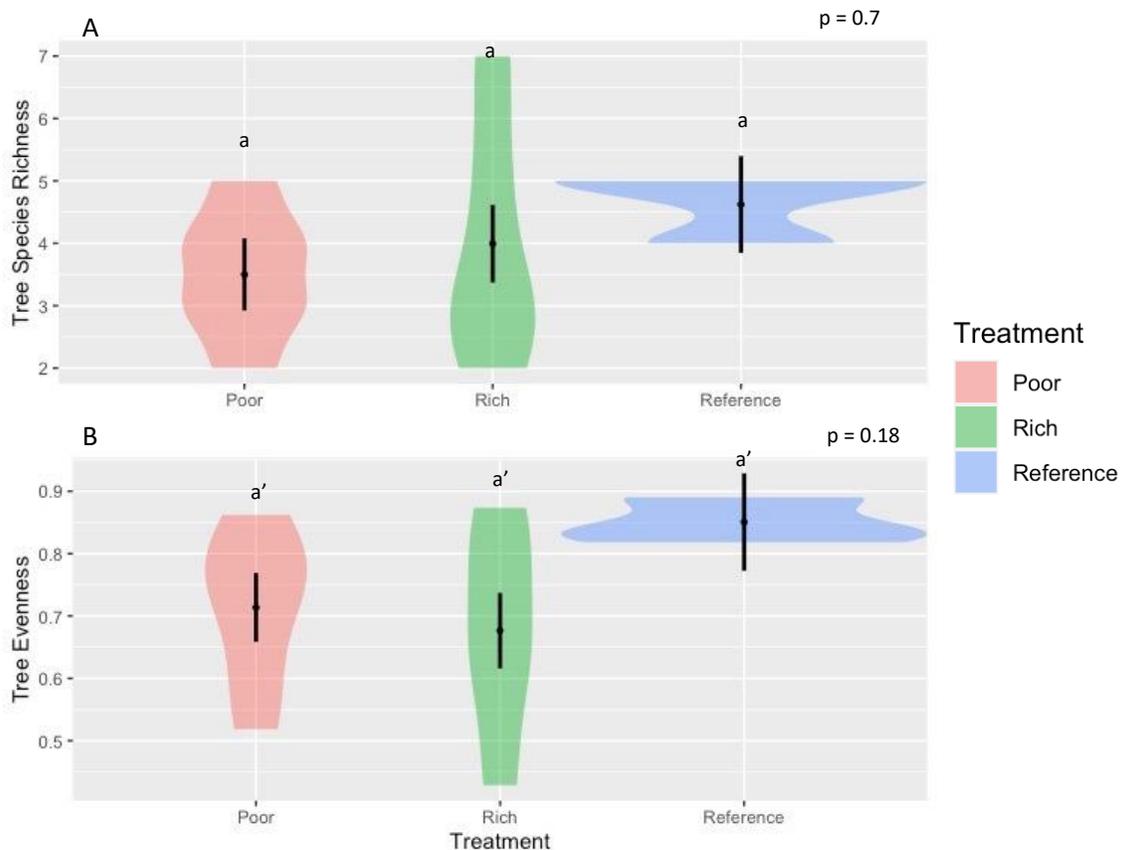
Figure 13: Comparison of tree species richness (A) and evenness (B) between the treatments (poor – rich – reference). The predicted value with the standard error of the GLMM is displayed in black. The model assumes a poisson and beta distribution, for richness and evenness, respectively and takes the site into account as random variable. The distribution of the raw data is represented by a violin plot for each treatment with poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test, done separately for both variables, are shown in small letters above each plot.

Treatment has a significant effect on the amount of poor and rich litter species present in the plot, approximated by basal area (m^2). The basal area for the poor-litter species is significantly higher in the poor treatments in comparison with the rich (9.89 ± 1.54, $p < 0.001$) and reference sites (13.00 ± 1.91, $p < 0.001$). But for the rich-litter species it is significantly lower in the poor sites than in the rich (-15.17 ± 1.79, $p < 0.001$) and reference (-14.05 ± 2.22, $p < 0.001$) (Figure 14).

Figure 14: Comparison of the basal area (m^2) for poor-litter species (A) and rich litter species (B) between the treatments (poor – rich – reference). The predicted value with the standard error of the LMM, which takes the site into account as random variable, is displayed in black. The distribution of the raw data is represented by a violin plot for each treatment with poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test, done separately for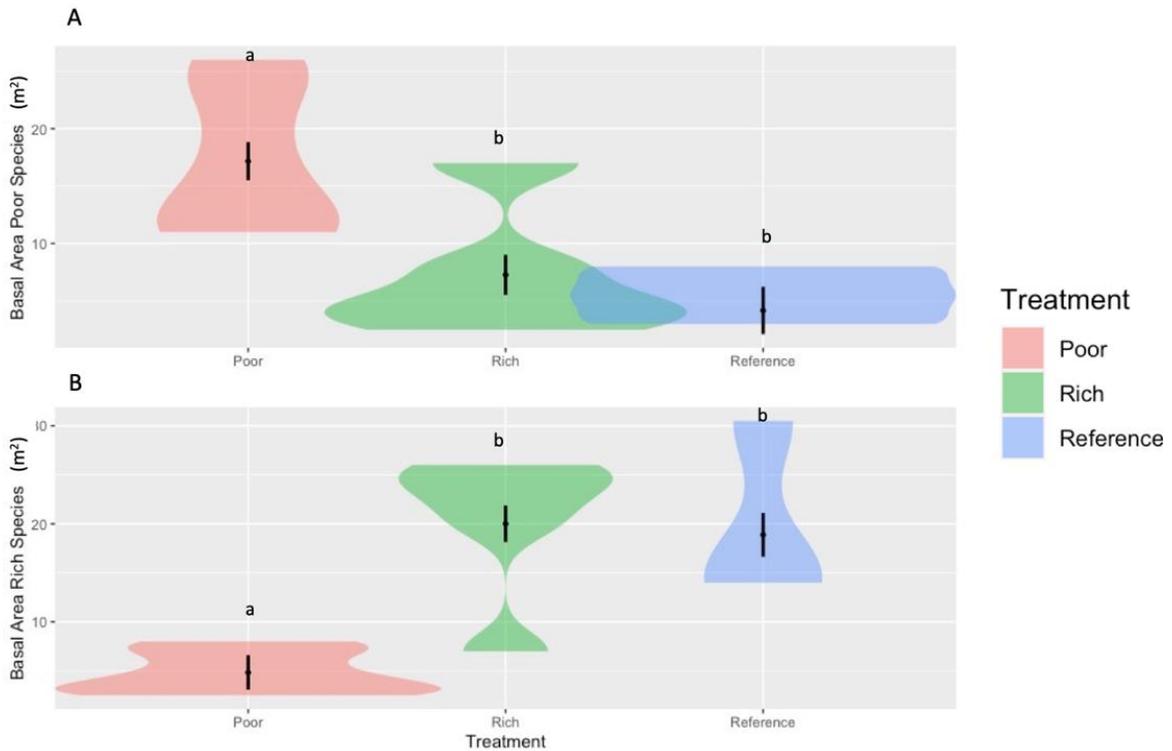 both variables, are shown in small letters above each plot. Here the poor treatment differs significantly from the rich and reference sites for both the poor and rich litter species.

The treatment also has a significant effect on the thickness of humus layer and is significantly higher in the poor sites compared to the rich (4.17 ± 1.44, p < 0.05) and reference sites (5.28 ± 1.68, p < 0.05) (Figure 15).
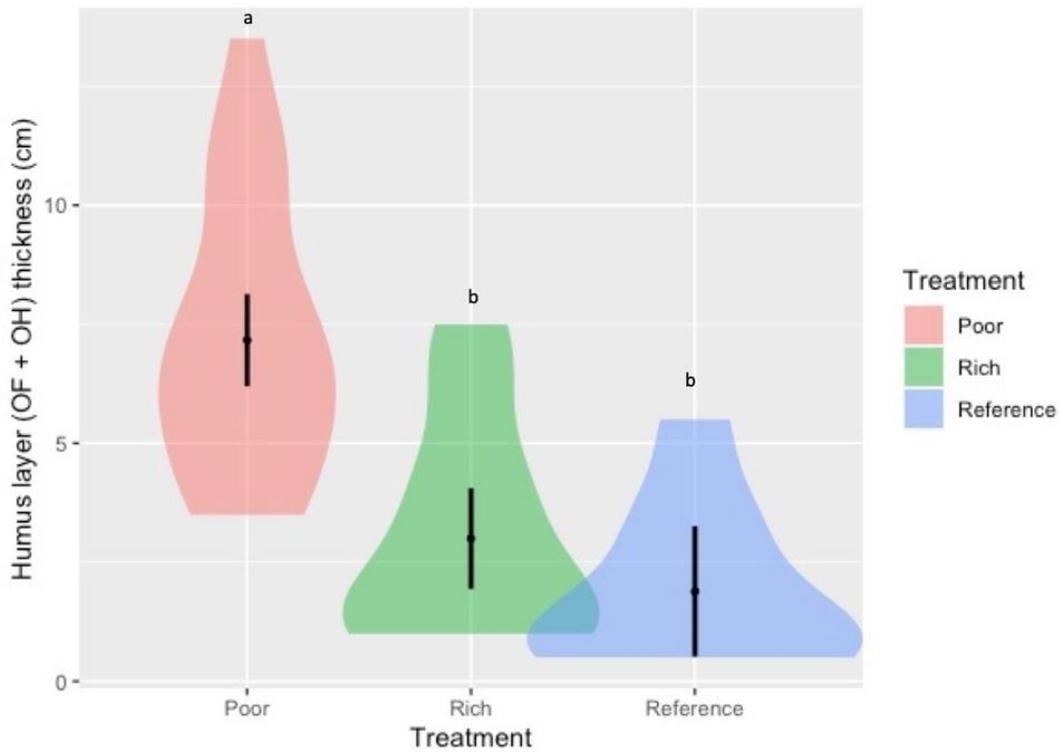
Figure 15: Comparison of the humus layer thickness between the treatments (poor – rich – reference). The humus layer is here defined as the OF and OH layer. The predicted value with the standard error of the LMM, which takes the site into account as random variable, is displayed in black. The distribution of the raw data is represented by a violin plot for poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test are shown in small letters above each plot.

The presence of soil biota is another indicator for soil health. The mesofauna, determined up to order, is tested for richness and the Shannon-Wiener Index, which also considers evenness. Neither showed significant differences between treatments. A higher number of orders can be found in the poor treatments, while there is less difference with rich and reference sites when evenness is accounted for (Figure 16).
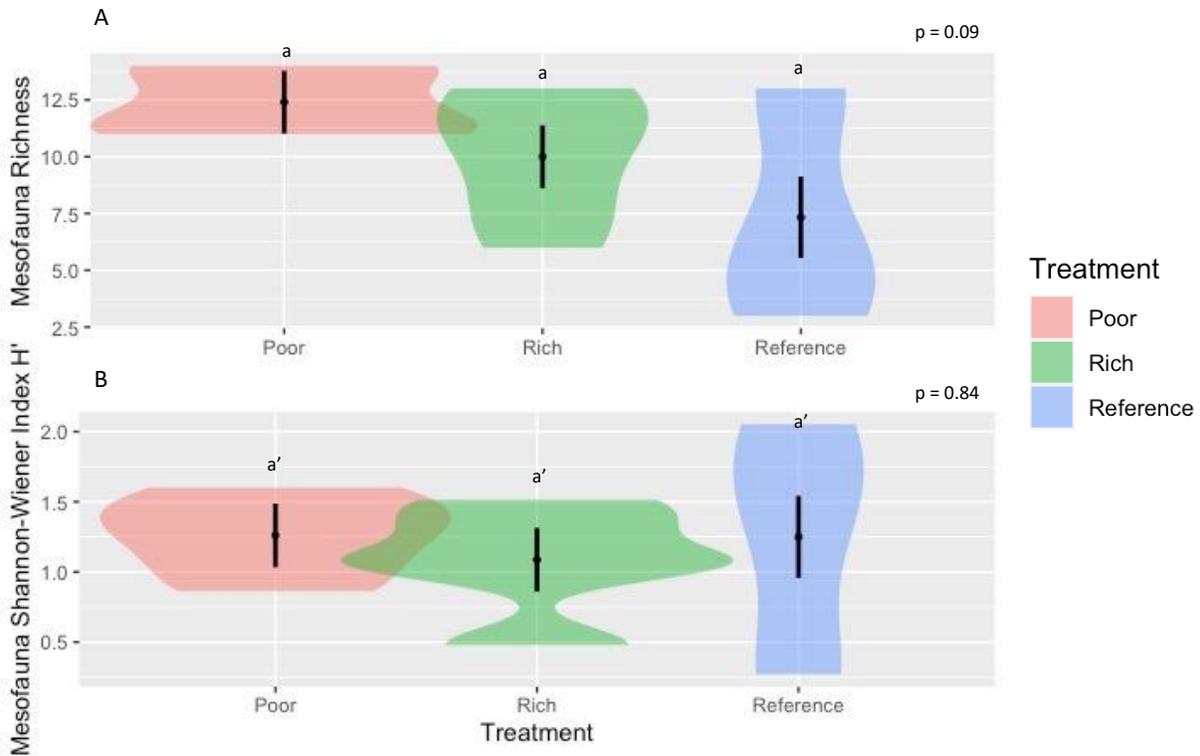
Figure 16: Comparison of the mesofauna richness (A) and Shannon-Wiener Index (H') (B) between the treatments (poor – rich – reference). The predicted value with the standard error of the GLMM, is displayed in black. The model used a Poisson and gamma distribution, for the richness and H' respectively, and accounts for site as a random effect. The distribution of the raw data is represented by a violin plot for poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test, done separately for both variables, are shown in small letters above each plot.

The mass of the earthworms is significantly lower in the poor plots, compared to the reference ones (-8.74 ± 2.13, $p < 0.001$) (Figure 17). This trend continues for the measured respiration from soil samples in the organic layer (-493.05 ± 50.0, $p < 0.001$) (Figure 18). It is also significantly lower for the rich treatment compared to the reference (-479.49 ± 52.5, $p < 0.001$). The differences in respiration between the layers is also significant for the organic layer and the subsoil (10-20 cm) for both the poor (134.03 ± 39.6, $p < 0.05$) and reference (633.86 ± 56.1, $p < 0.001$) sites.
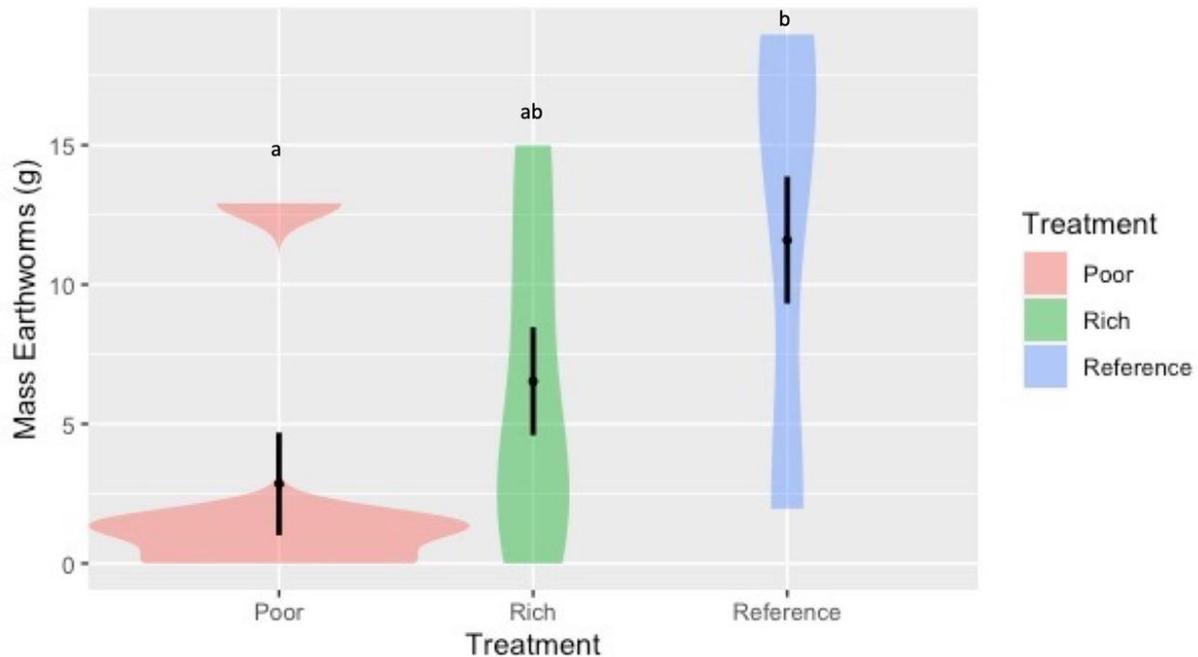
Figure 17: Comparison of the mass of earthworms between the treatments (poor – rich – reference). The predicted value with the standard error of the GLMM with a gamma distribution and the site as a random variable, is displayed in black. The distribution of the raw data is represented by a violin plot for poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test are shown in small letters above each plot.
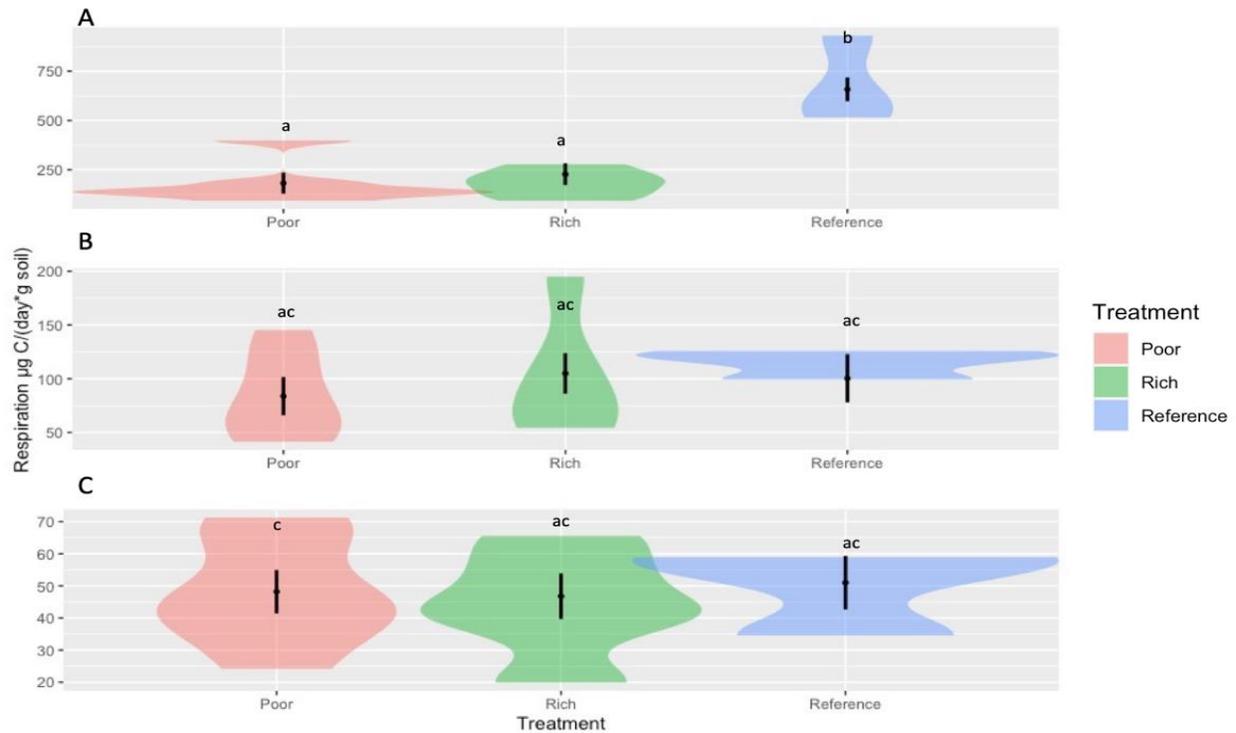
Figure 18: Comparison of the respiration (μg C / (day * g soil)) between the treatments for each soil layer: organic (A), topsoil (B) and subsoil (C). The predicted value with the standard error of the LMM, which takes the site into account as random variable, is displayed in black. The distribution of the raw data is represented by a violin plot for poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test are shown in small letters above each plot.

1.3 Carbon stock

The C stock is also tested between both treatment and soil layers (Figure 19). In the organic layer, the C stock is significantly higher in the poor treatment compared to the rich (42.46 ± 11.25, $p < 0.05$) and the reference plots (50.95 ± 12.40, $p < 0.01$). The poor treatment itself differs in soil layers, with the organic significantly higher than the top- (39.29 ± 9.94, $p < 0.05$) and subsoil (37.73 ± 9.94, $p < 0.05$).
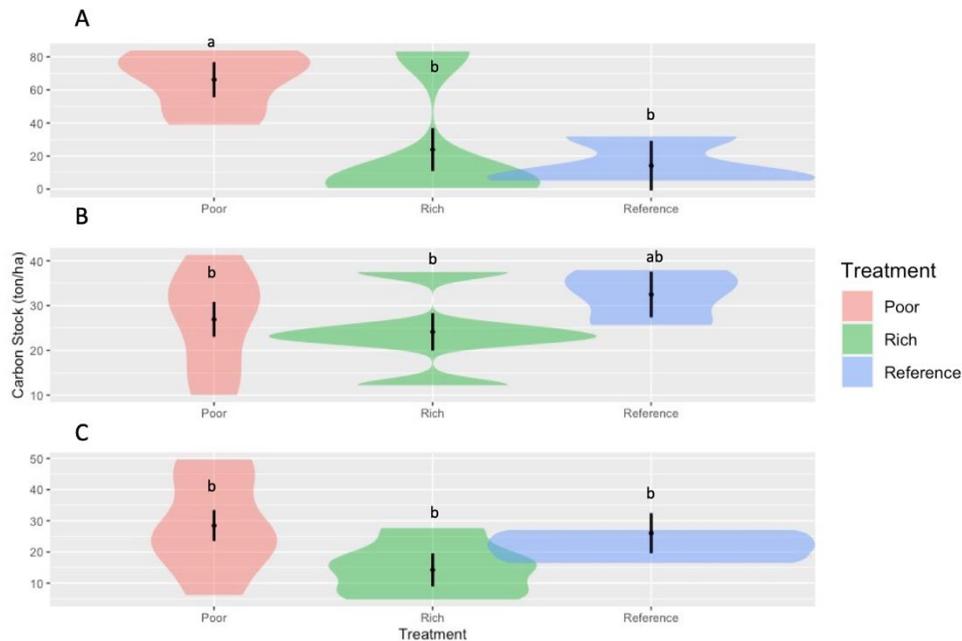
Figure 19: Comparison of the carbon stock (ton/ha) between the treatments (poor – rich – reference) for each soil layer: organic (A), topsoil (B) and subsoil (C). The predicted value with the standard error of the LMM, which takes the site into account as random variable, is displayed in black. The distribution of the raw data is represented by a violin plot for poor in red, rich in green and reference in blue. The results of a post-hoc Tukey test are shown in small letters above each plot.

2. Carbon stock analysis

With the help of LMM, which considers the dependence of variables from the same site, the relationship between C stock and the previously discussed abiotic and biotic variables is determined (Table 5A).

2.1 Abiotic variables

For all sampled soil layers, there is a negative relationship between C stock and pH (Figure 20). However, only for the organic layer, it is statistically significant (-0.86 ± 0.19, $p < 0.01$).
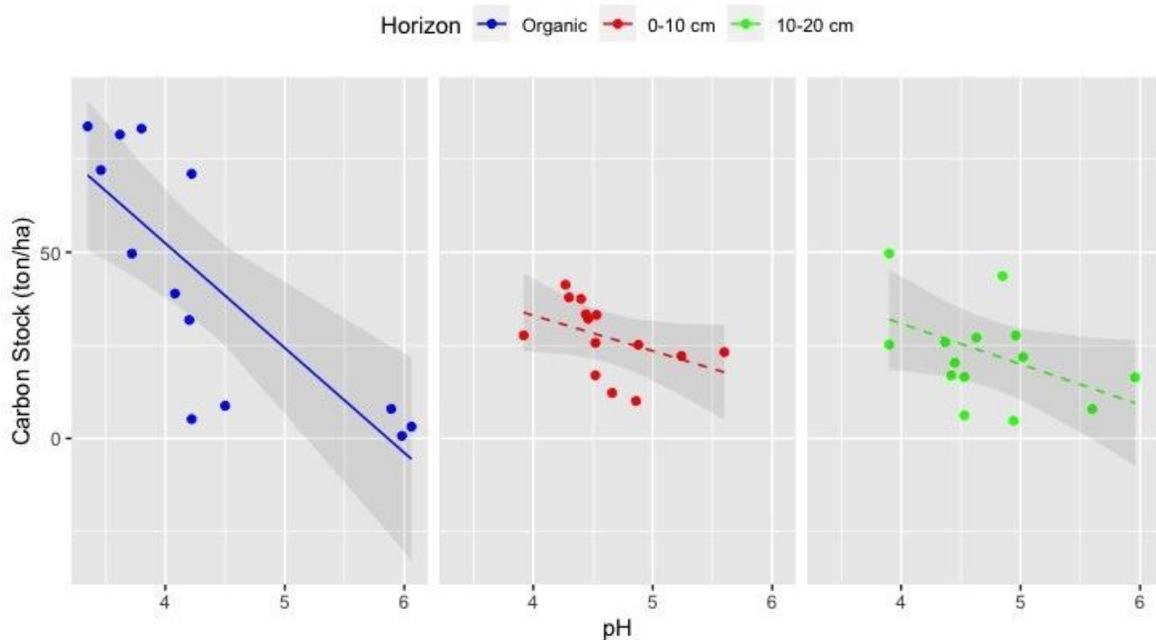
Figure 20: LMM showing a negative relationship between pH and carbon stock for the organic layer (blue), the topsoil (red) and the subsoil (green) (CI = 0.95). The slope for the organic layer is significant ($p < 0.01$) (solid), while this is not the case for the top- and subsoil (dashed).

Two other important abiotic variables are also discussed, namely BS and CEC, for the top- and subsoil (Figure 21). The organic layer could not be measured for these characteristics. The relationships between BS and the C stock are not significant for either soil layers. In the topsoil, we found a significant negative slope for CEC (-0.17 ± 0.08, $p < 0.05$), while in the subsoil it is positive (0.42 ± 0.17, $p < 0.05$). Most of the observations, however, are clustered around 0-9 cmol$_c$/kg.
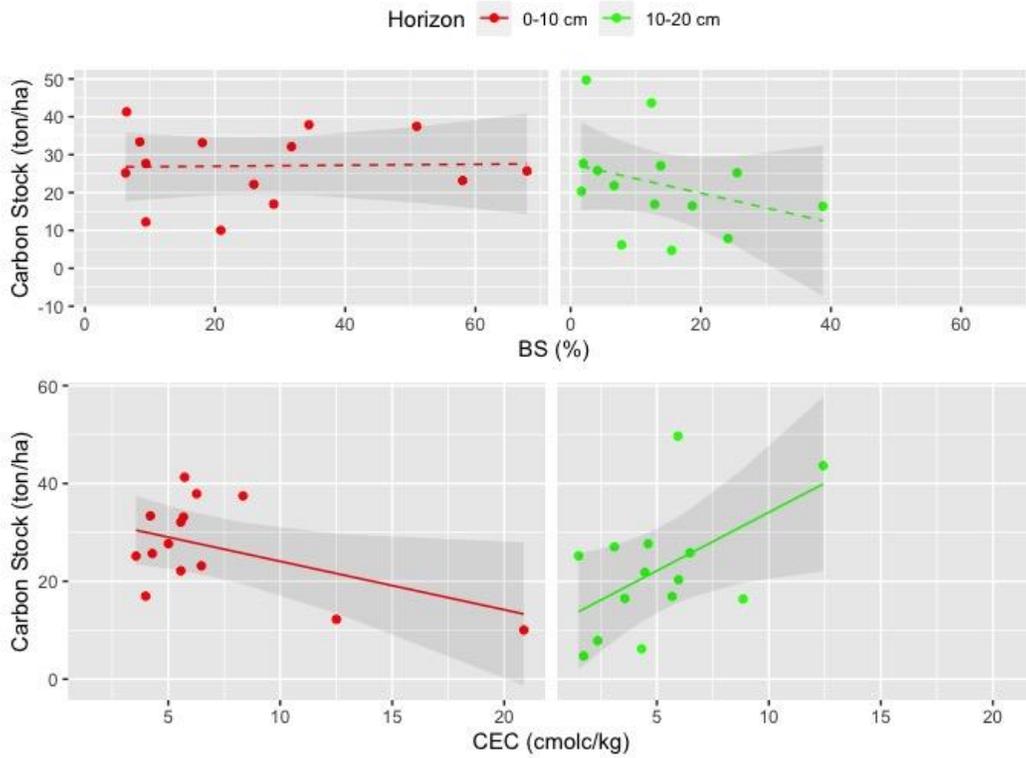
Figure 21: LMM showing the relationship between BS (%) and carbon stock (top) and between CEC (cmolc/kg) (bottom) for two soil layers: the topsoil (red) and the subsoil (green) (CI = 0.95). There is no significant relationship for BS (dashed). For CEC, in the topsoil there is a negative relationship, while in the subsoil, a positive one. Both slopes are significant (p <0.05) (solid).

2.2 Biotic variables

Biotic variables include diversity indices relating to mesofauna and tree species, litter quality and the resulting humus layer thickness, mass of earthworms found in the plots and the respiration measured in all three soil layers.

Tree species diversity is represented by both the species richness and evenness (Figure 22). Only richness shows a significant but small, negative relationship with C stock in the subsoil (-0.30 $\pm$ 0.14, $p < 0.05$). The evenness, on the other hand shows the opposite relationship. The variation in the organic layer is large, and also for the topsoil the slope is not pronounced.
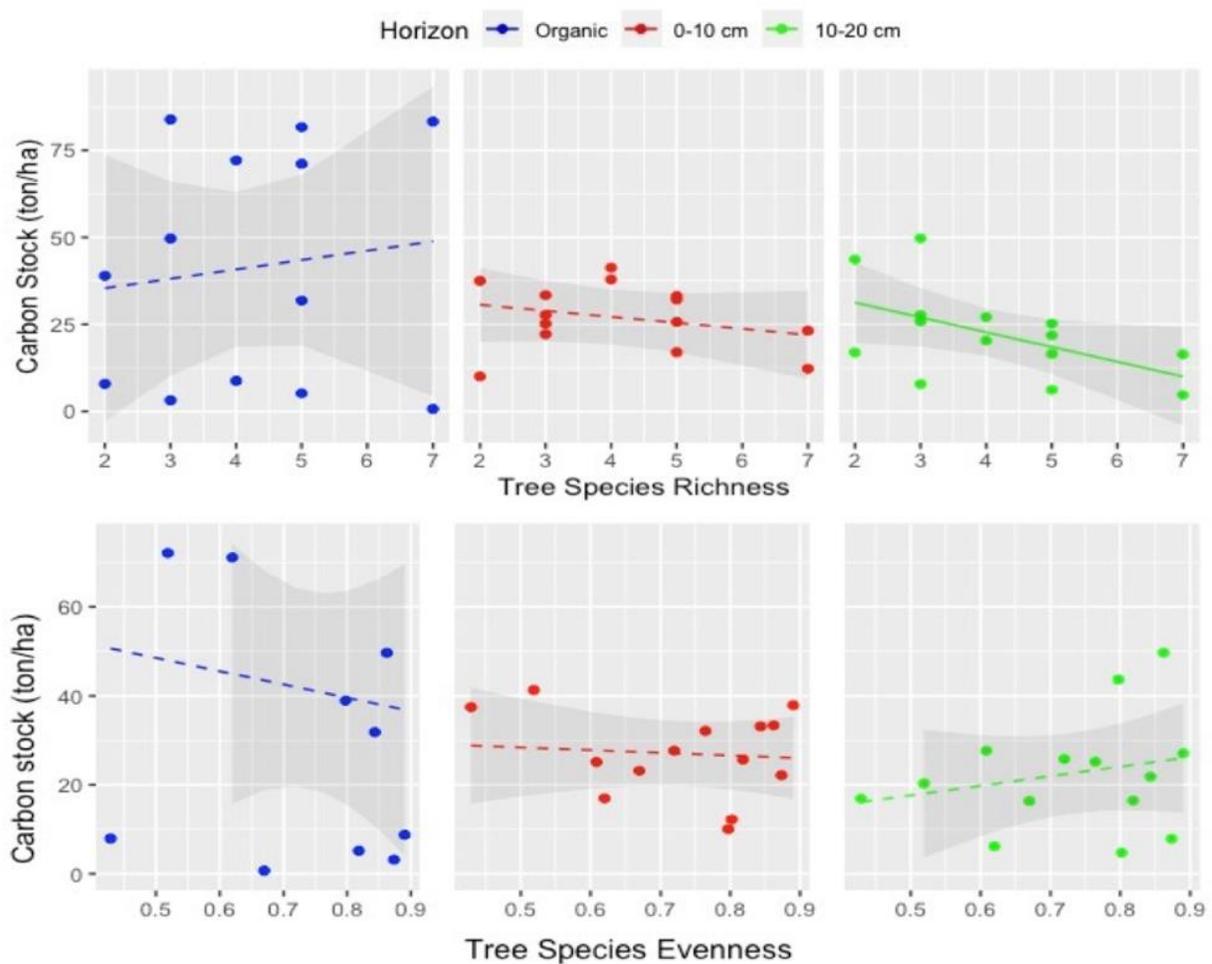
Figure 22: LMM showing the relationship between diversity indices for the tree species and the carbon stock, namely species richness (top) and evenness (bottom). The organic soil (blue), the topsoil (red) and the subsoil (green) are shown (CI = 0.95). Only the species richness shows a significant effect on C stock in the subsoil (solid).

The relationship between the C stock and litter quantity is also modelled, for both poor and rich litter (Figure 23). Basal area (m^2) is used as a proxy for the amount of litter. In organic soil, the slope is significant and positive for poor-litter species (1.25 ± 0.31, $p < 0.01$), while no relationship can be found for rich-litter species. In both the top- and subsoil, while none significant, the relationships are negative. Especially in the topsoil, there is a large variation in observations.
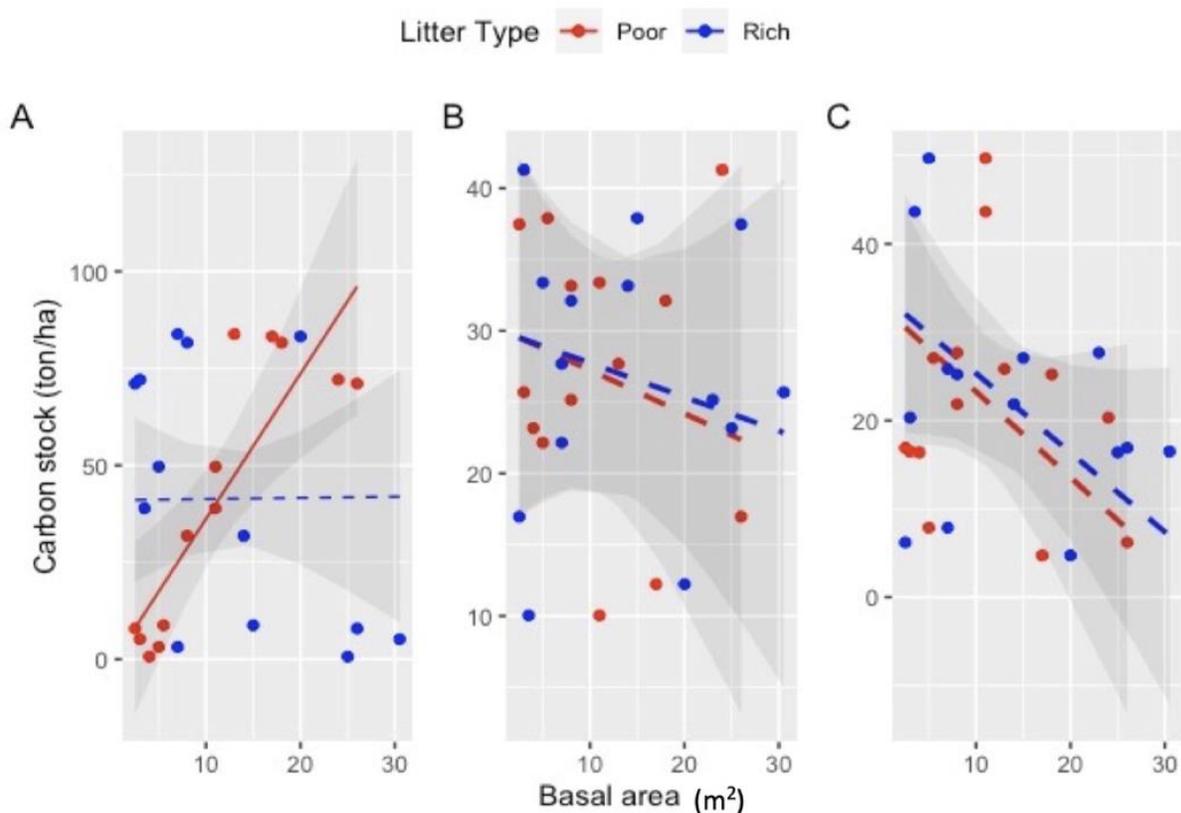
Figure 23: LMM showing the relationship between C stock and litter type, represented by the basal area (m^2) of poor (red) and rich (blue) litter species, for the organic layer (A) the topsoil (B) and the subsoil (C) (CI = 0.95). Only the basal area of poor litter quality is significant in the organic layer (solid).

The thickness of the humus layer, defined as the sum of OF and OH, has a significant positive relationship with the carbon stock for both the organic layer (0.80 ± 0.12, $p < 0.001$) and the subsoil (0.39 ± 0.11, $p < 0.001$). In the topsoil, there is no significant slope (Figure 24).
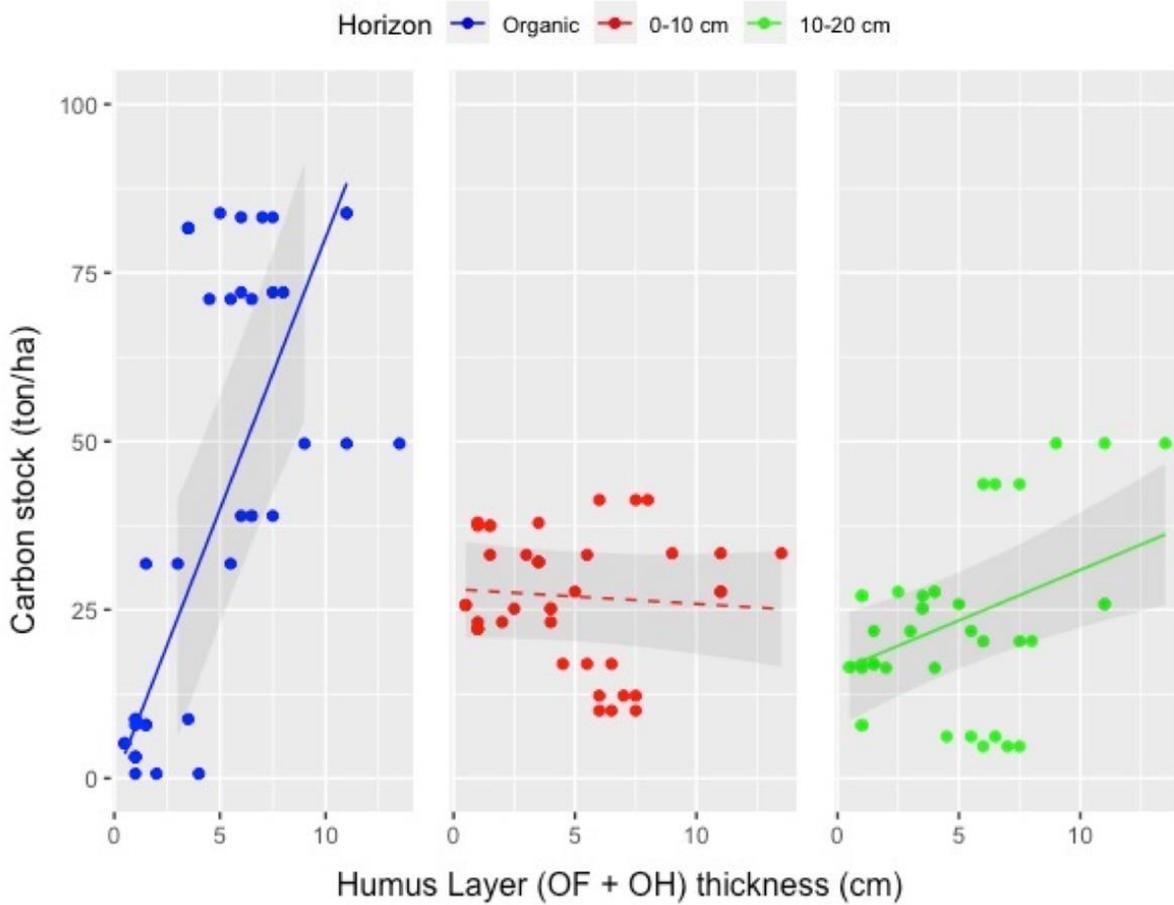
Figure 24: LMM showing the relationship between the thickness of the humus layer (cm) and the carbon stock, for three soil layers: the organic soil (blue), the topsoil (red) and the subsoil (green) (CI = 0.95). The humus layer consists of the OF and the OH layer. Both in the organic and subsoil, there is a significant positive effect on C stock (solid), but not in the topsoil (dashed).

The diversity of the soil mesofauna is represented by two indices, namely richness and Shannon-Wiener Index (H'). Their relationship between C stock is tested for all three sampled soil layers (Figure 25). For H', we found no significant slopes, while for mesofauna richness, there is a positive and significant relationship in the organic soil (0.73 ± 0.19, $p < 0.01$). The graphs of richness for each soil layer follow a similar course to those of H'.
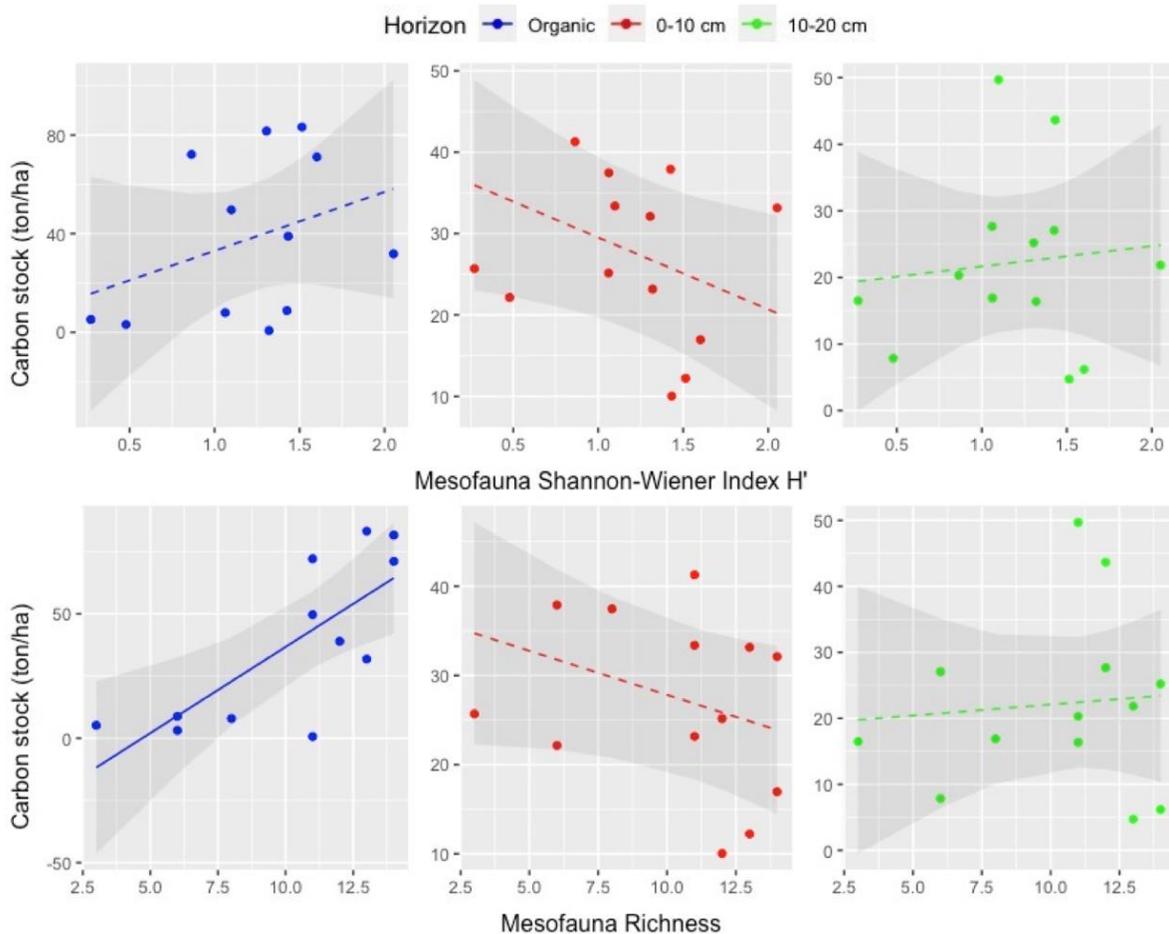
Figure 25: LMM showing the relationship between diversity indices for the soil mesofauna and the carbon stock, namely Shannon-Wiener Index (H') (top) and richness (bottom). The organic soil (blue), the topsoil (red) and the subsoil (green) are shown (CI = 0.95). Only H' shows a significant positive relationship with C stock in the organic layer (solid), while no other effects are significant (dashed).

There is significant negative relationship between C stock and the mass of earthworms in the organic soil (-0.82 ± 0.37, $p < 0.05$). In the top- and subsoil there is no significant effect due to the small effect size (0.04 ± 0.09 & -0.04 ± 0.16, respectively) (Figure 26). The respiration has no clear positive or negative relationship with the C stock in the organic layer due to large variations and small effect size (0.016 ± 0.12, $p = 0.90$). The latter phenomenon can also be found in the subsoil (-0.019 ± 0.21, $p = 0.93$). In the topsoil there is a significant positive relationship, but also with a relatively small slope (0.16 ± 0.05, $p < 0.05$) (Figure 27).
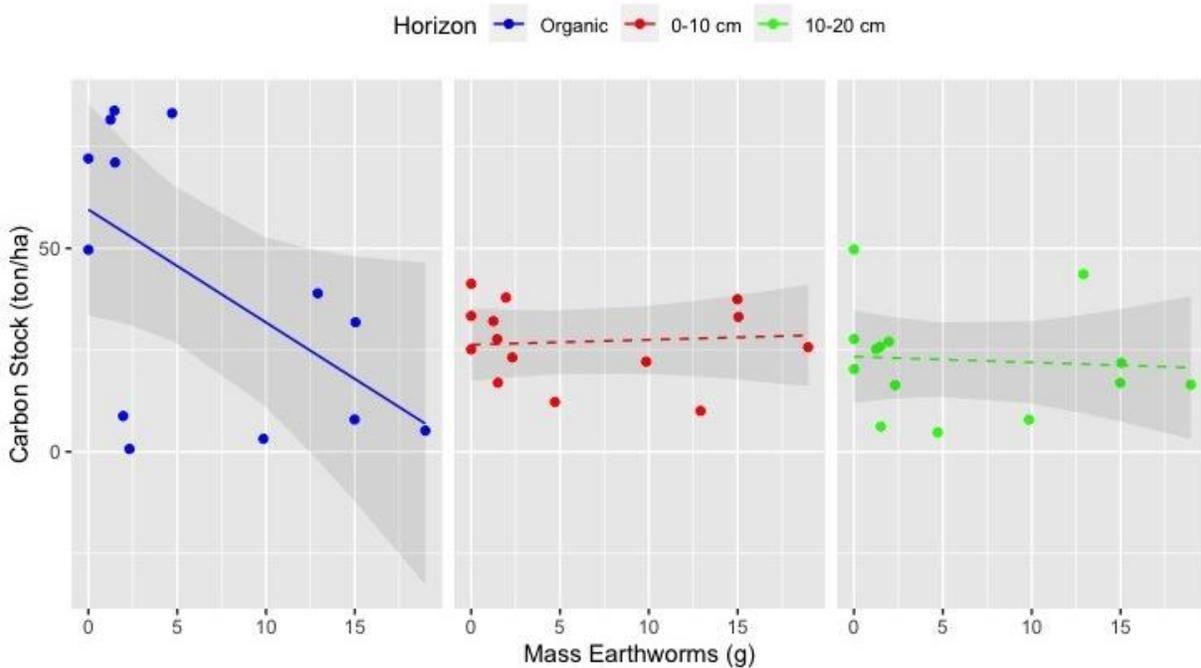
Figure 26: LMM showing the relationship between the mass of earthworms (g) and the carbon stock, for three soil layers: the organic soil (blue), the topsoil (red) and the subsoil (green) (CI = 0.95). In the organic layer, there is a significant negative effect on the C stock (solid), but not in the top- or subsoil (dashed).
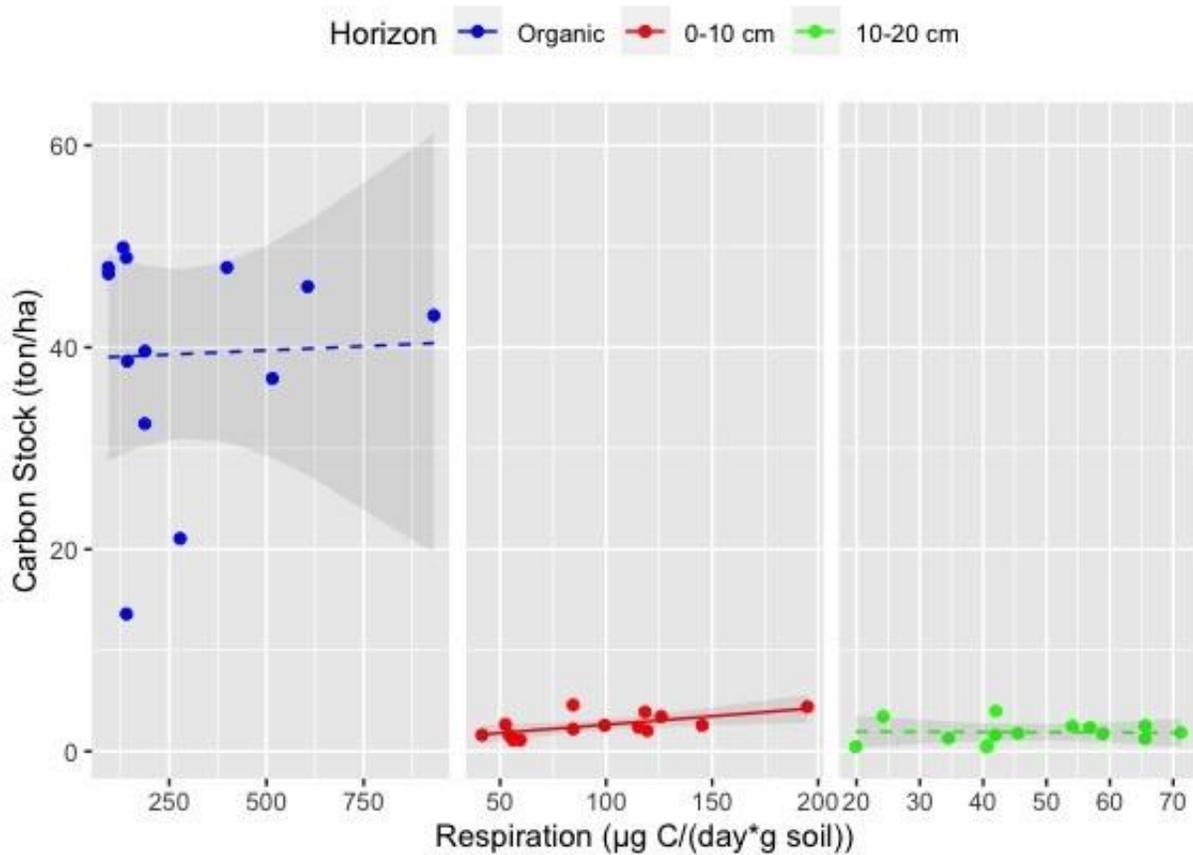
Figure 27: LMM showing the relationship between the mass of earthworms (g) and the carbon stock, for three soil layers: the organic soil (blue), the topsoil (red) and the subsoil (green) (CI = 0.95). Only in the topsoil, there is a small but significant relationship between respiration and C stock.

2.3 Carbon depth distribution

A depth profile for each site and treatment, is visually analysed for sand (%), organic C content (%) and pH for each horizon (Figure 28). Sand increases often with depth, with a steep decrease in the B-horizons and small differences between parent materials (C-horizons). There is an overall increase in pH with depth for all treatments. The carbon content decreases with depth, with high values in the humic mineral layer (Ah) and plaggic horizons (Ap). In the B horizon, we often see a small increase. If we look pairwise at the plots, there is often a slightly larger carbon content in the C-horizons in the rich and reference sites than in the poor ones.
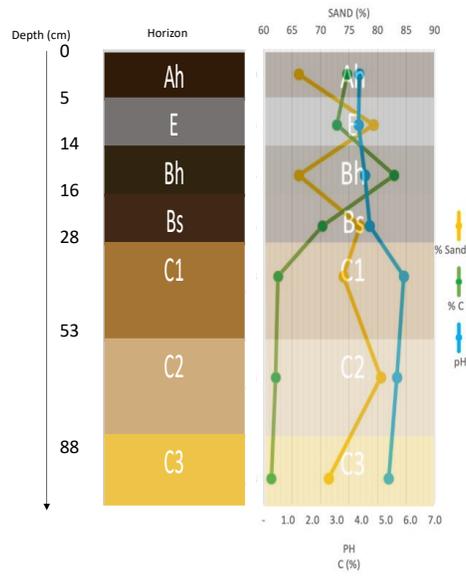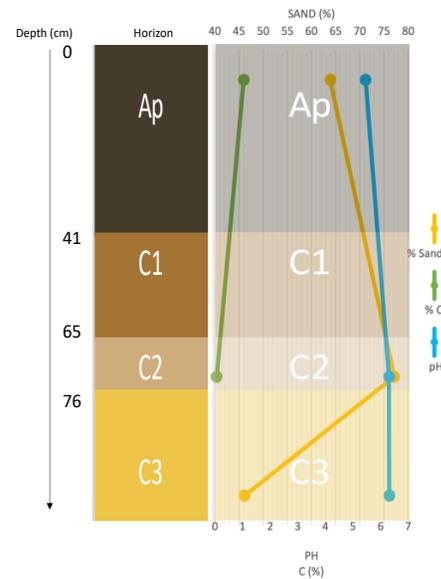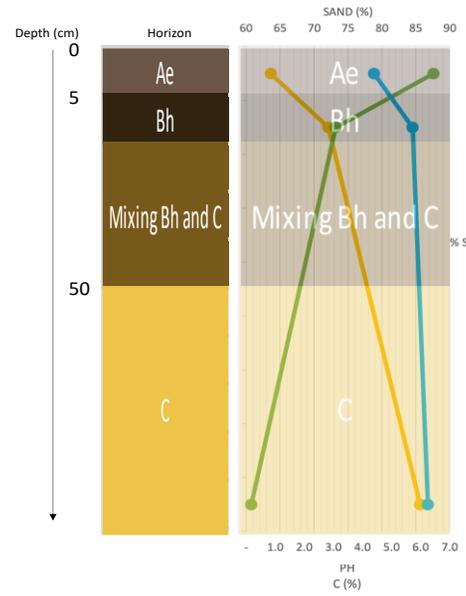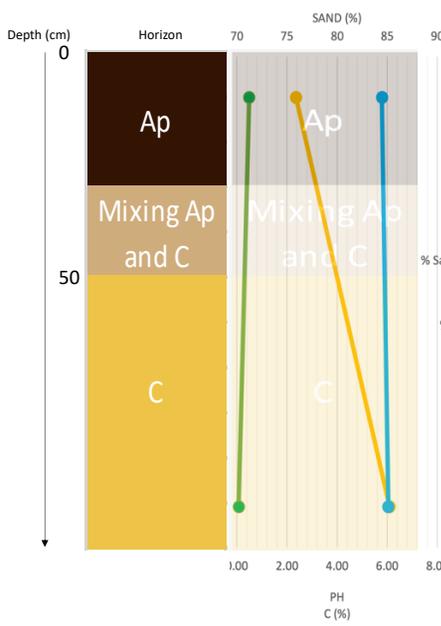
e)
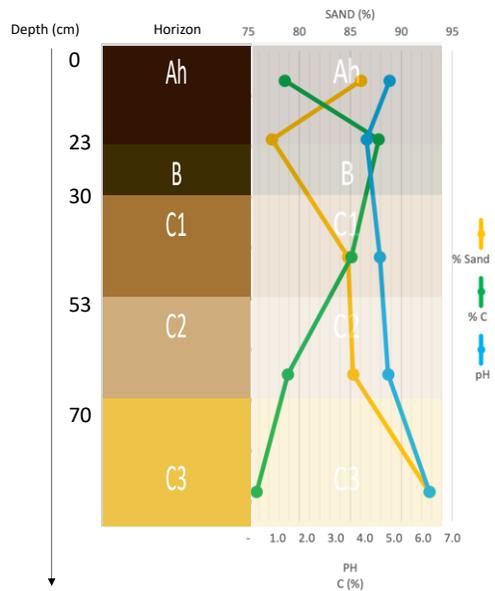
f)
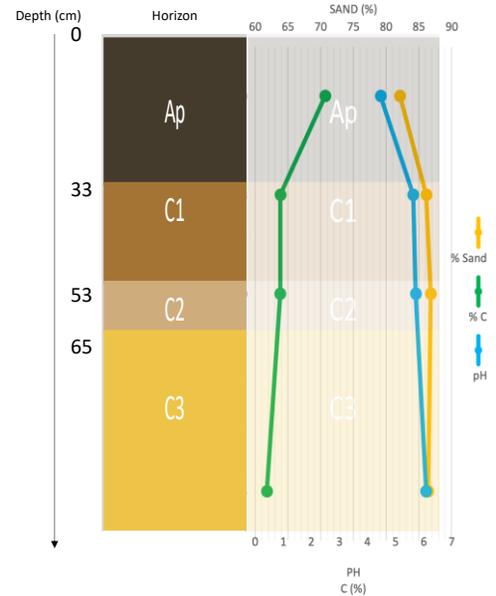
g)
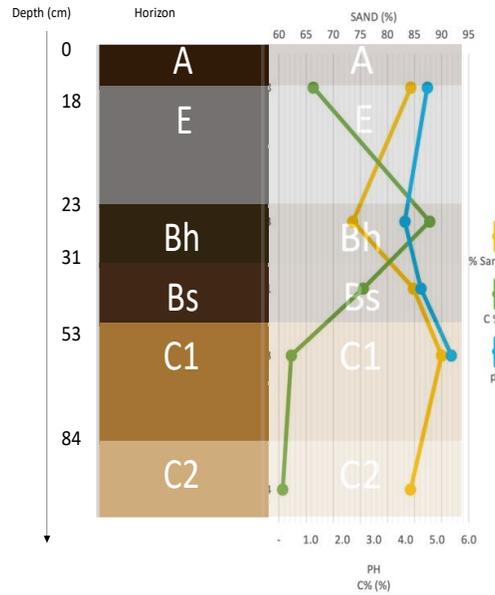
h)
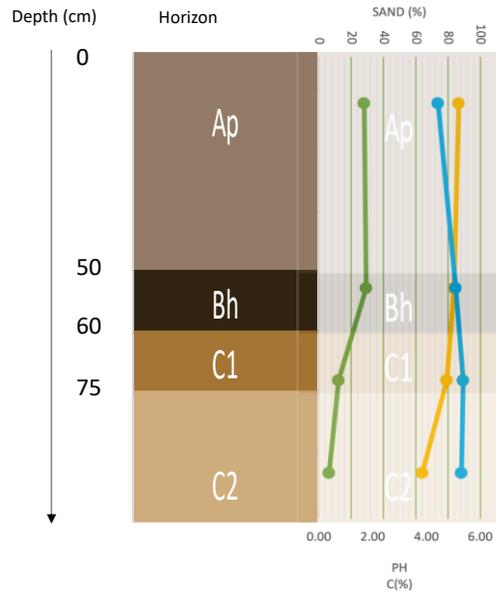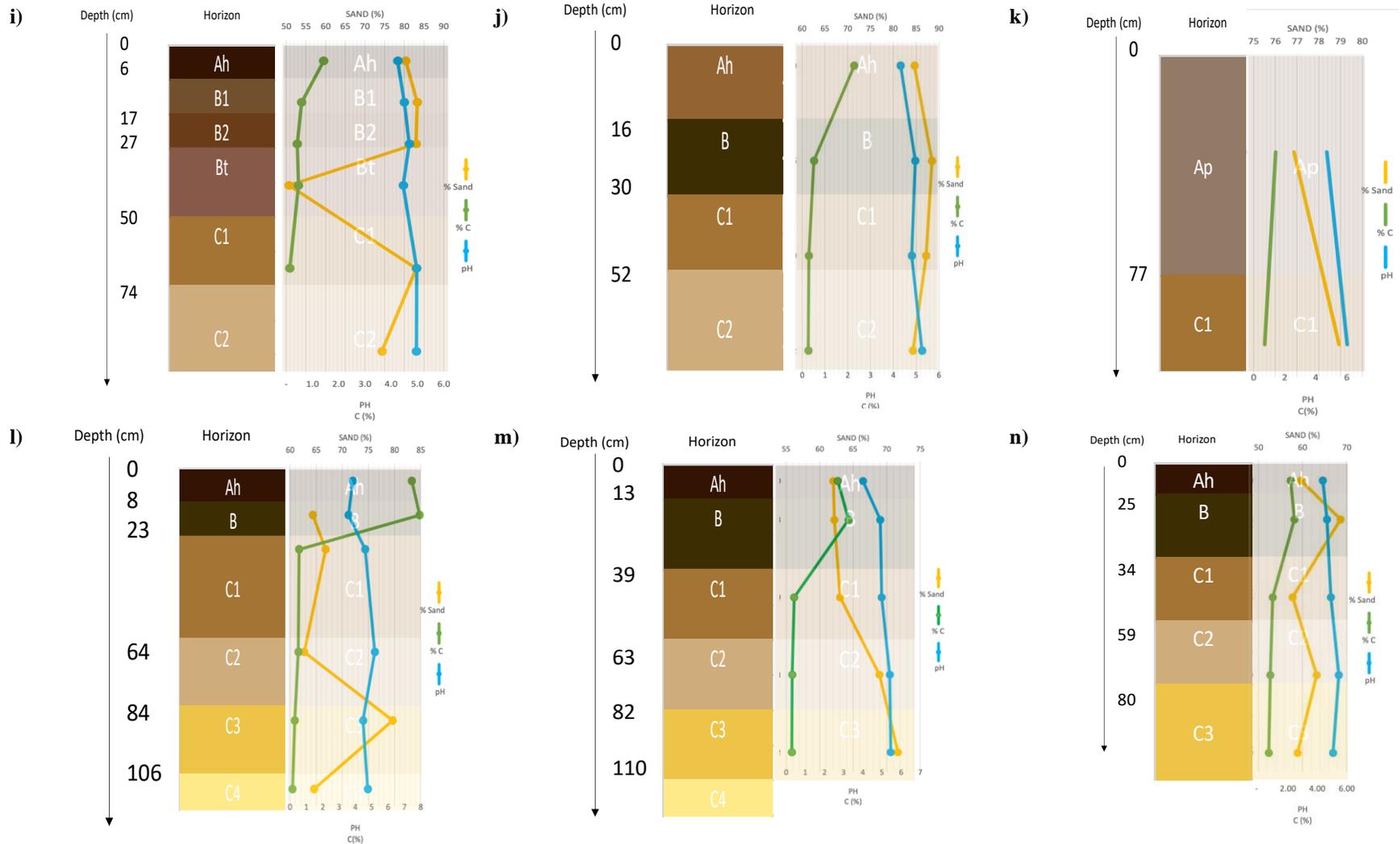
Figure 28: Soil depth profiles with on the left the depth (cm) and in the middle the horizons identified by color and type and. On the right a graph is given with on the upper vertical axis sand (%) (yellow) and on the lower vertical axis C content (%) (green) and the pH (blue). These profiles are given for poor (A) and rich (R) sites in Groote Heide (GH), Hechtel-Eksel (H) and Maashorst (M), for poor and reference (Ref) in Someren (S) and all treatments in Kasterlee (K) and Veldhoven (V): GHA (a), GHR (b), HA (c), HR (d), MA (e), MR (f), SA (g), SRef (h), KA (i), KR (j), Kref (k), VA (l), VR (m) and VRef (n).

D. Discussion

In this research, we tried to assess the impact of rich litter on the soil health by comparing the predicted values of the (G)LMM between the poor, rich and reference plots. Variables included abiotic soil characteristics but also soil biota community indices. Differences in the share of poor and rich litter, tree community and humus description were tested as well. These models took the dependency of the data within the same sites into account. Next, the relationships between these variables with the C stock were also modelled with G(LMM) and visualized.

1. Site evaluation

The three different forest types, i.e., those dominated by poor litter quality, rich litter quality and reference forests are discussed in terms of their biotic and abiotic conditions and how they differ relative to each other. In light of this, the suitability of the sites and their agreement with the theoretical conditions is argued.

1.1 Abiotic variables

The soil in our study area is described in terms of pH, texture, CEC and BS. These values are unexpectedly low in the rich and reference treatment. The organic layer in the plots, dominated by poor-litter species has a significantly lower pH than in the rich litter plots. This can be ascribed to the slower decomposition of poor litter, which results in larger acidification of the soil (den Ouden et al., 2010). Based on the effect of rich litter, which should elevate soil pH (Hommel et al., 2002), we expect significant differences between poor and reference treatment in the organic layer and topsoil layers, however, this is not the case. This phenomenon is also not present in deeper layers in the soil. While not significant, the soil pH ranges are overall higher in the rich sites than in the poor ones, but this is not the case for reference treatment, probably due to limited replications of the latter. The sand content in the sites ranges from 60 to 85%, with very low clay fractions. This results in texture classifications of sand, sandy loam or loamy sand, which might influence other soil characteristics. Next, the CEC, determined by particle size, organic matter content and pH, is an indicator of soil fertility and health (Saha et al., 2022). The typical range for sandy texture classes is between $1 - 10$ cmol$_c$/kg, which is also what we measured in the lab. These low values represent higher risk of nutrient leaching and lower buffer capacity (Saha et al., 2022). The BS, another soil health indicator, gives the percentage of CEC, occupied by the base cations (Ca^{2+}, Mg^{2+}, K^+). These values are, however, low in the studied plots, i.e. 4 - 12% in the topsoil and 2 - 10% for the subsoil, corresponding with the low soil pH. This points to a higher Al-toxicity (Saha

et al., 2022). Note, that there are no significant differences between different forest types and soil depths, which contradicts the hypothesis about the positive effects of rich litter on the soil in terms of CEC, BS and buffer capacity (Hommel et al., 2007). This might be explained by inaccuracies in the measurement of CEC. The cobalthexamine method relies on an exchange of at least 15% for accurate results, which was often lower in the subsoil samples, ranging between 4-9% (Table 4A). These values might thus be underestimated.

1.2 Biotic variables

The forest types are also compared in terms of biotic conditions relating to the tree and soil biota composition. While there are no significant differences between treatments in tree species richness and evenness, these indices are slightly higher in the reference treatment. This trend is also expected in the rich plots, compared to the monoculture pine plots (poor) but does not occur. There is large variation in tree species richness and evenness in the rich plots. There are, however, significant differences with poor sites in terms of tree litter quality. In the poor treatment, there are significantly more and/or larger trees with poor litter and significantly less or smaller trees with rich litter, compared to the rich and reference treatment. This corresponds with our desired setup of the study area. An indirect effect of litter quality is the thickness of the humus layer on the forest floor. In the poor sites, significantly thicker humus layers are found compared to rich and reference sites. An explanation might be found in the abundance of earthworms, here represented by the mass. Rich litter quality, with higher nutrient concentrations, especially in calcium, and a positive effect on pH, creates more suitable habitats for earthworms, enabling incorporation of organic matter in deeper soil layers (Hobbie et al., 2006). While this effect on pH is not established in the reference sites, there are significantly more earthworms present there, compared to the poor sites. Although earthworms are often considered sensitive to pH, some species might be acid-tolerant or ubiquitous (Edwards & Arancon, 2022; Piearce, 1972). Other research suggests acidity as an associated factor of earthworm's constraints but not the primary mechanism (Piearce, 1972; Ruiz et al., 2021). Ammer et al. (2006), found that introduction of rich-litter species only improves earthworm abundance in the presence of nutrient-rich mineral layers, in the case of converted Scots pine monocultures. This might explain the larger earthworm mass in the reference sites, which are characterized by an agricultural history (Currie et al., 2002). Another indicator of biological activity of soil organisms is respiration, which in this study is up to ten times larger in the organic

layer compared to the subsoil. According to Luo and Zhou (2006), there is a direct link between soil respiration and substrate supply from aboveground production. These values are also significantly higher in the reference plots for the organic layer compared to the poor plots. This could be described to a dominance of rich-litter tree species with fast-degrading litter and/or a larger microbial biomass (Luo & Zhou, 2006). It is important to note, however, that the measuring method has a large margin of error in the organic layer due to the small amount of sample in weight. Lastly the soil mesofauna is described by two indices. While no significant differences are found for either variable, there is slightly higher richness in the poor sites. This is explained by the larger volume of organic layer sampled in these sites, compared to often low volumes in the rich and reference sites.

1.3 Site evaluation

The poor sites are characterised by a low pH, CEC and BS, which is in line with our hypothesis. While Scots pine dominates, there are three other prominent species in the poor plots which explains a higher tree species richness and evenness than expected in a monoculture. Pedunculate oak, also a poor-litter species, and silver birch, a rich-litter species, are present in most plots. They can both grow well on dry sandy soils and are a common forest community on these soils in Flanders and the Netherlands (de Keersmaeker et al., 2010). Lastly, black cherry is a species introduced in the first half of the 20th century aiming at soil and litter improvement of pine monocultures on sandy soils but has since become an invasive species (Vanhellemont et al., n.d.). The soil biota activity, measured by the earthworm mass, respiration and mesofauna diversity, is also relatively low, which can be expected based on the poor soil health. The rich and reference sites do not differ significantly in general soil health from the poor sites. Although these sites are dominated by rich-litter species, the desired effect on soil characteristics is not (yet) achieved. The 'point-of-no-return' in terms of degradation, as mentioned by Hommel and de Waal (2003), could also be reached for some plots. Also the tree species richness and evenness found in the rich and reference plots are not significantly higher, while in general a higher biodiversity is expected. While these indices are slightly higher in rich and reference sites, the low number of sampling plots might explain a lack of significance. There is, however, no consistency in these treatments regarding presence of tree species so different interactions might be at play. Only the invasive species black cherry takes up a large share in most of the plots. In the rich site, there is also still a relatively large share of poor-litter species present, possibly affecting the restorative impact of the

rich-litter species. Texture and more specifically the fine fraction might also influence the restorative impact of rich litter (Desie et al., 2020b). Or possibly not enough time has passed to see the effects on soil health. The poor soil health, low biodiversity and interference of poor litter might explain the overall low soil biota activity.

2. Carbon stock analysis

The variation in site characteristics mentioned above translates into differences in SOC stocks, here analysed over three soil layers, namely the organic, top (0-10 cm) and subsoil (10-20 cm). In the topsoil, slightly higher values are found for the reference sites, but the C stock is lowest for the rich treatment in all soil layers. Only in the organic layer, there is a significantly larger C stock in the poor sites, which can be explained by the dominance of slow-degrading litter. The phenomenon of organic C accumulation on the forest floor does not occur in the rich or reference plots. This large C stock is also not further distributed to the mineral soil in the poor treatment, possibly due to soil biota activity dominated by fungi (Berg & McClaugherty, 2020; Mayer et al., 2020; Prescott, 2010). Further, we try to ascertain the driving factors of these C stocks and how these might impact deeper soil C through their depth distribution.

2.1 Effect of soil characteristics

Certain soil characteristics can either directly impact C storage or indirectly through their influence on both above- and belowground biotic conditions. Two important indicators of soil health, namely pH and CEC, are discussed here. The pH shows a negative relationship with the total C stock, for all sampled soil layers. The significant effect of pH in the organic layer can be explained by the highly unsuitable conditions for soil biota due to its high acidity, limiting soil microbial activity (Sitaula et al., 1995). Organic matter decomposition and incorporation into the mineral soil is slowed down, creating opportunity for carbon to accumulate on the forest floor (Schrijver et al., 2012). Janssens et al. (1999) suggest another possible mechanism for this negative relationship in the mineral soil. They found that soil pH is negatively correlated with aggregation, which in turn stimulates spatial protection of SOC (Elliott, 1986; Oades, 1984). Thus, the more acidic the soil, the larger the macroaggregate fractions and the more C is stored in the soil. However, also positive effects on the soil C stock by increased pH by liming have been reported, through increase in aboveground productivity (Paradelo et al., 2015).

The CEC shows both a negative and a positive significant relationship with C stock, for the top- and subsoil respectively. A positive relationship is also established by Solly et al. (2020) in the topsoil (0-30 cm), although weaker for acidic soils (pH < 5.5). They found a substantial contribution from the negative charges of SOM to the CEC. It is important to note however, that in this research the measurements for CEC are clustered around 0-9 $cmol_c/kg$, thus give no clear linear relationship.

2.2 Effect of tree species and litter quality

Tree species composition can directly affect decomposition and thus C storage through their litter quality, or indirectly by influencing the conditions for the microbial and faunal soil community. Literature often reports tree species diversity not to be a main mechanism behind soil C storage (Desie et al., 2023; Mayer et al., 2020), which is also the case in our research. Neither tree species richness nor evenness report a significant relationship, except for a negative but small effect of richness on C stock in the subsoil. More important are tree species characteristics, referring to certain traits such as N-fixing species or conifer versus broadleaved, and have been repeatedly discovered as a main driver (Dawud et al., 2016; Mayer et al., 2020). Here, the litter type is the main investigated characteristic. The significant positive effect of poor-litter species on C stock in the organic layer, might be connected to the effect seen for pH. Nutrient-poor litter and the associated fungi-dominated decomposition enhance the acidification during mineralization, decreasing the soil pH. Poor litter also has a higher AUR content, making it more decay-resistant (Prescott, 2010). The share of rich-litter species does not affect the carbon stock in the organic soil, since the litter is quickly decomposed, thus not accumulating on the forest floor. The effect of rich-litter species in the top- and subsoil, although insignificant, is negative, as opposed to literature. Most studies report a linkage between soil biota activity and high litter quality (dos Santos Nascimento et al., 2021; Prescott, 2010). The associated carbon fixation pathways and incorporation of C in the mineral soil through bioturbation should thus also be more prominent in forests dominated by rich-litter species (Prescott, 2010). In contradiction to our hypothesis, we do not see this trend in our data.

Litter quality affects decomposition rates, and indirectly thus also the thickness of the humus layer. A pronounced positive relationship with C stock is found for the organic layer. This trend can be ascribed to the calculations of the C stock in this layer based on the dry mass (Eq 2.1). It is sampled on a consistent area but differed in volume and thus dry mass, depending on the thickness of the

layer. The calculations for the top- and subsoil, on the other hand, are based on the bulk density and thus independent of volume. A significant positive relationship with C stock for the subsoil might mean that the more accumulation happens on the forest floor, the more is eventually incorporated into the subsoil. The opposite relationship might also be true where a larger C stock in the mineral soil results in higher productivity and thus more litter input.

2.3 Effect of soil biota

The soil biota community is composed of both microbial and faunal species, operating together in a cascading pathway of transforming, decomposing and fixating organic matter and carbon. While the microbial community plays a crucial role in the actual biochemical decomposition, the mesofauna is responsible for boosting these reactions through breakdown, preparation and incorporation of the litter (Jenny, 1980; Coleman et al., 2018). But also their contribution to physical stabilization through soil aggregate formation is important to consider. dos Santos Nascimento et al., (2021) found that stimulation of the release of binding agents by meso- and microfauna, during the decomposition process, form stable soil aggregates, which in turn store C. In our research, a higher mesofauna richness is significantly correlated to a larger C stock in the organic layer. This could be explained by the sampling method and calculation of the C stock, as mentioned in section D.2.2. The thicker the organic layer, the larger the C stock, but also to the larger the habitat and its heterogeneity, increasing the likelihood of different mesofaunal orders. Coûteaux et al. (1991) also found mesofauna to have a larger contribution to the decomposition of poor litter, responsible for the largest C stocks in the organic layer in our study. A larger amount of preferred substrate could thus attract more mesofauna. Furthermore, a significant negative relationship with C stock is found in the topsoil for both mesofauna richness and Shannon-Wiener Index. The incorporation of SOM into deeper soil layers results in a net reduction of soil C stocks due to increased faunal activity and the related facilitation of decomposition (Currie et al., 2002). This phenomenon, however, is not found in the subsoil. Next, we zoomed in on the effect of abundance of one macrofauna group, namely the earthworms, also a crucial agent in the process of bioturbation. There is still some debate on the net effect of earthworms on C storage. The findings by Currie et al. (2002) could thus also explain the significant negative correlation between earthworm biomass and C stock in the organic soil. Earthworms have also been found to stimulate microbial decomposition through addition of fresh organic matter as a product of egestion, called the "primer effect" (Bernard et al., 2012). Earlier research by Binet et al. (1998) found no

correlation between earthworm abundance and soil microbial activity. Some even reported a positive effect on C storage through physical protection from water-stable macro-aggregates, structures formed by earthworms (Blanchart et al., 2009; Coleman et al., 2018). Lastly, microbial activity, approximated by soil respiration has a small but significant positive relationship with C stock in the topsoil, but could be explained by both directions of effect. On the one hand, a larger carbon stock and thus substrate availability can result in more respiration (Luo & Zhou, 2006). On the other hand, a larger microbial community might have a larger contribution to SOM through either ex-vivo modification or in-vivo turnover as proposed by Liang et al. (2017) or even through enhanced physical protection from aggregation (Oades, 1984).

2.4 Carbon depth distribution

Differences in C stock are not only seen between the organic and top- or subsoil, but also deeper in the soil, as is shown in the depth profiles of the soil with each horizon characterized by organic C content (%), sand (%) and pH. We see an overall decrease of C (%) with depth. High values can be found in the topssoil, mainly in the Ah or Ap horizons. Ap or plaggic horizons are often enriched in C due to its agricultural history. Ah is connected with the forest floor where, especially in the poor plots, organic matter accumulates. This C is translocated to the mineral soil either as Dissolved Organic Carbon (DOC), which are soluble products of decomposition, or through bioturbation and other disturbances (Currie et al., 2002). The latter is more pronounced in rich-litter forests (Mayer et al., 2020; Prescott, 2010), while this vertical nutrient cycle is impeded in acidic poor-litter forests due to dominance of fungi in the soil biota community (de Keersmaeker et al., 2010; Sauren et al., n.d.). These phenomena might explain the slightly higher values of C in deeper mineral soil layers in rich and reference sites. For the latter, another explanation might be found in the plot's agricultural land use history, with a long period of fertile soil input, seen by the plagic horizons. It is thus important to note that the differences in C between plots might not be as a result of the environmental conditions mentioned above. In the B horizons, especially Bh, there is often a small increase in C due to illuviation of carbonates (Hartemink et al., 2020). The C horizons, representing different parent materials have relatively lower C, but often higher in reference and rich sites as mentioned above. We also see an overall increase in pH with depth due to less influence from acidification by forest litter on deeper layers.

3. Recommendations for further research

In this setup, the rich and reference treatment did not meet its theoretical requirements in terms of soil health. While thus the positive effects of the rich-litter species on soil pH and CEC could be questioned, there might be other interactions involved. The poor soils are still too hostile to allow complex and diverse soil biota communities. This makes it difficult to ascertain its effect on C stocks. Often the rich forest plots originated through natural regeneration or experimental introduction, with still a large share of poor-litter species. This resulted in a large diversity of rich-litter tree species, which makes it difficult to account for specific biotic interactions. Further research should sample plots more actively managed in terms of restoration measures to ensure an optimal effect of rich litter. Passive management, as was seen for this study area, is not enough to restore these forests (Hommel & de Waal, 2003; van den Burg et al., 2014). While these poor-litter species are not desirable in terms of soil conditions, conifer species such as Scots pine do have ecological value in terms of habitats for mosses, fungi etc. (de Keersmaeker et al., 2010). Therefore, they are preferably taken up into management targets to create a biodiverse and healthy ecosystem, able to withstand environmental and climatic disturbances. Also the number of samples in this research is relatively low and might explain the lack of a significant result. Especially the reference treatment with only 3 plots could show biased results.

Furthermore, there is the importance of stability in C storage. This refers to the physical or chemical protection of C from decomposition, substantially increasing their residence time in the soil. While our research points to a larger C stock in forests dominated by poor-litter species, these are mostly located on the forest floor. These C stocks, while more decay-resistant in case of poor litter, have shorter residence times than those in the mineral due to exposure to disturbances and changes in climatic conditions (Currie et al., 2002; Prescott, 2010). Climate change, with increases in temperature and variability in moisture, is expected to have a large positive impact on the decomposition rates, threatening the C reservoirs (Gholz et al., 2000; Zhang et al., 2008). The SOM on the forest floor is not only a C stock on itself, but also a main C source for the mineral soil layers, beside root input and microbial biomass (Liang et al., 2019; Mayer et al., 2020). Monocultures of Scots pine, characterised by low vitality, biodiversity and production potential, are highly susceptible to disturbances and climate change (Sauren et al., n.d.) and might thus not guarantee these C stocks in the future. Not taking the stability of C and the driving mechanisms behind it into account, might give a distorted view on the current status of C storage and even more

on future dynamics. Furthermore, a time lag on C storage should be considered, since its response to changes in environmental conditions is delayed. But for our study site, information on stand age is often unavailable. The focus on the impact of rich litter could also be redirected toward deeper soil layers, since broadleaved species have more impact on deeper C stocks as reported by (Mayer et al., 2020).

E. Conclusion

In this thesis, we tried to ascertain the existing C stocks in Scots pine forests on sandy soils and the effect of rich litter introduction. These Scots pine plots were characterised by poor soil conditions, with low pH and CEC values, which can be attributed to a long history of anthropogenic degradation and acidification. Based on other research, the soil health was expected to improve with a rich-litter tree community, as result of high-quality litter input. These effects were not found in the rich litter plots, contradicting our hypothesis. Measurement errors, interaction with texture and other environmental conditions or the 'point-of-no-return' status for some soils could be explanations. The rich and reference sites do also not perform better compared to the poor sites in terms of tree species diversity and the share of poor-litter species is often still high in rich plots. The introduction of rich-litter species also did not translate into a larger soil biota community with higher complexity, possibly due to these poor soil conditions.

In terms of the total soil C stocks, the poor Scots pine forests performed better. Even though these were for the most part stored in the organic layer on the forest floor and not further incorporated into the soil, they were still overall higher compared to the rich or reference plots. These thick humus layers are a result of the large poor, slow-degrading litter input, causing acidification and thus a hostile environment for soil biota. The absence of soil biota further slows down decomposition as well as bioturbation, thus allowing accumulation. There was also some evidence that these forest floor C stocks also result in belowground storage, with a larger C stock in the subsoil in case of a thicker humus layer. Tree species diversity did not seem to affect the C dynamics. As for the characteristics of the soil biota community, effects on C stock differed between variables as well as soil layers, making it difficult to support or reject our hypothesis. These findings confirmed that while the soil biota composition does influence C cycles, the interaction between all its members, from microbiota to mesofauna, as well as with environmental conditions, is challenging to disassemble into their different roles. These results incorporated measurements only up to 20 cm. In the soil depth profiles, we found in the C horizons often small differences in C, with higher values for rich and reference sites. This could possibly be attributed to higher soil biota activity deeper in the soil and/or larger input by roots in rich-litter forests. So broadening the study setup to deeper soil layers might be an interesting research focus to provide an even better understanding on the C stocks in these soils and the total effect of rich litter on soil

C storage. A time lag of the impact of rich litter on soil conditions, the soil biota community and thus ultimately the C stocks, should also be considered.

In conclusion, while the Scots pine forest currently seem to store more C, these C stocks, located in the forest floor, are unstable and vulnerable to climate change and other disturbances, thus not guaranteeing their existence in the future.

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G. Appendix

Table 1A: Study site characteristics per site (Kasterlee, Veldhoven, Hechtel-Eksel, Someren, Maashorst and Grooteh Heide) for each treatment (poor, rich and reference). Characteristics include coordinates and tree species composition. A proxy of their basal area, measured with bitterlich, is given between (). Species which were not accounted for with bitterlich but present in the plot are indicated by * and a distinction is made between litter type, with rich litter species underlined. Next, the forest type is determined based on share of basal area and classified accordingly.

POOR	Kasterlee	Veldhoven	Hechtel-Eksel	Someren	Maashorst	De Groote Heide
Coordinates	N 51°13'59,2" E 4°58'13.6"	N 51°25' 4.0" E 5°22'16.1"	N 51°10'10,8" E 5°23'44.3"	N 51°25'56,0" E 5°39'21.3"	N 51° 43' 24.0'' E 5° 36' 18.0''	N 51°25'04.0" E 5°22'16.1"
Tree species composition	• Pinus sylvestris (16) • Quercus robur L. (10) • <u>Betula pendula</u> (1) • <u>Coryllus avellana L.</u> (1) • <u>Tilia</u> (0,5) • <u>Acer pseudoplatanus*</u> • Quercus rubra* • <u>Sorbus aucuparia*</u>	• Pinus sylvestris (13) • Quercus robur L. (5) • <u>Prunus serotina</u> (4) • <u>Betula pendula</u> (3) • <u>Sorbus aucuparia</u> (1)	• Pinus sylvestris (11) • <u>Betula pendula</u> (3,5) • Ilex aquifolium* • Sorbus aucuparia* • Prunus serotina* • Rubus fruticosus* • Dryopteris dilatata*	• Pinus sylvestris (13) • <u>Prunus serotina</u> (6) • <u>Betula Pendula</u> (1) • <u>Sorbus aucuparia*</u>	• Pinus sylvestris (9) • <u>Prunus serotina</u> (5) • Quercus robur L. (2)	• Pinus sylvestris (23) • <u>Betula pendula</u> (3) • Frangula alnus (2) • Ilex aquifolium (1) • Quercus robur L.* • <u>Sorbus aucuparia*</u> • Pseudotsugam enziesii*
Forest type	Scots pine stand with pedunculate oak	Scots pine stand mixed with pedunculate oak, black cherry and silver birch	Scots pine stand mixed with silver birch	Scots pine stand with black cherry and low admixture of silver birh	Scots pin stand with black cherry mixed with pedunculate oak	Scots pine stand with admixture of silver birch
Humus type	Hemimor	Eumoder/ Dysmoder	Hemimoder/ Eumoder	Eumoder/ Hemimor	Eumor	Eumor

RICH	Kasterlee	Veldhoven	Hechtel-Eksel	Maashorst	De Groote Heide
Coordinates	N 51°13'57.8" E 4°58'7.7"	N 51°25'01.1" E 5°22'14.5"	N 51°10'12.9" E5°23'43.3"	N 51° 43' 24.0'' E 5° 36' 15.3''	N 51°23'17.9" E 5°33'06.3"
Tree species composition	• Pinus sylvestris (17) • Corylus avellana L. (10) • Tilia europeae (5) • Acer platanoides (3) • Amelanchier lamarckii (1) • Prunus serotina (1) • Carpinus betulus (1) • Ilex aquifolium* • Castanea sativa* • Sorbus aucuparia* • Quercus robur L.*	• Prunus serotina (26) • Pinus sylvestris (2,5) • Acer* pseudoplatanus* • Rubus fruticosus * • Ilex aquifolium*	• Tilia europea (7) • Quercus petraea (3) • Quercus robur L. (2) • Dryopteris dilatata* • Castanea sativa*	• Prunus serotina (23) • Pinus sylvestris (7) • Quercus robur L. (1)	• Acer pseudoplatanus L. (15) • Salix caprea (8,5) • Tsuga heterophyla/canadensis (2) • Fagus sylvatica (1) • Pseudotsugamenziesii (1) • Fraxinus excelsior (1) • Corylus avellana L. (0,5) • Larix Crataegus monogyna Jacq.* • Carpinus betulus*
Forest type	Mixture Scots pine and common hazel mixed with linden and with admixture of Norway maple	Black cherry stand	Common linden stand with sessile oak mixed with pedunculate oak	Black cherry mixed with Scots pine	Sycamore stand with pussy willow and admixture of hemlock
Humus type	Dysmoder	Hemimoder / Dysmull	Oligomull	Hemimoder/ Eumoder	Mesomull

REFERENCE	Kasterlee	Veldhoven	Someren
Coordinates	N 51° 10' 10.8" E 5°23'44.3"	N 51° 25' 1.85'' E 5° 22' 22.4''	N 51°25'55.7" E 5°39'13.1"
Tree species composition	• Tilia cordata (16) • Prunus avium (7,5) • Carpinus betulus (6) • Quercus robur L. (3) • Acer platanoides L. (1) • Prunus padus* • Coryllus avellana*	• Prunus serotina (7) • Coryllus avellana (7) • Quercus robur L. (5,5) • Sorbus aucuparia (1)	• Prunus serotina (9,5) • Quercus robur L. (5,5) • Acer pseudoplatanus L. (4,5) • Pinus sylvestris (1,5) • Quercus rubra (1) • Frangula alnus * • Sorbus aucuparia*
Forest type	Stand of small-leaved linden, mixed with wild cherry and common hornbeam and with admixture of pedunculate oak	Mixture of black cherry, common hazel and pedunculate oak with admixture of mountain ash	Stand of black cherry mixed with pedunculate oak and sycamore
Humus type	Oligomull	Oligomull	Eumoder

Table 2A: Measurements of pH, Cation Exchange Capacity (cmol$_c$/kg), Base Saturation (%), C stock (ton/ha) and sand (%) are given for each treatment (poor, rich and/or reference) in six different forests (Hechtel-Eksel, Someren, Groote Heide, Veldhoven, Kasterlee and Maashorst) and at three different soil depths: Organic, top (0-10 cm) and sub (10-20 cm). For CEC, BS and sand are not measured for the organic layer.

Site	Treatment	Soil depth	pH	CEC (cmol$_c$/kg)	BS (%)	C stock (ton/ha)	Sand (%)
Hechtel-Eksel	Poor	Organic	4.08	NA	NA	38.93	NA
	Poor	Top	4.86	20.88	20.88	10.04	70.31
	Poor	Sub	4.85	12.43	12.43	43.64	70.35
	Rich	Organic	6.06	NA	NA	3.16	NA
	Rich	Top	5.24	5.56	25.95	22.15	66.64
	Rich	Sub	5.60	2.36	24.18	7.86	76.79
Someren	Poor	Organic	3.35	NA	NA	83.84	NA
	Poor	Top	3.92	5.68	18.03	27.68	78.66
	Poor	Sub	4.37	4.47	6.69	25.83	79.60
	Reference	Organic	4.20	NA	NA	31.82	NA
	Reference	Top	4.53	5.01	9.36	33.15	72.35
	Reference	Sub	5.02	6.48	4.14	21.84	84.18
Groote Heide	Poor	Organic	3.46	NA	NA	72.09	NA
	Poor	Top	4.27	5.73	6.40	41.29	62.45
	Poor	Sub	4.45	5.97	1.65	20.32	63.78
	Rich	Organic	5.98	NA	NA	0.68	NA
	Rich	Top	5.60	6.48	58.04	23.17	57.23
	Rich	Sub	5.96	8.85	38.77	16.38	61.82

Site	Treatment	Soil depth	pH	CEC (cmol$_c$/kg)	BS (%)	C stock (ton/ha)	Sand (%)
Veldhoven	Poor	Organic	3.62	NA	NA	81.63	NA
	Poor	Top	4.46	5.56	31.75	32.11	62.56
	Poor	Sub	3.90	1.50	25.58	25.21	65.57
	Rich	Organic	5.89	NA	NA	7.92	NA
	Rich	Top	4.40	8.34	50.98	37.47	55.76
	Rich	Sub	4.42	5.68	12.92	16.91	58.75
	Reference	Organic	4.50	NA	NA	8.77	NA
	Reference	Top	4.30	6.27	34.42	37.90	63.43
	Reference	Sub	4.63	3.10	13.84	27.07	63.52
Kasterlee	Poor	Organic	4.22	NA	NA	71.09	NA
	Poor	Top	4.52	3.99	29.01	16.96	75.20
	Poor	Sub	4.53	4.32	7.85	6.19	82.79
	Rich	Organic	3.80	NA	NA	83.23	NA
	Rich	Top	4.66	12.51	9.36	12.23	85.60
	Rich	Sub	4.94	1.73	15.52	4.73	86.27
	Reference	Organic	4.22	NA	NA	5.18	NA
	Reference	Top	4.52	4.29	67.93	25.69	76.81
	Reference	Sub	4.53	3.57	18.71	16.49	77.49
Maashorst	Poor	Organic	3.72	NA	NA	49.65	NA
	Poor	Top	4.44	4.20	8.43	33.38	82.74
	Poor	Sub	3.90	5.94	2.40	49.69	84.34
	Rich	Organic	NA	NA	NA	NA	NA
	Rich	Top	4.88	3.56	6.24	25.16	69.70
	Rich	Sub	4.96	4.61	1.94	27.67	83.75

Table 3A: The thickness of the humus layer (cm), earthworm mass (g) and respiration (µg C / (day * g soil)) measurements are given for each treatment (poor, rich and/or reference) in six different forests (Hechtel-Eksel, Someren, Groote Heide, Veldhoven, Kasterlee and Maashorst). Humus thickness has three measurements per treatment in each site, while respiration is measured at three different soil depths: Organic, top (0-10 cm) and sub (10-20 cm). The mass of the earthworms comprises the masses of all earthworms found in each plot.

Site	Treatment	Humus thickness (cm)	Soil depth	$m_{earthworms}$ (g)	Respiration (µg C / (day * g soil))
Hechtel-Eksel	Poor	6	Organic		187.36
	Poor	6.5	Top	12.912	59.57
	Poor	7.5	Sub		24.18
	Rich	1	Organic		278.01
	Rich	1	Top	9.852	54.39
	Rich	1	Sub		40.58
Someren	Poor	5	Organic		398.43
	Poor	11	Top	1.465	119.38
	Poor	11	Sub		71.26
	Reference	1.5	Organic		930.46
	Reference	3	Top	15.041	118.39
	Reference	5.5	Sub		58.89
Groote Heide	Poor	8	Organic		139.98
	Poor	6	Top	0	84.47
	Poor	7.5	Sub		65.56
	Rich	4	Organic		139.98
	Rich	2	Top	2.313	84.47
	Rich	1	Sub		65.56

Site	Treatment	Humus thickness (cm)	Soil depth	$m_{earthworms}$ (g)	Respriation (μg C / (day * g soil))
Veldhoven	Poor	3.5	Organic		142.41
	Poor	3.5	Top	1.247	145.31
	Poor	3.5	Sub		45.48
	Rich	1	Organic		187.76
	Rich	1.5	Top	14.987	194.97
	Rich	1.5	Sub		41.91
	Reference	3.5	Organic		515.29
	Reference	1	Top	1.961	125.95
	Reference	1	Sub		56.86
Kasterlee	Poor	5.5	Organic		131.24
	Poor	6.5	Top	1.507	41.44
	Poor	4.5	Sub		40.58
	Rich	7.5	Organic		93.25
	Rich	6	Top	4.714	56.12
	Rich	7	Sub		19.86
	Reference	0.5	Organic		606.11
	Reference	0.5	Top	18.967	99.29
	Reference	0.5	Sub		34.54
Maashorst	Poor	13.5	Organic		93.79
	Poor	9	Top	0	52.54
	Poor	11	Sub		42.00
	Rich	4	Organic		218.67
	Rich	4	Top	0	115.23
	Rich	2.5	Sub		54.05

Table 4A: The cobalt exchanged during the cobalthexamine method to measure the CEC for each plot in the top (0-10 cm) and subsoil (10-20 cm).

Site	Treatment	Soil depth	Co-exchange (%)
Hechtel-Eksel	Poor	Top	21.4
	Poor	Sub	14.2
	Rich	Top	17.8
	Rich	Sub	7.6
Someren	Poor	Top	15.7
	Poor	Sub	20.6
	Reference	Top	17.9
	Reference	Sub	14
Maashorst	Poor	Top	13.6
	Poor	Sub	18.6
	Rich	Top	11.3
	Rich	Sub	14.2
Groote Heide	Poor	Top	17.9
	Poor	Sub	19.4
	Rich	Top	20.5
	Rich	Sub	28.6
Veldhoven	Poor	Top	17.4
	Poor	Sub	4.8
	Rich	Top	26.9
	Rich	Sub	18.2
	Reference	Top	20
	Reference	Sub	9.9
Kasterlee	Poor	Top	12.7
	Poor	Sub	13.7
	Rich	Top	13.5
	Rich	Sub	11.2
	Reference	Top	39.5
	Reference	Sub	5.6

Table 5A: Results of carbon stock analysis summarized for each variable tested with either a positive significant (++) or non-significant effect (+), a negative significant (- -) or non-significant effect (-) or a negligible effect (x).

Predictor variables		Carbon Stock		
		Organic	Top	Sub
pH		- -	-	-
CEC		/	- -	++
BS		/	x	-
Tree species	Richness	+	-	- -
	Evenness	-	x	+
Humus layer thickness		++	-	++
Mesofauna	Richness	++	-	x
	Shannon-Wiener	+	-	+
Earthworm biomass		- -	x	x
Respiration		x	++	x

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