Master's Thesis

The Effects of Northern Forage Crop Species on the Soil Carbon Sequestration and Microbial Community Structure

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8th November 2023

UNIVERSITY OF JYVÄSKYLÄ, Faculty of Mathematics and Science
Department of Biological and Environmental Science
Master's Degree Programme in Environmental Science

Anni Palvi	The Effects of Northern Forage Crop Species on the Soil Carbon Sequestration and Microbial Community Structure
MSci Thesis	62 p., 5 appendices (10 p.)
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November 2023	

Keywords: DNA sequencing, F:B-ratio, Finland, gas chromatography, legume, loss-of-ignition, milk production, perennial, PLFA analysis, polyculture, red clover, root biomass, diversity, tall fescue, timothy, yield

Grasslands play an essential role in Finnish agriculture, and carbon sequestration into the arable soils is suggested to be key to carbon-neutral milk production. Perennial grasslands can enhance soil carbon storage by influencing their surrounding microbiota through their root exudates. However, the soil microbial dynamics can be complex, and more research is needed to understand these processes in the cultivated grasslands in the distinctive northern conditions. In this study, I investigated the effects of different forage crop species (tall fescue, timothy, red clover) and their mixture (timothy + red clover) on yield and root biomass, soil carbon percent (C%), fungal:bacterial (F:B) -ratio, soil microbial diversity, and soil microbial community structure. The study was conducted as a part of the 'Sustainability from the Grasslands' research project by the Natural Resources Institute (Luke), funded by European Agricultural Fund for Rural Development. The cultivation was set up at the Luke Maaninka research station, where the sampling was carried out. The soil microbial community structure was identified using Ion Torrent PGM DNA sequencing, microbial biomass was estimated using PLFA-biomarkers, and C% was measured by loss-of-ignition. Additionally, Luke provided crop yield and root biomass data and comparative soil carbon measurement. Red clover stood out as a prominent contributor to carbon sequestration, particularly due to its substantial root biomass and extensive tap root system. Additionally, red clover showed soil stabilizing potential by increased abundance of Ascomycetes in the fungal community. While tall fescue topped the yield charts, its root biomass and soil C% did not meet the expectations. The study highlighted the potential of the forage crop species, like red clover, in enhancing soil carbon storage in northern grasslands. Further research is still needed to delve deeper into the mechanisms through which these crops influence soil microbial communities and, in turn, carbon sequestration.

JYVÄSKYLÄN YLIOPISTO, Matemaattis-luonnontieteellinen tiedekunta
Bio- ja ympäristötieteiden laitos
Ympäristötieteen maisteriohjelma

Anni Palvi	Pohjoisten nurmiviljelylajien vaikutukset maaperän hiilensidontaan ja mikrobiyhteisön rakenteeseen
Pro gradu tutkielma:	62 s., 5 liitettä (10 s.)
Työn ohjaajat:	Prof. Marja Tiirola, Prof. Perttu Virkajärvi (Luke), Apulaisprof. Sami Taipale, Väitöskirjatutkija Benjami Laine, Väitöskirjatutkija Sanna Kykkänen (Luke)
Tarkastajat:	FT, Dos. Riitta Nissinen, Apulaisprof. Suvi Ruuskanen
Marraskuu 2023	

Hakusanat: DNA-sekvensointi, hehkutusjäännös, juuribiomassa, kaasukromatografia, lajidiversiteetti, maidontuotanto, monivuotinen, PLFA-analyysi, puna-apila, ruokonata, sato, sieni:bakteeri-suhde, Suomi, timotei

Nurmimaiden maaperän hiilensidonta on oleellinen osa matkalla kohti hiilineutraalia maidontuotantoa. Monivuotiset nurmikasvit kasvattavat maaperän hiilivarastoa sitomalla hiilidioksidia ilmakehästä biomassaansa ja juuristonsa kautta osaksi maaperän substraatteja, eliöstöä ja mineraaliyhdisteitä. Juurieritteiden avulla kasvit muokkaavat ja säätelevät ympärillään olevaa mikrobiyhteisöä, mutta tietämys mikrobien ja kasvien välisen yhteistyön vaikutuksista erityisesti pohjoisten nurmimaiden hiilensidontaan on vielä verrattain rajallista. Tutkielmassani kysyn, miten nurmiviljelykasvit (ruokonata, timotei, puna-apila) ja niiden yhdistelmä (timotei ja puna-apila) vaikuttavat satoon ja juuribiomassaan, maaperän hiiliprosenttiin, sieni:bakteeri-suhteeseen sekä mikrobien lajidiversiteettiin ja rakenteeseen. Tutkimus toteutettiin osana Luonnonvarakeskuksen (Luke) Kestävyyttä Nurmesta -tutkimushanketta. Tutkimusasetelma sijaitsi Luken Maaningan tutkimusasemalla, josta keräsin näytteet. Selvitin maaperän mikrobiyhteisön rakenteen määrittämällä bakteerien ja sienien lajiston DNA-sekvensoinnilla Ion Torrent PGM -laitteistolla ja biomassan rasvahappoanalyysillä, sekä maaperän hiiliprosentin hehkutusjäännöksestä. Lisäksi Luke toimitti sato- ja juuribiomassa-aineiston. Tutkittavista lajeista puna-apila vaikutti edistävän maaperän hiilensidontaa sen kohonneen juuribiomassan ja paalujuuren ansiosta. Lisäksi puna-apila lisäsi sieniyhteisön Ascomycetes-sienten määrää, mikä voi vaikuttaa positiivisesti hiilen stabilointiin. Vaikka ruokonatan satotulos oli merkittävä, sen juuribiomassa ja maaperän hiiliprosentti eivät vastanneet odotuksia. Tutkimus loi kuvaa pohjoisten mikrobiyhteisöjen rakenteesta ja korosti nurmikasvien, kuten puna-apilan, potentiaalia nurmipeltojen maaperän hiilensidonnan edistämisessä pohjoisilla maatalousmailla. Lisää tutkimusta asian ympäriltä kuitenkin tarvitaan.

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TERMS AND ABBREVIATIONS

Terms

16S rDNA	Gene sequence used for bacterial identification
Agroecosystem	Human-managed ecological system focused on agricultural production of food, fodder, etc.
Carbon sequestration	Growing the carbon stocks
ITS rDNA	Gene sequence used for fungi identification
Metazoan	Macroscopic organisms, animals
Monoculture	Practice of growing one crop species extensively
Polyculture	Practice of growing multiple crop species together

Abbreviations

C	carbon
CUE	carbon use efficiency
eDNA	extracellular DNA
F:B-ratio	fungal:bacterial -ratio
FA	fatty acid
FAME	fatty acid methyl ester
GC-MS	gas chromatography-mass spectrometry
ISTD	internal standard
LOI	loss-of-ignition
MAOM	mineral-associated organic matter
N	nitrogen
NGS	next-generation sequencing
NTC	no template control
PCR	polymerase chain reaction
PLFA	phospholipid-delivered fatty acid
rDNA	ribosomal DNA
SD	standard deviation
SOC	soil organic carbon

1 INTRODUCTION

Natural grasslands hold a significant carbon (C) reservoir, globally constituting about one-third of terrestrial C stock, and thus, have the potential to accumulate significant amount of C in both above and belowground biomass (Bai & Contrufo 2022). Similarly, cultivated grasslands hold potential to enhance carbon sequestration from the atmosphere into the soil (Kätterer et al. 2013, Klumpp & Fornara 2018). In Finland, cultivated grasslands hold the share of 32% of the agricultural land, with 96% of it being intensively managed by ley rotation (Virkajärvi et al 2015). Therefore, grassland management is evaluated as one of the key players in reducing greenhouse gas emissions in mineral soils (Maanavilja et al. 2021). Still, the globally accepted agricultural management practices for carbon farming are not necessarily applicable in higher latitudes, where the weather and abiotic conditions differ considerably from Southern areas (Hengl et al. 2014). With soil C stocks and rates of climate warming already high in the north, there is also a remarkable risk of losing the considerably high soil C storage due to climate warming (Crowther et al. 2016). Thus, there is a significant knowledge cap concerning the northern cultivated grasslands under intensive fertilization, frequent tillage and short ley-rotation, and this calls for more research to find the right practices for these unique conditions. Furthermore, to broadly adopt these practices in Finland, changes to agricultural subsidies, policies, and regulations are necessary, calling for political backing (Poulton et al. 2018).

Microbes play a fundamental role in C cycling as they are involved in the processes of C fixation, respiration, and decomposition (Kirchman 2012). In soil, the most important processes of microbial C sequestration are decomposition and stabilization of organic matter (Liang et al. 2019, Islam et al. 2022). In the decomposition process, microbes not only break down the organic matter into simpler compounds but also sequester C by assimilating it to their biomass, and ultimately necromass (Liang et al. 2019, Kästner et al. 2021). Fungal necromass can cover more than 70% of the microbial soil organic carbon (SOC), even if the fungal biomass is relatively small compared to the total microbial biomass (Liang et al. 2019). Thereby, soil fungi have recently gained increasing attention and fungal:bacterial (F:B) -ratio has been suggested as a factor affecting the soil microbial C cumulation (Hannua & Morriën 2022). Still, the factors and relationships affecting the microbial C pool are complicated and not everything is yet known about the effects of the microbial community structure on C sequestration. Gaining a better insight into these mechanisms in the future holds particular significance in preserving C within soils.

In this study I ask, how does the cultivation of the northern forage crop species affect 1) yield and root biomass, 2) soil carbon percent (C%), 3) F:B-ratio, 4) soil microbial diversity and 5) soil microbial community structure. The

research set up was carried out as part of 'Sustainability from the Grasslands' research project by the Nature Resources Institutes (Luke). The selected forage crop species are among the most widely cultivated in northern grasslands (Virkajärvi et al. 2015): timothy (Phleum pratense), red clover (Trifolium pratense) and tall fescue (Festuca arundinacea), as well as polyculture of timothy and red clover. Tall fescue is known for its superior yield size (Cougnon et al. 2014) and root system (Kykkänen et al. 2022), so I expect tall fescue to outperform the monocultures of timothy and red clover in plant biomass. On the other hand, polycultures are known to result to a bigger yield size than monocultures (Sturludóttir et al. 2014) and therefore I also expect to see timothy-red clover mixture overyielding the monocultures. Tall fescue is known for its good C sequestration potential (Carter & Gregorich 2009), so I expect to find a greater soil C% with deep-rooted tall fescue than timothy and red clover, but since polycultures are also known to result into greater soil C input than monocultures (Yang et al. 2019), also timothy-red clover mixture is a good candidate for higher C%. Polyculture of multiple crops is known to increase microbial diversity (LeBlanc et al. 2015, Cline et al. 2018, Stefan et al. 2021), and therefore I expect the timothy-red clover mixture to hold higher microbial diversity than the monocultures.

To determine the microbial community structure, I combined next generation sequencing (NGS) techniques and phospholipid-delivered fatty acid (PLFA) analysis, as this combination is suggested as a reliable approach for studying microbial communities (Lewe et al. 2021, Chen et al. 2019). While PLFA analysis is useful for determining the living biomass and F:B -ratio, NGS is well suited for defining the microbial species, and diversity (Osburn et al. 2022). Thereby, combining the methods enables both taxonomic and quantitative characterization of microbial communities. Soil C% is examined by loss-of-ignition (LOI) method and compared to the C content data from Luke. Crop yield and root biomass data are gained as background information from Luke.

2 BACKGROUND

2.1 Carbon farming

Carbon farming involves employing crops and agricultural techniques to store C within the soil. The core idea is to increase crop diversity and create a balanced ecosystem with more fertile soil (Toensmeier 2016). By simplest this means a switch from monoculture to polyculture, where multiple species are cultivated at the same area instead of just one. Crop species richness has been associated with increased nutrient richness (LeBlanc et al. 2015), above- and underground biomass (Cline et al. 2018), root biomass (Oram et al. 2018), and soil C storage (Yang et al. 2019). Thus, by increased biomass, crop species richness could also affect the long-term C reservoirs of the soil (De Deyn et al. 2011). Perennial crops are favored over annual crops as they are known to enhance the soil C content (Heikkinen et al. 2020) mainly due to their extensive root systems and the reduced requirement for tillage (Toensmeier 2016), which disturbs the soil microbiome and, thus, release C back into the atmosphere (Wilman 2011, Amami et al. 2021, Cooper et al. 2021). For farmers, the benefits of carbon farming can also mean more income by bigger yield (Bareille & Dupraz 2020) and less costs by decreasing need for fertilizers and weeding (Isbell et al. 2017) by more fertile and high-quality soil (Tiefenbacher et al. 2021).

It is suggested that by implementing suitable carbon farming practices, it could be possible to store up to 1.1 tons of atmospheric C per hectare per year back into the agricultural soil (Tiefenbacher et al. 2021). However, climate warming is expected to deplete the sequestration potential and the rate may diminish significantly after the 2 °C warming gradient (IPCC 2023). By implementing the suitable agricultural management practices for carbon farming widely, it could be possible to reduce the agricultural emissions to counterbalance the effects of the warming gradients (Poeplay 2020, 2021) and even reabsorb the released C from the atmosphere back into the soil (Toensmeier 2016).

Carbon farming practices can vary substantially with increasing degrees of latitude and changing environmental and ecological factors. In northern regions, the unique cold climate, varying precipitation patterns and short growing season have slowed decomposition rates, and thereby the northern regions already possess significant soil C storage (Hengl et al. 2014). However, a lot has also been lost due to agriculture, especially due to land-use change and deforestation (IPCC 2023) and in Finland, for instance, agricultural greenhouse gas emissions amount to 6.4 million tons of carbon dioxide (CO_2) equivalents, about 13% of the total C emissions (including land use, land-use change, and forestry) in 2021 (Tilastokeskus 2023). A warming climate is projected to accelerate emissions and with the highest rates of climate warming in the north, these regions are at a

greater risk of losing considerable amounts of C compared to the rest of the world (Crowther et al. 2016). As the potential of carbon farming practices is not universal between different agroecosystems, in this study my primary focus is on northern soils.

2.1.1 Carbon cycling in agroecosystems

To grasp the mechanisms of carbon farming, it is crucial to first explore the C cycling within agroecosystems. Like terrestrial ecosystems in a broader context, C enters the soil through plants via primary production and exits mainly through microbial respiration (Figure 1; Bralower & Bice 2016). By photosynthesis, plants take up the atmospheric CO_2 and turn it into organic matter, the main resource of nutrition for all the terrestrial organisms. Most of the C is allocated to the plant growth, eventually becoming detritus in the soil, where microbial degradation is primarily driven by bacteria at a faster pace than fungal, yet fungi exhibit more efficient respiration in lower temperatures and arid conditions compared to bacteria (Kirchman 2012).

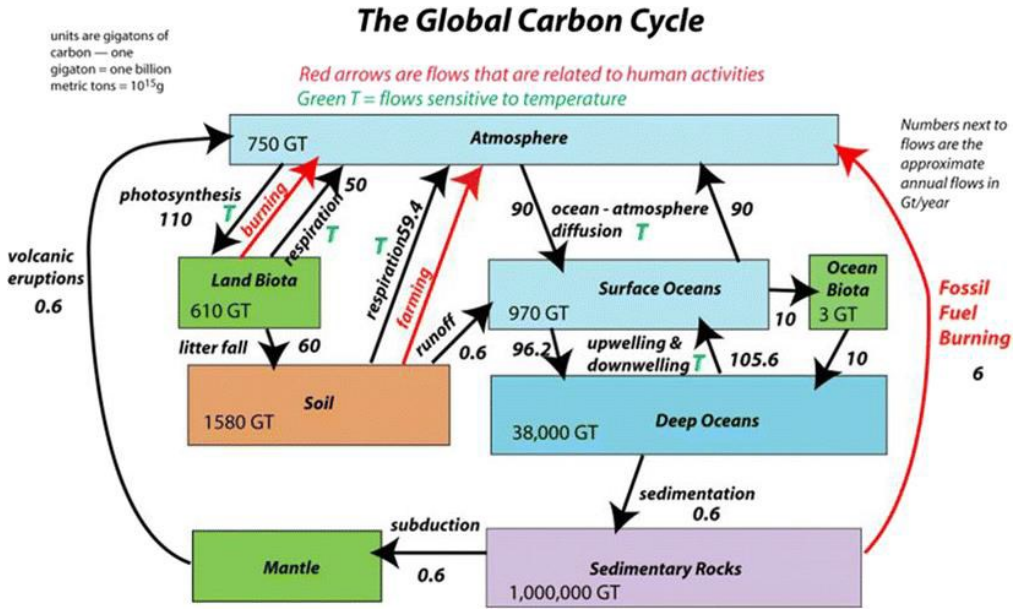

Figure 1. Terrestrial ecosystems gain all the C by photosynthesis from the atmospheric CO_2 and release it back mainly in the form of CO_2 by respiration. Farming and burning (land-use change) are increasing the C in the atmosphere and shaking the natural balance of C cycle. Still, the C reservoir in the soil is huge, about 1580 Gt. (Source: Bralower & Bice 2016)

Three of the most important C reservoirs in the soil are plant biomass, microbial carbon pump, and mineral-associated organic matter (MAOM; Figure 2). Plant biomass includes the roots and detritus, that is not yet decomposed. The microbial carbon pump refers to the microbial biomass production and stabilization into the necromass (Zhu et al. 2020, Kästner et al. 2021), a topic I will delve into in Chapter 2.2.2. Finally, MAOM is the long-term C reservoir in which soil C is adsorbed into the soil mineral particles forming complex soil C derivatives that microbes cannot dissolve (Kirchman 2012, Islam 2022). In this form, C can persist in the soil for hundreds, even thousands, of years (Dwivedi 2019).

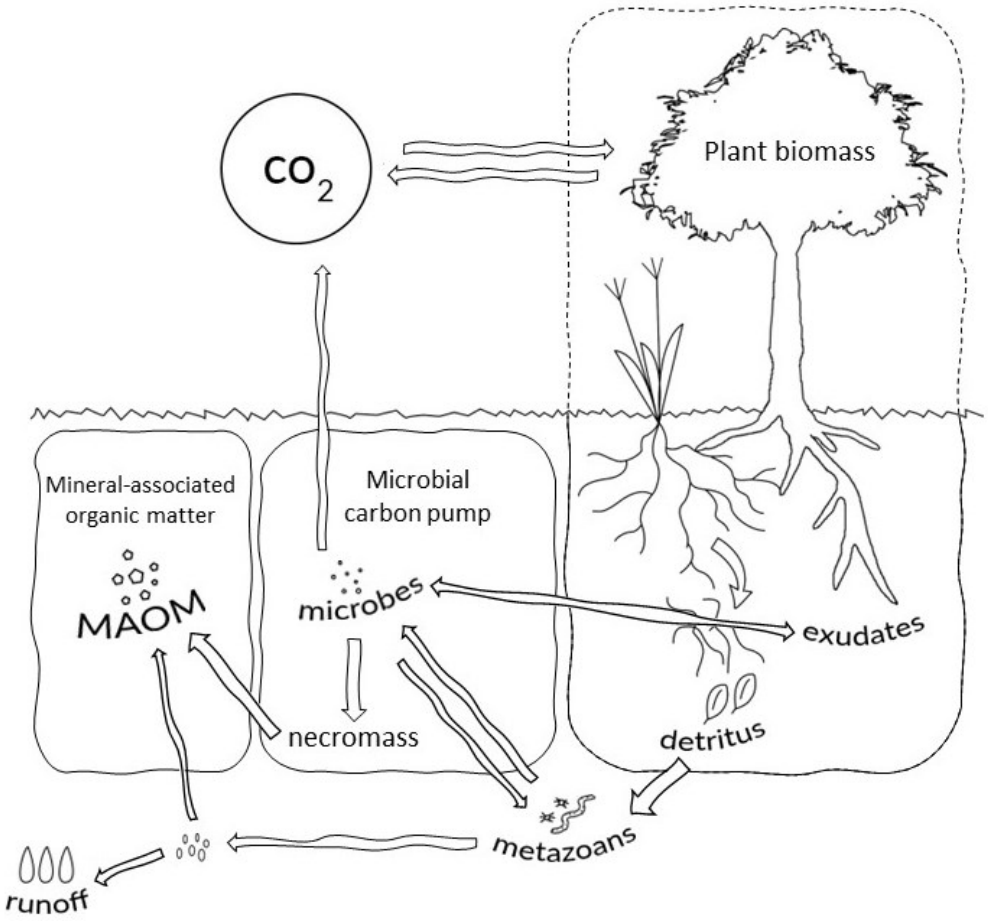

Figure 2. Three of the most important C stocks in soil are plant biomass, microbial carbon pump and MAOM. C enters the atmosphere by primary production and exits by respiration and runoff. The C pools are strongly interconnected by plant- and microbe exudates and chemical reactions between organic and mineral particles.

Agroecosystems are one of the most intensively managed ecosystems on Earth and thereby agricultural management practices are a single important factor separating agroecosystems from natural lands. These practices include mineral fertilization, crop residues, tillage, and plant cultivation, among others (Tiefenbacher et al. 2021). All these practices affect soil C cycling, which has a direct impact on soil C stocks and health (Poulton et al. 2018). Thus, implementing optimal agricultural practices for northern agriculture plays a vital role in C preservation and sequestration in the northern agroecosystems.

The land-use history greatly affects C cycling (Soussana et al. 2004). For instance, the long-term research by Bolinder et al. (2010) explains the soil organic C stock change of a clay soil within the 30-years of carbon farming experiment, where despite the management effort, the C stock was diminishing, probably because of the poor drainage of the field just years before the experiment and, thus, an existing substantial organic C storage in soil that started to deplete during the experiment (Bolinder et al. 2010). This highlights that regardless of the agricultural management practices employed, the land use history can play a more significant role in determining whether the field functions as a C source or sink. When transitioning to new management practices, establishing a new C equilibrium takes time, and the land use history has a significant influence, particularly in the initial years of adopting new practices (Soussana et al. 2004).

While the outcome of agricultural practices is challenging to predict due to the numerous factors influencing carbon cycling, one of the most interesting and significant variables is undoubtedly the uncertainty introduced by climate change. Elevated atmospheric CO_2 levels can stimulate plant and root growth, boosting soil C input, and as temperature rises, this effect could enhance grassland productivity and resilience against climate change (Soussana & Lusher 2007). Conversely, climate warming is also indicated to boost microbial respiration, potentially resulting in a reduction of the soil C pool (Lei et al. 2021). On the other hand, agricultural management practices are estimated to diminish SOC stocks more than climate change itself (Akujärvi et al. 2014).

2.1.2 Carbon farming in grasslands

Northern regions have a unique, yet limited selection of crops adapted to these harsh conditions, and therefore, the diversity in the grass seed mixtures is lower than in the southern regions (Virkajärvi et al. 2015). Timothy (*Phleum pratense*; Figure 3A) is a common forage grass with a shallow root system and good winter resistance and is undoubtedly the most popular forage crop in the northern regions (Höglind et al. 2010). It is often cultivated as a mixture with red clover (*Trifolium pratense*; Figure 3B), as growing grasses and legumes as mixtures has been found to increase the yield size (Sturludóttir et al. 2014), plant productivity and C input in soil (Bai & Contrufo 2022). Red clover is a leguminous species with the root nodules colonized by Rhizobia bacteria which fix N from the atmosphere and, thus, reduce the need for mineral fertilizers (McKenna et al. 2018). Red clover root biomass and microbial enzyme activity has been reported to be higher than of timothy, even though the roots of timothy go deeper and

wider than red clover (Niemi et al. 2005). Tall fescue (*Festuca arundinacea*; Figure 3C), on the other hand, serves as a carbon farming grass, enhancing soil C and N content through its deep root system (Carter & Gregorich 2009), and hence, its significance has been increasing in northern regions in recent years (Virkajärvi et al. 2015).

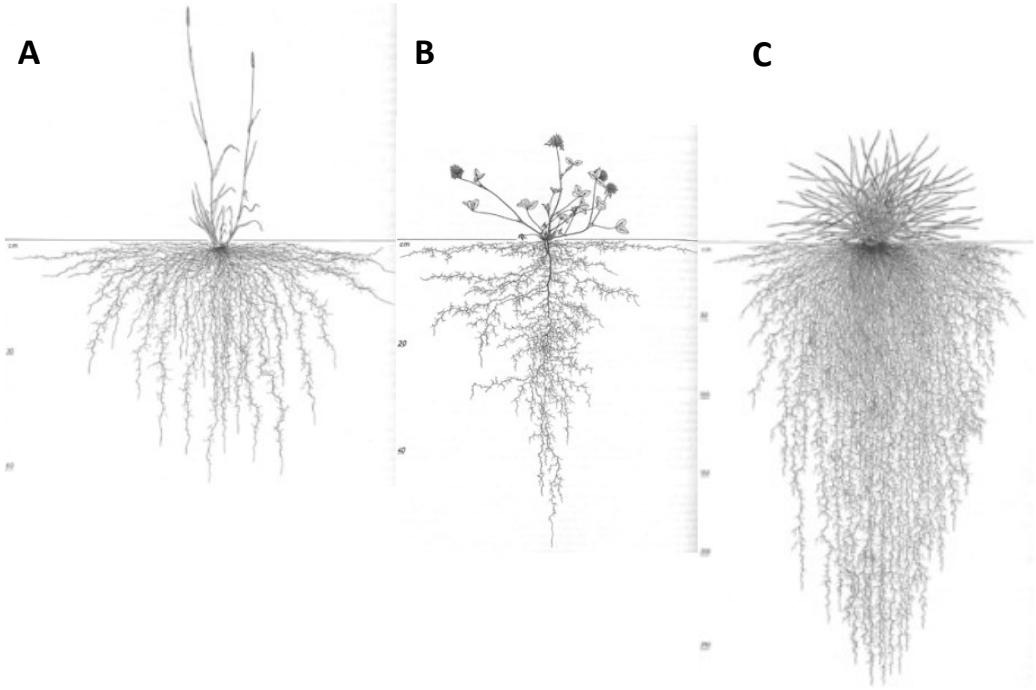

Figure 3. The root systems of A) timothy, B) red clover and C) tall fescue show different patterns and dimensions. While the root depths of both timothy and red clover are limited to approximately 60 cm, the roots of tall fescue extend to depths exceeding 250 cm. Please note that the scales presented in the figures are not consistent with each other. © Myllys et al. (2014)

Besides using the grass-legume seed mixtures, current understanding of the carbon farming practices in northern grasslands include mineral N fertilization, minimizing the time with bare soil (Kätterer et al. 2012), maximizing the years of grass rotation (Soussana et al. 2004) and reducing the tillage (Tiefenbacher et al. 2021). It is widely recognized that employing growth-limiting N as mineral fertilization enhances both above and belowground soil C stock due to increased production rates (Lu et al. 2011), and thus, can help increasing the SOC stocks (Cotrufo et al. 2019). Maximizing the years of grass rotation means less frequent regeneration of the managed grassland, which minimizes the emissions caused by tillage (Bolinder et al. 2010, Kätterer et al. 2013), although this effect is suspected to be limited in the north (Kätterer et al. 2012). Tillage is proposed to disturb the soil C reservoir (Wilman 2011, Cooper et al. 2021), but this impact differs based on temperature and humidity and thus, it is suggested that reducing tillage might not be effective for carbon farming in cooler environments

due to slower decomposition rates (Luo et al. 2010). This highlights the challenges of carbon farming in northern regions.

Grasslands could still provide a solution for carbon farming in northern croplands, as implementing lay-arable rotation in arable cultivation is suggested to compensate as much as 8% of the agricultural GHG emissions yearly (Poeplau & Don 2015). Besides the substantial soil C stock, the challenge in carbon farming in the north is that many of the practices, like N fertilization, have already been widely implemented (Virkajärvi et al. 2015, Poulton et al. 2018, Tiefenbacher et al. 2021) and, consequently, it is suggested that one method to grow C stocks would be removing the land from agriculture to forestry, which would have many unbeneficial effects like reduced food security (Soussana et al. 2004, Poulton et al. 2018). Utilization of grasses and legumes as cover crops could notably enhance C input and soil quality in between the cultivation of annual crops (Kätterer et al. 2013, Poeplau & Don 2015). Using grass-legume leys allows the soil to regenerate, build up organic matter, and enhance C input in cropland soils where SOC stocks are diminished (Kätterer et al. 2013).

2.2 The soil microbial community structure

Microbes are the most abundant species both in number and species richness, contain most of the C stored in biota, and are essential to the different ecosystems on Earth (Allison & Martiny 2008). Arable soils have distinct microbial communities that are important for soil fertility, nutrient cycling, and plant health, and in a single spoonful of soil there can be billions of microbes with an enormous genetical diversity that exceeds that of the aquatic ecosystems (Torsvik & Øvreås 2002). The most abundant microbial group in arable soils is bacteria due to their small size and high reproduction rates (Kirchman 2012). Another major group is fungi, that tends to be higher in number in soils with high organic matter content (Hannula & Morriën 2022). Even if fungi are less abundant in number than bacteria, due to their bigger size, the biomass of fungi can be as big as bacteria (Joergensen & Wichern 2008).

One of the most important microbial groups in arable soils is N fixers, as the N-fixing process helps enhance soil fertility and reduces the need for synthetic fertilizers (Wolińska et al. 2017). N-fixing microbes are thereby suggested to be used as organic fertilizers as inoculating plants with N-fixers has been noted to increase plant growth and yield sizes (Ashok et al. 2017, Backer et al. 2018, Zhang et al. 2018). One microbial group like this is Rhizobia, a group of bacteria that colonizes the plant roots and forms a symbiotic relationship with leguminous plant, like red clover, fixing atmospheric N and converting it into a form that can be readily used by the plant (Willems 2006). Another important N-fixer is arbuscular mycorrhizal fungi (AMF), which also forms symbiotic associations with plant roots, delivering N, phosphorus, and many growth-limiting nutrients to the plants and being therefore crucial for nutrient uptake in most of the agriculturally important crops (Smith & Smith 2011). Apart from Rhizobia and AMF, there are also a wide and diverse group of nonsymbiotic

bacteria, such as Cyanobacteria, that can fix atmospheric N in arable soils without establishing a symbiotic relationship with plants (Wolińska et al. 2017).

Plant growth-promoting rhizobacteria are another important microbial group that colonizes the rhizosphere and increases nutrient availability through enzyme activity that breaks down the molecule structures, making the nutrients available for plant consumption (Etesami & Maheshwari 2018). They also produce growth-promoting substances such as phytohormones, solubilize and mineralize nutrients, and protect plants from toxins and pathogens (Etesami & Maheshwari 2018). Decomposer microbes, on the other hand, includes a variety of bacteria and fungi that use their extracellular enzymes to break down crop residues and other organic materials, releasing nutrients and making them available for microbial uptake (Kaiser et al. 2015), enhancing soil structure and playing a crucial role in the soil C cycling (Rillig & Mummey 2006, Liang et al. 2019, Hannula & Morriën 2022). Denitrifying bacteria support N cycling by converting nitrate into N gas, and thereby reducing the potential negative impacts of nitrate runoff caused by excessive N fertilization (Zhou et al. 2019).

2.2.1 The soil microbiota relationships

The soil microbiome is not an isolated entity but rather an integral component of the complex network that makes up the soil ecosystem (Torsvik & Øvreås 2002). The interactions span across trophic levels and biogeochemical cycles, influencing nutrient cycling, soil structure maintenance, as well as plant health and productivity. The relationships between microbes, plants, and abiotic factors highlight the importance of viewing the soil microbiome as a dynamic and interconnected entity within the broader ecological context.

The role of fungi is particularly important to the well-being of the soil microbial community and their potential impacts on the entire soil ecosystem might be more important than we yet know (Hannula & Morriën 2022). The fungi form mycelian networks in soil, called hyphae, that function as transportation highways, making important organic nutrients to be available for bacteria (Rudnick et al. 2015) and helping bacteria communities to migrate to the new areas where they are needed for decomposing and keeping the soil healthy (Warmink et al. 2011). The fungi have different nutrient demands and can break down different, more complex organic compounds than bacteria (Keiblinger et al. 2010), so the interaction between bacteria and fungi promotes the efficient decomposition of organic material. Thus, understanding the fungal-bacterial relationship can provide an important insight into the impact of microbial communities on C cycling and help assess their role in C sequestration.

Many microbes, like Rhizobia or AMF mentioned above, are known to form symbiotic associations with plants (Huang et al. 2014). Microbes may produce signaling molecules that can be recognized by plant receptors, triggering specific plant responses and, similarly, plants can release signals that attract beneficial microbes or repel pathogens (Sanchez-Cañizares et al. 2017). Plants also control the microbiome by releasing a range of highly nutritious organic compounds, known as root exudates, into the rhizosphere, the soil surrounding the plant roots

(Backer et al. 2018). The microbes use the root exudates for growth and form wide food web structures, extending the nutrients beyond the rhizosphere (Esperschütz et al. 2009). On the other hand, microbes also secrete the exudates to influence the composition and quality of root exudates and plant metabolome, and, thus, modulating the plant-microbe interactions (Huang et al. 2014). The improved nutrient uptake can help to increase plant health and yield size, leading to better stress-tolerance and plant resilience to abiotic stresses, such as drought, salinity, and extreme temperature (Backer et al 2018). These interactions can help plants thrive even in challenging environments and eventually adapt to climate change.

Crop diversity plays a crucial role in shaping microbiota, which can have many benefits for soil health, plant disease control, and ecosystem resilience. Since the plants support a large community of microbes through root exudates, it leads to a higher microbial density, but lower microbial diversity compared to bulk soil outside the rhizosphere and therefore, growing the crops in monoculture can lead to the degradation of the microbial community and dysbiosis of the ecosystem (Bakker et al. 2020). Polyculture provides a wider range of resources for microbial communities, leading to increased soil microbial diversity (LeBlanc et al. 2015, Yang et al. 2017, Cline et al. 2018, Stefan et al. 2021). Each crop species interacts with the microbial community in a unique way and the variety of root exudates from diverse crops supports the growth and activity of different microbial groups.

Abiotic factors, meaning the physical and chemical components of the environment such as temperature, moisture, pH, and nutrients, profoundly influence the composition and functionality of the soil microbiome. Temperature orchestrates microbial activity and growth, as the maximum growth temperature is usually much higher than the usual soil mean temperature (Rousk et al. 2011). Moisture also influences microbial activity and growth, with wet conditions leading to more microbial biomass (Rousk et al. 2011). Soil pH acts as a master regulator for F:B-ratio, as increasing pH decreases the fungal and increases the bacterial growth, and vice versa (Rousk et al. 2011). Nutrient availability as well as the chemical composition of the soil intricately connects with the soil microbiome (Rousk et al. 2011). The interplay between the soil microbiome and abiotic factors is a dynamic process, with feedback loops and dynamics shaping the resilience and adaptability of soil microbial communities.

The relationship between microbiota, crop species and abiotic factors in soils is complex and not everything is yet known about how they influence each other. It has been suggested that abiotic factors, like fertilization and soil moisture, can play a more significant role for microbial composition than crop diversity (Stefan et al. 2021), but on the other hand, crop species richness also alters the abiotic factors like the organic substrates that further affect the microbial communities (Cline et al. 2018). More research is still needed to understand better the interactions between plants, microbes, fertilizers, temperature, moisture, and other influencing factors, and fully benefit from carbon farming practices for climate change mitigation.

2.2.2 Microbial carbon

When contemplating the C sequestration into the microbial biomass, an essential variable to consider is carbon use efficiency (CUE). It refers to the proportion of assimilated C retained by microbial biomass during metabolic processes, as opposed to being released back into the atmosphere through respiration (Kästner et al. 2021, Islam et al. 2022). High CUE values indicate efficient C utilization and biomass production by soil microbes, resulting in increased C input in the soil (Kästner et al. 2021). The CUE of soil microbial communities is influenced by various factors, including environmental conditions, like temperature, humidity, and pH (Zheng et al. 2019), availability of organic substrates (Kästner et al. 2021), substrate stoichiometry, quantity (Keiblinger et al. 2010), quality and complexity (Islam et al. 2022), together with the microbial community structure and diversity (Domeignoz-Horta 2020). On the other hand, various environmental factors can affect microbial growth rate itself, further affecting CUE (Zheng et al. 2019).

CUE is also linked to the C:N-ratio of the soil, as the availability of N is crucial for efficient decomposition of organic matter and successive SOC storage (Poeplau et al. 2019). The efficient microbial decomposition and storing of SOC into microbial biomass needs many N-rich compounds and thus, the addition of N fertilization has been perceived to increase SOC stocks (Cotrufo et al. 2019). Furthermore, cultivating leguminous plants, such as clovers, and other crops with Rhizobia and AMF relationship, promotes this complex balance by fixing atmospheric N, enriching the soil, and increasing the microbial anabolism and CUE (Poeplau 2020, 2021). This enhanced CUE, facilitated by increased N availability, increases microbial C sequestration potential, highlighting the relationship between N, microbial CUE, and C input (Poeplau et al. 2019). On the other hand, adding fresh C nutrients to the soil can slow down decomposition rate via reduced priming effect, whereby microbes can use the easily available fresh C, instead of decomposing the SOC (Fontaine et al. 2011).

Another important concept for microbial C sequestration is the soil microbial carbon pump, the process in which the SOC is first decomposed as the living fraction of microbial biomass, and thereafter stabilized as the microbial necromass, comprising the dead and inactive microbial biomass (Figure 4; Zhu et al. 2020, Kästner et al. 2021). Microbial necromass has been of particular interest regarding soil C stocks as it can cover over 60% of SOC of the grassland soil (Liang et al. 2019). Also, 76–92% of the additional SOC gained by successful carbon farming practices are suggested to be microbial necromass (Zhu et al. 2020). The microbial necromass C content is more stable than plant-originated SOC in general, as it contains complex organic molecules resistant to rapid decomposition by soil microbes (Kästner et al. 2021). These molecules also react easily with soil minerals, forming MAOM, which is the long-lasting C reservoir in soil (Islam et al. 2022). It is therefore suggested that the microbial necromass C pool is unaffected by grassland management practices like tillage and mowing, that usually lead to C losses (Buckeridge et al. 2020).

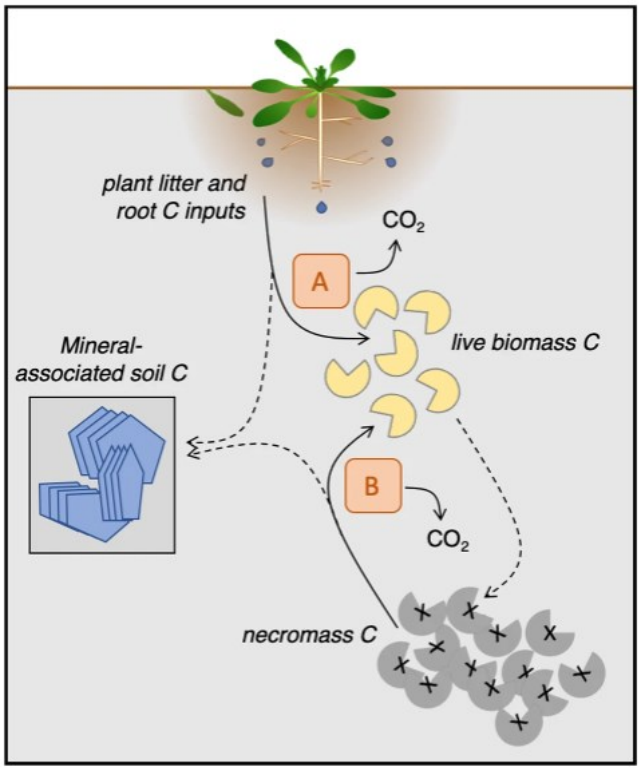

Figure 4. The soil microbial carbon pump is based on (A) decomposition of plant based organic matter into microbial biomass and (B) stabilization of microbial biomass into necromass. Microbial C input and stabilization is thereby dependent on (A) CUE and (B) microbial necromass. © Buckeridge et al. 2020.

Fungal hyphae have a key role in growing and stabilizing the soil C stocks and higher F:B-ratio is associated to a more efficient C input (Allison et al. 2005, Keiblinger et al. 2010, Hannula & Morrién 2022). Still, the mechanism is somewhat unclear. Previously, fungi have been suggested to have higher CUE than bacteria and therefore higher C sequestration potential, but this hypothesis has later been challenged (Allison et al. 2005, Thiet et al. 2006). A more credible mechanism is that fungi secrete degradation resisting compounds to form a stable soil structure that protects the SOC from respiration (Allison et al. 2005), and thus, builds up the soil C stock more efficiently than bacteria (Liang et al. 2019).

Mycorrhizal fungi are thought to be key players in protecting SOC as they affect the soil aggregation by secreting glomalin and other extracellular substances from their hyphae that binds the SOC into the soil (Rillig & Mummey 2006). AMF decreases the amount of C in the plant roots and, on the other hand, increases C in root exudates, rhizosphere, and soil in general by limiting decomposer activity (Zhou et al. 2020). On the other hand, ectomycorrhizal fungi have a notable role in storing C by forming stable SOC in their hyphae outside the plant roots (Ekblad et al. 2013). Thus, the C sequestration potential of fungi can be remarkably large, but more research is needed to understand the

underlying mechanisms and apply them for climate change mitigation (Hannula & Morrién 2022).

One suggested factor affecting the soil microbial C cumulation is the amount of so called 'cheaters', which means the microbes with lower enzyme activity that benefit from the enzyme activity of the decomposers (Kaiser et al. 2015). A model by Kaiser et al. (2015) suggests, that 'cheaters' can work as a C buffer, outbalancing the increasing enzyme efficiency by increasing the microbial biomass and thus, helping with accumulation on the microbial C in soil. In their model, the extracellular enzymes produced by the decomposing microbes make the C compounds available also for the other microbes nearby via diffusion, increasing the number of 'cheaters' and the amount of microbial biomass, and thereby decreasing the ratio between enzymes and microbial biomass. This is suggested to slow down the decomposing rates, further increasing microbial CUE and the accumulation of microbial biomass (Kaiser et al. 2015).

On the other hand, Heikkinen et al. (2021) suggests that the chemical composition and solubility of SOC affects the microbial community structure via decomposing processes, which are, on the other hand, controlled by microbial community structure and diversity. They divided SOC into four different forms of solubility: water, ethanol, acid and non-soluble, where water-soluble is the easiest to decompose and non-soluble the hardest. They showed that by adding organic matter with different fractions of solubilities into soil, it is possible to alter the microbial composition, inhibit the decomposition process and increase the C input. For example, red clover residues high with water and acid-soluble fragments resulted in lower microbial diversity and higher decomposition rate compared to materials high with non-soluble content, like wood chips, slurry, or sludge (Heikkinen et al. 2021).

2.2.3 Research methods

As the microbial world is not easily available for human eye, studying it has required the development of special kind of study methods. Therefore, we have only recently started to understand the abundance of microbes and the importance of it to life on Earth (Allison & Martiny 2008). Different methods have different pros and cons and thereby comparing and combining the different methods is important to better understand the structure and changes in microbial communities (Lewe et al. 2021, Osburn et al. 2022).

One common method to study microbial community is ribosomal RNA (rRNA) gene sequencing. With NGS techniques it is possible to efficiently provide detailed information about species, taxonomy and, thus, functionality of the microbial community and compare the microbial communities of different samples (Xun et al. 2021, Meeks et al. 2022, Vecere et al. 2022). The history of sequencing goes back to 1977 when the Sanger sequencing revolutionized the field of genomics with an enzymatic approach that uses the same technique to copy the DNA as the cell itself (McCombie et al. 2021). Thereafter, the method has been evolved to the massively parallel NGS techniques that can read huge amounts of sequences simultaneously, enabling the analysis of large number of

samples within a relatively short amount of time (McCombie et al. 2021). One of the NGS methods includes the Ion Torrent (Thermo Fisher) that measures the changing pH caused by the release of H+ ions in the synthesis reaction (Goodwin et al. 2016). The reactions are measured with an ion chip containing 1.2 million sensor wells, and, while each well allows one read, this method enables fast and cost-efficient reading of substantial size sequence libraries (Rothberg et al. 2011).

The nucleotide sequence of the rRNA gene contains both highly conserved regions, which are similar among different species, as well as species-specific regions, that are highly variable. The commonly used regions for microbial identification are 16S rRNA gene for bacteria (Weisburg et al. 1991), and internal transcribed spacer (ITS) region for fungi (Seifert 2009). The conserved regions are targeted with region specific primers and the sequences are amplified to observable amounts in the polymerase chain reaction (PCR; Goodwin et al. 2016). After the targeting and amplification of the specific region, it is possible to identify the species from the species-specific regions of the sequences.

Another commonly used method for studying microbial community structure is phospholipid fatty acid (PLFA) analysis. PLFAs are widely used as microbial biomarkers for their specificity and stability in the living microbial membranes and the method is accepted to be accurate for defining the microbial biomass and living community in soil (Zhang 2019) as well as the ratio of different microbial groups in the community (Willers et al. 2015). The method is based on the observation that the microbes contain specific types of PLFAs in their cell membranes, which can be used as biomarkers to define the microbial groups (Zelles 1999). Many monounsaturated fatty acids (FA) are usually found within gram-negative bacteria (Zelles 1999), while gram-positive bacteria have terminally branched saturated FAs in their cell membrain (Frostegård and Bååth 1996). The PLFA 18:2ω6 is accepted as a general fungal biomarker and can be used to calculate F:B-ratio in soil ecosystems (Frostegård and Bååth 1996), and PLFA 16:1ω5c together with PLFA 20:5 has been suggested as a specific biomarker of arbuscular mychorrizal fungi (Olsson et al. 1995).

The PLFAs are extracted from the samples using so called Folch-method, which is a traditional lipid extraction method (Folch et al. 1957), fractionated in solid-phase column, and finally the fatty acid methyl esters (FAMEs) are analyzed using gas chromatography-mass spectrometry (GC-MS; Willers et al. 2015). Gas chromatography separates the FAMEs based on their volatility and the separated compounds are ionized and fragmented into smaller ions by the mass spectrometer, producing a fragmentation pattern characteristic of each FAME (Bobbie & White 1980). The resulting mass spectra are analyzed to determine the molecular masses and structural information of the FAMEs (Bobbie & White 1980).

While NGS methods allow producing reliable data on the microbial species and, thus, accessing the qualitative information of microbial taxonomy, the quantitative information about the number of microbes and their abundance is highly inaccurate due to the unequal sequence amplification with PCR (Wintzingerode et al. 1997). PLFA analysis can be applied to gain a more accurate

picture of total microbial biomass. Moreover, PLFA analysis is recommended for accessing the information about the whole microbial community and the ratio of different microbial groups, but it cannot be used for identifying the individual species (Zelles 1999). By combining the NGS and PLFA methods, it is thereby possible to gain an accurate picture of the microbial community structure (Chen et al. 2019, Lewe et al. 2021, Osburn 2022).

3 MATERIALS AND METHODS

3.0 Research site and experimental design

This study is a part of Nature Resources Institute's (Luke) 'Sustainability from the Grasslands' research project funded by European Agricultural Fund for Rural Development. The research set up was established in 2020 in the cultivation plots of Luke research station in Maaninka, Finland (63°14'53''N, 27°35'54''E). The field soil type in the research site is sandy loam (sand 60.3%, silt 28.0%, clay 11.3%) with the organic matter content between 3–5%, and soil pH 6.2 ± 0.1. The soil analysis was performed from the samples collected on 18.5.2022 and analyzed by Eurofins Agro (Mikkeli, Finland).

The history of the area goes back to the 16th century when the plot was still a thick forest around lake Maaninkajärvi. The field was probably cleared somewhere between the 16th and 18th centuries when milk production started in the area (Martikainen et al. 2020). Nowadays the field is intensively cultivated with grass rotation of one year of cereal as a nurse crop and three years of grass.

The research design had four crop handling groups as treatments: (1) tall fescue (*Festuca arundinacea*, variety 'Retu'), (2) timothy (*Phleum pratense*, var. 'Tuure'), (3) red clover (*Trifolium pratense*, var. 'SW Yngve') and (4) timothy + red clover -mixture. Each treatment had four 1.5 x 8.0 m plot replicates in the research design (Figure 5). The nurse crop of cereal was used during the first year and the sampling was made during the second grass year.

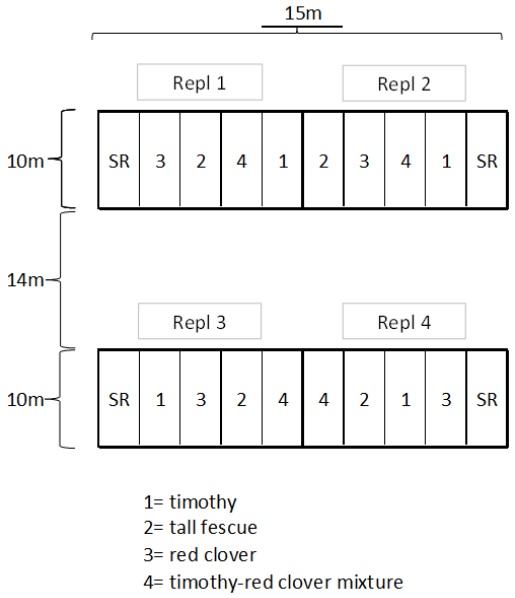

Figure 5. The research design has four crop handling groups as treatments and each treatment has four replicants (Repl) in the field.

Different amounts of mineral fertilization were used for the different treatments (Table 1) as the results and the amounts of fertilizers were meant to be directly applicable in practice. The content of soluble N in the fertilizer was 27% and both ammonium and nitrate form of N was included.

TABLE 1. The amount of fertilizers in the treatments during the sampling year of 2022. The spring fertilization was done on May 20th and the summer fertilization on June 21st after harvesting and sampling.

Treatment	Spring 2022 (kg/ha)			Summer 2022 (kg/ha)		
	Nitrogen	Phosphorus	Kalium	Nitrogen	Phosphorus	Kalium
Tall fescue	100	14	24	100	0	39
Timothy	100	14	24	100	0	39
Red clover	0	14	20	0	0	35
Timothy + Red clover	50	14	22	50	0	37

3.1 Soil sampling

The soil samples were collected during the summer 2022 in three time points: (1) after the first harvesting and before fertilizing (June 21st), (2) two weeks after the harvesting, and fertilizing (July 6th), and (3) after the second harvesting (July 29th). In each time point, 16 samples were collected, one from every species group from each of the four plot replicates. Altogether 48 samples were collected during the summer, 12 from each handling.

The soil sampling was done using a soil sampling drill of 20 mm diameter from 0–20 cm depth. After lifting the soil, 2 cm of the sample was removed from the top to avoid adding microbes in the soil surface into the sample. The drilling was done in eight different spots in each plot and the sample soil was collected in a plastic bag and roughly mixed there. The drilling spots were randomized by unfolding a measuring reel through the cultivation plot from corner to corner, and then drilling the holes in every 1 meter according to the measuring reel. This ensured that there was sampling both near the crop roots and in the bulk soil between the crops.

After sampling, the samples were homogenized by removing the roots and rocks, sieving the soil through 2 mm sieve, and storing each sample in a sealable plastic bag with air pressed out. For the DNA analysis, within 2 days after the sampling 2.0 g of the sample was weighted from the plastic bag to a Falcon tube (15 ml) and added 4.0 ml of the DNA/RNA Shield (Zymo Research, CA, U.S.A.)

as a preservative. All the samples were frozen at the temperature of -20 °C and after the final sampling, transported to Jyväskylä University in cold boxes, where they were again stored at -20 °C.

3.2 Plant and root biomass

The plant biomass was evaluated based on crop yield and root biomass data. The collection and analysis of data were conducted by Luke.

> Statistical analyses were calculated using ANOVA (SAS 9.4., Mixed-procedure). Pairwise comparisons were determined by Tukey–Kramer's test. Statistical significance was postulated at a probability of .05 (S. Kykkänen, personal communication, 29.9.2023).

3.3 Carbon percentage

C% was studied by LOI method during the February 2023 with the equipment in the premises of the Department of Biological and Environmental Sciences of the University of Jyväskylä (Finland). The LOI measurement was made by the standard protocol SFS 3008, but the higher sample mass of 20 g was used to minimize the variation of mass loss (Hoogsteen et al. 2015). The workflow started by weighing (Ohaus AP210, Switzerland) 20 ± 0.5 g of defrosted sample to a pre-weighted crucible and moving it to the oven to 105 ± 5 °C for 20 hours to evaporate the water content. After cooling down in a desiccator for 20 minutes, the samples were weighed to calculate the dry mass. Then, the samples were moved to the muffle furnace at 550 °C for 2 hours to burn out the organic content and cooled down in the desiccator for 1 hour before weighing.

The C% was calculated using Equation 1, where the amount of LOI was estimated to be the amount of organic matter, and 58% of the organic matter to be C:

$$C\% = \frac{DW - IR}{DW} \times 0{,}58 \times 100 \quad (1)$$

where DW is the dry weight of the sample soil and IR is the ignition residue. The conversion factor 0.58 was used for the conversion, which is derived from the van Bemmelen's factor of 1.724 (Heikkinen et al. 2020). It is a widely accepted conversion factor of soil organic matter to soil C content (Guo & Gifford 2002, De Vos et al. 2005, Rawlins et al. 2008) and was also recommended by Luke.

Lastly, the result was compared to Luke's C% data. Soil sampling for Luke's data was done on August 30th 2022 for the soil depths of 0–20 cm and 20–40 cm with the Elemental analyzer (LECO Corporation, Michigan, U.S.A.).

3.4 PLFA analysis

PLFA analysis was made in the premises of the Department of Biological and Environmental Sciences of the University of Jyväskylä (Finland) during the spring 2023.

3.4.1 Extraction and analysis of PLFAs

The samples were prepared for the GC-MS following a four-step process: (1) lipid extraction, (2) lipid fractioning, (3) derivatization by methylating the PLFAs to FAMEs, (4) final GC-MS instrumental analysis.

The samples were freeze-dried for two days and moved to the desiccator. From there, 2.0 ± 0.2 g of the freeze-dried sample soil was weighed (Mettler Toledo AG204) to clean chloroform-methanol (2:1) rinsed 30 ml Kimax glass tubes.

Each sample was blended with the internal standard (ISTD) solution prepared by mixing 10.074 mg of $PLFA_{19:0}$ (Larodan, Sweden) and 10.063 mg of $C_{23:0}$ free FA (Larodan, Sweden) with 10 ml of chloroform and vortexed thoroughly. $PLFA_{19:0}$ was used to evaluate acquisition of the PLFAs in the result, whereas $C_{23:0}$ was used to evaluate the amount of non-wanted FAs in the sample after the fractioning and, thus, the reliability of the results.

Lipids were extracted from the samples using the Folch method (Folch et al. 1957). For the lipid extraction, 9 ml of 2:1 chloroform-methanol solution, 2.25 ml sterile water and either 150 µl or 200 µl of room temperate ISTD solution was added to the tubes. Additionally, one no template control (NTC) without the soil was prepared, using 150 µl ISTD and following otherwise the same workflow as all the other samples. The samples were vortexed for 30 seconds, sonicated for 15 minutes in an ultrasonic water bath, and vortexed again for 30 seconds to enhance the extraction of the lipids to the chloroform. Finally, the samples were centrifuged at 3,000 rpm and 15 °C for 10 minutes to divide the sample into two liquid phases of water-soluble content and chloroform with lipids. The lower liquid phase of chloroform and the lipids was collected to a new clean chloroform-methanol (2:1) rinsed 12 ml Kimax glass tubes using disposable glass Pasteur pipettes. The extraction was then repeated to enhance the acquisition of the FAs, by adding 3 ml chloroform to the bigger Kimax tube containing the soil sample, vortexing the samples for 15 seconds, sonicating for 10 minutes, vortexing again briefly and centrifuging as previously. Once more, the lower liquid phase was collected with another clean disposable glass Pasteur pipettes, to the same 12 ml Kimax tube as the first extraction product. Lastly, the samples were evaporated to complete dryness under N_2 flow at 37.5 °C, added 400 µl of chloroform and stored in -20 °C.

In the lipid fractioning, the neutral lipids (NL), glycolipids (GL) and polar lipids (PL including PLFA) were separated by using 500 mg Bond Elut LRC-SI cartridge (Agilent Technologies). To do this, the cartridge was first activated by adding 3 ml of 1:1 chloroform-methanol solution into the cartridge with low vacuum. The sample was then added to the activated cartridge and, to ensure the

collection of all the lipids, the emptied sample Kimax tube was rinsed by adding 200 µl chloroform, vortexing briefly and pouring it to the cartridge. First, the NLs were eluted by adding 10 ml of chloroform into the cartridge under vacuum and collecting the filtered chloroform with eluted NLs into a Kimax tube. Secondly, GLs were eluted with 10 ml of acetone as previously to another Kimax tube. Finally, PLs were eluted with 10 ml of ethanol and collected to a new clean chloroform-methanol (2:1) rinsed 12 ml Kimax glass tubes. Since I only wanted to analyze FAs in PL fraction, the eluted NLs and GLs were discarded while the PLs (including PLFAs) were evaporated to complete dryness under N_2 flow at 40.0 °C, added 1 ml of toluene and stored in -20 °C.

The derivatization by methylation of the PLFAs was then done by adding the samples 3 ml of 1:100 H_2SO_4-methanol solution. After vortexing for 5 seconds, the samples were bathed in 50 °C water for 18 hours to improve the success of methylation process. The heat, in combination with the acidic conditions created by H_2SO_4, facilitated the addition of the methyl-group (CH_3) from methanol to the PLFAs, forming FAMEs. The methylation process increased the volatility and thermal stability of the compounds, making the biomarkers easier to analyse with GC-MS.

After cooling down at room temperature for 30 minutes, the samples were prepared for the second phase separation by adding 2 ml of sterile water and 2 ml of hexane. Samples were vortexed for 5 seconds and centrifuged at 2,000 rpm and 15 °C for 2 minutes, to separate them into two phases of hexane and water. Then the upper phase of hexane containing the FAMEs was collected using disposable glass Pasteur pipettes into new clean chloroform-methanol (2:1) rinsed 12 ml Kimax glass tubes, while the lower phase of unwanted water-soluble content was discarded. The samples were stored in -20 °C to wait for the GC-MS run. Finally, the samples were evaporated to complete dryness under N_2 flow at 40.0 °C, after which 800 µl of hexane was added, vortexed for 5 seconds and moved the whole sample into the clear GC-MS vials using disposable glass Pasteur pipettes. Hexane is highly volatile, so this step was taken to maintain a consistent volume for the GC-MS analysis.

The GC-MS instrumental analysis was performed with a gas chromatograph (Shimadzu Ultra, Kyoto, Japan) equipped with a mass detector. The column used was Agilent® (Santa Clara, California, U.S.A.) DB-FastFAME (30 m x 0.250 mm x 0.25 µm) and helium was used as a carrier gas (linear velocity = 36.3 cm/sec). The injection temperature was 260 °C, the total program time 25.38 min and the temperature program as follows: first 60 °C for 1 min, then the temperature was increased to 165 °C at 40 °C/min rate and held for 1 min, and finally increased to 230 °C at 4 °C/min rate and held for 4.5 min.

3.4.2 Data processing

The FAMEs were identified in GC Solution Postrun -software (Shimadzu) using the retention times (RT) of CG-MS curve and the chart of ions and their known characteristics.

The concentrations were calculated in GC Solution Browser -software using the FAME 566c standard mixture (GLC standard mixture 566c, Nu-Check Prep, MN, U.S.A.) and the standard concentrations of 50, 100, 250 and 500 ng/µl. The composition of the 566c mixture is shown in Appendix 2. The concentrations for the identified FAMEs were calculated using the slope of the calibration curve derived from the results of the FAME standard mixture. For each curve, the Pearson correlation coefficient was verified to be >0.99. In the case of the identified FAMEs that were not included in the FAME standard mixture, the slope of the most similar FAME in the standard mixture was employed.

After calculating the concentrations in the GC Solution Browser, the results were further analyzed in Microsoft Excel. First, the theoretical maximum concentration (C_{max}) for ISTDs was calculated using Equation 2 and the known amounts of ISTDs added to each sample:

$$C_{max} = \frac{V_{ISTD} \times C_{ISTD}}{V_{sample}} \times CF \quad (2)$$

where V_{ISTD} is volume of the ISTD added to the sample, C_{ISTD} is the concentration of the added ISTD, V_{sample} is the solvent volume in which the sample was diluted before CG-MS analysis and CF is the possible correction factor used. The C_{max} for the PLFAs in the result was calculated by using the concentration of nonadecanoic acid ($C_{19:0}$). For ISTD $PLFA_{19:0}$, the CF of 0.727 was added, reflecting the proportion of $C_{19:0}$ in $PLFA_{19:0}$. Conversely, for $C_{23:0}$ the CF was 1.

The percentage yields were calculated using the recoveries (R) of the PLFAs (Equation 3) using C_{max} and the concentration from the GC Solution Browser:

$$R = \frac{C_{sample}}{C_{max}} \quad (3)$$

where C_{sample} is the obtained concentration of the FA in the sample based on the calibration curves of the FAME standard mixture. The percentage yield (Y%) was determined by multiplying R by 100. The ISTD $PLFA_{19:0}$ percentage yield was 35.2 ± 4.8% for the qualified samples. Two samples of red clover with ISTD $PLFA_{19:0}$ percentage yield of ⩽ 2.0% and, thus, they were discarded for further analysis. For the ISTD $C_{23:0}$ free FA, the percentage yield was 2.8 ± 1.7% for the qualified samples, which was low enough for a reliable result of PLFA analysis. The NTC sample had higher numbers for both percentage yields, for $PLFA_{19:0}$ being 51.4% and for $C_{23:0}$ 14.9%. The sample-specific results are shown in Appendix 2.

The content of the found PLFAs in the samples (Equation 4) was calculated using the R of $C_{19:0}$ as a correction factors:

$$C_{PLFA} = \frac{C_{sample} \times V_{sample} \times \left(\frac{1}{R}\right)}{m_{sample}} \quad (4)$$

where C_{PLFA} is the content of the PLFA per soil sample dry weight (µg/g DW of soil), C_{sample} is the obtained concentration of the PLFA in the sample based on the calibration curves of the FAME standard mixture, V_{sample} is the solvent volume in which the sample was diluted before CG-MS analysis, R is the recovery and m_{sample} is the mass of the soil sample.

A total of 29 FAs were identified from the samples, and 17 of them were utilized as biomarkers for microbial cells (see Appendix 2). The $C_{16:0}$ and $C_{18:0}$ FAs were discarded as background noise, as the NTC sample contained a relatively high amount of them. Also, in addition to the microbial cell wall, both $C_{16:0}$ and $C_{18:0}$ are ubiquitously found in plants (Welti et al. 1999, Zelles 1999) so it is recommended to leave them out in the biomarker analysis (Willers et al. 2015, Zheng 2013).

Finally, the found PLFA biomarkers were assigned to the specific microbial group according to Table 2. To calculate the F:B-ratio, the contents of bacterial and fungal biomarker PLFAs were summed up and the results were compared by dividing the fungal content with bacterial content. The ratio count was the unit number of fungi to one unit of bacteria.

TABLE 2. Biomarker PLFAs used to describe the microbial community ratios (Frostegåård et Bååth 1993, 1996: Olsson et al. 1995; Zelles 1997, 1999). The names of the different FAs are presented in the form of CC:DBωOM, where CC is the number of carbons in the FA, DB is the number of double chains in the FA and OM is the location of the first double bond. The letter c in the end means cis isomerism of the doublebond. The isomers of saturated FAs are marked with i (iso) and a (anteiso) in the beginning, marking the position of the methyl-group in the FA (2nd C for iso- and 3rd C for anteiso). The abbreviation cy means cyclic FA.

Microbial group	Biomarker PLFAs
Bacteria - general marker	14:0, 15:0, 17:0
Gram -ve bacteria	16:1ω9c, 16:1ω7c, 18:1ω9c, 18:1ω7c, cy19:0, 20:0
Gram +ve bacteria	i14:0, i15:0, a15:0, i16:0, i17:0, a17:0
Fungi – general marker	18:2ω6c
Arbuscular mycorrhizal fungi	16:1ω5c

3.5 DNA sequencing

The DNA sequencing analysis consisted of three major stages: (1) preparing the sequence library, (2) reading the sequence library, and (3) data processing and analysis.

3.5.1 Preparing the sequence library

Preparation of the bacterial and fungal rRNA gene sequence libraries included six steps: (1) DNA extraction, (2) DNA isolation, (3) first-round PCR for gene amplification, (4) second-round PCR for sequence barcoding, (5) library preparation and (6) quality check. After the (2) DNA isolation, the bacterial and fungi DNA was targeted and amplified in separate PCR-runs to form two different sequencing libraries. In addition, three NTCs were prepared at the same time and the same way with the samples, but without the sample material. The laboratory analyzes were done in the premises of the Department of Biological and Environmental Sciences of the University of Jyväskylä (Finland) in winter 2022–2023, apart from (2) DNA isolation, which was done in the laboratory of BiopSense Oy (Jyväskylä, Finland).

The DNA extraction was done using bead mill homogenizer Bead Ruptor Elite 24 (Omni International Inc., GA, U.S.A.). Before extraction, the samples were allowed to thaw at room temperature, and then 1 g of 0.5 mm and 4 g of 0.1 mm beads were added to each sample to break the microbial cell walls and release the DNA. Next, the samples were homogenized using 4 m/s speed and 40 s time to release the DNA. The homogenized samples were then stored at -20 °C. Next day, the thawed samples were centrifuged at 2000 rpm and 4 °C for 10 minutes to separate the DNA from the beads and the soil material, and 300 µl of the supernatant per sample was collected to the 96-well plate. The plate of the extracted DNA was again stored at -20 °C to wait for further analysis.

The DNA isolation was done by using magnetic bead-based isolation using chemagic™ 360 Instrument (PerkinElmer Inc., MA, U.S.A.) and following the instructions of chemagic™ Viral DNA/RNA Kit (PerkinElmer Inc., MA, U.S.A.). The isolated product was then stored at -20 °C. After thawing, 10 µl of the isolated product per sample was purified with sparQ PureMag Beads (Quantabio, MA, U.S.A.) using the kit instructions. The purification was done before the first-round PCR to prevent PCR inhibition. Finally, the samples were eluted to 50 µl of HyClone™ HyPure™ Molecular Biology Grade Water (GE Healthcare Life Sciences, UT, U.S.A.).

In the first-round PCR, the genes were targeted for amplification using forward primer for 5′ to 3′ direction and reverse primer for 3′ to 5′ direction. For bacterial DNA, 16S ribosomal DNA (rDNA) gene were targeted using forward primer 515F (GTGYCAGCMGCCGCGGTAA; Parada et al. 2016) and reverse primer 806R (GGACTACHVGGGTWTCTAAT; Caporaso et al. 2012), whereas for fungi DNA, the ITS rDNA was targeted using forward primer ITS1F (CTTGGTCATTTAGAGGAAGTAA; Gardes & Bruns 1993) and reverse primer ITS2 (GCTGCGTTCTTCATCGATGC; White et al. 1990). The PCR-reactions were

prepared on two low-profile PCR 96-well plate, with 2 µl of the sample as template and 23 µl of the master mix consisting of 12.5 µl of SybGreen/Fluorescein qPCR Maxima Master Mix (2x) (Thermo Scientific, Lithuania), 0.5 µl of each primer and 9.5 µl of sterile water. Additionally, three no template controls (NTC) were prepared on each plate, using 23 µl of the Master Mix with no samples.

In the second-round PCR, the Ion Torrent sequencing adapters were adapted to the amplicon, as well as the individual barcodes for each sample. The used primers were forward fusion primer M13-515R (TGTAAAACGACGGCCAGT GTGYCAGCMGCCGCGGTAA) and reverse fusion primer P1-806R (CCTCTCTATGGGCAGTCGGTGAT GGACTACHVGGGTWTCTAAT) for 16S, and forward fusion primer M13-ITS1F (TGTAAAACGACGGCCAGT CTTGGTCATTTAGAGGAAGTAA) and reverse fusion primer P1-ITS2 (CCTCTCTATGGGCAGTCGGTGAT GCTGCGTTCTTCATCGATGC) for ITS. For the second PCR reaction, a new well-plate was prepared by adding 1 µl of the first-round PCR product and 24 µl of master mix consisting of 12.5 µl of the SybGreen/Fluorescein qPCR Maxima Master Mix (2x), 1 µl of 1 µM forward fusion primer and 1 µl of 10 µM reverse fusion primer. Lastly, to barcode the samples, 1 µl of 10 µM barcoded IonA-bc-M13 fusion primers was added to the samples so that each sample got an individual barcode. All primers were acquired from Metabion GmbH (www.metabion.com).

All the PCR-amplifications were performed with CFX96TM Real-Time System C1000TM PCR detection instrument (Bio-Rad, CA, U.S.A.). The reaction conditions were 95 °C for 10 min (initial denaturation), followed by 40 cycles for 30 sec (denaturation); 50 °C, 30 sec (annealing); 72 °C, 1 min (extension), a 5 min final extension at 72 °C, and finally 4 °C maintenance (Parada et al. 2016). However, the second-round PCR was performed with 15 cycles. This temperature program is recommended to be used for 16S rDNA sequences (Parada et al. 2016) and since it was very similar to the program recommended for ITS rDNA sequences (White et al. 1990) it was used for both PCR runs. CFX Maestro software was used to check the SYBR-fluorophore curves and to verify amplifications were successful. The curves are expressed in Appendix 1.

In addition, three NTCs were prepared for both 16S and ITS gene pools and the NTC quantification cycle (Cq) values from the PCR runs were compared with the sample Cq values to ensure the success of the PCR run. For the first-round PCR, the Cq values for the 16S samples were between 20.21–24.22 and for the ITS samples between 25.48–28.03, while Cq mean for 16S NTC was 32.96 and ITS NTC below the threshold (Appendix 1). According to the NTC Cq's, there was no need to discard any of the samples.

After performing the PCR reactions, the bacterial and fungal sequencing libraries were prepared by pooling the samples by adding an equal amount of DNA from each sample into one 1,5 ml Eppendorf. This was done by first quantifying the DNA concentrations of the samples using Invitrogen Qubit® 2.0 Fluorometer (Life Technologies Co., U.S.A.) and following QubitTM 1x dsDNA

HS Assay Kit (Life Technologies Co., UT, U.S.A.). Then the standards for calibration of the instruments were prepared by mixing 190 µl of the working solution and 10 µl of the standard. The samples were prepared for concentration measuring by mixing 195 µl of the working solution with 5 µl of the sample and performing the measurement with Qubit. After measuring the concentrations as ng/µl, it was possible to calculate the volume (µl) needed for each sample to contain equal amount (ng) of DNA. For bacterial sequencing library, 8 ng, and for the fungal sequencing library, 80 ng of DNA was added from each sample. Next, the final DNA concentrations were calculated with Qubit. For the bacterial sequencing library, the final concentration was 8.44 ng/µl and for the fungal sequencing library 2.41 ng/µl. In the end, both sequencing libraries were purified with sparQ PureMag Beads (Quantabio, MA, U.S.A.) using the kit instructions and the samples were eluted either to 75.2 µl (16S) or 384 µl (ITS) of HyCloneTM HyPureTM Molecular Biology Grade Water (GE Healthcare Life Sciences, UT, U.S.A.).

Finally, the quality of the sequencing libraries was measured to ensure they were suitable for further analysis. The quality check was done with Agilent 2200 TapeStation system (Agilent Technologies, CA, U.S.A.) following the High Sensitivity D1000 ScreenTape Assay Kit. Before the Tape Station run, the samples were diluted with sterile water to get a more reliable result, 16S pool by 1:10 and ITS pool by 1:2. After dilution, the Tape Station plate was prepared using 2 µl diluted sample and 2 µl buffer solution. The results of the quality check are expressed in Appendix 1.

3.5.2 Data processing

The Ion Torrent sequencing of the libraries was performed by the laboratory technician Elina Virtanen with the NGS platform Ion Torrent Personal Genome Machine (Life Technologies, CA, U.S.A.). The summary of the Ion Torrent sequencing run is shown in Appendix 1.

The resulting dataset was processed in CLC Genomics Workbench 12 software (Qiaqen, Germany). A total of 2,150,374 16S and ITS-sequences were processed altogether. In 16S sequence data, two samples from tall fescue handling group contained almost no sequences and, thus, were discarded.

The 16S and ITS data were processed and analyzed separately. First, the sequences were trimmed to discard the primers (515F and 806R for 16S and ITS1F and ITS2 for ITS) and sequences shorter than 250 bp in 16S and 200 bp in ITS. The sequences longer than 250bp were trimmed to the maximum length of 250bp. The trimming of 16S sequences resulted in 571,708 trimmed 16S sequences and ITS in 64,764 trimmed ITS sequences.

The operational taxonomic unit (OTU) clustering was performed based on SILVA 16S 99% database with a 99% similarity assumption, allowing the creation of new OTUs with 80% taxonomic similarity. The operational taxonomic unit (OTU) clustering with SILVA database resulted in obtaining 17,742 OTUs for 154,007 16S-sequences and 1,668 OTUs for 33,128 ITS-sequences.

Finally, the resulted datasets were analyzed in phylum and class -levels. In 16S sequence data, in phylum-level the 15 biggest groups were kept, as they covered 96.6% of all sequences, and in class-level the 25 biggest groups were kept, covering 90.8% of all sequences. In ITS sequence data, in phylum-level the three biggest groups (including the unidentified OTUs group) were kept, covering 99.4%, and class-level the nine biggest groups (including the unidentified OTUs group) were kept, covering 99.1% of all sequenced.

The ITS dataset had a lot of unidentified OTUs, so the pool was further investigated using Web BLAST standard database. Only OTUs constituting more than 0.4% of the total sequence pool were retained, accounting for 69.7% of all unidentified sequences at the phylum level and 68.6% at the class level. This manually built Web BLAST-dataset was then integrated with SILVA-data, forming a completed dataset for the following analysis. The data obtained from Web BLAST identification are shown in Appendix 5.

3.6 Statistical analysis

For the C% data, statistical analysis was done using R-Studio (version 2022.12.0). Two-way analysis of variance (ANOVA) was employed to compare four treatments based on two independent variables, assessing both the individual and combined effects of these variables (Moore et al. 2017). The Power Analysis was implemented to further investigate the result and the sample size. The statistical power of 85% was used, which means the probability of the groups to differ significantly with certain significance level (Moore et al. 2017). In addition, the effect size was calculated for the Power Analysis. The effect size is the magnitude of difference between the groups and η2-measurement is commonly used for 2-way ANOVA models (Emerson 2019).

The diversity analysis was made in alpha-level with the CLC Genomics Workbench 12 software (Qiaqen) separately for 16S and ITS OTUs using the genus-level. For 16S, 1877 sequences and for ITS, 231 sequences were used from every sample to balance the result.

All other statistical analysis was performed with Primer software v. 7 (Ivybridge, UK) with PERMANOVA+ add-on. The permutational multivariate analysis of variance (PERMANOVA) and the Monte Carlo -test (Anderson 2001) were applied, using Eucledian distance, type III sum of squares and unrestricted permutation of raw data, except for the microbial community structure the Bray-Curtis dissimilarity was used. For the results, the 95% confidence interval was applied. Where differences were found, Pair-wise test was used to find out which groups differed from each other.

The data from the DNA sequencing and PLFA-analysis were visually combined using a multidimensional scaling (MDS) plot. First, the profiles of 16S and ITS phyla-level abundances and the biomarker microbial groups were calculated for each treatment and sampling date, resulting in three microbial community profiles for each treatment. Then, the bubble plot was created to merge the biomass data into the plot, using the mean biomass content of each

sampling date. Bray-Curtis similarity threshold of 80% was used to investigate the differences between the treatment groups and sampling times. Similarly, another MDS plot was created using only the fungi class -level profile with the unidentified classes by SILVA defined by Web BLAST standard database.

3.7 AI-assisted methods

Artificial intelligence (AI) -based application ChatGPT-4 (OpenAI, CA, U.S.A.) was employed for drafting, brainstorming ideas, and language assistance.

In some sections of the introduction and background, the AI was used to deepen my understanding of the topic and concepts, and in assisting with the drafting of the basic content. This was done by entering queries related to the topic and then either writing based on those ideas, or asking the AI to draft a paragraph, either with its own ideas or giving a guidance, and further working with those ideas. After the AI-based drafting, the text was further revised by consulting and referencing the scientific articles. Using the AI substantially accelerated the writing process and helped to avoid the writer's block.

In the discussion and conclusion sections, the AI was employed for generating the ideas for the first draft. This was done by first providing the AI with the mean values of the results, followed by the abstract and the study questions. Next, the AI was consulted about the potential structuring of the discussion chapter, as well as the key findings. Finally, the draft was created by discussing the findings and concepts with the AI. This involved input queries and guidelines, directing the focus of the AI to specific areas of interest. Drafting with the AI helped combining and mixing the collected information and providing ideas to study further using reliable sources.

Throughout the paper, the AI served as a language consultant. Commands such as ‘rephrase’, ‘correct’, and ‘synonyms’ were used, followed by specific word(s) or the sentence(s) to be worked with, and the feedback from the AI was used as a guidance to enhance the text. At times, the AI was asked to suggest specific terms based on a conceptual description. Engaging with the AI notably improved not only the quality of the text but also my own language skills.

4 RESULTS

The weather conditions during the sampling times are reported according to the statistics of Finnish Meteorological Institute (2023) from the weather station of Kuopio Maaninka, which is located close to the sampling site. Before the first sampling day, the weekly average temperature was 12.9 °C and rainfall 3.9 mm/day, and during the first sampling day the daily average temperature was 15.9 °C without rainfall. Before the second sampling day, the weekly average temperature was 21.0 °C and rainfall 2.8 mm/day, and during the sampling day the daily average temperature was 16.6 °C and rainfall 12.3 mm. Before the third sampling day, the weekly average temperature was 17.0 °C and rainfall 3.4 mm/day, and during the sampling day the daily average temperature was 14.1 °C and rainfall 4.2 mm.

4.1 Plant and root biomass

The obtained crop yields varied between the treatments (ANOVA $p < 0.001$). There was a notable decrease of approximately 30% in yield from the first to the second cut across the treatments (Figure 6). Over both cuts, tall fescue stood out as the highest yielding species, having a yield size 17% greater than that of timothy (Tukey-Kramer $p = 0.006$), 47% greater than red clover (Tukey-Kramer $p < 0.001$), and 24% greater than the timothy-red clover mixture (Tukey-Kramer $p < 0.001$). The yield of the timothy-red clover mixture is approximately 5% greater than the average yield of the timothy (Tukey-Kramer $p = 0.455$) and red clover (Tukey-Kramer $p = 0.016$) monocultures combined.

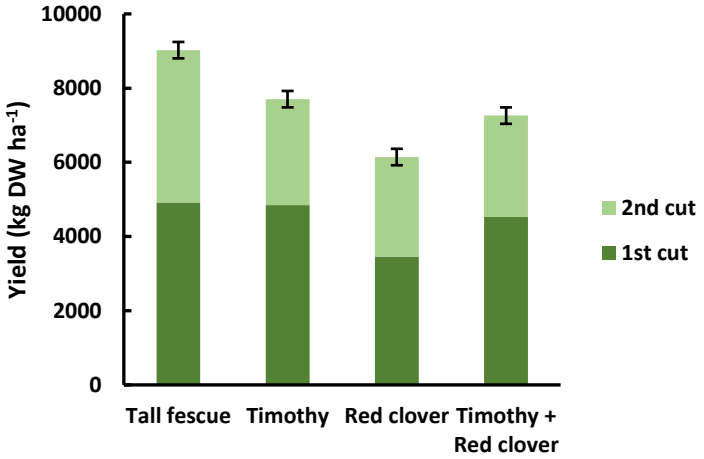

Figure 6. Crop yield is represented as mean values (kg DW/ha) for the different treatments, segmented by the yield from the first and second harvests. The standard error of the mean is represented for combined yield across the whole data. This yield serves as an approximation of the plant biomass.

In the first cut, timothy dominated the timothy-red clover mixture, providing 57 ± 14% of the yield, while red clover accounted for 35 ± 14%. By the second cut, the yield had shifted in favour of red clover, which constituted 48 ± 12% of the total, while the share of timothy decreased to 29 ± 6% timothy. The remainder was weeds.

Also, the obtained root biomasses varied between the treatments both with (ANOVA $p < 0.001$) and without (ANOVA $p = 0.003$) the red clover tap root included (Figure 7). Red clover exhibited the highest root biomass, being 51% higher than tall fescue (Tukey-Kramer $p = 0.001$), 100% higher than timothy (Tukey-Kramer $p < 0.001$), and 60% higher than timothy-red clover mixture (Tukey-Kramer $p < 0.001$), when the tap root was included. Even without the tap root, the root biomass of red clover was 49% higher than timothy (Tukey-Kramer $p = 0.014$), and 64% higher than timothy-red clover mixture (Tukey-Kramer $p = 0.005$). Tall fescue did not overperform compared to other treatments, but it had 46% higher root biomass than timothy-red clover mixture when the tap root was excluded (Tukey-Kramer $p = 0.033$). No other differences between the treatments were statistically significant.

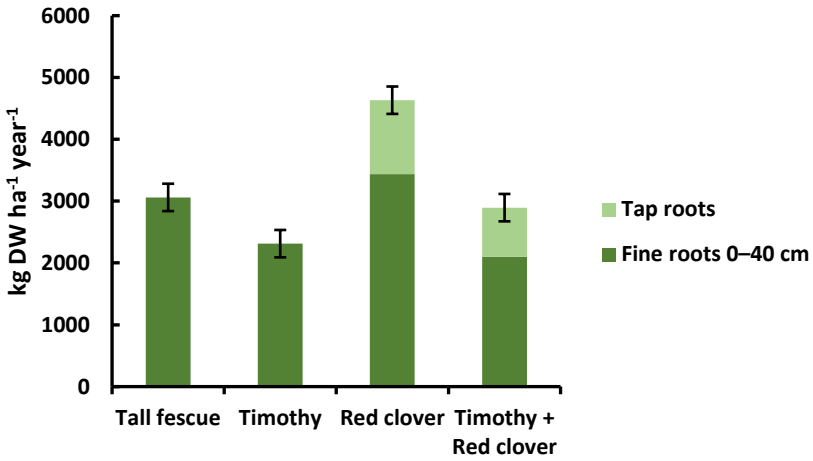

Figure 7. Root biomass is represented as mean values (kg DW/ha year) for the different treatments in the soil depth of 0–40 cm, distinguishing red clover tap roots from fine roots. The standard error of the mean is represented for combined root biomass across the whole data.

4.2 Carbon percentage

The total mean C% measured as LOI of 0–20 cm soil depth was 2.9% for all treatments with the standard deviation (SD) of 0.4% for tall fescue and red clover, and 0.5% for timothy and timothy + red clover -mixture. There was no difference in the C%, neither between the treatments (ANOVA $p = 0.993$, $df = 3$, $f = 0.030$), the sampling dates (ANOVA $p = 0.930$, $df = 2$, $f = 0{,}072$) nor the sampling dates

and treatments together (ANOVA p = 1.00, df = 6, f = 0.005). As seen in Figure 8, the change between the mean C% of the last and the first sampling date was highest in the tall fescue group, and, against the original hypothesis, smallest in the polyculture of timothy and red clover, but the change was still very small in all the treatments.

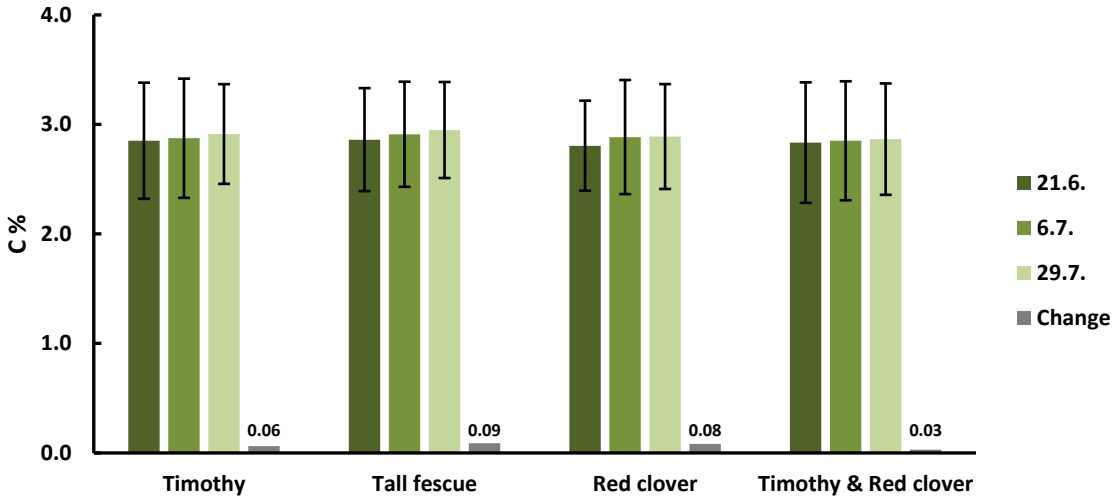

Figure 8. Soil C% means and SD's of the treatments and sampling dates in 0-20 cm soil profile. The change-value was calculated as the difference between the last and the first sampling date.

The result from Luke's data gave on average 0.7% lower C%s for the sampling depth of 0 – 20 cm (Figure 9). There was a variation between the sampling depths (ANOVA p < 0.001, df = 1, f = 61.581), 0–20 cm having 1.2% higher overall mean C% than 20–40 cm. Similarly, as above, there were no differences in C% between the treatments (ANOVA p = 0.962, df = 3, F = 0.096) nor the treatments and sampling depth together (ANOVA p = 0.892, df = 3, f = 0.205).

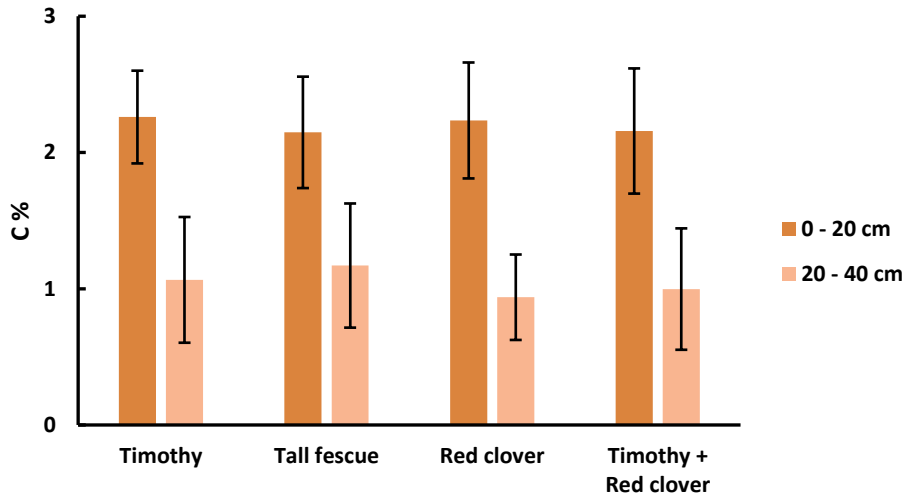

Figure 9. The result from Luke's C% data varied between the sampling depths of 0–20 cm and 20–40 cm (ANOVA $p < 0.001$, df = 1, f = 61.581), but not among the treatments (ANOVA p = 0.962, df = 3, F = 0.096) or the treatments and the sampling depth together (ANOVA p = 0.892, df = 3, f = 0.205).

The power level for treatments, sampling dates and both together were 0.05, meaning that there would be 5% probability to get a significant result with the used sample size (all in all 48 samples) and effect sizes. The effect sizes for the ANOVA-model were 2.52×10^{-3} for the treatments, 4.01×10^{-3} for the sampling dates and 7.88×10^{-4} for them both together (treatment x date). According to the sample size analysis, to get the power level of 0.85, all in all 161,422 samples would be needed, in other words, with 161,422 samples there would be 85% probability to get the groups to differ significantly.

4.3 Microbial biomass and F:B-ratio

The total PLFA content (Figure 10) was very similar between the treatments and the differences were not statistically significant (Appendix 4). The gram-negative bacteria (biomarker PLFAs: 16:1ω9c, 16:1ω7c, 18:1ω9c, 18:1ω7c, cy19:0, 20:0) were the most abundant microbial group, being 1.7 times higher than that of gram-positive bacteria (biomarker PLFAs: i14:0, i15:0, a15:0, i16:0, i17:0, a17:0). AMF was the most abundant fungi (biomarker PLFA: 16:1ω5c). Tall fescue had the biggest overall PLFA content, having 3.0% higher content compared to red clover, 7.7% to timothy, and 8.6% to the polyculture of timothy + red clover, that had the smallest PLFA content. In addition, a trace amount of methanotrophic bacteria biomarker (18:1ω6) was found within all the samples.

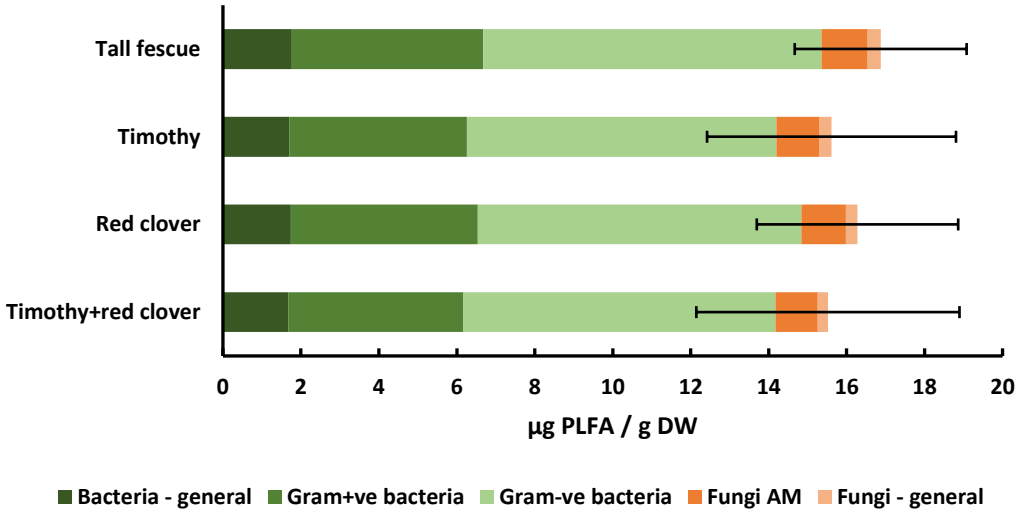

Figure 10. The total PLFA content based on microbial biomarkers (µg PLFA /g DW) of the microbial groups in the treatments.

The F:B-ratio was similar between the treatments, the bacteria being approximately 10 times more abundant than fungi in all groups (Table 4). There were no differences in the PLFA profile between the treatments (PERMANOVA: Pseudo-F = 0.198, p(MC) = 0.972).

TABLE 4. The biomass profile of the microbial community and F:B-ratio of the treatments.

Crop handling group	Fungi biomass (%)	Bacteria biomass (%)	F:B-ratio
Timothy	9.0	91.0	0.10
Tall fescue	8.9	91.1	0.10
Red clover	8.6	91.4	0.09
Timothy + Red clover	9.0	91.0	0.10

4.4 Microbial community analysis

The diversity and composition of the microbial communities was assessed to understand the effects of cultivating different northern forage crop species on the soil. While there were no differences in diversity and bacterial community among treatments, distinct variations emerged within the fungal communities. Notably, tall fescue demonstrated unique characteristics in its associated fungal community.

4.4.1 Diversity

The total number of OTUs were similar across the data of bacteria (Figure 11A) and fungi (Figure 11B). There were no differences in the total number of OTUs between the treatments neither in bacteria (PERMANOVA: Pseudo-F = 0.264, p(MC) = 0.863) nor fungi (PERMANOVA: Pseudo-F = 0.118, p(MC) = 0.954). Also, other calculated diversity indexes showed very similar results (Appendix 3).

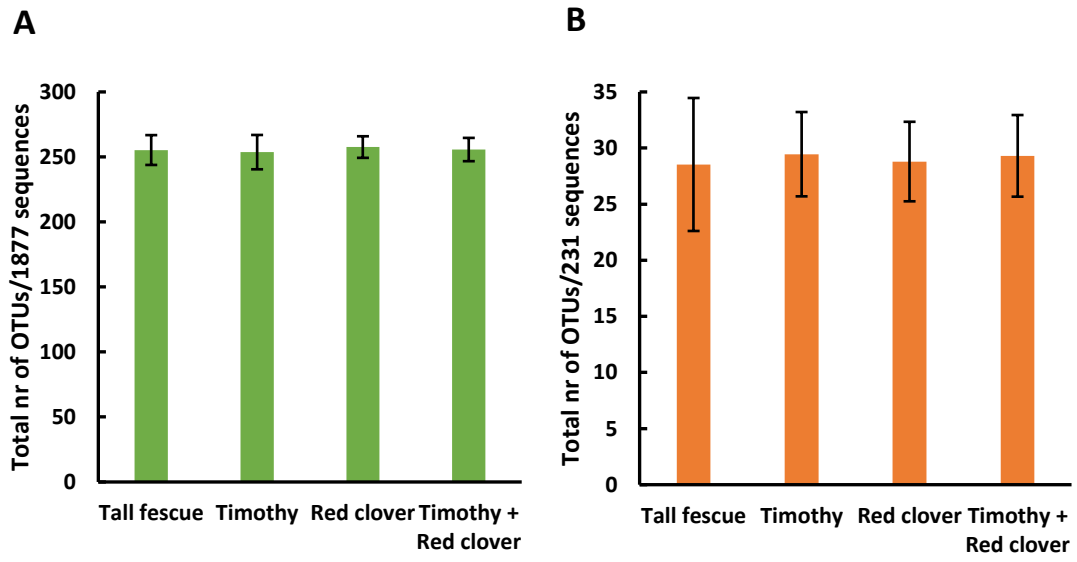

Figure 11. The total number of OTUs in A) 16S (bacteria) and B) ITS (fungi) rDNA sequence data. Due to the different number of sequences used, the scale between the figures is different.

4.4.2 Bacterial communities

Phylum-level bacterial communities were similar across all the treatments (PERMANOVA: Pseudo-F = 0.935, p(PERM) = 0.507, p(MC) = 0.492). For the whole data, Proteobacteria was the most abundant bacteria phylum, followed by Acidobacteriota, Verrucomicrobiota, Actinobacteriota, Chloroflexi and Planctomycetota (Figure 12).

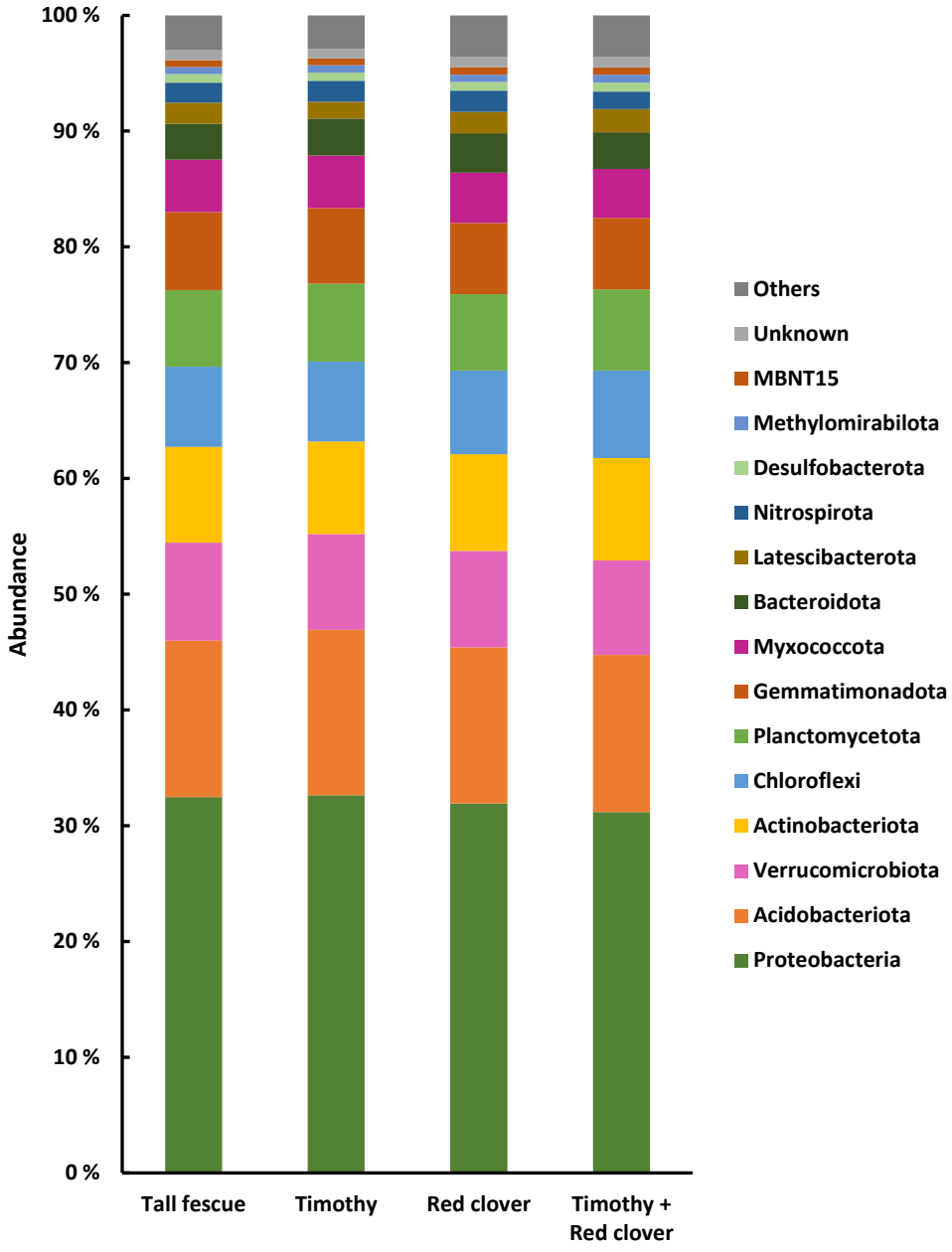

Figure 12. The relative mean abundances of the bacteria DNA sequences in phylum-level identification. 'Others' represent the bacterial phylum that were outside the 15 biggest bacterial phyla.

In the class-level, similarly than with the phylum level, the relative abundances of the major bacterial classes were similar across all the treatments (PERMANOVA: Pseudo-F = 0.952, p(PERM) = 0.534, p(MC) = 0.532). The most abundant bacteria-classes across the whole data were Gamma- and Alphaproteobacteria, followed by Verrucomicrobiota, Acidobacteriae and Gemmatimonadetes (Figure 13).

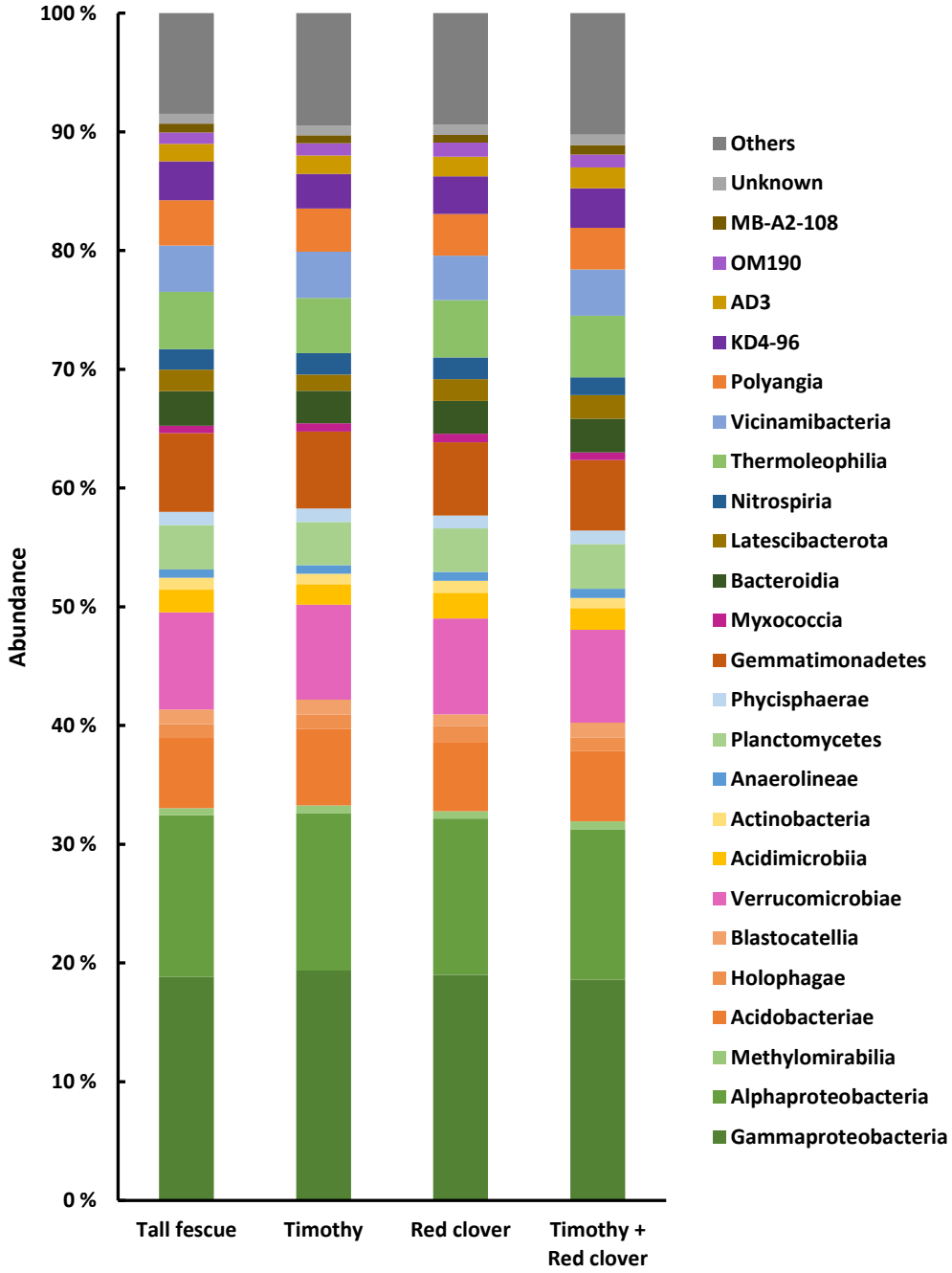

Figure 13. The relative mean abundances of the bacteria DNA sequences in class-level identification. 'Others' represent the bacterial classes that were outside the 25 biggest bacterial class.

4.4.3 Fungi communities

The treatment affected the phylum-level fungi communities according to the UNITY data (PERMANOVA: Pseudo-F = 3.082, p(MC) = 0.021) and after the Web BLAST identification (PERMANOVA: Pseudo-F = 2.499, p(MC) = 0.04). According to the SILVA data, the differences in fungi abundances were between tall fescue and timothy (Pair-wise: t = 1.79, p(MC) = 0.045), red clover (Pair-wise: t = 2.855, p(MC) = 0.003) and timothy + red clover mixture (Pair-wise: t = 2.1994, p(MC) = 0.018). After the Web BLAST -identification, the differences were between red clover and tall fescue (Pair-wise: t = 2.537, p(MC) = 0.016) and red clover and timothy + red clover mixture (Pair-wise: t = 2.024, p(MC) = 0.037). There were no significant differences between the rest of the treatments.

According to the SILVA data, the biggest phylum was Ascomycota, followed by Basidiomycota (Figure 14A). The unidentified fungi had the second greatest abundance of 40 ± 4%. Web BLAST identification showed that majority of the unidentified fungi also goes to these two major phyla and the abundance of 'Other'-fungi was more similar (15 ± 2%) across the treatments (Figure 14B). The observed differences between the treatments were in the distribution between Ascomycota and Basidiomycota, as tall fescue had the greatest abundance of Basidiomycota and least abundance of Ascomycota, while red clover had the opposite.

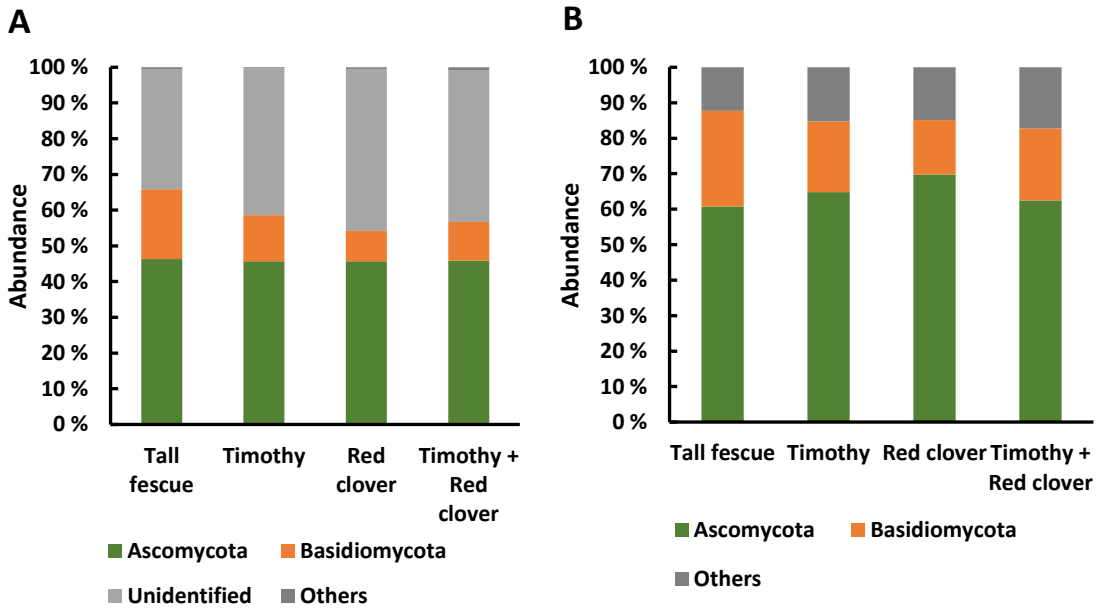

Figure 14. The relative mean abundances of the fungi DNA sequences in phylum-level identification A) before and B) after the identification of unidentified OTUs with Web BLAST standard database. 'Others' represent the bacterial phylum that were outside the two biggest fungi phylum and in B) also the unidentified OTUs with less than 0.4% combined abundance across the whole data. 'Unidentified' represents OTUs marked as 'Unidentified' or 'N/A' in OTU-clustering by SILVA database.

Similarly, in the class-level fungi, the treatment affected the fungi communities according to the SILVA data (PERMANOVA: Pseudo-F: 3.082, p(PERM) = 0.02, p(MC) = 0.018) and after the Web BLAST identification (PERMANOVA: Pseudo-F = 4.572, p(PERM) = 0.001, p(MC) = 0.001). According to the SILVA data, tall fescue fungi abundances differed from red clover (Pair-wise: t = 2.855, p(PERM) = 0.003, p(MC) = 0.006) and timothy + red clover mixture (Pair-wise: t = 2.199, p(PERM) = 0.021, p(MC) = 0.022), and the p-value with timothy was only slightly above the confidence level (Pair-wise: t = 1.79, p(PERM) = 0.06, p(MC) = 0.058). After the Web BLAST identification the effect was similar, as tall fescue differed from those of timothy (Pair-wise: t = 2.03, p(PERM) = 0.012, p(MC) = 0.014), red clover (Pair-wise: t = 2.19, p(PERM) = 0.001, p(MC) = 0.001) and timothy + red clover mixture (Pair-wise: t = 2.95, p(PERM) = 0.001, p(MC) = 0.001). There were no significant differences between the rest of the treatments.

According to SILVA data, the unidentified fungi had the greatest abundance of 44 ± 4%, followed by Sordariomycetes (Figure 15A). After the Web BLAST identification, the Sordariomycetes had the biggest abundance, and the abundance of other fungi was now smaller (21 ± 3%; Figure 15B). Dothideomycetes grew to be the second biggest fungi class, red clover having the biggest abundance that was three times bigger than that of tall fescue with the least abundance. Tall fescue, on the other hand, had the greatest abundance in Eurotiomycetes and Agaricomycetes, and least abundance in Tremellomycetes, compared to the other groups.

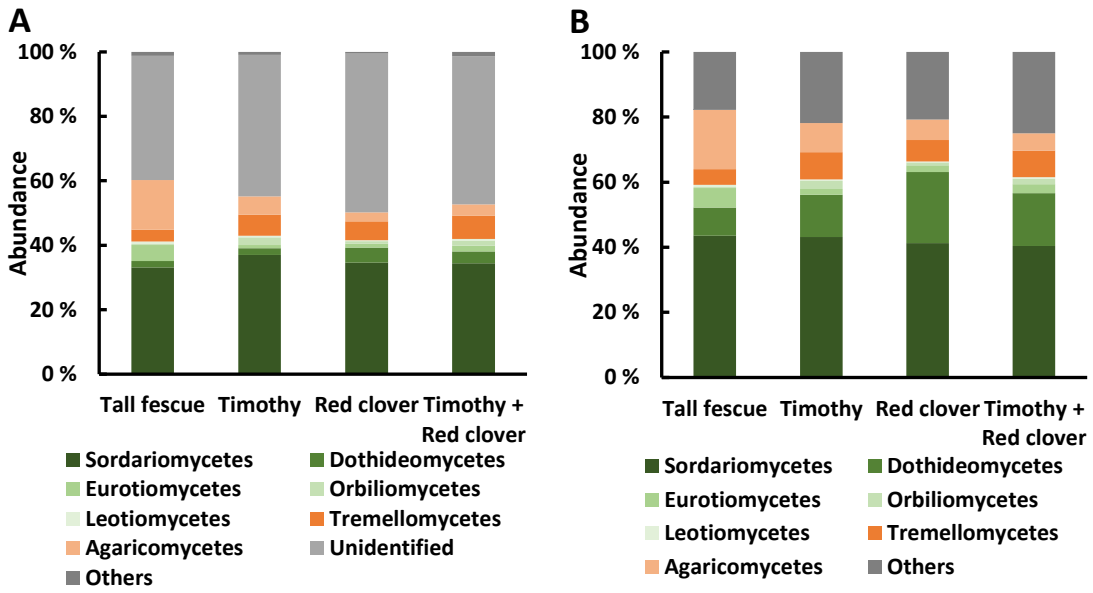

Figure 15. The relative mean abundances of the fungi DNA sequences in class-level identification A) before and B) after identification of the unidentified classes with Web BLAST. 'Others' represent the fungal classes outside the eight biggest fungi class and in A) also the unidentified OTUs with less than 0.4% combined abundance across the whole data. 'Unidentified' represents the OTUs marked as 'Unidentified' or 'N/A' in OTU-clustering by SILVA database.

The MDS plot shows that the fungal community composition from the 2nd and 3rd sampling dates of tall fescue is parted from the other samples, based on 80% Bray-Curtis similarity assumption (Figure 16). Tall fescue treatment increased the abundance of Agaricomycetes from Ascomycota-phylum and Eurotiomycetes and Leotiomycetes from Basidiomycota-phylum, while other groups had higher abundance of Sordariomycetes from Ascomycota-phylum and Tremellomycetes from Basidiomycota-phylum. The microbial composition of tall fescue also seemed to fluctuate more in time than the other treatments.

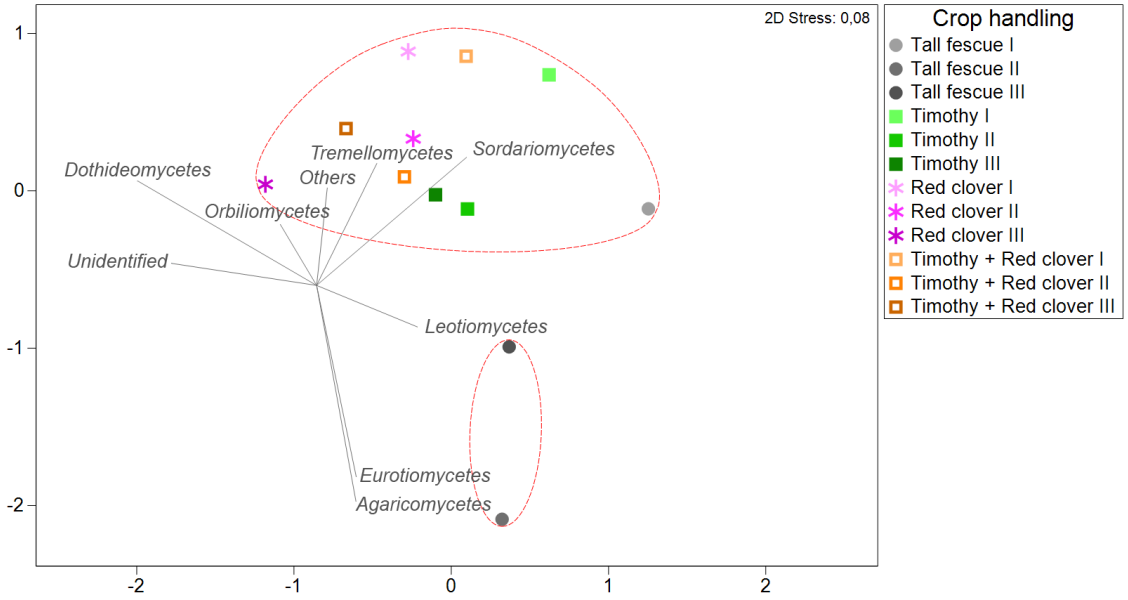

Figure 16. Multidimensional scaling (MDS) of the class-level fungi community composition, with different colors and shapes representing the treatments (crop handling) and the color shading the different sampling dates (light I = 21.6., medium II = 6.7. & dark III = 29.7.). The red dash line representing the 80% Bray-Curtis similarity shows that there are two groups with similar microbial community structures under 80% similarity assumption. The black lines represent the found classes of the fungi community.

4.4.4 Microbial community structure

There were no statistically significant differences between the microbial communities of the treatments in 80% Bray-Curtis similarity assumption. Still, even if the treatments do not show any clustering in Figure 17, a slight grouping can still be observed between the sampling dates. The samples of the 1st sampling date seemed to have a bigger abundance of Ascomycota, 2nd sampling date bigger abundance of Basidiomycota, and finally 3rd sampling date bigger abundance of the unidentified fungi and bacteria. Most of the differences in the figure can thus be seen in the fungi phylum levels, which point in separate directions in the MDS figure. All the fungi phyla also had a high MDS correlation (Pearson's r), Ascomycota having correlation of r = 0.915, Basidiomycota r = -0.900 and Unidentified r = 0.847.

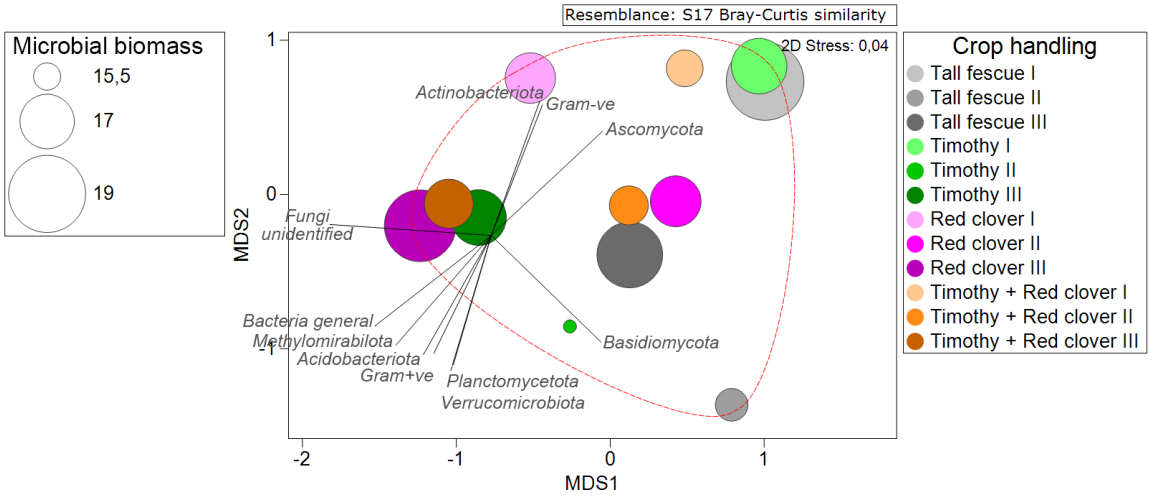

Figure 17. Multidimensional scaling (MDS) of the phylum and group -level microbial community composition (n = 3), with different colors representing the treatments (crop handling) and the color shading the different sampling dates (I = 21.6., II = 6.7. & III = 29.7.). The size of the bubble represents the overall microbial biomass defined by the PLFA content (µg/g DW) of the sample. The red dashed circle represents the 80% Bray-Curtis similarity between the samples, meaning, the samples inside the circle share similar microbial community structures under 80% similarity assumption. The black lines represent the phylum and microbial groups which most strongly explained the structural differences in microbial communities between treatments (Pearson correlation threshold value 0.8).

5 DISCUSSION

In this study, I delved into the effects of three commonly cultivated Northern forage crop species, timothy, red clover, and tall fescue, along with the combination of timothy and red clover, on the C sequestration and microbial community structure. C sequestration potential was drawn by examining the crop yield, root biomass and soil C%, while microbial community structure was perceived from bacterial and fungi phyla and classes, species diversity, biomasses and F:B-ratio. The results highlighted the complex interaction between various biotic and abiotic factors, and the challenges of C sequestration into the northern soils.

As expected, timothy did not stand out in any of the evaluated parameters, bolstering the idea that its primary advantage lies in its winter durability. While timothy can enhance stress-tolerance of grass mixtures, it does not necessarily contribute to soil C sequestration or stabilization. Surprisingly, red clover emerged as the standout performer in this study, revealing an intriguing soil C sequestration potential. Despite its modest yield, red clover's root system, particularly its robust taproot, had by far the highest biomass, and its fungi community indicated a more stable soil C. Conversely, tall fescue did not perform as expected. While it excelled in yield, it did not exceed the root biomass of red clover, and its microbial structure did not seem to favor C stability, either. I'll delve deeper into these findings in chapters 5.1 and 5.2. The polyculture of timothy and red clover also fell short in terms of the expectations of C sequestration and diversity, which I will explore further in chapter 5.3. Beyond these findings, no differences were found in C%, microbial diversity and biomasses, F:B-ratio or bacterial taxonomy.

As said, the bacterial community showed consistent results across the treatments, and no significant differences were observed between the cultivation set-ups. One factor to consider is the potential presence of background DNA in the samples. Some studies suggest that 40% of the soil DNA can be so-called relic DNA, referring to extracellular DNA (eDNA) from dead microbes that can stay in soil even for years, which can cause bias to the DNA sequencing data and cause the microbial diversity to be even 55% higher (Carini et al. 2016). Apart from the bias to microbial diversity, relic DNA can cause difficulties in detecting changes especially within the highly abundant bacterial taxon (Sun & Ge 2023). Given that my analysis was based on DNA method, there is a possibility that the samples might contain significant amount of relic DNA, not just causing bias to the observed taxonomic abundances but also fading out the possible differences between the living microbial communities. According to Gebhard & Smalla (1999), relic DNA can stay in soil for up to 2 years. Due to the cultivation period of two years in this study, there is a possibility that the history of the field might play a noteworthy role in the composition of microbial DNA in soil, and this effect could then have been further enhanced with the randomized sampling

method, where the subsamples taken from a barren spot might have caused the sample to contain a lot of bulk soil unaffected by the plant roots. Therefore, relic DNA might have caused dispersion in the sample due to the sampling method and short handling time and thus, the differences in the microbial communities between the treatments are not clear. A potential future alternative could be to employ an RNA-based approach rather than DNA to analyze the active microbial community. While this approach may not directly measure microbial activity (Carini et al. 2016), it does eliminate the eDNA that can preserve in soil and sediment for extended periods (Dlott et al. 2015). Another solution would have been to distinguish eDNA from viable versus dead cells during PCR by treating the samples with propidium monoazide prior to DNA extraction, as it selectively binds to and modifies eDNA from dead cells, inhibiting its amplification during PCR (Sun & Ge 2023). The third thing to consider would have been to use a more specific sampling technique, where bulk soil and rhizosphere are collected and analyzed separately, excluding the possibility of too much bulk soil causing the differences to disappear.

Even if the bacterial community held its constancy across the treatments, the fungal community was affected by the cultivated plant. This strengthens the idea that fungi have a pole position when it comes to the uptake of plant exudates, making them very effective in C sequestration (Hannula et Morriën 2022). Given their ability to establish symbiotic associations, this also suggests that fungi have a more pronounced relationship with plants than bacteria and they are highly responsive to the alterations in plant biota. This highlights the important role of fungi in shaping the rhizosphere and impacting soil health. Therefore, future research should focus on exploring the mechanisms through which the interaction of plants and fungi turn into enhanced carbon sequestration.

Even if the fungal taxa varied between the treatments, the initial DNA sequencing data yielded many unidentified sequences, suggesting that there is still much to study about the world of fungi. The Web BLAST identification decreased the number of unidentified sequences and provided a more detailed view of the taxonomical structure of the fungal community. Notably, Web BLAST identification also changed the fungal taxa composition. However, this method, while insightful, is time-consuming and unreliable as it is done manually from an unratified database. In addition, accessing the functional traits of the taxon is challenging yet fundamental. The functional traits of fungi affect both microbial community structure and ecosystem function, so understanding them can help to predict the effects of environmental changes on the whole ecosystem (Koide et al. 2014). Thus, gaining a better insight into fungal taxonomy and its functional traits is crucial for future research.

During the writing process of this study, I deployed the AI-assisted methods and the experience was altogether highly positive. This paper was my 2nd work where I employed the AI and throughout the process, I learned how to better work with it and where and how to deploy it. Using the AI enhanced my grasp of the subject, expanded my vocabulary, and improved my language skills efficiently. It helped with the writer's block by helping to get new ideas to study

further with reliable sources. Thus, using the AI significantly accelerated the writing process and made it easier to get started. For instance, I was able to draft the discussion with the AI in just three days, which then made it more straightforward to proceed with a more detailed examination of the results. It is still worth noting that the consulting and referencing of the scientific articles led to substantial revisions and refinements to the draft, enriching and deepening the work considerably. For instance, in the later revisions of the discussion, the structure was changed drastically, and the content was significantly enriched.

5.1 Soil carbon: Red clover stands out with its tap root

Red clover stood out for its notable root biomass, and particularly due to its substantial tap root. Surprisingly, tall fescue did not exceed red clovers root biomass even when the tap root was not considered, and did not enhance the soil C, either. Nevertheless, tall fescue stood out in terms of yield performance in both the first and second cuts, exceeding the yields of red clover, timothy, and their mixture, and aligning with my hypothesis regarding its higher yield performance. This finding also aligns with the study by Cougnon et al. (2014), although they did not compare the yield of tall fescue with timothy or red clover, but with perennial ryegrass (*Lolium perenne*), instead.

Despite the differences between the treatments in the yield and root biomass, this study could not reveal any shortcut for enhancing C input into the soil of northern grasslands. All the treatments showed similar soil C% values, suggesting that the short-term influence of plant species on SOC may be more complex than previously assumed. The mean C% of 2.9% observed in this study is notably below the 3.8–4.0% documented by Kätterer et al. (2013) concerning the top layers of mineral soil in Sweden with 4–6-year ley rotations, and 3.5–4.8% reported by Salonen et al. (2023) following 24 years of 5-year crop rotation on intensively managed glaciofluvial clay soil in Finland. On the other hand, Heikkinen et al. (2020) reported slightly lower C% values of 2.2–2.6% for the grass fields across Finland. Nevertheless, it is noteworthy that the content of clay and silt, along with the amount of aluminum and iron, significantly impacts the potential for C preservation (Salonen et al. 2023), so the differences in C% can simply rise from the differences in the soil texture. All in all, the soil C% of 2.9% observed in this study could be described as typical value to Finnish soils. Additionally, Yang and the group (2019) observed the increased soil C only after 13 years of grassland biodiversity restoration in Minnesota, USA, suggesting that longer cultivation time and ley rotation may provide an enhanced window of time for organic matter accumulation and stabilization, while our two-year cultivation might not have allowed sufficient time for notable changes in C levels to occur.

Carter & Gregorich (2009) reported an increased higher C storage of tall fescue after seven years of cultivation, which could not be observed in this study. This supports the idea that the two years of cultivation were not long enough for this effect to be seen. On the other hand, Carter & Gregorich (2009) did not report

any significant increases within soil layers of 10–40 cm, but most of the differences were either on very shallow soil surface of 0-10 cm or deeper than 40 cm in the soil. This study focused on the 0–20 and 0–40 cm layer, excluding the deeper soil layers affected by tall fescue, and possibly fading potential changes in the surface layer.

Furthermore, the consistency in soil C% over the sampling times describes the area's fertile soil and stabile C reservoir, that could increase on time. This also reflects the area's long history of grassland cultivation with intensive fertilization, compared to for example a recently cleared forest or drained swamp, which would likely show a decline in C% (eg. Bolinder et al. 2010). On the other hand, Hasslink et al. (1997) suggests that soil possesses an upper limit for C stabilization, and the comparatively low C% result in this study indicates that there is still room for more C storage within longer ley rotation. The marginal increase in C% over the sampling times also hints at the possibility that ongoing cultivation could have enhanced soil C% over time, despite the cultivated forage plant. Even if the power analysis suggested a vigorous sample size of 161,422 with the power-level 0.85, it is noteworthy that even a minor positive swift in C% occurred within a brief 1.5-month span. This indicates that extending the study and sampling duration and increasing the sample size could lead to a significant finding, potentially revealing an increase in C% and possibly also differences among the handling groups. Heikkinen et al. (2020) suggested that the minimum sample size of several hundred would be needed, and the sample size increases with the soil C content. Yet, this number is significantly lower than the impractically large sample size found in the power analysis of this study.

5.2 Microbiome: Red clover reveals carbon stability potential

Ascomycota was the major fungi phylum across the treatments, with Sordariomycota and Dothideomycota being the most abundant fungi classes. Ascomycota is recognized as the most abundant soil fungi worldwide, with Sordariomycota and Dothideomycota being among the most common classes (Edigi et al. 2019), so the result corresponds with the previous findings. Ascomycetes are generalists with different types of lifestyles, including decomposers, parasites as well as symbionts (Webster & Weber 2007). They are suggested to dominate in soils over Basidiomycetes due to their better stress-tolerance partly due to accumulation of melanin into their hyphae (Koide et al. 2014, Egidi et al. 2019). Melanin, also described as 'fungal analogue to lignin', is a group of complex polymers found in fungal cell wall that are hard to break down and thus, they are implied to enhance the soil C stock (Fernandez & Koide 2014).

A greater abundance of Ascomycetes, more specifically Dothideomycetes, were found within red clover. This could indicate a more stable soil C content, potentially resulting from the increased buildup of Ascomycota necromass. This could mean that despite the similar soil C% among the treatments, the red clover could help with retaining the gained C in the soil even after the cultivation. This

result, among the finding of higher root biomass in red clover, could strengthen the idea of red clover being an apt choice for carbon farming in the northern grasslands, further endorsing its inclusion in seed mixtures. Still, more research would be needed to confirm this assumption. First, the taxonomy of Ascomycota could be investigated in more detail, and second, it could be further investigated if the red clover indeed accumulated more melanin into the soil. Furthermore, it should be noted that cultivating red clover as a monoculture might not be advisable, as the larger field area required to achieve a comparable yield to grasses could easily offset the benefits of carbon stabilization.

The increased abundance of Agaricomycetes within tall fescue would further support the hypothesis of faster C turnover in tall fescue than red clover. Agaricomycetes, from the Basidiomycota phylum, are recognized as effective decomposers of complex organic compounds (Liers et al. 2013, Mali et al. 2017), which might signal more efficient decomposing of organic matter and thus, increased release of C into the atmosphere. Even if the below and underground biomass of tall fescue increased the SOC stock, the C of tall fescue cultivation might not be as stable as within red clover.

Yet, this hypothesis of more stable C within red clover is not directly supported by the consistency of F:B-ratio across the treatments. Higher F:B-ratio is typically linked to more stable C storage due to increased microbial necromass and a healthy soil food web (Allison et al. 2005, Keiblinger et al. 2010, Hannula & Morriën 2022). Despite the differences in the fungal community structure, the microbial biomasses and F:B-ratio were similar across the treatments, indicating a stable soil microbial community that maintains its balance regardless of the type of forage crop cultivated. The stable F:B-ratio also indicates the functional redundancy of the soil, which means that while the microbial composition might be differing, the microbes may perform similar functions, leading to a stable soil ecosystem. Functional redundancy is usually more conserved in soil microbial communities than taxonomical redundancy, and thereby these both should be considered when studying microbial community structures (Chen et al. 2022). Nonetheless, the consistency in F:B-ratio does not necessarily invalidate the hypothesis of red clover having more stable C stock, as they are indicators of different kinds of stability. Also, there still were marginal differences between microbial biomasses, even if not statistically significant. Another potential reason for the consistent F:B-ratio could be pH, which profoundly influences the F:B-ratio (De Vries et al. 2006, Rousk et al. 2011, Yu et al. 2022). Thus, the consistency in the F:B-ratio implies that the abiotic properties of the soil might have provided a buffer against the changes in F:B-ratio.

Moreover, the average F:B-ratio of 0.10 reported in this study appears to be on the higher side when compared to other studies. For instance, Malik et al. (2016) studied two different sandy loam fields in Germany, revealing F:B ratios of 0.03 and 0.04, respectively. However, their reliance on 18:2ω6c as a fungal biomarker, in contrast to my use of both 18:2ω6c and 16:1ω5c, could draw bias when making comparisons. Conversely, a recent study by Siles et al. (2023), converging the European Union, including Finland and Sweden, reported an

average F:B-ratio of <0.10 in cold climate grasslands. This aligns with the 0.10 ratio from this study, suggesting its validity. They also found that grasslands typically exhibit lower F:B-ratios compared to forests or even croplands, but the total microbial biomass in the grasslands is higher than in croplands (Siles et al. 2023). Furthermore, Yu et al. (2022) reported fungal dominance in soils with a pH <5-6. Given that the research site of this study has a pH of 6.2, only slightly above this range, it is plausible that the higher-than-average F:B-ratio could be attributed to the conditions favored by fungi. On the other hand, mineral N fertilization is believed to rapidly decrease the fungal biomass and F:B-ratio shortly after N application, and the inclusion of clover with grass cultivation can further amplify this effect (De Vries et al. 2006). This might have influenced the results in this study as well, considering that the applied levels of fertilization were used. The variation in N fertilization could have been balanced by the N fixed by the red clover root nodules, leading to a similar effect of decreased F:B-ratio across all treatments.

Apart from pH, other abiotic conditions like temperature and moisture also influence microbial activity and community composition as well as soil C dynamics (Cates et al. 2019). Different sampling times could have, thus, captured the soil microbial community in different states influenced by changes in temperature and soil moisture content as well as growth stages of plants. The first sampling was conducted during a cooler period with moderate rainfall, but by the time of the second sampling, the region experienced a warmer phase of a heatwave, leading to drier conditions despite the rainfall. The third sampling period presented a more humid period with moderate temperatures and consistent rainfall. The MDS analysis indicated that the similarities between the sampling times could be more distinct than those between the treatments, suggesting that the environmental conditions linked to the sampling times may have played a role in shaping the microbial composition. The microbial biomasses seemed to show a slight decrease during the second sampling time, which would be in line with the previous findings of warm and dry conditions decreasing the microbial biomass (Cates et al. 2019). This suggests that abiotic factors should be reviewed more closely in future research. It is also possible that taking samples during multiple time points might have caused more deviation to the samples and, thus, no differences between the communities were observed. This tells the story of how challenging field study can be as the abiotic factors are hard to control. Thus, it would be important to acknowledge the abiotic factors in better detail in the future.

Within the bacteria communities, Proteobacteria, particularly Gamma- and Alphaproteobacteria, dominated across the treatments. This result was expected, as Proteobacteria are the most abundant and diverse bacteria on Earth with various roles and functions in the soil (Kormas 2011), including the N fixating bacteria like Rhizobia (Velázquez et al. 2011), among others. Previous studies have shown varied results for the abundance of Proteobacteria in soil, for example Spain et al. (2009) reported 25–40% abundance in undisturbed tall-grass prairie in Oklahoma, while Chen et al. (2019) reported abundance of only 15.5%

in semi-arid grassland in Mongolia. Both sites represent natural, unfertilized grasslands, while Proteobacteria average abundance of 32% observed in this study shows a result from intensive cultivated northern grassland. N fertilization is suggested to increase the abundance of Proteobacteria in soil (Dai et al. 2018), which could be seen when making the comparison between natural grasslands and the grasslands under intensive fertilization.

In contrast to the finding of increased abundance of Proteobacteria withing N fertilization (Dai et al. 2018), the levels of Proteobacteria and the whole bacterial community remained constant across the treatments, despite the varying N fertilization levels. In this study, the applied levels of mineral fertilization were used: timothy and tall fescue received the full dose, the timothy-red clover mixture received half and red clover received no N fertilization. It is a common farming practice to replace some or all the additive N by cultivating the N fixing legumes like red clover. The enhanced N by red clover could, thus, work similarly than N fertilization, increasing the amount of Proteobacteria and thus, the bacteria communities do not show any differences.

5.3 Diversity: Polyculture underperformed

Contrary to the hypothesis, the timothy-red clover mixture did not outperform the monocultures in this study. The yield of the mixture was somewhat of an average of the two crops when grown individually, root biomass even less than the average, and the C% did not show any significant increase compared to the monocultures. Thus, I could not witness the synergistic effect, where growing grasses and legumes in polyculture leads to bigger crop yield (Sturludóttir et al. 2014) and enhanced soil C input (Bai & Contrufo 2022) compared to monocultures. Previous study suggests a clearer increase in soil C with higher plant biodiversity (Yang et al. 2019), particularly when the cultivation included woody plants (Kreitzman et al. 2022), suggesting that more than two crops could have been needed for the effect to show.

On the other hand, Cougnon et al. (2013) compared the yield of tall fescue to the polyculture tall fescue-white clover (*Trifolium repens*) and did not find a synergistic effect, either, which aligns with the result in this study with timothy and red clover. Interestingly, they used applied levels of fertilization like the one used in this study, with whole dose (300 kg N ha^{-1} $year^{-1}$) for the tall fescue and approximately half dose (165 kg N ha^{-1} $year^{-1}$) for the mixture with clover, although the annual N amounts in this study were 1/3 lower. Grasses react strongly to fertilizing, reaching their full yield potential with these amounts of fertilizers used, and thus, adding low-yielding clover does not further increase the herbage yield. Instead, it reduces the need for N fertilization, which has economic benefits for the farmer, as well as positive environmental effects with reduced N runoff.

Microbial diversity is often linked to better plant productivity (Yang et al. 2017, Sanchez-Cañizares et al. 2017, Stefan et al. 2021), but even if the biomass differed among the treatments, the soil microbial diversity showed no difference.

Furthermore, polycultures are associated with enhanced microbial diversity, as higher selection of crops release a more diverse set of root exudates into the soil, enabling environmental conditions for a wider range of soil microbes (Bakker et al. 2020). This has been shown in previous studies, where growing plants in polyculture have indeed led to higher microbial diversity (LeBlanc et al. 2015, Cline et al. 2018, Stefan et al. 2021). However, in this study the polyculture of timothy and red clover did not exhibit a significant effect in microbial diversity. This discrepancy could result from various factors, one being the quantity of species in the mixture. For instance, both Le Blanc's (2015) and Cline's (2018) research groups showed the effect by comparing the monoculture to the polyculture of 16 forage crop species, while Stefan et al. (2021) used eight annual crop species, all using much higher selection of crops than the two species used in this study. Another influencing factor might be the two years duration of the study, as LeBlanc's group (2015) had a duration of 15 years and Cline's group (2018) 20 years, both significantly longer than two years in this study. On the other hand, Stefan et al. (2021) was able to show the connection between crop and microbial diversity with their annual crop cultivation set up in just 3–4 months of cultivation, challenging this idea. Lastly, the randomized sampling technique employed in this study might have influenced the result, as the sample could have included a significant amount of bulk soil, potentially masking any variations in the microbial composition. While Le Blanc et al. (2015) collected the samples from the rhizosphere of selected crop species, both Cline's (2018) and Stefan's (2021) research groups described the pooling of four or five soil samples into one sample, showing similar type of sampling than in this study, and challenging the idea of ineffective sampling technique.

Abiotic factors may also play a role in this effect. Cline et al. (2018) was able to show a microbiome responding to crop diversity in Switzerland but not in Spain, the two sites that differed in terms of precipitation, nutrient availability, and soil texture. They suggested that higher humidity, fertilization, and clay content increased the microbial biomass, noting that these factors exerted a more significant impact on microbiome than crop diversity itself (Cline et al. 2018). Knowing these factors, the crop species richness of this mixture of grass and legume might be overshadowed by the long history of intensive fertilization, stabile clay content, as well as relatively humid climate in the northern research area of this study.

Another contributing factor might be related to the concept of plant functional type, which means categorization of plants based on their functional attributes and ecological responses, rather than their taxonomic classification (Keddy 1992). These attributes can include many types of traits related to, for example, plant size, flowering, or stress-tolerance (Louault et al. 2005) and those traits are furthermore affected by the agricultural management practices employed (Kahmen & Poschlod 2008). Thus, when designing polycultures, it is essential to consider these plant functional types as opposed to the on-going agricultural practices. While timothy and red clover differ in their functional type in many ways, such as their plant family, root and leaf structure, flowering

patterns, stress-tolerance, and symbiotic N-fixing ability, they also possess several shared functional traits and ecological niches. Both are herbaceous perennials that utilize C_3 photosynthesis, are well-adapted to cold climates, and are cultivated for forage. Additionally, they both grow as understory plants, in contrast to, for instance, woody plants or vine vegetation. These similarities might weaken the benefits of polyculture compared to the results gained from previous studies. For instance, the research groups of Le Blanc (2015) and Cline (2018) used crop species from four different functional groups, including C_4 grasses, C_3 grasses, legumes, and non-legume forbs, including woody plants. Also, Stefan et al. (2021) had four different functional groups, including C_3 grasses, legumes, superrosids, and superasterids. All these set-ups show significantly higher plant diversity than used in this study, both taxonomically and functionally.

Still, cultivating timothy and red clover mixture instead of monocultures could offer economic advantages for farmers due to red clover's ability to reduce the need for mineral fertilization. In the agricultural practice of intensive fertilization, the soil becomes rich with N, allowing the grasses to thrive and produce their full potential even in monocultures. As a result, while the yield differences between monocultures and polycultures might not be seen, the benefit lies in the reduced need for fertilizing. Farmers have also noted several advantages of red clover, such as improved soil quality through increased SOC, soil microbiome and earthworm population, improved soil structure and water balance, and an ability to inhibit weed growth (Peltonen-Sainio et al. 2022).

6 CONCLUSIONS

In this study, I set out to understand the impact of different northern forage crop species on various aspects of C sequestration and microbial community. I found that different crop species had distinct influences on the crop yield, root biomass and soil fungal community structure, though soil C%, microbial diversity, F:B ratio and bacterial taxonomy remained unchanged. Interestingly, tall fescue emerged as notably advantageous in terms of yield, while red clover showed potential for C accumulation in the root biomass, and stabilization with the increased amount of Ascomycota in soil, but more research is needed to confirm this assumption. While my expectations regarding polyculture of timothy and red clover enhancing microbial diversity were not met, it is possible that the synergistic benefits of polyculture require more complex plant interactions than the two-species mixture studied. Additionally, years of fertilization in the intensive cultivated grassland could overshadow the advantages of polyculture. However, even though the plant and root biomass varied, the soil C% remained consistent across the treatments and sampling times. This could be attributed to the relatively short cultivation time of two years, as C accumulation from roots to soil might require a more extended period. These findings highlight the complex interactions between plants and soil microbes and underline the need for more research to define the agricultural practices for carbon farming in the northern regions.

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to the Natural Resources Institute for granting access to the cultivation setup, covering the costs of molecular and fatty acid analyses, and for their assistance with sampling, as well as Jyväskylä University for providing their laboratory facilities for analyses. I also thank K. Ristiniemi for the help with graphic design. Finally, my gratitude goes to the European Agricultural Fund for Rural Development for funding the 'Sustainability from Grasslands' -project.

Vantaa November 8th, 2023
Anni Palvi

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APPENDIX 1. Supplement data of the rDNA gene sequencing

The results of the first and second-round PCRs, library quality check, and NGS run summary for 16S rRNA and ITS gene sequencing are shown in Figures 1–4.

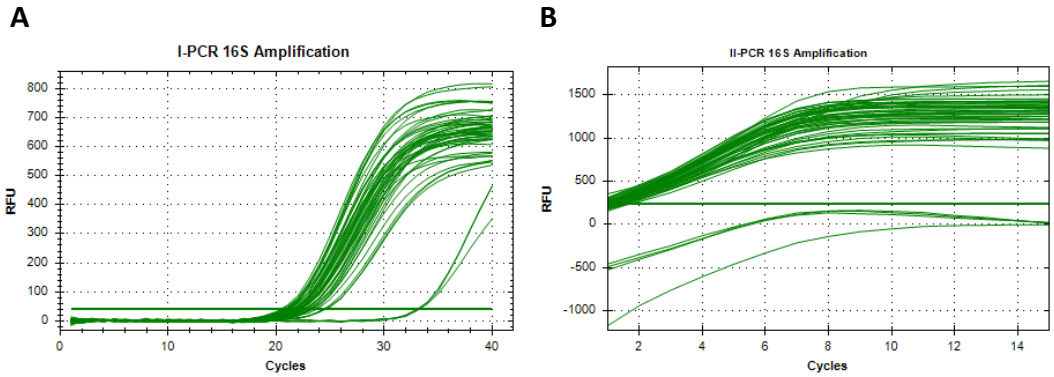

Figure 1. The 16S rRNA gene A) first- and B) second-round PCRs result graphs show a successful result. In the A) first-round PCR the no template control samples exceeded the threshold only after 32nd round of amplification so the result can be considered as a good quality.

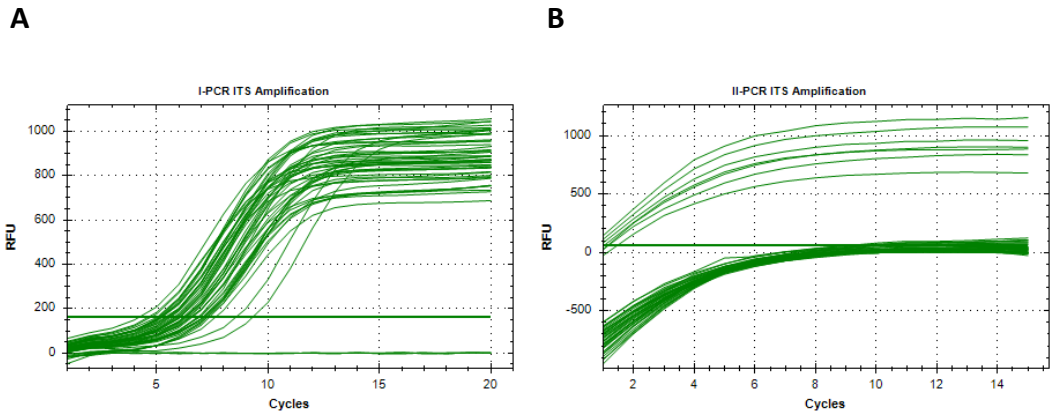

Figure 2. The ITS A) first- and B) second-round PCRs result graphs show a good quality result. Due to the software error the A) first-round PCR was made in two consecutive runs so the graph shows the second run cycles 21–40.

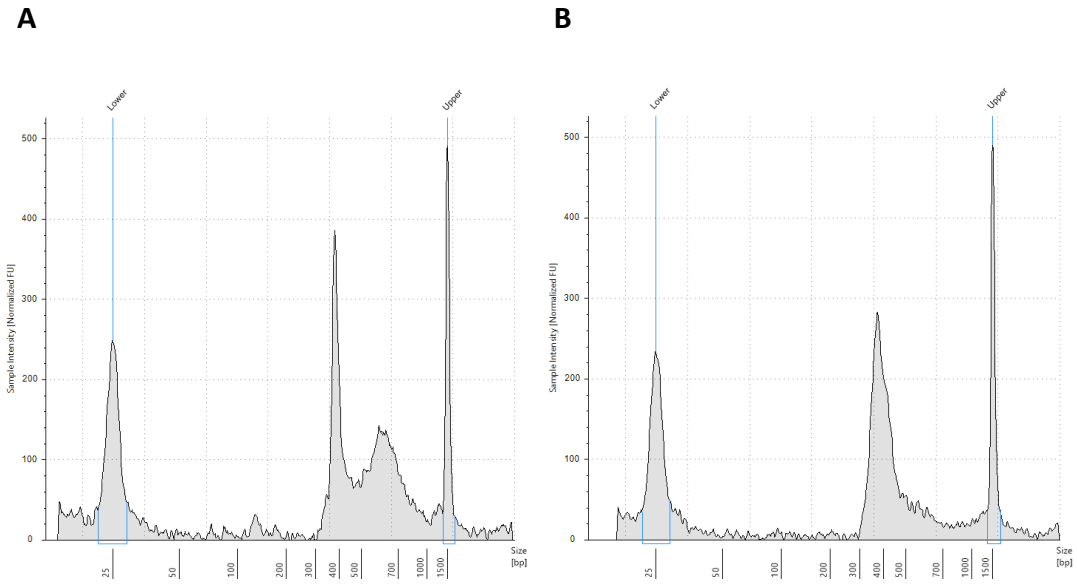

Figure 3. The Tape Station run was performed to the A) 16S rRNA gene and B) ITS libraries. The data was confirmed to be good quality as the majority of the sequences were >300 bp in length.

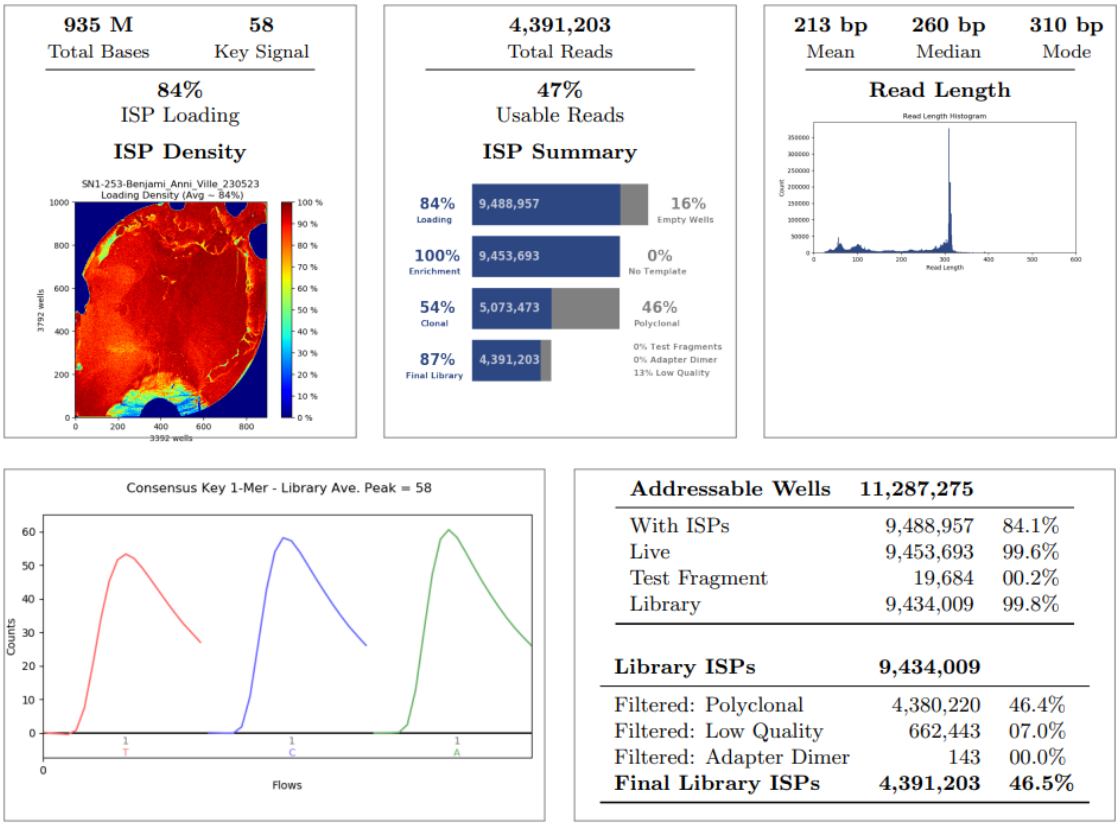

Figure 4. Summary of the Ion Torrent sequencing run.

APPENDIX 2. Supplement data of the PLFA-analysis

Table 1 shows the identified fatty acids (FA) from the samples and their information. The composition of the 566c FAME standard mixture can be seen in Table 2. The obtained internal standard (ISTD) yields can be seen in Table 3.

TABLE 1. All in all, 29 FAs were identified from the samples, including ISTDs. 17 of them were identified as a biomarker for microbial cells (Frostegåård et Bååth 1993, 1996: Olsson et al. 1995; Zelles 1997, 1999), and were used for analysis. Two of them were identified to origin from background or plant cells and were discarded.

Fatty acid	Retention time	Major peak	Reference ions (m/z)	Biomarker status
13:1	6.00	69	55, 226	
i14:0	6.07	74	242	gram+ bacteria
14:0	6.40	74	242	general bacteria
i15:0	6.79	74	256	gram+ bacteria
a15:0	6.93	74	256	gram+ bacteria
15:0	7.20	74	256	general bacteria
i16:0	7.65	74	270	gram+ bacteria
16:0	8.1	74	270	background/plant cell
16:1ω9	8.25	69	55, 268, 192	gram- bacteria
16:1ω7c	8.45	69	55, 268, 164	gram- bacteria
16:1ω6c	8.58	69	55, 268, 150	
i17:0	8.65	74	284	gram+ bacteria
a17:0	8.85	74	284	gram+ bacteria
16:1ω5c	8.98	69	55, 268, 136	AM fungi
17:0	9.20	74	284	general bacteria
17:1ω9c	9.57	69	55, 282, 192	
18:0	10.37	74	298	background/plant cell
18:1ω9c	10.70	69	55, 296, 192	gram- bacteria
18:1ω7c	10.79	69	55, 296, 164	gram- bacteria
18:1ω6c	10.95	69	55, 296, 150	methanotroph
18:2ω6c	11.35	67	81, 294, 150	general fungi
19:0	11.63	74	312	ISTD
cy19:0	12.06	74	312	gram- bacteria
20:0	12.98	74	326	gram- bacteria
22:0	15.68	74	354	
22:1ω9c	16.12	69	55, 352, 192	
23:0	17.08	74	368	ISTD
24:0	18.43	74	382	
26:0	21.065	74	410	

TABLE 2. The composition of the 566c FAME standard mixture (Nu-Check Prep, Elysian, MN, U.S.A.).

Fatty acid methyl ester	C:D	Proportion by mass (%)
Methyl octanoate	8:0	1
Methyl nonanoate	9:0	1
Methyl decanoate	10:0	2
Methyl undecanoate	11:0	3
Methyl undecanoate	11:1	1
Methyl laurate	12:0	4
Methyl tridecanoate	13:0	1
Methyl tridecanoate	13:1	2
Methyl myristate	14:0	4
Methyl myristoleate	14:1	1
Methyl pentadecanoate	15:0	3
Methyl palmitate	16:0	5
Methyl palmitoleate	16:1	1
Methyl heptadecanoate	17:0	2
Methyl heptadecanoate	17:1	1
Methyl stearate	18:0	3
Methyl oleate	18:1	2
Methyl vaccenate	18:1	2
Methyl linoleate	18:2	4
Methyl gamma linolenate	18:3	3
Methyl nonadecanoate	19:0	2
Methyl -linolenate	18:3	1
Methyl arachidate	20:0	4
Methyl 11-eicosenoate	20:1	1
Methyl 11-14-eicosadienoate	20:2	3
Methyl homogamma linolenate	20:3	2
Methyl arachidonate	20:4	5
Methyl 11-14-17-eicosatrienoate	20:3	1
Methyl behenate	22:0	1
Methyl erucate	22:1	4
Methyl eicosapentaenoate	20:5	3
Methyl docosadienoate	22:2	2
Methyl docosatrienoate	22:3	1
Methyl docosatetraenoate	22:4	4
N3 methyl docosapentaenooate	22:5	2
N6 methyl docosapentaenoate	22:5	3
Methyl docosahexaenoate	22:6	5
Methyl tricosanoate	23:0	3
Methyl lignocerate	24:0	1
Methyl nervonate	24:1	3

TABLE 3. The obtained yields of the ISTDs from the PLFA-analysis show a good reliability of the result. The $C_{19:0}$ yield resembles the obtained amount of PLFAs from the samples, while $C_{23:0}$ resembles the amount of neural lipids and glycolipids in the sample, removed by fractioning. Two samples from the red clover group were discarded due to the $C_{19:0}$ percentage yield ≤ 2.0% (marked in red). On the other hand, the yield for $C_{23:0}$ was < 8.0 % and, thus, small enough for the result to be reliable for rest of the samples. The mean percentage yield for $C_{19:0}$ was 34.1% with standard deviation (SD) of 8.5% and for $C_{23:0}$ 2.9% with SD 2.4%.

Crop handling group	Sampling time	Replication	$C_{19:0}$ yield (%)	$C_{23:0}$ yield (%)
Timothy	June 21st 2023	1	41.2%	1.8%
Timothy	June 21st 2023	2	38.8%	3.4%
Timothy	June 21st 2023	3	42.4%	7.0%
Timothy	June 21st 2023	4	47.5%	7.9%
Timothy	July 6th 2023	1	26.0%	2.8%
Timothy	July 6th 2023	2	34.2%	1.4%
Timothy	July 6th 2023	3	35.3%	2.8%
Timothy	July 6th 2023	4	36.1%	2.6%
Timothy	July 29th 2023	1	34.3%	2.1%
Timothy	July 29th 2023	2	27.5%	1.5%
Timothy	July 29th 2023	3	36.3%	2.4%
Timothy	July 29th 2023	4	37.6%	1.5%
Tall fescue	June 21st 2023	1	36.7%	3.0%
Tall fescue	June 21st 2023	2	28.3%	1.8%
Tall fescue	June 21st 2023	3	36.1%	2.9%
Tall fescue	June 21st 2023	4	41.0%	2.9%
Tall fescue	July 6th 2023	1	26.2%	0.9%
Tall fescue	July 6th 2023	2	28.2%	0.9%
Tall fescue	July 6th 2023	3	32.6%	3.6%
Tall fescue	July 6th 2023	4	32.8%	1.6%
Tall fescue	July 29th 2023	1	29.9%	1.0%
Tall fescue	July 29th 2023	2	36.9%	3.9%
Tall fescue	July 29th 2023	3	37.3%	1.9%
Tall fescue	July 29th 2023	4	39.4%	1.7%
Red clover	June 21st 2023	1	38.2%	2.9%
Red clover	June 21st 2023	2	38.9%	3.5%
Red clover	June 21st 2023	3	38.7%	4.0%
Red clover	June 21st 2023	4	39.6%	2.7%
Red clover	July 6th 2023	1	24.5%	2.1%
Red clover	July 6th 2023	2	36.4%	3.4%
Red clover	July 6th 2023	3	35.6%	1.6%
Red clover	July 6th 2023	4	34.0%	2.8%
Red clover	July 29th 2023	1	33.2%	0.2%
Red clover	July 29th 2023	2	29.7%	1.0%
Red clover	July 29th 2023	3	2.0%	0.2%
Red clover	July 29th 2023	4	1.2%	0.1%

Table 3. (Continued)

Timothy+Red clover	June 21st 2023	1	34.9%	2.0%
Timothy+Red clover	June 21st 2023	2	31.2%	2.1%
Timothy+Red clover	June 21st 2023	3	35.5%	6.4%
Timothy+Red clover	June 21st 2023	4	39.2%	4.3%
Timothy+Red clover	July 6th 2023	1	25.6%	1.1%
Timothy+Red clover	July 6th 2023	2	33.9%	0.9%
Timothy+Red clover	July 6th 2023	3	39.6%	6.6%
Timothy+Red clover	July 6th 2023	4	37.1%	2.2%
Timothy+Red clover	July 29th 2023	1	34.6%	2.4%
Timothy+Red clover	July 29th 2023	2	36.1%	5.3%
Timothy+Red clover	July 29th 2023	3	39.7%	4.3%
Timothy+Red clover	July 29th 2023	4	39.5%	3.6%
No Template Control (NTC)			51.4%	14.9%

APPENDIX 3. Results of the rDNA gene sequencing

The summary of diversity indicators for both bacteria and fungi OTUs in different crop handling groups is shown in Table 1.

TABLE 1. The diversity indicators (mean ± SD) for bacteria and fungi OTUs in the different crop handling groups (n = 12, except for bacteria in tall fescue and timothy n= 11). Chao1 index for fungi gave many infinite values so it was left out from the summary.

	Tall fescue	Timothy	Red clover	Timothy + Red clover
Bacteria				
Chao1	360.4 ± 23.9	361.4 ± 21.1	370.2 ± 14.7	367.3 ± 13.4
Simpson's index	0.98 ± 0.00	0.98 ± 0.00	0.98 ± 0.00	0.98 ± 0.00
Shannon entropy	6.7 ± 0.1	6.7 ± 0.1	6.8 ± 0.1	6.8 ± 0.1
Total number of OTUs	255 ± 11	254 ± 13	258 ± 8	256 ± 9
Fungi				
Simpson's index	0.84 ± 0.05	0.81 ± 0.05	0.77 ± 0.09	0.80 ± 0.07
Shannon entropy	3.5 ± 0.4	3.3 ± 0.3	3.2 ± 0.5	3.4 ± 0.4
Total number of OTUs	29 ± 6	30 ± 4	29 ± 4	29 ± 4

APPENDIX 4. Results of the PLFA-analysis

The phospholipid-delivered fatty acid (PLFA) contents of the microbial groups within the treatments are shown in Table 1, and p-values from statistical tests in table 2.

TABLE 1. The PLFA content (µg/g DW; mean ± SD) of the crops handling groups.

PLFA content (µg/g DW)	Timothy	Tall fescue	Red clover	Timothy + Red clover
Bacteria general	1.70 ± 0.33	1.77 ± 0.35	1.75 ± 0.24	1.67 ± 0.23
Bacteria gram+ve	4.56 ± 0.89	4.91 ± 0.88	4.79 ± 0.69	4.50 ± 0.54
Bacteria gram-ve	7.95 ± 1.51	8.68 ± 1.86	8.30 ± 1.32	8.01 ± 1.01
Fungi general	0.32 ± 0.17	0.34 ± 0.17	0.30 ± 0.10	0.26 ± 0.06
Fungi AM	1.09 ± 0.22	1.18 ± 0.28	1.14 ±0.21	1.08 ± 0.16

TABLE 2. Summary of the results of PERMANOVA and Monte Carlo test for PLFA content data.

	Pseudo-F	p (PERMANOVA)	p (Monte Carlo)
Bacteria			
general	0.503	0.668	0.679
gram-ve	0.564	0.640	0.634
gram+ve	0.751	0.544	0.545
all	0.623	0.623	0.609
Fungi			
general	0.730	0.584	0.542
AMF	0.498	0.718	0.705
all	0.882	0.461	0.467
Total content	0.645	0.598	0.565

APPENDIX 5. Data from Web BLAST standard database

The ITS sequences that were unidentified by SILVA database, were further identified by Web BLAST standard database. Table 1 shows the phylum and class used for the most abundant unidentified sequences, their combined abundance, and the information from Web BLAST on which the identification was based on.

TABLE 1. The phylums and class used for the most abundant unidentified ITS sequences and the reference identification from Web BLASR standard database. Similarity means the similarity between the unidentified and reference sequence and accession is the identification code of the sequence.

Phylum	Class	BLAST reference	Similarity (per ident)	Accession	Combined abundance
Ascomycota	Dothideomycetes	Plectosphaerella cucumerina	100.00%	MT852207.1	3095
*Ascomycota	Dothideomycetes	Myrmecridium schulzeri	100.00%	MT446214.1	715
Ascomycota	Dothideomycetes	Didymosphaeriaceae	94.20%	MT353370.1	207
*Ascomycota	Eurotiomycetes	Paecilomyces	100.00%	HG936908.1	246
Ascomycota	Eurotiomycetes	Mrakia	96.36%	LM655262.1	61
Ascomycota	Lecanoromycetes	Porpidia	95.08%	OQ745868.1	90
*Ascomycota	Pezizomycetes	Pezizomycotina	96.38%	AB986461.1	115
Ascomycota	Pezizomycetes	Tarzetta	100.00%	OP339671.1	66
Ascomycota	Sordariomycetes	Coniochaetales	97.67%	OM744795.1	999
Ascomycota	Sordariomycetes	Sordariomycetes	99.62%	OM745232.1	344
Ascomycota	Sordariomycetes	Lambertella	95.00%	AB705254.1	291
Ascomycota	Sordariomycetes	Trichocladium	100.00%	MT348606.1	163
Ascomycota	Sordariomycetes	Sordariales	99.51%	MZ650998.1	126
Ascomycota	Sordariomycetes	Xylaria	92.54%	MT925469.1	113
Ascomycota	Sordariomycetes	Sordaria	100.00%	HG937124.1	88
Ascomycota	Sordariomycetes	Sordariomycetes	100.00%	OM745232.1	79
Ascomycota	Sordariomycetes	Eucasphaeria rustici	81.25%	KY173410.1	72
Ascomycota	Unidentified	Ascomycota	100.00%	MF534499.1	193
Ascomycota	Unidentified	Ascomycota	98.56%	MF534748.1	152

*phylum-level identified by SILVA

Table 1. (continued)

Phylum	Class	BLAST reference	Similarity (per ident)	Accession	Combined abundance
Basidiomycota	Agaricomycetes	Auricularia	100.00%	JX135079.1	757
Basidiomycota	Agaricomycetes	Basidiodendron globisporum	96.61%	MT040884.1	86
Basidiomycota	Agaricomycetes	Basidiodendron globisporum	96.61%	NR_172177.1	72
Basidiomycota	Agaricomycetes	Dictyophora rubro-volvata	100.00%	CP114124.1	61
Basidiomycota	Agaricomycetes	Rusavskia elegans	96.61%	MN103182.1	60
Basidiomycota	Pucciniomycetes	Puccinia graminis	91.95%	MT924529.1	394
Basidiomycota	Pucciniomycetes	Puccinia graminis	92.77%	MT924529.1	162
Basidiomycota	Pucciniomycetes	Puccinia graminis	79.25%	MT924592.1	77
Basidiomycota	Tremellomycetes	Tremella subalpina	86.34%	NR_155908.1	303
Basidiomycota	Tremellomycetes	Cryptococcus	100.00%	HG937127.1	91
Basidiomycota	Unidentified	Basidiomycota	98.68%	MF534498.1	254
Basidiomycota	Unidentified	Basidiomycota	90.09%	MF534498.1	152
Zygomycota	Zygomycetes	Mortierella	87.50%	MK281958.1	168
Zygomycota	Zygomycetes	Basidiobolus	96.72%	OQ378885.1	131
Unidentified	Unidentified	Unidentified			306
Unidentified	Unidentified	Unidentified			76