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Soil microbe communities and their interactions with understorey plants in temperate forests

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR (PHD) IN APPLIED BIOLOGICAL SCIENCES:
FOREST AND NATURE MANAGEMENT

Dutch translation of the title:
De microbiële bodemgemeenschap in gematigd bos, in interactie met de kruidlaag

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Illustrations on the cover:
Understorey plants and open-top champers in a temperate forest in Belgium
Photo credit: Bin Wang

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Contents

Summary

Global change, including climate change, nitrogen (N) deposition and land-use change, drives species dynamics both aboveground and belowground in terrestrial ecosystems. In temperate forests, forest understoreys and their associated soil microbes are understudied despite their substantial contribution to forest plant species diversity and for maintaining multiple forest functions. Understanding how soil microbes differ between understorey plant species, how the two communities (understorey plants and soil microbes) change if environmental conditions are altered, and the feedbacks between understorey plants and microbes, is important to be able to maintain future forest ecosystem functioning. Yet, knowledge about these aspects is largely lacking, limiting our ability to project plant and soil microbe dynamics in the face of global change.

In this thesis, we will help filling this knowledge gap using observational studies, *in-situ* and greenhouse experiments set-up along large-scale environmental gradients. First, soil microbes under individual plant species were assessed along a large latitudinal gradient to study the effect of biotic and abiotic driving factors on the assemblage of soil microbes under different plant species. Then, an *in-situ* experiment was set up to explore the plant and soil microbial community responses to multiple environmental change drivers and the connection between the two communities. Last, a plant-soil feedback experiment was conducted in a greenhouse to demonstrate the magnitude of soil microbes' effects on understorey plant regeneration.

We found that soil microbial community composition (soil microbial biomass and bacterial community composition) in the rhizosphere soil under different understorey species along the latitudinal gradient differed significantly. Even for conspecific species, plant individuals established at different latitudes underwent distinct soil microbial community composition, mainly due to differences in local soil characteristics and soil acidity in particular. The importance of larger-scale environmental conditions (mean annual temperature, precipitation and N deposition) depended on species identity. Specifically, soil microbial biomass was significantly affected in graminoids only, i.e., *Milium effusum* and *Poa nemoralis* and this effect correlated with soil chemistry. Soil bacterial community composition was significantly affected by larger-scale environmental conditions in *Stachys sylvatica* only and this effect was not correlated with soil chemistry. Soil microbial community composition under all species did not differ between ancient and recent forests.

In the study of multiple environmental change drivers' effects on the plant-soil continuum, we found light availability emerged as the most important driver in changing plant and soil bacterial

community composition, as well as in decreasing soil microbial biomass, and it was key in explaining the significant co-structure between plant and soil bacterial communities. Warming and N addition did not affect plant community composition nor soil microbial biomass. However, irrespective of illumination, the soil bacterial community composition responded significantly to warming. In addition, significant interactions of illumination with warming and N addition were observed in shifting the soil bacterial community.

In the plant-soil feedback experiment, we found that soil microbes affected plant growth negatively, with plants showing higher plant height and biomass in sterilized soil. The presence and absence of soil microbes altered the effects of soil chemistry on plant emergence and growth when seeds were grown in home and away soil.

We conclude that the interactions and responses of understorey plants to environmental conditions (soil characteristics, light avaibility and temperature) are likely the most important drivers for shaping distinct patterns of soil microbial diversity and community composition. In turn, the soil microbial community is linked with plants and their dynamics can determine plant establishment. The alteration of understorey habitat conditions by global change and forest management will impact the plant-soil continuum and thus alter forest ecosystem functioning.

Samenvatting

Soorten van terrestrische ecosystemen worden, zowel boven- als ondergronds, beïnvloed door globale veranderingen zoals klimaatverandering, stikstofdepositie en verandering in landgebruik. De kruidlaag en geassocieerde micro-organismen in de bodem zijn belangrijk voor de biodiversiteit en het functioneren van bossen, maar zijn tot nog toe niet gedetailleerd bestudeerd in gematigd bos. Om te kunnen voorspellen hoe planten en de microbiële bodemgemeenschap zullen reageren op toekomstige globale veranderingen hebben we inzicht nodig in de koppeling tussen plantensoorten en micro-organismen en de invloed van bv. klimaatverandering op de bovengrondse plantengemeenschap en de ondergrondse microbiële gemeenschap.

In deze thesis bestudeerden we de invloed van milieugradiënten in een observationele studie, een veld- en een serre-experiment. Eerst bestudeerden we de samenstelling van de gemeenschap micro-organismen onder specifieke plantensoorten in de kruidlaag van bossen langs een latitudinale gradiënt. Daarna bekeken we in een veldexperiment hoe de kruidlaag en de gemeenschap van micro-organismen reageren op veranderingen in verschillende milieuvariabelen. Tot slot bekeken we in een serre-experiment de invloed van micro-organismen op de verjonging van kruidlaagsoorten.

De rizosfeer van de kruidlaagsoorten langsheen de latitudinale gradient toonde verschillen in microbiële biomassa en bacteriële soortensamenstelling tussen de kruidlaagsoorten en tussen groeiplaatsen verschillend in bodem(zuurtegraad). De invloed van grootschalige milieuvariabelen (temperatuur, neerslag, stikstofdepositie) verschilde tussen soorten. Grassen (*Milium effusum* en *Poa nemoralis*) vertoonden een milieu-effect voor microbiële biomassa, in correlatie met bodemchemie. *Stachys sylvatica* vertoonde een milieu-effect voor de samenstelling van de microbiële gemeenschap (onafhankelijk van bodemchemie). Er was geen verschil in de microbiële gemeenschappen in oud of recent bos voor de bestudeerde kruidlaagsoorten.

In het veldexperiment had lichtbeschikbaarheid de grootste invloed op de samenstelling van de kruidlaag en microbiële gemeenschap en de microbiële biomassa. Lichtbeschikbaarheid was bovendien essentieel voor het verklaren van de co-structuur van kruidlaag en microbiële gemeenschap. Verhogen van de temperatuur en toevoegen van stikstof hadden geen invloed op de samenstelling van de kruidlaag of de microbiële biomassa. De bacteriële soortensamenstelling daarentegen werd wel beïnvloed door temperatuur, in interactie met lichtbeschikbaarheid en stikstofadditie.

In het serre-experiment hadden bodemmicro-organismen een negatieve invloed op de groei van de zaailingen; hoogte en biomassa waren hoger bij planten in gesteriliseerde bodem. De invloed van bodemchemie op kieming van zaad en groei van de zaailingen werd beïnvloed door de aanwezigheid van de micro-organismen in bodem met dezelfde of een andere herkomst als het zaad.

De diversiteit en samenstelling van de microbiële gemeenschap van de rizosfeer van de bestudeerde kruidlaagsoorten werden vooral beïnvloed door de veranderingen en verschillen in de kruidlaag zelf, als gevolg van verschillen in bodem, lichtbeschikbaarheid en temperatuur. De microbiële gemeenschap beïnvloedde, op zijn beurt, de vestiging van kruidlaagsoorten. Verandering van de groeiomstandigheden in de onderetage van bossen kan zo een invloed hebben op het samenspel van plant en bodem en daardoor ook op het functioneren van het bosecosysteem.

List of Abbreviations and Symbols

Abbreviations

COIA	CO-Inertia Analysis
Df	The Degree of freedom
GLM	Generalized Linear Model
IPCC	Intergovernmental Panel on Climate Change
MAP	Mean Annual Precipitation
MAT	Mean Annual Temperature
MET	Mean Emergence Time
Ndep	Nitrogen deposition
NGS	Next-generation Sequencing
NMDS	Non-metric Multidimensional Scaling
ns	non-significant ($P > 0.1$)
NPP	Net Primary Productivity
OTU	Operational Taxonomic Unit
P	Significance of statistical test
PCA	Principal Component Analysis
PLFA	Phospholipid Fatty Acid
PSF	Plant-soil Feedback
R^2 value	The coefficient of determination
RDA	Redundancy Analysis
RV	Coefficient Values
SLA	Specific Leaf Area

Symbols

§	Log10-transformed
£	Sqrt-transformed
¶	A Poisson error distribution was applied
χ^2	Likelihood ratio test statistics
*	$P < 0.05$
**	$P < 0.01$
***	$P < 0.001$

Chapter 1

General introduction

Temperate forests occupy about 1097 million hectares worldwide and mainly distribute in East Asia, Europe and North America (**Fig. 1.1**), contributing substantially to biodiversity, ecosystem functions and services (Pan, Birdsey, Phillips & Jackson 2013). Plants and soil microbes are the two most abundant communities in forests, as well as on the earth (Bar-On, Phillips & Milo 2018). The two communities are responsible for large parts of forest biodiversity and biogeochemical cycling. Any changes in their community composition can disturb these functions. Structurally, temperate forests include tree, shrub and understorey layer. Compared to the large attention on forest trees, studies on the ecological significance of the understorey are scarce, despite the critical role understorey plants can have in maintaining biodiversity and other ecological functions (e.g., water purification, nursing tree regeneration) (Gilliam 2007).

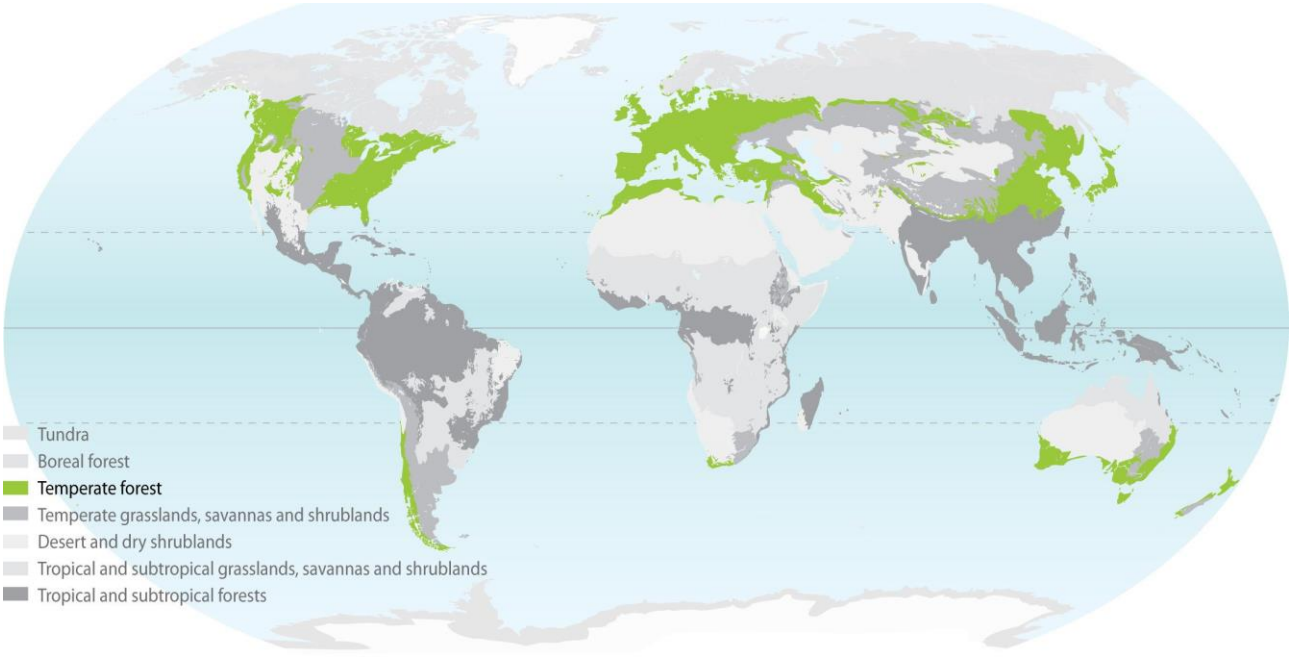


Fig. 1.1 The distributional map of temperate forests (the green parts) across the globe. This map was generated by Trumper (2009) according to data published by Olson *et al.* (2001).

Currently, global change threatens forest ecosystem functioning through its influences on biotic and abiotic conditions. These influences vary greatly among ecosystems and geographical scales. Additionally, the interaction between trophic levels, including plants and soil microbes, hinders our insight in global change effects. Hence, documenting and understanding the response of plants and

their related soil microbes to global change at different geographical scales is still one of the main tasks for forest ecologists.

1.1 Current global change and influences

Terrestrial ecosystems are facing multiple global change pressures, such as climate change, increased nitrogen (N) deposition loads and atmospheric CO_2 concentrations, land-use change and species invasion. Among those, climate change, N deposition and land-use change are the predominant drivers for ecosystem biodiversity (Sala *et al.* 2000). Enhanced emissions of greenhouse gases have led to global increases of temperature, changing precipitation patterns and extreme weather frequencies (Crowther *et al.* 2016; An, Ji & Zhang 2018). The temperature in a preindustrial period (most likely 1720-1800) was 0.55 ℃-0.80 ℃ cooler than 1986-2005. Furthermore, 2015 was the first year in which global average temperature increased more than 1 ℃ compared to preindustrial levels (Hawkins *et al.* 2017). According to Hoegh-Guldberg *et al.* (2018) (a special report from IPCC, 2018), limiting global warming to 1.5 ℃ over the preindustrial levels by 2100 rather than 2 ℃ is a challenge but is highly required for reducing risks of extreme weather to human and natural systems. In the north of temperate regions, the mean annual temperature of the year 2010 has increased by ca. 1 ℃ compared to that of 1980 (Dillon, Wang & Huey 2010). Climate models have predicted that the climate in central Sweden (Stockholm) by the end of 21st will become similar to the current climate of northern France (IPCC 2014). In a warmer world, extreme precipitation shows linear relationships with the magnitude of global warming (Wang, Jiang & Lang 2017). Climate change in the way of temperature and precipitation is partly responsible for species diversity loss, plant migration and land-use change (Thuiller, Lavorel, Araujo, Sykes & Prentice 2005; Allen *et al.* 2010; Hawkins *et al.* 2017). Climate warming can drive aboveground and belowground community assembly because plant species may migrate to higher latitudes or altitudes to follow the shifting isotherms (Parolo & Rossi 2008) or adapt to current warming conditions via gaining warm-adapted species and populations (Crowther & Bradford 2013). Soil microbes can shift their community structure and carbon (C) use efficiency to adapt to decreased soil C caused by increased temperature (Melillo *et al.* 2017).

N deposition is known to be harmful to terrestrial ecosystems worldwide, and can alter the vegetation composition and microbial activity. N is an essential component of plant nutrient resource. In the preindustrial period, many ecosystems are adapted to low N conditions and this situation leads to high species diversity (Bobbink, Hornung & Roelofs 1998). By 1860, natural N processes, i.e., biological N fixation (BNF), dominated the globe (~120 teragram N/yr) and anthropogenic inputs only counted 13% of total BNF (Galloway *et al.* 2004). By 2005,

anthropogenic processes overcame the natural processes and reached to ~210 teragram per year (Galloway *et al.* 2008). Unfortunately, the trend of increased atmospheric N deposition is projected to continuously occur in many regions until 2030 (Dentener *et al.* 2006). Long-term accumulation of excessive N deposition loads facilitates a few competitive plant species but impairs species that are not that competitive, causing competition exclusion (Hautier, Niklaus & Hector 2009). Studies on vegetation dynamics in distinct ecosystems (tundra, boreal forest, temperate forest, temperate grassland, mediterranean) reported a common shift trend of species composition due to local increased N deposition loads (Bobbink *et al.* 2010).

Additionally, land-use change (e.g., deforestation and reforestation) is a key force changing biogeochemical cycles and biodiversity across the globe (Foley *et al.* 2005; Turner, Lambin & Reenberg 2007; Le Quere *et al.* 2016). Goldewijk (2001) estimated land-use change between the year of 1700 and 1990 and reported that a global loss of forest areas due to their usage transformation to croplands, despite this global loss pattern was not applicable for regional scales, i.e., forest gain occurred in regions such as Europe among this studied period. According to a recent report by Song *et al.* (2018), a global estimation of land-use change using satellite observations of three land categories (tall vegetation cover (tree cover; ≥ 5m in height), short vegetation cover and bare ground) reported an increase of the total area of tree cover by 2.24 million km^2 from 1982 to 2016. Meanwhile they attributed 60% of the cause of land-use change to human-related activities (direct) and 40% to climatic change (indirect), and the largest gain was undergone by temperate forests (+726 000 km^2, +33%). As the action of conservation in 19th century, many of the current temperate forests, especially in northwestern Europe and northeastern North America, are developed from agricultural lands. Land-use change can result in positive or negative influences on C emission to the atmosphere and global C storage, depending on change directions, i.e., crop to forest or the opposite (Guo & Gifford 2002), as well as divergent habitat conditions with respect to abiotic and biotic aspects (Perring *et al.* 2016; Abadie *et al.* 2018).

1.2 Understorey plants

Understorey plants, also known as herbaceous layer plants or ground vegetation, grow in the soil under the canopies of trees and shrubs (Gilliam 2007). An inclusive definition of this community commonly refers to all vascular plants lower than 1 m height, including true herb species, ferns and also seedlings and saplings of woody species. The definition of understorey communities varies among studies depending on the threshold of setting plant height (e.g., max. 2 m), and on whether non-vascular plants (e.g. mosses) or woody species are considered.

1.2.1 Understorey diversity and ecosystem functioning

Understorey plants represent the largest proportion of plant biodiversity in temperate forest ecosystems, accounting for more than 80% richness of all plant species and 3- to 9-fold higher richness than tree species' (**Fig. 1.2**) (Gilliam 2007). While multiple environmental drivers are threatening their diversity and composition. Threats to understorey biodiversity loss have been highlighted to be more severe than that of woody tree species and gymnosperms (Levin & Wilson 1976; Jolis 2003). Given the ecological importance of the understorey community in temperate forests, the diversity loss of the understorey plant community can further impair the stability of forest services and functions.

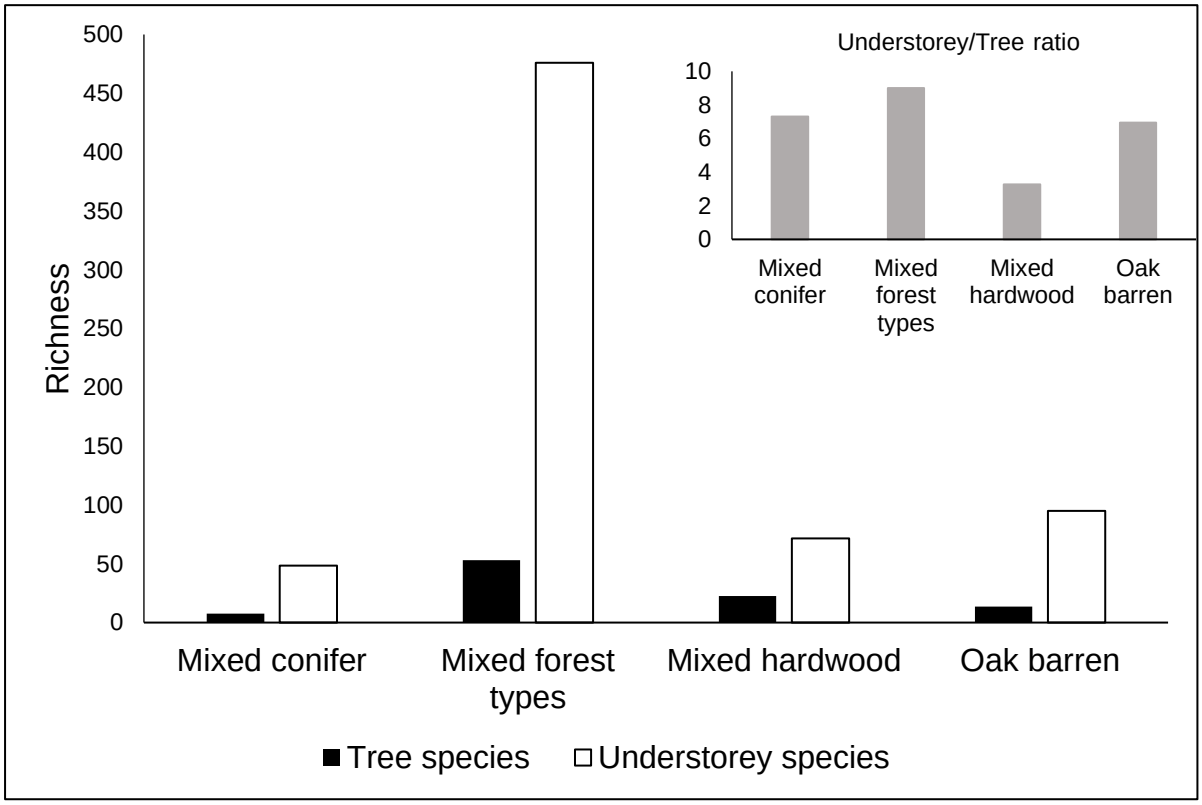


Fig. 1.2 Mean understorey (white) and tree (black) species richness in different temperate forests. The ratio of the mean understorey species richness to tree species richness in each temperate forest type is shown in grey. Data were extracted from Gilliam (2007).

Understorey plants are involved in many ecosystem functions, such as primary production, energy flow, forest evapotranspiration and forest regeneration. Despite the fact that its aboveground biomass can be less than 1% of a whole forest, its contribution to net primary productivity (NPP) is quantifiably significant. Understorey plants are also a source of soil organic matter and affect soil nutrient cycling. The characteristics of higher litter quality and nutrient concentrations, but lower concentrations of cellulose and lignin of understorey vegetation indicate a fast decomposition rate by soil decomposers and thereby driving the structure of soil microbes (e.g., the ratio of fungi to

bacteria), the process of soil biogeochemical cycling, sapling growth and NPP (Melillo *et al.* 1989). In addition, understorey vegetation contributes substantially to water cycling and dynamics in forest ecosystems via evapotranspiration. The percentage of evapotranspiration by understorey vegetation varies between 10-15% in dense forests and 40% in open forests (Vincke, Breda, Granier & Devillez 2005; Soubie, Heinesch, Granier, Aubinet & Vincke 2016; Oishi *et al.* 2018). For tree regeneration, studies have shown that increased understorey plant cover or density suppressed seedling growth mainly due to competition for light and nutrients (Collins 2003; Gaudio *et al.* 2011). Apart from the presence of understorey plants, functional types of understorey plant species are also critical in affecting seedling mortality (Dolling 1996). Last but not least, understorey layer can harbour some exclusive species, which need specific resources, and these exclusive species are important indicators with respect to site condition, forest management, ecosystem health and conservation (Spyreas & Matthews 2006; Gracia, Montane, Pique & Retana 2007; Chavez & Macdonald 2012).

1.2.2 Impacted by multiple drivers

Understorey plant communities are affected by multiple biotic and abiotic drivers. Their composition also differs among temperate forests depending on the dominant tree species within a specific stand (e.g., evergreen broadleaved stand, deciduous stand or mixed plant stand). For instance, Mestre *et al.* (2017) compared the understorey plant species diversity under three forest stands, i.e., pure evergreen broadleaved, deciduous and mixed stand, and found that the mixed stand experienced higher diversity than that of the pure deciduous and evergreen broadleaved stand. In the comparison of tree composition effects on understorey plant diversity, coniferous forests, in most cases, have less understorey vascular species diversity than that in broadleaved forests (Barbier, Gosselin & Balandier 2008), although exceptional cases exist with no difference between the two types of forests (Nagaike 2002) or higher diversity observed under coniferous forests (Augusto, Dupouey & Ranger 2003). Overstorey species identities and composition influence understorey plant biomass, production and turnover rates of understorey plants by altering quality and quantity of litters, availability of light, water and soil nutrients (Barbier, Gosselin & Balandier 2008; Kumar, Chen, Searle & Shahi 2018). It has been shown that litter resourced from deciduous forests contain higher pH and base cations than which in coniferous forests, thereby favouring the establishment of herbaceous and woody plants but not bryophytes (Hart & Chen 2006). However, tree species composition and canopy change are closely correlated with forest management and global change drivers, such as climatic regimes and land-use change.

Environmental changes, such as light reduction because of dense canopies and increased temperature can affect plant diversity and community composition in forest ecosystems. Dense

canopies reduce light availability for understorey plants and light is proved as one of the most limiting resources for understorey communities. Owing to fierce competition among understorey plants, understorey plant community composition gradually shifts to more shade-tolerant plant species and populations with large size (Strengbom, Näsholm & Ericson 2004). In response to climate warming, many plants have adjusted their phenology or physiological mechanisms to a warmer environment (De Frenne *et al.* 2013b; Bellemare & Moeller 2014). Thuiller, Lavorel, Araujo, Sykes and Prentice (2005) investigated 1350 plant species under seven climate change scenarios in Europe and found that more than half of the studied species could be vulnerable or threatened by 2080. They also found that the degree of changes in temperature and moisture conditions was the main predictor of modelled species loss and turnover. However, dense canopies caused microclimate in temperate forests may attenuate the response of understorey communities to climate change, and the microclimate at understorey layers, to some extent, depends on forest management (De Frenne *et al.* 2013b).

Plant diversity and composition are also susceptible to increased atmospheric N deposition and land-use change. A twenty-five-year observation of understorey plants to N addition elucidated a plant community shift to nitrophilic species (Gilliam *et al.* 2016). Yet, plants' responses to elevated N deposition are context-dependent, e.g., differences in landscape conditions and historical land use and management (de la Pena *et al.* 2016; Perring *et al.* 2018b). In addition, warming and increased N deposition loads are interacted in terms of their impacts on plant dynamics. For instance, nutrient-demanding forbs and grasses in temperature-increased habitats benefit from eutrophication and thereby increase their cover and biomass (Hedwall & Mikusinski 2015). Legacies of land-use change have profound effects on plant assembly attributing the divergent habitats in terms of abiotic and biotic conditions (Perring *et al.* 2016). Understorey plant communities assembled in post-agricultural soils might be less diverse (Hermy & Verheyen 2007) and more susceptible to aboveground herbivores when compared to ancient forests (de la Pena *et al.* 2016). Yet, any shifts in understorey plant community composition can trigger or be a consequence of changes in underground soil microbial community.

1.3 Soil microbes

1.3.1 Composition, diversity and ecosystem functioning

Soil biota include fungi, bacteria, archaea, viruses, micro- and macro-arthropods, etc. Among which, soil fungi and bacteria (hereafter referred as soil microbes) are probably the most diverse and abundant communities on Earth, and govern biogeochemical cycling and pathology (Tedersoo *et al.* 2014; Delgado-Baquerizo *et al.* 2018). An old microbiological tenet "Everything is everywhere, but,

the environment selects", formulated by Bass Becking in the early 20th century (Becking 1934), conveys the idea of worldwide distribution of all microbial life, while in a given environmental context many of the soil microbial species are only latently present (below the limit of detection) (de Wit & Bouvier 2006). Current evidence confirms the hypothesis that "the environment selects" and contributes partially to explain the distributional diversity of soil microbial species at geographical scales (Martiny *et al.* 2006). While the part "Everything is everywhere" is challenged during the past two decades, because of the evidence of dispersal limitation (Telford, Vandvik & Birks 2006) and the degree of provincialism (restricted to particular areas) promoted potentially by genetic isolation (Rout & Callaway 2012). With the advent of molecular microbiology, which allows the detection and taxonomic discrimination of soil microbes without culturing, it is possible to characterize the full extent of soil microbial diversity and community composition across continents (**Fig. 1.3**) (Delgado-Baquerizo *et al.* 2018).

It has been estimated that 10^{10}-10^{11} bacteria, 6000-50 000 bacterial species, and up to 200 m fungal hyphae can be dwelled in just one gram of soil (Curtis, Sloan & Scannell 2002; Horner-Devine, Leibold, Smith & Bohannan 2003; Leake *et al.* 2004). The diversity and composition of soil microbial community in different ecosystems or habitats differ significantly owing to distinct environmental properties (Tedersoo *et al.* 2014; Davison *et al.* 2015; Delgado-Baquerizo *et al.* 2018). This is confirmed by a global estimation of soil bacterial community distribution, in which 25224 bacterial phylotypes were found in six continents across the globe, while only 511 phylotypes (accounted 2% of the total phylotype) were relatively abundant and occurred in all soils. Meanwhile, this study also illustrated that dry and temperate forests contained a higher relative abundance of bacteria compared to cold, tropical forests and grasslands (Delgado-Baquerizo *et al.* 2018). At a smaller scale, the differences of the abundance and diversity of soil microbial community reflect on spatial heterogeneity in different soil depths and the distance to roots, with higher abundance in the topsoil (generally 0-10 cm depth) and the rhizosphere soil (the zone closes to roots) (Guo, Wang, Luo & Wu 2015; Nacke *et al.* 2016). Changes in soil microbial diversity and composition can affect multiple ecological processes, such as nutrient retention and cycling, because of their critical role in decomposition and mineralization (Wagg, Bender, Widmer & van der Heijden 2014).

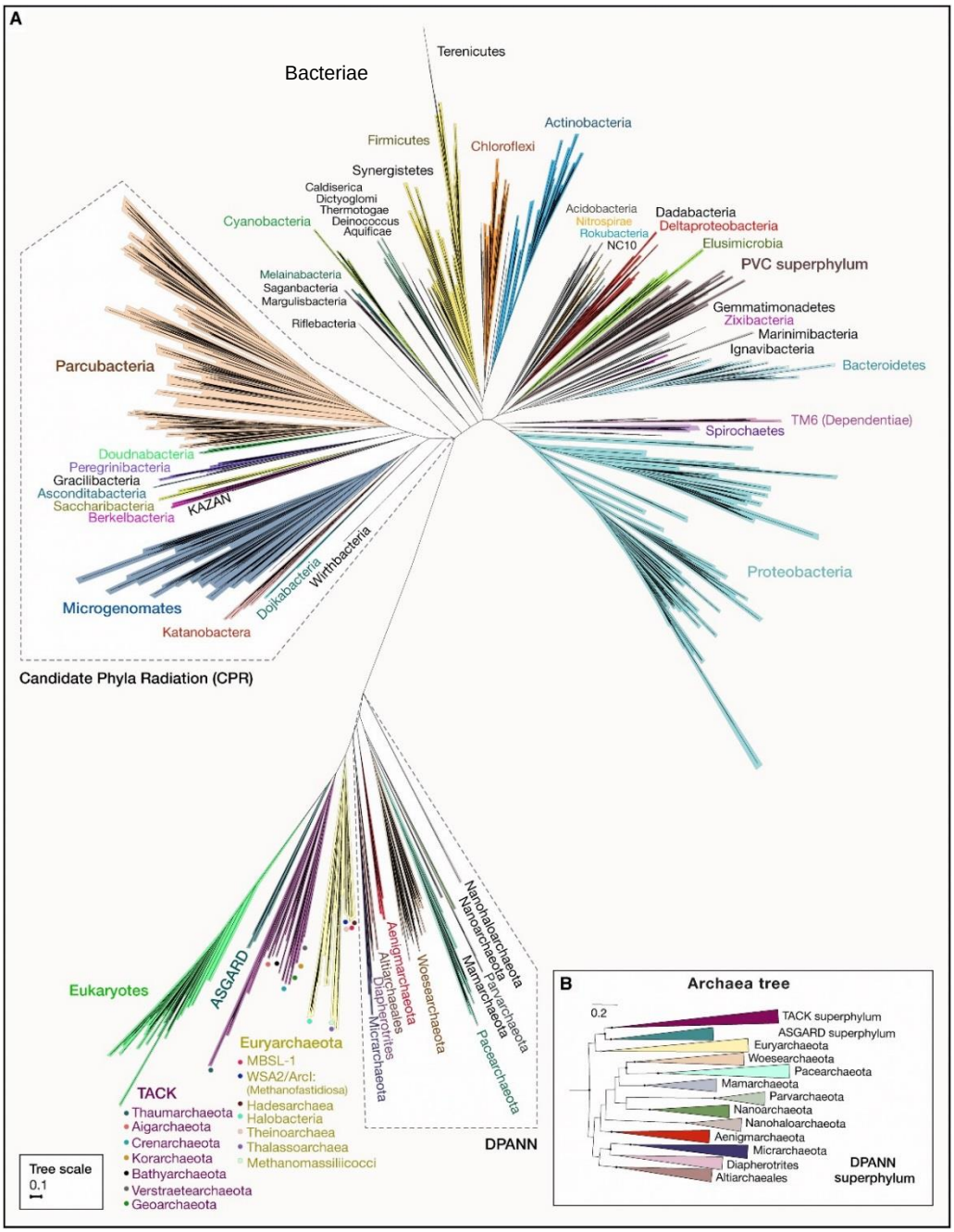


Fig. 1.3 A: The tree of life includes bacterial, archaeal and eukaryotic (five super groups) phyla. B: A reconstruction of the monophyly of the DPANN clade. This figure was edited from Hug *et al.* (2016) by Castelle and Banfield (2018).

Soil microbes are the primary decomposers of soil organic matter and plant residuals in terrestrial ecosystems, and the decomposition of which is the main step of returning organic materials to re-usable resources for both plants and soil microbes (Moore, Trofymow, Prescott, Fyles & Titus 2006; Garcia-Pausas & Paterson 2011; Bani *et al.* 2018). While different chemical compounds (e.g., amino acids, sugar, cellulose and lignin) in litter complex the decomposition process led by soil fungi and bacteria, which requires diverse soil enzymes and microbes (Bani *et al.* 2018). Therefore, soil microbial status (e.g., functional diversity, abundance, activity), is, at least partially, responsible for plant diversity and productivity (van der Heijden, Bardgett & van Straalen 2008b). Indeed, it has been shown that, in temperate and boreal forests, the majority of plant annual N- and P-uptake (ca. 80% N and up to 75% P) is supplied by mycorrhizal fungi and N-fixing bacteria via mineralisation (van der Heijden, Bardgett & van Straalen 2008b). Apart from the function of driving energy flow from soil organic matter to plant entities, soil microbes use the decomposed C resources to synthesize their own biomass and thus store a certain amount of soil organic matter. Soil microbes per se as a primary pathway of soil organic matter formation has been directly proved recently in maintaining the stability of soil nutrient pool (Kallenbach, Frey & Grandy 2016). However, soil microbes involved soil chemistry regulation can be disrupted by environmental and land-use change, causing positive or negative to ecosystem functioning.

1.3.2 Impacted by multiple drivers

Given the functional role of soil microbes in biogeochemical cycles, many biotic and abiotic drivers can also affect soil microbial community assembly. Aboveground plants are the main determinant for the chemical compounds released via roots and litter resources. Higher plant biodiversity can increase C inputs used by soil microbes, leading to increased microbial activity and soil C storage. Increased microbial activity can also lead to a higher contribution of microbes to C sequestration. (Lange *et al.* 2015). Moreover, plant beta diversity can be used as a predictor of beta diversity of soil microbes (Prober *et al.* 2015). Besides the biotic influences of plants, soil predators (including fungivores, other protists' predators and nematodes) interfere soil microbial community structure and composition significantly (Anderson, Juhl & Bock 2018; Geisen & Bonkowski 2018).

Abiotic drivers in different ecosystems differ substantially with respect to edaphic characteristics, atmospheric CO_2, temperature and precipitation regimes (Gray, Classen, Kardol, Yermakov & Miller 2011; Bissett, Brown, Siciliano & Thrall 2013). Soil chemistry, particularly pH, is generally regarded as one of the most important environmental factors in driving soil microbial diversity and community composition at global scales (Lauber, Hamady, Knight & Fierer 2009). However, this is not consistent in other studies. Zhou *et al.* (2016) examined forest soil microbial diversity at North America, and found that temperature, but not other environmental characteristics, was the main

determinant of soil microbial community composition. Different results suggest high variation of soil microbes' responses to abiotic conditions in different habitats. Soil differences in terms of drainage capacity can also regulate microbial dynamics through effects on soil moisture conditions, aerobic and anaerobic processes (Yang, Weber & Silver 2012). Understoreys in temperate forests bear more complexity in the aspect of abiotic effects on soil microbial community composition due to microclimate conditions that caused by tree canopies' buffering. Similar to lag responses of plants to climate change, soil microbes' responses might be mitigated as well (De Frenne *et al.* 2013b). Additionally, belowground soil microbes were found with considerable differences between ancient and post-agricultural forests because of distinct resource components (de la Pena *et al.* 2016).

1.4 Plant-soil interactions

Aboveground plants and belowground soil biota are depending on each other (**Fig. 1.4**). Biotic interactions between aboveground and belowground components have been recognized as a key mechanism in driving ecosystem biodiversity and functioning (Wardle *et al.* 2004; Wardle 2006). Individual plants as well as plant communities and interactions between aboveground plants and animals, all have influences on belowground soil biota. In soil food webs, trophic interactions reflect on taxa that are resource-regulated or consumer-regulated (Coleman *et al.* 1978; Ruess *et al.* 2005). These interactions include many processes, for instance, competition, facilitation and predation, and are linked with plant communities. Plant-soil interactions generally include two pathways (Wardle *et al.* 2004). First, direct interactions exist between root systems and soil microbes such as pathogens and symbionts. Second, indirect interactions between plants and decomposers with respect to soil formation and nutrient cycling. Global change drivers, such as climate change and N deposition, shift the composition of both plants and soil microbes and thereby modulating the interacting relationships (van Grunsven, van der Putten, Bezemer, Berendse & Veenendaal 2010; Bardgett, Manning, Morrien & De Vries 2013).

Plant-soil feedback is a well-known process to unravel the relationships between plants and their associated soil microbes. In the process of plant-soil feedbacks, plants alter the soils in which they grow by releasing chemical compounds (e.g., phenolics, hormones, organic acids and sugars) via roots (Bais, Weir, Perry, Gilroy & Vivanco 2006). Therefore, plants can shape the assembly of soil biota that deal with these plant products. Soil biota, such as fungi and bacteria, in turn modify plant performance via, for instance, accumulated pathogens (Berendsen, Pieterse & Bakker 2012) and changes in physical and chemical soil characteristics (Schnitzer *et al.* 2011).

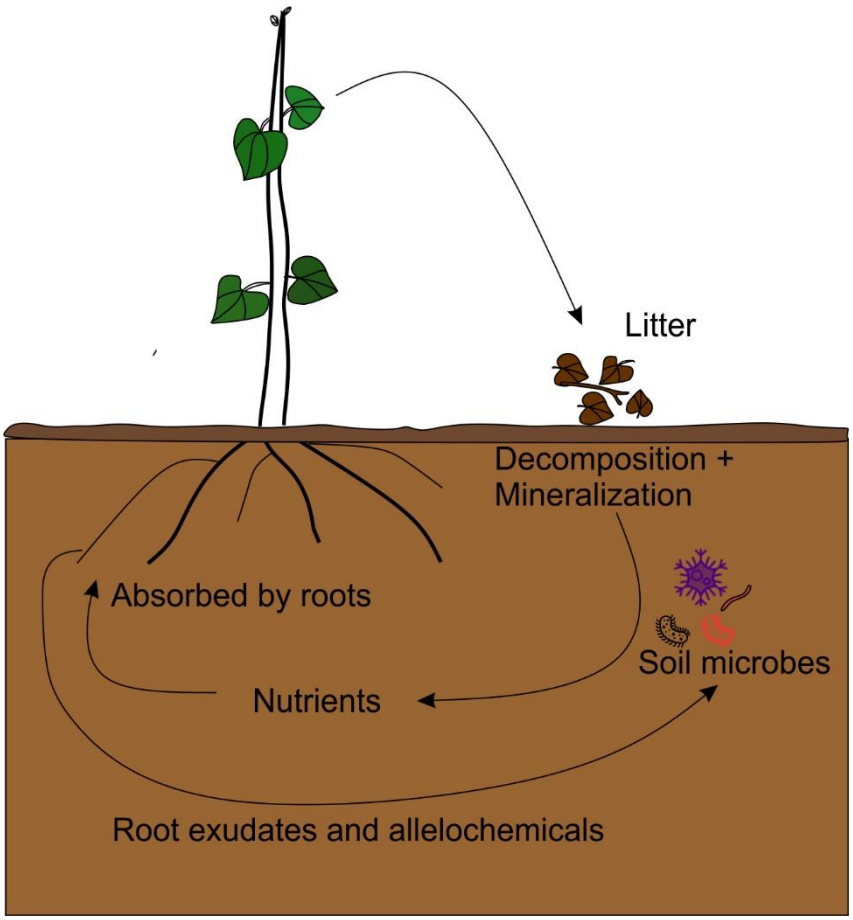


Fig. 1.4 A schematic diagram showing interactions between plants and soil microbes studied here.

Plant-soil feedbacks play roles in evolutionary trajectories because they are partly responsible for changes in plant fitness and phenotypes (vanderPutten 1997). This mechanism might ultimately induce diversification (in both conspecifics and heterospecifics), genetic divergence among populations, and adaptation or evolution (Bailey *et al.* 2014; Schweitzer *et al.* 2014). Plant-soil feedbacks can be evaluated in multiple ways. The methodology of a typical plant-soil feedback experiment commonly includes a conditioning and response phase. In the conditioning phase, plants grow in soils in the field or greenhouse to alter soil characteristics. In the response phase, the conditioned soil is used to grow a new set of plants, and these plants' performance is compared between conspecific- and heterospecific-conditioned soils.

1.5 Research gaps, objectives and outline of the thesis

Soil microbial communities are extremely diverse in different ecosystems and driving factors for microbial community assembly also differ among habitats (Fierer & Jackson 2006; Shen, Ni, Liang, Wang & Chu 2015). In temperate forests, understorey is key for biodiversity and ecosystem functioning (e.g., biogeochemical cycling and NPP). Yet, knowledge about soil microbes' dynamics

and their correlation with associated aboveground understorey plants is limited, not only in the context of European scale, but also in the context of facing multiple global change drivers.

Plants and soil microbes co-occur and their spontaneous responses to multiple biotic and abiotic factors can help a better understanding of ecological process and predicting dynamics. However, these interactions are confounded when facing multiple environmental drivers, because the response to one environmental driver can be counteracted or accelerated by another (Leuzinger *et al.* 2011). Understanding the dynamics of co-occurring understorey plants and soil microbes to multiple environmental drivers underpins more realistic conclusions that are instructive for maintaining forest biodiversity and ecosystem functioning. Furthermore, if understorey plants together with ambient conditions modify the assemblage of soil microbes, to which degree that these structured soil microbes can affect the regeneration of understorey plant species is largely unknown.

The specific objectives of the thesis were to:

1) uncover the effects of contrasting abiotic and biotic conditions on soil microbial community composition and diversity across European temperate forests to get insight in the diversity and structure of soil microbe communities under understorey plant species in temperate European forests.
2) explore potential shifts in the community composition of aboveground plants and belowground soil microbes when facing multiple environmental changes, i.e., canopy opening (increased light availability), climate warming and increased N deposition.
3) reflect on potential distribution range changes of understorey plant species in the future by assessing the influences of nonlocal soil microbial conditions on understorey plant species.

The thesis comprises three parts (**Fig. 1.5**)

The first part (**Chapter 2 and Chapter 3**) describes the results of two observational experiments, in which we concentrated on the variation of soil microbial community and diversity along a 1700 km latitudinal gradient in Europe. **Chapter 2** reports the whole soil microbial community composition using phospholipid fatty acids and its correlation with abiotic conditions. In **Chapter 3**, based on the high abundance of soil bacterial community in the community, we specifically elucidated the community composition and diversity of soil bacteria at the highest possible taxonomic levels using high throughput sequencing and investigated relationships with biotic and abiotic conditions.

The second part (**Chapter 4 and Chapter 5**) reports the results of two experiments, one conducted in the field and one in a greenhouse. In **Chapter 4**, we simulated global change with three-way factorial treatments of N addition (simulating N deposition), warming temperatures (simulating

climate change) and increasing light availability (simulating enhanced harvests of woody biomass) under field conditions in a temperate forest in Gontrode, Belgium. We then studied the responses of understorey plants and the soil microbial community to the three environmental drivers. In **Chapter 5**, we conducted a plant-soil feedback experiment in a greenhouse setting using soil and seeds collected from understorey species *Milium effusum* and *Stachys sylvatica* along a latitudinal gradient from northern France to central Sweden. Soil microbes were eliminated by using Gamma irradiation (sterilization). Soil microbes' change was generated by inoculating with home and foreign soil to sterilized soil. Seeds from each origin were sown in both home and foreign soils. This experiment was designed to study the net effect of soil microbes on plant regeneration (net plant-soil feedback) and the mechanism of local adaptation in explaining such effect.

The last part (**Chapter 6**), we summarise all findings in the thesis and discuss the consistence and differentiation with other studies. We also give suggestions for maintaining ecosystem biodiversity and perspectives on further studies.

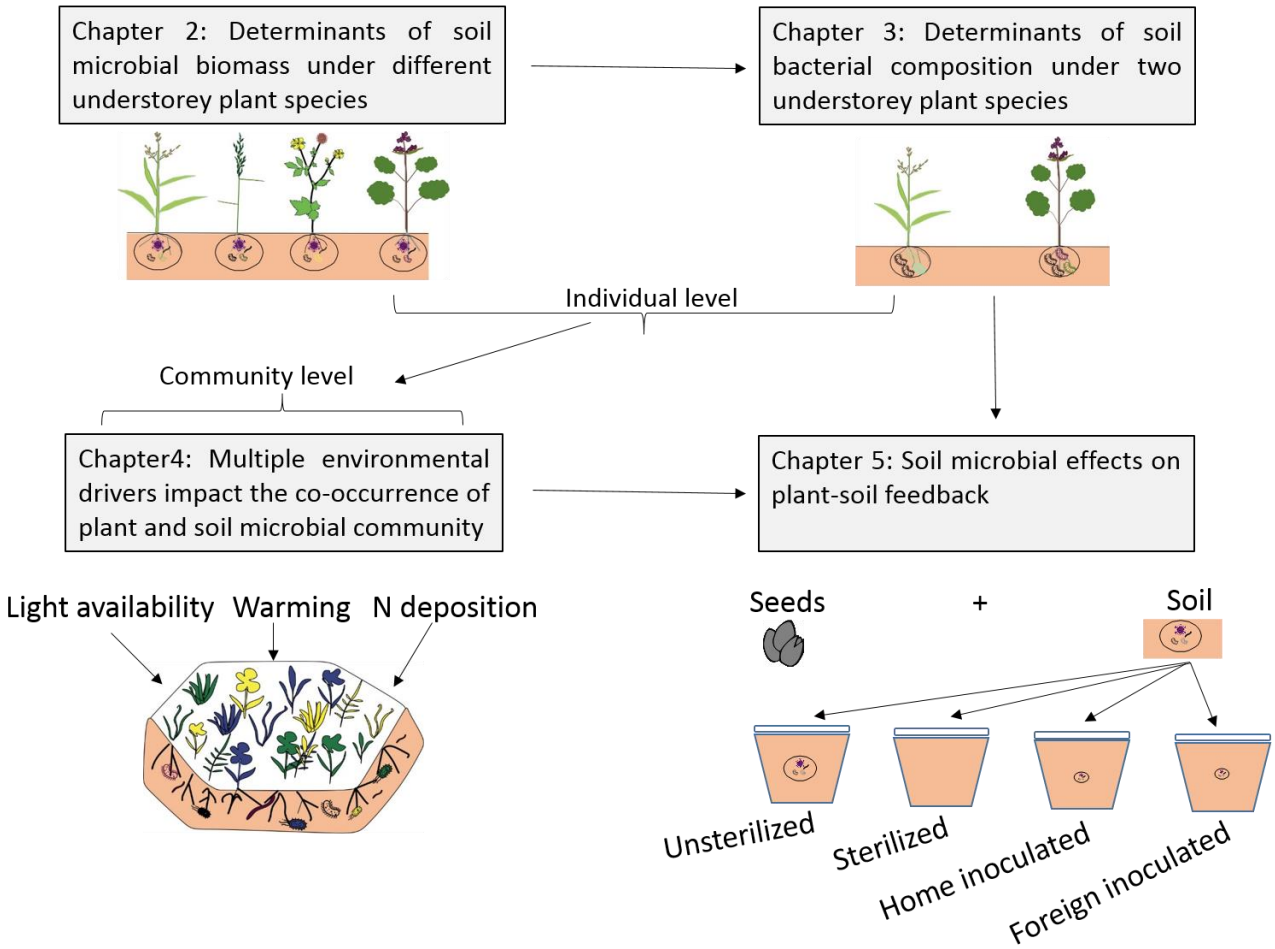


Fig. 1.5 Outline of the main four chapters in the thesis.

Chapter 2

Local soil characteristics determine the microbial communities under forest understorey plants along a latitudinal gradient

After: Ma S, De Frenne P, Vanhellemont M, Wasof S, Boeckx P, Brunet J, Cousins SAO, Decocq G, Kolb A, Lemke I, Liira J, Naaf T, Orczewska A, Plue J, Wulf M and. Verheyen K. Local soil characteristics determine the microbial communities under forest understorey plants along a latitudinal gradient. *Basic and Applied Ecology*, resubmitted after revisions.

2.1 Abstract

The soil microbial community is essential for maintaining ecosystem functioning and is intimately linked with the plant community. Yet, little is known on how soil microbial communities in the rhizosphere vary at continental scales within species. Here we assess the effects of soil chemistry, large-scale environmental conditions (i.e. temperature, precipitation and nitrogen deposition) and forest land-use history on the soil microbial communities (measured by phospholipid fatty acids) in the rhizosphere soils of four plant species (*Geum urbanum*, *Milium effusum*, *Poa nemoralis* and *Stachys sylvatica*) in forests along a 1700 km latitudinal gradient in Europe.

Soil microbial communities differed significantly among plant species, and soil chemistry was the main determinant of the microbial community composition within each plant species. Influential soil chemical variables for microbial communities were plant-species specific; soil acidity, however, was often an important driver. Large-scale environmental conditions, together with soil chemistry, only explained the microbial community composition in *M. effusum* and *P. nemoralis*. Forest land-use history did not affect the soil microbial community composition.

Our results underpin the dominant role of soil chemistry in shaping microbial community composition variation within plant species at the continental scale, and provide insights into the diversity and functionality of soil microbial communities in forest ecosystems.

2.2 Introduction

Understorey plant species in temperate forests play a critical role in maintaining forest ecosystem functioning. Yet, little is known about the soil microbial communities under the understorey plant species (Gilliam 2007). Soil microbial communities such as fungi and bacteria are highly diverse and are essential for key ecosystem functions such as carbon (C) and nutrient cycling (Paul 2014; Wagg, Bender, Widmer & van der Heijden 2014). The key aspect of elucidating the ecological role of soil microbial communities under plants is to find the driving factor for their compositional differences. Many studies have focused on the soil microbial community composition between different plant species and found that plant species identity is a critical driver for soil microbial community composition mainly through root exudates and litter chemistry (Bakker, Bradeen & Kinkel 2013; Martinez-Garcia, Richardson, Tylianakis, Peltzer & Dickie 2015). Within species, populations occurring in different regions experience sometimes very contrasting resources and conditions. These resources and conditions, such as soil chemistry, climate, nitrogen (N) deposition and land-use history, can strongly affect the intraspecific variation in terms of soil microbe communities of the root zone. Yet, knowledge on the importance of these factors in driving soil microbial community composition within plant species is largely lacking.

Among the abiotic environmental drivers, soil chemistry can affect soil microbial communities substantially because it relates to many essential resources for soil fungi and bacteria (e.g., the available soil C, N, phosphorus (P) and many other macro- and microelements) (Schmidt *et al.* 2011; Brockett, Prescott & Grayston 2012; Paul 2014). Soil nutrient availability (e.g., N and P) and acidity drive the activities of soil fungi and bacteria with respect to nutrient utilization and decomposition rate (Yin, Phillips, Liang, Xu & Liu 2016). For instance, lower pH generally benefits soil fungi while higher pH facilitates soil bacteria (Stevens *et al.* 2011). In addition, the composition of microbial communities can shift from fungi-dominated to bacteria-dominated with lower soil C concentrations (Hu *et al.* 2017). In another study, Richter, Schoning, Kahl, Bauhus and Ruess (2018) examined fungi and bacterial biomass in the mineral soil (0-10 cm) in 150 temperate forests across Germany and found that soil nutrients and pH are strongly correlated with the abundance of Gram-positive bacteria and *Actinobacteria*.

At larger geographical scales, forests along latitudinal gradients vary in terms of their climatic conditions and N deposition loads. Forest soil fungal and bacterial communities may shift corresponding to the variation in temperature, precipitation and atmospheric N deposition (Staddon, Trevors, Duchesne & Colombo 1998; Dentener *et al.* 2006; Castro, Classen, Austin, Norby & Schadt 2010). Staddon, Trevors, Duchesne and Colombo (1998) found ambient temperature to be

one of the main determinants for the decreasing diversity of the soil microbial community along an 800 km latitudinal gradient in Western Canada. Likewise, different precipitation regimes altered the fungal community composition and the relative abundance of two dominant bacterial phyla (i.e., *Acidobacteria* and *Proteobacteria*) in a deciduous forest ecosystem in Eastern America (Castro, Classen, Austin, Norby & Schadt 2010). Many non-crop vegetation in Western and Eastern Europe experience exceedingly high N deposition loads (higher than 10 kg N ha^{-1} yr^{-1}) (Dentener *et al.* 2006), and the high N deposition shifts the composition of plant and soil microbial communities (Fierer *et al.* 2012). Collectively, these large-scale environmental conditions may contribute in explaining soil microbial community composition.

Finally, in Europe and eastern North America, many temperate forests are post-agricultural and bear the imprint of previous land use. Previous land use shapes specific soil abiotic and biotic conditions (Jangid *et al.* (2011), and the legacy of land-use change on plant and soil microbial communities may persist for decades to centuries (Aggemyr & Cousins 2012; Bachelot *et al.* 2016). Thus, the soil microbial community of ancient forests (i.e. those forests existed continuously based on the oldest historical maps available, mainly range from the middle to the late 18th century), and more recently established forests on former agricultural land (did not occur on the oldest maps but established at the beginning of 19th century) (Hermy & Verheyen 2007), may differ. Surprisingly, despite its ecological importance, the soil microbial community (with a focus on fungi and bacteria) has never been compared between ancient and recent temperate forests at the European scale. Tracking the impact strength of land-use history on soil microbial community can create baselines to refer to and further our understanding on the importance of soil abiotic and biotic conditions with respect to temporal dynamics of soil biota.

Here we focused on the effects of abiotic factors (soil chemistry, large-scale environmental conditions) and land-use history on soil microbial community composition within plant species. We focused on four understorey plant species that ranged in their colonization rates and life form. Phospholipid fatty acid-based soil microbial community composition was determined using rhizosphere soils under each plant species, collected along a 1700 km latitudinal gradient in Europe. We hypothesized that (1) soil microbial community composition differs between the four plant species; (2) soil microbial community composition in each plant species is significantly driven by three factors (soil chemistry, large-scale environmental conditions and land-use history); and (3) higher soil nutrients and pH can facilitate and/or decline specific fungi and bacteria groups, resulting shifts in soil microbial community composition.

2.3 Materials and Methods

2.3.1 Study species and area

We selected *Geum urbanum* L. (Rosaceae), *Milium effusum* L. (Poaceae), *Poa nemoralis* L. (Poaceae) and *Stachys sylvatica* L. (Lamiaceae) across eight regions as study species based on their occurrence along the latitudinal gradient. The selected species cover two plant life forms, i.e., grasses (*M. effusum* and *P. nemoralis*) and forbs (*G. urbanum* and *S. sylvatica*), and have different capacities to colonize ecotones of ancient and recent (land-use history) deciduous forests. We focused on deciduous forests because they are the most abundant forest type in rural landscape across Europe and have a highly diverse understorey species composition. Within each region, forest continuity based on existing evidence was used to distinguish ancient and recent forests. Ancient forests were those forested continuously since at least a specified date, but this date varies among regions depending on the availability of historical site information and ranges from 1600-1820 (Hermy, Honnay, Firbank, Grashof-Bokdam & Lawesson 1999). Recent forests were those afforested on previously agricultural lands (Flinn & Vellend 2005) and mostly established in the 19th century except in Poland, where recent forests were established 15-40 years ago. In several regions, only a small fraction of the actual forest cover can be referred to as 'ancient'. These ancient forests (AF) have no historical record (mainly cartographical) of agricultural land use and have generally been continuously wooded for at least ca. 150–400 years (Hermy, Honnay, Firbank, Grashof-Bokdam & Lawesson 1999; Flinn & Vellend 2005; Hermy & Verheyen 2007; De Frenne *et al.* 2011a). Within each forest, we sampled the entire patch until we found the four species, and the sampling site for each species within the patch was at least 50 m away from each other. When the four species did not occur in the same forest patch in a certain region, we sampled from other patches but with consistent land-use history within the region. For each sampling site, the focal plant was surrounded by multiple individuals of the same species. Trees were not close to focal individuals but often partially (canopies) distributed within a diameter of 5 m (See **Appendix 2.1** for tree species composition).

2.3.2 Soil sampling

We collected soil samples in eight regions (**Fig. 2.1**), i.e., Northern France (NF), Belgium (Be), Poland (Po), Western Germany (WG), Eastern Germany (EG), Southern Sweden (SS), Central Sweden (CS) and Estonia (Es) between June and August 2015. Within each region, soil samples for each species were taken from two pairs of forest patches differing in their time of origin, i.e., each pair consisted one ancient (having existed continuously as forest based on the oldest maps (ca. 1750) in Europe, (Hermy & Verheyen 2007)) and one recent (established on former agricultural lands, (De Frenne *et al.* 2011a)). In each forest, we selected healthy individuals (with no signs of damage from

herbivores or pathogens) of each understorey species, growing at least 10 m away from the nearest forest edge. Tree species composition within a 5 × 5 m^2 range around each sampling site was recorded to assess the forest uniformity across the sites. This record was used as background information but not a determinant of site selection because it was not possible to select focal individuals with consistent tree species composition across all regions.

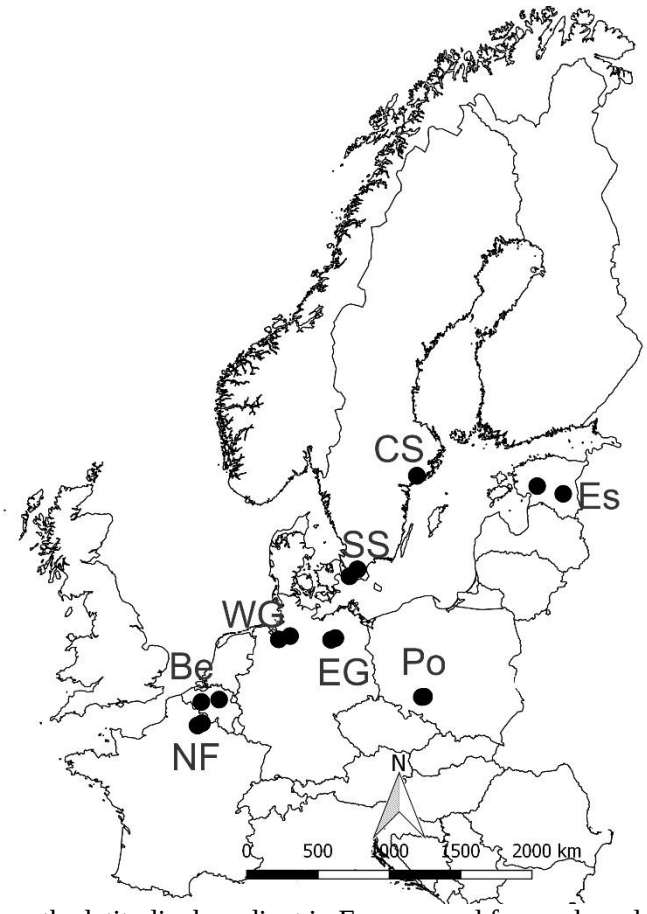


Fig. 2.1 Sampling regions along the latitudinal gradient in Europe used for seeds and soil collection in **Chapter 2**, **3**, **5**. Northern France (NF), Belgium (Be), Poland (Po), Western Germany (WG), Eastern Germany (EG), Southern Sweden (SS), Central Sweden (CS) and Estonia (Es). Note that only three regions were used in **Chapter 5**.

We cut the stem of each herb plant at about 1 cm above the ground. Soil samples were taken with augers with a diameter of 2.7 cm at 0-10 cm depth after removing of the litter layer around the base of the plant stem between June and August 2015 across all sampling regions. Differences in terms of sampling time depended on the maturation of seeds among species and regions. Debris and stones were removed from the soil samples immediately. Soil was brushed off from the plant root systems and this soil used for further analyses. We defined the used soil here as rhizosphere soil because of their distances to plant roots (max. 10 mm) and the high likelihood that this soil is affected by complex root systems (McNear Jr 2013). In total, we had 118 soil samples instead of the expected 128 samples (8 regions × 4 sites × 4 species) because some species were absent in some

forests in the region. Specifically, *M. effusum* was absent in recent forests in Belgium and instead we sampled from three ancient forests. *P. nemoralis* was absent in one ancient and recent forests in Belgium, two recent forests in Poland, and no samples for this species in Western Germany. *S. sylvatica* was absent in one recent forest in Estonia. Soil samples were stored at 4 °C and transported with plastic bags until treatments in the central lab in Belgium. All samples were immediately sieved through a 1-mm mesh upon arrival (the mesh was cleaned and sterilized with 75% ethanol in between samples) and stored at -18 ℃ until the start of soil microbial biomass measurement and soil DNA extraction (**Chapter 3**). A subsample of each soil sample was taken and dried at 40 ℃ for 48 h for subsequent chemical analyses.

2.3.3 Soil microbial biomass

Phospholipid fatty acids (PLFAs) were extracted and determined following Huygens *et al.* (2011). In brief, total lipids were extracted from 6 g freeze-dried soil using phosphate buffer/chloroform/methanol (0.9:1:2) before being loaded on a silica-filled solid-phase extraction column for purification. Neutral- and glycol-lipids were washed off from the extraction column using chloroform and acetone, respectively, before eluting the phospholipids with methanol. Phospholipids were subsequently transmethylated using methanolic KOH and the prepared fatty acids methyl esters (FAME) were quantified using gas chromatography-mass spectrometry (GC-MS, Trace GC-DSQ, Thermo Fisher, USA) equipped with a VF 23-MS column (60 m, 0.25 mm i.d., 0.25 µm film thickness; Varian, USA). Methylnonadecanoic acid (Me19:0) was used as internal standard. The biomass represented by each biomarker was calculated based on the PLFA concentrations (µg/g). In total, 35 PLFA biomarkers were detected. We did not consider three of the biomarkers because of the low frequency in our samples (two biomarkers) and the unrecognizable identity (one biomarker). The retained 32 useful biomarkers accounted for 89-94% of the total biomass. We classified 17 biomarkers in different functional groups (*Actinobacteria*, non-specific bacteria, Gram-positive bacteria, Gram-negative bacteria and fungi) and the remaining 15 biomarkers as unclassified (**Appendix 2.2**).

2.3.4 Soil chemistry

Soils were combusted at 1200 °C, and the gases were measured using a thermal conductivity detector in a CNS elemental analyser (vario Macro Cube, Elementar, Germany) for total carbon (C) and nitrogen (N). Total phosphorus (P) was measured after complete destruction of the soil samples with $HClO_4$ (65%), HNO_3 (70%) and H_2SO_4 (98%) in teflon bombs for 4 h at 150 °C. The concentrations of total P were measured colorimetrically according to the malachite green procedure (Lajtha, Driscoll, Jarrell & Elliott 1999). Bioavailable P (Olsen P) was measured by using extraction in $NaHCO_3$ (according to ISO 11263:1994 (E)) and colorimetric measurement according to the

malachite green procedure (Lajtha, Driscoll, Jarrell & Elliott 1999). Potassium (K), calcium (Ca), magnesium (Mg) and aluminum (Al) were measured by extracting soil samples with NH_4 Ac-EDTA and by analysing with atomic absorption spectrophotometry. Soil pH-H_2O was measured by shaking a 1:5 ratio soil/H_2O mixture for 5 min at 300 rpm and using a pH meter Orion 920A (with pH electrode model Ross sure-flow 8172 BNWP, Thermo Scientific Orion, USA). All chemical elements are shown in **Appendix 2.3**.

2.3.5 Large-scale environmental conditions

We calculated mean annual temperature and precipitation at the scale of 30 arc-seconds (approximately 1 km²) using WorldClim version 2 (http://worldclim.org/version2) (Fick & Hijmans 2017). Atmospheric N deposition at each sampling site was calculated for the year 2015 as the sum of wet and dry depositions of oxidised (NO_y) and reduced (NH_x) N based on modelled EMEP deposition data; and the model results of the 2016 version (data edition 2015v2016, 50 km resolution; http://www.emep.int/mscw/mscw_ydata.html#NCdata; **Appendix 2.4**).

2.3.6 Data analysis

All data were analysed in R version 3.4.3 (R Core Team 2017). To explore the data, we calculated Spearman correlations of pairs of variables in soil chemistry and large-scale environmental conditions (**Appendix 2.5**) using the function *cor* in the *corrplot* package (Taiyun & Viliam 2017). We then used linear mixed-effects models (*site* nested within *region* as random factor) to test chemical soil differences between the four plant species (**Appendix 2.6**) as well as between ancient and recent forests (**Appendix 2.7**). Models were generated by using the function *lmer* in the package *lme4* (Bates, Machler, Bolker & Walker 2015). Data of chemical soil variables were log10- or sqrt-transformed to meet the normality assumption of the statistical tests. We did not include latitude and longitude in our data analyses because spatial autocorrelation tests by using Mantel correlogram were not significant. Additionally, trees can significantly affect soil chemistry (De Schrijver *et al.* 2012). Therefore, we also considered the effect of tree species composition on soil microbial biomass due to tree compositional divergence among sampling sites. Litter quality (LQ) scores were used as an indicator of litter decomposition rate. The score for individual tree species ranges from 1 to 5 (1: very low decomposition rate, 5: very high decomposition rate) (**Appendix 2.8**). At each sampling site, tree canopy cover weighted average of LQ score was calculated and used for further data analyses (**Appendix 2.9**).

To test the compositional differences of soil microbial communities between the four plant species (the first hypothesis), we used non-metric multidimensional scaling (NMDS) with 999 runs using the function *metaMDS* (distance = Bray-Curtis) in the *vegan* package (Oksanen *et al.* 2016).

Objects that are ordinated closer are more similar than to those further apart. The stress plot was shown in **Appendix 2.10**. The distinction was tested using PERMANOVA and pairwise PERMANOVA. To demonstrate which PLFA biomarker differs between the four plant species, we applied linear mixed-effects models (*site* nested within *region* as random factor) and multiple pairwise comparisons on each model using function *glht* (Tukey contrasts) in the *multcomp* package (Torsten, Frank & Peter 2008) (**Appendix 2.11**). Data of each PLFA biomarker were log10- or sqrt-transformed to meet the normality assumption of the statistical tests.

To quantify the explanatory power of soil chemistry, large-scale environmental conditions and land-use history (three data groups) for the soil microbial community composition (the second hypothesis), redundancy analysis (RDA) based variation partitioning was applied per plant species. First, the PLFA data was *Hellinger* transformed as this transformation produces more accurate estimates of R^2 values (Peres-Neto, Legendre, Dray & Borcard 2006). Then, variation partitioning with three explanatory factors, i.e., soil chemistry, large-scale environmental conditions and land-use history, was applied to each plant species using function *varpart* (package *vegan*). Extra variation partitioning was applied to consider the explanatory power of plant species identity (**Appendix 2.12**) and tree species composition (**Appendix 2.13**) in the variation of soil microbial community composition. We used adjusted R^2 values to express explained variations because of the unbalanced numbers of variables in the three explanatory factors, and the significance of each factor's explanatory power was tested using the function *anova.cca*.

To find the specific abiotic effects on soil microbial community composition under different plant species (the third hypothesis), we first used forward selection on all chemical soil variables in each plant species and retained the significant soil variables. Then, the significant soil chemistry variables were used in RDA for each plant species to assess their correlations with PLFA biomarkers. We plotted the correlation of the significant soil chemistry variables with specific PLFA biomarkers using species and biplot scores from RDA (function *rda* in package *vegan*) by using package *ggplot2* (Wickham 2009).

2.4 Results

2.4.1 Differences of soil microbial communities among plant species

The composition of the soil microbial community represented by PLFA biomarkers differed significantly among the plant species (**Fig. 2.2,** $P < 0.001$). Pairwise PERMANOVA showed that the soil microbial community composition under *M. effusum* differed significantly from the other three plant species (**Appendix 2.14**). At the biomarker level, there were 24 biomarkers showed

significant differences among plant species **Appendix 2.11**). For instance, aC16:0 was absent in the soils of *M. effusum*, C24.1ω15c only occurred in the soils of *P. nemoralis*, and 16:1ω7t only occurred in the soils of *P. nemoralis* and *S. sylvatica* (and in one soil of *G. urbanum*).

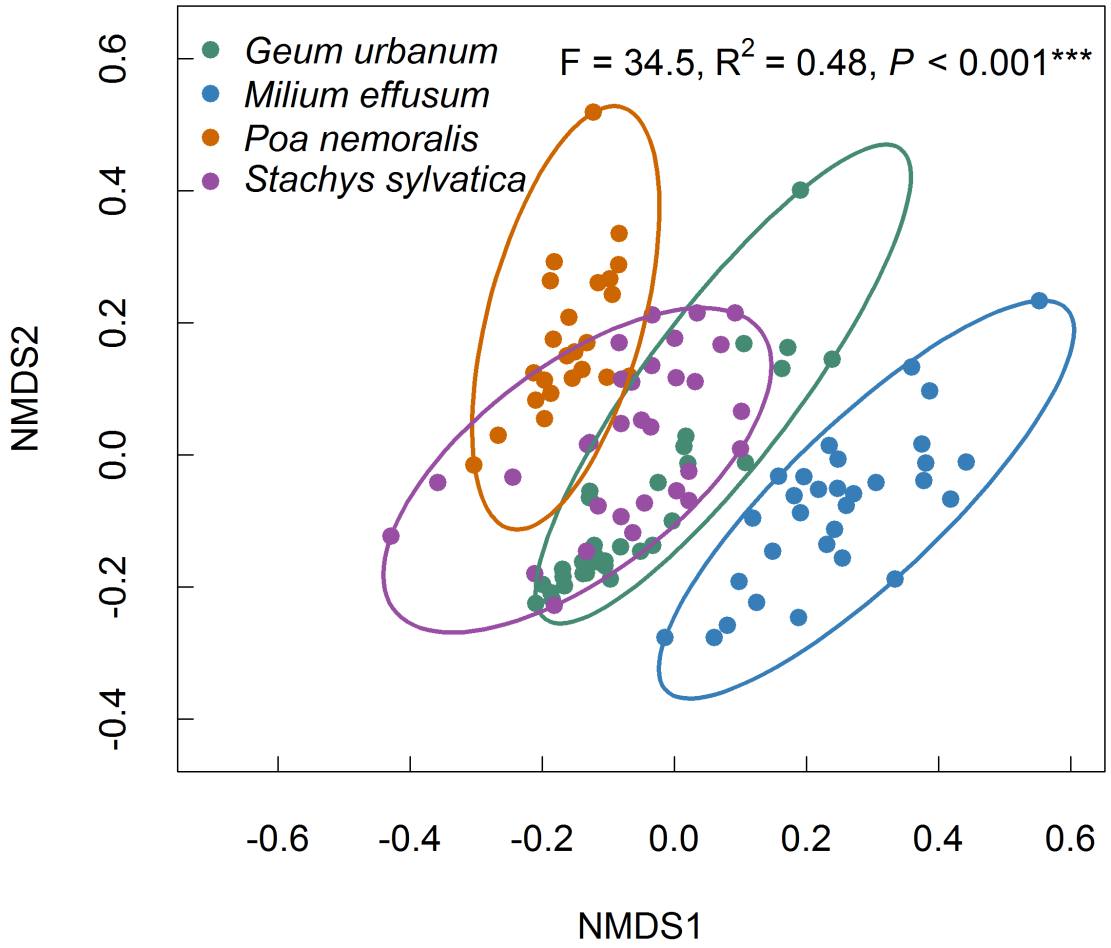


Fig. 2.2 Composition of the soil microbial community based on PLFAs and analysed with non-metric multidimensional scaling (NMDS) of all PLFA biomarkers for four species (distance = Bray-Curtis). The stress value is 0.17. Ellipsoid hulls were added to enclose all points in each plant species. The significance test was based on a PERMANOVA.

2.4.2 Factors determining soil microbial community composition within each plant species

Across the four understorey species, soil chemistry explained more variation in the soil microbial community composition than the large-scale environmental conditions and land-use history (**Fig. 2.3**). For the forbs *G. urbanum* and *S. sylvatica*, soil chemistry significantly and purely explained 0.44 and 0.36 of the variation in soil microbial community composition respectively (Fig. 2.3). For the grasses, i.e., *M. effusum* and *P. nemoralis*, both soil chemistry and large-scale environmental conditions significantly explained the variation in soil microbial community composition. The joint explanation by the two factors was 0.10 in *M. effusum* and 0.30 in *P. nemoralis*. The pure explanation by soil chemistry in the two grasses accounted to 0.27 (*M. effusum*) and 0.21 (*P. nemoralis*). Land-use history did not significantly explain any of the variation in soil microbial community composition in each of the investigated plant species. Similar insignificant patterns

were found for tree species composition in three plant species, i.e., *G. urbanum*, *M. effusum* and *P. nemoralis*. However, tree species composition significantly affected soil microbial community composition under *S. sylvatica* (**Appendix 2.13**).

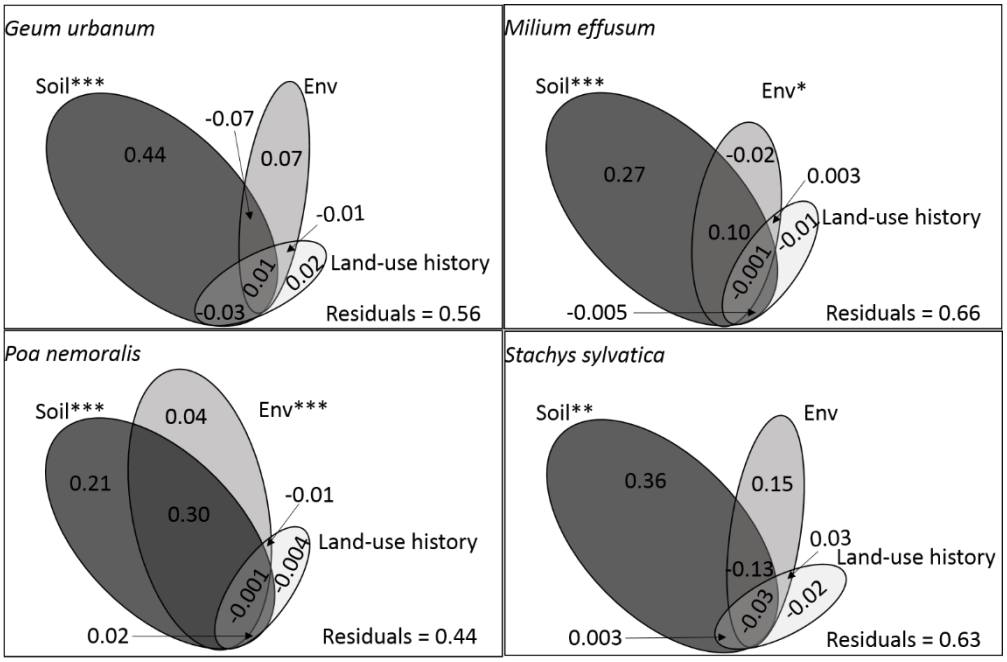


Fig. 2.3 Variation in soil microbial community composition explained by three factors, i.e., soil chemistry (Soil), large-scale environmental conditions (Env) and land-use history for each plant species. Adjusted R^2 values in each fraction indicate the explained percentage of the variation. Residuals indicate the unexplained variation. Adjusted R^2 values may cause small negative values. Asterisks show the significance of the permutation tests for each explanatory factor.

2.4.3 Correlations between soil chemistry and PLFA biomarkers

Across the four understorey species, the significant chemical soil variables contributed to explain the variation in soil microbial communities were soil K, Ca, Mg, Al, Olsen P and pH, which associated with biomarkers classified into different microbial functional groups (*Actinobacteria*, non-specific bacteria, Gram-positive and Gram-negative bacteria and fungi) (**Fig. 2.4 and Appendix 2.15**). In *G. urbanum*, soil Olsen P, Mg and Al concentration were the significant soil variables correlated with the soil microbial community composition. The biomarkers of non-specific bacteria and fungi were positively correlated with Olsen P, while biomarkers of *Actinobacteria*, Gram-positive bacteria and Gram-negative bacteria (C18:1ω7t) were negatively correlated with Olsen P concentration. Gram-negative bacteria (C16:1ω7c) and fungi were positively correlated with soil Mg concentration, while negatively correlated with soil Al concentration. In *M. effusum*, soil Ca concentration and pH were significantly correlated with the soil microbial communities. Gram-negative bacteria (C16:1ω7c) was positively related to soil Ca concentration; Gram-positive bacteria (iC16:0) was negatively related to Ca concentration and soil

pH. In *P. nemoralis*, five soil variables significantly predicted the soil microbial community composition, i.e., P, Olsen P, Ca, Al and pH. Gram-negative bacteria (C16:1ω7c) was positively correlated with Olsen P, while Gram-negative bacteria (C18:1ω7t) showed a negative relationship with Olsen P. In *S. sylvatica*, soil microbial PLFA biomarkers correlated with soil K and Al concentration, which were mostly driven by non-specific bacteria (C16:0) and Gram-positive bacteria (iC15:0 and iC16:0).

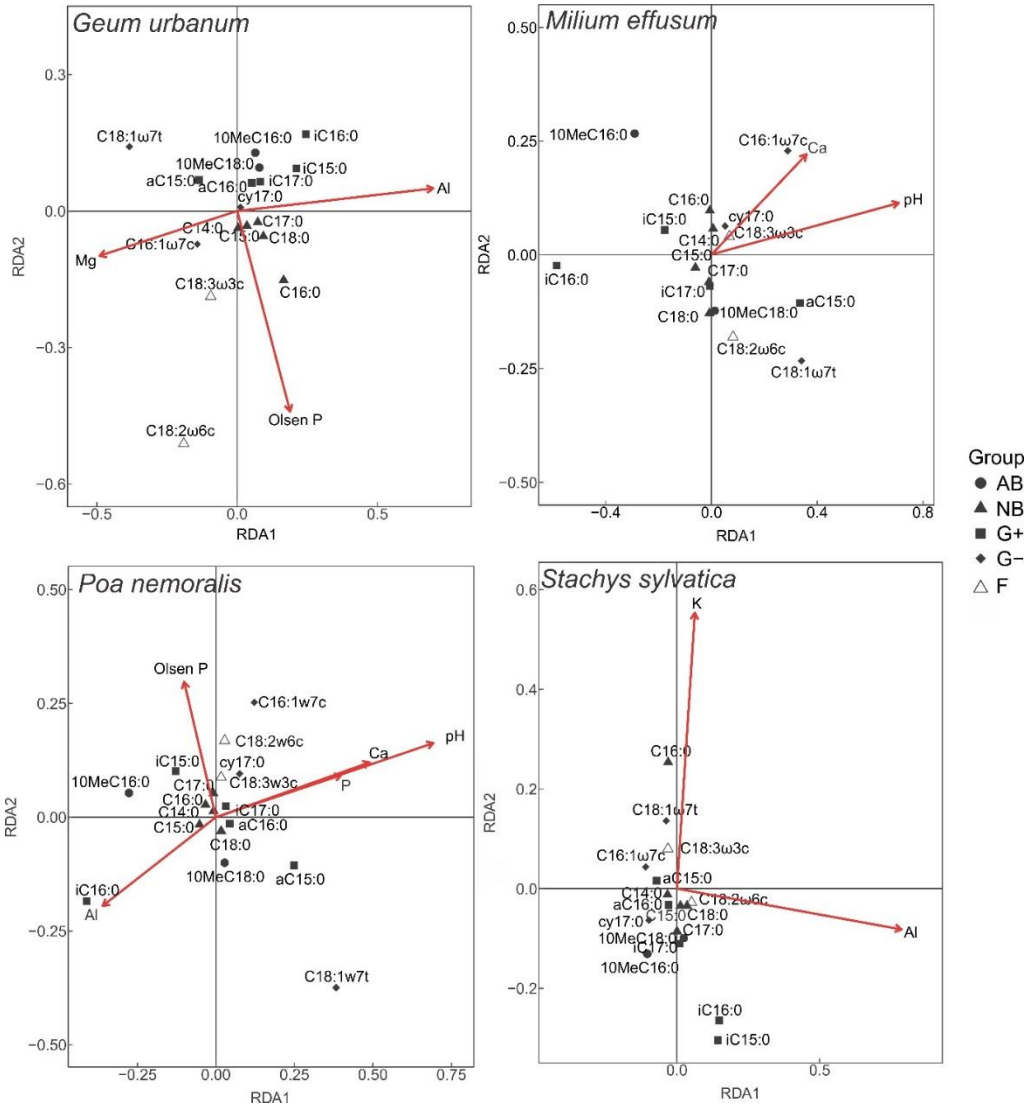


Fig. 2.4 Redundancy analysis (RDA) of soil microbial community composition in the rhizosphere soil of *Geum urbanum*, *Milium effusum*, *Poa nemoralis* and *Stachys sylvatica* with only significant explanatory chemical soil variables indicated in the plots. PLFA biomarkers indicated by five microbial functional groups were shown in the figure. Different symbols indicate the classified microbial functional groups. AB: *Actinobacteria*; NB: non-specific bacteria; G+: Gram-positive bacteria; G-: Gram-negative bacteria; F: fungi.

2.5 Discussion

We conducted an observational study on rhizospheric soil microbial community composition under four understorey plant species across Europe. Confirming our first hypothesis, soil microbial

community composition under the studied plant species differed significantly between *M. effusum* and the other three plant species. The importance of plant species in determining soil microbial community composition is also reflected by the significant explanation of plant species in the variation partitioning of the soil microbial community composition (**Appendix 2.12)**. This finding is consistent with many studies confirming the ecological linkages between aboveground plants and belowground microbes (Wardle, Yeates, Williamson & Bonner 2003; Philippot, Raaijmakers, Lemanceau & van der Putten 2013; Burns, Anacker, Strauss & Burke 2015). The four plant species harboured different soil microbial communities possibly induced by different root exudates and soil nutrients (Martinez-Garcia, Richardson, Tylianakis, Peltzer & Dickie 2015). Interestingly, soil Ca and Al concentration were chemical characteristics determining microbial community composition in *G. urbanum, M. effusum* and *P. nemoralis* but not in *S. sylvatica*. Instead, we found a significant role of soil K in this species. Although there is no study indicating that *S. sylvatica* is K-limited in our forests, Taylor and Rowland (2010) found Ca concentrations in plant tissue of *S. sylvatica* to be lower than in most herb species. This partly explains the absence of effects of soil Ca concentration on soil microbial community composition under this species. Microbial-mediated K cycling under *S. sylvatica* might be more active because of the characteristic fast elongation of the stem of this species (much K is needed for fast stem elongation (Taylor & Rowland 2010)). Most importantly, our study was designed to assess the effects of multiple factors in driving microbial community composition within plant species across Europe. Soil chemistry and large-scale environmental conditions, but not land-use history, were the main determinants of the rhizospheric soil microbial community composition across all plant species. Particular chemical soil variables showed correlations with specific soil microbial communities, and these soil variables were mostly indicators for soil acidity.

2.5.1 Three explanatory factors for soil microbial community composition

Our second hypothesis that soil chemistry, large-scale environmental conditions and land-use history are significant factors in determining soil microbial community composition in each plant species was partly supported. We only found that soil chemistry significantly determined soil microbial community composition within each plant species. Its explanation power ranged from 21% to 44% across the four study plant species. The significant influence of soil chemistry on microbial community composition is congruent with the previous study at a smaller spatial scale, which focused on the chemical soil conditions of under forest tree (birch) in regulating the bacterial and fungal community composition (Mitchell *et al.* 2010). Soil chemistry includes many components and its influences on soil fungi and bacteria via, for instance, soil enzymes and microbial activity can ultimately lead to compositional shifts in soil microbial communities (Schappe *et al.* 2017;

Waldrop *et al.* 2017). In turn, chemical soil conditions can also be modified through the activities of different soil microbes (Souza-Alonso, Novoa & Gonzalez 2014).

The large-scale environmental conditions (climate and N deposition) explained a significant part of the soil microbial community composition of the two studied grasses (*M. effusum* and *P. nemoralis*). Mean annual temperature and N deposition are both positively correlated with mean annual precipitation across the studied latitudinal gradient (**Appendix 2.5**). Changes in precipitation regimes have been considered as one of the main factors for soil microbial community composition and diversity (Brockett, Prescott & Grayston 2012). Less precipitation benefits the assembly of Gram-negative bacteria and fungi, while higher precipitation may result in more Gram-positive, anaerobic and sulphate-reducing bacteria (Drenovsky, Steenwerth, Jackson & Scow 2010). Even the historical precipitation regime matters for the contemporary dynamics of the microbial community and therefore the biogeochemical cycling (Evans & Wallenstein 2012). The large-scale environmental conditions were not important for the soil microbial community composition in the two forbs we studied (*G. urbanum* and *S. sylvatica*). Given the inconsistent responses to environmental conditions for the soil microbial communities in the four studied plant species, more studies are needed to understand the determinants of large-scale environmental conditions on soil microbial community composition harboured by different host plants and ecosystems. The joint explanation, which can be explained by both soil chemistry and large-scale environmental conditions together, was 23% in *P. nemoralis* suggesting a strong correlation between the two factors in this species. Large-scale environmental conditions can affect soil microbial community indirectly through changes in soil chemistry. For instance, increased N deposition can result in lower pH and decrease the concentrations of soil base cations, while increase the concentration of soil Al (Shi *et al.* 2018). We indeed observed a negative correlation between N deposition and soil Mg concentrations (**Appendix 2.5**).

Surprisingly, we found no significant effect of land-use history on soil microbial communities, and thus microbial community composition was found to be similar in ancient and recent forests in our pan-European study. This finding contradicts some previous studies (Ma, Guo, Lu, Yuan & Wang 2015; de la Pena *et al.* 2016) that demonstrated that land-use legacies (mainly soil N and P concentration) affect the composition and activity of soil microbial communities. In the study of Ma, Guo, Lu, Yuan and Wang (2015), soil microbial community composition in soil cores (5 cm diameter and 0-15 cm depth) was assessed at a regional scale in Northeastern China and they found that land-use change but not soil chemistry was one of the main factor in determining soil microbial community composition. Yet, our study corroborates the results displayed by Jangid et al. (2011), in which the soil microbial communities in soil cores (5 cm diameter and 0-10 cm depth) are similar in

ancient forests and post-agricultural forests that had been established ca. 60 years ago. In our study, except Poland (recent forest age ranges between 15-45 years), all recent forests in the other seven regions are established at the beginning of the 19th century and thus stand for ca. 200 years. Long-term succession after land-use change may explain the similarity between the soil microbial communities. Additionally, the four study species occurred both in recent and ancient forests, which partly support the absence of microbial composition difference between the two types of forests. There was unexplained variation in soil microbial community composition in our study, suggesting that other potential factors should be included, for instance, historic management and tree species composition nearby. Given that we only found significant effects of the tree species in *S. sylvatica*, but not in the other three plant species, interspecific variation should be considered as well, which can be due to variation in plant traits.

2.5.2 The correlation between soil microbial community and soil characteristics

Soil pH, Ca, and Al concentrations, which are indicators for soil acidity, and Olsen P, were among the soil chemical variables correlated with soil microbial community composition. Thus, our third hypothesis was supported. Soil microbial activities, such as microbial catabolic diversity (microbial responses to the addition of C-rich substrates), can be driven by soil pH and Ca concentration (Gartzia-Bengoetxea, Kandeler, de Arano & Arias-Gonzalez 2016). Assessing soil micorbial activity (although not available in this study) can facilitate the understanding of biochemcial cycling and soil micorbial community dynamics and could be the focus of future research. In our study, soil Al concentration showed a negative relationship with Gram-negative bacteria in three species (*G. urbanum*, *P. nemoralis* and *S. sylvatica*). Free Al ions are toxic and may affect the effectiveness of biochemical cycling; Gram-negative bacteria are essential to trigger the catalytic properties of enzymes necessary for the decomposition of cellulose, hemicellulose, starch and monophosphoesters (Tischer, Blagodatskaya & Hamer 2015). Previous studies have observed an increased biomass of Gram-negative bacteria with the availability of organic substrates. The correlations between soil microbes and Olsen P mostly varied between *G. urbanum* and *P. nemoralis*, but consistent correlation trends in both plant species did occur, for instance, between soil total P and Gram-negative bacteria (C18:1ω7t, negative correlation) and fungal PLFAs (C18:2ω6c and C18:3ω3c, positive correlation). Interestingly, we observed a similar concentration range of Olsen P (19.7-51.6 mg kg^{-1} for *G. urbanum* and 17.8-52.7 mg kg^{-1} for *P. nemoralis*) (**Appendix 2.3**). Fungi and Gram-negative bacteria are both involved in soil P solubilisation and mineralization (Chatli, Beri & Sidhu 2008). Sufficient Olsen P may ease resource competition between fungi and Gram-negative bacteria (C16:1ω7c), but Gram-negative bacteria (C18:1ω7t) will

be inhibited by higher soil P concentrations under *G. urbanum* and *P. nemoralis*, which implies a threat to microbial composition changes in P-eutrophic forests (Liu, Gundersen, Zhang & Mo 2012).

2.6 Conclusions

Soil microbial community composition in the plant root zone varied substantially between plant species, as well as within populations of conspecifics at different latitudes. Chemical soil characteristics, particularly soil acidity and bioavailable phosphorus, along the studied latitudinal gradient were most important explaining the distinct microbial community composition under each plant species, suggesting an important role of local adaptation, inter- and intraspecific variation. Recovery with respect to soil microbial community diversity in post-agricultural lands might be expected after several decades because plants establish gradually and can facilitate the assemblage of soil microbes. With all these effects of abiotic and biotic factors on soil microbial community composition, we advocate the importance of maintaining the diversity of forest understorey species as it can lead to a divergent soil microbial community composition. Our results also imply the possibility of using biogeochemical conditions to predict distribution patterns and dynamics of soil microbes across the globe and when facing environmental changes. Additionally, given the major contribution of soil bacteria to detected PLFA biomarkers in this study, further studies on the correlation between bacterial diversity and abiotic and biotic conditions will facilitate the understanding of biogeochemical cycling driven by soil bacteria in forested ecosystems.

Appendix 2.1

Tree species composition and cover within a 5 x 5 m² range at each sampling site within each region: Northern France (NF), Belgium (Be), Poland (Po), Western Germany (WG), Eastern Germany (EG), Southern Sweden (SS), Central Sweden (CS) and Estonia (Es)

Site_ID	Region	Land-use history	Tree species (cover%)
Geum urbanum			
G1	Es	Ancient	*Picea abies* (5); *Pinus sylvestris* (10); *Populus tremula* (30); *Quercus robur* (15); *Sorbus aucuparia* (20)
G2	Es	Recent	*Betula pendula* (40); *Picea abies* (15); *Populus tremula* (25); *Sorbus aucuparia* (10)
G3	Es	Ancient	*Alnus incana* (1); *Betula pendula* (30); *Populus tremula* (20); *Quercus robur* (50)
G4	Es	Recent	*Alnus glutinosa* (20); *Alnus incana* (60); *Betula pubescens* (10)
G5	CS	Ancient	*Betulla pendula* (2); *Corylus avellana* (90); *Picea abies* (10)
G6	CS	Recent	*Corylus avellana* (75); *Quercus robur* (50)
G7	CS	Ancient	*Corylus avellana* (10); *Fraxinus excelsior* (50)
G8	CS	Recent	*Corylus avellana* (10); *Malus sylvestris* (60); *Quercus robur* (50)
G9	SS	Ancient	*Fagus sylvatica* (60); *Fraxinus excelsior* (10)
G10	SS	Recent	*Fagus sylvatica* (80)
G11	SS	Ancient	*Fagus sylvatica* (40); *Fraxinus excelsior* (20)
G12	SS	Recent	*Acer platanoides* (30); *Acer pseudoplatanus* (40); *Fraxinus excelsior* (30); *Ulmus glabra* (20)
G13	EG	Ancient	*Quercus robur* (65)
G14	EG	Recent	*Alnus glutinosa* (90)
G15	EG	Ancient	*Alnus glutinosa* (65)
G16	EG	Recent	*Alnus glutinosa* (80)
G17	WG	Ancient	*Fraxinus excelsior* (30); *Picea abies* (20)
G18	WG	Recent	*Alnus glutinosa* (20); *Fraxinus excelsior* (50)
G19	WG	Ancient	*Fagus sylvatica* (90); *Fraxinus excelsior* (10)
G20	WG	Recent	*Carpinus betulus* (30); *Quercus robur* (80)
G21	Po	Ancient	*Alnus glutinosa* (40); *Prunus padus* (15); *Rhamnus cathartica* (5)
G22	Po	Recent	*Alnus glutinosa* (70)
G23	Po	Ancient	*Acer pseudoplatanus* (10); *Fagus sylvatica* (10); Fraxinus excelsior (5); *Carpinus betulus* (5); *Quercus robur* (60)
G24	Po	Recent	*Alnus glutinosa* (80)
G25	Be	Recent	*Populus tremula* (60)
G26	Be	Ancient	*Populus tremula* (40)

Appendix 2.1 (continued)

Site_ID	Region	Land-use history	Tree species (cover%)
G27	Be	Ancient	*Alnus glutinosa* (40); *Fraxinus excelsior* (50)
G28	Be	Recent	*Fraxinus excelsior* (70); *Quercus robur*(30)
G29	NF	Ancient	*Carpinus betulus* (95); *Acer pseudoplatanus* (70)
G30	NF	Recent	*Fraxinus excelsior* (90); *Quercus robur* (20); *Ulmus minor* (15)
G31	NF	Ancient	*Acer pseudoplatanus* (90); *Tilia cordata* (15)
G32	NF	Recent	*Acer pseudoplatanus* (65); *Carpinus betulus* (35); *Quercus robur* (30)
Milium effusum			
M1	Es	Ancient	*Acer platanoides* (10); *Pinus sylvestris* (40); *Populus tremula* (50)
M2	Es	Recent	*Picea abies* (15); *Populus tremula* (50); *Quercus robur* (10); *Sorbus aucuparia* (20)
M3	Es	Ancient	*Alnus glutinosa* (10); *Betula pubescens* (35); *Picea abies* (20); *Pinus sylvestris* (10); *Populus tremula* (15); *Quercus robur* (20)
M4	Es	Recent	*Alnus glutinosa* (10); *Betula pendua* (10); *Picea abies* (5); *Populus tremula* (65); *Sorbus aucuparia* (1)
M5	CS	Ancient	*Betula pendula* (75); *Corylus avellana* (30); *Picea abies* (40)
M6	CS	Recent	*Betula pendula* (50); *Quercus robur* (10)
M7	CS	Ancient	*Acer pseudoplatanus* (30); *Corylus avellana* (25); *Fraxinus excelsior* (5); *Sorbus aucuparia* (10)
M8	CS	Recent	*Betula pendula* (40); *Quercus robur* (30)
M9	SS	Ancient	*Fagus sylvatica* (30); *Ulmus glabra* (30)
M10	SS	Recent	*Fagus sylvatica* (80)
M11	SS	Ancient	*Fagus sylvatica* (80); *Fraxinus excelsior* (10)
M12	SS	Recent	*Fagus sylvatica* (80)
M13	EG	Ancient	*Carpinus betulus* (90)
M14	EG	Recent	*Alnus glutinosa* (75); *Prunus padus* (15)
M15	EG	Ancient	*Alnus glutinosa* (55)
M16	EG	Recent	*Betula pubescens* (60); *Quercus robur*(10); *Sorbus aucuparia* (25)
M17	WG	Ancient	*Fraxinus excelsior* (50)
M18	WG	Recent	*Alnus glutinosa* (20); *Fraxinus excelsior* (50)
M19	WG	Ancient	*Alnus glutinosa* (50); *Fraxinus excelsior* (10)
M20	WG	Recent	*Carpinus betulus* (90); *Quercus robur* (20)
M21	Po	Ancient	*Alnus glutinosa* (80)
M22	Po	Recent	*Alnus glutinosa* (70)

Appendix 2.1 (continued)

Site_ID	Region	Land-use history	Tree species (cover%)
M23	Po	Ancient	*Acer pseudoplatanus* (10); *Carpinus betulus* (80); *Quercus robur* (20)
M24	Po	Recent	*Alnus glutinosa* (80); *Fraxinus excelsior* (10)
M25	Be	Ancient	*Fagus sylvatica* (85)
M26	Be	Ancient	*Quercus robur* (40); Populus alba (15); *Populus* × *canescens* (50)
M27	Be	Ancient	*Acer pseudoplatanus* (55); *Alnus glutinosa* (35)
M28	NF	Ancient	*Acer pseudoplatanus* (70); *Corylus avellana* (60); *Fraxinus excelsior* (35); *Hedera helix* (1)
M29	NF	Recent	*Carpinus betulus* (80); *Corylus avellana* (10); *Fraxinus excelsior* (10); *Ulmus minor* (5)
M30	NF	Ancient	*Picea abies* (70)
M31	NF	Recent	*Acer pseudoplatanus* (10); *Betula pendula* (55); *Carpinus betulus* (90); *Fraxinus excelsior* (20)
Poa nemoralis			
P1	Es	Ancient	*Picea abies* (15); *Populus tremula* (50); *Quercus robur* (10); *Sorbus aucuparia* (20)
P2	Es	Recent	*Betula pendula* (40); *Picea abies* (15); *Populus tremula* (25); *Sorbus aucuparia* (10)
P3	Es	Ancient	*Populus tremula* (20); *Quercus robur* (50); *Tilia cordata* (1)
P4	Es	Recent	*Alnus glutinosa* (60); *Alnus incana* (2); *Betula pubescens* (10)
P5	CS	Ancient	*Betulla pendula* (2); *Corylus avellana* (5); *Sorbus aucuparia* (10)
P6	CS	Recent	*Populus tremula* (30)
P7	CS	Ancient	*Betula pendula* (5); *Populus tremula* (5)
P8	CS	Recent	*Corylus avellana* (10); *Quercus robur* (50); *Malus sylvestris* (60)
P9	SS	Ancient	*Fagus sylvatica* (70)
P10	SS	Recent	*Fagus sylvatica* (90)
P11	SS	Ancient	*Fagus sylvatica* (40); *Fraxinus excelsior* (20)
P12	SS	Recent	*Fagus sylvatica* (90)
P13	EG	Ancient	*Carpinus betulus* (85); *Fagus sylvatica* (3); *Quercus robur* (10)
P14	EG	Recent	*Fagus sylvatica* (2); *Quercus robur* (65)
P15	EG	Ancient	*Quercus robur* (80)
P16	EG	Recent	*Alnus glutinosa* (2); *Betula pendula* (2); *Populus tremula* (60); *Quercus robur* (25)
P17	Po	Ancient	*Acer pseudoplatanus* (5); *Corylus avellana* (10); *Fraxinus excelsior* (10); *Prunus serotina* (10); *Quercus robur* (70)
P18	Po	Ancient	*Fagus sylvatica* (90); *Tilia cordata* (10)
P19	Be	Ancient	*Populus tremula* (90); *Quercus robur*(60)
P20	Be	Recent	*Fagus sylvatica* (90)

Appendix 2.1 (continued)

Site_ID	Region	Land-use history	Tree species (cover%)
P21	NF	Ancient	*Carpinus betulus* (70); *Fraxinus excelsior* (40)
P22	NF	Recent	*Clematis vitalba* (10); *Corylus avellana* (70)
P23	NF	Ancient	*Carpinus betulus* (15); *Tilia cordata* (100)
P24	NF	Recent	*Betula pendula* (10); *Carpinus betulus* (70); *Corylus avellana* (30); *Fraxinus excelsior* (40); *Quercus robur* (20)
Stachys sylvatica			
S1	Es	Ancient	*Populus tremula* (60); *Picea abies* (20); *Acer platanoides* (5)
S2	Es	Recent	*Betula pendula* (80); *Populus tremula* (10); *Alnus incana* (5)
S3	Es	Ancient	*Alnus incana* (5); *Populus tremula* (20); *Quercus robur* (60)
S4	CS	Ancient	*Betula pendula* (5); *Populus tremula* (5)
S5	CS	Recent	*Betula pendula* (10); *Salix caprea* (10); *Tilia cordata* (10)
S6	CS	Ancient	*Alnus glutinosa* (5)
S7	CS	Recent	*Populus tremula* (3)
S8	SS	Ancient	*Fagus sylvatica* (50)
S9	SS	Recent	*Fagus sylvatica* (50); *Quercus robur* (30)
S10	SS	Ancient	*Fagus sylvatica* (60); *Fraxinus excelsior* (20)
S11	SS	Recent	*Acer pseudoplatanus* (60); *Tilia cordata* (20)
S12	EG	Ancient	*Carpinus betulus* (90); *Quercus robur* (5)
S13	EG	Recent	*Alnus glutinosa* (5)
S14	EG	Ancient	*Alnus glutinosa* (60); *Fraxinus excelsior* (5)
S15	EG	Recent	*Alnus glutinosa* (40); *Fagus sylvatica* (30); *Quercus robur* (15)
S16	WG	Ancient	*Fraxinus excelsior* (70)
S17	WG	Recent	*Quercus robur* (70)
S18	WG	Ancient	*Alnus glutinosa* (60); *Fraxinus excelsior* (10)
S19	WG	Recent	*Carpinus betulus* (80); *Quercus robur* (40)
S20	Po	Ancient	*Alnus glutinosa* (80); *Prunus padus* (20)
S21	Po	Recent	*Alnus glutinosa* (80); *Sambucus nigra* (10)
S22	Po	Ancient	*Carpinus betulus* (10); *Fagus sylvatica* (40); *Pinus sylvestris* (5); *Quercus robur* (50); *Ulmus laevis* (10)
S23	Po	Recent	*Alnus glutinosa* (75); *Fraxinus excelsior* (5)
S24	Be	Recent	*Populus tremula* (40); *Quercus robur* (60)
S25	Be	Ancient	*Populus tremula* (60)

Appendix 2.1 (continued)

Site_ID	Region	Land-use history	Tree species (cover%)
S26	Be	Ancient	*Alnus glutinosa* (50); *Fraxinus excelsior* (40); *Quercus robur* (10)
S27	Be	Recent	*Alnus glutinosa* (70); *Robinia pseudoacacia* (10)
S28	NF	Ancient	*Fraxinus excelsior* (50)
S29	NF	Recent	*Acer pseudoplatanus* (15); *Fraxinus excelsior* (75)
S30	NF	Ancient	*Corylus avellana* (40); *Fraxinus excelsior* (45)
S31	NF	Recent	*Prunus domestica* ssp. Insititia (90)

Appendix 2.2

The assignment of functional groups based on the biomarkers of phospholipid fatty acids (PLFAs)

Functional group	Biomarker	Reference
Actinobacteria (AB)	10MeC16:0; 10MeC18:0	Turpeinen, Kairesalo and Haggblom (2004) Bach, Baer, Meyer and Six (2010)
Non-specific bacteria (NB)	C14:0; C15:0; C16:0; C17:0; C18:0	Bossio and Scow (1998) Fuchslueger, Bahn, Fritz, Hasibeder and Richter (2014) Kaiser *et al.* (2015)
Gram-positive bacteria (G+)	iC15:0; aC15:0; iC16:0; aC16:0; iC17:0	Bach, Baer, Meyer and Six (2010) Fuchslueger, Bahn, Fritz, Hasibeder and Richter (2014) Kaiser *et al.* (2015)
Gram-negative bacteria (G-)	16:1ω7c; cy17:0; C18:1ω7t	McKinley, Peacock and White (2005) Helfrich, Ludwig, Thoms, Gleixner and Flessa (2015) Kaiser *et al.* (2015)
Fungi	C18:2ω6c; C18:3ω3c	Kaiser *et al.* (2015) Bai, Liang, Bode, Huygens and Boeckx (2016)

Appendix 2.3

Mean values of chemical soil variables (calculated from four samples) for the soil sampled below each study species in each region

Region	C %	N %	C/N	P (mg/kg)	Olsen P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Al (mg/kg)	pH (H_2O)
Geum urbanum										
NF	5.4	0.4	13.4	709.1	19.7	161.0	21596.8	215.6	129.8	6.1
Be	3.6	0.3	11.5	692.7	41.8	152.5	2082.4	192.3	131.0	5.4
Po	5.2	0.4	12.0	653.8	27.7	57.5	2174.4	148.6	178.4	5.1
WG	5.1	0.4	13.0	558.8	39.7	172.3	2558.8	125.4	101.9	5.5
EG	11.	0.8	14.0	662.6	29.2	242.1	4691.8	224.2	175.5	5.9
SS	4.1	0.3	12.9	673.9	24.3	100.0	1747.3	135.4	198.1	5.7
CS	7.5	0.5	14.2	731.8	34.9	347.8	3009.8	522.7	134.6	5.4
Es	9.0	0.5	14.8	751.1	51.6	224.3	2727.6	396.6	241.2	5.6
Milium effusum										
NF	4.6	0.3	13.3	664.2	26.6	135.5	16963.2	150.4	154.1	6.1
Be[a]	2.9	0.2	12.6	347.8	21.6	112.1	428.9	71.6	450.9	4.8
Po	4.9	0.3	14.4	411.1	34.2	70.0	771.0	62.9	309.0	4.4
WG	4.8	0.3	14.1	406.0	27.2	85.9	859.3	68.6	301.7	4.7
EG	6.7	0.4	15.7	355.0	36.8	86.7	1145.7	68.8	458.4	4.5
SS	4.7	0.3	14.6	602.9	41.0	110.2	755.9	77.2	417.7	4.9
CS[b]	18.	0.9	18.8	511.5	69.7	398.3	1702.6	318.2	499.6	4.9
Es	5.6	0.4	15.3	463.1	43.9	99.4	1624.7	196.6	336.3	5.3
Poa nemoralis										
NF	3.6	0.3	11.6	449.3	17.8	247.4	3455.5	175.9	164.4	5.9
Be[c]	3.0	0.3	11.8	728.0	52.7	118.3	3140.2	230.5	122.9	6.4
Po[c]	4.1	0.3	15.3	462.5	43.2	72.8	619.8	50.5	353.8	4.8
WG	–	–	–	–	–	–	–	–	–	–
EG	6.4	0.4	15.1	369.6	33.0	113.6	669.1	80.9	381.5	4.6
SS	3.3	0.3	12.6	585.9	32.9	105.6	804.0	86.3	353.5	5.2
CS	8.1	0.5	14.6	651.4	29.2	259.1	3148.8	506.1	293.9	5.8
Es	4.5	0.3	13.0	509.1	42.4	109.9	1579.3	244.8	262.7	5.5
Stachys sylvatica										
NF	2.9	0.3	11.2	585.0	32.3	181.6	2095.6	192.8	126.9	5.7
Be	5.7	0.4	12.9	705.4	32.3	121.2	2672.4	184.1	205.6	6.0
Po	3.6	0.3	12.2	463.4	24.6	51.0	1422.4	76.6	168.5	5.1
WG	5.8	0.4	14.1	729.0	25.0	135.1	3183.1	147.2	207.6	5.4
EG	5.8	0.4	13.4	639.5	24.8	152.5	2490.3	142.4	263.1	5.4
SS	3.6	0.3	12.6	799.8	54.9	207.0	1632.6	127.8	240.2	5.9
CS	6.5	0.5	13.8	717.9	28.7	291.7	2613.9	374.5	228.4	5.6
Es	9.8	0.7	13.9	858.1	28.5	104.7	4574.5	433.7	234.5	5.6

[a]Not found in recent forests, soil samples were collected from three ancient forests. [b]The mean values of P and Al are based on two soil samples; the means for the other seven soil variables were calculated based on three soil samples. [c]Soil samples were collected from one ancient and one recent forests. –: Not found in Western Germany.

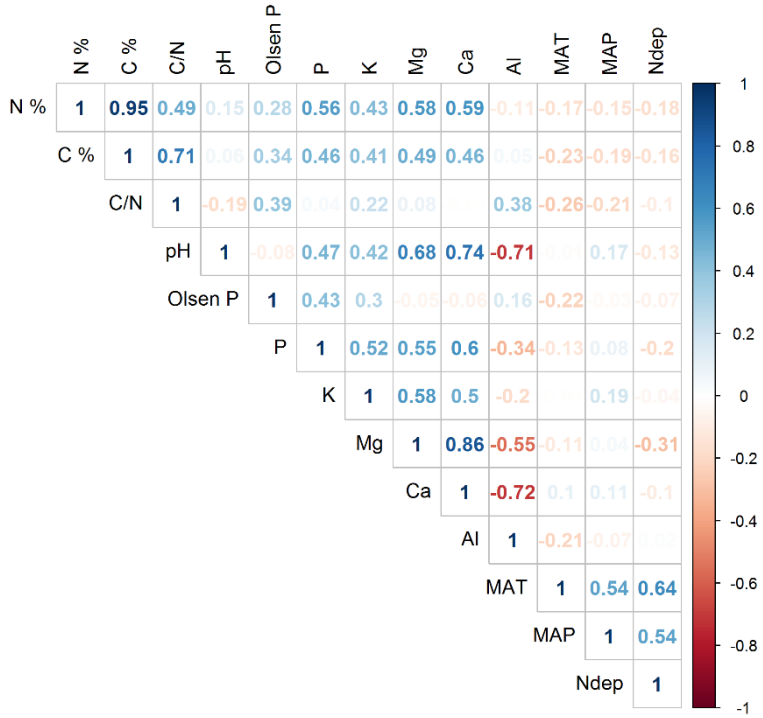
Appendix 2.4

Mean values of environmental conditions (MAT: mean annual temperature, MAP: mean annual precipitation and Ndep: N deposition) calculated from four sampling sites for each study species in each region

Region	MAT (℃)	MAP (mm)	Ndep (kg/ha)
Geum urbanum			
NF	9.7	773.8	13.3
Be	10.2	775.5	13.4
Po	8.4	580.8	11.1
WG	8.9	732.0	24.7
EG	8.6	621.0	15.1
SS	7.5	697.3	11.9
CS	6.3	589.7	5.3
Es	5.4	661.3	6.0
Milium effusum			
NF	9.8	743.5	12.1
Be[a]	10.2	779.7	13.2
Po	8.4	583.3	11.1
WG	8.9	732.0	24.7
EG	8.6	620.5	15.1
SS	7.5	697.3	11.9
CS	6.3	591.2	5.3
Es	5.4	666.8	5.9
Poa nemoralis			
NF	9.7	771.3	13.3
Be[b]	10.0	795.0	13.7
Po[b]	8.4	583.5	11.1
WG	–	–	–
EG	8.5	623.2	15.0
SS	7.5	697.3	11.9
CS	6.3	589.7	5.3
Es	5.4	661.3	6.0
Stachys sylvatica			
NF	9.6	787.5	13.3
Be	10.2	775.7	13.4
Po	8.4	581.0	11.1
WG	8.9	732.0	24.7
EG	8.6	622.0	15.1
SS	7.5	697.3	11.9
CS	6.3	591.2	5.3
Es	5.4	646.7	6.0

[a]Not found in recent forests, soil samples were collected from three ancient forests. [b]Soil samples were collected from one ancient and one recent forests –: Not found in Western Germany.

Appendix 2.5



The Spearman correlation matrix of variables in soil chemistry and large-scale environmental conditions (MAT: mean annual temperature, MAP: mean annual precipitation and Ndep: N deposition). Values with different colour hues indicate positive (blue) or negative (red) correlations between pairs of variables.

Appendix 2.6

Differences of chemical soil variables between the four study species based on linear mixed-effects models. Mean values (standard errors) in the columns of plant species (no. of soil samples) were calculated from all soil samples within each plant species. Each chemical variable as one response variable in the linear mixed-effects model against plant species, and values in the columns of Df and χ^2-value were obtained from each model comparison to a blank model by using maximum likelihood estimation. Superscript letters indicate the significant differences between the four plant species based on multiple pairwise comparisons

Variable	*Geum urbanum* (32)	*Milium effusum* (31)	*Poa nemoralis* (24)	*Stachys sylvatica* (31)	Df	χ^2-value
C% §	6.4 (0.9)	6.3 (1.3)	4.9 (0.7)	5.3 (0.6)	3	3.3 ns
N% §	0.5 (0.1)	0.4 (0.1)	0.4 (0)	0.4 (0)	3	4.4 ns
C/N §	13.2 (0.4)b	14.8 (0.4)a	13.4 (0.5)b	13 (0.3)b	3	17.4 ***
P (mg/kg) §	679.2 (40.8)a	471.6 (34.3)b	526.8 (38.7)b	681.7 (51.6)a	3	25.7 ***
Olsen P (mg/kg) §	33.6 (4.1)	37.1 (5.2)	33.9 (4.7)	31.5 (3.6)	3	1.3 ns
K (mg/kg) §	182.2 (23.5)	129.4 (27.6)	155.2 (22.9)	157.2 (23.3)	3	6.5 ns
Ca (mg/kg) §	5073.6 (1761.2)a	3162.4 (1507.1)b	1922.8 (369.4)bc	2521.4 (352.3)ac	3	17.3 ***
Mg (mg/kg) §	245.1 (33.7)a	122.3 (22.2)b	205.7 (44.9)ab	202.7 (33.7)a	3	19.4 ***
Al (mg/kg) £	161.3 (21.2)c	353.8 (36.8)a	282.4 (33.3)ab	208.5 (25.7)bc	3	33.1 ***
pH £	5.6 (0.1)a	4.9 (0.1)b	5.4 (0.2)ab	5.6 (0.1)a	3	20.6 ***

Appendix 2.7

Differences of chemical soil variables between the ancient and recent forests (land-use history) based on linear mixed models. Mean values (standard errors) in the columns of ancient and recent (no. of soil samples) were calculated from all soil samples within each forest type. Each chemical variable as one response variable in the linear mixed model against land-use history, and values in the columns of Df and χ^2-value were obtained from each model comparison to a blank model

Variable	Ancient (62)	Recent (56)	Df	χ^2-value
C% §	5.5 (0.4)	6.1 (0.9)	1	0.1 ns
N% §	0.4 (0)	0.4 (0)	1	0.0 ns
C/N §	13.8 (0.3)	13.4 (0.3)	1	1.3 ns
P (mg/kg) §	555 (29.1)	642.4 (34.8)	1	2.4 ns
Olsen P (mg/kg) §	30.9 (2.4)	37.4 (3.7)	1	1.0 ns
K (mg/kg) §	127.7 (10.9)	187.9 (22.1)	1	4.1 *
Ca (mg/kg) §	2455.5 (440.8)	4138.5 (1224.2)	1	0.6 ns
Mg (mg/kg) §	182.9 (23.3)	206.7 (25.2)	1	0.4 ns
Al (mg/kg) £	271 (24)	220.6 (20.4)	1	0.8 ns
pH £	5.3 (0.1)	5.5 (0.1)	1	0.4 ns

Appendix 2.8

Overview of the litter quality (LQ) scores of each tree species (1: very low decomposition rate, 5: very high decomposition rate)

Tree species	LQ	Tree species	LQ
Acer platanoides	3	*Populus tremula*	*3*
Acer pseudoplatanus	3	*Prunus domestica* ssp. Insititia	*4*
Alnus glutinosa	4	*Prunus padus*	*4*
Alnus incana	3	*Prunus serotina*	*4*
Betula pendula	2	*Quercus robur*	*1*
Betula Pubescens	2	*Rhamnus cathartica*	-
Carpinus betulus	3	*Salix caprea*	*3*
Corylus avellana	3	*Sambucus nigra*	*5*
Fagus sylvatica	1	*Sorbus aucuparia*	*3*
Fraxinus excelsior	5	*Tilia cordata*	*4*
Malus sylvestris	-	*Ulmus glabra*	*5*
Picea abies	1	*Ulmus laevis*	*5*
Pinus sylvestris	2.5	*Ulmus minor*	*5*
Populus × canescens	3.5		

-: Data were not available. Sources of the different LQ scores were collected from Baeten *et al.* (2009), Hermy (1985), Van Calster *et al.* (2008) and (Verheyen *et al.* 2012). When different LQ scores for the same species occurred in different sources, the average score was used.

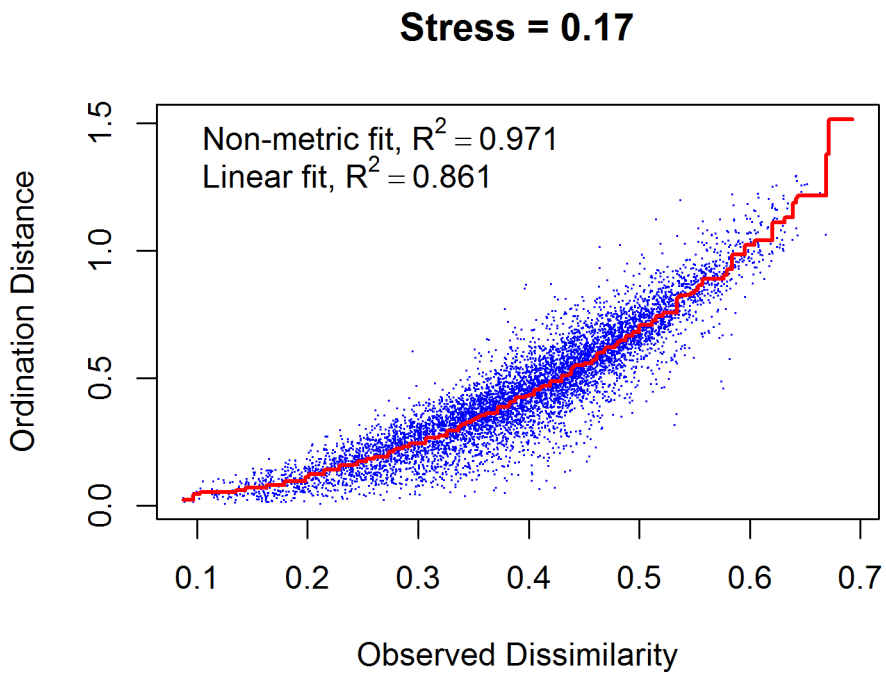
Appendix 2.9

Average litter quality (LQ) score of tree species at each sampling site

Geum urbanum		*Poa nemoralis*		*Milium effusum*		*Stachys sylvatica*	
Site_ID	LQ	Site_ID	LQ	Site_ID	LQ	Site_ID	LQ
G1	2.4	P1	2.5	M1	2.8	S1	2.5
G2	2.2	P2	2.2	M2	2.3	S2	2.2
G3	1.7	P3	1.6	M3	2.0	S3	1.6
G4	3.1	P4	3.7	M4	2.8	S4	2.5
G5	2.8	P5	2.9	M5	1.9	S5	3.0
G6	2.2	P6	3.0	M6	1.8	S6	4.0
G7	4.7	P7	2.5	M7	3.2	S7	3.0
G8	-	P8	-	M8	1.6	S8	1.0
G9	1.6	P9	1.0	M9	3.0	S9	1.0
G10	1.0	P10	1.0	M10	1.0	S10	2.0
G11	2.3	P11	2.3	M11	1.4	S11	3.3
G12	3.8	P12	1.0	M12	1.0	S12	2.9
G13	1.0	P13	2.7	M13	3.0	S13	4.0
G14	4.0	P14	1.0	M14	4.0	S14	4.1
G15	4.0	P15	1.0	M15	4.0	S15	2.4
G16	4.0	P16	2.4	M16	1.9	S16	5.0
G17	3.4	P17	2.0	M17	5.0	S17	1.0
G18	4.7	P18	1.3	M18	4.7	S18	4.1
G19	1.4	P19	2.2	M19	4.2	S19	2.3
G20	1.5	P20	1.0	M20	2.6	S20	4.0
G21	4.0	P21	3.7	M21	4.0	S21	4.1
G22	4.0	P22	3.0	M22	4.0	S22	1.6
G23	1.6	P23	3.9	M23	2.6	S23	4.1
G24	4.0	P24	3.2	M24	4.1	S24	1.8
G25	3.0			M25	1.0	S25	3.0
G26	3.0			M26	2.4	S26	3.5
G27	4.6			M27	4.0	S27	4.0
G28	3.8			M28	3.4	S28	5.0
G29	3.0			M29	3.3	S29	4.7
G30	4.4			M30	1.0	S30	4.1
G31	3.1			M31	2.9	S31	4.0
G32	2.5						

-: Average LQ scores were not calculated because sites contained tree species which LQ scores were not found, see **Appendix 2.8**.

Appendix 2.10



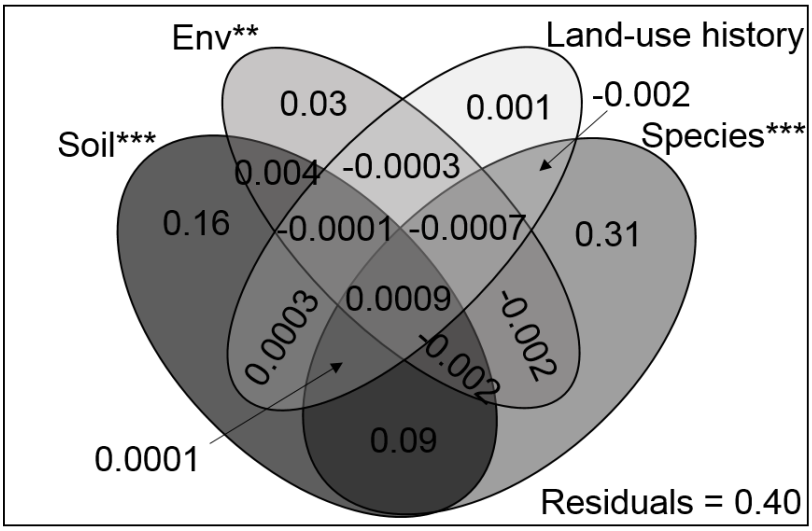
The stress plot of non-metric multidimensional scaling (NMDS, distance = Bray-Curtis).

Appendix 2.11

Differences of PLFA biomarkers between the four study species based on linear mixed-effects models. Mean values (standard errors) in the columns of plant species (no. of soil samples) were calculated from all soil samples within each plant species. Each PLFA biomarker as one response variable in the linear mixed-effects model against plant species, and values in the columns of Df and χ^2-value were obtained from each model comparison to a blank model by using maximum likelihood estimation. Superscript letters indicate the significant differences between the four plant species based on multiple pairwise comparisons

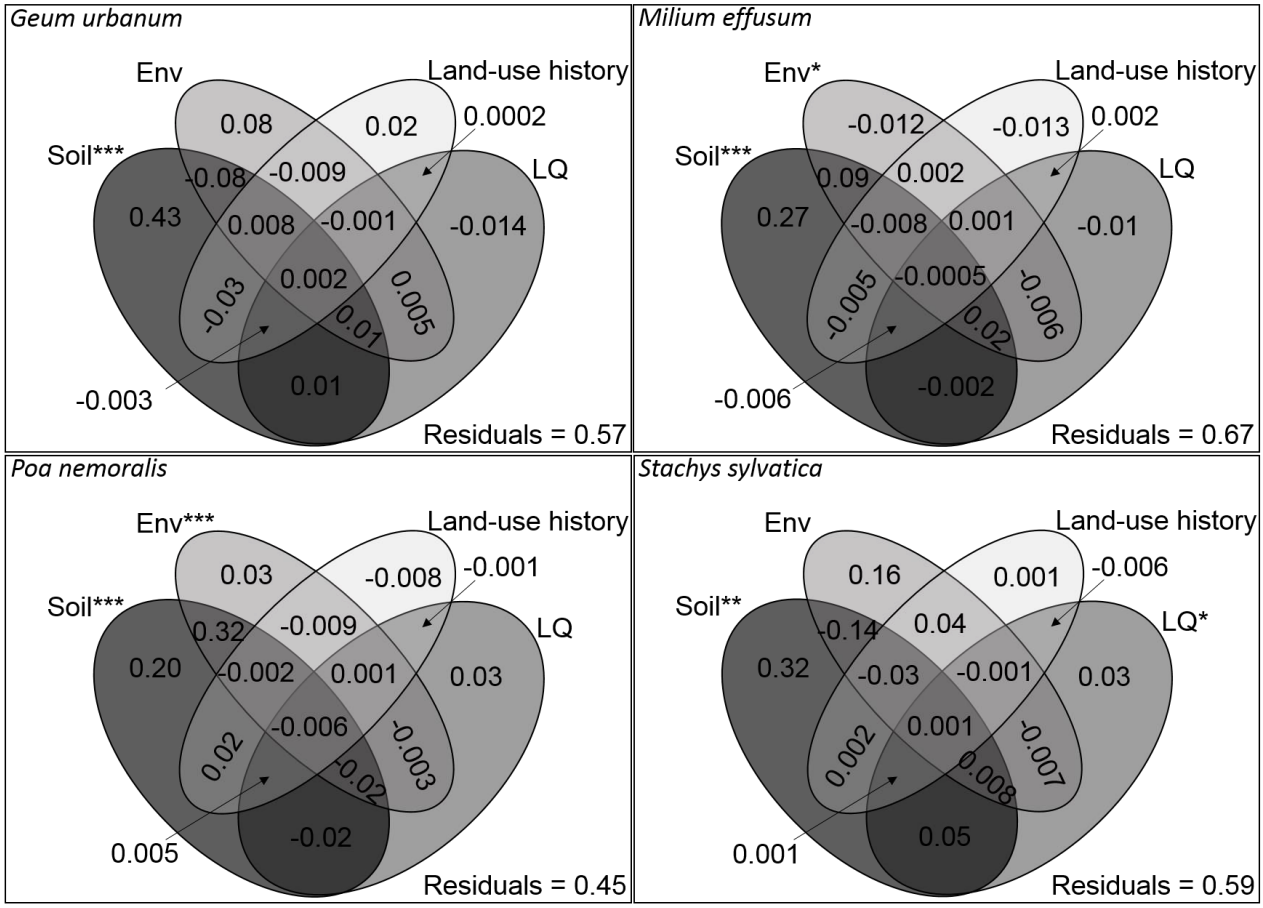
Biomarkers (µg/g)	*Geum urbanum* (32)	*Milium effusum* (31)	*Poa nemoralis* (24)	*Stachys sylvatica* (31)	χ^2-value (Df =3)
10MeC16:0	0.77 (0.07)	0.69 (0.1)	0.78 (0.08)	0.66 (0.07)	1.9 ns
10MeC18:0§	0.44 (0.03)a	0.26 (0.05)b	0.4 (0.03)a	0.35 (0.02)a	34.7 ***
aC15:0§	1.75 (0.17)a	0.95 (0.23)b	1.35 (0.19)a	1.21 (0.11)a	34.9 ***
aC16:0	0.49 (0.04)a	0 (0)c	0.37 (0.04)b	0.35 (0.03)b	108.0 ***
C12:0£	0.02 (0)ab	0.02 (0)b	0.02 (0)a	0.02 (0)ab	11.5 **
C13:0£	0.01 (0)ab	0.01 (0)bc	0.01 (0)a	0 (0)c	20.8 ***
C14:0£	0.28 (0.02)a	0.21 (0.05)b	0.26 (0.03)b	0.24 (0.02)b	11.1 *
C15:0£	0.19 (0.01)a	0.14 (0.03)b	0.18 (0.01)a	0.14 (0.01)ab	11.1 *
C15:1ω10c	0.13 (0.02)a	0.05 (0.01)bc	0 (0)c	0.1 (0.02)ab	33.6 ***
C16:0§	2.78 (0.2)b	3.03 (0.94)a	2.62 (0.22)ab	2.91 (0.36)b	11.3 **
C16:1ω7c§	1.42 (0.13)a	1 (0.32)b	1.17 (0.11)a	1.1 (0.1)a	25.0 ***
C16:1ω7t	0.01 (0.01)c	0 (0)c	0.61 (0.06)a	0.3 (0.06)b	98.0 ***
C17:0§	0.27 (0.02)a	0.19 (0.04)b	0.23 (0.02)a	0.22 (0.02)a	22.6 ***
C18:0£	0.6 (0.04)a	0.42 (0.1)b	0.53 (0.06)a	0.47 (0.04)ab	16.5 ***
C18:1ω7t§	3.61 (0.33)a	1.97 (0.6)b	2.94 (0.44)a	2.53 (0.24)a	36.9 ***
C18:1ω9t	0.32 (0.04)a	0.18 (0.04)b	0.27 (0.03)ab	0.24 (0.03)ab	10.2 *
C18:2ω6c£	0.66 (0.07)	0.63 (0.21)	0.7 (0.07)	0.44 (0.04)	7.3 ns
C18:3ω3c£	0.15 (0.01)a	0.13 (0.06)b	0.1 (0.01)ab	0.09 (0.01)ab	8.3 *
C20:0£	0.12 (0.01)a	0.1 (0.02)b	0.12 (0.01)a	0.1 (0.01)ab	10.2 *
C20:1ω9c	0.06 (0.01)a	0.01 (0.01)b	0.01 (0.01)b	0.03 (0.01)b	24.4 ***
C20:4ω6c	0.04 (0.01)	0.04 (0.03)	0.05 (0.01)	0.04 (0.01)	0.1 ns
C20:5ω3c	0.04 (0.01)	0.04 (0.02)	0.03 (0.01)	0.03 (0.01)	1.3 ns
C21:0£	0.01 (0)c	0.02 (0)b	0.03 (0)a	0.02 (0)b	35.1 ***
C22:0£	0.14 (0.01)ab	0.13 (0.03)b	0.17 (0.01)a	0.12 (0.01)ab	10.4 *
C23:0£	0.05 (0)	0.05 (0.01)	0.06 (0)	0.05 (0)	7.1 ns
C24:0£	0.11 (0.01)	0.12 (0.03)	0.14 (0.01)	0.11 (0.01)	5.6 ns
C24:1ω15c	0 (0)b	0 (0)b	0.09 (0.01)a	0 (0)b	141.1 ***
cyC17:0£	0.77 (0.06)a	0.53 (0.12)b	0.71 (0.07)a	0.68 (0.07)a	14.3 **
iC13:0£	0.04 (0)c	0.05 (0.01)a	0.04 (0)bc	0.03 (0)b	24.6 ***
iC15:0£	1.98 (0.15)	1.99 (0.49)	1.79 (0.12)	1.53 (0.12)	3.3 ns
iC16:0£	0.95 (0.06)	1.09 (0.18)	1.13 (0.1)	0.79 (0.07)	5.7 ns
iC17:0£	0.55 (0.05)a	0.29 (0.06)b	0.43 (0.04)a	0.42 (0.03)a	32.3 ***

Appendix 2.12



Variation in soil microbial community composition explained by four factors, i.e., soil chemistry (Soil), large-scale environmental conditions (Env), litter quality (LQ) and plant species (Species). Adjusted R^2 values in each fraction indicate the explained percentage of the variation. Residuals indicate the unexplained variation. Adjusted R^2 values may cause small negative values. Asterisks show the significance of the permutation tests for each explanatory factor.

Appendix 2.13



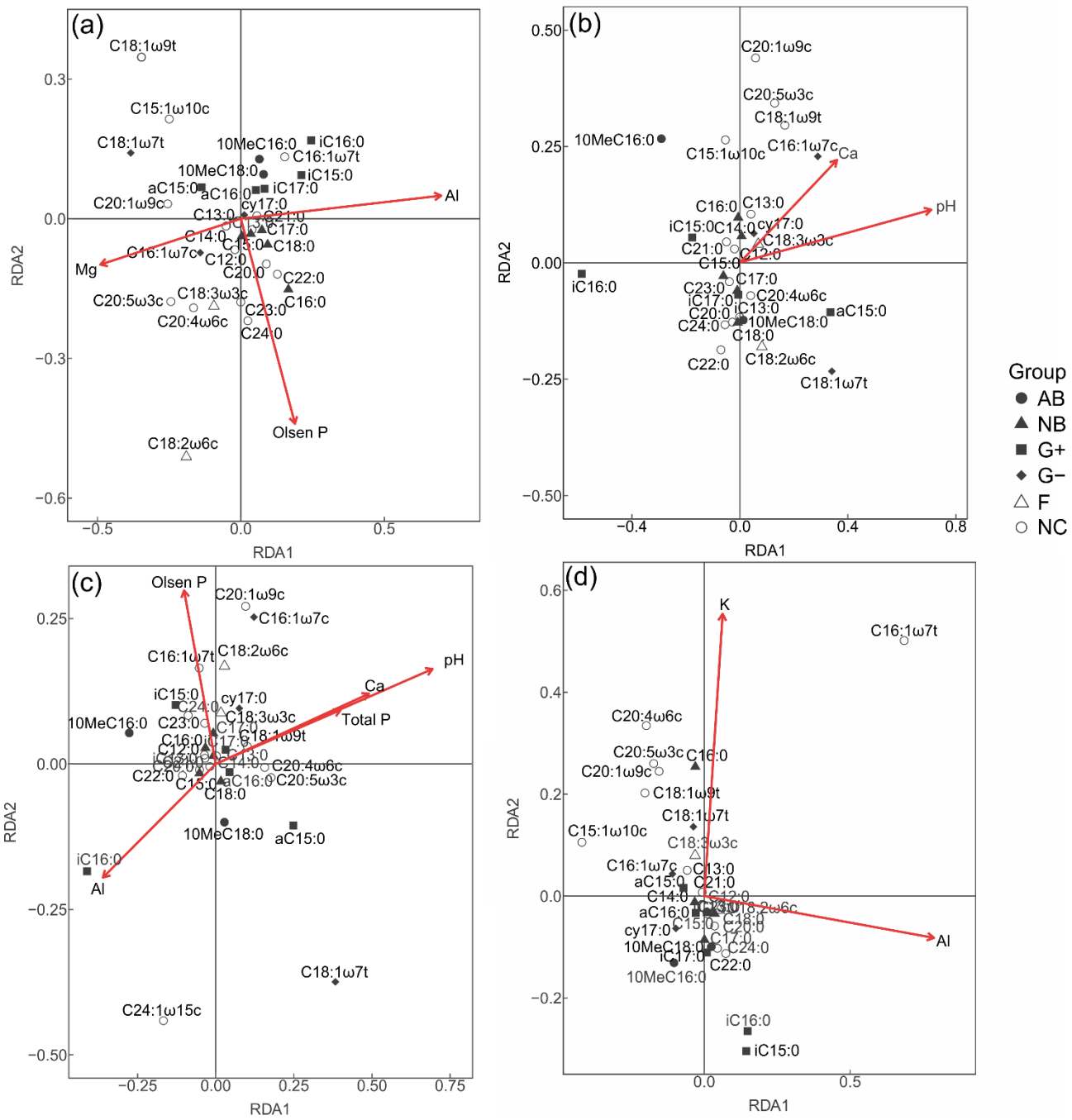
Variation in soil microbial community composition in each plant species explained by four factors, i.e., soil chemistry (Soil), large-scale environmental conditions (Env), land-use history and litter quality (LQ). Adjusted R^2 values in each fraction indicate the explained percentage of the variation. Residuals indicate the unexplained variation. Adjusted R^2 values may cause small negative values. Asterisks show the significance of the permutation tests for each explanatory factor.

Appendix 2.14

Results of pairwise PERMANOVA on soil microbial community composition between the four plant species. *P* values were adjusted using "bonferroni"

Pairs	F value	R^2	*P* value	*P*-adjusted
Milium effusum vs *Poa. nemoralis*	9.7	0.15	0.001	0.006**
Milium effusum vs *Geum urbanum*	11.7	0.16	0.001	0.006**
Milium effusum vs *Stachys sylvatica*	7.1	0.11	0.001	0.006**
Poa nemoralis vs *Geum urbanum*	4.0	0.07	0.029	0.174
Poa nemoralis vs *Stachys sylvatica*	2.5	0.04	0.083	0.498
Geum urbanum vs *Stachys sylvatica*	4.3	0.06	0.045	0.270

Appendix 2.15



Redundancy analysis (RDA) of PLFA profiles for soil samples collected around (a) *Geum urbanum*; (b) *Milium effusum*; (c) *Poa nemoralis* and (d) *Stachys sylvatica* using five microbial functional groups and non-classified PLFAs and significant soil explanatory variables. Different symbols indicate classified groups. AB: Actinobacteria; NB: non-specific bacteria; G+: Gram-positive bacteria; G-: Gram-negative bacteria; F: fungi. NC: non-classified biomarkers.

Chapter 3

Plant species identity and soil characteristics determine rhizosphere soil bacteria community composition in European temperate forests

After: Ma S, De Frenne P, Boon N, Brunet J, Cousins SAO, Decocq G, Kolb A, Lemke I, Liira J, Naaf T, Orczewska A, Plue J, Wulf M and. Verheyen K. Plant species identity and soil characteristics determine rhizosphere soil bacteria community composition in European temperate forests. *FEMS Microbiology Ecology*, resubmitted after revisions.

3.1 Abstract

Soil bacteria and understorey plants interact and drive forest ecosystem functioning. Yet, knowledge about biotic and abiotic factors that affect the composition of the bacterial community in the rhizosphere of understorey plants is largely lacking.

Here, we assessed the effects of plant species identity (*Milium effusum* vs *Stachys sylvatica)*, local soil characteristics, large-scale environmental conditions (temperature, precipitation and nitrogen (N) deposition), and land-use history (ancient vs recent forests) on bacterial community composition in rhizosphere soil in temperate forests along a 1700 km latitudinal gradient in Europe.

The dominant bacterial phyla in the rhizosphere soil of both plant species were *Acidobacteria*, *Actinobacteria* and *Proteobacteria*. Bacterial community composition differed significantly between the two plant species. Within plant species, soil chemistry was the most important factor determining soil bacterial community composition. More precisely, soil acidity correlated with the presence of multiple phyla, e.g., *Acidobacteria* (negatively), *Chlamydiae* (negatively) and *Nitrospirae* (positively), in both plant species. Large-scale environmental conditions were only important in *S. sylvatica* and land-use history was not important in any of the plant species.

The observed role of understorey plant identity and local soil characteristics in determining soil bacterial community composition extends our understanding of plant-soil bacteria interactions in forest ecosystem functioning.

3.2 Introduction

Understorey plants in temperate forest ecosystems play a critical role for the maintenance of biodiversity, nutrient and carbon (C) cycling, evapotranspiration and tree regeneration (Gilliam 2007). Soil bacteria are one of the most abundant and diverse organisms on earth (Bardgett & van der Putten 2014; Delgado-Baquerizo *et al.* 2018). Despite their importance for ecological processes, rhizospheric bacterial diversity under different understorey plant species in temperate forests are less studied. The ecological importance of soil bacteria (e.g., in biogeochemical cycling) in different ecosystems has only being elucidated recently because of advanced analytical methods (reviewed in Llado, Lopez-Mondejar & Baldrian 2017). In forest ecosystems, bacteria are among the most abundant microorganisms in soils (Lauber, Hamady, Knight & Fierer 2009), and their composition varies substantially across the globe owing to distinct biotic and abiotic conditions in different habitats (Delgado-Baquerizo *et al.* 2018). Understanding the driving factors for soil bacterial diversity and composition is essential for maintaining ecosystem functioning because the dynamics of the soil bacterial community determines multiple ecological processes, for instance, litter decomposition and N fixation (Bardgett & van der Putten 2014; Wu, Huang, Huang, Li & Liao 2018). Yet, knowledge about the extent to which soil bacterial community can be affected by understorey plant species and associated abiotic environmental drivers, such as soil chemistry, climatic conditions, nitrogen (N) deposition and land-use history, in temperate forests is still limited.

Plant species can affect the soil bacterial community around the root systems via rhizosphere resources (e.g., root exudates, allelochemicals and soil nutrients) (Haichar *et al.* 2008; Eilers, Lauber, Knight & Fierer 2010). Root exudates produced by different plant species may result in species-specific interactions with different soil microbial groups and finally shift bacterial community composition. For instance, nitrification inhibitory compounds can constrain the activity and change the composition of nitrifying bacteria (Gopalakrishnan *et al.* 2009). Additionally, plants can modify soil bacterial community composition through effects on soil C resources and nutrients because plants translocate up to 16% of plant N and 40% of photosynthetically fixed C into the soil (Berendsen, Pieterse & Bakker 2012; Bulgarelli, Schlaeppi, Spaepen, van Themaat & Schulze-Lefert 2013). However, there are inconsistent results of plant species effects on soil bacterial community composition. Dawson, Hor, Egert, van Kleunen and Pester (2017) compared soil bacterial community composition under 19 herbaceous grassland species and found that plant species identity was not a significant factor in explaining the composition of soil bacterial community. In forest understoreys, studies have mainly focused on the driving effects of understorey plant species diversity, richness and abundance on soil microbial community assembly (Wardle, Yeates, Barker & Bonner 2006; McIntosh, Macdonald & Quideau 2013). Studies on a

specific understorey plant species affecting its rhizosphere bacterial community composition will help to understand the underlying mechanisms of plant-bacteria interaction in temperate ecosystems.

Abiotic conditions are highly correlated with the dynamics and activity of soil microbes (Bissett, Brown, Siciliano & Thrall 2013). Among those, soil chemistry is one of the overarching reasons that drive soil microbial community assembly. For instance, soil pH and nutrients are key drivers for microbial catabolic activities (the process of breaking down molecules into small units) and nutrient utilisation (Lauber, Hamady, Knight & Fierer 2009; Klimek *et al.* 2015). Lauber, Hamady, Knight and Fierer (2009) specifically focused on soil bacteria and characterized soil bacteria community composition across North and South America and found that the relative abundance of three phyla (i.e., *Acidobacteria*, *Actinobacteria* and *Bacteroidetes*) was strongly affected by soil pH. Klimek *et al.* (2015) compared soil bacteria along an altitudinal gradient and indicated that the utilization of amines differs significantly in bacterial communities that inhabited at different sites. These biogeographical patterns between soil chemistry and bacteria can be used to predict soil bacterial dynamics at large geographical scales. Recently, a global-scale study on soil bacterial abundance and diversity again advocated that the high divergence of habitat preferences in terms of chemical and climatic characteristics is responsible for soil bacterial community assembly (Delgado-Baquerizo *et al.* 2018).

At larger spatial scales, also climate and N deposition loads affect the plant as well as the soil bacterial community, both directly and indirectly. The direct effects of soil temperature and moisture capacity on the activity of soil bacteria and the structure of the soil bacterial community composition are evidenced as explanatory mechanisms for soil microbial diversity change and biochemical kinetics (Avrahami & Bohannan 2007; Santana & Gonzalez 2015; Borowik & Wyszkowska 2016). Similarly, increased N deposition may favour copiotrophic bacteria taxa (species that have a high requirement of N concentration) but suppress the diversity of oligotrophic bacteria taxa (Fierer *et al.* 2012). Indirectly, soil bacterial community assembly can be affected through changes in aboveground plant community composition (Zak, Pregitzer, Burton, Edwards & Kellner 2011). As such, soil microbes inhibited under understorey plants are influenced by resource inputs and allelochemicals.

Finally, past land use is also a key driver of forest communities (Perring *et al.* 2018a). The past land use influences both biotic and abiotic conditions in forests and past use imprints can persist for decades to centuries, reflecting on dissimilarities in vegetation, and chemical and physical soil properties (Jangid *et al.* 2011; Aggemyr & Cousins 2012; Bachelot *et al.* 2016). These dissimilarities, together with current climate change and N deposition, can modulate the activity and

composition of soil microorganisms (Dupouey, Dambrine, Laffite & Moares 2002; Ma *et al.* 2018). Thus, the soil bacterial community may differ between ancient forests (i.e., those forests already present on the oldest available land use maps, typically > 200 years old (Hermy & Verheyen 2007), and more recently established forests on former agricultural land. Apart from knowing the difference of soil bacterial assemblages between ancient and recent forests, the most important rational is to understand and predict soil microbial succession in the face of land fragmentation and land-use change relative to the formation of understorey vegetation.

Here, we used a 16S rRNA gene marker approach to assess soil bacterial community composition under two widespread temperate forest understorey plant species (*Milium effusum* and *Stachys sylvatica*). Rhizosphere soils under each plant species were sampled in ancient and recent forests along a 1700 km latitudinal gradient in Europe. The importance of the four factors, i.e., plant species identity, soil chemistry, large-scale environmental conditions and land-use history, on differences in soil bacterial community composition was assessed at the European scale. Our aims were to address (1) whether the soil bacteria community is affected by the plant species identity, and (2) within each plant species, which factors affect the soil bacterial community composition.

3.3 Materials and Methods

3.3.1 Study species

Milium effusum L. (Poaceae), and *Stachys sylvatica* L. (Lamiaceae), covering distinct plant functional types, are both perennial and rhizomatous understorey plant species. *Milium effusum* is a hemicryptophyte, early-summer flowing grass; *S. sylvatica* is a protohemicryptophyte, early-summer forb (Taylor & Rowland 2010; De Frenne *et al.* 2017). Both plant species are characteristic for the understorey layer across European temperate forests and may be used, in some regions, as indicators for ancient forests (Wulf 1997). They have different colonization capacity: *S. sylvatica* is a somewhat faster colonizer than *M. effusum* across ancient-recent forest ecotones in temperate forests (De Frenne *et al.* 2011a; Brunet, De Frenne, Holmstrom & Mayr 2012). In addition, their interaction with soil microbes may differ owing to different habitat preferences in physicochemical conditions. The seeds of both species are wind- and gravity-dispersed, but epizoochory also occurs (Hermy, Honnay, Firbank, Grashof-Bokdam & Lawesson 1999; Graae 2002; Grime, Hodgson & Hunt 2007). Reproduction from seed is the main regeneration mode, but *M. effusum* also produces stolons for vegetative spread and stolons of *S. sylvatica* have been found to be effective for local clonal reproduction (Taylor & Rowland 2010; De Frenne *et al.* 2011b).

3.3.2 Soil sampling

Soil samples were taken in eight regions. More information about the sampling methods and sites for *M. effusum* and *S. sylvatica* can be found in section 2.3.2.

3.3.3 Soil bacterial community

Total DNA extraction from soil samples was carried out with the PowerSoil®DNA Isolation kit and purified by means of the Wizard®DNA Clean-Up System, following the manufacturer's instructions. The 16S rRNA gene v3-v4 region was amplified by PCR using the barcoded versions of the primers described by Klindworth *et al.* (2013). The PCR mix included 1 µl of DNA extract, 15 pmol of both the forward primer 341F 5'- NNNNNNNNNTCCTACGGGNGGCWGCAG-3' and the reverse primer 785R 5'- NNNNNNNNNNTGACTACHVGGGTATCTAAKCC-3' in 20 µL volume of MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 µl of BioStabII PCR Enhancer (Sigma). For each extracted sample, the forward and reverse primers had the same unique 10-nt barcode sequence. PCRs were carried out with an initial denaturation of 2 min at 96 °C, followed by 20 cycles of 15 s at 96 °C, 30 s at 50 °C, 90 s at 70 °C, and a final extension of 5 min at 72 °C.

The DNA concentration of the amplicons of interest was determined by gel electrophoresis. Next, 20 ng amplicon DNA of each sample was pooled. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on MinElute columns (Qiagen). Finally, about 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries by means of adaptor ligation using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq platform.

Contigs were created by merging paired-end reads based on the Phred quality score (of both reads) heuristic (Schloss, Gevers & Westcott 2011; Kozich, Westcott, Baxter, Highlander & Schloss 2013), in *MOTHUR* (v.1.38) (Schloss *et al.* 2009). Contigs were aligned to the Silva database (v123), and filtered from those with (i) very divergent lengths (outside of the 2.5% - 97.5%) and ambiguous bases, (ii) sequences falling outside of the alignment space and with more homopolymers (maximum of the alignment database), (iii) those not corresponding to the v3-v4 region. The aligned sequences were filtered and dereplicated, while sequencing errors were removed using the *pre.cluster* command. Chimera removal was performed with the *uchime* command. The sequences were compared to RDP 16S rRNA reference version 10 and clustered into operational taxonomic units (OTUs) at 97% similarity with the *cluster* command (opticlust algorithm). Soil bacterial

biomass was determined by assessing phospholipid fatty acids. A full description about this method can be found in section 2.3.3.

3.3.4 Soil chemistry

Soil chemistry including ten variables was assessed. More information about this assessment is given in section 2.3.4 and **Appendix 2.3**.

3.3.5 Large-scale environmental conditions

Data about the mean annual temperature and precipitation and N deposition of each sampling site were extracted from online databases. See details in section 2.3.5 and **Appendix 2.4**.

3.3.6 Data analysis

Data analyses of the bacterial community were conducted after proportional normalization. We normalized OTUs data by first taking the proportion of each OTU's read in the total number of reads, then multiplying the result with the minimum sample size (3338 reads) and rounding to the nearest integer to account for sample size differences (McMurdie & Holmes 2014). This resulted in a scaled taxon-abundance matrix comprised of 10729 OTUs which were classified into 38 phyla. OTU reads in each soil sample were multiplied with bacterial biomass (abundance) in the corresponding soil sample to calculate the absolute abundance of read count. Comparing with traditional relative abundance based on OTU reads, the action of multiplication considers one more parameter, i.e., bacterial abundance (Props *et al.* 2017). The absolute abundance was used for all composition and diversity analyses. Predictors were plant species identity (biotic), two abiotic factor groups (soil chemistry and large-scale environmental conditions) and land-use history. We did not include latitude and longitude in our data analyses because spatial autocorrelation tests were not significant. Litter quality (LQ) assessment can be found in section 2.3.6, **Appendix 2.8** and **Appendix 2.9**.

To visualise the composition of soil bacterial community, we calculated the absolute abundance of shared OTUs and unique OTUs (Schmidt, White & Denef 2016) in *M. effusum* and *S. sylvatica*, following a chi-square test to assess differences in the number of unique OTUs between the two plant species. Phylum composition in the shared and unique group was plotted separately using the *ggplot2* package (Wickham 2009). Non-metric multidimensional scaling (NMDS) was used to visualize the difference of soil bacterial community composition (both OTU and taxonomic phylum level) between the two plant species. The significance of the difference was tested using PERMANOVA (*site* nested within *region*) with Bray-Curtis distance and performed within the function *adonis* implemented in the *vegan* package (Oksanen *et al.* 2016).

Three alpha diversity indices, i.e., species richness, Shannon diversity and the inverse Simpson index were calculated at the OTU level. We assessed the effects of plant species, land-use history, all variables in soil chemistry, large-scale environmental conditions and tree species composition on each alpha diversity index, using functions *glmer* (species richness) and *lmer* (Shannon diversity and inverse Simpson) (*site* nested within *region*) in the *lme4* package (Bates, Machler, Bolker & Walker 2015). Each variable was tested in isolation. Values of Shannon diversity and inverse Simpson were log10- or sqrt- transformed to meet the assumptions of the statistical tests. Significances were determined with likelihood ratio tests.

Variation partitioning based on redundancy analysis (RDA) was used to evaluate the strength of plant species identity, soil chemistry, large-scale environmental conditions and land-use history, as well as the two abiotic factor groups, land-use history and one biotic factor (tree species composition) within each plant species, in explaining the variation in soil bacterial community composition (phylum level). Phylum data were *Hellinger*-transformed before variation partitioning. Adjusted R^2 values were used due to the unbalanced number of variables in each variable category. The significance of each factor was tested using function *anova.cca* in the *vegan* package (Oksanen *et al.* 2016).

In order to find the most significant abiotic drivers determining soil bacterial community composition in each plant species, all variables in soil chemistry, large-scale environmental conditions and land-use history were combined and then were selected using the function *forward.sel* in the package *adespatial* with 999 permutations. Site scores were extracted from the RDA results and selected significant variables were plotted using the package *ggplot2* (Wickham 2009).

3.4 Results

3.4.1 Soil bacterial community composition

The main bacteria phyla in our samples were *Acidobacteria*, *Actinobacteria* and *Proteobacteria*. These three phyla accounted for 76% of the absolute abundance of all OTUs in *Milium effusum* and 72% in *Stachys sylvatica*. The number of unique OTUs was significantly ($P < 0.001$) higher in *M. effusum* (60712) than *S. sylvatica* (45048) (**Fig. 3.1a,b**). The absolute abundance of each phylum in the unique OTUs showed evident differences between the two plant species (**Fig. 3.1b**). The absolute abundance of shared OTUs accounted for 93% (*M. effusum*) and 95% (*S. sylvatica*) of the overall abundance (**Fig. 3.1a,b,c**).

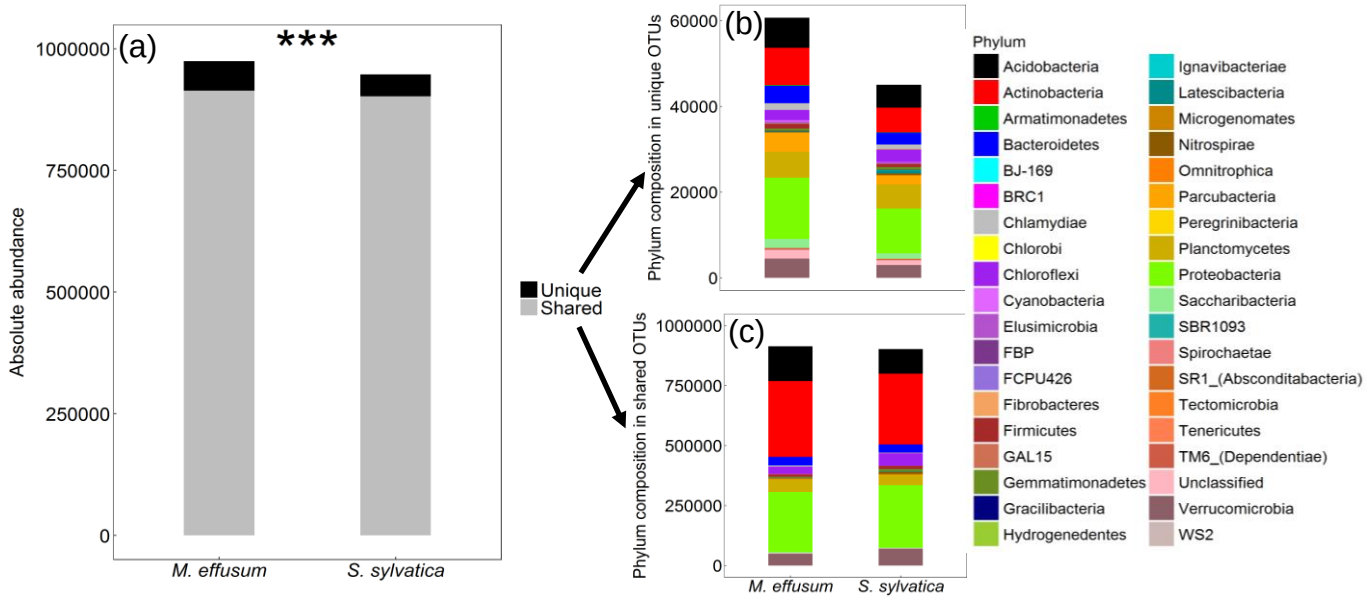


Fig. 3.1 The absolute abundance of the number of unique and shared operational taxonomic units (OTUs) in rhizosphere soil samples of *Milium effusum* and *Stachys sylvatica* (a). The community composition of bacterial phyla in unique (b) and shared (c) OTUs in *M. effusum* and *S. sylvatica*. *** indicates a significant difference in unique OTUs between the two plant species based on a chi-square test ($P < 0.001$).

3.4.2 Soil bacterial community differed between the plant species

Soil bacterial community represented by OTUs (**Fig. 3.2a**) and phylum (**Fig. 3.2b**) both differed significantly between *M. effusum* and *S. sylvatica* (PERMANOVA, $P < 0.001$). Twelve phyla showed significant differences between the two plant species (**Appendix 3.1**). For instance, one of the main phyla *Acidobacteria* was significantly more abundant under *M. effusum*, while nitrite-oxidizer bacteria *Nitrospirae* was nearly four times as abundant in *S. sylvatica*.

3.4.3 The effect of biotic and abiotic factors on alpha diversity

For the three alpha diversity indices, richness was the most affected index, with significant effects of understorey plant species identity, multiple chemical characteristics, mean annual precipitation and litter quality (**Table 3.1**). Land-use history had no influence on any of the measured indices. Soil C/N ratio and mean annual precipitation had contrast effects and were the only two factors that had impacts on all alpha diversity indices, i.e., indices' values increased with the C/N ratio, whereas decreased with mean annual precipitation.

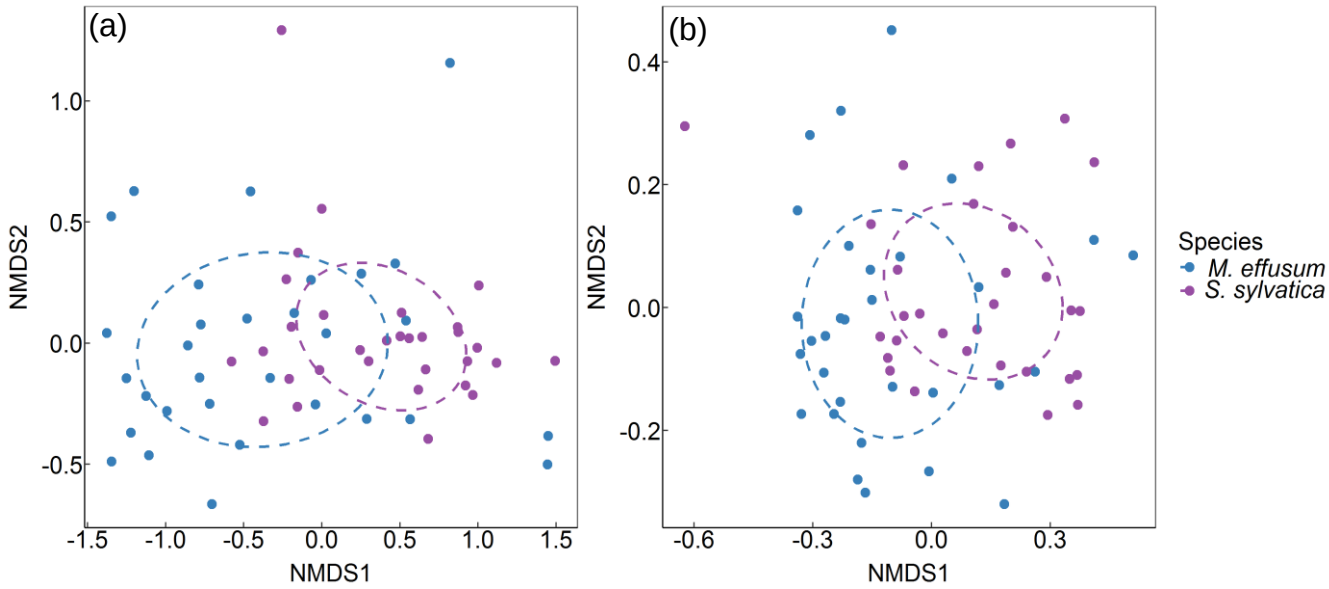


Fig. 3.2 The compositional differences in the rhizosphere soil bacterial community based on non-metric multidimensional scaling (NMDS) in *Milium effusum* and *Stachys sylvatica* (distance metric = Bray). Data used in (a) were the absolute abundance of all OTUs, and in (b) only per phylum, see **Fig. 3.1** for the phylum composition. The stress value is 0.10 (a) and 0.15 (b). Significance tests (PERMANOVA) for (a) and (b) $P <$ 0.001***. Ellipses represent standard deviation of the samples within the species.

Table 3.1 Effects of biotic and abiotic driving variables on alpha diversity of soil bacterial community represented by three indexes (species richness, Shannon index and inverse Simpson) based on generalized linear and linear mixed-effects models. Values were χ^2-values extracted from likelihood ratio tests

Variable	Richness (Df =1) ¶	Shannon index (Df=1)	Inverse Simpson (Df =1)
Plant species	↑26.4***	2.8 ns §	0.6 ns §
Land-use history	<0.1 ns	<0.1 ns §	0.2 ns §
C%	↓53.9***	2.2 ns §	1.6 ns §
N%	↓56.2***	1.6 ns §	1.4 ns §
C/N	↓38.5***	↓12.2*** §	↓8.2** §
P (mg/kg)	↓28.1***	0.3 ns §	0.6 ns §
Olsen P (mg/kg)	↓10.6***	0.1 ns §	<0.1 ns §
K (mg/kg)	↓10.8***	0.8 ns §	2.3 ns §
Ca (mg/kg)	↓25.1***	1.5 ns §	1.1 ns §
Mg (mg/kg)	↓34.6***	0.1 ns £	1.5 ns §
Al (mg/kg)	19.5ns	3.6 ns §	0.1 ns §
pH (H_2O)	3.0 ns	2.3 ns §	0.3 ns §
MAT (°C yr^{-1})	2.4 ns	<0.1 ns §	0.8 ns §
MAP (mm yr^{-1})	↑17.0***	↑6.0* §	↑3.0(*) §
Ndep (kg/ha)	2.7 ns	0.1 ns §	0.8 ns §
LQ	↑11.0***	0.5 ns	0.8 ns §

The direction of the effect is displayed as an arrow: ↑ indicates a positive effect and ↓ indicates a negative effect, ↑ in the row of plant species indicates higher species richness in *S. sylvatica*.

3.4.4 Soil bacterial community composition is mainly driven by plant species identity and soil chemistry

Taking all biotic and abiotic factors together, plant species identity, soil chemistry and large-scale environmental conditions were significant drivers in the variation of soil bacterial community composition (**Fig. 3.3**). Plant species identity with soil chemistry jointly explained 8%, soil chemistry purely explained 27% and large-scale environmental conditions purely explained 5% of the variation in bacterial community composition. Within each plant species, soil chemistry explained nearly half (47%) of the variation in *M. effusum*, and 27% in *S. sylvatica*. Large-scale environmental conditions explained only the variation in bacterial community composition in *S. sylvatica* significantly (11%). Land-use history did not result in differences in terms of soil chemistry (**Appendix 3.2**) and was not significant in explaining the variation neither in *M. effusum* nor *in S. sylvatica* (**Fig. 3.3**). This absence of evidence was also found for litter quality (**Appendix 3.3**).

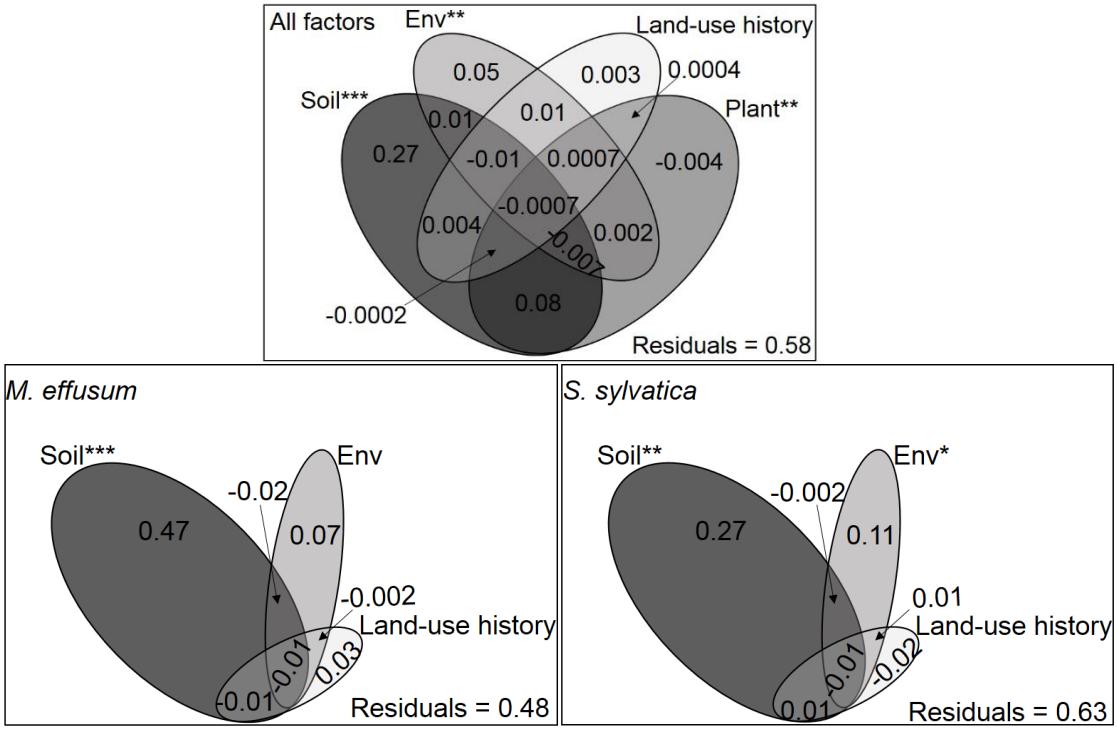


Fig. 3.3 Variation partitioning of the rhizosphere soil bacterial community composition explained by all investigated factors, i.e., plant species identity (Plant), soil chemistry (Soil), large-scale environmental conditions (Env) and land-use history, and by two abiotic factors and land-use history in each plant species. Adjusted R^2 values in each fraction indicate the explained proportion of the variation for that variable category. Residuals indicate the unexplained variation. Adjusted R^2 values may cause small negative values. Asterisks show the significance of permutation tests for each explanatory factor.

3.4.5 The correlation between soil bacteria and significant abiotic drivers

Soil pH, Ca and total N concentration were the significant drivers for bacterial community composition in *M. effusum*, and pH and Al concentration were important drivers in *S. sylvatica* (**Fig. 3.4**). All significant chemical drivers were associated with soil acidity. *Nitrospirae* was positively correlated with soil pH in both plant species, while *Actinobacteria* showed a positive correlation with pH in *M. effusum*, but a negative correlation with pH in *S. sylvatica*. *Acidobacteria*, *Chlamydiae*, *Firmicutes* and *Proteobacteria* all had negative correlation with soil pH in both plant species.

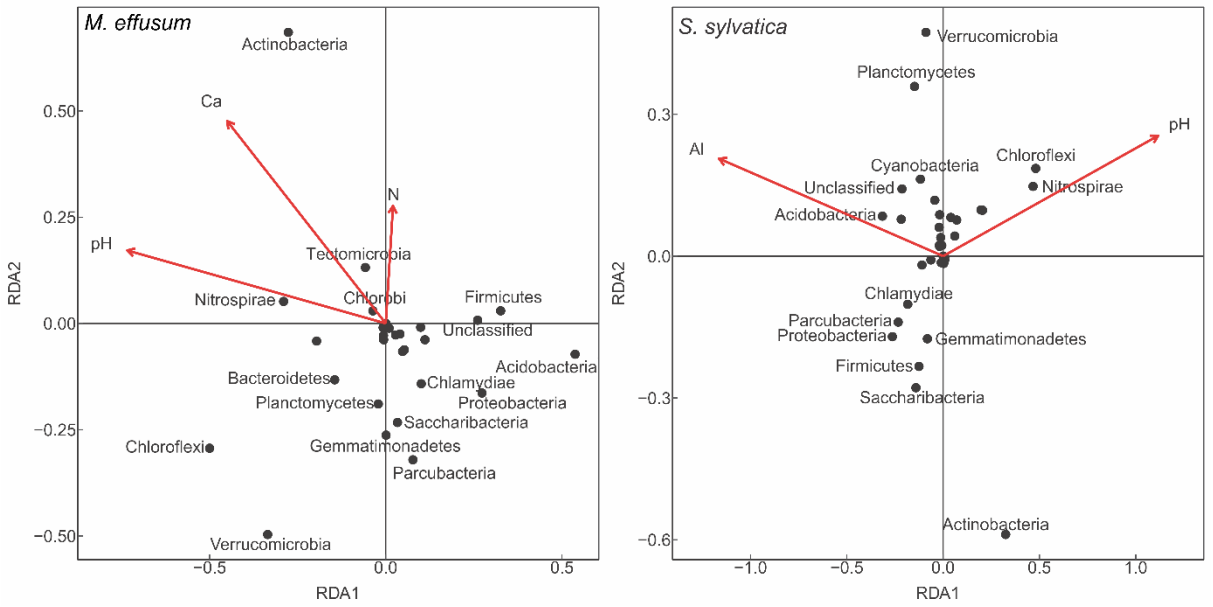


Fig. 3.4 Redundancy analysis (RDA) of soil bacterial taxa in the rhizosphere soils of *Milium effusum* and *Stachys sylvatica* with significant explanatory variables produced by forward selection. The predictor variables in these analyses included fourteen variables, i.e., land-use history, soil C, total N, C/N ratio, total P, Olsen P, K, Ca, Mg, Al, pH, MAT, MAP, and Ndep. Each dot represents a bacterial taxon. To avoid text overlapping, dots located around the centre were not aligned with taxon names.

3.5 Discussion

In this study, we assessed soil bacterial community composition in the rhizosphere of two common understorey plant species with distinct functional types at the continental scale. The results demonstrated that local factors, i.e., plant species identity and associated soil chemistry, were the main determinants of soil bacterial community diversity and composition. Each plant species formed specific composition of bacterial phyla and this formation likely correlated with soil acidity. Large-scale factors, i.e., climate and N deposition loads were only important in determining soil bacterial community composition in *S. sylvatica.*

3.5.1 Plant species identity and soil bacteria community composition

The dominant phyla were the same in both *M. effusum* and *S. sylvatica*, i.e., *Acidobacteria*, *Actinobacteria* and *Proteobacteria* and most OTUs were shared between the two plant species. This observation is consistent with the main trend of dominant bacteria in forest ecosystems (Lauber, Hamady, Knight & Fierer 2009; Ma *et al.* 2018). However, the two plant species did show differences with respect to bacterial phylum abundance and uniqueness, and plant species identity significantly explained variation in soil bacterial community composition. To our knowledge, this is the first study demonstrating rhizospheric bacterial community composition under individual understorey plant species in temperate forests. Our results are consistent with other studies, which have shown that tree (Chodak, Klimek, Azarbad & Jazwa 2015), moss (Bach, Frostegard & Ohlson 2009) and fern (Liu, Wu, Zhou, Lin & Fu 2012) identity have significant influences on soil microbial community composition in various ecosystems (i.e., temperate forests, grasslands, tundra). In grasslands, Dassen *et al.* (2017) conducted comparisons of soil bacterial community composition among legumes, grasses, small and large herbs and found that distinct soil bacterial communities occur especially between small herbs and grasses. We observed significant effects of understorey plant species identity on bacterial community composition but not litter quality of tree layers (although a significant effect on species richness was found) implying strong correlations between soil bacteria and the root surface. One of the mechanisms is that litter quality produced by different plant species is highly selective in soil decomposers, leading to distinct soil microbial assemblage between plant species (De Deyn, Raaijmakers, van Ruijven, Berendse & van der Putten 2004; Sayer *et al.* 2017). Although we observed minor influences of tree species composition on soil bacterial community composition, the selective role of litter quality and quantity produced by tree layers cannot be overlooked because trees usually dominant forest ecosystems and have strong influences on both biotic and abiotic conditions for all biomes at large scales (Lu, Turkington & Zhou 2016). Whether litter quality or chemical composition of root exudates is the main mechanism is still open for the discussion. However, both are directly correlated with plant identity and soil chemistry of the rhizosphere (see **Appendix 3.4** for the assessed chemical differences between the two plant species). Therefore, the effects of plant species identity on soil bacterial diversity and composition demonstrated here facilitate our understanding of plant-soil interactions and soil physicochemical cycling. Yet, our study only focused on soil bacterial community. Meanwhile, there is a large number of fungi, protozoa, archaea and soil fauna that occur spontaneously with bacteria. Changes in one community can lead to shifts in other communities, resulting in complex biotic interactions. Therefore, further studies are needed to disentangle plant-soil feedbacks in the face of global change.

3.5.2 Soil chemistry and bacterial community composition

Apart from the biotic factor of plant species identity, we assessed three groups of abiotic factors and found that soil chemistry was the most important determinant in soil bacterial community composition in each plant species. The critical role of soil chemistry is not surprising as soil bacteria and chemical properties are intimately correlated. Specifically, soil pH was one of the most influential chemical soil properties in the correlation with soil bacteria. This is consistent with previous studies conducted at different large geographical scales (Lauber, Hamady, Knight & Fierer 2009; Iovieno, Alfani & Baath 2010; Shen *et al.* 2013). In our study, the two plant species spontaneously displayed a positive relationship between the bacterial phylum *Nitrospirae* and soil pH. *Nitrospirae* is one of the main nitrite-oxidizer bacterial phylum and occurs widely in terrestrial and aquatic ecosystems (Lucker *et al.* 2010). Thus, the process of ammonia and nitrite conversion is partly regulated by the abundance of nitrite-oxidizers (Lucker *et al.* 2010). Soil pH has been widely used as the best predictor for *Acidobacteria* (Jones *et al.* 2009), with negative relationship between the abundance of *Acidobacteria* and soil pH (the same pattern as we observed). *Chlamydiae*, a bacterial phylum including many pathogens, was also negatively correlated with soil pH. Soil N was a significant factor driving soil bacterial community composition in *M. effusum* but not in *S. sylvatica*. This may result from a regional variation in terms of soil N between the eight regions. We found the highest soil N concentration in central Sweden in *M. effusum*. In addition, soil Ca in the rhizosphere of *M. effusum* and Al in *S. sylvatica* were also significantly correlated with certain bacteria, for instance *Actinobacteria*. However, the correlation was positive in *M. effusum*, while negative in *S. sylvatica*, suggesting the dissimilarity of bacterial community composition between different plant species. This again supports the importance of taking understorey plant species identity into consideration when exploring the effects of abiotic factors on soil microbial dynamics. Within the selected study sites, soil Al concentration was negatively correlated with pH, while soil Ca concentration showed a positive correlation with pH. All these soil chemical properties are good indicators of soil acidity (**Appendix 3.5**). Furthermore, soil pH may affect the structure and diversity of soil bacteria through dissolved organic matter (DOM) because low pH values are likely to reduce the bioavailable substrates for microbes by coagulating the surface charge and in DOM. Despite the large differences of the mean values of each chemical characteristic, there was no statistical significance (except C/N ratio) among regions, probably due to a large variation of soil chemistry within regions.

3.5.3 Large-scale environmental conditions and soil bacterial community composition

The effects of large-scale environmental conditions (temperature, precipitation and N deposition loads) on soil bacterial community composition depended on plant species, with significant effects

under *S. sylvatica*. This observation, to some extent, partly supports the mechanism of divergent soil bacterial community assembly in rhizosphere soil due to the ability of each plant species in selecting specific soil microbes and accumulating different pathogens (Dassen *et al.* 2017; Dawson, Hor, Egert, van Kleunen & Pester 2017). Across all studied sites, temperature and N deposition loads were positively correlated with precipitation (**Appendix 3.5**). Different precipitation regimes can have selective effects on specific bacteria. For instance, less precipitation facilitates the assembly of Gram-positive bacteria, while higher precipitation increases the abundance of Gram-negative, anaerobic and sulphate-reducing bacteria (Drenovsky, Steenwerth, Jackson & Scow 2010). In our study, precipitation did increase bacteria richness and alpha diversity across the two studied plant species (**Table 3.1**). The underlying mechanisms between precipitation regimes on specific bacteria taxa are yet unknown. Sampling regions along the latitudinal gradient differ significantly with respect to temperature and N deposition (**Appendix 2.4**). The two drivers can affect bacterial taxa which are involved in the processes of nitrification (sequential oxidation of ammonia to nitrate) and subsequently nutrient transportation (Lucker *et al.* 2010; Osborne, Baron & Wallenstein 2016). Unexpectedly, when combined with chemical soil variables, the three drivers in large-scale environmental conditions were cancelled out because of less contribution relative to soil acidic drivers in explaining the variation of soil bacterial community composition. Yet, we cannot exclude that there might be indirect effects of temperature, precipitation and N deposition among studied regions on rhizosphere soil bacterial community composition through changes in aboveground plant community composition and local edaphic properties (reviewed in Classen *et al.* 2015).

3.5.4 Land-use history and soil bacterial community composition

As for land-use history, we observed weak influence on soil bacterial community composition, which was out of our expectation. Previous studies have shown that the legacy effect of land-use change on biodiversity (e.g., plants, soil microbes) is profound, in both enduring and multifaceted point of view (Goodale & Aber 2001; Aggemyr & Cousins 2012). While forest age matters in the process of plants and soil microbes' recovery (Krause, Pugh, Bayer, Lindeskog & Arneth 2016). In the study of Jangid *et al.* (2011), post-agricultural lands which were established in 1951 showed high similarity in terms of soil microbial community composition with that in ancient forest. Moreover, the attribute of short life turnover of soil bacteria can also contribute to fast recovery after land-use change or disturbance (Baath 1998). In our study, recent forests were mostly afforested in the early 20th century (except the sites in Poland, which are younger and their age ranges from 15 to 40 years) and thus stand more than one century. Long-term succession in recent forests may be one of the main reasons of lack of differences in soil bacteria between ancient and recent forests. Additionally, soil chemistry differences between ancient and recent forests partly

supported this insight, as we observed no significant differences between the two types of forests (**Appendix 3.2**). The unexplained variation in the two species indicates the importance of other factors in determining soil bacterial community composition, for instance soil physical properties, and the presence of competitors and predators.

3.6 Conclusions

We assessed the rhizospheric soil bacterial community composition in temperate forests across Europe, and found the community be dominated by *Acidobacteria*, *Actinobacteria* and *Proteobacteria*. Soil bacterial community composition in the rhizosphere soil was strongly affected by plant species identity and soil acidity (as overarching characteristic of the soil chemistry). For different plant species, different chemical characteristics help to predict specific bacterial abundance. A long time after land-use change (more than one century), the rhizospheric soil bacterial community showed no difference between ancient and recent forests. Our results further our understanding of interaction mechanisms between the microbial community and soil chemistry, and elucidate the ecological importance of understorey plants in determining microbial diversity. The observed correlations between N fixation bacteria and chemical soil characteristics can be a focus for further bacterial functioning research in forests.

Appendix 3.1

The mean values (with standard error) of the absolute abundance of 38 phyla in *Milium effusum* (31 soil samples) and *Stachys sylvatica* (31 soil samples) and likelihood ratio tests for the significance of the absolute abundance of each phylum between the two plant species based on linear mixed-effects models

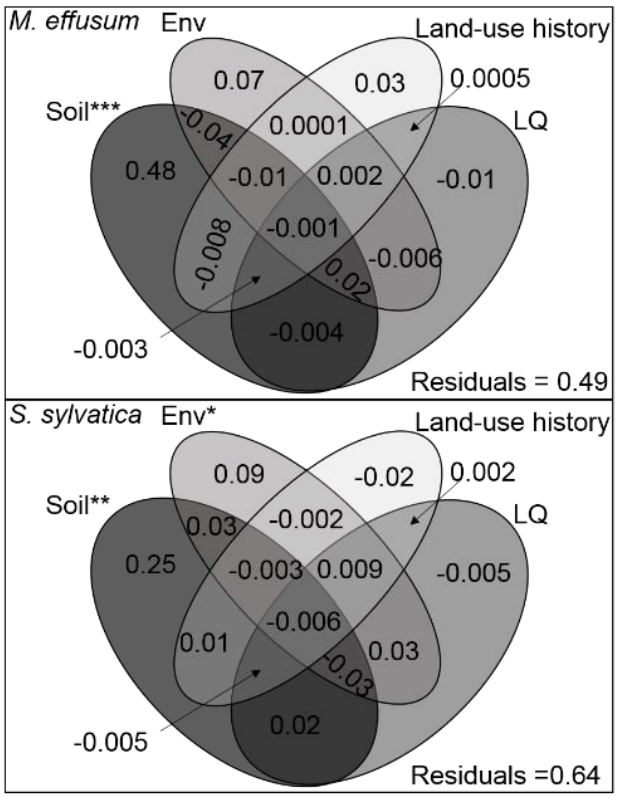
Phylum	*M. effusum* (31)	*S. sylvatica* (31)	χ^2-value (Df = 1)
Acidobacteria	4909 (934.4)	3477.9 (390.5)	21.5***
Actinobacteria	10474 (2853.7)	9693.4 (942.2)	1.3ns
Armatimonadetes	9.4 (3.9)	3 (1.5)	7.5**
Bacteroidetes	1287 (457.1)	1177.5 (125)	2.4ns
BJ.169	0.2 (0.2)	0.2 (0.2)	< 0.1ns
BRC1	0.2 (0.2)	0.2 (0.2)	< 0.1ns
Chlamydiae	190.9 (94.9)	83.5 (20.8)	5.4*
Chlorobi	1.1 (0.7)	5.9 (3.3)	1.4ns
Chloroflexi	1035.8 (328.6)	1792.1 (242.5)	14.6***
Cyanobacteria	40.8 (12.3)	15.3 (4.5)	9.6**
Elusimicrobia	27.3 (5.9)	25.1 (7.3)	1.8ns
FBP	0.7 (0.3)	0.7 (0.5)	0.5ns
FCPU426	17.4 (4)	8 (2.7)	9.0**
Fibrobacteres	5 (2.6)	2.3 (0.9)	1.6ns
Firmicutes	389.5 (70.1)	464.1 (84.8)	0.2ns
GAL15	3.4 (1.1)	0.7 (0.5)	9.1**
Gemmatimonadetes	207.8 (27.7)	328.7 (32.9)	1.8ns
Gracilibacteria	2.8 (2.6)	0.5 (0.5)	0.3ns
Hydrogenedentes	0.2 (0.2)	0.2 (0.2)	< 0.1ns
Ignavibacteriae	0 (0)	2.4 (2.1)	1.8ns
Latescibacteria	17.1 (5.4)	88 (22.5)	12.6***
Microgenomates	0 (0)	0.2 (0.2)	1.0ns
Nitrospirae	86.4 (34.5)	330.6 (82.3)	14.8***
Omnitrophica	0.6 (0.4)	1.7 (0.9)	1.9ns
Parcubacteria	266.5 (71.8)	180.4 (46)	2.6ns
Peregrinibacteria	0.2 (0.2)	0.6 (0.6)	0.2ns
Planctomycetes	1821.6 (439.1)	1536.5 (177.1)	2.1ns
Proteobacteria	8526.1 (1939.6)	8721.1 (889.4)	0.3ns
Saccharibacteria	218.5 (46.7)	161.8 (25.8)	3.6ns
SBR1093	0 (0)	0.8 (0.8)	< 0.1ns
Spirochaetae	1.7 (1.1)	0.9 (0.5)	1.1ns
SR1_Absconditabacteria	0.3 (0.3)	0 (0)	1.0ns
Tectomicrobia	6.4 (6.2)	5.8 (3)	0.1ns
Tenericutes	1.3 (0.9)	0.7 (0.7)	0.4ns
TM6_Dependentiae	17.6 (4)	16.3 (4.3)	4.9*
Unclassified	158.6 (32.8)	85.9 (25)	9.2**
Verrucomicrobia	1717.6 (364.1)	2339.7 (405.9)	5.0*
WS2	0.2 (0.2)	0.2 (0.2)	< 0.1ns

Appendix 3.2

The mean values (with standard error) of soil chemistry in ancient (33 soil samples) and recent (29 soil samples) forests (land-use history) and likelihood ratio tests for the significance of the soil chemistry between the two types of forests based on linear mixed-effects models

Variable	Ancient (33)	Recent (29)	χ^2-value (Df = 1)
C% §	5.3 (0.4)	6.4 (1.4)	< 0.1 ns
N% §	0.4 (0)	0.4 (0.1)	<0.1 ns
C/N §	13.9 (0.3)	13.9 (0.5)	0.1 ns
P (mg/kg) §	551.1 (44.4)	613.4 (52.4)	0.8 ns
Olsen P (mg/kg) §	30.4 (3.1)	38.5 (5.6)	0.8 ns
K (mg/kg) §	116 (13.1)	174 (34.3)	1.3 ns
Ca (mg/kg) §	2621.6 (781.3)	3074 (1354.2)	< 0.1 ns
Mg (mg/kg) §	151.7 (27)	175.7 (32.4)	0.2 ns
Al (mg/kg) £	289.4 (35)	266.6 (32.8)	0.1 ns
pH £	5.2 (0.1)	5.3 (0.1)	0.1 ns

Appendix 3.3



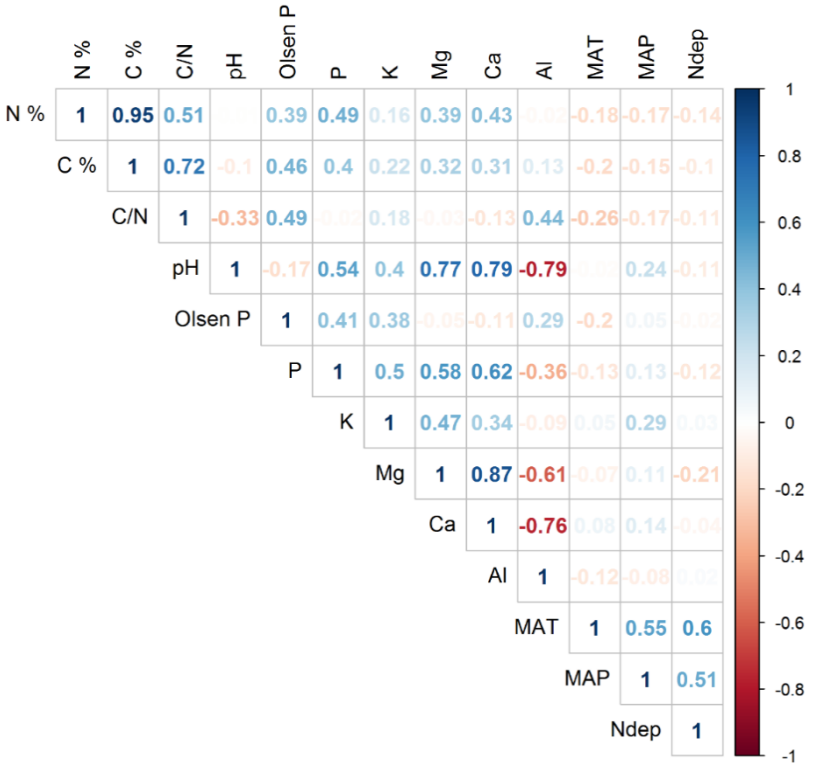
Variation partitioning of the rhizosphere soil bacterial community composition explained by soil chemistry (Soil), large-scale environmental conditions (Env), land-use history and litter quality (LQ) in each understorey plant species. Adjusted R^2 values in each fraction indicate the explained proportion of the variation for that variable category. Residuals indicate the unexplained variation. Adjusted R^2 values may cause small negative values. Asterisks show the significance of permutation tests for each explanatory factor. * P < 0.05; ** P < 0.01; *** P < 0.001.

Appendix 3.4

The mean values (with standard error) of soil chemistry in *Milium effusum* (31 soil samples) and *Stachys sylvatica* (31 soil samples) and likelihood ratio tests for the significance of the soil chemistry between the two plant species based on linear mixed-effects models

Variable	*M. effusum* (31)	*S. sylvatica* (31)	χ^2-value (Df = 1)
C% §	6.3 (1.3)	5.3 (0.6)	0.3ns
N% §	0.4 (0.1)	0.4 (0)	0.2ns
C/N §	14.8 (0.4)	13 (0.3)	13.8***
P (mg/kg) §	471.6 (34.3)	681.7 (51.6)	12.4***
Olsen P (mg/kg) §	37.1 (5.2)	31.5 (3.6)	0.9ns
K (mg/kg) §	129.4 (27.6)	157.2 (23.3)	1.8ns
Ca (mg/kg) §	3162.4 (1507.1)	2521.4 (352.3)	6.8*
Mg (mg/kg) §	122.3 (22.2)	202.7 (33.7)	10.3**
Al (mg/kg) £	353.8 (36.8)	208.5 (25.7)	9.9**
pH £	4.9 (0.1)	5.6 (0.1)	14.2***

Appendix 3.5



The Spearman correlation matrix of variables in soil chemistry and large-scale environmental conditions. Values with different colour hues indicate positive (blue) or negative (red) correlations between pairs of variables.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Chapter 4

Plant and soil microbe responses to light, warming and nitrogen addition in a temperate forest

After: Ma, S., Verheyen, K., Props, R., Wasof, S., Vanhellemont, M., Boeckx, P., Boon, N. and De Frenne, P., 2018. Plant and soil microbe responses to light, warming and nitrogen addition in a temperate forest. Functional Ecology, 32(5), pp.1293-1303.

4.1 Abstract

Temperate forests across Europe and eastern North America have become denser since the 1950s due to less intensive forest management and global environmental changes such as nitrogen (N) deposition and climate warming. Denser tree canopies result in lower light availability at the forest floor. This shade may buffer the effects of N deposition and climate warming on understorey plant communities. We conducted an innovative *in-situ* field experiment to study the responses of co-occurring soil microbial and understorey plant communities to N addition, enhanced light availability, and experimental warming in a full-factorial design. We determined the effects of multiple environmental drivers and their interactions on the soil microbial and understorey plant communities, and assessed to what extent the soil microbial and understorey plant communities co-vary.

High light led to lower biomass of the soil microbes (analysed by phospholipid fatty acids), but the soil microbial structure, i.e., the ratio of fungal biomass to bacterial biomass, was not affected by light availability. The composition of the soil bacterial community (analysed by NGS, i.e., next-generation sequencing) was affected by both light availability and warming (and their interaction), but not by N addition. Yet, the number of unique operational taxonomic units (OTUs) was higher in plots with N addition, and there were significant interactive effects of light and N addition. Light availability also determined the composition of the plant community; no effects of N addition and warming were observed. The soil bacterial and plant communities were co-structured, and light availability explained a large part of the variance of this co-structure.

We provide robust evidence for the key role of light in affecting both the soil microbial and plant communities in forest understoreys. Our results advocate for more multifactor global-change experiments that investigate the mechanism underlying the (in)direct effects of light on the plant-soil continuum in forests.

4.2 Introduction

Global change is affecting ecosystems through multiple environmental drivers (Vitousek 1994; Schroter *et al.* 2005). Increased atmospheric nitrogen (N) deposition and climate warming are amongst the most important ones, and they interact in complex ways (Sala *et al.* 2000). In temperate forests across Europe and eastern North America, the canopy cover has been increasing since the 1950s, mainly caused by changes in forest management, atmospheric N deposition, and climate (Schroter *et al.* 2005; Gold, Korotkov & Sasse 2006; McMahon, Parker & Miller 2010; Rautiainen, Wernick, Waggoner, Ausubel & Kauppi 2011; Kauppi *et al.* 2015). Denser forest canopies cause more shading and lower ground-level temperatures and may buffer the effects of both N deposition and climate warming on the forest understorey. Denser canopies can thus buffer the temperature experienced by understorey plants (De Frenne *et al.* 2013b; Melin *et al.* 2014; Scheffers, Edwards, Diesmos, Williams & Evans 2014; Valladares, Laanisto, Niinemets & Zavala 2016), which can explain the lagged response of understorey plants to climate warming (Bertrand *et al.* 2011). In addition, shade potentially limits plant responses to increased N deposition (Hautier, Niklaus & Hector 2009; Verheyen *et al.* 2012; Borer *et al.* 2014; Farrer & Suding 2016; Walter *et al.* 2016).

To date, studies on the effect of global environmental change have involved various drivers, such as N deposition, climate warming, precipitation, and CO_2 concentration (Ciais *et al.* 2005; Hyvonen *et al.* 2007; Ramirez, Craine & Fierer 2012; von Rein *et al.* 2016). However, the role of light has rarely been considered, in spite of its importance in light-limited habitats such as the forest understorey. The few light-related studies showed that enhanced light availability can increase the cover of *Rubus* spp. (Walter *et al.* 2016), and enhance plant community responses to climate warming (De Frenne *et al.*, 2015). A direct experimental test of the effects of light, integrated with other global environmental change drivers, is still lacking, for both the soil microbial and understorey plant communities in temperate forests.

The soil microbial community can be severely influenced by environmental change drivers such as enhanced atmospheric N deposition and climate warming (Yergeau *et al.* 2012; De Vries, Dobbertin, Solberg, Van Dobben & Schaub 2014; Carey 2016; Farrer & Suding 2016). For example, in plots with N addition, N-demanding taxa may become more abundant than oligotrophic taxa, which eventually affects the metabolic capabilities of the soil microbial community (Ramirez, Craine & Fierer 2012). Experiments showed that climate warming may cause shifts in soil microbial composition or function that affect the global carbon (C) and N cycles via changes in, e.g., the biomass of microbial decomposers (Allison, Wallenstein & Bradford 2010), the rate of heterotrophic respiration (Suseela, Conant, Wallenstein & Dukes 2012), and the efficiency of

recalcitrant substrate utilization (Frey, Lee, Melillo & Six 2013). In natural conditions, multiple global environmental change drivers co-occur (Leuzinger *et al.* 2011), and the influence of multiple, combined global environmental change drivers on soil microbial communities may differ from the influences of the drivers applied in isolation (e.g., effects may be additive or one driver may dominate the overall effect).

Changes in the soil microbial community composition and structure can affect the plant community through, for instance, plant-soil feedbacks associated with the processes of soil organic matter decomposition and mineralization, or pathogenic and beneficial interactions (De Deyn & van der Putten 2005; Bardgett & van der Putten 2014; Larios & Suding 2015; van der Putten, Bradford, Brinkman, van de Voorde & Veen 2016). Likewise, changes in plant community composition and diversity can also modulate soil microbial activities (Lange *et al.* 2015). To better understand the mechanisms behind the combined effects of multiple environmental drivers on light-limited ecosystems such as forest understoreys, it is important to not only look at the responses of the understorey plants or the responses of the soil microbes and the understorey plants separately, but to also quantify co-occurring responses (von Rein *et al.* 2016). Hence, studies should investigate the plant-soil continuum together to make realistic predictions on the effects of global environmental changes (von Rein *et al.* 2016). Plants and soil microbes might respond differently to different global environmental change drivers (Farrar & Suding, 2016), and the interaction between the multiple drivers may attenuate or increase the magnitude of the responses of both plants and soil microbes. A recent meta-analysis of a large database of studies on multiple environmental drivers showed that global change drivers indeed affect terrestrial ecosystems and that the effects may be less dramatic than expected if several drivers occur concurrently (Leuzinger *et al.* 2011).

Here we report findings from an innovative, *in-situ* experiment under a scenario of multiple environmental changes. We assessed the individual and interactive effects of N deposition, light availability, and climate warming on the biomass of the soil microbes, as well as the composition and diversity of the soil bacterial and understorey plant communities in a full-factorial experiment in an ancient temperate deciduous forest. We hypothesized that: (i) illumination and warming under the dense canopy of the studied ancient forest will shift the composition of the soil microbial and understorey plant communities. Meanwhile, we expect weak or no effects of N addition because of N saturation in the study area; (ii) the interactive effects of two or three simultaneously applied drivers on the composition of soil microbial and plant communities will differ from the effects of the drivers applied in isolation. We specifically expected illumination to enhance community responses to N and warming because of increased inputs of chemical compounds to the soil via enhanced photosynthesis; (iii) the soil microbial and plant community will co-vary (an interplay

between the two communities), as both communities are expected to respond concurrently to the applied global environmental change drivers.

4.3 Materials and Methods

4.3.1 Study area

The experiment was performed in the Aelmoeseneie forest, a temperate deciduous forest in Belgium (50.97°N, 3.81°E) owned by Ghent University, which has been forested since at least the 1770s. Ash (*Fraxinus excelsior* L.) and pedunculate oak (*Quercus robur* L.) were the dominant tree species. The soil developed from a quaternary layer of sandy loam on a shallow impermeable clay and sand complex of tertiary origin (Vanhellemont, Baeten & Verheyen 2014). The recorded mean annual temperature and mean annual precipitation (1981-2010) were 10.6 ℃ and 786 mm, with the precipitation evenly distributed over the year and the mean temperature of the warmest and coldest month 16.8 ℃ and 2.4 ℃. The N deposition in 2009 was 25.3 kg ha^{-1} (Verstraeten, Sioen, Neirynck, Roskams & Hens 2012).

4.3.2 Experimental design

Forty 0.5 m × 0.5 m plots with a similar canopy cover and without any disturbance were randomly established in 2011. The distance between two plots ranged from 5 m to 200 m (the closest two plots – the farthest two plots). The similar canopy cover ensured homogenized natural light availability for all plots. There were no statistically significant differences in plant species richness, evenness, and composition between the plots at the beginning of the experiment (De Frenne *et al.* 2015). The three manipulated environmental drivers were N addition (N), illumination (L), and warming (W). A full-factorial design resulted in eight possible combinations: all drivers applied in isolation, the two-factor and three-factor interactions (with five replicates each, i.e., 40 plots in total).

Transplantation. In addition to the naturally occurring species, we introduced three characteristic, relatively tall, competitive native plant species (*Urtica dioica* L., *Rubus fruticosus* agg. and *Aegopodium podagraria* L.) at the start of the experiment in September 2011 in all plots. Three rhizome fragments of ca. 7 cm length per species were transplanted along the two diagonal axes of each plot (min. 10 cm away from the corners of the plot). The rhizomes of each of these species were collected within the same forest at < 500 m distance. This was key for the experiment because these species are expected to strongly respond to resource alterations and affect the understorey dynamics, but did not occur in any of the plots due to closed-canopy conditions before the experiment started (except for *A. podagraria* 3% ground cover in one single plot and *R. fruticosus* 0.5% and 2% ground cover in two of the 40 plots, repectively). Transplantation was thus needed to overcome dispersal

limitation because the selected plots are surrounded by dense forest and these species would most likely not naturally colonize the plots within the timeframe of this study. By transplanting these light-demanding plants to the naturally light-limited study area, we can assess the effects of resource manipulation on plant community change unequivocally. Interestingly, the transplanted species disappeared almost completely in the plots without resource manipulation. More description of the full experiment design can be found in De Frenne *et al.* (2015).

N addition. In half of the plots, we added 10 g N m^{-2} $year^{-1}$. Ammonium nitrate was dissolved in 400 ml distilled water and applied four times per year (Jan., Apr., Jun., and Sep.). After the solution was sprayed onto the plots, 200 ml distilled water was immediately added to avoid salt deposition on the plants. Plots without N addition received 600 ml distilled water. Each spraying amounted to 0.33% of the total mean annual precipitation. This resulted in a mean top soil concentration increase of 4.39 mg inorganic-N kg^{-1} dry soil in N added plots.

Illumination. Additional light was provided by two 14 W fluorescent tubes (coolwhite 4000 K, spectral peaks at 546 nm and 611 nm), which were suspended at 65 cm above the forest floor with a wooden frame and protected by a plastic cover in 20 plots (one plot in treatment L was destroyed, leaving 19 plots for data analyses). The lamps switched on at sunrise and off at sunset to simulate the natural photoperiod of the study area. The timing was adjusted every two or three weeks throughout the whole year to match the seasonal variation in day length. Unilluminated plots were equipped with the same frame and cover but with dummy lamps. Photosynthetically active radiation (PAR) was measured after canopy flush on 14 June 2012 under cloudless conditions at five height intervals (20 cm, 30 cm, 40 cm, 50 cm, and 60 cm above the soil surface) in three plots per treatment using PAR Quantum sensors connected to Spectrosense 2+ meters (Skye Instruments, UK). The illuminated and unilluminated plots differed significantly in PAR influx (76.4±10.8 μmol m^{-2} s^{-1} vs. 6.7±0.4 μmol m^{-2} s^{-1}), and there was no effect of illumination on the measured temperatures (De Frenne *et al.* 2015). The applied increase in light availability is similar to the difference between denser canopy conditions in deciduous forest during the growing season and the typical light intensity in small clearings or more open forest types. During the growing season, the PAR levels in the understorey of temperate deciduous forests with a high leaf area index typically vary between 5-77 μmol m^{-2} s^{-1}, whereas the PAR levels in small clearings or in forests with a lower leaf area index vary between 11-121 μmol m^{-2} s^{-1} (Hutchison & Matt 1977; Clinton 1995; Nilsen *et al.* 2001; Fladeland, Ashton & Lee 2003; Augspurger, Cheeseman & Salk 2005).

Warming. Hexagonal open-top chambers (OTCs) passively warmed the air and soil temperatures. The OTCs were 60 cm high, consisted of six inclined plexiglas walls and an open top, and covered a

ground surface area of 1.15 m^2. We set our 0.5 m × 0.5 m plots right beneath the open top, in the core area of the open-top chamber, to avoid the effects of the inclined OTC walls on precipitation, soil moisture, and air humidity. We installed three Type T miniature thermocouples (TC Direct, Nederweert, NL) in one unwarmed-unilluminated plot; one unwarmed-illuminated plot; one warmed-unilluminated plot and one warmed-illuminated plot (12 sensors in total). The temperatures were logged at 5 min intervals at 20 cm above the soil surface, at the soil surface and at 5 cm below the soil surface. Overall, the mean temperature (14 Sep. 2011-01 Apr. 2015) was raised by 1.43 ℃ (20 cm), 1.26 ℃ (soil surface), and 3.21 ℃ (-5 cm) in the OTCs compared to unwarmed plots, i.e., plots without OTC. The mean daily minimum and maximum temperatures were raised by 1.63 ℃ and 1.46 ℃ (20 cm), 0.85 ℃ and 1.79 ℃ (soil surface), 3.72 ℃ and 2.59 ℃ (-5 cm).

4.3.3 Data collection

Soil sampling. Soil samples for the analyses of microbial biomass and bacterial community composition were taken with a 2.7 cm diameter auger from 0-10 cm soil depth on 2 April 2015. In each plot, two opposite sample sites were selected at 5 cm distance from the plot centre. The two soil samples from one plot were pooled and homogenized. The pooled and homogenized samples were immediately sieved through a 1-mm mesh and stored at -18 ℃ until the start of the extraction. To avoid cross contamination, the auger was sterilized with 75% ethanol between the plots.

Soil microbial biomass. Phospholipid fatty acids (PLFAs) were extracted and derived according to Huygens *et al.* (2011). More information on this extraction is given in section 2.3.3. In total, 42 PLFA biomarkers were detected. We selected 19 PLFAs as useful biomarkers, which accounted for 91% of the detected biomass. Those selected biomarkers were assigned to different functional microbial groups (**Appendix 4.1**). Soil microbial structure was calculated as the ratio of total fungal biomass to total bacterial biomass.

Soil bacterial community. The bacteria dominated the microbial community in the experimental plots; they made up 90.7% to 91.9% of the total extracted PLFAs in the different treatments. Hence, we only quantified the composition of the soil bacterial community, using next-generation sequencing (NGS). The total DNA extraction from the soil samples was carried out with the PowerSoil®DNA Isolation kit and purified by means of the Wizard®DNA Clean-Up System, following the manufacturer's instructions. The 16 S rRNA gene v3-v4 region was amplified by PCR using the barcoded versions of the primers described by Klindworth *et al.* (2013). Sequencing was done on an Illumina MiSeq platform. The operational taxonomic units (OTUs) table was created using *MOTHUR* (v.1.38) (Schloss *et al.* 2009). See section 3.3.3 for sequencing and OTUs table obtainment.

Plant community. The cover of all plants below 1 m height in each plot was assessed in 2015, in April for *Anemone nemorosa* L. and *Ranunculus ficaria* L. and in June for all other species.

4.3.4 Data analysis

We investigated how the three drivers - N addition, illumination, and warming - (interactively) affected soil microbial biomass, soil bacterial community composition, and plant community composition. Next, we investigated whether and how the aboveground (plants) and belowground (soil microbes) communities were linked. All data analyses were performed in R 3.3.2 (R Core Team 2016).

Soil microbial biomass. We used one-way analysis of variance (ANOVA) to test for the effects of the treatments on the biomass of each functional microbial group and the microbial structure (PLFA data), and Tukey's post-hoc test to investigate the differences among the treatments. We then applied three-way ANOVA to test for the main and interactive effects of the three drivers on each functional microbial group (the three-way interaction was never significant and therefore omitted).

Shared and unique OTUs of the bacterial community. The analyses of the bacterial community (NGS data) were conducted after proportional normalization. The abundance of each OTU was rescaled by taking the proportion of the OTU's read in the total reads, multiplying the result with the minimum sample size (1803 reads) and then rounding to the nearest integer to account for sample size differences (McMurdie & Holmes 2014). This resulted in a scaled taxon-abundance matrix comprised of 4110 OTUs. We calculated the number of shared and unique OTUs between the pairs of treatments: 'N added' vs. 'No N added', 'Illuminated' vs. 'Unilluminated', and 'Warmed' vs. 'Unwarmed'. We used chi-square tests to assess differences in the number of unique OTUs in each pair of treatments (Schmidt, White & Denef 2016).

Alpha diversity of the soil bacterial and plant community. We calculated two indices of alpha diversity: (i) the species richness, i.e., the number of species or OTUs in a plot, and (ii) the inverse Simpson diversity index, taking into account the number of plant species or OTUs present and the relative abundance of each plant species or OTU, using the package *vegan* (Oksanen *et al.* 2016). Three-way ANOVA was used to characterize the individual and interacting effects of the three drivers (the three-way interaction was never significant and therefore omitted).

Beta diversity of the soil bacterial and plant community. We used model-based multivariate abundance analysis to test beta diversity of the soil bacterial and the plant community (Wang, Naumann, Wright & Warton 2012). The model fits individual generalized linear models (GLMs) to each plant or bacterial species (OTU) and then uses these models to make community-level

inferences about the importance of the model predictors based on resampling. The multivariate abundance models were constructed using a forward selection procedure, i.e., by systematically adding variables to the null model and testing for significant model improvement between nested models by likelihood-ratio tests (*anova.manyglm* function, 500 for the bacteria community, 5,000 PIT-trap resampling runs for the plant community). Model improvements were considered significant at the 0.05 significance level. Mean-variance relationships were modelled by a negative binomial model, and Dunn-Smythe residuals of each model were evaluated for normality and homoscedasticity. The significance of the final model parameters was used to assess community-level effects of the model predictors.

The linkage of the soil bacterial and plant community. We conducted co-inertia analysis (hereafter referred to as COIA), a general and flexible eigenvector framework with no constraint regarding the number of variables that allows to measure the concordance (i.e. co-structure) between two multivariate datasets that share the same objects (plots in our case) (Dolédec and Chessel, 1994; Dray et al., 2003). The method finds a common space into which the plots and species of the datasets can be projected and compared (the distance between plots reflects their similarity). We used the *ade4* package (Dray et al., 2007) to apply COIA to two pairs of datasets: i.e., plant data vs. PLFA biomarkers and plant data vs. bacterial OTU data (at phylum level). We also applied COIA to two subsets of the PLFA data and looked at plant data vs. fungal biomarkers and plant data vs. bacterial biomarkers. Prior to the COIA, we performed Principal Component Analysis (PCA) on the Hellinger-transformed community datasets. We evaluated the strength of the coupling between each pair of datasets with the RV coefficient, which is a multivariate generalization of the Pearson correlation coefficient. The RV coefficient gives a measure of the global similarity of the two datasets between 0 and 1: the closer the coefficient is to 1, the stronger the correlation between the datasets. We then used the Monte-Carlo test (with 999 random permutations) to assess the significance of the co-structure between the datasets.

4.4 Results

4.4.1 Soil microbial biomass

The biomass of the functional microbial groups differed significantly between the treatments (**Fig. 4.1** and **Appendix 4.2**).The highest total microbial biomass occurred in the treatment of warming and N addition combined (WN), while the lowest total microbial biomass occurred in the treatment of N addition, illumination, and warming combined (WLN). The WN treatment significantly differed from the WLN treatment for all functional microbial groups. Three other significant differences in the biomass of functional microbial groups between treatments were found. First, the WN treatment

differed from the LN treatment (combined illumination and N addition), with significantly higher biomasses of Gram-negative bacteria, total biomass of bacteria and total biomass of the microbial community in the WN treatment (i.e., the treatment without illumination). Second, the WN treatment differed from the L treatment, with a significantly higher biomass of Gram-negative bacteria in the WN treatment (i.e., the treatment without illumination). Third, the W treatment differed from the WLN treatment, with a significantly higher biomass of Gram-positive bacteria and *Actinobacteria* in the W treatment (i.e., the treatment without illumination). Illumination was the only driver that showed a strong negative effect on all functional microbial groups ($P < 0.001$); neither N addition, warming, nor the two-way interactions had an effect (**Fig. 4.1** and **Appendix 4.3**). The three drivers did not affect the soil microbial structure (**Appendix 4.3**).

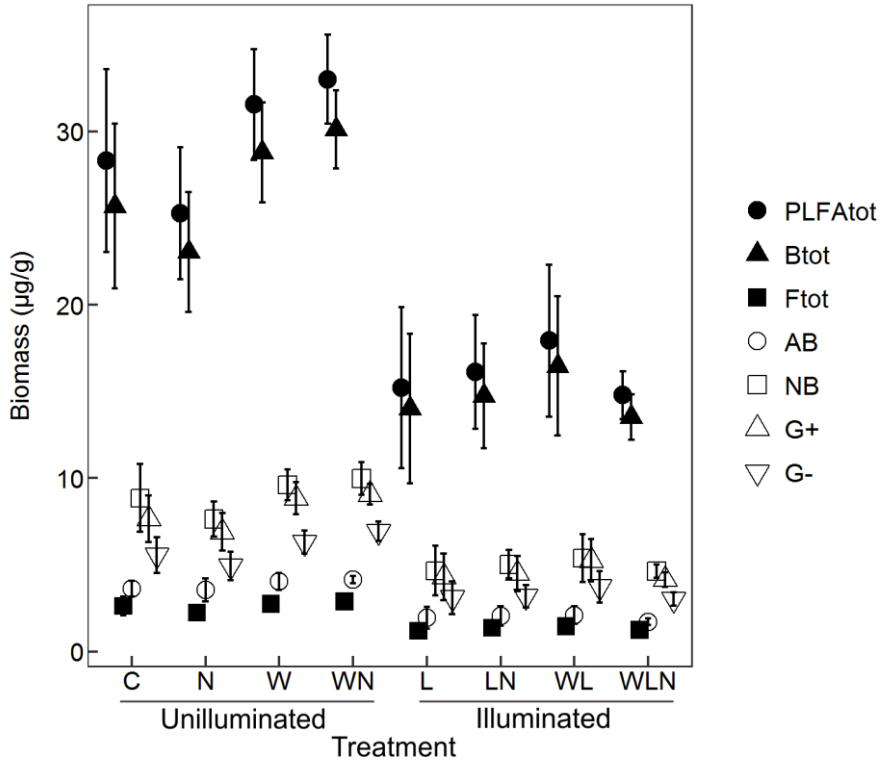


Fig. 4.1 Biomass (mean ± standard error) of the microbial functional groups based on PLFA concentrations. The treatments are control (C), N addition (N), illumination (L), warming (W), warming + N addition (WN), warming + illumination (WL), illumination + N addition (LN) and warming + illumination + N addition (WLN). Each symbol represents a functional microbial group: PLFAtot (total biomass of the microbial community), Btot (total biomass of bacteria), Ftot (total biomass of fungi), AB (*Actinobacteria*), NB (Non-specific bacteria), G+ (Gram-positive bacteria) and G- (Gram-negative bacteria).

4.4.2 Composition and diversity of the soil bacterial and plant communities

Seventeen phyla were identified in the soil bacterial community. *Acidobacteria, Actinobacteria,* and *Proteobacteria* accounted for 94% of all reads and were the main phyla in all treatments (unclassified phyla were not taken into account) (**Appendix 4.4**). Concerning alpha diversity, none of the three drivers had an effect on phylum richness, but the interactive effect of N addition and

warming was significant for the inverse Simpson index (P = 0.031). The beta diversity of the soil bacterial community was significantly affected by illumination (P = 0.004), warming (P = 0.012) (**Table 4.1** and **Appendix 4.5**), the interaction between illumination and warming (P = 0.006), and the interaction between illumination and N addition (P = 0.002) (**Table 4.1**). N addition did not significantly affect the soil bacterial community composition (P = 0.09). The three drivers also affected the number of unique OTUs found in the plots. The number of unique OTUs was significantly (P < 0.001) higher in plots in which the driver was manipulated, i.e., in the plots with N addition, in illuminated plots, and in warmed plots (**Fig. 4.2a**), and the highest number of unique OTUs was found in the plots with N addition (**Fig. 4.2a**). The shared OTUs made up between 92.6 and 95.6% of the overall OTU abundance (**Fig. 4.2b**), and there was no significant effect of the three drivers on the number of shared OTUs (**Fig. 4.2a**).

Table 4.1 Final model testing the effects of nitrogen addition (N), illumination (L), and warming (W) on the soil bacterial community composition

Driver	Df	Test statistic (LRTs)	P value
N	37	5571.543	0.088
L	36	5642.737	0.004**
W	35	5358.399	0.012*
L:W	34	1754.798	0.006**
N:W	33	1474.774	0.076
L:N	32	2080.481	0.002**

The full model selection is available in **Appendix 4.5.**

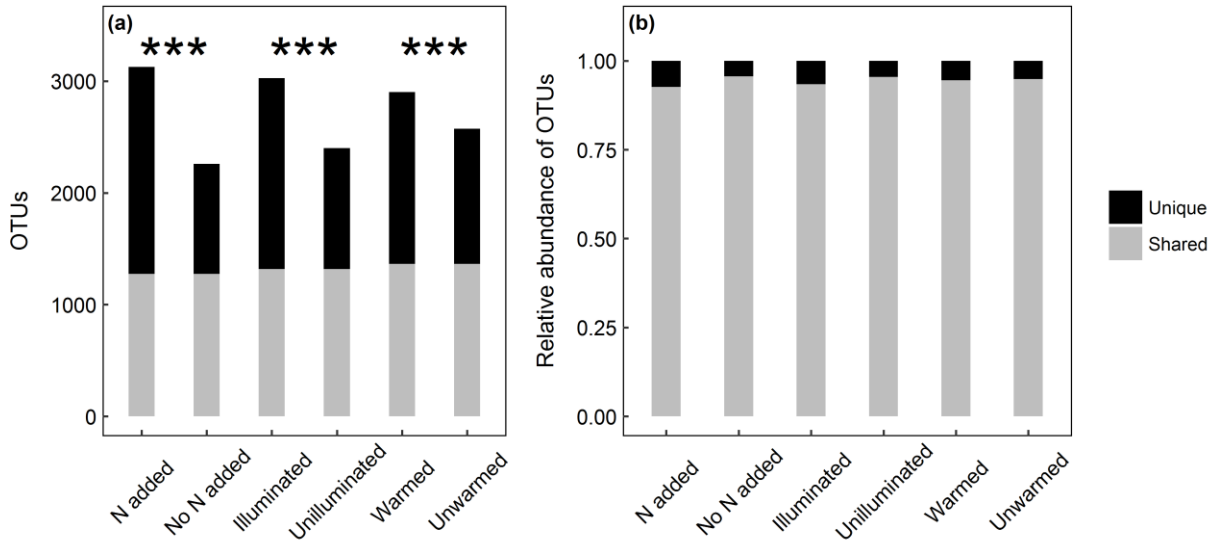


Fig. 4.2 The number of unique and shared operational taxonomic units (OTUs) for the treatments in which a specific driver is manipulated or not manipulated (a). The relative abundance of shared and unique OTUs within each group of treatments (b). *** Indicates significant differences in the number of unique OTUs between the pair of treatments based on a chi-square test (P < 0.001).

Fourteen plant species were observed across all plots. The two most abundant species were *Anemone nemorosa* L. and *Oxalis acetosella* L. (**Appendix 4.6**). The alpha diversity of the plant community was not significantly affected by the three drivers, but the effect of the two-way interaction of N addition and warming on plant species richness was marginal significant (P = 0.063). The beta diversity of the plant community was significantly affected by illumination only (P = 0.026, **Appendix 4.5**).

4.4.3 Co-structure between the soil microbial and the plant community

There was no significant co-structure between the soil microbial (measured as PLFA biomarkers) and plant communities or the fungal biomarkers (**Table 4.2**) in all plots. Yet, we saw significant co-structure between the soil bacterial and plant communities, for both the bacterial biomarkers (PLFA data, **Table 4.2**) and the bacterial phyla (OTU data, **Table 4.2**, **Fig. 4.3**). When we split the dataset into illuminated plots and unilluminated plots, we only saw co-structure in the illuminated plots, between the plant community on the one hand and the overall soil microbial community (19 PLFA biomarkers) and the soil bacterial community (15 PLFA biomarkers, 17 phyla) on the other hand (**Table 4.2**).

Table 4.2 The co-structures between the soil microbial (PLFA data, i.e., four fungal biomarkers and fifteen bacterial biomarkers; OTU data of the bacterial community, i.e., 17 phyla) and plant communities (14 species) in all plots and subset plots with and without illumination

Soil microbial communities	RV value		
	All plots	Unilluminated plots	Illuminated plots
PLFA biomarkers (19)	0.25	0.31	0.44*
- Fungal biomarkers (4)	0.13	0.14	0.28
- Bacterial biomarkers (15)	0.24*	0.29	0.43*
Bacterial OTUs (17 phyla)	0.31**	0.38	0.43*

Significance was obtained from Monte-Carlo tests.

4.5 Discussion

Light availability emerged as a critical driver for both soil microbes and understorey plants in our experiment. Denser canopies in forests do not merely reduce light availability in the understorey but may also affect, for instance, light quality, temperature, humidity, soil mineralisation, and decomposition (Neufeld & Young 2003; Valladares, Laanisto, Niinemets & Zavala 2016). In our full-factorial experiment, we were able to separate the effects of N availability, light availability, and temperature in the understorey unequivocally, one of the strengths of our study.

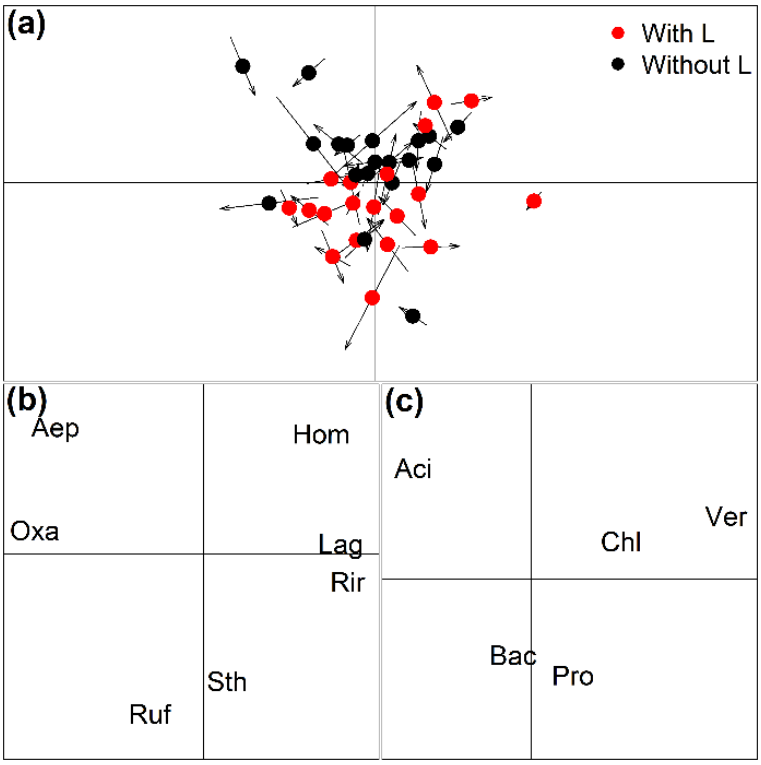


Fig. 4.3 The co-inertia analysis of the soil bacterial (17 phyla) and plant community compositions across all plots. (a) The mutual ordination of the plots as a function of both the soil bacterial and plant community compositions, (b) projection of the plant species, and (c) projection of the bacterial phyla. Illuminated plots (with L) are shown in red; unilluminated plots (without L) are shown in black. The two first canonical axes account for 78.3**%** of total co-inertia and the RV coefficient is 0.31 (P = 0.002). The arrow length in (a) is proportional to the difference between the ordinations of the plant and bacteria data; the position of the arrow tails is determined by the ordination of the plant community data, the arrowheads by the bacterial data. Only the plant species and bacterial phyla with the highest ordination scores are shown in figures (b) and (c). The abbreviations of the plant species in (b): Aep *Aegopodium podagraria* L., Hom *Holcus mollis* L., Lag *Lamium galeobdolon* (L.) Crantz, Oxa *Oxalis acetosella* L., Rir *Ribes rubrum* L., Rur *Rubus fruticosus* agg., Sth *Stellaria holostea* L. The abbreviations of the bacterial phyla in (c): Aci Acidobacteria, Bac Bacteroidetes, Chl Chlamydiae, Pro Proteobacteria, Ver Verrucomicrobia.

4.5.1 Light effects on plant and soil microbial community composition

In illuminated plots, the soil microbial biomass was significantly lower and the composition of the soil bacterial community and the plant community differed when compared to unilluminated plots. The response to light of the soil microbial and plant communities may be explained in two (non-exclusive) ways. (i) Light drives the species composition of the plant community through asymmetric resource competition (Valladares, Laanisto, Niinemets & Zavala 2016), and differences in plant community composition result in a different quality and quantity of litter, which in turn affects the nutrient supply to the soil microbial community (Strickland, McCulley, Nelson & Bradford 2015). (ii) Light availability affects the allocation of the photosynthetic products in plants, resulting in, e.g., more

investment in aboveground plant biomass and less belowground C allocation (to root exudates and rhizodeposition), which eventually results in reduced soil microbial biomass (Drake *et al.* 2013; Balasooriya, Denef, Huygens & Boeckx 2014). It is likely that the plant community in our plots changed first, as a result of illumination, and that this change in the plant community then affected the soil microbial community composition. We speculate that the time scale in which soil microbial community starts to change is after 2.5-year manipulation as we have documented increases of plant heights under illuminated plots than in unilluminated plots in 2014 (De Frenne *et al.* 2015). Light availability is a key environmental driver in shaping plant community composition and diversity, and in linking the aboveground and belowground communities, in various ecosystems. In grasslands, for instance, taller plants are more competitive and reduce the light availability for smaller plants, which results in changes in the plant community composition and diversity loss with fertilization (Hautier, Niklaus & Hector 2009). An altered plant community can affect soil heterotrophic activities via, for instance, litter decomposition (Spehn, Joshi, Schmid, Alphei & Korner 2000). In an agro-ecosystem for example, Lau *et al.* (2012) found that the aboveground biomass of soybean significantly increased with light availability (PAR 279.12±44.65 μmol m^{-2} s^{-1} vs. 51.25±8.45 μmol m^{-2} s^{-1}) most likely through *Rhizobia*. Because the process of N fixation by *Rhizobia* is highly correlated with the products of photosynthesis. Hence, light availability can affect both aboveground and belowground communities in direct and indirect ways in different ecosystems. In this study, the transplanted species are competitors, stress-tolerator and ruderal species and can rapidly colonize open forests (De Frenne *et al.* 2015). Manipulated resources in this study might stimulate the priming effects of these competitive species by adjusting their physical metabolism, resulting in community shifts (both aboveground and belowground) and fierce resource completion between organisms. The co-structure we found between the soil bacterial and plant communities in illuminated plots supports the idea that the abiotic factor, light availability affects the soil bacteria through its effect on the understorey plants. We found no co-structure between the soil fungal and plant communities. Cassman *et al.* (2016), on the contrary, found co-structures between the plant and soil fungal communities but not between the plant and soil bacterial communities in grassland ecosystems with long-term N addition. The difference between our study and Cassman *et al.* (2016) may be caused by the differences in resource limitation and the composition of the soil microbial and plant communities in different ecosystem types, e.g., in forests vs. grasslands (Nacke *et al.* 2011; Kaiser *et al.* 2016).

4.5.2 Interaction effects of multiple environmental change drivers on plant and soil microbial community composition

The composition of the bacterial community was more responsive to the manipulated drivers than the plant community and the soil microbial biomass. Both illumination and warming significantly affected the composition of the soil bacterial community. Light availability most likely affects the soil bacterial community in an indirect way, via root exudates for instance. Warming, on the other hand, can affect the soil bacterial community in a direct way, via metabolic C (Schindlbacher *et al.* 2011). We also observed pairwise interaction effects of the three drivers on the composition of the bacterial community, whereas we saw only a main effect (of illumination) for the plant community. There were interactive effects of illumination with N addition and with warming, while N addition alone did not affect the bacterial community. These results support our second hypothesis that when N addition and illumination are applied simultaneously, the interactive effects of the two drivers on the composition of the soil bacterial community differ from the effect of N addition alone. In N-saturated soils, the competition for N between the soil microbial community and plant roots is lower than that in N-limited soils (Kuzyakov & Xu 2013). In soils, however, all N transformation and uptake processes are correlated with soil C resources and regulated by soil microbes (Geisseler, Horwath, Joergensen & Ludwig 2010). Light availability can affect soil C sources via changes in the composition of aboveground plant community or their photosynthesis (Raven & Karley 2006; Strickland, McCulley, Nelson & Bradford 2015; Valladares, Laanisto, Niinemets & Zavala 2016). Similarly, increased temperatures can affect the decomposition rate of soil organic matter and thus potentially affect the composition of the soil bacterial community via the soil nutrient pool (Hopkins *et al.* 2014; Sierra, Trumbore, Davidson, Vicca & Janssens 2015). Warming and enhanced light also interactively affected the composition of the soil bacterial community in our study. Yet, to which degree the two drivers are synergetic or antagonistic in terms of affecting the soil bacterial community needs further research. von Rein *et al.* (2016), in a warming experiment in an incubator, showed interactive effects of the global change drivers warming and drought on the composition of the soil microbial community, with no effect of warming alone. Hence, when two or more global change drivers are considered simultaneously, the interaction of the drivers may result in additive or attenuated effects and thus cause different responses of the studied community when compared to drivers applied in isolation. The differences in the soil bacterial community composition between the treatments in our study (the unique OTUs, see **Fig. 4.2**) suggest a compositional trajectory of change in the bacterial community and a taxon-specific succession under different environmental drivers.

4.5.3 Lacking responses of soil microbial biomass and plant community composition to N addition and warming

We observed no response of the soil microbial biomass and the plant community composition to N addition and warming. Many temperate forests in Europe and North America are N saturated, and this may cause the lacking response in the soil microbial biomass and plant community to extra N addition (De Schrijver *et al.* 2008; De Schrijver *et al.* 2011; Verstraeten *et al.* 2012; Verstraeten *et al.* 2017). With regard to global warming, a lagged response of plant communities has been observed in lowland areas (Brohan, Kennedy, Harris, Tett & Jones 2006; Bertrand *et al.* 2011). The lagged response to warming is at least partly due to the large proportion of cosmopolitan and thermophilous species in these forests, which results in a higher tolerance to increased temperatures (Bertrand *et al.* 2011; De Frenne *et al.* 2015). The response of soil microbial biomass and plant community composition to N addition and warming may vary over time (Smith 2011; Contosta, Frey & Cooper 2015; Shi *et al.* 2015), which underpins the importance of the temporal scale in environmental change studies. At the global scale, warming is expected to change plant communities (Hooper *et al.* 2012). The lack in response of the plants to N addition and warming in our four-year study does not imply that these environmental changes will not affect the soil microbial biomass and understorey plants in the longer run. Chronical warming and accumulation of N (i.e., over more than ten years) can shift the composition and interaction of the soil microbial and plant communities in forests (Bradford *et al.* 2008), especially if the canopy is opened up (Verheyen et al. 2012).

4.6 Conclusions

In our four-year experiment in which we simultaneously manipulated three global change drivers in an ancient temperate deciduous forest, light availability emerged as a critical driver for both soil microbes and plants. Under additional illumination, the soil microbial biomass was lower, the composition of the soil bacterial and plant communities was different, and the composition of the soil bacterial and plant communities was co-structured. N addition and warming did not significantly affect the soil microbial biomass and plant community composition, but warming significantly altered the composition of the soil bacterial community. Our results underpin the need to concurrently investigate several communities of the plant-soil continuum and to integrate multiple environmental drivers when studying the effects of global environmental change on ecosystem functioning. In addition, the mechanisms underlying our results also merit further investigation.

Data accessibility

Data of soil microbial biomass, bacterial sequencing and plant cover deposited in the Dryad Digital Repository: https://doi:10.5061/dryad.q6789. The sequencing data are also available from NCBI BioProject under accession ID: PRJNA429723.

https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA429723

Appendix 4.1

The functional microbial group assignment based on the biomarkers of phospholipid fatty acids (PLFAs)

Functional microbial group	Biomarkers	References
G+ (Gram-positive bacteria)	iC15:0; aC15:0; iC16:0; iC17:0; aC17:0	Fuchslueger, Bahn, Fritz, Hasibeder and Richter (2014), Kaiser *et al.* (2015)
G- (Gram-negative bacteria)	16:1ω7c; cy17:0; cy19:0	Helfrich, Ludwig, Thoms, Gleixner and Flessa (2015), Kaiser *et al.* (2015)
AB (*Actinobacteria*)	10MeC16:0; 10MeC18:0	Turpeinen, Kairesalo and Haggblom (2004)
NB (Non-specific bacteria)	C14:0; C15:0; C16:0; C17:0; C18:1ω11c	Fuchslueger, Bahn, Fritz, Hasibeder and Richter (2014), Kaiser *et al.* (2015)
Ftot (Fungi)	C18:1ω9c; C18:2ω6c; C18:3ω6c; C18:3ω3c	Kaiser *et al.* (2015), Bai, Liang, Bode, Huygens and Boeckx (2016)
Btot (Total bacteria)	All biomarkers of G+, G-, AB, and NB	
PLFAtot (Total microbes)	All biomarkers of Btot, and Ftot	Frostegard *et al.* (2011)

Appendix 4.2

Mean biomass (µg/g) of each functional microbial group and the soil microbial structures for the different treatments. The last column shows the F values and significances of the one-way ANOVA analyses. Within the same row, means followed by the same superscript letter are not significantly different at $P < 0.05$, using Post-hoc analyses (TukeyHSD, adjusted *P* value, level = 0.05). The functional microbial groups are PLFAtot (total biomass of the microbial community), Btot (total biomass of bacteria), Ftot (total biomass of fungi), AB (*Actinobacteria*), NB (Non-specific bacteria), G+ (Gram-positive bacteria) and G- (Gram-negative bacteria). F/B (soil microbial structure) was the ratio Ftot to Btot. The treatments are control (C), N addition (N), illumination (L), warming (W) and their combinations: warming + illumination (WL), warming + N addition (WN), illumination + N addition (LN), and warming + illumination + N addition (WLN)

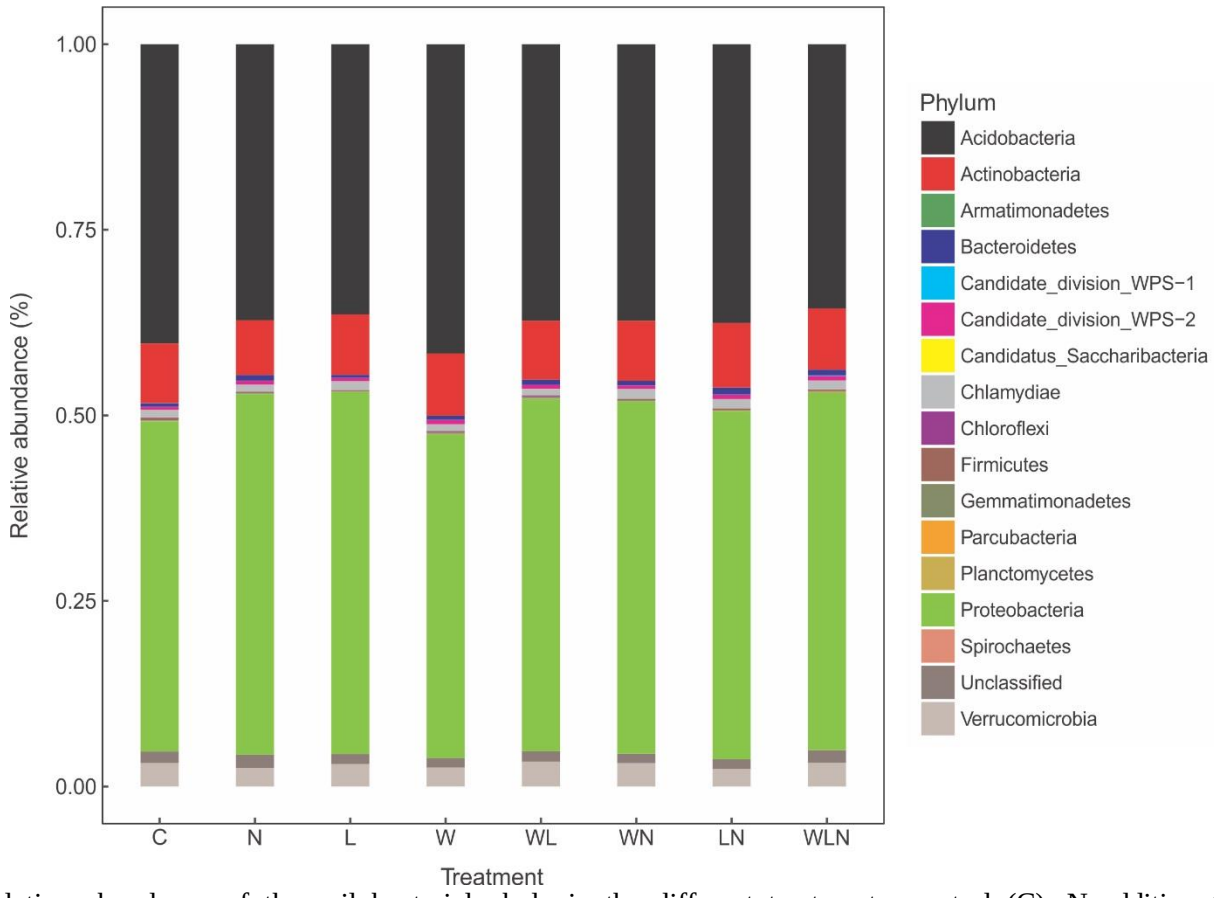
Microbial group	C	N	L	W	WL	WN	LN	WLN	F value
PLFAtot	28.3^{ab}	25.3^{ab}	15.2^{ab}	31.5^{ab}	17.9^{ab}	33.0^{a}	16.1^{b}	14.8^{b}	4.2 **
Btot	25.7^{ab}	23.0^{ab}	14.03^{ab}	28.8^{ab}	16.47^{ab}	30.1^{a}	14.8^{b}	13.5^{b}	4.2 **
Ftot	2.6^{ab}	2.2^{ab}	1.2^{ab}	2.7^{ab}	1.4^{ab}	2.9^{a}	1.4^{ab}	1.2^{b}	4.2 **
AB	3.6^{ab}	3.5^{ab}	2.0^{ab}	4.0^{a}	2.1^{ab}	4.1^{a}	2.0^{ab}	1.7^{b}	4.6 **
NB	8.9^{ab}	7.6^{ab}	4.6^{ab}	9.6^{ab}	5.4^{ab}	10.0^{a}	5.0^{ab}	4.6^{b}	3.8 **
G+	7.6^{ab}	6.9^{ab}	4.3^{ab}	8.8^{a}	5.3^{ab}	9.1^{a}	4.5^{ab}	4.1^{b}	3.9 **
G-	5.6^{ab}	4.9^{ab}	3.1^{b}	6.3^{ab}	3.7^{ab}	6.9^{a}	3.2^{b}	3.0^{b}	4.1 **
F/B	0.5	0.5	0.4	0.5	0.4	0.5	0.5	0.5	0.8

Appendix 4.3

The effects of N addition (N), illumination (L), warming (W) and their interactions on the biomass of each functional microbial group and the soil microbial structure. F values are given as the results of the three-way ANOVA analyses. See **Appendix 4.1** for the abbreviations of functional microbial groups and the soil microbial structure.

Drivers	PLFAtot	Btot	Ftot	AB	NB	G+	G-	F/B
N	0.3	0.3	0.2	0.1	0.3	0.4	0.2	0.2
L	26.5***	26.2***	27.1***	31.2***	24.0***	23.9***	24.2***	3.6
W	1.4	1.5	0.8	0.4	1.1	1.9	2.2	0.6
L:W	0.9	0.9	0.4	0.8	0.7	0.9	1.1	0.3
N:W	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2
L:N	0.0	0.1	0.0	0.1	0.0	0.0	0.1	1.2

Appendix 4.4



Relative abundance of the soil bacterial phyla in the different treatments: control (C), N addition (N), illumination (L), warming (W), warming + illumination (WL), warming + N addition (WN), illumination + N addition (LN), and warming + illumination + N addition (WLN).

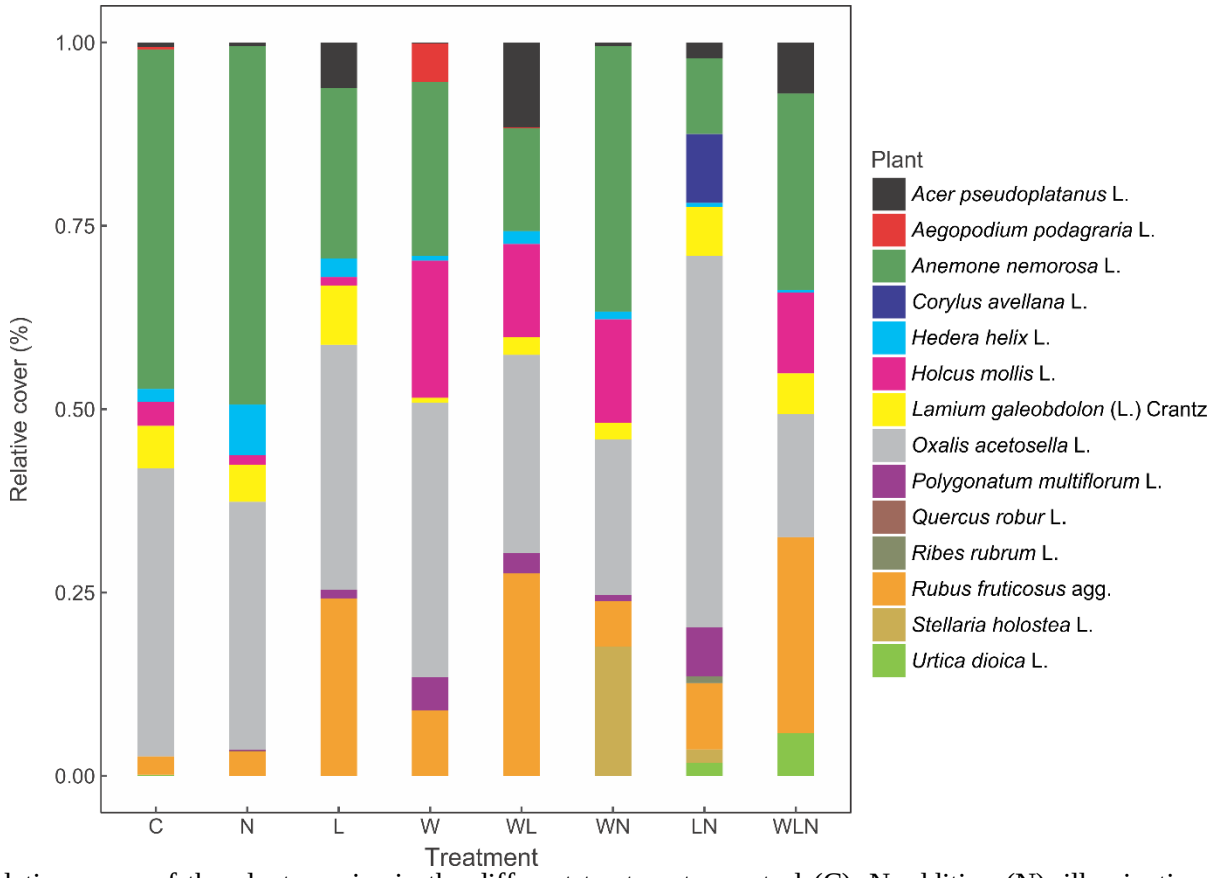
Appendix 4.5

Multivariate abundance testing of N addition (N), illumination (L), warming (W) and the two-way interactions for the beta diversity of the soil bacterial and the plant communities. Statistics for the forward model selection through comparison of nested models by likelihood ratio tests (LRTs)

Community	Step	Model	Residual df	Test statistic (LRTs)	*P* value
Soil bacteria	0	Intercept	38	NA	NA
	1	0 + N	37	5571.543	0.060
	2	1 + L	36	5578.111	0.016*
	3	2 + W	35	5642.737	0.002**
	4	3 + L:W	34	1754.798	0.010**
	5	4 + N:W	33	1474.774	0.052
	6	5 + L:N	32	2080.481	0.002**
Plant	0	Intercept	38	NA	NA
	1	0 + L	37	29.55706	0.026*

Indicates significant differences between the model and the model above ($P < 0.05$, ** $P < 0.01$). NA: not available because LRTs test for nested models.

Appendix 4.6



Relative cover of the plant species in the different treatments: control (C), N addition (N), illumination (L), warming (W), warming + illumination (WL), warming + N addition (WN), illumination + N addition (LN), and warming + illumination + N addition (WLN).

Chapter 5

Plant-soil feedbacks of forest understorey plants transplanted in nonlocal soils along a latitudinal gradient

After: Ma S, De Frenne P, Wasof S, Brunet J, Cousins SAO, Decocq G, Kolb A, Lemke I, Liira J, Naaf T, Orczewska A, Plue J, Wulf M and. Verheyen K. Plant-soil feedbacks of forest understorey plants transplanted in nonlocal soils along a latitudinal gradient. *Plant Biology*, resubmitted after minor revisions.

5.1 Abstract

Climate change is driving movements of many plants beyond, as well as within, their current distributional ranges. Even migrant plants moving within their current range may experience different plant-soil feedbacks (PSFs) because of divergent nonlocal biotic soil conditions. Yet, our understanding to what extent soil biotic conditions can affect the performance of within-range migrant plants is still very limited. We assessed the emergence and growth of migrant forest herbs (*Milium effusum* and *Stachys sylvatica*) using soils and seeds collected along a 1700 km latitudinal gradient across Europe. Soil biota were manipulated through four soil treatments, i.e., unsterilized control soil (PSF_{US}), sterilized soil (PSF_{S}), sterilized soil inoculated with unsterilized home soil (PSF_{S+HI}) and sterilized soil inoculated with unsterilized foreign soil (PSF_{S+FI}, expected to occur when both plants and soil biota track climate change).

Compared to PSF_{S}, PSF_{US} had negative effects on the growth but not emergence of both species, while PSF_{S+FI} only affected *S. sylvatica* across all seed provenances. When considering seeds' origins, seedling emergence and growth responses to nonlocal soils depended on soil biotic conditions. The home-away distance effect on seedling emergence differed among the four treatments, and significant responses to chemistry either disappeared (*M. effusum*) or changed (*S. sylvatica*) from PSF_{US} to PSF_{S}.

Soil biota emerges as an important driver of the estimated plant migration success. Our results of the effects of soil microorganisms on plant establishment provide relevant information for predictions of the distribution and dynamics of plant species in a changing climate.

5.2 Introduction

Climate change influences both plant and soil microbial communities in terrestrial ecosystems (Classen *et al.* 2015). Many studies have demonstrated that some plant species can track their climatic envelope in space (Thuiller, Lavorel, Araujo, Sykes & Prentice 2005; Klausmeyer & Shaw 2009; Leithead, Anand & Silva 2010; Engler *et al.* 2011; Fisichelli, Frelich & Reich 2014). Also within species' distributional ranges, plant movements occur, for instance, more southern populations may be more adapted to warmer temperatures and track the shifting isotherms towards the north. Such within-range shifts are often ignored, although it is likely that many species are exhibiting such behaviour (Davis & Shaw 2001; Berg *et al.* 2010; De Frenne *et al.* 2014). If plant populations move within their current distribution range, it is likely that interactions occur with multiple factors such as abiotic and biotic soil characteristics and novel interactions with other species within the ecosystem. For instance, abiotic soil characteristics (e.g., soil chemistry) can significantly affect plant movements (De Frenne *et al.* 2014). However, to which extent soil biota, both alone and together with soil chemistry, can affect within-species plant movements and plant performance of moving plants is still limited (reviewed in van der Putten, Macel & Visser 2010).

There is a growing consensus that plants and their associated soil microorganisms do not migrate at the same rate (Berg *et al.* 2010). Soil microorganisms are expected to be poorer dispersers and assumed to migrate at lower rates than plants (Berg *et al.* 2010; van der Putten 2012). Due to the differential dispersal rates and responses to climate change of plants and soil microorganisms, trophic interactions can be, at least temporarily, disrupted while novel interactions establish (van Grunsven *et al.* 2007; van der Putten, Macel & Visser 2010; Alexander, Diez & Levine 2015; Classen *et al.* 2015). It is not clear yet how the decoupling of plant and soil microbial community responses to climate change may affect understorey plant establishment in nonlocal sites (De Frenne *et al.* 2014; Classen *et al.* 2015).

The microorganisms that mostly determine plant-soil feedbacks are soil pathogens, symbionts, decomposers and herbivores. For plants, negative plant-soil feedbacks occur when soil enemies (i.e., pathogens) accumulate near a given individual and subsequently suppress the growth of conspecific juveniles. Negative plant-soil feedbacks play a dominant role in terrestrial ecosystems (Kulmatiski, Beard, Stevens & Cobbold 2008; Anacker, Klironomos, Maherali, Reinhart & Strauss 2014) and can be tested by comparing plant performance in conditioned and unconditioned or sterilized soil. When species migrate and colonize nonlocal (away) soils, the biotic and abiotic characteristics of away soils may differ from home soils. Potential negative plant-soil feedbacks from the home site may thus become less negative when plants establish in nonlocal soils due to less accumulated

amounts of soil pathogens (van Grunsven *et al.* 2007; Dostalek, Munzbergova, Kladivova & Macel 2016). Such home and away effects can be tested via the geographical distance between soil and seed samples, i.e., the latitudinal distance between seed and soil origins. Furthermore, soil biota plays a key role in the energy transfer and nutrient cycling via e.g., decomposition and mineralization (Bardgett & van der Putten 2014). Thus, abiotic characteristics (e.g., soil chemistry) and soil biota can interact in affecting the performance of migrant plants (Levine 2000). This abiotic effect, as well as the interaction with soil biota, can be tested via the ecological distance between local and nonlocal soil, e.g., the difference of soil chemistry between local and nonlocal soil, in conditioned and unconditioned or sterilized soil (reviewed in Kawecki & Ebert 2004). Investigating the soil biotic constraints and abiotic drivers is necessary for a more accurate prediction of future plant distributions under climate change (van der Putten, Macel & Visser 2010).

To simulate plant movements and growth responses to nonlocal soils, we set up a greenhouse experiment with soil and seeds of two forest understorey plant species, i.e., *Milium effusum* L. and *Stachys sylvatica* L., sampled in forests along a 1700 km long latitudinal gradient in northwestern Europe (France, Germany and Sweden). Current climate models predict that the climate in Stockholm in 2100 will become as in northern France now (IPCC 2014). We then developed virtual movement scenarios of plants and soil microorganisms by using soil sterilization and inoculation and fully crossed seeds and soils, i.e., sowing seeds from each site in soils from each site. This design allowed us to investigate migrants' responses to climate change within the current distribution range of the species. We monitored seedling emergence and growth to address the following three hypotheses: (1) as negative plant-soil feedbacks are prevalent in terrestrial ecosystems, we expected that the performance of the studied species was enhanced in sterilized soil compared to unsterilized and inoculated soils, regardless of whether plants grow in local or nonlocal soils; (2) when migrant plants grow in nonlocal soils, they may benefit from soil pathogen release (in sterilized soil) or, on the other hand, lose the advantages of local adaptation. These two contrary theories may result in better, worse or indifferent plant performance in nonlocal compared to plants grown in local soils; (3) migrants may also experience different abiotic conditions in nonlocal soils, for instance, due to soil chemistry. Soil microorganisms play key roles in regulating chemical soil conditions. When migrant plants grow in nonlocal soils with contrasting composition of soil microorganisms, i.e., presence vs. absence, the effects of chemical differences between local and nonlocal soils on plant performance may differ between the two scenarios of soil microbial community composition.

5.3 Materials and Methods

5.3.1 Study species

We selected *Milium effusum* L. and *Stachys sylvatica* L.as study species. More information on the two study species can be found in section 3.3.1.

5.3.2 Seed and soil sampling

We selected three regions along a 1700 km latitudinal gradient, corresponding to the northern, central and southern part of the distribution range of the study species in Europe (i.e., Central Sweden, Eastern Germany, Northern France) (**Fig. 2.1** and **Fig. 5.1a**). In each region, we sampled in two separate landscape windows of circa 5 × 5 km². In each window and for each study species, we sampled two forests (to achieve maximal generality, always one ancient woodland, forested since at least the oldest available land use maps, which is generally >200 years old, and one recent, post-agricultural woodland, generally afforested after the 1800s) (Hermy, Honnay, Firbank, Grashof-Bokdam & Lawesson 1999; Verheyen, Bossuyt, Honnay & Hermy 2003). Thus, for each species in each window, we obtained 6 seed and soil origins (3 regions × 2 forests). There were, in total, 24 sampling sites (6 origins × 2 windows × 2 species).

We collected seeds and soil in August 2015. To collect a sufficient number of seeds for the sowing experiment, we sampled at least 10 healthy plants per species and site at the moment of seed maturity. The selected sites were undisturbed and were at least 10 m away from the nearest forest edge. We also recorded the tree species composition and cover within a 5 × 5 m^2 range around each sampling site (see **Appendix 2.1**). The collected seeds were stored at room temperature until the start of the sowing experiment. In addition to seeds, we collected mineral topsoil (1.7 L per site) in the immediate vicinity (within a radius of 20 cm) of the sampled plants at 0-10 cm depth. All soil samples were sieved through a 2-mm mesh (the mesh was cleaned and sterilized by 75% ethanol between samples) to remove stones, macrolitter and macrofauna (e.g., earthworms, insects). Each soil had a specific origin and was not mixed with any others. Soil samples were stored at 4 ℃ until the start of the experiment. A subsample of each soil sample was taken to quantify chemical soil properties. For extractable ammonium (NH_4^+) and nitrate (NO_3^-), circa 10 g fresh soil was shaken in 20 ml 1 M KCL for 1 h. The solution was filtered, the concentration of NH_4^+ was determined by the salicylate-nitroprusside method on an auto-analyzer (AA3, Bran & Luebbe, Germany), and the NO_3^- concentration was measured using the same auto-analyzer after reduction of nitrate to nitrite in a Cu-Cd column followed by the reaction of nitrate with N-(1-napthyl) ethylenediamine dihydrochloride to produce a chromophore. The subsamples were dried at 40 ℃ for 48 h for the next analyses. More information about chemical soil measurements is given in the section 2.3.4 and

Appendix 5.1. In addition, we calculated the atmospheric N deposition for each sampling site as the sum of wet and dry depositions of oxidised (NO_y) and reduced (NH_x) N. More information about the data resource is listed in section 2.3.5 and **Appendix 2.4**.

5.3.3 Experimental design and setup

We applied four experimental treatments: (1) unsterilized soil (PSF_{US}), which still contained the home (original) soil microbes; (2) sterilized soil (PSF_S), in which soil microbes were eliminated by Gamma irradiation with a dose range of 28.4-29.9 kGy (which was specifically used to balance the successful mortality of as much soil biota as possible but at the same time not affecting soil N cycling too much) (McNamara, Black, Beresford & Parekh 2003; Buchan, Moeskops, Ameloot, De Neve & Sleutel 2012); (3) home-inoculated soil (PSF_{S+HI}), in which sterilized soils were used as main substrate and then inoculated with their own unsterilized home soil, e.g., French sterilized soils were inoculated with French unsterilized inoculum; and (4) foreign-inoculated soil (PSF_{S+FI}), in which unsterilized foreign soil inoculum from a southern region was transplanted into sterilized soil from a northern region (9% mass based) to mimic the scenario of soil microbes change when tracking the isotherm shifts to higher latitudes (**Fig. 5.1b**). In other words, soil from France was inoculated into sterilized German and Swedish soil (91 mass%), and from Germany to sterilized Swedish soil. To avoid any side effects between ancient and recent forests, we only inoculated sterilized soil of ancient forests with unsterilized soil from ancient forests (similar for recent forests).

To investigate the potential future performance of the study species within their current range, we developed virtual movement scenarios simulating sowing seeds from all sampling sites in soil from each sampling site. To mimic natural seed dispersal and avoid side effects of disinfection, seeds were not disinfected.

We used trays with 72 cone-shaped pots (pot size: 16 cm^2 surface area and 4.5 cm depth). Before inoculation, soils from each origin were mixed homogenously. A cylindrical lid (3.3 cm diameter × 1.7 cm height) was attached to the bottom of each pot to avoid cross contamination among the pots while watering them but which still allowed drainage of surplus water. Each pot contained 13 g soil and five seeds. For inoculated soil, 11.8 g sterilized soil was compiled with 1.2 g unsterilized soil. After inoculation, the sterilized (11.8 g) and unsterilized soil (1.2 g) were also mixed well to make sure that the inoculum was evenly distributed in each pot. For one recent forest from Germany and one recent forest from Sweden, only four seeds were sown per pot due to low seed availability in the sampled forest sites. For each plant species and soil treatment, we had 72 pots (6 soil origins × 6 seed origins × 2 windows). We used three replicate trays of this transplantation setup, and the

locations of each seed and soil combination were randomized within trays. In total, 1296 pots (72 × 3 replicates × 3 soil treatments × 2 plant species) were used in the experiment.

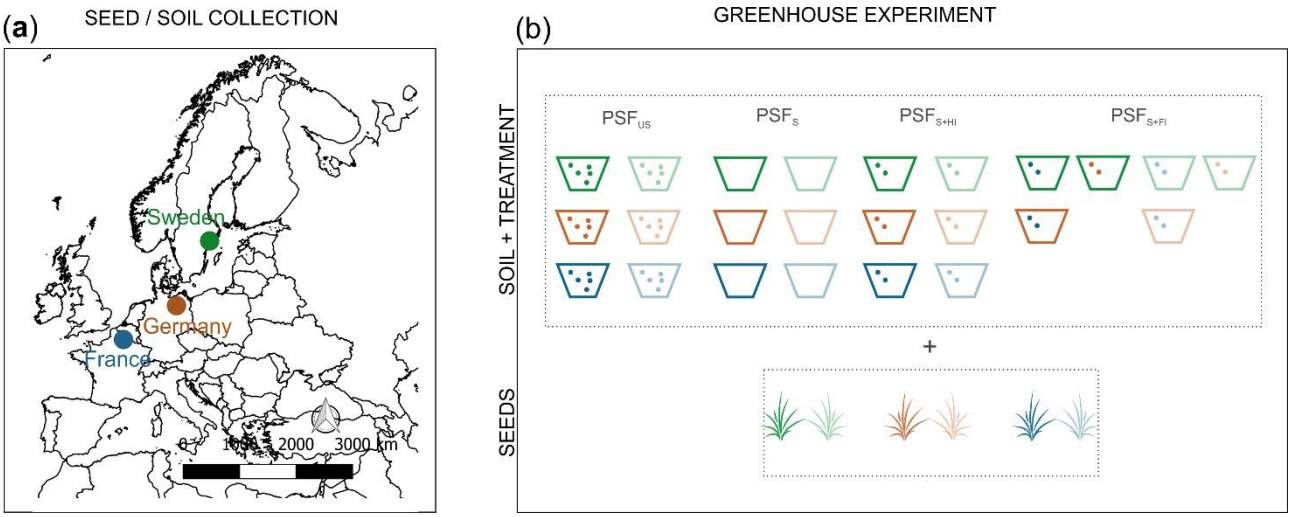


Fig. 5.1 Design of the seed and soil sampling across Europe and the greenhouse experiment. The three regions of seed and soil sampling (a). The design of the greenhouse experiment. Six seed origins from the three regions (represented by the different colours; each colour contains one bright and one light hue to indicate two forest types in each region) were sown into unsterilized control soil (PSF_{US}), sterilized soil (PSF_{S}), home-inoculated soil (PSF_{S+HI}) and foreign-inoculated soil (PSF_{S+FI}) from six soil origins (b). Dots in the pots representing soil microbes and their provenance (also represented by the different colours and hues). We applied this experimental setup for each of the two landscape windows per region.

After filling the pots on 12 November 2015, we watered the trays thoroughly with distilled water. The trays were incubated for eight weeks in a cold room at 5 ℃ for cold seed stratification and then moved to a greenhouse on 4 January 2016. The greenhouse temperature was around 10 ℃ at night and 20 ℃ during the day; the average air humidity was 61%. We equally sprayed distilled water every other day to all trays throughout the experiment and shifted the position of the trays randomly every week. We removed any plants other than the study species germinating from the seed bank.

5.3.4 Plant measurements

We recorded seedling emergence weekly and terminated the greenhouse experiment at the beginning of May (*M. effusum*) and June (*S. sylvatica*) 2016. We calculated the seedling emergence percentage by dividing the number of emerged seedlings by the number of seeds sown in each pot. The mean emergence time (MET) was calculated as (Deines, Rosentreter, Eldridge & Serpe 2007; De Frenne *et al.* 2012):

$$\mathrm{MET} = \sum_{i=1}^{N} n_i t_i \,/N$$

Where n_i is the number of emerged seedlings within consecutive intervals of time, t_i is the time between the beginning of the experiment and the end of a particular interval (in weeks), and N is the total number of emerged seedlings at the end of the experiment.

In *S. sylvatica*, when multiple seeds germinated in one pot, we often observed that seedlings were small below the canopy of the tallest seedling. Therefore, we decided to use the tallest seedling to measure the following functional traits that affect plant growth, survival and reproduction in both *M. effusum* and *S. sylvatica*: plant height, specific leaf area, shoot biomass, root biomass, and the root:shoot ratio. We measured plant height from the base of the seedling to the top of the longest, fully expanded and healthy leaf. The specific leaf area of each selected seedling was determined for the largest and healthy leaf by using a Li-Cor Portable Area Meter Li-3000 (Li-Cor Biosciences, NE, USA). We weighed the shoot biomass and the root biomass (after washing off the soil from the roots using a 1 mm mesh) separately after drying the harvested seedlings for three days at 50 ℃. The root:shoot ratio of each seedling was calculated as the ratio of dry root biomass to dry shoot biomass.

5.3.5 Data analysis

We calculated the mean plant performance in each treatment including emergence and growth (mean emergence time and plant functional traits (**Appendix 5.2**). The three hypotheses were tested separately. For the first hypothesis, the negative PSF was tested under two situations: net PSF using two treatments, i.e., PSF_{US} (unsterilized home soil), and PSF_S (sterilized soil); net PSF in the context of soil biota change using three treatments, i.e., PSF_S, PSF_{S+HI} and PSF_{S+FI}. To test the second hypothesis, we calculated home-away distance, i.e., latitudinal differences between soil and seed origins, and tested its effects on plant performance in each treatment. In the treatments PSF_{S+HI} and PSF_{S+FI}, the latitudinal differences were calculated based on the soil origins of the substrate part. Seeds sown in their home soil had a home-away distance of zero; positive home-away distances denote that seeds experienced northward movements while negative distances denote southward movements. The home-away distance is a common metric in common garden and transplant studies across latitudes (De Frenne *et al.* 2013a). To test the third hypothesis, i.e., whether the effects of soil chemistry differences between local and nonlocal soil on PSF depended on the presence of soil biota, we calculated eleven different 'soil chemistry difference' variables (10 measured chemical soil properties as well as the estimated atmospheric N deposition) as the difference between the soil chemistry of the site of soil sampling and the site of seed sampling for each seed-soil origin combination (i.e., exactly as for the home-away distance) in PSF_{US} and PSF_S. We tested the effects of each variable on plant performance in PSF_{US} and PSF_S separately. We did not calculate these differences for PSF_{S+HI} and PSF_{S+FI} because they consisted of a small portion of soil biota and two

different soil origins (only in PSF_{S+FI}), i.e., the sterilized 91% mass and unsterilized 9% mass. Positive values of the soil chemical differences reflect that seeds were sown in soil with higher nutrient concentrations or in soil collected from sites with higher N deposition.

All tests were analysed with (1) generalized linear mixed-effects models (*glmer* function, Binomial error distribution) for emergence and (2) linear mixed-effects models (*lmer* function, Gaussian error distributions) for growth, using the *lme4*-package (Bates, Machler, Bolker & Walker 2015) in R 3.3.3 (R Core Team 2017). To address nested data structure, we used two random effects: *tray* and *seed origin* (*window* nested within *region*). Mean emergence time and functional traits were log10- or sqrt-transformed before the analyses to meet the normality assumption of the statistical tests. To measure the effects of each predict variable on each response variable, we constructed a model with a response variable and a predict variable, and compared this to a blank (intercept-only) model with the same response variable using likelihood ratio test. We did not include interactions in the models because of the complexity of this multifactor experiment.

5.4 Results

5.4.1 Negative plant-soil feedback

Negative plant-soil feedbacks were found for seedling growth, but no such effect was found for seedling emergence in both plant species. Specifically, in the comparisons of unsterilized to sterilized soil, the mean emergence time and the aboveground performance, i.e., seedling height, specific leaf area and shoot biomass of the two species were significantly increased in sterilized soil (**Fig. 5.2** and **Fig. 5.3**). For root-related functional traits, only *S. sylvatica* showed a significant increase in root biomass, while both species showed a substantial decrease in root: shoot ratio (**Fig. 5.2** and **Fig. 5.3**). In the comparisons among sterilized, home-inoculated and foreign-inoculated soil, the two plant species showed different plant-soil feedbacks when facing soil biota change (**Table 5.1**). In *M. effusum*, plant performance showed no differences between the three treatments. In *S. sylvatica*, foreign-inoculated soil had significantly negative effects on plant height, shoot biomass and root biomass, while a positive effect on root: shoot ratio when compared with PSF_S. There was no plant performance difference between home-inoculated and foreign-inoculated soil.

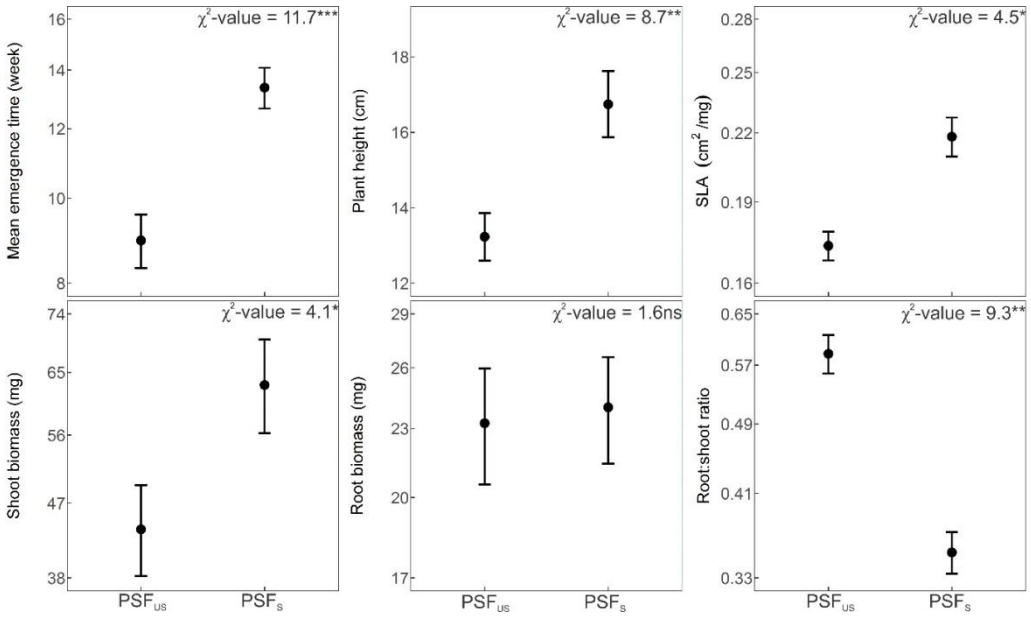


Fig. 5.2 Plant-soil feedback (PSF) assessment based on the comparison between treatments PSF_{US} (unsterilized soil) and PSF_S (sterilized soil) in terms of mean emergence time and functional traits (plant height, SLA, shoot biomass, root biomass and root:shoot ratio) in *Milium effusum*. Points indicate the mean values of emergence and growth traits. Error bars indicate standard error. χ^2-values and asterisks represented significance were extracted from the likelihood ratio tests of linear mixed-effects models. Df is 1. Note the data transformation for response variables.

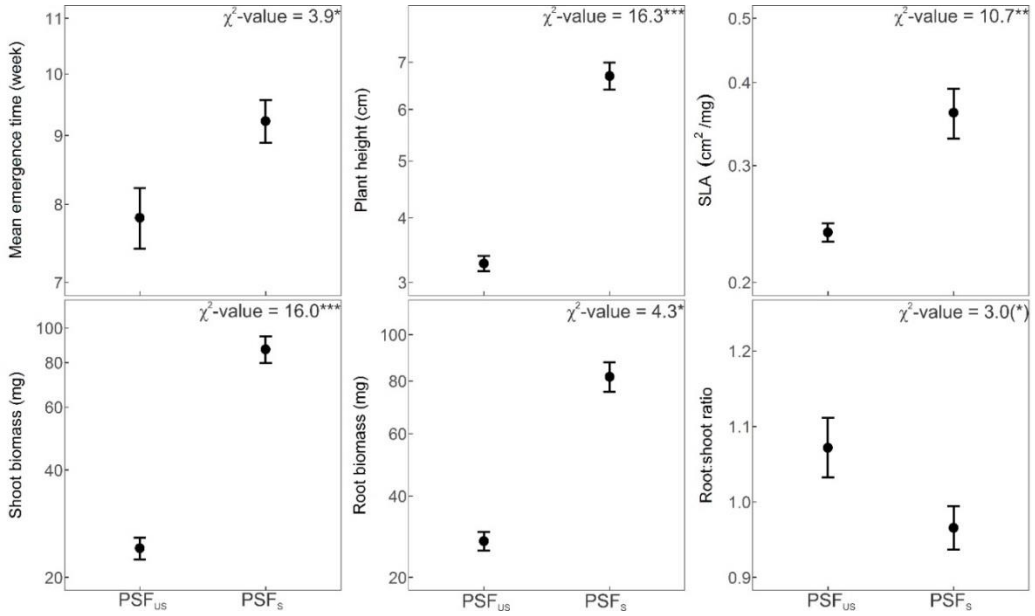


Fig. 5.3 Plant-soil feedback (PSF) assessment based on the comparison between treatments unsterilized soil (PSF_{US}) and sterilized soil (PSF_S) in terms of mean emergence time and functional traits (plant height, SLA, shoot biomass, root biomass and root:shoot ratio) in *Stachys sylvatica*. Points indicate the mean values of emergence and growth traits. Error bars indicate standard error. χ^2-values and asterisks represented significance were extracted from the likelihood ratio tests of linear mixed-effects models. Df is 1. Note the data transformation for response variables.

Table 5.1 The mean (with standard error) seedling emergence and growth of *Milium effusum* and *Stachys sylvatica* and likelihood ratio tests for the effects of soil microbe change on plant-soil feedback (PSF) of the two plant species based on (generalized) linear mixed-effects models. The tests were conducted among sterilized soil (PSF_S), home-inoculated soil (PSF_{S+HI}) and foreign-inoculated soil (PSF_{S+FI}). χ^2-values and asterisks represented significance were extracted from the likelihood ratio tests of linear mixed-effects models. Small letters indicate the results of multiple comparisons based on the models

	PSF_S	PSF_{S+HI}	PSF_{S+FI}	Df	χ^2-value
		Milium effusum			
Emergence (%) ¶	9.4 (1.2)	7.2 (1.0)	6.4 (0.9)	2	4.2ns
MET (week) §	13.4 (0.7)	12.5 (0.7)	12.8 (0.7)	2	2.5ns
Height (cm)	16.7 (0.9)	14.5 (0.9)	16.5 (1.1)	2	2.5ns
SLA (cm^2 mg^{-1}) §	0.22 (0.01)	0.23 (0.01)	0.23 (0.02)	2	1.3ns
Shoot biomass (mg) £	63.1 (6.9)	52.9 (7.2)	74.6 (11.1)	2	1.2ns
Root biomass (mg) §	24.0 (2.6)	20.2 (2.6)	27.3 (4.1)	2	0.3ns
Root:shoot ratio §	0.35 (0.02)	0.37 (0.02)	0.34 (0.01)	2	0.7ns
Stachys sylvatica					
Emergence (%) ¶	20.1 (1.5)	13.1 (1.2)	13.0 (1.4)	2	4.8ns
MET (week) §	9.2 (0.3)	9.5 (0.5)	9.3 (0.5)	2	1.5ns
Height (cm) £	6.7 (0.3)a	5.4 (0.2)b	5.6 (0.3)b	2	6.5*
SLA (cm^2 mg^{-1}) §	0.4 (0.03)	0.4 (0.04)	0.4 (0.04)	2	4.1ns
Shoot biomass (mg) §	87.1 (7.4)a	34 (4.2)b	39.1 (4.8)b	2	10.6**
Root biomass (mg) §	81.7 (6.1)a	43.5 (5.4)b	45.9 (5.0)b	2	9.4**
Root:shoot ratio	1.0 (0.03)b	1.1 (0.04)a	1.1 (0.04)a	2	10.5**

5.4.2 The effect of home-away distance

The home-away distance mainly affected the seedling emergence of *M. effusum* in unsterilized and sterilized soil, and the seedling emergence of *S. sylvatica* in foreign-inoculated soil (**Fig. 5.4** and **Appendix 5.3**). In unsterilized soil, the emergence of *M. effusum* increased, while the mean emergence time decreased with the home-away distance, with higher seedling emergence and shorter mean emergence time when seeds were sown in Swedish soils. In sterilized soil, the emergence of *M. effusum* and root: shoot ratio of *S. sylvatica* decreased with the home-away distance, with higher emergence and root: shoot ratio, respectively, when seeds were sown in French soils. In home-inoculated soil, specific leaf area of *M. effusum* and plant height of *S. sylvatica* increased when grown in Swedish soils. In foreign-inoculated soil, only the emergence of *S. sylvatica* showed a negative response to the home-away distance, with higher emergence in German soils than that in Swedish soils.

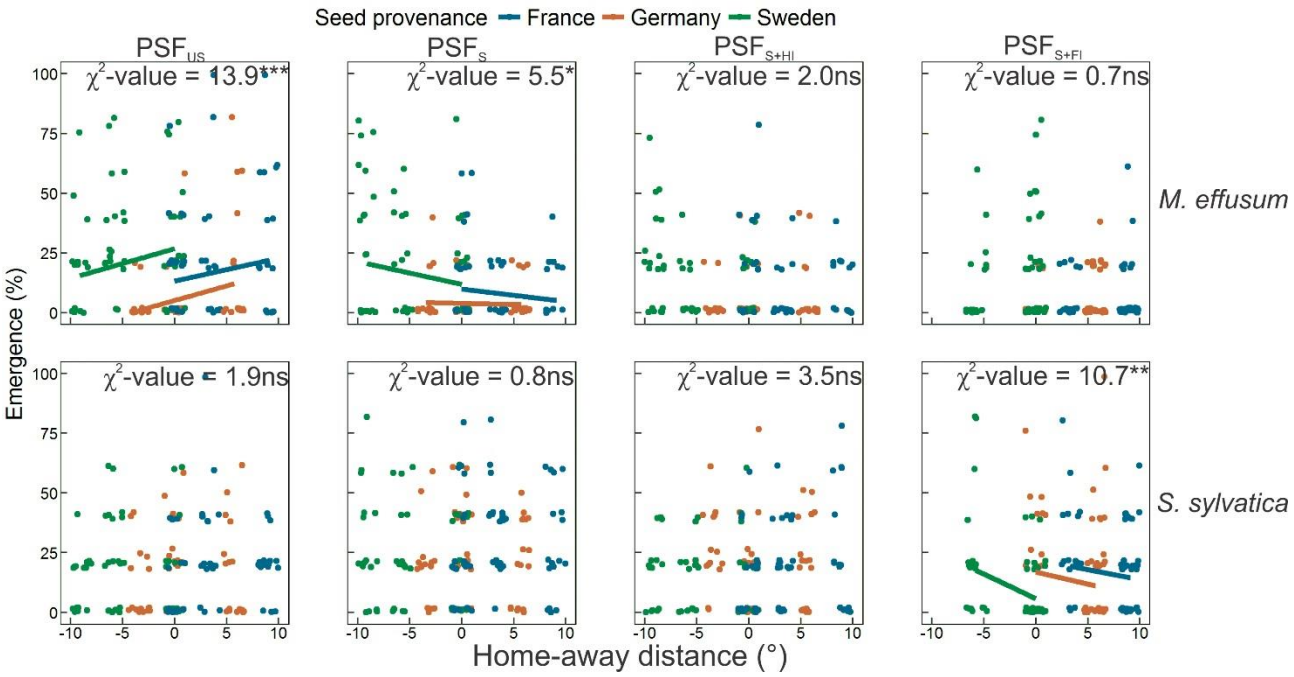


Fig. 5.4 The effects of local adaptation, displayed as the home-away distance (in degrees latitude), on seedling emergence of *Milium effusum* and *Stachys sylvatica* in the four experimental treatments: unsterilized soil (PSF_{US}), sterilized soil (PSF_{S}), home-inoculated soil (PSF_{S+HI}) and foreign-inoculated soil (PSF_{S+FI}). Positive home-away distances represent southern seeds planted into northern soils, negative distances represent northern seeds planted into southern soils. Lines were drawn when home-away distance significantly affected seedling emergence, and the lines were coloured based on the seed provenances.

5.4.3 Abiotic soil chemistry differences

The differences in soil chemistry between the sites of soil and seed origin affected seedling emergence and growth of both species, but the effects differed between unsterilized and sterilized soil (**Table 5.2**). In unsterilized soil, the seedling emergence, height, shoot and root biomass of both species increased when plants were grown in soils containing a rich supply of soil C and N resources. In sterilized soil, most significant effects on both species that were observed in unsterilized soil disappeared. Conversely, the root: shoot ratio of both species, for instance, was significantly affected by soil chemistry in sterilized but not in unsterilized soil.

Table 5.2 The effects of soil chemistry differences between the sites of soil and seed collection (a reflection of changes in chemical conditions between home and foreign soil) on plant performance in unsterilized (PSF_{US}) and sterilized (PSF_S) soil. Values and asterisk symbols are χ^2-values and significances, respectively, from the likelihood ratio tests of the (generalized) linear mixed-effects models

Soil chemistry difference	*Milium effusum*							*Stachys sylvatica*						
	Emergence (%) ¶	MET (week)	Height (cm)	SLA(cm^2 mg^{-1})	Shoot biomass	Root Biomass	Root: shoot	Emergence (%) ¶	MET (week)	Height (cm)	SLA(cm^2 mg^{-1})	Shoot biomass	Root Biomass	Root: shoot
PSF_{US}														
NH_4^+	↑9.9**	2.4ns	1.2ns	↓4.7*	1.2ns	1.8ns	0.1ns	0.1ns	0.0ns	0.0ns	0.8ns	0.9ns	1.2ns	0.4ns
NO_3^-	↑ 14.0 ***	1.5ns	↑19.3*	0.8ns	↑20.0**	↑9.4** §	0.5ns	↑12.9***	↑4.1*	↑6.1*	↓7.4**	↑7.5**	↑6.5*	0.5ns
P	↑19.8***	0.9ns	3.1ns	2.7ns	↑6.7** £	↑5.3* £	1.3ns	0.0ns	0.1ns	0.6ns	3.5ns £	↓4.9*	2.3ns	1.5ns
K	↑21.0***	0.2ns	↑13.1*	0.1ns	↑17.8**	↑16.5**	0.1ns	↑18.3***	0.2ns	1.5ns	2.5ns	2.9ns	0.1ns	1.0ns
Ca	↓5.5*	0.9ns	↑4.3*	↑4.0*	↑5.5*	2.5ns £	3.6ns	↑17.0***	1.0ns	↑9.4**	0.5ns	↑4.0*	3.5ns	1.3ns
Mg	↑14.9***	0.4ns	↑18.7*	0.0ns	↑22.6**	↑20.2**	0.6ns	1.2ns	0.1ns	1.8ns	0.1ns	1.4ns	2.2ns	0.2ns
pH	↓6.9**	0.6ns	↑4.6*	↑5.5* §	2.4ns §	0.6ns §	0.6ns	0.8ns	0.3ns	↑3.9*	2.0ns	0.1ns	0.4ns	0.1ns
C	↑20.9***	1.6ns	↑4.2*	1.3ns	↑8.2** £	↑6.5* £	0.3ns	↑11.6***	↑4.0*	↑11.2*	3.5ns £	↑9.0**	↑11.2**	1.4ns
N	↑21.7***	1.6ns	↑5.6*	0.9ns	↑10.2**	↑8.3***	0.4ns	↑10.9***	3.7ns	↑9.9**	↓3.9* £	↑9.1**	↑11.6**	n1.5s
C/N	0.0ns	0.8ns	↓5.0*	↓9.9**	↓4.3* £	2.6ns £	0.0ns	↑10.6**	1.1ns	↑9.9**	0.2ns	3.4ns	3.8ns £	0.7ns
Ndep	0.0ns	0.7ns	2.8ns	0.3ns	↓4.8*	3.5ns £	0.6ns	1.3ns	0.1ns	0.2ns	0.0ns £	0.0ns	0.4ns £	0.1ns
PSF_S														
NH_4^+	0.8ns	0.7ns	1.3ns	0.3ns	0.7ns	0.6ns	↓4.1*	0.3ns	0.0ns	0.6ns	0.7ns	0.2ns	0.4ns	0.9ns
NO_3^-	1.0ns	0.9ns	0.1ns	2.8ns	2.1ns £	2.3ns £	0.4ns	0.3ns	0.5ns	↑5.3*	2.1ns	1.3ns	0.0ns	↓12.3
P	2.9ns	0.3ns	2.9ns	0.0ns	1.2ns £	0.8ns £	↓4.9*	0.1ns	0.0ns	↓4.7*	1.8ns §	0.6ns	0.1ns	↑4.0*
K	0.0ns	0.0ns	0.5ns	0.8ns	0.0ns £	1.6ns £	↓4.5*	↓6.7**	0.0ns	↓5.3*	2.1ns §	↓9.0**	↓4.6**	2.7ns
Ca	0.0ns	0.9ns	0.0ns	1.0ns §	0.4ns £	0.0ns £	2.4ns	↑15.5***	1.0ns	↑6.5*	0.5ns §	↑4.3*	3.8ns	0.3ns
Mg	1.5ns	1.3ns	0.0ns	1.8ns	0.5ns £	1.8ns £	1.0ns	1.2ns	0.4ns	3.4ns	0.2ns §	1.2ns	2.2ns	0.0ns
pH	0.1ns	1.1ns	0.2ns	0.9ns §	0.8ns £	0.1ns £	2.6ns	↑10.3**	1.7ns	0.0ns	0.1ns §	0.1ns	0.1ns	0.5ns
C	1.4ns	0.3ns	0.8ns	0.3ns	0.2ns £	2.0ns £	↓5.2*	2.1ns	1.0ns	↑4.5*	2.1ns §	0.3ns	0.1ns	↓11.4
N	1.2ns	0.1ns	0.7ns	0.5ns £	0.1ns £	1.9ns £	↓4.9*	1.1ns	1.1ns	↑4.1*	2.7ns §	0.4ns	0.1ns	↓12.6
C/N	2.8ns	0.4ns	0.0ns	0.1ns §	0.0ns £	0.7ns £	1.0ns	↑12.5**	2.0ns	1.1ns	0.6ns §	0.5ns	0.3ns	0.2ns
Ndep	0.0ns	0.3ns	0.5ns	0.1ns £	0.0ns £	0.2ns £	0.0ns	↑4.9*	0.4ns	0.0ns	0.8ns §	1.6ns	1.4ns	0.5ns

The direction of the effect is displayed as an arrow: ↑ indicates higher values in higher soil chemistry differences between the sites of soil and seed collection, and ↓ lower values in higher soil chemistry differences between the sites of soil and seed collection. Df is 1.

5.5 Discussion

Efforts to identify the effects of soil biota on plants have underpinned the importance of understanding the effect of soil biota on plant movements (van der Putten, Macel & Visser 2010; van der Heijden, de Bruin, Luckerhoff, van Logtestijn & Schlaeppi 2016). Yet, the effect of soil microbes on plant regeneration is hard to assess in the field because of the potential interaction with other biotic and abiotic factors (van der Putten, Macel & Visser 2010; Walck, Hidayati, Dixon, Thompson & Poschlod 2011; Bellard, Bertelsmeier, Leadley, Thuiller & Courchamp 2012). Our greenhouse experiment allowed us to focus on the effects of soil microbes on seedling emergence and growth using sterilized and inoculated soils, and virtual movement scenarios (assessed by means of the home-away distance) while taking abiotic soil chemical differences into account.

5.5.1 Negative plant soil-feedbacks

Seedling growth but not seedling emergence of the two studied species was improved by killing-off soil microorganisms via sterilization. Our first hypothesis about negative plant-soil feedbacks was thus partly supported. Across all sites of soil and seed collection, seedlings of both species experienced a longer emergence time and grew better in sterilized soil compared to unsterilized soil (**Fig. 5.2** and **Fig. 5.3**). We found taller seedlings with a higher shoot biomass and higher specific leaf area in sterilized soil. The lower growth in unsterilized soil may suggest that soil microorganisms negatively affect plants, which appears to be the predominant situation in nature (Kulmatiski, Beard, Stevens & Cobbold 2008; Anacker, Klironomos, Maherali, Reinhart & Strauss 2014), driven by soil enemies such as pathogens (Reinhart & Callaway 2006). This is consistent with previous findings of negative plant-soil feedbacks in other forest understorey species (Smith & Reynolds 2015). Our observed negative plant-soil feedbacks underpin the likely mechanism of co-existence of multiple plant species in forest ecosystems and imply potential opportunities for invasive plant species to establish successfully in nonlocal sites, which might be a threat to maintaining forest plant species diversity and structure (Bonanomi, Giannino & Mazzoleni 2005; Smith & Reynolds 2015; Gioria & Pysek 2016).

Though the dose used in Gamma irradiation was carefully selected to minimize the side effects of Gamma irradiation on soil chemistry, we did not examine microbial cell lysis, which may result in increased immobilized nutrients. However, significant soil nutrient change caused by Gamma irradiation on plant-soil feedbacks can most likely be eliminated: when sterilized soils were inoculated with 9% of home soil, most plant traits in home-inoculated soil showed similar performance with that in unsterilized soil, particularly in *S. sylvatica* (**Appendix 5.4** and **Appendix 5.5**). This indicates that plant performance was likely not affected by sterilization due to a flush in

nutrients, but by the occurrence of soil microorganisms. Additionally, despite the soil and seeds being sampled from ancient and recent forests (land-use history), plant performance was not affected by land-use history (except the emergence of *S. sylvatica*) (**Appendix 5.6**). This is consistent with our previous study that there is no difference in terms of soil microbial community composition, bacterial diversity and composition between the two types of forests (**Chapter 2** and **Chapter 3**).

Negative plant-soil feedbacks in the context of soil biota change (foreign-inoculated soil) were mainly found in *S. sylvatica*. This implies that if soil microorganisms and host plant species disperse together to higher latitudes, the negative effects on the growth of the dispersed plants can persist. When soil microorganisms track the movements of plants, there is often a lag time between the arrival of new soil biota and ecological effects (Schwartz *et al.* 2006). In our study, the effect of southern soil microorganisms inoculated in sterilized northern soil probably depended on the sensitivity of the plant species to soil microorganisms (van der Heijden, Bardgett & van Straalen 2008a). *Milium effusum* seemed to be less sensitive to the inoculants than *S. sylvatica*, showing no growth differences between the three experimental treatments (**Table 5.1**). *Milium effusum* in Sweden experienced a much higher abundance of soil microorganisms than that of *S. sylvatica* (**Appendix 5.7**), indicating more intensive competition for nutrients between microorganisms and plants (Kuster, Wilkinson, Hill, Jones & Bardgett 2016). Therefore, a sharp decrease in microbial abundance in inoculated soil (9%) may not trigger negative effects of inoculated soil microorganisms on plant performance. This was also confirmed in the home-away distance effects, where no response of *M. effusum* was observed in home-inoculated or foreign-inoculated soil (**Fig. 5.4**).

In this study, we treated soil microorganisms as an integral community and disentangled their effects on plant performance. Further studies should explore whether different microbial communities (e.g., fungi vs. bacteria), and communities with different richness or abundances have a contrasting influence on plant performance. The analysis of phospholipid fatty acids and/or high throughput sequencing of bacterial or fungal DNA could be highly valuable in this respect (Ma *et al.* 2018).

5.5.2 Home-away distance

Compared to home soil, seedlings established in away soil may benefit from soil pathogen-release (in sterilized soil) but may also lose the advantages of local adaptation. These two contrary theories resulted in no differences in terms of seedling growth, but significant differences in seedling emergence for the two studied plant species (the second hypothesis was supported). Seedling

emergence, the first critical step in plant establishment and migration, was affected by the combination of soil and seeds' origins, i.e., home-away distance, but the effect differed among the four experimental treatments and between the two plant species.

In unsterilized soil, the seedling emergence of *M. effusum* was higher when sown in northern soils, while *S. sylvatica* showed no response to the home-away distance. The higher emergence in northern soils may be attributed to differences in seed provenances and soil microbial conditions (Grassein, Lavorel & Till-Bottraud 2014). Swedish seeds showed an overall higher germination capacity in unsterilized soil (**Fig. 5.4**). However, we did not test the variation of seed provenances to avoid statistical complexity. Whether the better germination capacity is associated with mother-plant genotypes or local adaptation needs more exploration. Yet, we observed significant soil origin effects on plant performance (**Appendix 5.8**). There was a substantial regional variation in terms of soil microbial biomass (**Appendix 5.7**) and bacterial community composition **Appendix 5.9**). The abundance of soil microorganisms in German and Swedish soil was higher than that in French soil, especially in *M. effusum*. Differences in temperatures between regions cause divergent assembly in soil microbial communities (Liang *et al.* 2015). In warmer environments, soil pathogens are expected to be more prevalent than in colder environments (van der Putten, Macel & Visser 2010; Roos, Hopkins, Kvarnheden & Dixelius 2011). In addition, the abundance of mycorrhizal fungi decreases with nitrogen deposition (van Diepen, Lilleskov, Pregitzer & Miller 2010; Chen *et al.* 2017), and the southern sites experienced a much higher nitrogen deposition load than the Swedish sites (France: 12.09 kg ha^{-1}, Germany: 15.10 kg ha^{-1} vs. Sweden: 5.28 kg ha^{-1}). Thus, northern, Swedish soil may have contained less soil pathogens and more mycorrhizal fungi, which may have enhanced seedling emergence. Nonetheless, more data on the community composition and functioning of these microbial groups between regions are needed.

In sterilized soil, the seedling emergence of *M. effusum* was higher in the soil from France, opposite to the result in unsterilized soil. Release from soil-borne pathogens from the home range or interactions with novel soil mutualists in the new environment have been found to help explain the success of invasive plants (Reinhart & Callaway 2006). In the soil collected from France, soil pathogens most probably had a stronger effect on the plant-soil feedback than mutualists, which resulted in higher seedling emergence in sterilized soil. When foreign soil microbes were inoculated, *S. sylvatica* but not *M. effusum* increased its emergence in southern soil. The different patterns between the two species indicate that there is an interspecific variation in the sensitivity of plant species to soil biotic change. Total soil microbial biomass was higher in *M. effusum* (mainly due to the patterns in Swedish soils) when compared to *S. sylvatica* (**Appendix 5.7**). The relatively low

microbial differences between soil and seeds' origins did not cause any performance change in *S. sylvatica* (**Appendix 5.10**).

5.5.3 Soil chemistry and the presence and absence of soil microorganisms

Except biotic effects, we also observed mainly positive soil chemistry effects on seedling growth when grown in soils with higher energy resources (e.g., soil C and nutrients) and the presence of soil microorganisms but most of these positive effects disappeared when soil microorganisms were absent (the third hypothesis was supported). In unsterilized soil, the soil chemistry differences between the sites of soil and seed collection affected the growth of the studied species in spite of the observed overall lower seedling growth in the unsterilized soil than the sterilized soil. It is likely that seeds sown in nonlocal soils and with higher soil nutrients performed better, showing higher seedling height and aboveground biomass in unsterilized soil, while these effects changed when soil microorganisms were removed, i.e., in sterilized soil. In addition to the pathogen-release theory (see above), priming effects can also contribute to explain the observed different responses between the soil with and without soil microorganisms (Murphy, Baggs, Morley, Wall & Paterson 2017). The higher C and N concentrations in the northern soils may have increased the activity of soil microorganisms and improved the degradation of soil organic matter, resulting in sufficient nutrient uptake by plant roots (Chen *et al.* 2014; Michalet & Pugnaire 2016). This is a reciprocal sowing experiment of *M. effusum* individuals collected in Belgium, Poland and Sweden found enhanced growth in soils that were richer in nutrients (De Frenne *et al.* 2014). Despite the different seed and soil provenances used in the study of De Frenne *et al.* (2014) and in the present study, both highlight that species moving north may encounter favourable soil conditions that promote plant establishment. Most importantly, our study underpins the necessity to consider soil microorganisms when studying plant movements as a result of climate change.

5.6 Conclusions

Our results emphasize the important role of soil microorganisms as mediator of plant regeneration from seed. Release from soil microorganisms facilitated the establishment of the plants, corresponding to the prevalent negative plant-soil feedbacks in terrestrial ecosystems. Seedling emergence of *M. effusum* and *S. sylvatica* was different in home and away soil, and these effects differed substantially between both plant species and the four soil treatments. Migrants established better in the soil containing higher energy resources, but the enhanced establishment depended on the presence of soil biota. Given the observed role of soil biotic control for plant-soil feedbacks during plant movements, we suggest to better take into account soil microbial composition and dynamics when predicting plant species distribution ranges in the face of climate change.

Appendix 5.1

The mean values (with standard error) and multiple comparisons of soil chemistry in different regions (bold texts) and the mean values of soil chemistry in ancient and recent forest soils (land-use history) within each region. The mean values of each region were calculated from four soil samples (two ancient and two recent forests). ANOVA and multiple comparisons (results were indicated by small letters) were used for testing the significance of regional differences (Kruskal and Dunn were used when data were not normal distributed). Significant differences were shown in red. The mean values of ancient and recent forest soils in each region were calculated from two soil samples

Region	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})	Olsen P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Al (mg kg^{-1})	pH (H_2O)	C (%)	N (%)	C/N	Ndep (kg ha^{-1})
						Milium effusum						
France	**14.7 (5.5)a**	**19.8 (7.2)a**	**21.1 (5.7)a**	**140.9 (34.4)a**	**14777.4 (8130.1)a**	**146.7 (28.1)a**	**145.1 (74.8)a**	**6.1 (0.7)a**	**5.1 (0.8)b**	**0.4 (0)a**	**13.5 (0.8)a**	**12.1 (1.2)ab**
Ancient	12.3	17.0	19.2	101.3	11272.8	108.0	141.1	6.0	4.3	0.3	12.7	10.8
Recent	17.1	22.6	23.1	180.4	18282.1	185.4	149.0	6.3	5.9	0.4	14.4	13.3
Germany	**34.7 (11.2)a**	**29.8 (7.7)a**	**36.4 (5.8)a**	**114.3 (20.3)a**	**1469.7 (631.2)a**	**88.3 (16.6)a**	**395.7 (29)a**	**4.4 (0.3)a**	**9.4 (1.2)ab**	**0.6 (0)a**	**16.3 (1)a**	**15.1 (0.1)a**
Ancient	19.2	36.1	30.0	85.0	2086.9	110.4	387.4	4.7	8.5	0.5	15.6	15.0
Recent	50.3	23.6	42.7	143.7	852.5	66.3	404.0	4.2	10.3	0.6	17.0	15.2
Sweden	**38.4 (9.2)a**	**21.4 (15.1)a**	**41.5 (15.3)a**	**367.3 (178.2)a**	**2468.1 (1365.9)a**	**433.4 (230.7)a**	**495.8 (212.2)a**	**4.8 (0.4)a**	**22.5 (9.1)a**	**1.4 (0.6)a**	**16.5 (0.5)a**	**5.3 (0)b**
Ancient	37.9	33.3	44.7	473.0	3277.6	573.7	484.6	4.8	24.2	1.6	16.1	5.3
Recent	38.9	9.5	38.3	261.7	1658.7	293.2	506.9	4.9	20.7	1.2	16.9	5.3
						Stachys sylvatica						
France	**17.4 (2.8)a**	**10.9 (4.5)b**	**28.1 (11.7)a**	**177.5 (47.4)a**	**2291.4 (571.1)a**	**202.8 (50.7)a**	**123 (46)a**	**5.3 (0.5)a**	**3.5 (0.6)a**	**0.3 (0)a**	**11.8 (1)a**	**13.3 (1.3)a**
Ancient	21.3	4.1	34.3	203.7	2137.6	147.5	158.5	5.7	3.4	0.3	12.0	13.3
Recent	13.6	17.8	21.9	151.4	2445.2	258.1	87.5	4.9	3.6	0.3	11.6	13.3
Germany	**13.6 (2.4)a**	**53.6 (19)a**	**21.2 (3.6)a**	**91.3 (15.4)a**	**2601 (809.4)a**	**153.6 (35.9)a**	**280.4 (38.2)a**	**5.1 (0.2)a**	**7.2 (2)a**	**0.5 (0.1)a**	**13.2 (0.6)a**	**15.1 (0.1)a**
Ancient	13.0	66.0	23.7	85.6	3692.9	202.3	256.9	5.3	9.5	0.7	14.0	15.0
Recent	14.2	41.1	18.8	97.0	1509.1	104.9	304.0	4.8	4.9	0.4	12.3	15.2
Sweden	**22.9 (4.1)a**	**21.8 (6.2)ab**	**19.9 (4.1)a**	**228.8 (95)a**	**2647 (931.7)a**	**406.1 (151.6)a**	**240.8 (71.7)a**	**5.5 (0.3)a**	**7.3 (1.2)a**	**0.5 (0.1)a**	**13.8 (0.4)a**	**5.3 (0)b**
Ancient	29.7	18.8	17.5	156.6	2931.6	443.8	231.4	5.5	7.1	0.5	13.3	5.3
Recent	16.1	24.7	22.2	301.0	2362.4	368.4	250.1	5.6	7.5	0.5	14.2	5.3

Appendix 5.2

The mean plant performance (with standard error) of the two plant species, and multiple comparisons of the means in unsterilized soil (PSF_{US}), sterilized soil (PSF_{S}), home-inoculated soil (PSF_{S+HI}) and foreign-inoculated soil (PSF_{S+FI}) based on (generalized) linear mixed-effects models

Plant performance	PSF_{US}	PSF_{S}	PSF_{S+HI}	PSF_{S+FI}
		Milium effusum		
Emergence (%) ¶	14.7 (1.6)a	9.4 (1.2)ab	7.2 (1.0)b	6.4 (0.9)b
MET (week) §	8.9 (0.6)b	13.4 (0.7)a	12.5 (0.7)a	12.8 (0.7)a
Height (cm)	13.2 (0.6)b	16.7 (0.9)a	14.5 (0.9)ab	16.5 (1.1)ab
SLA (cm^2 mg^{-1}) §	0.2 (0.00)b	0.22 (0.01)a	0.23 (0.01)a	0.23 (0.02)a
Shoot biomass (mg) £	43.7 (5.5)a	63.1 (6.9)a	52.9 (7.2)a	74.6 (11.1)a
Root biomass (mg) §	23.2 (2.7)a	24.0 (2.6)a	20.2 (2.6)a	27.3 (4.1)a
Root:shoot ratio §	0.6 (0.03)a	0.35 (0.02)b	0.37 (0.02)b	0.34 (0.01)b
		Stachys sylvatica		
Emergence (%) ¶	13.5 (1.2)a	20.1 (1.5)a	13.1 (1.2)a	13.0 (1.4)a
MET (week) §	7.8 (0.4)a	9.2 (0.3)a	9.5 (0.5)a	9.3 (0.5)a
Height (cm) £	3.3 (0.1)c	6.7 (0.3)a	5.4 (0.2)b	5.6 (0.3)b
SLA (cm^2 mg^{-1}) §	0.2 (0.01)b	0.4 (0.03)a	0.4 (0.04)a	0.4 (0.04)a
Shoot biomass (mg) §	24.1 (1.7)b	87.1 (7.4)a	34 (4.2)b	39.1 (4.8)b
Root biomass (mg) §	28.1 (2.3)b	81.7 (6.1)a	43.5 (5.4)b	45.9 (5.0)b
Root:shoot ratio	1.1 (0.04)ab	1.0 (0.03)b	1.1 (0.04)a	1.1 (0.04)a

One singularity SLA in *S. sylvatica* was removed.

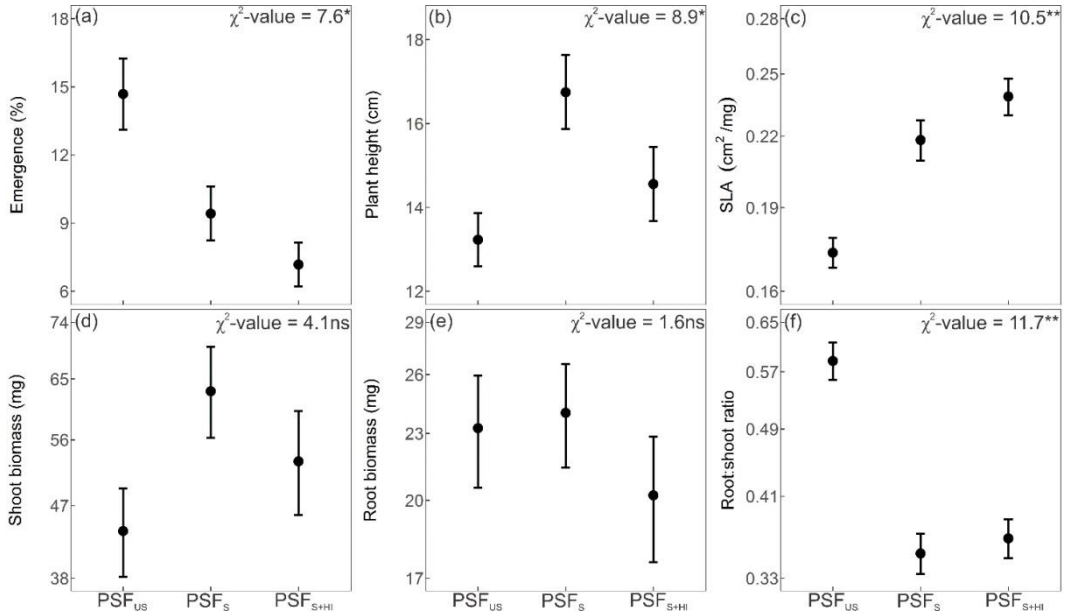
Appendix 5.3

The effect of home-away distance on plant performance of *Milium effusum* and *Stachys sylvatica* in unsterilized soil (PSF_{US}), sterilized soil (PSF_S), home-inoculated soil (PSF_{S+HI}) and foreign-inoculated soil (PSF_{S+FI}). Values and asterisk symbols are χ^2-values and significances, respectively, from the likelihood ratio tests of the (generalized) linear mixed-effects models. Df is 1

Plant performance	Home-away distance (°)			
	PSF_{US}	PSF_S	PSF_{S+HI}	PSF_{S+FI}
		Milium effusum		
Emergence (%) ¶	↑13.9***	↓5.5*	2.0ns	0.7ns
MET (week)	↓5.6*	0.6ns	0.0ns	0.2ns
Height (cm)	1.9ns	1.0ns	0.0ns	1.6ns
SLA (cm^2 mg^{-1})	0.8ns §	0.6ns £	↑4.7* §	2.0ns §
Shoot biomass (mg)	0.2ns §	0.4ns £	0.4ns §	0.7ns §
Root biomass (mg)	3.6ns	1.3ns	2.2ns £	0.0ns §
Root:shoot ratio	0.2ns §	0.3ns §	0.2ns §	2.2ns §
		Stachys sylvatica		
Emergence (%) ¶	1.9ns	1.9ns	3.5ns	↓10.7**
MET (week)	0.3ns	1.1ns	1.3ns	0.1ns
Height (cm)	1.1ns	1.3ns §	↑3.9* §	2.7ns §
SLA (cm^2 mg^{-1})	0.8ns	0.0ns §	0.1ns §	0.3ns §
Shoot biomass (mg)	0.7ns £	0.0ns §	0.3ns §	1.0ns §
Root biomass (mg)	2.9ns £	0.1ns	0.3ns §	0.1ns £
Root:shoot ratio	0.0ns	↓5.6*	2.9ns	0.1ns

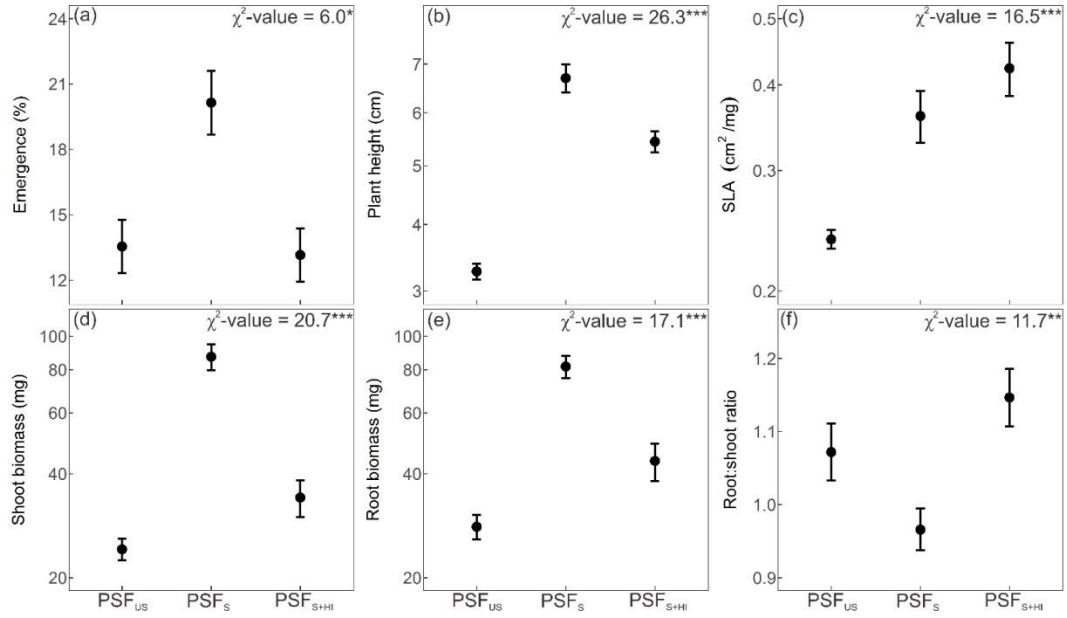
The direction of the effect is displayed as an arrow: ↑ indicates higher values in northern soil and ↓ lower values in northern soil.

Appendix 5.4



The effect of treatments: unsterilized (PSF_{US}), sterilized (PSF_S) and home-inoculated soil (PSF_{S+HI}) on seedling emergence and functional traits (plant height, SLA, shoot biomass, root biomass and root:shoot ratio) in *Milium effusum*. Points indicate the mean values of emergence and growth traits. Error bars indicate standard error. χ^2-values and asterisks represented significance were extracted from the likelihood ratio tests of linear mixed-effects models. Df is 2. Note the data transformation for response variables.

Appendix 5.5



The effect of treatments: unsterilized (PSF_{US}), sterilized (PSF_S) and home-inoculated soil (PSF_{S+HI}) on seedling emergence and functional traits (plant height, SLA, shoot biomass, root biomass and root:shoot ratio) in *Stachys sylvatica*. Points indicate the mean values of emergence and growth traits. Error bars indicate standard error. χ^2-values and asterisks represented significance were extracted from the likelihood ratio tests of linear mixed-effects models. Df is 2. Note the data transformation for response variables.

Appendix 5.6

The mean plant performance (with standard error) of *Milium effusum* and *Stachys sylvatica* with different land-use history (ancient and recent forests) and likelihood ratio tests for the effects of land-use history on plant performance of both plant species based on (generalized) linear mixed-effects models

Species	Plant performance	Ancient	Recent	χ^2-value
M. effusum	Emergence (%) ¶	9.2 (0.9)	9.7 (0.8)	0.5ns
	MET (week) §	11.9 (0.5)	11.3 (0.5)	0.01ns
	Height (cm)	15.8 (0.7)	14.3 (0.6)	2.8ns
	SLA (cm^2 mg^{-1}) §	0.2 (0)	0.2 (0)	0.6ns
	Shoot biomass (mg) £	63.2 (6.1)	50.3 (4.6)	2.5ns
	Root biomass (mg) §	27.1 (2.6)	20.3 (1.8)	1.2ns
	Root:shoot ratio §	0.4 (0.0)	0.4 (0.0)	1.3ns
S. sylvatica	Emergence (%) ¶	16.0 (1.0)	13.9 (0.9)	8.3**
	MET (week) §	8.9 (0.3)	9.0 (0.3)	3.8ns
	Height (cm)	5.5 (0.2)	5.2 (0.2)	0.1ns
	SLA (cm^2 mg^{-1}) §	0.3 (0.0)	0.4 (0.0)	0.1ns
	Shoot biomass (mg) £	50.7 (4.3)	48 (4.4)	0.3ns
	Root biomass (mg) §	51.5 (3.7)	53.2 (4.3)	1.0ns
	Root:shoot ratio §	1.0 (0)	1.1 (0)	3.7ns

Data in unsterilized soil (PSF_{US}) were used. Df is 1.

Appendix 5.7

The mean soil microbial biomass (with standard error) and multiple comparisons between regions in *Milium effusum* and *Stahcys sylvatica* based on linear mixed-effects models

Species	Microbial group	France (µg/g)	Germany (µg/g)	Sweden (µg/g)
M. effusum	*Actinobacteria* §	1.0 (0.3)a	0.8 (0.2)a	2.2 (0.7)a
	Non-specific bacteria §	3.5 (1.2)ab	2.0 (0.7)b	15.4 (7)a
	Gram-positive bacteria £	4.1 (1.3)ab	2.8 (0.9)b	12.8 (5.7)a
	Gram-negative bacteria §	3.2 (1.2)ab	1.7 (0.7)b	12.5 (6.9)a
	Total bacteria §	11.8 (4)ab	7.3 (2.5)b	43.0 (20.1)a
	Total fungi §	0.5 (0.1)b	0.3 (0.1)b	3.5 (1.7)a
	Total microbes §	13.0 (4.3)ab	8.0 (2.8)b	49.5 (23.1)a
S. sylvatica	*Actinobacteria* §	0.9 (0.1)a	0.9 (0.2)a	1.0 (0.4)a
	Non-specific bacteria	3.6 (0.7)a	3.4 (0.7)a	5.3 (2.4)a
	Gram-positive bacteria §	4.0 (0.6)a	4.1 (0.6)a	4.7 (1.8)a
	Gram-negative bacteria §	4.1 (0.9)a	3.7 (0.8)a	5.2 (2.1)a
	Total bacteria §	12.6 (2.4)a	12.1 (2.3)a	16.2 (6.7)a
	Total fungi §	0.4 (0.1)a	0.6 (0.1)a	0.6 (0.2)a
	Total microbes	14.3 (2.8)a	13.8 (2.5)a	18.3 (7.6)a

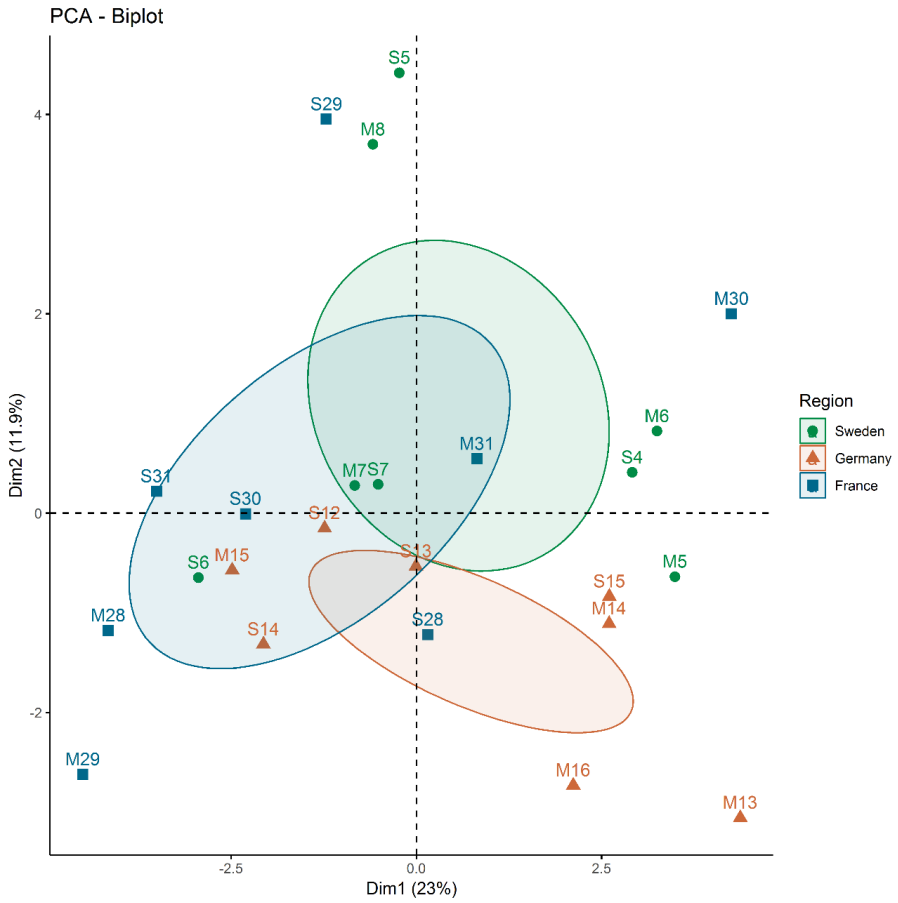
Microbial biomass was determined using PLFA. Small letters indicate the results of multiple comparisons.

Appendix 5.8

The mean plant performance (with standard error) of *Milium effusum* and *Stachys sylvatica* with different soil regions and likelihood ratio tests for the effects of soil regions on plant performance of both plant species in each soil treatment based on (generalized) linear mixed-effects models. Small letters indicate the results of multiple comparisons based on the models

	M. effusum				*S. sylvatica*			
Plant performance	France	Germany	Sweden	χ^2-value	France	Germany	Sweden	χ^2-value
Unsterilized soil (Df =2)								
Emergence (%)¶	9.2 (2.0)b	14.9 (2.8)a	19.9 (3.1)a	16.8***	7.9 (1.5)b	20 (2.4)a	12.7 (2.1)b	22.7***
MET (week)	9.9 (1.0)	9.4 (0.9)	8.0 (1.2)	1.8ns	8.6 (0.9)	7.5 (0.5)	7.6 (0.8)	1.6ns
Height (cm)	13.5 (1.4)	12.5 (0.7)	13.6 (1.2)	0.6ns	2.9 (0.1)	3.4 (0.2)	3.4 (0.2)	§2.9ns
SLA (cm^2 mg^{-1})	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	§2.0ns	0.3 (0.0)a	0.2 (0.0)b	0.2 (0.0)b	§13.6**
Shoot biomass (mg)	43.3 (9.2)	27.5 (4.4)	57 (11.3)	£2.8ns	14.2 (2.3)b	28 (2.5)a	25.9 (3.4)a	£13.6**
Root biomass (mg)	20.9 (3.5)	16.6 (2.7)	30.1 (5.9)	§0.7ns	15.3 (2.9)b	31.4 (3)a	33 (5.3)a	£15.7***
Root:shoot	0.6 (0.1)	0.6 (0)	0.6 (0.1)	0.4ns	1.0 (0.1)	1.1 (0.0)	1.1 (0.1)	1.4ns
Sterilized soil (Df =2)								
Emergence (%)¶	13.5 (2.6)a	6.9 (1.6)b	7.9 (1.7)ab	9.3**	19.9 (2.7)ab	24.1 (2.4)a	16.4 (2.4)b	7.0*
MET (week)	12.7 (1.3)	14.7 (1)	13 (1.4)	1.5ns	8.8 (0.6)	9.5 (0.6)	9.3 (0.6)	1.0ns
Height (cm)	18.2 (1.2)	16.4 (1.5)	15.5 (2)	1.2ns	5.6 (0.3)b	7.3 (0.5)a	7.1 (0.7)ab	§6.7*
SLA (cm^2 mg^{-1})	0.2 (0)	0.2 (0)	0.2 (0)	§0.5ns	0.4 (0.1)	0.4 (0)	0.3 (0)	§0.4ns
Shoot biomass (mg)	74.3 (12.3)	49.8 (8.6)	62.8 (14.2)	£1.8ns	62.2 (9.1)	103.4 (12.5)	93.3 (16.6)	§1.7ns
Root biomass (mg)	31.7 (5.1)	20.5 (4.1)	18.5 (4.1)	§4.0ns	72.1 (10.3)	86.5 (8.8)	86.6 (14.1)	0.6ns
Root:shoot	0.4 (0)	0.3 (0)	0.3 (0)	§4.4ns	1.1 (0.1)a	0.9 (0)b	0.9 (0)b	12.6**
Home-inoculated soil (Df =2)								
Emergence (%)¶	11.2 (2.2)a	4.2 (1.2)b	6.2 (1.4)ab	9.9**	11.8 (1.9)	13.7 (2.0)	14.0 (2.4)	0.7ns
MET (week)	11.9 (1.1)	12.7 (1.8)	13 (1.1)	0.4ns	8.5 (0.7)	9.7 (0.8)	10.1 (0.8)	1.9ns
Height (cm)	14.1 (1.3)	16.8 (1.8)	13.6 (1.7)	1.9ns	4.8 (0.2)	5.4 (0.3)	6.2 (0.4)	§3.8ns
SLA (cm^2 mg^{-1})	0.2 (0)b	0.3 (0)a	0.3 (0)a	§8.0*	0.4 (0)	0.4 (0)	0.5 (0.1)	§1.7ns
Shoot biomass (mg)	51.9 (9.6)	57.9 (15.6)	51 (15)	£0.3ns	28.6 (4.3)	33.3 (5.7)	40.3 (10.6)	§0.5ns
Root biomass (mg)	23.7 (4.3)	19.9 (5.4)	16.3 (4.7)	§3.0ns	40.1 (6.6)	41.3 (6.9)	49.5 (14.1)	§0.7ns
Root:shoot	0.4 (0)	0.3 (0)	0.3 (0)	§4.2ns	1.3 (0.1)	1.1 (0.1)	1.1 (0.1)	4.7ns
Foreign-inoculated soil (soil origin of the substrate, Df =1)								
Emergence (%)¶		4.8 (1.3)	7.3 (1.3)	3.0ns		17.8 (2.6)	10.5 (1.5)	17.0***
MET (week)		14.1 (1.5)	12.3 (0.9)	0.9ns		9.1 (0.8)	9.5 (0.7)	0.2ns
Height (cm)		13 (1.8)	17.9 (1.3)	3.4ns		5.4 (0.3)	5.8 (0.4)	§1.1ns
SLA (cm^2 mg^{-1})		0.2 (0)	0.2 (0)	§1.4ns		0.4 (0.1)	0.4 (0.1)	§0.6ns
Shoot biomass (mg)		40.5 (13.4)	87.7 (14.3)	£2.6ns		42.3 (5.8)	36.6 (7.3)	£0.1ns
Root biomass (mg)		15.5 (4.8)	31.6 (5.4)	§0.8ns		52.7 (7.7)	40.6 (6.5)	§0.4ns
Root:shoot		0.4 (0)	0.3 (0)	§2.1ns		1.1 (0.1)	1.1 (0.1)	0.4ns
Foreign-inoculated soil (soil origin of the foreign-inoculants, Df =1)								
Emergence (%)¶	6.9 (1.3)	5.6 (1.4)		0.3ns	12.1 (1.6)	14.7 (2.5)		0.9ns
MET (week)	13.1 (1)	12.3 (1)		0.4ns	8.4 (0.6)b	10.7 (0.8)a		5.3*
Height (cm)	16.9 (1.5)	15.8 (1.6)		0.2ns	6 (0.4)	5 (0.3)		§3.0ns
SLA (cm^2 mg^{-1})	0.2 (0)	0.3 (0)		§3.4ns	0.4 (0.1)	0.5 (0.1)		£2.4ns
Shoot biomass (mg)	85.8 (15.9)	52.9 (11.5)		£0.8ns	50.2 (7.2)a	21.7 (3.5)b		§9.6**
Root biomass (mg)	32.8 (6.1)	17.2 (3.8)		§1.8ns	57.3 (7.1)a	27.9 (5.1)b		§6.8**
Root:shoot	0.4 (0)	0.3 (0)		§2.9ns	1.1 (0.1)	1.1 (0.1)		0.0ns

Appendix 5.9



The compositional difference of soil bacterial community based on a PCA for 24 sampling sites. OTU Data were obtained from NGS. Regions were displayed by different colours; texts for each point were Site_ID (see **Appendix 2.1** for details). Ellipses were plotted with the confidence of the mean (level = 0.95).

Appendix 5.10

The effects of ΔMicrobial biomass (the difference of soil microbial biomass between home and away soil) on plant performance of *Milium effusum* and *Stachys sylvatica* in unsterilized soil (PSF_{US}). Values and asterisk symbols are χ^2-values and significances, respectively, from the likelihood ratio tests of the (generalized) linear mixed-effects models. Df is 1

Species	Plant performance	ΔMicrobial biomass (µg/g)
M. effusum	Emergence (%) ¶	↑18.9***
	MET (week)	2.0ns
	Height (cm)	↑11.3***
	SLA (cm^2 mg^{-1})	0.4ns
	Shoot biomass (mg) £	↑17.5***
	Root biomass (mg) £	↑15.6***
	Root:shoot §	1.2ns
S. sylvatica	Emergence (%) ¶	0.4ns
	MET (week)	0.02ns
	Height (cm)	1.0ns
	SLA (cm^2 mg^{-1}) £	0.3 ns
	Shoot biomass (mg) £	0.3ns
	Root biomass (mg) £	1.7ns
	Root:shoot	1.9ns

The direction of the effect is displayed as an arrow: ↑ indicates higher values in soils with higher amount of soil microbial biomass.

Chapter 6

General discussion

Understorey plant species diversity and community composition merit large attention due to their ecological importance in forests (Gilliam 2007; Bellemare & Moeller 2014). However, how different soil microbial communities occurring under understorey plants establish is hardly ever studied, nor to which extent plants interact with the soil microbial communities in the context of global change which hinders our understanding of forest ecosystem functioning. In this thesis, we used observational studies, *in-situ* field and greenhouse experiments to obtain a relatively comprehensive and systematic understanding of the dynamics of understorey plants and soil microbes facing distinct environmental conditions and environmental changes (**Fig. 6.1**).

We found that the differences in soil microbial community composition under conspecifics and heterospecifics within their current distributional range in Europe mainly depended on understorey plant species identity and local soil characteristics (**Chapter 2** and **Chapter 3**). The plant-soil continuum responded significantly to increased light availability, which was also the main driver for the significant co-structure between understorey plants and soil microbes. While warming and the interaction of illumination and nitrogen (N) addition only affected soil microbial community composition (**Chapter 4**). In the plant-soil feedback experiment, soil microbes' presence or change to nonlocal communities affected plant performance significantly with respect to seedling emergence and growth (**Chapter 5**). In following sections, we will discuss these findings and compare them with previous studies. Finally, we will summarize the limitation of our study, thereby proposing perspectives for further research.

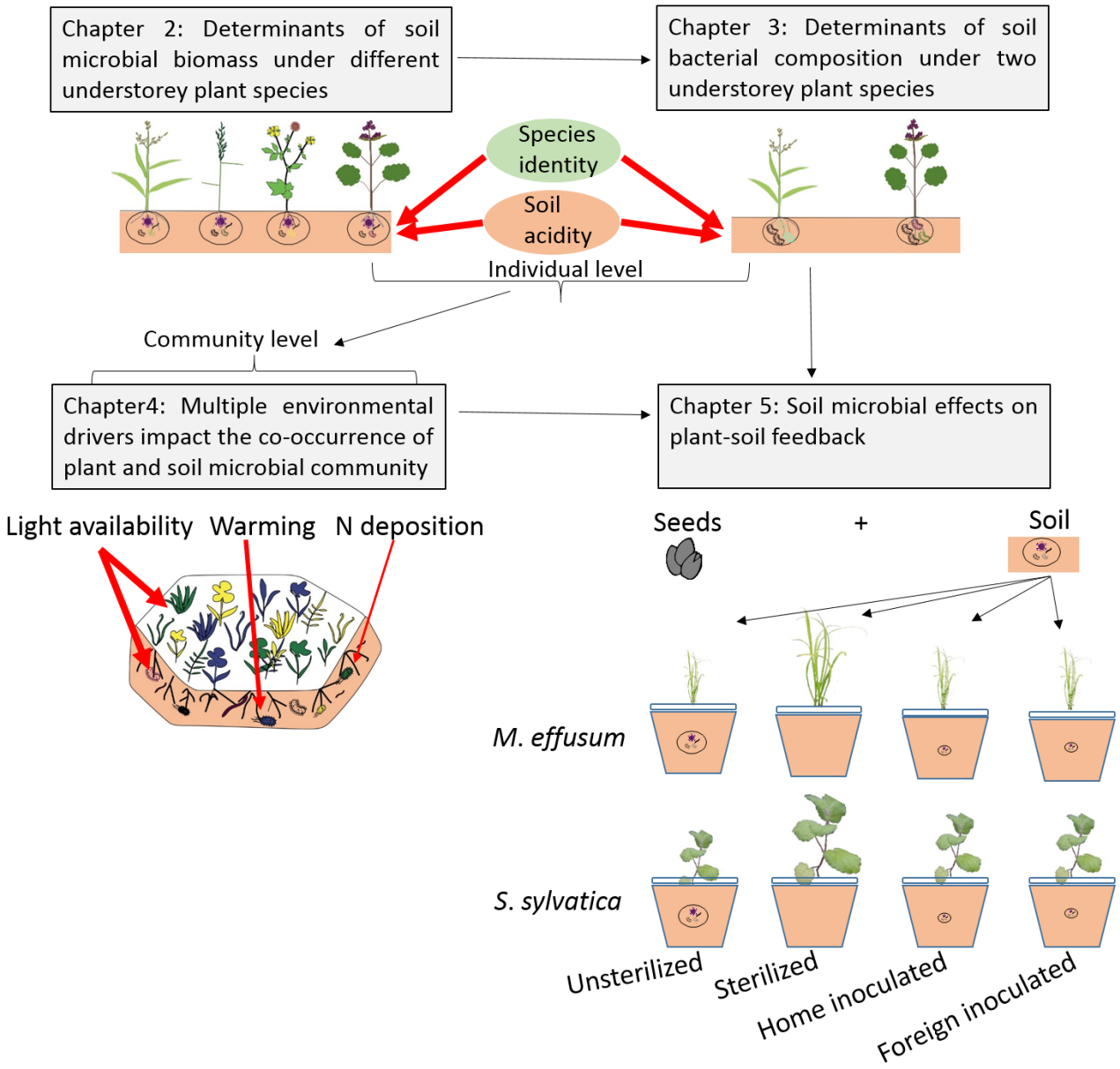


Fig. 6.1 An overview of the study results. The effects of the most significant factors/drivers on plant or soil microbial community composition are indicated with red arrows. Lacking effects on soil microbial biomass (warming and N deposition) and bacterial community composition (N deposition) are indicated with thinner red arrows. Soil microbes' effects on plant performance are reflected on seedling sizes.

6.1 Soil microbial biomass and dominant bacterial taxa in temperate forests

We characterized five functional groups of soil microbial communities, i.e., fungi, *Actinobacteria*, Gram-positive bacteria, Gram-negative bacteria and non-specific bacteria (**Chapter 2** and **Chapter 4**). Along the large geographical scale, the abundance of specific biomarkers in each functional group differed significantly under different understorey plant species (**Chapter 2**). In the *in-situ* experiment, the same five functional groups were found (**Chapter 4**) when three environmental

factors were conditioned. Yet, biomarkers in the functional group fungi, Gram-negative bacteria and non-specific bacteria differed between the two studies. Compared to other studies, despite the similar categorized functional groups, the detected PLFAs showed large variation in identified biomarkers (Feng & Simpson 2009; Schindlbacher *et al.* 2011) suggesting a compositional and functional discrepancy of soil microbes corresponding to a certain forest environment. In different ecosystems, soil microbial biomass differs even more due to vegetation caused differences in soil organic carbon (C) and nutrient resources.

We detected three dominant bacterial taxa in both the observational study and *in-situ* experiment, i.e., *Acidobacteria*, *Actinobacteria* and *Proteobacteria* (**Table 6.1**). This is consistent with large geographical-scale studies, in which a global assessment of bacterial distribution was determined and shown that despite the overwhelming diversity of soil bacterial community, only a few bacterial taxa showed dominant patterns across the globe (Nemergut *et al.* 2011; Delgado-Baquerizo *et al.* 2018). In our results, apart from the three dominant bacterial taxa, many bacterial phyla showed distinct composition when compared between regions and experimental treatments, for instance, the unique OTUs characterized into the phyla of *Chlamydiae*, *Nitrospirae* and *Planctomycetes*, indicating a specific assemblage of soil microbes in different habitat conditions (Bonito *et al.* 2014; DeAngelis *et al.* 2015). These results about the main bacterial taxa and distinct assemblage of the immense bacterial community may narrow down studies in the future that address functional roles of certain and important taxa in forests.

Table 6.1 An overview of the relative abundance of soil bacterial taxa sampled in **Chapter 3** and **4**

Phylum	Chapter 3	Chapter 4	Associated ecosystem function
Acidobacteria	0.15	0.38	Acidophilic
Actinobacteria	0.31	0.08	G+; N-fixer
Armatimonadetes	<0.01	<0.01	G-
Bacteroidetes	0.04	0.01	G-
BJ-169	<0.01	-	Understudied
BRC1	<0.01	-	Understudied
Candidate_division_WPS-1	-	<0.01	Understudied
Candidate_division_WPS-2	-	0.01	Understudied
Candidatus_Saccharibacteria	-	<0.01	Understudied
Chlamydiae	<0.01	0.01	G-; obligate intracellular bacteria
Chlorobi	<0.01	-	Green sulfur + non-photosynthetic bacteria
Chloroflexi	0.04	<0.01	G-; aerobic thermophiles
Cyanobacteria	<0.01	-	Phototrophic eukaryotes
Elusimicrobia	<0.01	-	Understudied
FBP	<0.01	-	Understudied
FCPU426	<0.01	-	Understudied
Fibrobacteres	<0.01	-	Cellulose decomposer
Firmicutes	0.02	<0.01	G+; pathogen; resistant to desiccation
GAL15	<0.01	-	Understudied
Gemmatimonadetes	0.01	<0.01	G-; adapt to low soil moisture
Gracilibacteria	<0.01	-	Understudied
Hydrogenedentes	<0.01	-	Understudied
Ignavibacteriae	<0.01	-	Understudied
Latescibacteria	<0.01	-	Understudied
Microgenomates	<0.01	-	Understudied
Nitrospirae	0.01	-	Nitrite-oxidizing
Omnitrophica	<0.01	-	Understudied
Parcubacteria	0.01	<0.01	Understudied
Peregrinibacteria	<0.01	-	Understudied
Planctomycetes	0.05	<0.01	G-
Proteobacteria	0.28	0.47	G-; pathogen; N-fixer
Saccharibacteria	0.01	-	Degrade toluene
SBR1093	<0.01	-	Understudied
Spirochaetes	<0.01	<0.01	Understudied
SR1_(Absconditabacteria)	<0.01	-	Understudied
Tectomicrobia	<0.01	-	Understudied
Tenericutes	<0.01	-	Understudied
TM6_(Dependentiae)	<0.01	-	Understudied
Unclassified	0.01	0.01	Unclassified
Verrucomicrobia	0.07	0.03	Methane oxidation
WS2	<0.01	-	Understudied

-: Not found; G+: Gram-positive bacteria; G-: Gram-negative bacteria

6.2 The main determinants of soil microbial community composition at a continental scale

6.2.1 Local soil characteristics

Soil microbes in the rhizosphere soil can be affected by multiple factors. Compared to macroclimatic conditions, N deposition load and land-use history, local soil characteristics emerged as the main factor determining soil microbial community composition along the studied latitudinal gradient (**Chapter 2** and **Chapter 3**). Specifically, soil microbial biomass and bacterial community diversity and composition were significantly affected by soil acidity and related chemical properties. Soil pH, the concentration of total P, Ca and Al captured the largest proportion of soil microbial biomass variation across the four studied plant species. Although the specific chemical determinants varied, consistent correlation patterns were found among plant species, such as the PLFA C16:1ω7c, which was negatively correlated with soil Al concentration but positively correlated with soil Ca and pH in all plant species. The latter correlation is consistent with a previous study in which soil pH was increased in limed forests and thereby changed soil microbial structure (Frostegard, Baath & Tunlid 1993). Previous studies stressed the importance of soil pH as one of the most important determinants of soil microbial community composition (Lauber, Hamady, Knight & Fierer 2009; Delgado-Baquerizo *et al.* 2018), and projected that higher soil pH tends to shift the microbial structure to bacteria-preferred communities, while lower soil pH enhances the abundance of soil fungi (Baath & Anderson 2003). Indeed, soil pH had a positive effect on bacterial species richness spanning the 1700-km latitude (**Chapter 3**). We found that bacterial richness increased linearly with pH between 4.1 (the lowest observation in this study) and 6.5, while decreasing slightly between 6.5 and 7.5 (although only four soil samples fell into this pH range). Therefore, the effects of soil pH on microbial community (structure, abundance and activity) should be explored along a broader pH range.

We combined the PLFA and sequencing methods and found that the variation in soil bacterial taxa was consistently explained by soil pH. Meanwhile, we observed contrasting correlations of different bacterial taxa with soil pH, indicating total soil bacterial biomass change (increase, decrease or neutral) may hinder the abundance of a specific bacterial taxa change. This highlights the necessity of exploring functional specialisation of different bacterial species. Furthermore, chemical element cycling, including litter decomposition, mineralization and nutrient immobilization, is closely linked with microbial structure and activity (Fu *et al.* 2015). Yet, the observed correlation patterns between specific bacteria taxa and chemical soil elements might be context-dependent because different land-use types underwent divergent management strategies and shaped distinct habitat

conditions, including soil characteristics and climatic conditions.(Roesch *et al.* 2007; Drenovsky, Steenwerth, Jackson & Scow 2010). Our *in-situ* experiment (**Chapter 4**) partially supported this point seeing the influence of changes in local light availability, temperature and N deposition on soil bacterial diversity and community composition shifts.

6.2.2 Plant species identity

The studied grasses and forbs shaped soil microbial community divergently. Some detected biomarkers even only occurred under specific plant species, e.g., the fatty acid C15:1ω10c (not in *P. nemoralis*) and C24:1ω15c (only in *P. nemoralis*). In addition, the assessed biomass of each biomarker characterized into different functional groups also differed significantly among species, probably due to different root exudates and allelochemicals between the four plant species. Unfortunately, since this was beyond the scope of the present project, we could not unravel the underlying mechanisms on how plants release distinct root exudates to rhizosphere soil and affect soil microbial biomass.

Next to rhizodeposition (e.g., root cap and border cell loss, root exudates), litter inputs by understorey plants are also important ways to connect plant species identity and soil microbial communities. Understorey plants contribute up to 20% of foliar litter to the forest floor (Muller 2003), and litter chemical traits of different resources are related to the decomposition rate and thereby driving the function and composition of soil decomposers. For bacterial taxa, *Chloroflexi*, *Nitrospirae* and *Verrucomicrobia* were more abundant in *S. sylvatica* than in *M. effusum*, whereas the abundance of *Acidobacteria* was significantly higher in the latter. *Acidobacteria* and *Nitrospirae* are two important bacterial phylum with low-pH tolerance (Sait, Davis & Janssen 2006) and nitrite-oxidizer (Tyson *et al.* 2005), respectively. This observation is congruent with soil property differentiation under the two plant species as soils under *M. effusum* possessed a higher C/N ratio and Al concentrations, while lower soil P-concentrations and pH were observed. Our results of plant species identity effects on soil microbial biomass and bacterial diversity and composition suggest the necessity of maintaining the understorey plants in forests as their absence and diversity loss may result in substantial changes in soil microbial community composition and eventually interfere soil C stock and nutrient cycling (Meier & Bowman 2008; Schimel & Schaeffer 2012).

6.2.3 Large-scale environmental conditions (temperature, precipitation and N deposition)

Along the studied latitudinal gradient, large-scale environmental conditions, i.e., temperature, precipitation and N deposition were positively correlated with each other. The variation of soil microbial community composition explained by these factors varied among plant species. For soil microbial biomass, large-scale environmental conditions only significantly explained the variation in graminoids, i.e., *M. effusum* and *P. nemoralis*, but not in the two forbs. Moreover, the joint explanation by large-scale environmental conditions and soil chemistry suggests a close correlation between the two factors. This is underpinned by a study in which positive effects of increased temperature and N deposition on soil base saturation and N concentration in soil solution, respectively, were found in temperate forest ecosystems (Gaudio *et al.* 2015). For bacterial taxa, large-scale environmental conditions explained the variation significantly and purely in *S. sylvatica* but not in *M. effusum*. Combining these patterns with the assessed lower soil P in the two grass species, we suggest that further studies uncovering the correlation between climatic conditions and N deposition should consider interspecific and intraspecific variation.

6.2.4 Land-use history

Post-agricultural forests bear the imprints of past land use, leading to different soil microbial community composition when compared to ancient forests (de la Pena *et al.* 2016). In contrast, we did not observe any soil microbial biomass or bacterial community compositional differences between the studied ancient and recent forests, suggesting a complete recovery of belowground soil biota in post-agricultural forests after decades. This soil microbial recovery phenomenon was also observed in other studies. For example, soil microbial community composition was similar between primary (ancient) and secondary (recent) forests (Jesus, Marsh, Tiedje & Moreira 2009); Sixty-year-old post-agricultural forests tended to harbour the same soil microbial communities as relatively pristine deciduous forests (Jangid *et al.* 2011). Likely, succession stage and/or forest age are important in determining soil microbes' compositional differences. In the studied regions, most recent forests (relative to the studied ancient forests) were established more than one hundred years (except in Poland). Additionally, since we found soil characteristics were the main determinant for soil microbial community composition, and there were no statistically significant differences of soil chemistry between the ancient and recent forests, the absence of land-use history effects on soil microbial community composition was not unexpected. In turn, if a post-agricultural forest contains less tree species and distinct chemical soil characteristics (e.g., total soil N, P and Mg concentrations), the potential legacy effects on ectomycorrhizal community composition can persist

after centuries (Diedhiou *et al.* 2009; Diedhiou *et al.* 2010). Based on these results, forest age and soil characteristics after land-use change can be combined when evaluating forest succession status in terms of soil microbial community composition.

6.3 Multiple environmental changes affect the plant-soil continuum

At a continental scale, interspecific variation together with distinct local soil characteristics were important drivers for soil microbial community composition (Wardle, Yeates, Williamson & Bonner 2003; Loranger-Merciris, Barthes, Gastine & Leadley 2006). Nevertheless, these drivers can be influenced by climate change and increased N deposition. At a smaller regional scale, environmental changes of a certain habitat, including light availability, temperature and N deposition, affect both plant and soil microbial community composition (De Frenne *et al.* 2015; von Rein *et al.* 2016; Liu *et al.* 2017). Our full factorial design allowed us to not only tease apart the effects of individual environmental drivers but also combine interaction effects of multiple drivers on the plant-soil continuum in understorey layer.

Using lamp-enhanced illumination in a forest context to mimic increased light availability in a dense forest, we observed compositional shifts in both plant and soil microbial communities and a reduction of soil microbial biomass in illuminated plots. Under closed canopies, light availability is one of the most severe constraints of understorey plants. During the study period, plants in illuminated plots received nearly ten times more photosynthetically active radiation than those in unilluminated plots. Increased light availability might affect plant growth through altered photosynthetic processes because photosynthetic rate per unit leaf area assessed for plant grown in high light conditions can reach three times higher than for those low-light-grown plants (Evans & Poorter 2001). Concomitantly, different light regimes modify specific leaf area and N partitioning, and thereby affecting soil microbial community composition most likely via changes in plant community composition, root exudates and allelochemicals (Lillo 2008). The substantial reduction of soil microbial biomass may be a result of insufficient energy resources, such as soil C and P, because plants may utilize more of these resources when light availability increased (Jonasson, Michelsen, Schmidt & Nielsen 1999). Therefore, we found a strong explanation of light availability for the significant linkage between plants and soil microbes.

During four years of treatment, mean temperatures in warmed plots increased by 1.43 ℃ and 3.21 ℃ at 20 cm above and -5 cm below soil surface, respectively. Soil bacterial community composition shifted while no changes in plant community composition and soil microbial biomass. Long-term soil warming has been projected to elevate CO_2 emission and decrease soil organic matter in

temperate forest ecosystems (Frey, Lee, Melillo & Six 2013; DeAngelis *et al.* 2015). However, it is more likely, in our case, that the warming effects were accelerated by illumination, as plots with the treatment combination of illumination and warming experienced a significant reduction in soil P (1.36 mg P kg^{-1} dry soil in control plots vs. 0.29 mg P kg^{-1} dry soil in the combined treatment) and microbial biomass. If this is true, the underlying mechanisms affecting the magnitude of reduction in soil energy resources and soil microbial dynamics deserve more exploration. Because canopy gaps caused by management or disturbance may happen frequently and these gaps subsequently alter original microclimate in the understorey layer towards a warmer and higher light transmission environmental conditions. Although we failed in detecting warming effects (neither in isolation nor combination) on plant community composition shifts, a tendency of increasing dominance of warmth-preferring taxa was reported and this thermophilization signal was again driven by illumination (De Frenne *et al.* 2015).

We found unexpected results that N addition alone had little influence on plant and soil microbial community composition. Understorey plant cover, soil microbial biomass and bacterial community composition were indistinguishable from those in N added plots after the four-year treatment. Why did soil microbial biomass and bacterial composition not respond to N addition when many studies have already reported such responses (e.g., Leff *et al.* (2015))? There are several potential explanations. First, N enrichment due to chronical accumulation of the relatively high N deposition in this dense ancient forest (25.3 kg N ha^{-1} in 2009 (Verstraeten *et al.* 2012) or high nutrient inputs via sufficient litter fall from the canopy layer. The enrichment did not cause N-resource constraints for plants and soil microbes, and thereby we observed no compositional difference with respect to N-fixing bacteria between plots with and without N-addition (**Chapter 4**). Lacking effects of different N deposition loads on soil bacterial richness and alpha diversity was also observed in **Chapter 3**. Second, it might be that the availability of another resource, e.g. light, limits the processes of N utilization as we found soil bacterial community composition shifted when illumination and N addition applied in combination. Meanwhile, soil microbial biomass in this combined treatment was also decreased significantly. These effects suggest that plant-soil interaction in the process of resource distribution (Winsome *et al.* 2017). Third, it has been shown that N addition can affect plant and soil microbial community composition via indirect effects on soil pH (Hallin, Jones, Schloter & Philippot 2009; Zhalnina *et al.* 2015), while in this study N-added plots did not show any change in soil pH, possibly due to the buffering capacity of soil calcium carbonate. Fourth, it is possible that shifts in plant and soil microbial community composition caused by N addition may take longer time to emerge than the implemented four years.

Our observation of the crucial role of light availability for understorey plant and soil microbial community composition may give hints for forest management. Light availability in understorey layer is strongly determined by forest management, e.g., via thinning operations. Canopy opening may result in less soil organic matter immobilized by soil microbes, at least at a four-year temporal scale. Understorey plants contribute foliar litter to the forest floor and this is generally better decomposable than tree litter (Muller 2003; Zhao *et al.* 2012; Pan *et al.* 2018). These attributes can substantially and positively affect soil C and N stocks (Winsome *et al.* 2017; Pan *et al.* 2018). The action of simple understorey removal to increase the survival possibility of tree seedlings or saplings should be avoid. Furthermore, canopy opening can also modify many other environmental conditions, e.g., CO_2 concentration and soil moisture in the understorey layer, which can be included in further studies.

6.4 Soil microbes feedback to plants

Soil microbial community shifted readily in different environmental conditions as seen in **Chapters 2, 3** and **4**. Different plant species producing exudates and allelochemicals around root systems promote the assemblage of specific soil microbes, including harmful pathogens for plant growth (Haichar *et al.* 2008). Soil microbes conditioned by different plant individuals and species can affect subsequent plant establishment positively or negatively, owing to their important roles in regulating biogeochemical cycling and their attributes of causing plant diseases. In the implemented plant-soil feedback experiment, soil microbes suppressed plant performance showing better growth in sterilized soils (**Chapter 5**), which is consistent with the prevalent negative plant-soil feedback in terrestrial ecosystems (van de Voorde, van der Putten & Bezemer 2011; van der Putten *et al.* 2013).

There were large differences in mean emergence time and plant functional traits between unsterilized (microbe-present) and sterilized (microbe-absent) soil. In sterilized soil, seeds of *M. effusum* and *S. sylvatica* experienced a longer time to germinate while seedlings were taller and the biomass of both shoots and roots increased compared to those grown in unsterilized soils. This negative plant-soil feedback with respect to plant functional traits persisted even when soil microbes were changed, i.e., inoculated with new soil microbes from southern regions. Negative plant-soil feedback is commonly driven by soil pathogens (van der Heijden, Bardgett & van Straalen 2008b). *Chlamydiae*, a bacterial taxa containing many pathogens, was found in the rhizosphere soil under the two studied plant species (**Chapter 4**). Negative plant-soil feedbacks imply a mechanism of plant species co-existence because the presence of soil microbes balances the growth of different plant individuals, leading to species co-existence and higher species diversity (Bonanomi, Giannino & Mazzoleni 2005). However, this mechanism may also cause diversity loss when considering invasive species. Because invasive species take the advantage of losing negative

plant-soil feedbacks and grow better in new habitats, which eventually result in changes in local plant species structures (Gaertner *et al.* 2014; Gioria & Pysek 2016).

Plants established in nonlocal soil depend on both biotic and abiotic conditions. Seeds from southern regions sown in soil from northern regions showed a higher emergence rate in *M. effusum*. The differences of soil chemistry between home and away soils affected seedling emergence and growth. When seeds were sown in soil with higher nutrients, both species showed a higher emergence rate and increased plant height and biomass. Yet, all these effects depended on the presence of soil microbes. In general, Swedish soil contained more soil nutrients and C resources. Meanwhile, we found higher soil microbial biomass in Swedish soil as well, especially for *M. effusum* (**Chapter 2**). This observation again supports our previous discussion that soil microbes can affect plant growth via partitioning energy resources (e.g., soil C and P utilization for the establishment of soil microbes per se).

Apart from the above discussion about the observed results, it is necessary to discuss the sterilization method we used in this plant-soil feedback experiment. Because this is a key step in plant-soil feedback research and how soil microbes are removed is influential for subsequent feedback phases. Currently, Gamma irradiation and autoclaving are the most recommended methods to remove soil microbes. However, the latter one has strongly disruptive effects on soil physical properties (Lotrario *et al.* 1995). Therefore, Gamma irradiation is often used attributing its high effectiveness in soil sterilization. However, side effects of Gamma irradiation on chemical properties emerge gradually due to nutrient release from dead soil microbes (McNamara, Black, Beresford & Parekh 2003). Yet, side effects are not consistently observed and studies have shown that a well-balanced dose of Gamma irradiation could minimize these side effects (Berns *et al.* 2008; Buchan, Moeskops, Ameloot, De Neve & Sleutel 2012). A high dose can reach up to 70 kGy. A proper dose also depends on the amount of soil. We aimed to sterilize ca. 24 kg soil. It has been suggested that a dose of ca. 30 kGy can be used to achieve the successful mortality of as much soil biota as possible but at the same time reduce side effects on soil N conditions (McNamara, Black, Beresford & Parekh 2003; Buchan, Moeskops, Ameloot, De Neve & Sleutel 2012). Furthermore, we distributed soil samples in multiple plastic bags to maximize the exposure of all soils to Gamma irradiation. Although we selected a relatively effective dose (28.4-29.9 kGy) and indirectly proved there was no side effect of nutrient change on the observed negative plant-soil feedbacks, a re-assessment of chemical soil characteristics after Gamma irradiation can be useful for tracking the chemical status. Alternatively, if a nutrient increase after sterilization is recorded, a period of incubation may mitigate this increase (Buchan, Moeskops, Ameloot, De Neve & Sleutel 2012).

6.5 Recommendations for further research

Understorey plant species diversity and compositional dynamics are closely associated with forest functioning. Soil microbes inhabited under these understorey plants are highly diverse and important for biogeochemical cycling. Our results showed the effects of local soil characteristics and multiple environmental change drivers on soil microbial community composition and the feedback of soil microbes on understorey plant regeneration. However, the response of plant and soil microbial community composition to environmental change is context-dependent. Based on our results, several recommendations can be made for further studies:

(1) Parallel studies at the global scale

Temperate forests in Europe, Eastern Asia and North America differ in tree, shrub and understorey species composition and environmental conditions. To obtain a conclusive result about variation in soil microbial community composition in temperate forests across continents, more parallel studies at different locations are needed. In addition, studies in contrasting ecosystems, such as tropical forests vs. temperate forests, at large geographical scales and along broad environmental gradients (sampling the environmental space) can facilitate a comprehensive understanding of the diversity and dynamics of terrestrial ecosystems.

(2) Different combinations of multiple environmental drivers and multiple responses

Except light, temperature and N deposition, there are other environmental drivers, such as changes in precipitation regimes (e.g., by applying rainout shelters or irrigation) and CO_2 concentration (e.g., by applying a free-air CO_2 enrichment, FACE, experiment), that can affect plant and soil microbial community composition separately or in combination with other drivers. Moreover, plant physiological characteristics including water and C use efficiency can also respond to multiple environmental drivers. Comprehensive experiments with multiple drivers and divergent response variables can capture more realistic responses of plants and soil microbes in the face of global change.

(3) Manipulating contrasting magnitudes of environmental conditions

Research on environmental change drivers showed inconclusive results of a certain driver on plant and soil microbial community composition. This is often caused by different magnitudes of manipulation, for instance, warming in different studies can vary from 1 up to 5 ℃. N addition varies even more. Inconsistent results caused by different magnitudes complex our understanding and predicting of biodiversity dynamics. Comparing multiple magnitudes of

environmental changes and discovering a threshold of each environmental driver would be helpful to make right management strategies for the conservation of forest biodiversity and productivity.

The mechanisms underlying the responses of plants and soil microbes to environmental change and the effects of soil microbes on plant performance are interesting and can be studied from several aspects:

(1) How can soil microbes regulate soil C and N resources?

We reported that there was a strong correlation between soil C/N ratio and soil microbial community composition. Microbial necromasses (dead microbes) contribute significantly to stabilized soil organic matter. Isotope probing and markers can be used to quantify soil C and N resources utilized and released by soil microbes. Additionally, soil enzyme activity and microbial respiration rates in different forest ecosystems and different plant species are essential for understanding biogeochemical processes between aboveground and belowground organisms.

(2) How can light decrease soil microbial biomass and alter soil bacterial community composition?

Light was the most important driver affecting plant and soil microbial community composition. The observed microbial biomass reduction and soil bacterial community composition shifts are most likely connected with C resources, plant root exudates, allelochemicals and plant litter. How these chemical compounds and resource inputs affect soil microbial community composition needs further studies. Additionally, whether increased light availability can promote P-uptake by plants remains unclear.

(3) The functional diversity of soil bacteria?

We have observed a correlation of specific functional group, e.g., Gram-positive and Gram-negative bacterial, and specifically functional bacterial species, e.g., nitrite-oxidizing bacteria *Nitrospirae*, with soil acidity. It would be interesting to focus on the function of these specific bacterial groups and species in the nutrient synthesis and biogeochemical cycles.

Finally, yet importantly, we only focused on the soil bacterial community in the assessment of taxonomic composition because of their main contribution to soil microbial biomass. There are many other soil organisms, such as fungi, archaea and nematodes, co-occurring with soil bacteria in soil food webs. Moreover, although large descriptive NGS data are increasingly available, soil

microbial diversity and composition using sequencing are still not enough to unveil soil microbial metabolisms. A combination of metagenomics (determining the functional potential of the microbiome) and metaphenomics (determining the actual functions of active cells under given environmental conditions) will be interesting for further research (Jansson & Hofmockel 2018).

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Curriculum vitae

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Education

2011-2014	MSc in Forest Genetics and Forest Tree Breeding, Sichuan Agricultural University, Sichuan, China
2008-2011	BSc in Forestry, Sichuan Agricultural University, Sichuan, China

Professional experience

September 2014-to date	PhD research at Forest & Nature Lab, Faculty of Bioscience Engineering, Ghent University

Scientific publications

International journals with peer review (IF: impact factor in 2017)

Ma, S., Verheyen, K., Props, R., Wasof, S., Vanhellemont, M., Boeckx, P., Boon, N. and De Frenne, P., 2018. Plant and soil microbe responses to light, warming and nitrogen addition in a temperate forest. *Functional Ecology*, *32*(5), pp.1293-1303. (IF = 5.49)

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National journals with peer review

Ma, S., Zhou, L., Xiong, H., Pu, G., Liu, H., Cai, L., 2014. 四川 5 种杉科植物的核型分析 (Karyotypes of five Taxodiaceae species in Sichuan). Journal of Northwest A&F University (Natural Science Edition). 42(9), pp.63-69.

Ma, S., Zhou, L., Pu, G., Lai, T., Cai, L., Wang B., 2014. 贴梗海棠花粉生活力与贮藏性研究 (Pollen viability and Storage methods of Chaenomeles speciose). Northern Horticulture. (16), pp.81-85.

Scientific activities

Participation in conferences with oral presentation

24 March 2017	Startersdag in het natuur- en bosonderzoek, Ghent, Belgium
11-14 December 2017	British Ecological Society: ecology across borders, Ghent Belgium

Participation in conferences with poster presentation

23-29 July 2017	XIX International Botanical congress, Shenzhen, China
17-21 June 2018	Ecology of Soil Microorganisms, Helsinki, Finland

Participation in symposium

17 May 2017	Frontiers in Ecology, Wageningen, the Netherlands

Participation in specialist courses

22-30 October 2014	Introduction to R by the Institute for Continuing Education in Science of Ghent University
18 November-9 December, 2014	Advanced Academic English Presentation skills by the Doctoral School of Ghent University
18 May-2 June 2017	Graphics in R organized by FLAMES (Flanders Training Network for Methodology and Statistics
12-14 June 2017	Tools for multivariate analysis by FLAMES
December 2017-January 2018	Effective Scientific Communication by the Doctoral School of Ghent University