UNIVERSIDAD POLITÉCNICA DE MADRID
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA DE
MONTES, FORESTAL Y DEL MEDIO NATURAL

**Soil functioning in the ecotone between
Scots pine and Pyrenean oak forests:
effects of tree species on the soil carbon
and nitrogen cycling**

TESIS DOCTORAL

María José Fernández Alonso

Ingeniera Agrónoma

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Table of Contents

Abstract	1
Keywords	2
Resumen	3
Palabras clave	4
Abbreviations and symbols	5
Chapter 1: Introduction	7
Setting the context: The global change	9
The role of forests in the C cycle	15
Plant-soil-microorganism interactions on soil carbon dynamics	20
Mitigating global change through forest management	22
Chapter 2: Objectives	25
Chapter 3: Materials and methods	29
Land-use history and known aspects of C cycle on the Guadarrama Range	31
General methods	35
Chapter 4: Disentangling the effects of tree species and microclimate on heterotrophic and autotrophic soil respiration in a Mediterranean ecotone forest	41
Abstract	43
Keywords	43
Highlights	44
Introduction	44
Materials and methods	46
Results	52
Discussion	62
Conclusions	65

Acknowledgements	66
<i>Chapter 5: Mechanisms driving soil respiration in response to short-term nitrogen additions in a Scots pine-Pyrenean oak ecotone forest</i>	67
Abstract	69
Keywords	69
Highlights	70
Introduction	70
Materials and methods	72
Results	77
Discussion	85
Conclusions	89
Acknowledgements	90
Supplementary information	91
<i>Chapter 6: Tree species shape the assembly of microbial decomposer communities during oak leaf and pine needle litter decomposition</i>	93
Abstract	95
Keywords	95
Highlights	96
Introduction	96
Materials and Methods	98
Results	102
Discussion	110
Conclusions	114
Acknowledgments	114
Supplementary information	115
<i>Chapter 7: Changes in litter chemistry associated with global change-driven forest succession resulted in time-decoupled responses of soil carbon and nitrogen cycles</i>	121
Abstract	123

Keywords:	123
Highlights	124
Graphical abstract	124
Introduction	125
Materials and methods	126
Results	132
Discussion	144
Conclusions	147
Acknowledgments	147
Chapter 8: General discussion	149
Soil CO ₂ efflux in the Scots pine – Pyrenean oak ecotone	151
Global change-driven forest succession: the linkage between green and brown worlds	153
Methodological remarks	155
New challenges for the forestry sector: including soil C sequestration into forest management planning and decision-making	156
Chapter 9: General conclusions	159
Chapter 10: References	163
Chapter 11: Annexes	185
Experimental plots	187
Scientific production derived from this PhD Thesis	189
Acknowledgements	191

Abstract

Background: Current composition and structure of the Scots pine-Pyrenean oak ecotone forest in the Guadarrama Range are the result of an intense and lasting human management. Although Scots pine forests have shown millennial stability in these Mountains, the global change may involve vegetation dynamics mainly fostering shifts of the dominant tree species in their lowest altitudinal range.

Aim: To assess future consequences driven by shifts in forest composition in the amount of carbon sequestered in the soil, we are interested in understanding the soil functioning in the ecotone to disclosure the effects of tree species, namely Scots pine or Pyrenean oak, and atmospheric nitrogen deposition that influence the belowground carbon and nitrogen cycling.

Methods: Three experimental plots were established in a pure Pyrenean oak forest, mixed oak-pine forest and pure Scots pine forest along a north-to-south transect at 1325 m a.s.l in the Valsaín Mountains (Guadarrama Range, Central Spain). Experiments at stand scale were combined with microcosm incubations and laboratory analysis to: **(Chapter 4)** disentangle tree species effects on the microclimate dependence of autotrophic and heterotrophic respiration components, **(Chapter 5)** identify how increases in atmospheric nitrogen decomposition may affect the causal relationships between edaphic properties and soil respiration, **(Chapter 6)** analyse how tree species influence litter decomposition, and **(Chapter 7)** understand how belowground carbon and nitrogen cycles could shift along the transitional stages of the forest vegetation succession from pine to oak.

Results: Our results revealed tree species-specific responses of soil respiration components to soil microclimate conditions being particularly important the higher sensitivity of the pine autotrophic respiration to summer drought and the oak autotrophic respiration to low winter temperatures. Moreover, the strong and inverse water and thermal seasonality of the Mediterranean climate strongly constrained the heterotrophic microbial activity in the ecotone. Carbon turnover of soil and foliar litter from the pine forest were faster than values found from the oak forest, which may have contributed to the higher carbon stocks in both topsoil and forest floor beneath the Scots pine.

Overall, three-years of nitrogen additions simulating the increased in atmospheric deposition caused soil acidification, leaching of potassium base cation, changes in extractable organic carbon and substantial decrease in soil microbial biomass, although soil respiration was rather resilient. It seems that soil stoichiometry, seasonal climatic constraints for biological activity and doses of nitrogen fertilizer were factors that modulated the response of soil respiration

to nitrogen additions and caused pine soil respiration to be more sensible to soil nitrogen enrichment.

Tree species directly affected the decomposition constants and N dynamics of decaying litter through the different litter chemistry between oak leaves and pine needles but also indirectly by affecting the colonization of microbial functional groups (K-strategists vs. r-strategists). Consequently, depending on whether the soil organic matter origin derives from pine or from oak the effects on the ecology and physiology of soil microbial communities will be different, causing the carbon and nitrogen cycling to decouple throughout the forest secondary succession from pine to oak. In the first stage of forest succession, an acceleration of the soil carbon cycling was found in the mixed forest with regard to pure pine forest together with a shift toward lower fungal to bacterial ratios. However, soil processes leading to an acceleration of the nitrogen cycling occurred later in the succession when there were only soil organic matter from oak. Hence, when secondary succession was completely accomplished, we found an alleviation of the nitrogen limitation for microbial growth, greater abundance of gram-negative bacteria, an accelerated carbon turnover and suppressed methane consumption.

Conclusions: In view of the different functionality of the plant-soil-microorganism system beneath pine and oak stands, the forest secondary succession from Scots pine to Pyrenean oak would have remarkable implications in the belowground carbon and nitrogen cycles that should be included as practical considerations in forest management.

Keywords

Temperate forests, Mediterranean basin, global change, forest secondary succession, autotrophic respiration, heterotrophic respiration, atmospheric nitrogen deposition, litter decomposition, ecological stoichiometry, functional microbial groups, carbon cycling, carbon sequestration, nitrogen cycling, greenhouse gases.

Resumen

Antecedentes: La actual composición y estructura del bosque ecotonal entre el pino silvestre y el roble pirenaico en la Sierra de Guadarrama es el resultado de una larga e intensa gestión forestal. A pesar de que los pinares de silvestre han mostrado una estabilidad milenaria en estos montes, el cambio global puede desencadenar dinámicas en la vegetación que resulten en cambios en la especie vegetal dominante especialmente en su límite altitudinal más bajo.

Objetivo: Para evaluar las futuras consecuencias de los cambios en la composición del bosque sobre la cantidad de carbono almacenado en el suelo, estamos interesados en entender cómo es el funcionamiento del suelo en el ecotono y en revelar los efectos de la especie, es decir si se trata de pino o roble, y de la deposición atmosférica de nitrógeno que determinan la tasa de renovación del carbono y nitrógeno del suelo.

Metodología: Tres parcelas experimentales fueron establecidas en un robledal puro, en una masa mixta de pino y roble y un pinar puro, a lo largo de un transecto norte-sur a 1325 m s.n.m. en los Montes de Valsaín (Sierra de Guadarrama, Sistema Central). Se combinaron experimentos en campo con incubaciones de microcosmos y análisis de laboratorio para: **(Chapter 4)** desentrañar el efecto de la especie sobre la dependencia microclimática de las componentes de la respiración autotrófica y heterotrófica, **(Chapter 5)** identificar cómo incrementos en la deposición de nitrógeno pueden afectar a las relaciones causales entre las propiedades edáficas y la respiración del suelo, **(Chapter 6)** analizar cómo la especie influencia la descomposición de la hojarasca y **(Chapter 7)** entender cómo los ciclos subterráneos del carbono y nitrógeno pueden alterarse a lo largo de la sucesión secundaria de pinar a robledal.

Resultados: Nuestros resultados revelaron respuestas específicas para cada especie de la respiración del suelo a las condiciones microclimáticas edáficas, siendo particularmente importantes la mayor sensibilidad de la respiración autotrófica del pinar a la sequía estival y la del rebollar a las bajas temperaturas invernales. Además, la fuerte e inversa estacionalidad hídrica y térmica del clima mediterráneo restringió fuertemente la actividad heterotrófica en el ecotono. Las tasas de renovación del carbono del suelo y de la hojarasca foliar del bosque de pino fueron más rápidas que los valores encontrados para el bosque de rebollo, lo cual puede haber contribuido al mayor contenido de carbono tanto en las capas superiores del suelo como en el horizonte forestal del pinar.

En general, tres años de adiciones de nitrógeno simulando el aumento en la deposición atmosférica provocaron acidificación del suelo, lixiviación de cationes base como el potasio, cambios en el carbono extraíble y disminución substancial de la biomasa microbiana del suelo,

aunque la respiración del suelo fue bastante resiliente. Parece que la estequiometría del suelo, las restricciones climáticas estacionales para la actividad biológica y las dosis de fertilización fueron factores que modularon la respuesta de la respiración del suelo a las adiciones de nitrógeno y que por tanto fueron responsables de la mayor sensibilidad mostrada por la respiración del suelo de pinar.

Las especies forestales afectaron el proceso de descomposición de la hojarasca a través de la distinta composición química de hojas y acículas, pero también a través de la diferente colonización de grupos funcionales microbianos (K estrategias vs. r estrategias). En consecuencia, dependiendo de si el origen de la materia orgánica del suelo deriva del pino o del roble los efectos en la ecología y fisiología de las comunidades microbianas del suelo serán diferentes, provocando que los ciclos del carbono y el nitrógeno se desacoplen a través de la sucesión secundaria del bosque de pinar a robledal. En la primera etapa de la sucesión forestal, encontramos una aceleración en el reciclado del carbono del suelo en el bosque mixto con respecto al bosque puro de pinar, junto con un cambio hacia una menor relación entre hongos y bacterias. Sin embargo, los procesos del suelo que provocaron una aceleración del reciclado del nitrógeno ocurrieron más tarde en la sucesión, cuando solo hubo materia orgánica procedente del roble y estuvieron asociados con una mayor abundancia de bacterias gram negativas, una aun mayor aceleración del reciclado del carbono y una reducción en la absorción de metano del suelo.

Conclusión: En vista de la diferente funcionalidad del sistema planta-suelo-microorganismo en pinar y robledal, la sucesión secundaria de la vegetación de pino silvestre a roble pirenaico tendría implicaciones notables en los ciclos subterráneos del carbono y nitrógeno que deberían incluirse como consideraciones prácticas en el manejo forestal.

Palabras clave

Bosque templado, Cuenca Mediterránea, cambio global, sucesión secundaria del bosque, respiración autótrofa, respiración heterotrófica, deposición atmosférica de nitrógeno, descomposición de hojarasca, estequiometría ecológica, grupos funcionales microbianos, ciclo del carbono, secuestro de carbono, ciclo del nitrógeno, gases de efecto invernadero.

Abbreviations and symbols

ρ	Spearman's rank correlation
AICc	Corrected Akaike Information Criterion
AU	Autumn
C	Carbon
CH ₄	Methane
CH ₄ -C	Carbon derived from CH ₄ fluxes
C _{mic}	Microbial biomass carbon
CO ₂	Carbon dioxide
CO ₂ -C	Carbon derived from CO ₂ efflux
D ²	Explained deviance
DOY	Day of year
EOC	Extractable organic carbon
ET ₀	Daily potential evapotranspiration
FAME	Fatty acid methyl esters
F:B	Fungal to bacterial ratio
FID	Flame ionization detector in gas chromatography
GHG	Greenhouse gas
GLM	Generalized linear mixed model
GP:GN	Gram-positive to gram-negative bacterial ratio
HFA	Home-field advantage theory
K-W	Non-parametric Kruskal-Wallis test
LME	Linear mixed effect model
LULCC	Land use and land cover change
L _v	Leaf litter from Pyrenean oaks
ML _v	Pyrenean oak leaves from mixed needle-leaf litter
MN _d	Scots pine needles from mixed needle-leaf litter
M _x	Mixed 1:1 litter from Scots pine and Pyrenean oak
N	Nitrogen
N _d	Needle litter from Scots pines
N _{mic}	Microbial biomass nitrogen
N ₂ O	Nitrous oxide
N ₂ O-N	Nitrogen derived from N ₂ O fluxes
N _r	Reactive nitrogen
P	Daily precipitation
PLFA	Phospholipid fatty acids
PLFA _{mic}	Total microbial biomass
Ps	Forest of <i>Pinus sylvestris</i>
qCO ₂	Metabolic quotient

Qp	Forest of <i>Quercus pyrenaica</i>
r	Pearson's product-moment correlation coefficient
R _A	Autotrophic respiration
RDA	Redundancy analysis
R _H	Heterotrophic respiration
R _S	Soil Respiration
SM	Soil sample from mixed Scots pine-Pyrenean oak forest
SO	Soil sample from Pyrenean oak forest
SOM	Soil organic matter
SP	Soil sample from Scots pine forest (Chapter 7)
SP	Spring (Chapter 4 and 5)
SU	Summer
SWB	Soil water balance
SWC	Soil water content
T	Soil temperature
TDN	Total dissolved nitrogen
TN	Total nitrogen
TOC	Total organic carbon
Tto	Treatment
W _{AIC}	Akaike weights
WHC	Soil water holding capacity
WI	Winter

Chapter 1

General introduction

Setting the context: The global change

The huge growth of the human population size and the overuse of natural resources through the combustion of fossil fuels in combination with the intensification of industrial and agricultural activities (including forestry, grazing and fishing) trigger global environmental changes (Hughes et al., 2003; Tilman et al., 2001; Turner and Meyer, 1991). The main determining factors of the ongoing global change comprise the rising atmospheric carbon dioxide (CO_2) concentration, alterations in the biogeochemical cycles of nitrogen (N) and other nutrients, widespread changes in both land use and land cover and biological invasions of exotic species (Vitousek et al., 1997b). These multiple human-induced forces interact and are in their turn the major drivers of the well-documented changes in global climate (IPCC, 2014) and the losses of biological diversity across the globe (Sala et al., 2000).

Figure 1.1. Globally averaged atmospheric concentrations of greenhouse gases. Source: IPCC (2014).

Global climate change

Anthropogenic greenhouse gas (GHG) emissions have risen since the pre-industrial era (Figure 1.1) leading to unprecedented levels in the atmospheric concentrations of CO_2 , methane (CH_4) and nitrous oxide (N_2O) in at least the last 800,000 years (IPCC, 2014). This has provoked the increase of the radiative forcing¹ of the climate system that has altered the Earth's energy budget producing a surface warming of approximately 0.6°C to 0.7°C over the past 100 years (Hartmann et al., 2013). In addition to the warming tendency, since the late 1960s there has been a lengthening of freeze-free periods that has resulted in the rise in mean sea level and the reduction

¹ The balance between incoming energy absorbed by the Earth from insolation and the energy reflected back to space. Radiative forcing above zero leads to the warming whereas radiative forcing smaller than zero leads to the cooling of the Earth's surface (Hartmann et al., 2013).

of snow cover and ice extent (Walther et al., 2002). At regional scale, there are consistent evidences of diverging patterns of the water cycle in response to the warming (Trenberth et al., 2014). With respect to the European continent, there is likely a north-south dipole in precipitation and evapotranspiration showing enhanced drought frequencies in southern regions, especially in the Mediterranean area, and wetter conditions in northern Europe (Spinoni et al., 2017; Stagge et al., 2017). Moreover, climate-related extreme events such as heat waves, droughts, heavy precipitations, floods and wildfires have become more frequent, intense and longer-lasting on a global scale (IPCC, 2014).

Land-use and land cover changes

The primary GHG responsible for the observed warming over the past century (80 % contribution to the anthropogenic radiative forcing) is the atmospheric concentration of CO₂ (Hartmann et al., 2013) that has increased from 277 ppm in 1750 to 402.8 ppm in 2016 (Le Quéré et al., 2018). After the burning of fossil fuels (88 % contribution for the last decade), the second largest source of the atmospheric CO₂ growth is the carbon (C) emission from land-use and land cover change (LULCC contributes 12 %, Figure 1.2). The LULCC accounts for the opposing dynamics in the rapid C losses –deforestation from natural to managed land, logging and forest degradation (including wood harvesting, fires and storms) and shifting cultivation (cycle of cutting forest for agriculture and subsequent abandoning)– and slow C gains –afforestation following wood harvest or agriculture abandonment (Arnell et al., 2017). Over the past century, there has been a relatively constant effect of LULCC on the global C cycle with global averaged emission rates of 1.3 Gt C year⁻¹ (Le Quéré et al., 2018). Recent C emissions from LULCC include areas acting as a net source in tropics, as a consequence of deforestation, and zones operating as a net sink in northern mid-latitudes, due to both the increased C density from forest recovery and the expanded areas of secondary forest caused by management changes (Houghton and Nassikas, 2017). It is worth to mention the existence of the net residual sink in the global C budget (the budget imbalance in Figure 1.2) that is mostly attributable to environmental changes, meaning the interactions between land-use and climate-C cycle feedbacks such as CO₂-enhanced productivity of secondary forests (Houghton, 2018).

Figure 1.2. Perturbation of the global carbon cycle caused by anthropogenic activities averaged globally for the decade 2007-2016. Numbers represent fluxes in Gt CO₂ year⁻¹. The budget imbalance accounts for the mismatch between estimated emissions from both fossil fuels and land-use changes and the total sinks including the atmosphere. Source: Le Quéré et al. (2018).

Alterations of the global nitrogen cycle

In the absence of human-caused changes to global N cycle, the creation of reactive nitrogen² (Nr) is entirely by two natural processes: the biological N fixation in terrestrial and aquatic ecosystems and the atmosphere lighting (Figure 1.3). Once created, the transfer of atmospheric Nr into ecosystems could be by either wet or dry deposition (Galloway et al., 2004). In the current world, the increased anthropogenic production of Nr to fulfil human demands for food and energy (Bobbink et al., 2010) has largely increased both the availability and mobility of N resulting in a massive acceleration of the N cycle of the entire Earth (Gruber and Galloway, 2008). There are three major sources of the anthropogenic Nr (Ciais et al., 2013; Vitousek et al., 1997a). Firstly, the combustion of fossil fuels and biomass that emits nitrogen oxides (NO_x) to the atmosphere. Secondly, the symbiotic N fixation by cultivation systems such as leguminous crops and forages. And lastly, the industrial production of synthetic N fertilizers –ammonia (NH₃), ammonium (NH₄⁺) and nitrate (NO₃⁻)– and also their application along with manure in agriculture,

² The Nr comprises oxidized (e.g. NO_x, HNO₃, N₂O, NO₃⁻), reduced (e.g. NH₃, NH₄⁺) and organic (e.g. urea, amines, proteins) nitrogenous compounds in opposition to unreactive molecular N (N₂) which cannot be used directly by most organisms (Bobbink et al., 2010).

that further increases the emissions of N_2O and nitric oxide (NO) via nitrification and denitrification pathways in soils as well as NH_3 volatilization from urea.

Figure 1.3. Global nitrogen cycle on land and ocean. Numbers represent fluxes in Tg N yr^{-1} for the 1990s. The blue color denotes “natural” fluxes whereas red color denotes anthropogenic fluxes. Source: Gruber and Galloway (2008).

In the year 2010, these anthropogenic activities added to the environment $\sim 210 \text{ Tg}$ of Nr per year, which is two times larger the natural rates of terrestrial Nr supply (Ciais et al., 2013). The excess Nr accumulation in the environment has the potential to negatively affect ecosystems causing soil acidification and mineral depletion, eutrophication and losses of biodiversity (Galloway and Cowling, 2002). But in addition to the detrimental effects, there are also beneficial consequences since the N is a limiting nutrient for plant growth in most agricultural and forestry ecosystems (Vitousek et al., 1997a). Hence, elevated atmospheric CO_2 and nitrogen deposition stimulate the biosphere productivity and thereby enhance the ecosystem C uptake from the atmosphere resulting in a positive feedback on global terrestrial C sequestration (De Vries et al., 2009; Hyvönen et al., 2008). This involves that human perturbations of the global N cycle interact with the also altered C cycle since both biogeochemical cycles are tightly coupled (Figure 1.3, Gruber and Galloway, 2008). However, the increased C sink could be partially offset because anthropogenic N enrichment may also increase the production of other GHGs such as the CH_4 and N_2O (Liu and Greaver, 2009). Overall, there are still large uncertainties in the understanding of N-C-climate interactions and their concomitant impacts on humans and ecosystems (Gruber and Galloway, 2008; Hyvönen et al., 2007).

Effects of global change on terrestrial ecosystems

Global change impacts natural systems by altering biodiversity and species ecology and causing changes in ecosystem processes, such as those involved in productivity and nutrient biogeochemical cycling (Ciais et al., 2005; Gruber and Galloway, 2008; Walther et al., 2002). The timing of seasonal activity of plants and animals is intimately linked to the climate and, as a result, in recent decades shifts in plant phenology have been related to climate change (Cleland et al., 2007). Further, the increasing concentration of atmospheric CO₂ and anthropogenic N deposition may directly alter the litter chemistry (i.e. C:N ratios), the short-term growth rates of plant species and the activity of microbial decomposers (Janssens et al., 2010; Liu and Greaver, 2009; Vitousek et al., 1997a). The variations within species in physiological properties and among species in phenology may produce cascade effects on the biotic interactions inside a community (e.g. disturbances in the life cycle coordination between species, trophic levels including soil decomposers, competitive imbalances and pathogen infections) (Tylianakis et al., 2008). Such alterations in biotic interactions in response to global environmental changes may further exert important shifts on the structure and composition of communities, species abundances and distribution ranges or even may lead to species extinctions (Cleland et al., 2007; Walther et al., 2002).

Recent studies highlight a substantial variability in the observed changes in terrestrial ecosystems due to the different drivers of the environmental changes that may interplay and their wide variation in both direction and intensity among regions (Walther et al., 2002). On the other hand, not all the species nor ecosystems are equally sensitive to global change factors since differing levels of fitness, physiological thresholds of tolerance, evolution and plasticity also condition their adaptive capacity and resilience (Parmesan, 2006). Despite of this, a number of sources have shown clear evidence of increasing episodes of forest die-off followed by tree mortality associated with climate-induced physiological stress in many regions even in those that are not water limited (Allen et al., 2010; Breshears et al., 2005). The forest may become more vulnerable to heat and drought and thereby distributional shifts have been observed in some forest ecosystems in response to the current general warming trends towards the Polar latitudes and the higher altitudes (Parmesan, 2006). In some areas such as the Mediterranean basin, the forest dieback have occurred with consecutive changes in overstory and understory species better physiologically adapted to dry and warm conditions (Curiel Yuste et al., 2012; Ruiz-Labourdette et al., 2012). The ecological implications of the climate-induced broad-scale tree die-off and the subsequent impacts on the terrestrial C budgets and fluxes remain still largely unassessed (Breshears and Allen, 2002).

The uncertainties regarding the alterations in the natural earth functioning need to be reduced because it may also have important impacts on the human well-being by directly affecting

human health (Galloway and Cowling, 2002; Gasparrini et al., 2017), but also by indirectly altering the provision of ecosystem services such as water supply and crop and livestock production (Schröter et al., 2005). In addition, there are less tangible forest services for societies –e.g. recreational, aesthetic or spiritual benefits– that are important to preserve as well (Allen et al., 2010). The ability of ecosystems and society to respond and adapt to the new environmental conditions is a challenge due to the magnitude and the rapid rate of the environmental shifts (Ciais et al., 2013). Therefore, it is crucial to understand the effects caused by the global change that is currently unavoidable in order to be able to anticipate and adapt to them, but above all to undertake future societal actions and policies involving reduction and mitigation strategies through sustainable land-management.

The ecotone forest between Scots pine and Pyrenean oak in the Iberian Peninsula as study case

Of particular concern in recent decades are the reported changes in the composition, structure and dynamics of Scots pine forests (*Pinus sylvestris* L.) located near the geographic and elevational range margins of this pine species in the Iberian Peninsula (Galiano et al., 2010; Hereş et al., 2012; Martínez-Vilalta and Piñol, 2002; Vilà-Cabrera et al., 2013). The warmer temperatures combined with severe drought events (Martínez-Vilalta and Piñol, 2002) are main factors behind the crown defoliation and mortality of Scots pine trees. In Mediterranean ecotone forests, the patterns of Scots pine mortality may be followed by subsequent seedling recruitment limitation due to drought stress, shade-intolerance and disturbances such as fires, land-use or forestry changes (Alvarez et al., 2014; Carnicer et al., 2014). If the pine mortality is not compensated by the recruitment, the dieback of the Scots pine stands may further result in a vegetation shift (Lloret et al., 2012). Late-successional species, for example those belonging to the genus *Quercus* sp., may lead to the progressive replacement of Scots pine as the dominant overstory species according to the findings of several studies (Barba et al., 2016; Carnicer et al., 2014; Galiano et al., 2010; Peñuelas et al., 2001). In this respect, a potential vegetation succession in the context of global change could be the altitudinal upwards migration of Pyrenean oak (*Quercus pyrenaica* Willd) favoured by Scots pine die-off in the very dry edge of the ecological distribution of the latter one. Such a global change-driven secondary forest succession from pine to oak could likely happen in the Scots pine-Pyrenean oak ecotone in the Guadarrama Range (Alvarez et al., 2014; Díaz-Pinés et al., 2014). At this emplacement, the structure and composition of pure Scots pine forests have been resilient to environmental disturbances in the past (Morales-Molino et al., 2017) and currently the pine persistence does not seem to be in risk. However, those Scots pine stands located in the lowermost altitudinal limits –where climatic factors (particularly water stress) are presumed to be already limiting (Allen et al., 2010)– may likely suffer a shrinkage associated with the interaction of present climate tendencies and management history.

The legacy of past human activities determines the current composition and structure of the forests in the ecotone between the Scots pine and Pyrenean in Guadarrama Range (Díaz-Pinés et al., 2014) that has significant implications for soil properties and functioning affecting future responses to the global change-induced vegetation succession. The high-density of Scots pine stands has led to scarce presence of understory vegetation and nutrient-poor soils but with huge amount of stored C in the forest floor and topsoil. On the contrary, Pyrenean oak stands in Valsaín suffered a heavy decline during the last 700 years as a consequence of the intensive coppice use and, to a lesser extent, overgrazing (Salomón et al., 2016a). Soils under Pyrenean oak stands have better nutrient status but comparative far lower stored C than soils from pine stands (Díaz-Pinés, 2011; Díaz-Pinés et al., 2011b). Consequently, One of the major risk of this tree species distribution shift in the Guadarrama Range, that has also been suggested for other Mediterranean forests, is the potential release of huge amounts of the C sequestered in Scots pine soils and the further associated positive feedbacks on climate change (Allen et al., 2010; Díaz-Pinés, 2011).

The role of forests in the C cycle

For the last decades, land ecosystems have been absorbing ~34 % of anthropogenic CO₂ emissions each year (Figure 1.2), with most of this uptake occurring through the net biome production in forests (Figure 1.4) that amounts on average 4 Gt C year⁻¹ (Birdsey and Pan, 2015; Ciais et al., 2013). Initially, the assimilated C from photosynthesis (gross primary production, 123 Gt C year⁻¹, (Beer et al., 2010)) is allocated to the autotrophic respiration (R_A, ca. 50 %, (Group and IGBP Terrestrial Carbon Working Group, 1998)) and the net primary production meaning the aboveground (foliage, fruits, branches, stems and epiphytes) and belowground tissue production (roots, exudates and mycorrhizae). Part of this C enters the soil as above and belowground detritus, where it is decomposed by biotic processes (i.e. microbial heterotrophic activity) and to a minor extent by abiotic processes (e.g. photodegradation) releasing nutrients to the soil and CO₂ to the atmosphere. The remaining C after these emissions is the net ecosystem production that is partly lost by natural and artificial disturbances (e.g. wood harvesting, fires, heavy storms, extreme droughts, etc.) eventually resulting in the net biome production.

Figure 1.4. Schematic of global C fluxes in terrestrial ecosystems. GPP: Gross primary production; NPP: Net primary production; NEP: Net Ecosystem Production; NBP: Net Biome Production; R_A: autotrophic respiration from belowground and aboveground tissues; R_H: microbial heterotrophic respiration; D: disturbances. Adapted from: (Beer et al., 2010; Birdsey and Pan, 2015; Ciais et al., 2013; Group and IGBP Terrestrial Carbon Working Group, 1998).

The net biome production is the rate of long-term organic C sequestration that includes the stored C in both phytomass and soils. Currently, the forest C stock is estimated to be 861 Gt C with 383 Gt C (44 %) in soil, 116 Gt C (14 %) in organic matter and 363 (42 %) Gt C in live biomass (Pan et al., 2011). Regarding the role of C sequestration of terrestrial ecosystems, the belowground compartment (soils and roots) is especially important since exceeds the amount of C stored in aboveground biomass and has lower turnover rates being more chemical and physically protected from degradation (Scharlemann et al., 2014). The soil C stocks vary widely depending on climatic regions (polar, boreal, temperate, and tropical) and forest cover (conifers, broadleaves and evergreen broadleaves). For instance, the largest soil C stocks are in northern permafrost regions and under conifer species (Chiti et al., 2012; Pan et al., 2011). Several authors have argued that the respiration of terrestrial ecosystems, including microbial decomposition of soil organic matter (SOM), would be more sensitive to global change than gross primary production (Davidson and Janssens, 2006). This is a critical issue because soils in forest ecosystems could turn into C sources when changes occur in climatic conditions and land cover contributing to positive C-climate feedbacks (Ciais et al., 2005; Reichstein et al., 2013).

Despite of the great deal of research on global soil C stocks, there currently remains substantially uncertainty on their responses to climate change and anthropogenic LULCC (Scharlemann et al., 2014). The decomposition of SOM is a very complex process involving environmental controlling factors such as soil moisture and temperature and intrinsic characteristics of the organic substrate (Davidson and Janssens, 2006). Regarding their important role in mitigating global change, a better understanding of the factors controlling the C dynamics

in soils, especially the soil-plant-microorganisms interactions in a changing environment (Aponte et al., 2011), is needed in order to successfully adapt forest management.

Soil respiration and its drivers

Soil respiration (R_s) accounts for all the CO_2 efflux originated in metabolic processes that take place within the soil. There are five biogenic sources of R_s that commonly are grouped in two terms according to the residence time of the respired C in the soil: one autotrophic (R_A , including living roots respiration, rhizomicrobial respiration and mycorrhizal respiration) and the other heterotrophic (R_H , respiration of dead plant tissues and SOM by microbes and saprophytic fungi) (Kuzyakov, 2006).

Soil respiration in forests is mainly controlled by soil microclimate conditions (i.e. soil temperature and soil water content (SWC)) and by vegetation activity (Curiel Yuste et al., 2004; Raich and Schlesinger, 1992). In the absence of other limiting factors, warmer temperatures are expected to increase both microbial and plant respiration (Ciais et al., 2005) since enzyme kinetics involved in carbohydrates oxidation by roots or in decomposition by microbes are extremely dependent of soil temperature (Davidson and Janssens, 2006). Nevertheless, the temperature-dependence of R_s not always holds true because strongly interacts with the SWC that in turn may have a bimodal effect on both respiratory components (Lellei-Kovács et al., 2016). Under soil saturated conditions R_s is inhibited due to limitations in oxygen diffusion, but besides under dryness conditions the plant and microbial activities are suppressed due to water stress (Davidson and Janssens, 2006).

There are additional factors related to the vegetation type that may also modulate the response of R_s to climatic conditions in forests. In this regard, vegetation influences R_s directly via seasonal patterns of plant activity and indirectly through the timing and chemistry of plant litter and the structure and physiology of the associated microbial decomposer community (Curiel Yuste et al., 2004; Strickland et al., 2009a; Ushio et al., 2010). Consequently, how abiotic and biotic drivers influence R_s strongly depend on the spatial (mainly due to vegetation-determined properties in soils) and temporal scales (seasonal evolution of soil microclimate) (Barba et al., 2016, 2013). Furthermore, the above-mentioned biotic and environmental drivers control differently the R_A and R_H in soils (Bond-Lamberty et al., 2004a; Schindlbacher et al., 2009), which adds more complexity to the understanding of the variability of soil C fluxes in forest ecosystems. Hence, determining the mechanisms and drivers responsible for R_s in the study area –the ecotone between the Scots pine-Pyrenean oak in the Guadarrama range– is necessary to reduce uncertainties when estimating C emissions in these forests soils, which is a prerequisite for accurately forecasting the potential impacts of global change in the soil C efflux.

Other greenhouse gas fluxes in soils

Soils in natural and artificial systems interchange CH_4 and N_2O with the atmosphere as a consequence of the heterotrophic and autotrophic activity of microbial communities. Although CH_4 and N_2O fluxes are quantitatively much lower than CO_2 efflux in soils, they have a significant contribution to the total global warming potential of a soil given their inherent warming potential (Díaz-Pinés et al., 2014). Therefore is crucial to understand if changes in soil microbial processes induced by global change may lead to a net positive or negative effect on CH_4 and N_2O emissions in terrestrial ecosystems (Singh et al., 2010).

Soils can act as sinks or sources of CH_4 and hence the net soil-atmosphere CH_4 exchange depends on the balance between microbial CH_4 consumption and production (Le Mer and Roger, 2001). The metabolic process by which anaerobic microorganisms, mainly from the *Archaea* domain, produces CH_4 for yielding energy from organic substrates (i.e. carbohydrates) is call methanogenesis (Figure 1.5, Singh et al., 2010). Part of the CH_4 produced in soils and the CH_4 already in the atmosphere may be consumed by methanotrophic bacteria, although this oxidation process requires the presence of oxygen. In addition to the constraining effect of soil anaerobic conditions (e.g. water-saturated and clayey soils), an inhibitory effect of NH_4^+ on CH_4 oxidation in soils have also been described (Bodelier and Laanbroek, 2004). Given the environmental conditions necessary for these microbial processes –i.e. anoxic conditions during methanogenesis and well-aerated soils during methane oxidation–, most temperate forest soils are net CH_4 sinks (Schaufli et al., 2010).

Figure 1.5. Microbial processes involved in methane (CH_4) production and consumption in soils. Source: Singh et al., (2010).

The N_2O release from soils to the atmosphere (Figure 1.6) is the result of a series of N turnover processes, which can be either oxidative or reductive. Nitrification is the processes by which NH_4^+ is oxidized to NO_3^- under aerobic conditions (Butterbach-Bahl et al., 2013). On the other hand, denitrification takes place in the absence of oxygen in order to reduce both NO_3^- and NO_2^- to molecular N (N_2). Denitrification may be a chemical (chemodenitrification) or a biological process; both denitrification and denitrification processes are strongly dependent on the availability of the specific substrates that is NH_4^+ and NO_3^- , respectively. In consequence, soils with low C:N ratios promote N_2O emissions whereas high soil C:N ratios lead to microbial N immobilisation (Díaz-Pinés, 2011).

Similarly to CO_2 efflux in soils, the exchange of CH_4 and N_2O between the soil and the atmosphere is strongly influenced by the vegetation cover through the root density, N input and associated microbial communities (Raich and Tufekcioglu, 2000), along with the anthropogenic management practices (Moreno-Fernández et al., 2015; Singh et al., 2010). In the same way, environmental factors (e.g. soil microclimate, soil texture, pH, C:N ratio) have an impact on the CH_4 and N_2O emissions and uptakes via their effects on the metabolic activity of both roots and microbes (Schaufler et al., 2010). Considering the essential role of vegetation in soil GHG emissions, is crucial to understand the impact of the global change-driven Scots pine succession by Pyrenean oak on the ecology and function of soil microbial communities in order to enable a better mitigation management in the study ecotone.

Figure 1.6. Nitrification and denitrification processes underlying the production of N_2O fluxes in soils. Source: Singh et al., (2010).

Plant-soil-microorganism interactions on soil carbon dynamics

The soil matrix is a complex system that is biologically active and where several living components, such as soil microorganisms and plants, interplay with other abiotic elements (e.g. minerals, rocks and substrates) and environmental factors (e.g. acidity, salinity, light irradiance, soil temperature and SWC). The plant cover and their cause-and-effect relationships with microbial communities configure soil physical and chemical characteristics (e.g. pH, nutrient availabilities, organic matter fractions, aggregate stability and moisture content among others) and altogether determine the soil system functioning (Figure 1.7). This way, changes in soil physicochemical properties derived from shifts in tree species composition and structure may affect ecosystem services such as nutrient cycling and soil fertility through alterations in the mechanism underlying plant-soil microorganism interactions (Aponte et al., 2011).

Figure 1.7. A schematic of plant-soil-microorganism interactions. Vegetation provides substrates for microbial decomposers via exudation of labile C and input of plant detritus and also modifies key soil properties and environmental conditions. Consequently, plant cover determines a specific soil microbial community structure and physiology that, in turn, control nutrient availability for plants by carrying out litter decomposition and soil organic matter (SOM) mineralization processes. These interactions constitute the mechanism underlying ecosystem processes such as the biogeochemical cycling that holds forest productivity and controls greenhouse gas (GHG) fluxes.

Vegetation directly influences the habitat and resources of soil organisms through the rhizodeposition of recently assimilated C and the quality (i.e. composition and stoichiometry) and quantity of the litter provided to the soil (Curiel Yuste et al., 2012), as well as by impacting

hydrological processes and surface soil temperatures (Van der Putten et al., 2013). Thus, plant species determine the composition and physiology of the soil decomposer community that, in turn exert an important role in biogeochemical cycles and forest productivity through the nutrient supply derived from litter decomposition and SOM mineralization processes (Kaiser et al., 2010). Consequently, there are specie-specific functional linkages between vegetation and soil microbial communities in decomposing litter, forest floors, mineral soil and rhizosphere through which arise the soil processes (Prescott and Grayston, 2013). Main determining factors associated with differences in microbial communities are soil pH, base cation content and whether the trees are broadleaf or coniferous (Prescott and Grayston, 2013; Rousk et al., 2010). The functional traits of needleleaf and broadleaf litter develop distinct microbial communities during their decomposition that are further adapted to decompose the type of litter produced on their environments (i.e. the home-field advantage, HFA), so litter decomposes faster on their own site due to this specialization (Ayres et al., 2009; Barba et al., 2015; Veen et al., 2015).

Normally, soils with large amounts of available organic C and nutrients hold copiotrophic communities –i.e. r-strategist– that have high growth rates and low resource use efficiency, whereas soils where organic C quality and/or quantity is low hold oligotrophic microbial communities –i.e. K-strategists– that are organisms with low growth rates (De Vries and Shade, 2013; Fierer et al., 2007). Then, microbial communities that have a high proportion of fungi compared to bacteria and a high proportion of gram-positive bacteria compared to gram-negative bacteria are associated with nutrient poor conditions –i.e. high soil C:N ratio– and recalcitrant soil C (De Vries and Shade, 2013; Six et al., 2006). In this regard, coniferous species are generally considered to be especially effective at storing C in soils in part because of the high abundance of fungal groups in the soil relative to bacteria (Díaz-Pinés et al., 2014) that are indeed associated to the large contents of recalcitrant compounds in their litter (Schneider et al., 2012; Stockmann et al., 2013). However, recent studies highlight that plant-soil-microorganism interactions are much more complicated than those relationships studied so far, especially if responses to changing environmental conditions are taken into account (Barba et al., 2016; Creamer et al., 2015; Curiel Yuste et al., 2010; Sardans and Peñuelas, 2013).

Soil microbial community may show an array of responses when challenged with disturbances expected with global environmental shifts: resistance –microbial structure remains unchanged–, resilience –microbial structure changes but recovers after the disturbance–, adaptation –microbial structure changes along with its functionality–, and redundancy –microbial structure changes without shifts in functionality (Allison and Martiny, 2008). It is supposed that the relative dominance of r- and K-strategists in the soil environment may give clues for predicting the response of soil functioning to global change although this still needs to be validated (De Vries and Shade, 2013). Therefore, significant challenges remains both in characterizing the microbial

communities in the ecotone under study and in understanding how forest secondary succession might affect long-term processes such as litter decomposition and mineralization of soil stored C.

Mitigating global change through forest management

Most temperate forests are currently accumulating C due to elevated atmospheric CO₂ concentration and N fertilization effect on productivity although there have been episodes of severe heatwaves that already reversed this tendency (e.g. the reduction in forest primary productivity caused by the heat and drought in 2003, Ciais et al., 2005). Moreover, there are evidences of an increasing risk of losing the C stored in standing trees and soils through the greater incidence of wildfires and forest dieback caused by the more frequent extreme climatic events, which are directly related with tree age, density and temperature in dry areas (Carnicer et al., 2011; Neumann et al., 2017). These perturbations may further be followed by pest outbreaks hindering the entirely recover of forests (Carnicer et al., 2011). An appropriate forest management including adaptation and mitigation strategies is essential to improve the resistance and resilience of forests to projected increases in climate stress and associated global changes. Although managers should carry out a flexible and adapted management to each species and geographical areas, general recommendations for Mediterranean forests are (Bravo et al., 2008; Sabaté et al., 2002; Vayreda et al., 2012):

- To adequately select species and regions of provenance for forestry taking into account the forecasted scenarios of climate change.
- To increase the density during plantation and regeneration stages and hence subsequently increase the opportunity to select better adapted trees. In later rotation stages, to reduce competition for resources by increasing the thinning regime and hence diminish the fire risk, avoid forest dieback, stimulate the growth of surviving trees and decrease subsequent mortality.
- To convert abandoned coppice stands to high forests by thinning.
- To shorten the harvest cycle and adapt the regenerative cuttings –e.g. lengthening the regeneration period or use nurse species.
- In forests where the risk of disturbances is low, a more conservative management may be recommended maintaining extended rotation periods and hence achieving longer residence time of the standing C stock.
- To increase the number of age classes and foster irregular structures.

- To promote mixed forest stands including coniferous and broadleaf species.
- To promote afforestation.
- To use predominantly recycled materials and renewable energy.

These options may reduce the potentially negative impacts derived from the global change in forest ecosystems by increasing the forest health, decreasing the mortality rates at the same time that increase the final wood yield (Sabaté et al., 2002). However, general management recommendations for increasing the amount of C stored belowground are not so obvious when should be applied to local forests and hence a better knowledge of both the current soil functioning and the potential impacts of forest secondary succession in belowground C and N dynamics is required to give more accurate recommendations in this respect.

Chapter 2

Objectives

The main objective of this thesis is to evaluate how the replacement of pure Scots pine forest by the more drought-tolerant Pyrenean oak may affect the functionality of the plant-soil-microorganism system considering the implications for the belowground biogeochemical cycles. Specifically, we address the effects of two main factors of global change, the vegetation shifts and the increased atmospheric N deposition, on soil ecological dynamics. To that end, we focused on studying GHG emissions, functioning and activity of microbial communities and properties of both, litter and soils, in an ecotonal transect between a pure Pyrenean oak forest and a pure Scots pine forest located in the Guadarrama Range (Central Iberian Peninsula). We combined experiments at stand scale with microcosm incubations (mixing soils and litter types) and laboratory analysis.

Former studies at the ecotone under study have provided relevant and high-quality knowledge about several aspects of the C balance at ecosystem level. In each forest type, the aboveground biomass C stocks, net C uptake, litterfall C rates, soil C stocks, mineral-stabilisation and biochemical recalcitrance of SOM and composition of soil microbial communities (for details see Chapter 3) have been already well characterised. At this point, it is critical to make progress on this line of research but with particular attention on the current soil system functioning and the potential impacts of global change on key functional soil processes.

To successfully accomplish the overall goal of this thesis, we established four specific objectives that were approached in different chapters following the conceptual outline of Figure 2.1:

1. Determine how seasonality and environmental conditions influence soil CO₂ efflux depending on tree species (Scots pine vs. Pyrenean oak) in pure forest stands. In each forest type, we distinguish the contribution of the autotrophic and heterotrophic components of R_s throughout a full year, determine their microclimate-dependence and quantify the apparent turnover rates of soil organic C. These forest stands allow to study seasonal dynamics of R_s since they share similar environmental characteristics being separated only by 1400 m distance (Chapter 4, blue line in Figure 2.1).
2. Identify the direct and indirect influence of soil properties on R_s and determine how increases in atmospheric N deposition may affect these causal relationships in monospecific stands of pine and oak. We are especially interested on evaluating the extent to which the anthropogenic acceleration of the N cycle affects the C cycle. This issue is particularly interesting in our study site since different responses could appear depending on the level of soil N availability –pine soils are N limited to a greater extent than oak soils– (Chapter 5, green line in Figure 2.1).

3. Analyse how litter decomposition takes place in terms of microbial community assembly, mass loss rates and nutrient release. It is also intended to know the influence of sites (pure Scots pine vs. pure Pyrenean oak forests) and litter types (only-leaves, leaf:needle litter mixture and only-needles) on decomposition (Chapter 6, yellow line in Figure 2.1).
4. Disentangle how C and N biogeochemical cycles could shift along the transitional stages of the global-change driven vegetation succession from pure Scots pine forest to pure Pyrenean oak forest. We evaluate the changes in microbial ecology and decomposition patterns (GHGs fluxes and nutrient cycling) derived from the input of litter with different chemical features (deciduous leaves vs. conifer needles) in soil inoculums that a priori hold different decomposing functionalities (Chapter 7, red line in Figure 2.1).

The acquired knowledge in this thesis will help forest managers and policy makers in the decision-making toward a management that includes not only production purposes or biodiversity promotion, but also other ecosystem services such as climate change mitigation through soil C sequestration.

Figure 2.1. Conceptual outline of the PhD thesis structure.

Chapter 3

Materials and methods

Land-use history and known aspects of C cycle on the Guadarrama Range

This PhD thesis focuses in a specific ecotonal area between Pyrenean oak –an endemic marcescent oak from the Iberian Peninsula with high root-resprouting capability– and the widely distributed Scots pine in the Guadarrama mountains, which are located on the northern slopes of the Central System Range in Spain (Figure 3.1). This experimental area was chosen because ecotones between ecosystems are highly suited for testing global change impacts on forests since avoid potential bias due to variabilities in site-specific factors (Neilson, 1993) such as parent material (Rasmussen et al., 2007), soil properties (Delgado-Baquerizo et al., 2015), macro-climate (Berger et al., 2015) and former land-use (Ortiz et al., 2016). Moreover, forest dynamics are expected to be faster in Mediterranean ecotones because constitute highly sensitive areas to climatic changes in particular due to both multi-year droughts and recurrent summer droughts (Allen et al., 2010). Specifically, our study site hosts one of the Scots pine lowermost altitudinal and southernmost geographical distribution limits (Ruiz de la Torre, 2006) making these pine stands more vulnerable to the future dryer and warmer conditions. Overall, this location is highly suitable to fulfil the objectives of this thesis described in Chapter 2.

Figure 3.1. Location of the experimental forest stands in the M.U.P. N° 1 Matas (1300-1350 m.a.s.l. 40° 51' N, 4° 3' W; H30, ETRS89), inside the Sierra the Guadarrama National Park (Real Sitio de San Ildefonso Municipality, Segovia Province, Spain).

The Scots pine forests in Valsain mountains have shown millennial stability only suffering decline episodes caused by the combined effects of fire and grazing (Morales-Molino et al., 2017). At 1890, the preservation and distribution of pine stands were favoured by planned forest management to timber production of high economic value (Alvarez et al., 2014). At low altitudes, the Pyrenean oak stems were removed when appeared interspersed with the pine to foster pure Scots pine woodlands. A forest reserve was established in 1980s and the frequency and intensity of selective cuttings in pine stands were reduced in order to give priority to other ecosystem services (Sánchez-Salguero et al., 2015b). On the other hand, the Pyrenean oak forests had a multi-centennial intensive coppicing with 8-13 year turns mainly to provide charcoal, firewood, woody forage and other derived products such as tannins (Ximénez de Embún, 1977). In the 1970s, the abandonment of ancestral practices with the rural exodus led the Pyrenean oak stands to have excessive stem density, over-aging steams, low forest structure, disequilibrium between belowground and aboveground tree organs, and low genetic diversity since root-resprouting was more frequent than sexual regeneration from acorn (Salomón et al., 2013). Nowadays, intensive thinning operations are being developed in an attempt to convert Pyrenean oak coppices into high forest (Alvarez et al., 2014). Thus, the long history of intense human interventions configure the current distribution, composition and structure of the ecotone forest under study conditioning the future vegetation dynamics when facing the new environmental conditions driven by the global change.

Figure 3.2. Climatic diagram of the experimental site for the study period 2014-2016. The orange line represents mean monthly air temperature and the blue bars denote mean monthly precipitation. Meteorological data were obtained from the meteorological station “Embalse del Pontón Alto” that is run by the Spanish Meteorological Agency and is located 9 km away.

In the study site, the Scots pine forests occupy areas between 1,400 m a.s.l., or 1,250 m along north facing humid valleys, and timberline at 1,900 m (Morales-Molino et al., 2017). The altitudinal belt of Pyrenean oak forests are placed at lower altitudes mostly located underneath 1,600 m (Costa et al., 2005). At these stands, the presence of understory is scarce except in open

areas where commonly appear the following species: *Crataegus monogyna* Jacq., *Cytisus oromediterraneus* Rivas Mart. et al., *Cytisus scoparius* (L.) Link, *Genista florida* L., *Cistus laurifolius* L., *Erica arborea* L., *Rosa* spp and *Rubus* spp. This emplacement presents a continental Mediterranean mountain climate with an inverse seasonality in precipitations and temperatures (Figure 3.2). Soils are shallow and acidic (pH values in KCl of 4.3 and 5.1 in topsoils under pine and oak, respectively) being predominantly Humic Cambisols (Soil Survey Staff, 2014) with sandy loam textures and having granites and gneisses as bedrocks. Previous studies carried out in the same experimental stands highlighted differences between forest types in soil physical properties that are summarised in Table 3.1.

Table 3.1. Main physical parameters of the first 50 cm of mineral soil at the experimental forest stands. Clay, silt and sand content (%) following USDA (Soil Survey Staff, 2014). Information collected from Díaz-Pinés et al. (2011).

Forest	Depth (cm)	Bulk density (g cm ⁻³)	Clay (%)	Silt (%)	Sand (%)	Fine gravels (< 6 mm) (g kg ⁻¹ soil)	Coarse gravels (< 6 mm) (g kg ⁻¹ soil)
Pyrenean oak	0-5	1.15	40	430	530	184	190
	5-10	1.33	40	450	510	175	224
	10-20	1.18	120	270	610	162	174
	20-30	1.13	100	280	620	172	158
	30-50	1.19	100	260	640	158	249
Mixed	0-5	0.73	150	270	580	182	58
	5-10	1.07	120	290	590	189	121
	10-20	0.98	130	270	600	130	170
	20-30	1.00	130	280	590	171	143
	30-50	1.00	120	250	630	172	167
Scots pine	0-5	0.68	100	320	580	155	118
	5-10	0.99	120	280	590	151	113
	10-20	1.00	140	270	590	128	158
	20-30	1.01	140	260	600	148	159
	30-50	1.10	100	240	660	174	165

In the experimental forest area, several aspects related to the C cycle at ecosystem level are already well characterized.

- Díaz-Pinés (2011) found that aboveground biomass C stocks were higher in the Scots pine stand than in the Pyrenean oak stand and intermediate in the mixed forest (158, 41 and 64 Mg C ha⁻¹, respectively). The legacy of past use on the general silvicultural characteristics in each experimental forest stand can be seen in Table 3.2.

Table 3.2. Main silvicultural characteristics of the experimental forest stands. Information collected from Díaz-Pinés et al. (2011).

Forest	Basal area (m ² ha ⁻¹)		Diameter at breast height (cm)		Density (Stems ha ⁻¹)		Height (m)	
	Oak	Pine	Oak	Pine	Oak	Pine	Oak	Pine
Pyrenean oak	17.2	-	14.5	-	932	-	9.7	-
Mixed	4.4	26.2	12.8	48.2	331	132	9.3	19.9
Scots pine	-	64.3	-	32.4	-	718	-	19.0

- The application of growth models demonstrated a higher net C uptake in the mixed forest than in Scots pine and Pyrenean oak ones (Díaz-Pinés, 2011). The combination of different functional traits of both tree species once they are successfully established may be behind the greatest growth rate and C uptake in the mixed forest, whereas again the historical coppice management in Pyrenean oak forest limits the C allocation to growth in the pure oak stand (Salomón et al., 2016a).
- According to the productivity and the basal area of the three forest stands, the highest litterfall C rate takes place in the pine stand (Díaz-Pinés et al., 2011a). Different litterfall patterns occur in the ecotone as maximum leaf-fall occurs in early-winter, due to the marcescent character of the Pyrenean oak that maintains its withered leaves in the branches to protect its buds from freezing (Costa et al., 2005), whereas the water stress that suffers the Scots pine produces the highest needle-fall period in summer (Díaz-Pinés et al., 2011a).
- The accumulation of C and N in the top 50 cm of soil (see Table 3.3) is strongly influenced by the tree species litterfall rates. Hence, C stocks are far greater in the pine than in oak forest, and intermediate in mixed forest (Díaz-Pinés et al., 2011b).
- The same study suggested that abiotic factors such as the background level of the bedrock may be driven the rather stable mineral-stabilisation degree of SOM across forests types, however some indicators suggested that the particulate organic matter from Scots pine soils is more biochemical recalcitrant than those from Pyrenean oak.
- The last aspect that is worth to be mentioned is the contrasting microbial community composition between the soils based on the phospholipid fatty acid profile (Díaz-Pinés et al., 2014). These authors indicated as possible main factors contributing to the most abundance of fungal groups in pine soils the different nature of litter provided to the soil by each forest type (marcescent-leaves vs. coniferous-needles), the contrasting C stocks across forest soils and the above-mentioned issues related to both the stabilisation and recalcitrance of SOM.

Once these general aspects of the C cycle at the ecotone are well characterised, new experiments are necessary to deepen in the specific drivers that are currently controlling the

belowground C turnover rate under each pure forest and in the potential impacts of a future upward shift of the oak into the pure pine stand.

Table 3.3. Soil C and N stocks in the experimental forest stands (forest floor plus first 50 cm of mineral soil). Information adapted from Díaz-Pinés et al. (2011).

Forest	Depth. (cm)	Soil C stock (Mg ha ⁻¹)	Soil N stock (Mg ha ⁻¹)	Soil C:N ratio
Pyrenean oak	Forest floor	1.3 ± 0.7	- -	-
	0-5	14.0 ± 2.1	1.1 ± 0.2	13.0
	5-10	7.0 ± 0.8	0.6 ± 0.1	11.5
	10-20	8.1 ± 2.7	1.0 ± 0.4	8.2
	20-30	5.8 ± 2.5	0.7 ± 0.2	8.4
	30-50	4.3 ± 1.3	0.8 ± 0.4	5.4
	Total	40.6 ± 6.2	4.2 ± 0.8	9.4
Mixed	Forest floor	2.7 ± 2.2	- -	-
	0-5	20.3 ± 4.1	1.2 ± 0.3	16.9
	5-10	16.3 ± 1.7	1.2 ± 0.2	13.4
	10-20	15.4 ± 7.3	1.4 ± 0.6	10.8
	20-30	11.5 ± 7.2	1.1 ± 0.7	10.2
	30-50	16.0 ± 7.9	1.5 ± 0.7	10.5
	Total	82.2 ± 19.4	6.5 ± 1.6	12.3
Scots pine	Forest floor	9.7 ± 4.2	- -	-
	0-5	27.1 ± 4.2	1.1 ± 0.2	24.2
	5-10	19.8 ± 1.7	1.0 ± 0.2	19.1
	10-20	13.8 ± 3.4	1.1 ± 0.2	12.0
	20-30	10.4 ± 3.6	0.9 ± 0.3	11.4
	30-50	12.2 ± 3.3	1.2 ± 0.3	10.4
	Total	93.1 ± 9.2	5.4 ± 0.7	15.5

General methods

Experimental setup

The experiments carried out in this thesis took place at 1300-1350 m altitude in three circular experimental plots (40 m radius) established in a pure Pyrenean oak forest, mixed Pyrenean oak-Scots pine forest and pure Scots pine forest along a north-to-south transect of 1400 m length (Figure 3.1). In the two selected monospecific forests (i.e. the multi-stem Pyrenean oak forest and the even aged Scots pine forest, Figure 3.1), four different treatments designated as ‘control’, ‘root-exclusion’, ‘medium-fertilization’ and ‘high-fertilization’ were applied in four replicated squared plots each (1 m × 1 m). The 16 plots of each pure stand were established in January 2014 at a distance less than 2.5 m from tree stems and were randomly assigned to the treatments (mean distance between same treatment plots was 30 m, see Experimental plots in Annexes).

Two polyvinylchloride respiratory collars (20 cm diameter) per plot were inserted to a depth of 2 cm into the mineral soil. In total, 64 respiratory collars were permanently installed from 2014 to 2017 in order to monthly monitor soil CO₂ efflux using non-steady-state through-flow chambers coupled to a portable infrared gas analyser (Figure 3.3a). In addition to the measurements of soil CO₂ efflux in the square plots, three annual soil samplings were performed in the early spring of the years 2015, 2016, 2017 using core samplers and two sampling depths 0-5 cm and 5-10 cm (Figure 3.3b-c). Soil chemical (C and N pools, nutrients and pH) and biological properties (microbial biomass, microbial C:N ratio and root biomass) were analysed in collected soil samples in order to evaluate the effects of the applied treatments. Soil temperature was monitored on hourly basis throughout the study period using three iButton sensors per pure forest stand (for more details see Chapter 4 and Chapter 5).

Figure 3.3. Details of the fieldwork carried out during a) the measurements of soil CO₂ efflux and b-c) the annual soil samplings.

Root-exclusion treatment

The root-exclusion treatment (Figure 3.4) was used to physically isolate the respiration of tree root systems from total soil respiration as proposed (Hanson et al., 2000). This methodology was used for *in situ* studying total soil respiration (R_s , measured directly in control plots), heterotrophic respiration (R_H , measured directly in trenched plots) and autotrophic respiration (R_A , calculated as the different between the total and heterotrophic components). The main advantage with this technique is to consider the effect of the seasonal variability. At the same time, it is cheap and reproducible allowing the comparison between different ecosystems. However, it is relatively severe since alters the plant-soil system and may therefore produce disturbances in the soil water balance as well as in the soil microbial community (Díaz-Pinés et al., 2010).

In January 2014, a narrow ditch was dug 40 cm down at trenched plots and all roots entering the inner plot were cut. To prevent the ingrowth of new roots, an anti-root geotextile was vertically placed and then the trench was refilled with the previously extracted soil. A clipping of the surface vegetation was periodically performed during the experiment, to ensure that weeds

did not develop in the trenched plots. The monthly measurements of soil CO₂ efflux in both trenched and control plots began after 4 months of the root-trenching, this way the pulse of CO₂ released from the decomposition of severed roots was not registered (Butler et al., 2012; Hanson et al., 2000), and continued throughout the following 36 months (April 2014 – April 2017).

Figure 3.4. Implementation stages of the root-exclusion treatment in trenched plots at the Pyrenean oak stand: a) delimitation of the square plot and installation of respiratory collars, b) 40-cm trench excavation at the plot boundary, c) vertical placement of the anti-root geotextile, d) trench backfilling using the same soil previously digged and e) surface clipping of weeds (additional details are provided in Chapter 4).

Nitrogen fertilization treatment

One of the major components of the anthropogenic global change is the acceleration of the N cycle (Phoenix et al., 2012). Accordingly, forest ecosystems are subjected to increased atmospheric N deposition that may affect both their structure and functioning (Ochoa-Hueso et al., 2011). A field experimental N manipulation study was conducted to assess the short-term effects of the rising N atmospheric deposition on soil respiration and soil properties from both the pure Scots pine and Pyrenean oak stands. Along three years of experiment, from the beginning of June 2014 to the ending of May 2017, three doses of ammonium nitrate fertilizer were supplied according to the most pessimistic scenario SRES A2 forecasted by the IPCC at the Mediterranean basin in 2030 that is 20 kg N ha⁻¹ year⁻¹ (Bobbink et al., 2010; Phoenix et al., 2012, 2006). Given the 10-15 kg N ha⁻¹ year⁻¹ of background N deposition for the study site (García-Gómez et al., 2014), the control, medium- and high-fertilization plots were treated with 0 (water-only), 10 and 40 kg N ha⁻¹ year⁻¹, respectively. Annual N additions were split in quarterly applications (March, June, September and December, working around these dates when climatic conditions made it necessary). The N fertilizer was dissolved in 2 L of water and then a backpack sprayer was used to evenly apply the solution as wet deposition over an area of 2 m × 2 m and thus avoid the edge

effect on the 1-m² treatment plots. Once the experiment was underway, the soil CO₂ efflux were monthly measured in every respiratory collar, besides additional measurements were carried out within the following 24 hours after individual application of N fertilizer. Moreover, the soil properties in each treatment plot were annually determined (see Chapter 5 for more details).

In situ decomposition experiment using litterbags

During the global change driven secondary forest succession from pine to oak, the forest floor horizon may be physically and chemically modified by the different litter nature that each vegetation type provides to the soil. An experimental study using litterbags was performed to assess the alterations in the activity and structure of the litter-specific microbial communities and to evaluate the tree-species influence along the litter decomposition processes (Figure 3.5).

Figure 3.5. Litterbags (20 × 20 cm containing 10 g of air-dried senescent litter) with identification metal labels and disposed in triplets (only-leaves, leaf:needle litter mixture 1:1 and only-needles) joined together with a polyethylene rope and anchored to the soil at a) pure Scots pine stand and b) Pyrenean oak stand.

Specifically, three types of litterbags (only-leaves, leaf:needle litter mixture 1:1 and only-needles) from both tree species were placed beneath the pure experimental stands of Scots pine and Pyrenean oak (Figure 3.1). The litterbags were retrieved from the field at four-month intervals along two years of decomposition (from April 2014 until April 2016). A total of 144 litterbags (2 habitats × 3 litter types × 4 replicates × 6 collections) were analysed for changes in the microbial community composition, mass loss, total contents of C and N, chemical composition and thermal stability indices of the litter (see Chapter 6 for further information).

Laboratory incubation experiment using microcosms

The oak altitudinal expansion into the pure pine forest may also have important consequences for the belowground C and N cycling that currently are difficult to predict. A full-factorial microcosm approach was performed to detect how changes in aboveground litter input caused by tree species replacement could affect soil functioning as well as C and N pools. In order to isolate this particular climate-change related perturbation from the rest of the potential sources of variability that occur in the field, we incubated (20 °C and 50% water-filled pore space) 12 types of microcosms consisted of stainless steel cylinders (187.3 cm³) filled with field-moist soil inoculums and mixed with senescent litter.

Figure 3.6. Subset of the microcosms representing the sequential habitat of the vegetation succession. Soils from oak forest (SO), mixed forest (SM) and pine forest (SP) amended with leaf (Lv), mixed (Mx) and needle (Nd) litter.

Soil inoculums were collected from the three experimental stands identified in the study ecotone transect (pure Pyrenean oak forest, mixed pine-oak forest and pure Scots pine forest). The soil inoculums were independently amended with four types of withered litter (oak leaves, mixed 1:1 needles-leaves, pine needles and control with no litter). Specifically, a subset of the microcosms were used to recreate the chronological sequence of the vegetation change (Figure 3.6). After a 54-day incubation period, we examined the changes across the chronosequence on microbial community structure, total and extractable C and N contents and the production of soil GHGs (CO₂, CH₄ and N₂O). This experiment was developed at the facilities of the Austrian Research Centre for Forests (Department of Forest Ecology and Soils, Vienna, Austria) under the supervision of Dra. Kitzler (see Chapter 7 for more details).

Data analysis

The statistical techniques used to analyse the dataset of each experiment were mainly parametric models, ranging from simple linear models to generalized linear models, including mixed models whenever there was a hierarchical or grouping structure in the data. In those cases where the parametric assumptions were not complied, the Kruskal-Wallis test was used instead. Moreover, *post hoc* tests were performed to further detect any significant differences among factor levels. In addition, multivariate statistical methods (e.g. principal component analysis and redundancy analysis) were applied on soil microbial communities. The Spearman's rank correlation coefficient and the Pearson's product-moment correlation coefficient were also calculated between some study variables. On the other hand, structural equation modelling (SEM) combined with multigroup analysis were used to evaluate the direct and indirect influence of environmental variables on soil respiration and to determine how N supply may affect these causal relationships.

The fit and evaluation of SEMs were performed with the Amos 22.0.0 software (SPSS, IBM Corporation, Meadville, USA) while the rest of the statistical analyses as well as most of the plots in this document were conducted in the statistical platform R version 3.4.0. (R Core Team, 2017) using a large battery of R packages (e.g. *ggplot2*, *lattice*, *car*, *stats*, *PMCR*, *nlme*, *lsmeans*, *Hmisc* and *vegan*, among others). A more detailed description of the data analysis is included under the appropriate section of the Chapter 4, Chapter 5, Chapter 6 and Chapter 7.

Chapter 4

Disentangling the effects of tree species and microclimate on
heterotrophic and autotrophic soil respiration in a
Mediterranean ecotone forest

Abstract

Understanding how forest ecosystems influence the response of soil respiration (R_s) to climate drivers is essential to accurately predict soil carbon dioxide (CO_2) fluxes in a changing environment. This is particularly crucial in areas of contact between coniferous and broadleaved forests in the Mediterranean region which are already experiencing a warmer and drier climate. We present a case study in a Mediterranean ecotone forest where R_s components –heterotrophic (R_H) and autotrophic (R_A)– were monitored beneath pure Scots pine (*Pinus sylvestris* L.) and Pyrenean oak (*Quercus pyrenaica* Willd.) stands using the root-trenching method. We used generalized linear models to predict the soil CO_2 efflux based on interactions between soil water content (SWC) and soil temperature. Regardless of the tree species, we found that the strong and inverse water availability and thermal seasonality of the Mediterranean climate intensely constrained soil microbial activity. The incorporation of the soil temperature-moisture covariation thus greatly improved the quality of the models compared to approaches that consider soil temperature alone. We also identified species-specific responses influencing both the total amount of R_A and its sensitivity to environmental variables. Root respiration in the Scots pine stand showed greater vulnerability to the decline in SWC throughout the summer than in the oak stand. The R_A in the Pyrenean oak stand was mainly limited by low soil temperatures in winter, indicating low maintenance rates during vegetative dormancy. Mean annual R_H rates were highest in the Scots pine stand, probably driven by the larger litterfall rates and soil carbon (C) stocks; however, the apparent turnover rate of soil organic C in the oak stand was almost twice as fast as in the pine stand. While our observations are limited to a case study, our work shows that both soil moisture and forest composition can significantly control the temperature dependence of R_s components under a Mediterranean climate.

Keywords

Soil CO_2 efflux, soil temperature and moisture, root-trenching, carbon turnover rate, forest succession, global change, ecosystem ecology.

Highlights

- Soil temperature and SWC covariation improved the quality of R_s models.
- R_s components showed tree species-specific responses to soil microclimate conditions.
- Pine R_A was more vulnerable to soil water deficit during summer than oak R_A .
- Soil microclimate constraints led to strong R_H seasonality.
- Turnover rate of soil C in the oak forest was twice that of pine soils.

Introduction

Land cover changes in forests occur to a large extent as a result of climate change and modifications in forest practices (Allen et al., 2010; Peñuelas and Boada, 2003). Alterations in the dominant tree species can significantly alter soil respiration (R_s), which in turn affects the global carbon (C) cycle, since soils are responsible for 60–90 % of the carbon dioxide (CO_2) released into the atmosphere in terrestrial ecosystems (Goulden et al., 1996). Soil CO_2 efflux is the outcome of the metabolic processes of plant roots (autotrophic respiration, R_A) and microbial communities (heterotrophic respiration, R_H) (Hanson et al., 2000; Kuzyakov, 2006). It is therefore important to determine the contribution of each component to R_s and their dependence on climate drivers to gain a deeper insight into the effects of changes in forest composition on soil C cycling and respiration (Barba et al., 2016; Bond-Lamberty et al., 2004a).

It is well known that temperature is the main factor controlling soil metabolic activity, so the kinetics of released CO_2 have usually been modelled by a broad set of temperature-dependent functions (Fang and Moncrieff, 2001; Kirschbaum, 1995; O'Connell, 1990; Tuomi et al., 2008). However, the temporal variability of R_H observed in field experiments captures not only its dependence on soil temperature and resource quality, but also reflects the limitations on decomposition caused by soil water content (SWC) (Chang et al., 2014; Davidson and Janssens, 2006; Lellei-Kovács et al., 2016); e.g. drought restricts substrate availability while excess water slows oxygen diffusion for decay processes. Soil microclimate limitations at the field scale can also cause physiological stress to both microbes and plants, which may display different response behaviours in R_H and R_A (Bond-Lamberty et al., 2004a; Galiano et al., 2011; Manzoni et al., 2014). Studies investigating the combined effects of soil temperature and SWC on R_s components are scarce in Mediterranean and semiarid ecosystems, although both climate drivers may significantly influence soil C efflux (Chang et al., 2014; Lellei-Kovács et al., 2016; Rey et al., 2002; Tedeschi et al., 2006). There is therefore a need for experimental approaches that address

the dynamic interaction between soil temperature and SWC in decomposition processes and root activities in order to understand the seasonal variability of R_s in forest ecosystems.

Vegetation also controls R_s and its fractions through its influence on litter quantity and quality, nutrient availability, microbial decomposer community, carbohydrate allocation patterns, root biomass, hydrological processes and surface soil temperatures (Bardgett and Wardle, 2010; Fernández-Alonso et al., 2018a; Högberg et al., 2001; Salomón et al., 2015). On large spatial scales, the length of the plant-growing season is one of the major drivers of R_s (Chang et al., 2014; Goulden et al., 1996) as it regulates the gross primary production of the ecosystems (Reichstein et al., 2003). Gross primary production stimulates the total metabolic activity in the soil through the net primary production, which provides the inputs of above- and belowground litter for microbial decomposition (Chang et al., 2014; Reichstein et al., 2003), and through the fresh photosynthetic products or tree carbohydrate reserves that fuel the metabolic activity of the root systems (Phillips et al., 2016). Variations in seasonal patterns of R_s across species may thus be the result of differences in the response of R_A and R_H to soil microclimate variables (Bond-Lamberty et al., 2004a). These differences in the relative contribution of R_A and R_H to R_s could be particularly significant when comparing vegetation species with differences in leaf longevity such as coniferous and broadleaved forests (Curiel Yuste et al., 2004). It is therefore crucial to study the seasonal evolution of R_A and R_H in each forest type individually in order to improve the forecasts of R_s rates with the predicted changes in species distribution.

The ecotones between conifers and broadleaved species in the Mediterranean basin conform a particularly relevant case where the effects of plant functional traits and environmental constraints on R_s overlap. The contact limit between Scots pine forests (*Pinus sylvestris* L.) and Pyrenean oak forests (*Quercus pyrenainca* Willd.) on the Iberian Peninsula is a representative example of these ecotones. In these sites the Scots pine is located at one of its southernmost geographical distribution limits, and locally in its lowermost altitudinal range (Martínez García and Montero, 2000), hence extreme drought events in the summer can potentially lead to episodes of pine die-off (Allen et al., 2010; Martínez-Vilalta and Piñol, 2002). The ongoing intensification of climate-change-driven drought in southern Europe (Stagge et al., 2017) is aggravating water stress in Scots pine and leading to its replacement by oak species (Galiano et al., 2010) such as the Pyrenean oak, which is better adapted to physiological drought and continental temperatures (Sánchez Palomares et al., 2008).

A three-year long case study was conducted to subtract *in situ* R_A from R_s using the root-exclusion method (Ewel et al., 1987), and to monitor the soil CO_2 efflux in a specific ecotonal area between Scots pine and Pyrenean oak in the Valsaín mountains (central Iberian Peninsula). We aim to assess how seasonality and environmental conditions influence soil CO_2 efflux depending on the functional composition of the forest (Scots pine vs. Pyrenean oak stands). First,

we modelled the interaction between soil temperature and SWC on the R_s components under pine and oak stands. We expected species-dependent effects, since the relevance and role of environmental drivers differ between tree species composition given their contrasting ecological strategies. Second, we used fitted models to predict the daily estimates of R_A and R_H for both tree species from daily soil temperature and SWC. Third, we calculated annual cumulative R_H fluxes and compared the apparent soil C turnover rate in both forest types. We hypothesized that annual R_H would be greatest in the pine stand, coinciding with the substantial amount of C stored in this soil as shown in previous studies. We further hypothesized that soil C turnover would be higher in the oak soil since soil organic matter (SOM) is believed to be more stable in pine stands.

Materials and methods

Study site

This study was conducted in the Valsaín forests (40° 51' N, 4° 3' W, ETRS89) located on the north-western slopes of the Sierra de Guadarrama National Park (Central Range, Spain). The study area is at an altitude of about 1350 m a.s.l., where both Pyrenean oak and Scots pine stands are common. The mean annual temperature was 11.0 °C and the mean annual precipitation 621 mm for the study period (Embalse del Pontón Alto weather station, National Meteorological Agency, 9 km away from the experimental sites). Soils are shallow and acidic (pH values in KCl of 4.3 and 5.1 in topsoils under pine and oak respectively (Díaz-Pinés et al., 2011b)) and are Humic Cambisols (Soil Survey Staff, 2014) with sandy loam texture developed on siliceous bedrock, mostly granite and gneiss.

We selected two monospecific forests of Scots pine and Pyrenean oak that were representative in terms of past and current management –oak coppicing for charcoal and pine shelterwood for timber production– on stand structures in the Guadarrama Range, with reduced environmental confounding factors (e.g. slope, altitude, bedrock and climate) since they were 1400 m apart. Soil organic matter content varied widely between the forests, especially in the upper soil layers (Table 4.1), resulting in a stock of 40.6 Mg C ha⁻¹ in the oak stand and 91.7 Mg C ha⁻¹ in the pine stand (forest floor and 0-50 cm of mineral soil (Díaz-Pinés et al., 2011b)). The Scots pine stand is managed under a uniform shelterwood system. The Pyrenean oak stand has traditionally been coppiced since the 12th century (Ximénez de Embún, 1977), although this practice was abandoned in the 1970s. The oak stand has around 60-year old stems, 17.2 m² ha⁻¹ basal area, 932 stems ha⁻¹ and 9.7 m mean height; the pine stand has about 80-year old trees, basal area of 64.3 m² ha⁻¹, 718 trees ha⁻¹ and 19 m mean tree height (Díaz-Pinés et al., 2011b).

Experimental design

The root-trenching method was used to measure *in situ* R_H and R_S following the protocol of Hanson et al. (2000). In January 2014, we established two different rooting treatments, designated as ‘control’ and ‘root-exclusion’, with four replicated plots each (1 m $\times$ 1 m) inside each forest stand at a distance of less than 2.5 m from the tree stems and with a mean distance of 30 m between the same treatment plots. In the root-exclusion treatment, we dug a ditch around the plot boundaries to physically isolate the soil matrix from the tree root system. A trench 40-cm deep was considered sufficient to gauge the extent of the effective root depth, since the soil depth ranges from 20 to 50-60 cm, and most of the roots are in the uppermost 15-20 cm of the soil profile. An anti-root geotextile was then placed vertically in the ditch and the outer part of the ditch was refilled with the previously extracted soil material. The surface was periodically clipped to ensure that herbs did not develop inside the trenched plots. Control plots –without the excision of roots– served to monitor R_S . In the root-exclusion treatment it was assumed that R_A was completely suppressed and hence solely R_H occurred –i.e. CO_2 efflux derived from the microbial decomposition of SOM and plant litter (Kuzyakov, 2006). The term R_A refers to root-derived CO_2 but may also comprise contributions from rhizomicrobial respiration from mycorrhizal fungi (Bond-Lamberty et al., 2004a; Kuzyakov, 2006).

Soil CO_2 efflux measurements

Two polyvinylchloride respiratory collars (20 cm in diameter) were inserted to a depth of 2 cm in the mineral soil in each plot. A four-month period was allowed to elapse before beginning soil respiration measurements in order to reduce the overestimation of R_H while the severed pre-existing roots were decaying (Butler et al., 2012; Hanson et al., 2000). The 32 respiratory collars (2 forest types $\times$ 2 treatments $\times$ 4 replicated plots $\times$ 2 respiratory collars) were monitored for soil CO_2 efflux on a monthly basis from April 2014 to April 2017 (35 measurement campaigns). The influence of additional C efflux from old root decomposition in our field-registered values should be minimal, since the measurements presented here lasted three years (Díaz-Pinés et al., 2010). Manual measurements of soil CO_2 efflux were taken using a non-steady-state through-flow chamber (Pumpanen et al., 2004). The respiratory collars were closed with a self-made chamber lid (10 cm height and 4.04 l volume) equipped with a rubber septum in the top where a stainless-steel needle vent was inserted to avoid overpressure and disturbing the CO_2 diffusion from the soil into the chamber headspace (Hutchinson and Livingston, 2001). The respiratory chamber was also fitted with outlet and inlet Teflon tubes (2.5 mm inner diameter) coupled to a portable infrared gas analyser WMA-4 (PP Systems, Hertfordshire, United Kingdom). A pump circulated the air through the closed-path system at a constant rate of 0.5 l min^{-1} during the closed-chamber period (5 min). The headspace CO_2 concentration was recorded

from one minute after the chamber closure to discard fluctuations immediately after the lid onset at 30-second measurement intervals. Soil respiration rates were calculated as the linear increase in CO₂ concentration in the time after the chamber closure, considering chamber volume, soil area, air temperature and air pressure, and expressed as mg CO₂-C m⁻² h⁻¹. No signs of flattening of the CO₂ concentration in the headspace were observed throughout the closure time. The portable analyser was calibrated prior to the sampling events using 400 µL L⁻¹ of standard CO₂ gas (Air Liquide, Madrid, Spain) under ambient air pressure. Soil respiration was measured between 07:30 and 16:30 hours (local time). Although diel patterns are unlikely to occur in closed canopy stands, special care was taken to fully randomize the sampling sequence across plots in each measurement campaign to account for potential diel fluctuations. Average soil CO₂ efflux at the plot level was computed for each measurement campaign to avoid within-group correlation in the statistical models during data analysis (n = 2 respiratory collars).

During the soil CO₂ efflux measurement campaigns, air and soil temperature and volumetric SWC were measured on the individual plots using probes, a Termistor Vertex 5989M (Herter Instruments, Barcelona, Spain) and a time-domain reflectometer (Field Scout TDR 100, Spectrum Technologies Inc., Aurora, IL, USA) respectively. Soil temperature was recorded on an hourly basis by means of three sensors randomly installed in each forest stand (DS1922L Thermochron, iButtonLink LCC, Whitewater, WI, USA). Soil temperature and SWC were measured at 10 cm depth in the mineral soil.

The root-exclusion treatment disrupts the root water uptake and may therefore produce disturbances in the soil water regime (Díaz-Pinés et al., 2010; Hanson et al., 2000). In order to avoid potential bias on measured CO₂ efflux caused by altered SWC in the trenched plots, we modelled the response surfaces of both R_S and R_H to the interaction effects between soil temperature and moisture measured in each sampling event (see data analysis in page 49). We subsequently used the fitted models to predict soil CO₂ efflux throughout the study period using corrected values of daily SWC calculated with a soil water balance (SWB) model.

Soil water balance for estimating soil water content

The daily SWC during the study period was estimated with a SWB model (Thornthwaite and Mather, 1955). The input variables for the model were: potential evapotranspiration (ET_o), maximum soil water holding capacity (WHC) and precipitation (P) in both forest stands. The model assumes that the depletion of SWC occurs exponentially on dry days when P < ET_o, whereas there is a direct increase in SWC on humid days when P > ET_o (Botey et al., 2011). The ET_o was calculated according to the modified equation of Penman-Monteith (Allen et al., 1998), based on meteorological variables registered at a nearby weather station (Embalse del Pontón Alto). Given that soil forests may present irregular properties at depth, the WHC was calculated

from texture, organic matter content, slope and permeability of soil layers at different soil depths (0-10, 0-20, 0-30 and 0-50 cm) following the physical model proposed by Gandullo, (1994) (see also Domingo et al., 2006). The SWB was calculated from January 2014 (four months before the start of soil CO₂ measurements) coinciding with water-saturated conditions (SWC = WHC) in soils, as is common in winter in both forests. After initializing the SWB model for 4 months, we assumed the values predicted for daily SWCs were already adjusted to current field conditions at the onset of the soil CO₂ measurements. Estimated SWCs calculated with the WHC for soil properties in the first 30-cm of soil (Table 4.1) gave the best coefficients of determination with measured SWCs using the TDR probe ($R^2 = 0.77$ and 0.79 in oak and pine forest stands respectively; p -value < 0.001).

Table 4.1. Soil parameters required to calculate soil water holding capacity (WHC) in each forest stand. Compiled with data from Díaz-Pinés et al. (2011b) and methodology from Gandullo (1994).

Tree specie	Slope	Depth (cm)	Clay (%)	Silt (%)	SOM (%)	Fine-earth (< 2 mm)	WHC (mm H ₂ O m ⁻¹ depth)
Oak	0.18	0-5	4	43	3.9	73	27.3
		5-10	4	45	1.8	71	24.2
		10-20	12	27	1.0	75	45.7
		20-30	10	28	0.8	75	43.5
		0-30					140.7
Pine	0.21	0-5	10	32	12.8	73	40.5
		5-10	12	28	5.5	74	29.7
		10-20	14	27	2.4	74	51.4
		20-30	14	26	2.1	74	49.6
		0-30					171.2

SOM: Soil organic matter content.

Data analysis

Differences in soil temperature and SWC across seasons both within and between tree species were evaluated separately with the non-parametric Kruskal-Wallis test, using Dunn's test for pairwise comparisons of mean rank sums (*PMCR* package, Pohlert, 2015). Annual and seasonal differences in soil microclimate variables between treatments were also assessed using the non-parametric Kruskal-Wallis test. Spearman's rank correlation (ρ) was performed between soil temperature and SWC for each forest stand. Seasonal delimitations were established according to their astronomical definition (March 21st, June 21st, September 21st and December 21st) since they fit the seasonal fluctuations of soil microclimate conditions.

We used generalized linear models with gamma error distribution to relate soil CO₂ efflux to soil temperature, SWC and treatment (*stats* package, (R Core Team, 2017)). An

individual model was built for each forest to account for differences in the relationships between R_S components and soil microclimate. We tested two biological responses as a function of soil temperature: (1) an exponential increase in R_S components –biological activity increases without any limit–; and (2) a second-order exponential growth of R_S components according to the Gaussian function (O’Connell, 1990) – R_S reaches its maximum at a given temperature, above which it declines with any further increases in soil temperature. We used the logarithmic link function in the models to linearize the exponential relationship between R_S and the independent variables. We included the categorical variable Treatment and its interaction terms with all the independent variables in the model formulations. We fitted additional generalized linear models to examine the potential interactive effects between SWC and soil temperature on R_S components. We introduced the SWC as either first- or second-order exponential functions. The combination of the two temperature-dependence functions with no effect, a linear effect or a quadratic effect of SWC resulted in six separate fitted models for each forest (Table 4.3).

In a second step, we compared the performance of the models in terms of the reduction in the corrected Akaike Information Criterion (AICc) (Akaike, 1973; Burnham and Anderson, 2002). The models were selected by calculating the Akaike weights (W_{AIC}) (Johnson and Omland, 2004) based on two sets of candidates (Lellei-Kovács et al., 2016): models that considered only temperature-dependence functions, and the six models together. The confidence set of best candidate models for the observed data included all the models with a W_{AIC} within 10 % of the highest. We examined the explained deviance (D^2) and the significance of each fitted coefficient in the model selected. An iterative process of stepwise removal of the least important terms was performed to select the optimal reduced model. The procedure was repeated until only significant coefficients remained in the model (Table 4.4, $p \leq 0.1$). After each step, we inspected the D^2 , the normal probability plot and the residual plot against the predicted values for the fitted models. We also examined the residual variability against the explanatory variables and the plots, and considered them to be a qualitative factor since measurements of soil CO₂ efflux were repeated on the same plots throughout the study. Residual heterogeneities were similar between plots in the fitted models, and we therefore assumed that the variation was ecologically irrelevant and do not consider plots as a random factor in the models (Bolker, 2008).

The optimal reduced models were used to estimate daily R_S and R_H for the whole study period (April 2014 – April 2017) and thus to correct any possible bias introduced on the measured soil CO₂ efflux by the higher SWC in root-exclusion plots. We used the daily average soil temperature (registered with the iButton sensors) and daily SWC (estimated by means of the SWB in page 48) as input data for the models. Daily rates of R_A were calculated by subtracting modelled R_H from modelled R_S . We aggregated the daily values of the R_S components to obtain cumulative seasonal and cumulative annual estimates, and calculated the average values for each day of the

year (DOY: 0 - 365 days, $n = 3$ growing seasons). We then estimated the mean relative contribution of R_H to R_S throughout DOYs. We also calculated the turnover rate for the soil C as the annual amount of R_H per unit of C stored in the forest floor, plus 0-50 cm of mineral soil (Metcalf et al., 2007; Olsen and Van Miegroet, 2010).

Annual and seasonal amounts of R_S , R_H and R_A were tested for differences between tree species using linear models (*stats* package, R Core Team, 2017). Linear models were also applied to test for differences in R_S components across seasons for each tree species. Normality and homogeneity of residuals for the linear fitted models were checked by the Shapiro-Wilk and Levene test (*car* package, Fox and Weisberg, 2011) respectively. Multiple comparisons between the means of the factor levels were made using Tukey's post hoc analyses.

All analyses were performed in the statistical platform R version 3.4.0. (R Core Team, 2017). The *ggplot2* package (Wickham, 2009) was used to generate 2D general plots, and 3D plots were created with the *lattice* package (Sarkar, 2008).

Figure 4.1. Seasonal evolution (SP: spring, SU: summer, AU: autumn and WI: winter) of environmental variables throughout the study period (Abril 2014 – April 2017). (a) Daily soil temperature (average values for each forest stand from data collected with iButton sensors at 10 cm in the mineral soil, $n = 3$). (b) Daily soil water content (points represent average values ($n = 4$) for each forest stand with bars indicating standard deviation from data collected with a TDR probe at control plots during measurement campaigns; lines represent daily estimated values from soil water balances (SWB)). (c) Daily precipitation (registered in the nearby weather station, Embalse del Pontón Alto).

Results

Variability of soil microclimate and soil CO₂ efflux over the study period

Soil microclimate variables at the study site showed pronounced seasonal fluctuations typical of the Mediterranean climate (Figure 4.1). Measured soil temperature and SWC had opposing seasonal patterns and hence were negatively correlated ($\rho = -0.85$ and $\rho = -0.73$ in Scots pine and Pyrenean oak forests respectively, p -value < 0.001). The SWC in both forests peaked in mid-winter (Figure 4.1), and then underwent a progressive decrease in spring until reaching its minimum value for the year with the onset of the free-rain period in July, when it remained low until October. Soil water content increased progressively with the start of the autumn rainfall until the soil moisture reached the WHC in winter in both forests. Soil temperature increased in spring, peaked around July and then gradually decreased in autumn, and remained low all winter. On the study site, annual average soil temperature was 11.8 ± 0.7 °C and annual average SWC was 8.2 ± 1.6 %, without any differences between tree species. With regard to seasons, in winter, soil temperature was higher in the pine than in the oak stand, whereas the opposite occurred in spring (Table 4.2). The SWC during the winter was higher in the oak than in the pine stand (Table 4.2). The effect of the root-exclusion treatment on the SWC was only significant in the Scots pine forest (Table 4.2), which showed higher SWCs in trenched plots than in control plots.

Soil respiration rates varied between forest types and showed large seasonal dynamics, from minimum values in winter to maximum values in spring (Figure 4.2a). Soil respiration ranged from 49.0 to 494.8 mg CO₂-C m⁻² h⁻¹ in pine and 35.4 to 572.1 mg CO₂-C m⁻² h⁻¹ in oak stands. Measured R_H showed a similar seasonal pattern to R_S with average values during the measurement period of 138.8 ± 5.3 mg CO₂-C m⁻² h⁻¹ in pine and 106.8 ± 13.5 mg CO₂-C m⁻² h⁻¹ in oak forests (Figure 4.2b).

Table 4.2. Mean $\pm$ standard deviations of hourly values of soil temperature (T, n = 3) and soil water content (SWC, n = 4) in both forests.

Soil variables	Forest	Treatment	Winter	Spring	Summer	Autumn	Annual
T (°C)	Pine	-	4.7 $\pm$ 0.9 A d	11.8 $\pm$ 0.7 B b	18.4 $\pm$ 1.1 a	9.8 $\pm$ 0.4 c	11.7 $\pm$ 0.7
	Oak	-	3.9 $\pm$ 1.2 B d	13.3 $\pm$ 0.8 A b	18.4 $\pm$ 0.6 a	10.0 $\pm$ 0.5 c	11.9 $\pm$ 0.9
SWC (%)	Pine	Control	11.4 $\pm$ 2.3 B a	10.6 $\pm$ 3.5 a	2.1 $\pm$ 1.0* b	8.7 $\pm$ 1.7 a	7.7 $\pm$ 2.1*
		Root-exclusion	12.3 $\pm$ 2.2 a	12.5 $\pm$ 2.1 a	4.5 $\pm$ 0.7* A c	9.2 $\pm$ 2.2 b	9.3 $\pm$ 1.6*
	Oak	Control	13.5 $\pm$ 1.4 A a	10.2 $\pm$ 1.5 b	2.7 $\pm$ 0.6 c	10.3 $\pm$ 0.8 b	8.7 $\pm$ 1.0
		Root-exclusion	13.6 $\pm$ 0.4 a	10.2 $\pm$ 1.9 b	3.3 $\pm$ 1.0 B c	10.8 $\pm$ 1.1 b	9.0 $\pm$ 1.1

Different lowercase letters denote significant differences in average values across seasons within the same tree species, while capital letters denote significant differences between tree species within the same season (Kruskal-Wallis, $p < 0.05$). Asterisks indicate significant differences in the SWC between control and root-exclusion treatment (Kruskal-Wallis, $p < 0.05$).

Figure 4.2. Soil CO₂ efflux measured over the 35 measurement campaigns in (a) the control plots (R_s : total soil respiration); and (b) the plots with root-exclusion treatment (R_H : heterotrophic respiration) under Scots pine (green circle) and Pyrenean oak cover (red triangle). Data are mean values of plots with bars representing standard deviation ($n = 4$). SP: spring, SU: summer, AU: autumn and WI: winter.

Choosing the statistical model for soil CO₂ efflux in each forest

In both forests, the quadratic temperature dependence of soil CO₂ efflux was shown unambiguously to be the model that best fitted the empirical data when considering this climate variable exclusively (Table 4.3), meaning that increased soil temperatures above an optimum temperature resulted in lower soil CO₂ efflux.

The models that included the interactive effects of soil temperature and SWC on soil CO₂ efflux greatly improved the quality of fit, as seen from the lower AICc values in comparison with models that do not consider SWC (Table 4.3). In the Pyrenean oak forest, the model for the interaction effect between linear SWC and quadratic soil temperature (model 4 in Table 4.3b) unequivocally showed the best fit ($W_{AIC} > 0.99$), while in the Scots pine forest all models with interactive SWC effects performed equally, irrespective of the relationship between soil temperature and SWC ($W_{AIC} > 0.01$). In most cases, the model choice strongly affected the predicted values of soil CO₂ efflux at the extremes of the ranges of the data set used in the model fitting procedure (Bond-Lamberty et al., 2004a). The values near the range limits of the soil microclimate variables are normally underrepresented in the data and this may hamper the detection of differences in the shape of the model under these circumstances. We chose the model

containing the quadratic interaction effect of soil temperature and SWC to explain soil CO₂ efflux in pine forests (model 6 in Table 4.3a) because it showed the narrowest confidence intervals with the soil at both its WHC in winter and its wilting point in the free-rain period (data not shown).

Optimal reduced models with all terms statistically significant at $p < 0.1$ for Scots pine and Pyrenean oak forests accounted for 64.8 % and 63.7 % of the variability in soil CO₂ efflux respectively (Table 4.4). The main effects of soil temperature explained most of the D^2 in the models of both forest stands (32.4 % in pine and 31 % in oak). The interaction effects between soil temperature and either SWC or Treatment in the oak stand suggest that optimal temperatures for soil CO₂ efflux vary differently with diverse levels of SWC in R_H and R_A components (Figure 4.3b, d and f). The main effects of SWC explained less D^2 in the pine (6.2 %) than in the oak stand (14.7 %). We only found an optimal SWC for soil CO₂ efflux in the pine stand, which was also dependent on soil temperature since the interaction effects between both microclimate variables significantly accounted for 2.4 % of the model D^2 (Table 4.4a). The SWC sensitivity of soil CO₂ efflux varied between treatments in the pine stand (Table 4.4a), indicating both a restriction of R_H above the soil WHC (Figure 4.3c), and a restriction on R_A at the soil wilting point (Figure 4.3e).

Table 4.3. Results of model comparisons in (a) Scots pine and (b) Pyrenean oak forests.

Tree species	Generalized linear models		Res. dev.	AICc	d.f.	D ² (%)	W _{AIC}	
(a) Scots pine	Null ~ 1		64.05	2830.1	254			
SWC is not considered								
Linear effect of T	T × Tto	(1)	52.66	2784.4	251	17.78	< 0.01	< 0.01
Quadratic effect of T	T × Tto + T ² × Tto	(2)	42.97	2735.1	249	32.92	1.000	< 0.01
Linear SWC interaction effect								
Linear effect of T	T × Tto × SWC	(3)	23.47	2581.8	247	63.37	-	0.196
Quadratic effect of T	T × Tto × SWC + T ² × Tto × SWC	(4)	22.6	2580.5	243	64.77	-	0.391
Quadratic SWC interaction effect								
Linear effect of T	T × Tto × SWC + T × Tto × SWC ²	(5)	22.81	2583.2	243	64.69	-	0.099
→ Quadratic effect of T	T × Tto × SWC + T ² × Tto × SWC + T × Tto × SWC ² + T ² × Tto × SWC ²	(6)	21.45	2580.9	237	66.51	-	0.314
(b) Pyrenean oak	Null ~ 1		82.83	3081.7	278			
SWC is not considered								
Linear effect of T	T × Tto	(1)	62.88	3007.7	275	24.08	< 0.01	< 0.01
Quadratic effect of T	T × Tto + T ² × Tto	(2)	45.11	2916.2	273	45.54	1.000	< 0.01
Linear SWC interaction effect								
Linear effect of T	T × Tto × SWC	(3)	35.44	2851.6	271	57.21	-	< 0.01
→ Quadratic effect of T	T × Tto × SWC + T ² × Tto × SWC	(4)	29.41	2807.1	267	64.50	-	0.997
Quadratic SWC interaction effect								
Linear effect of T	T × Tto × SWC + T × Tto × SWC ²	(5)	32.78	2838.0	267	60.43	-	< 0.01
Quadratic effect of T	T × Tto × SWC + T ² × Tto × SWC + T × Tto × SWC ² + T ² × Tto × SWC ²	(6)	29.23	2818.9	261	64.71	-	< 0.01

The W_{AIC} of the best candidate models are shown in bold. Arrows indicate the models selected for each tree species. T: daily soil temperature, Tto: treatment (control, root-exclusion), SWC: daily soil water content. Res. dev.: residual deviance, AICc: corrected Akaike information criterion, d.f.: degrees of freedom, D²: explained deviance, W_{AIC}: Akaike weights.

Table 4.4. Parameter estimates of optimal reduced generalized linear models for (a) Scots pine and (b) Pyrenean oak forests.

Terms	AICc	D ²	d.f.	Deviance	Estimate	SE	t-value	Sign.
(a) Scots pine model								
	2578.3	64.79	244					
Intercept		0.00	254	0.00	3.25	$4.10 \cdot 10^{-1}$	7.92	***
Treatment		0.13	253	0.08	$-2.11 \cdot 10^{-2}$	$7.72 \cdot 10^{-2}$	-0.27	
T		42.17	252	26.93	$1.61 \cdot 10^{-1}$	$5.46 \cdot 10^{-2}$	2.95	**
SWC		48.25	251	3.89	$1.23 \cdot 10^{-1}$	$6.79 \cdot 10^{-2}$	1.82	•
T ²		56.77	250	5.46	$-4.85 \cdot 10^{-3}$	$1.75 \cdot 10^{-3}$	-2.77	**
SWC ²		60.32	249	2.27	$-4.65 \cdot 10^{-3}$	$3.07 \cdot 10^{-3}$	-1.51	
SWC × Treatment		60.99	248	0.43				
SWC × control					-	-	-	
SWC × root-exclusion					$-1.22 \cdot 10^{-2}$	$7.46 \cdot 10^{-3}$	-1.63	•
T × SWC		63.07	247	1.33	$-1.73 \cdot 10^{-2}$	$1.04 \cdot 10^{-2}$	-1.67	•
T ² × SWC		64.14	245	0.68	$1.06 \cdot 10^{-3}$	$4.01 \cdot 10^{-4}$	2.64	**
T × SWC ²		64.16	245	0.02	$1.01 \cdot 10^{-3}$	$5.52 \cdot 10^{-4}$	1.83	•
T ² × SWC ²		64.79	244	0.40	$-5.18 \cdot 10^{-5}$	$2.53 \cdot 10^{-5}$	-2.05	*
(b) Pyrenean oak model								
	2804.76	63.72	271					
Intercept		0.00	278	0.00	2.81	$1.71 \cdot 10^{-1}$	16.42	***
Treatment		7.36	277	6.10	$8.14 \cdot 10^{-2}$	$1.48 \cdot 10^{-1}$	0.55	
T		23.71	276	13.54	$2.24 \cdot 10^{-1}$	$2.38 \cdot 10^{-2}$	9.41	***
SWC		41.41	275	14.66	$4.40 \cdot 10^{-2}$	$6.64 \cdot 10^{-3}$	6.63	***
T ²		62.49	274	17.46	$-6.21 \cdot 10^{-3}$	$8.74 \cdot 10^{-4}$	-7.11	***
T × Treatment		62.89	273	0.33				
T × control					-	-	-	
T × root-exclusion					$-5.68 \cdot 10^{-2}$	$2.66 \cdot 10^{-2}$	-2.13	*
T ² × Treatment		63.38	272	0.41				
T ² × control					-	-	-	
T ² × root-exclusion					$1.90 \cdot 10^{-3}$	$1.05 \cdot 10^{-3}$	1.81	•
T ² × SWC		63.72	271	0.28	$5.30 \cdot 10^{-5}$	$3.29 \cdot 10^{-5}$	1.61	•

AICc: corrected Akaike information criterion, D²: explained deviance, d.f.: degrees of freedom, SE: standard error, Sign.: significance codes (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, • $p < 0.1$). Explanatory variables (T: soil temperature, SWC: soil water content).

Figure 4.3. Modelled response of soil CO₂ efflux (Rs: total soil respiration, RH: heterotrophic respiration, RA: autotrophic respiration) to the continuous interaction effect between soil temperature and soil water content (SWC) in Scots pine (a, c, e, respectively) and Pyrenean oak forests (b, d, f, respectively), according to the fitted models in Table 4.4.

Figure 4.4. Modelled daily rates of heterotrophic respiration (R_H) according to the parameterized models in Table 4.4; and estimated daily rates of autotrophic respiration (R_A) as the difference between modelled R_S and R_H in Scots pine (a) and Pyrenean oak forest (b). Lines represent mean values, and ribbons show the upper and lower 95 % confidence intervals predicted by the models. SP: spring, SU: summer, AU: autumn and WI: winter.

Soil respiratory components: annual and seasonal patterns

Estimated daily rates of R_A throughout the study period differed between forest types and underwent strong seasonal fluctuations, revealing marked variations between the forests (Figure 4.4). R_A ranged from almost negligible values in both stands to 47.6 and 65.9 $\text{mg CO}_2\text{-C m}^{-2} \text{ h}^{-1}$ in pine and oak respectively. R_H followed similar patterns in soils from pine and oak stands (Figure 4.4) with minimum rates in winter (52.3 and 35.4 $\text{mg CO}_2\text{-C m}^{-2} \text{ h}^{-1}$ respectively) and maximum rates in late spring (246.5 and 161.8 $\text{mg CO}_2\text{-C m}^{-2} \text{ h}^{-1}$ respectively). The relative contribution of each soil respiratory component to R_S varied throughout the year (Figure 4.5). The highest contribution of R_A to R_S in the pine forest occurred in the period between mid-October (DOY 281) and end June (DOY 183), while R_A had a greater relative importance in the oak forest from April (DOY 83) to October (DOY 330).

Figure 4.5. Relative contribution of soil heterotrophic respiration to total soil respiration under Scots pine and Pyrenean oak tree species. Lines represent daily means of predicted values for the study period (April 2014 to April 2017, $n = 3$ growing seasons). WI: winter, SP: spring, SU: summer and AU: autumn.

For the study period (April 2014 – April 2017), annual predicted R_S was similar in the Pyrenean oak and Scots pine forests ($p = 0.125$, Table 4.5). R_A was significantly higher in the oak than in the pine stand ($p < 0.001$), whereas the opposite occurred for R_H , which showed lower values in the oak than in the pine stand ($p < 0.01$). However, the R_H for the stored C in the soils in the study (Díaz-Pinés et al., 2011b) pointed to a significantly faster turnover rate of soil C in the oak than in the pine forest ($p < 0.001$). On average, R_H accounted for 88.2 % of total R_S in pine and 75.8 % in oak stands.

Table 4.5. Seasonal and annual total soil respiration (R_S) and its components (R_A : autotrophic, R_H : heterotrophic) estimated for each tree species (mean $\pm$ SD, $n = 3$) and given as $\text{g CO}_2\text{-C m}^{-2}$. Turnover rate of soil C was calculated by considering the stored C in the forest floor plus 0-50 cm of mineral soil with stocks from Díaz-Pinés et al. (2011b).

Soil CO ₂ efflux	Winter	Spring	Summer	Autumn	Annual ($\text{g m}^{-2} \text{ year}^{-1}$)	Turnover rate (year^{-1})
Scots pine						
R_S	225 $\pm$ 25	A b 352 $\pm$ 26	a 229 $\pm$ 9.6	B b 247 $\pm$ 35	b 1.05 $\cdot 10^3 \pm$ 61	
R_A	41 $\pm$ 2 (18 %)	A ab 50 $\pm$ 8 (14 %)	B a 7 $\pm$ 1.9 (3 %)	B c 27 $\pm$ 11 (11 %)	B b 124 $\pm$ 13 (12 %)	B
R_H	184 $\pm$ 23 (82 %)	b 302 $\pm$ 18 (86 %)	A a 222 $\pm$ 7.8 (97 %)	A b 220 $\pm$ 25 (89 %)	b 929 $\pm$ 48 (88 %)	A 0.10 $\pm$ 0.01 B
Pyrenean oak						
R_S	167 $\pm$ 23	B c 313 $\pm$ 18	a 280 $\pm$ 2.3	A ab 243 $\pm$ 23	b 1.00 $\cdot 10^3 \pm$ 29	
R_A	25 $\pm$ 8 (15 %)	B c 84 $\pm$ 6 (27 %)	A a 74 $\pm$ 3.0 (26 %)	A ab 60 $\pm$ 5 (25 %)	A b 243 $\pm$ 7 (24 %)	A
R_H	142 $\pm$ 15 (85 %)	c 229 $\pm$ 13 (73 %)	B a 206 $\pm$ 0.9 (74 %)	B ab 183 $\pm$ 19 (75 %)	b 761 $\pm$ 22 (76 %)	B 0.19 $\pm$ 0.01 A

Different lowercase letters denote significant differences across seasons within the same tree species (linear models, $p < 0.05$). Capital letters denote significant differences between tree species within the same period.

Discussion

Modelling soil microclimate control on soil CO₂ efflux

Estimates of annual R_s in this study were $1.03 \text{ kg CO}_2\text{-C m}^{-2} \text{ year}^{-1}$ irrespective of the tree species, which is similar to reported values for other pine and oak forests in the Mediterranean basin (Barba et al., 2016; Rey et al., 2002). However, our results clearly showed that tree species largely controlled the temporal dynamics of the soil CO₂ efflux in the ecotone through specific responses of R_A and R_H to soil microclimate conditions. We were also able to detect common patterns in the response of CO₂ efflux to the environmental conditions, which can be attributed to the particular climate characteristics of the Mediterranean region. Soil temperature in both forests was largely the best predictor of soil CO₂ efflux; however the inclusion of the soil temperature-SWC covariation critically improved our modelling capability in this drought-prone ecotone.

Independently of the tree species, a strong seasonality of modelled R_s was observed, suggesting an apparent limitation to soil metabolic activity all year round except in spring, the only period when optimal soil temperatures and SWC occur simultaneously. The pattern observed in R_s may be basically explained by the temporal variability of R_H , since it accounted for more than 70 % of the total R_s in both forest stands. We also observed peaks of modelled R_H in autumn produced by occasional rainfall events at the beginning of the wet season (Almagro et al., 2009), when temperatures are still warm. This seasonal pattern for R_H in our study has been previously reported in other forests in the Mediterranean basin (Chang et al., 2014; Lellei-Kovács et al., 2016; Rey et al., 2002; Tedeschi et al., 2006). We identified low winter temperatures and low summer SWCs as important climate constraints that dampen the apparent temperature sensitivity of R_H and other factors that stimulate microbial decomposition, such as the timing of leaf and needle fall when there is an extra input of fresh organic matter (Saiz et al., 2007) and/or high-quality litter (Raich and Tufekcioglu, 2000).

Our results also suggest a higher sensitivity of R_A to low values of SWC in pine stands, which agrees with the known vulnerability of Scots pine to drought events (Sabaté et al., 2002; Vayreda et al., 2012). The low SWC values observed here are probably a combined result of the strong seasonality of the precipitation and the low WHC of these coarse-textured soils. High sand contents are common in large areas of the Iberian Peninsula (Ballabio et al., 2016) so we presume these respiratory patterns are occurring in other Scots pine and Pyrenean oak forests. However, additional case studies specifically addressing a broad range of soil textures are highly recommended to assess the extent of the effects of drought on R_A and R_H at the regional level.

Seasonal patterns of soil respiration components modulated by tree species composition

Although annual R_A made a modest contribution to total soil CO_2 efflux in our study (12-24 %), the differences in this component likely balanced out those of R_H , so similar annual R_S were found in both forest types. Two mechanisms are proposed to explain these differences in R_A : (1) the different root-to-shoot ratios derived from historical management (0.54 - 0.96 in Pyrenean oak coppices (Salomón et al., 2016a) vs 0.28 in Scots pine forests (Ruiz-Peinado et al., 2011)); and (2) the fact that physiological activity (including roots) is more severely constrained in pine than oak during the dry season, resulting in higher R_A rates in the Pyrenean oak stands. The R_A accounted for 24 % of R_S in the Pyrenean oak forest, similar to other oak coppices under a Mediterranean climate (Rey et al., 2002; Tedeschi et al., 2006). The 12 % annual contribution of R_A to R_S in Scots pine is also similar to data from old conifer stands in boreal forests (Bond-Lamberty et al., 2004a), but considerably lower than the 36 % contribution reported for Scots pine suffering die-off on the north-eastern Iberian Peninsula (Barba et al., 2016), or estimates from warm temperate forests (Bond-Lamberty et al., 2004b). The R_A contribution in both forests falls within the lowest values reported in previous research, which varied from 10 % to 90 % across vegetation types according to Hanson et al., (2000). To the best of our knowledge, our results for pine are among the lowest estimates in forest ecosystems, which may be due to the low volumetric SWCs (rarely above 14 %) which appear to have a more marked effect on R_A than on R_H .

The seasonal patterns for R_A were also significantly different depending on tree species. Water availability has been reported in previous research as a limiting resource for Scots pine growth in Mediterranean climates, mainly at its lowest elevations (Bogino et al., 2009; Camarero et al., 2010; Sánchez-Salguero et al., 2015a), which is consistent with the pattern of pine R_A throughout the year. High and sustained R_A rates occurred for pine whenever water was available even outside its growing season (Camarero et al., 2010). Rates of R_A in winter declined by only 20 % compared to rates in spring, probably because winter photosynthesis enables the pine to produce and accumulate carbohydrates during this unfavourable season (Sánchez-Salguero et al., 2015a). Since SWC in winter was close to WHC, the soil temperature was the constraining factor for pine physiological activity as shown by the R_A . Warm winters therefore appeared to promote higher rates of root physiological activity, and presumably have a positive effect on pine growth (Bogino et al., 2009). In early spring the warmer temperatures and high SWCs resulted in maximum rates of R_A , coinciding with the resumption of growth in the pines (Camarero et al., 2010). The seasonal trend in R_A then reached a turning point and showed minimum rates in summer, a period with overlapping soil water deficit and high evaporative demand. These minimum R_A rates are consistent with other ecophysiological responses observed in pine trees under summer drought, such as efficient stomatal closure to reduce transpiration (Poyatos et al.,

2005), low growth rates (Camarero et al., 2010; Sabaté et al., 2002; Vayreda et al., 2012) and needle fall period from May to September (Díaz-Pinés et al., 2011a). The abundant precipitation in the first half of the year may delay the time at which drought stress becomes critical in the pine growing season. Soil temperature throughout winter and soil water availability throughout summer therefore constitute the main limitations for R_A in Scots pine, closely linked to the main climate constraining factors for pine radial growth (Sánchez-Salguero et al., 2015a).

The Pyrenean oak stand showed a distinct temporal evolution of R_A throughout the year, which we assume can be attributed to different leaf strategies influencing root phenological patterns (Curiel Yuste et al., 2004). Thus, the lowest rates of R_A were registered during tree dormancy in winter. In early spring, with increasing soil temperatures and optimum soil moisture conditions, we found a progressive rise in R_A when the oak became phenologically active (Salomón et al., 2016b). This rise in R_A peaked roughly three weeks before budburst (bud break occurs around DOY 145), probably fuelled by the mobilization of carbohydrate reserves in roots (Zhu et al., 2012) since there was no fresh assimilation of photosynthates or sap flow before leaf expansion (Salomón et al., 2016b). In June, R_A contributed up to ca. 30 % to R_S , the highest proportion of the year. This is a time of high metabolic demand for leaf production (Lee et al., 2010) which lasts until photosynthetic activity is reactivated with leaf unfolding. Later in the summer, R_A rates decreased again across the drought period, which could be explained by the low fine root density and biomass attributed to the depletion of SWC in Mediterranean oak forests (López et al., 1998; Rey et al., 2002). The maximum contribution of R_A to R_S was again reached in mid-September, coinciding with the decreasing temperatures and the arrival of the autumn rainfall. We registered maintenance R_A rates when the oak entered dormancy during its litterfall period from October to December (Díaz-Pinés et al., 2011a).

Differences in R_A rates between species may not be completely reduced to plant phenology, since other plant traits and processes may also be involved, such as transport patterns of root-respired CO_2 through the xylem, or different physiological strategies to deal with summer drought (Grossiord et al., 2012; Poyatos et al., 2005; Ubierna et al., 2009). We show strong evidence here that SWC and forest functional composition strongly influenced the temperature dependence of R_A and thereby R_S , providing important insights into the possible consequences of changes in forest composition. However, these major differences in R_S between oak and pine observed in our study site may have been particularly apparent due to the mild winters favouring Scots pine root activity and the intense summer drought causing evident R_A responses. The generalisation of our results should therefore be treated with caution.

Soil C stocks and cycling

Confirming our hypothesis, annual R_H was highest in the pine stand, where litterfall rates (Díaz-Pinés et al., 2011a) and C stored in the topsoil (Díaz-Pinés et al., 2011b) are significantly higher than in the oak stand. However, the C turnover rate in soils under pines was roughly twice that of oaks, reflecting the functional traits of leaves and needles –i.e. the nitrogen and lignin content of plant detritus in the litter layer (Gallardo and Merino, 1992; Melillo et al., 1982)– which is closely linked to tree C and its strategy for obtaining nutrients (Cornwell et al., 2008; Fernández-Alonso et al., 2018a). This result contradicts the similarity in the potential decomposability of soil C between forest types that was found during an incubation experiment using mineral topsoil samples from the same study site (Díaz-Pinés et al., 2014). Some of the multiple factors affecting the apparent decomposability of soil C in the field and which may lead to differences in the results obtained under controlled conditions include changes in the composition and activity of the soil microbial community throughout the year, the presence of C pools of varying decomposability and physical protection, and the SWB. The lower decomposability of SOM of pine origin may thus have contributed over centuries to the accumulation of greater soil C stocks than in the oak stand (Curiel Yuste et al., 2005). Our findings suggest that an upward shift by oak in the ecotone, induced by either climate change or forest management, will affect the soil's capacity to store C; however, the magnitude of the impact could be partly offset by a further intensification of soil microclimate constraints for R_H –i.e. higher temperatures and extended periods of drought–, which may in turn slow the decomposition and release of soil C pools.

Conclusions

Our results clearly show different seasonal dynamics in soil metabolic activity between Pyrenean oak and Scots pine stands in a Mediterranean mountain area. Common patterns were also detected in the ecotone of both tree species, highlighting how the strong and inverse water and thermal seasonality of the Mediterranean climate constrains both microbial decomposition activity and root respiration. The consideration of the interaction effect between soil temperature and SWC was therefore critical for appropriately modelling the soil CO_2 efflux, and enabled us to detect a higher sensitivity of pine R_A than oak R_A to the summer drought period. The slow turnover rate of pine litter compared to oak-derived detritus may also have promoted soil C accumulation over the centuries. This greater understanding of the plant-soil system in the ecotone offers an insight into upcoming patterns that must be taken into account in future forest management. The forest's capacity to store soil C could be undermined by an oak expansion driven by current changing environmental conditions; however, the magnitude of the impact could be buffered by the further intensification of soil microclimate constraints on R_H .

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Chapter 5

Mechanisms driving soil respiration in response to short-term nitrogen additions in a Scots pine-Pyrenean oak ecotone forest

Abstract

Soil respiration (R_s) in forest ecosystems is strongly determined by nitrogen (N) availability. We investigated the causal influences between changes in soil properties (microbial biomass, total roots, pH, nitrate, ammonium, phosphate, potassium, total and extractable carbon and N) and the response of R_s to short-term N enrichment in the ecotone between the Scots pine and Pyrenean oak forests in Central Spain. We conducted a manipulative experiment using three doses of ammonium nitrate fertilizer to simulate enhanced N deposition rates: 0 (control), 10 (medium) and 40 kg N ha⁻¹ year⁻¹ (high) over the background deposition. Throughout three years of experiment, we monitored R_s and developed annual soil samplings. To test the causal relationships among studied soil variables in response to N fertilization we used structural equation models. We observed a pulse of R_s within the following 24 hours after the N-fertilizer spreading that only in the Scots pine forest displayed significant differences among treatments. The high-dose fertilization produced smaller stimulation of R_s than the medium-fertilization, which could be explained by negative effects of soil acidification and high concentration of inorganic N. The lower sensitivity of oak R_s to N additions may reflect differences in native soil stoichiometry between the forests –i.e. pine soils are N limited to a greater extent than oak soils. Moreover, we found significant differences in monthly R_s among treatments from February to May at the Scots pine stand, suggesting the strong climate seasonality as a potential factor constraining the effects of N enrichment through the limitation of soil biological activity. Hence, N additions were not related to significant changes on cumulative R_s in any of the forests. We further found that soil temperature explained larger variation of oak R_s indicating a greater dependency on the oak growing season than on N fertilization. Overall, our findings stressed the high R_s stability to short-term N enrichment in this Mediterranean ecotone forest. However, soil acidification and the substantial decrease in soil microbial biomass suggest that stronger effects in the C cycle may occur if chronic N enrichment persists in a longer run.

Keywords

Nitrogen deposition, fertilization, temperate forests, soil acidification, microbial biomass, carbon pools.

Highlights

- Three-level N fertilization experiment was conducted in a Mediterranean ecotone forest.
- Nitrogen additions caused soil acidification and decreased microbial biomass carbon.
- Cumulative R_s in the ecotone was resilient to short-term N additions.
- The response of R_s to N addition were conditioned by soil microclimate seasonality.
- Extractable organic carbon decreased following N additions only in pine soils.

Introduction

The use of fossil fuels and nitrogen (N) fertilizers for food production has significantly increased the transfer of reactive N from the atmosphere to terrestrial ecosystems (Galloway et al., 2008), including unintentional deposition in forest systems. The resultant increment in N availability may lead to soil acidification, biodiversity loss and leaching of base cations (Bobbink et al., 2010), especially if N saturation thresholds are surpassed. Furthermore, changes in the C cycling as a consequence of higher N inputs have been reported, namely in the form of enhanced C stocks including the living plant biomass and the soil profile (De Vries et al., 2009). There are two major mechanisms that may explain the larger C sequestration rates usually observed in temperate forests subjected to high N deposition rates: the increased wood formation and the slowdown of litter and soil organic matter (SOM) decomposition (Pregitzer et al., 2008). These mechanisms in turn lead to important variations in soil respiration (R_s) in most forests through both of its components autotrophic and heterotrophic –i.e. microbial and root respired CO_2 from soils to atmosphere (Janssens et al., 2010). However, responses between ecosystems are highly divergent and there is still a high uncertainty in the identification of the biological and chemical processes involved in the changes of soil CO_2 efflux with increased anthropogenic N deposition rates (Riggs and Hobbie, 2016; Waldrop and Zak, 2006).

Several meta-analysis investigating R_s in N manipulation experiments and along N-deposition gradients found different trends among forests biomes, ranging from declines of 10 % to unchanged effects over R_s (Janssens et al., 2010; Zhong et al., 2016; Zhou et al., 2014). Nitrogen addition mostly impedes R_s through three main aspects of the belowground C cycle: (1) declined rhizosphere activity with plant reductions in belowground C allocation in favour of aboveground tissue production (De Vries et al., 2009; Pregitzer et al., 2008), (2) changes in the abundance, composition and/or enzymatic spectra of soil saprophytic communities (Frey et al., 2004; Treseder, 2008), and (3) SOM stabilization via soil acidification and chemical protection

(Averill and Waring, 2018; Janssens et al., 2010). All these aspects support a negative relationship between R_s and N enrichment rates (Zhong et al., 2016) that are not universal since competing mechanisms with different responses of R_s have been described in N-limited as well as in N-saturated forest ecosystems (Högberg et al., 2006). Other specific environmental factors such as soil moisture and temperature may also contribute to disagreements in the effects of N on R_s among studies, due to constraints of soil microclimate conditions on plants and microbial activities (Liu and Greaver, 2009; Zhong et al., 2016; Zhou et al., 2014). Therefore, the prediction of R_s with N additions in forests containing different levels of N availability and whose biological activity is climate-constrained (e.g. the summer drought in the Mediterranean basin) is a challenge and needs further investigation.

Opposing effects of rising N deposition on belowground C cycling in temperate forests appear to result from site-specific characteristic associated with tree species such as biochemistry of detritus inputs, microbial community, soil pH and N availability (Averill and Waring, 2018; Bobbink et al., 2010; Yang et al., 2014; Zhong et al., 2016). In the broadleaved-coniferous ecotone forests between the Pyrenean oak (*Quercus pyrenainca* Willd.) and the Scots pine (*Pinus sylvestris* L.) in the Iberian Peninsula, N deposition has the potential to differently affect soil properties and R_s since the functionally dissimilar forest composition lead to differences in soil pH, N status and C decomposability (Díaz-Pinés et al., 2011b; Fernández-Alonso et al., 2018a). Moreover, at these emplacements successional changes are currently taking place as a result of Scots pine dieback induced by the combined effects of the increasingly harsher drought periods and land-use and forest management changes (Galiano et al., 2010; Ruiz-Benito et al., 2017). The ongoing vegetation shifts from pine to oak may have important consequences in the whole ecosystem functioning. It is therefore important to explore the mechanistic links between N-induced effects in R_s and tree species at these sensitive areas.

In this study, we conducted a multi-level manipulation fertilization experiment over three years to simulate the increment of atmospheric N deposition in soils from two neighbouring pure stands of Scots pine and Pyrenean oak at their altitudinal contact limit in Central Spain. We combined field measurements of R_s with soil sampling in order to evaluate if the short-term impact of anthropogenic N deposition on R_s could be explained through changes on soil microbes (soil microbial biomass and microbial C:N ratio) and soil characteristics (pH, nutrients, total and extractable organic C and N), while considering the different nature of the forests. We seek to elucidate whether the direct and indirect effects induced by N deposition in soil properties and R_s vary as a function of the plant-soil system in the ecotone. We conducted a three-level experiment of N addition: 0 (control), 10 (medium) and 40 kg N ha⁻¹ year⁻¹ (high) over the background N deposition. Our specific objectives were to (1) determine to which extent the simulated N deposition rates alter R_s in each forest; (2) analyse the changes produced in soil properties with

the N-enrichment; and (3) explore the causal influences between changes in soil properties and the R_s response to anthropogenic N deposition. We hypothesized that i) N deposition will reduce R_s through reduced microbial biomass and soil pH, but the magnitude of R_s changes would depend on both the initial soil N status and the fertilization level. Therefore, we further expected that ii) R_s would show different sensitivity to external N inputs between oligotrophic soils under pine and copiotrophic soils under oak; likewise, we hypothesized iii) a larger decrease in R_s with the high-fertilization treatment. Finally, we also anticipated iv) a high temporal variability in the response of R_s to N additions because of the marked seasonality of the Mediterranean climate.

Materials and methods

Study site

The N fertilization experiment was carried out in Valsaín mountains (Guadarrama Range, Central Spain, 40° 51' N, 4° 3' W, ETRS89) at 1350 m a.s.l., at the contact limit between pure forest of Pyrenean oak and Scots pine. This area has a continental Mediterranean mountain climate characterized by a mean annual precipitation of 648 mm and a mean annual temperature of 10.4 °C (mean for the study period 2014-2017). There is a strong an inverse water and thermal seasonality in which precipitation concentrates mostly from late autumn to mid spring (Chapter 3). The summer is warm and has a marked drought that lasts roughly 3 months (from July to September). The winter is cold and a thin snow cover may be present up to several weeks, although it is highly variable between years.

Experimental design

We chose two representative areas separated by a distance of 1400 m: a multi-stemmed Pyrenean oak forest and an-even aged Scots pine forest. The oak stand present a density of 932 stems ha^{-1} of around 60-year old, resulting of an earlier use as a coppice (Ximénez de Embún, 1977). The Scots pine stand is currently under management involving uniform shelterwood system that leads to 80 years old trees with a density of 718 trees ha^{-1} (Díaz-Pinés et al., 2011b). Soils are Humic Cambisols (Soil Survey Staff, 2014) formed on a siliceous bedrock with silty clay loam texture in the oak stand and sandy loam texture in the pine stand. The background atmospheric N deposition in this area ranges from 10 to 15 $\text{kg N ha}^{-1} \text{ year}^{-1}$ (García-Gómez et al., 2014). The IPCC scenarios forecasts atmospheric deposition rates up to 20 $\text{kg N ha}^{-1} \text{ year}^{-1}$ in the Mediterranean basin by 2030 (SRES A2, Bobbink et al., 2010; Phoenix et al., 2012, 2006).

We experimentally simulated rising N deposition by spreading fertilizer of ammonium nitrate (NH_4NO_3 34.5 % N, Fertiberia S.A., Puertollano, Spain) to squared plots (2 m x 2 m) in

the two adjacent Mediterranean forest stands. A total of 24 plots (2 forest stands $\times$ 3 treatments $\times$ 4 replicates) were established in January 2014 and randomly assigned to each one of the N fertilization treatments: control, medium-fertilization and high-fertilization (0, 10 and 40 kg N ha⁻¹ year⁻¹, respectively). Considering the background deposition (García-Gómez et al., 2014), plots were receiving approximately 10-15, 20-25 and 50-55 kg N ha⁻¹ year⁻¹, according to each treatment. Fertilization was split in doses applied in March, June, September and December; in each of which the N fertilizer was evenly sprayed over the whole surface of the plots as wet deposition using 2 L of NH₄NO₃ solution (equivalent to 0.5 mm rainfall). Untreated control plots received an equivalent application of water-only in every fertilization campaign. The experiment involved 12 fertilization events in three years, from June 2014 to May 2017.

Soil CO₂ efflux

Two polyvinylchloride respiratory collars (20 cm diameter) were installed in each plot at a depth of 2 cm into the mineral soil. To avoid edge effects, the collars were onset in the inner 1 m $\times$ 1 m area. The respiratory collars were monthly monitored throughout the experimental period (June 2014 – May 2017); additionally Rs measurements were also conducted 24 hours after each N-fertilization event, thus making a total of 45 measurement campaigns. The monitoring of Rs was carried out between 7:30 and 16:30 hours (local time) fully randomizing the sampling sequence across plots to account for potential diel fluctuations. We used a self-made chamber lid (10 cm height and 4.04 l volume; additional details of chamber design are provided in page 47) to create a non-steady-state through-flow chamber (Pumpanen et al., 2004) during Rs measurements. The chamber was connected to a portable infrared gas analyser WMA-4 (PP Systems, Hertfordshire, United Kingdom) with air circulating with a constant rate set to 0.5 l min⁻¹. The air CO₂ concentration was recorded every 30 seconds over a measurement period of 5 min. Soil respiration rates were calculated as the linear increase of CO₂ concentration in the chamber headspace, since no levelling off in the CO₂ concentration was observed over time. The first minute after chamber closure was discarded for the calculations to avoid initial fluctuations when closing the chamber. The individual plots were also monitored for soil moisture at 10 cm depth with a time-domain reflectometer (Field Scout TDR 100, Spectrum Technologies Inc, Illinois, USA) and surface air and soil temperatures at 10 cm depth with a Termistor Vertix 5989M (Herter Instruments, Barcelona, Spain). In each measurement campaign, Rs rates were averaged at the plot level to avoid spatial correlation in the statistical models in the data analysis (n = 2 respiratory collars). We computed averaged rates of Rs for every month of the year (Mg CO₂-C ha⁻¹ month⁻¹) and cumulative Rs for the whole study period using linear interpolation between consecutive measurement campaigns (Mg CO₂-C ha⁻¹).

Soil sampling and laboratory analyses

Three soil sampling campaigns were performed in the springs of 2015, 2016 and 2017, 24 hours after the application of N-fertilizer. We used a 1 m² sampling area centred in each plot and divided into a regular grid (0.10 m x 0.10 m). In each sampling day, any litter layer was removed and three cores of mineral soil (5 cm diameter) were taken in randomly selected spots to constitute a composite sample. The empty holes were filled with inert gravel material. Composite samples were split into two depths (0-5 cm and 5-10 cm), delivered in ice to the lab, sieved (mesh size < 2 mm) and stored at 4 °C until soil extractions for microbial biomass, ammonium (NH₄⁺) and nitrate (NO₃⁻) determinations were performed. Then, soil extracts were stored at -20 °C until analysis. Air-dried soil samples were used for the rest of the analysis.

Soil extracts with 0.5 M K₂SO₄ (1:5, w:v) were analysed using the salicylate method (Kempers and Zweers, 1986) for extractable NH₄⁺ concentration and the vanadium (III) chloride method (Doane and Horwáth, 2003) for NO₃⁻ concentration. Microbial biomass C and N were calculated as the difference in K₂SO₄-extractable organic C (EOC) and N between chloroform-fumigated samples and control samples, respectively (Schinner et al., 1996; Vance et al., 1987). Soil pH was determined with a calibrated pH-meter (micropH-2000, Crison Instruments SA, Spain) in 1M KCl at a soil:suspension ratio of 1:2.5 (w:v) (McLean, 1982). Potassium (K⁺) was determined on soil extracts (1:10, w:v) with 1 N ammonium acetate (Pratt, 1965) using a flame photometer (PFP-7, Jenway, UK). Soil phosphate (PO₄³⁻) concentration was measured spectrophotometrically (Evolution 201, Thermo Scientific, USA) following the modified ascorbic acid method (Olsen and Sommers, 1982). Concentrations of TOC and TN were determined in soil samples using an elemental analyser (CNS 2000, LECO Corporation, USA). Roots were picked up by hand from the soil samples during sieving and total root biomass weight was determined after oven drying for 48 h at 65 °C. Soil water contents of field-moist and air-dried soil samples were determined gravimetrically by oven drying soil aliquots at 105 °C to constant mass.

All chemical components were expressed on a soil dry-weight basis except for the microbial biomass C and the EOC that were expressed per unit of soil organic C (mg g⁻¹ SOC). Carbon and N stocks in soils at both sampling depths were calculated based on TOC and TN concentrations and with the mean values of soil bulk density, fine earth and stone contents of each forest estimated by Díaz-Pinés et al. (2011) in the same experimental forest stands.

Data analysis

We evaluated the effects of 3-years of N fertilization –i.e. short-term responses– on R_s and soil properties in Scots pine and Pyrenean oak forests using separated linear mixed effect (LME) models for each sampling depth. In all LME models we included the interaction effect

between 'Forest' and 'Treatment', including the 'Plot' as a random factor given that data consisted of repeated measurements (*nlme* package, Pinheiro et al., (2017)). Since the seasonal evolution of R_s could obscure the effect of N fertilization treatments, we used separated LME models to evaluate differences in monthly values of R_s between treatments within each forest, and between forests within the same treatment, including the random effect of 'Plot'. Additionally, we used a linear model to test the interaction effect between 'Forest' and 'Treatment' in the cumulative R_s throughout the whole study period (*stats* package, R Core Team, (2017)). We inspected the general assumptions of normality and homoscedasticity in the models by plotting the residual values versus predicted values and the quantile-quantile residual plots for each fitted model. We conducted generalized linear mixed (GLM) models with gamma error distribution and the logarithmic link function to satisfy the assumptions in the analysis of the interaction effect between the 'Moment of measurement' (before fertilization and 24 h after the N-fertilizer spreading) and the 'Treatment' on R_s . GLM models were run independently for each forest including again the 'Plot' as a random factor (*lme4* package, Bates et al., (2014)). Pairwise comparisons using Tukey's honestly significant differences of least-square means (*lsmeans* package, (Lenth, 2016)) were performed whenever significant factor effects were detected.

Structural equation modelling (SEM; Shipley, 2004) was used to evaluate the direct and indirect influence of soil properties on R_s and to determine how N supply may affect these causal relationships. SEM is a multivariate statistical method that compares the observed covariances among measured variables to those expected based on an aprioristic model in which causal networks are explicitly depicted (Grace and Jutla, 1999; Iriondo et al., 2003). The hypothesized relationships among variables were based on literature and previous statistical analysis (LME models). First, we hypothesized that R_s would depend on ST and SWC (Almagro et al., 2009), and that both predictor variables would be correlated. Second, we anticipated that R_s would be influenced by abiotic and biotic conditions in soils, namely pH (Rousk et al., 2010; Xu and Qi, 2001), EOC (Søe and Buchmann, 2005), inorganic N concentrations (Sun et al., 2014), microbial biomass (Liu and Greaver, 2010), and that all these variables in their turn would be affected by soil C storage (De Vries et al., 2012) and fertilization treatments (Janssens et al., 2010; Riggs and Hobbie, 2016; Treseder, 2008). The hypothesized SEM is shown in Figure 5.1. The N fertilization treatment was encoded as an ordinal variable (control, 0; medium, 1; high, 2). To test the hypothesized model, we supplemented the dataset of the analysed soil properties for the top 0-5 cm with all the measurements of R_s developed 24 hours after each N-fertilizer spreading. Separated SEM were run for the dataset of each forest type, containing all variables z-standardized. Model estimations were performed using maximum likelihood methodology, and the adequacy of the fit was evaluated by χ^2 goodness of fit and other statistics often used in SEM (Browne and Cudeck, 1993; Shipley, 2004), such as the Bentler and Bonnett's normed fit index

(NFI), the goodness of fit index (GFI) and the root mean square error approximation (RMSE). We tested for both univariate and multivariate normality and evaluated the statistical significance of each regression weight through bootstrapping (Finney and DiStefano, 2006). Furthermore, we explored if there were differences in the influence of N fertilization on R_s between forests through the explicit comparisons performed with a multi-group analysis (Shipley, 2004). We applied the Bonferroni correction to adjust the overall significance level to the number of cases tested in these multi-group comparisons.

The fit and evaluation of SEMs and the multi-group analyses were performed with the Amos 22.0.0 software (SPSS, IBM Corporation, Meadville, USA) whereas the rest of the analyses were carried out in the statistical platform R version 3.4.0. (R Core Team, 2017).

Figure 5.1. Hypothesized structural equation model for determinants of soil respiration (R_s) in the studied forest systems. Rectangles represent the variables: Fertilization = N fertilization treatments (control, medium and high); NH_4^+ and NO_3^- = concentrations of N-inorganic forms; R_s = soil respiration, SWC = soil water content; ST = soil temperature; Cmic = microbial biomass carbon; EOC = extractable organic carbon; pH = soil pH in KCl and Stock C = soil stored carbon for the top 0-5 cm. U_1 - U_6 represent the unexplained variance of the model to each dependent variable. Single-headed arrows indicate dependence relationships between predictor and predicted variables while double-headed arrows show correlations between independent variables.

Results

Soil climate variables and soil respiration response to N additions

Mean soil temperature was 12.2 ± 0.6 °C (ranging from 1.4 °C in winter to 23.8 °C in summer) while mean soil moisture was 8.2 ± 1.4 % vol. (ranging from < 1 % in summer to 21.3 % in late winter, Figure 5.2). In both studied forests, monthly averaged R_s rates (Table 5.1) peaked in the period March-July and again in October, coinciding with optimal values for soil temperature and soil moisture. LME models showed that N fertilization significantly increased R_s compared to control plots in the Scots pine stand from February to May and again in September; in the oak forest, fertilization significantly increased R_s only in April. During the period of warmer temperatures (May-August) R_s was higher in the oak than in the pine forest. The treatments effects on cumulative R_s throughout the entire study period were not statistically significant in any of the forests (Figure 5.3a). However, significant effects of N additions were found 24 hours after the N-fertilizer spreading in the pine stand, with the medium-fertilization showing higher rates of R_s than the control treatment (Figure 5.3b). In the oak stand, both the medium- and high-fertilization increased their R_s rates 24 hours after the application of N-fertilizer with regard to their R_s values prior the N additions, although these differences were not significant among treatments.

Changes in soil properties with N additions

Across the three years of experiment, the N additions decreased significantly the microbial biomass C in both forests and in the two analysed soil depths (Figure 5.4a, b). Furthermore, in all cases, microbial biomass C was lower in the pine soils than in oak soils. Microbial biomass N and the microbial biomass C:N ratio were not influenced by N fertilization in any of the studied soil layers, showing significant differences only between forest types (Table S5.1). Similarly, total root biomass was not affected by N additions, although there was a significantly higher amount of roots in oak than in pine topsoils.

The C and N stocks did not significantly change after three years of N fertilization, but there were differences regarding the forest type (Table S5.2). The stored C in the top 10-cm of mineral soil was significantly higher in the pine (44.2 ± 14.7 Mg C ha⁻¹) than in the oak forest (33.8 ± 5.2 Mg C ha⁻¹). On the contrary, the stock of N was greater in the oak stand (2.5 ± 0.4 Mg N ha⁻¹) in comparison with the pine stand (2.0 ± 0.6 Mg N ha⁻¹). Thus, average soil C:N ratio in the pine forests nearly doubled the one in the oak forests (22.7 and 13.4, respectively).

Table 5.1. Monthly averaged soil respiration ($\text{Mg CO}_2\text{-C ha}^{-1} \text{ month}^{-1}$).

Month	s	Scots pine			Pyrenean oak		
		Control	Medium-fert.	High-fert.	Control	Medium-fert.	High-fert.
Jan	4	0.56 ± 0.15	0.59 ± 0.21	0.60 ± 0.21	0.54 ± 0.25	0.64 ± 0.41	0.60 ± 0.32
Feb	2	0.53 ± 0.11 b	0.62 ± 0.07 ab	0.73 ± 0.15 a	0.51 ± 0.22	0.57 ± 0.25	0.55 ± 0.25
Mar	3	1.21 ± 0.32 b	1.57 ± 0.26 a A	1.29 ± 0.39 ab	1.04 ± 0.24	1.18 ± 0.25 B	1.18 ± 0.21
Apr	3	0.82 ± 0.24 b	1.21 ± 0.37 a	0.97 ± 0.37 ab	0.98 ± 0.33 b	1.29 ± 0.45 a	1.22 ± 0.42 ab
May	3	1.35 ± 0.42 b B	1.91 ± 0.93 a	1.74 ± 0.71 ab	1.99 ± 0.98 A	2.12 ± 0.82	2.16 ± 1.07
Jun	6	1.31 ± 0.73 B	1.45 ± 0.75	1.32 ± 0.58 B	1.52 ± 0.26 A	1.47 ± 0.34	1.48 ± 0.31 A
Jul	5	1.08 ± 0.56 B	1.19 ± 0.76 B	1.10 ± 0.63 B	1.36 ± 0.46 A	1.42 ± 0.51 A	1.32 ± 0.50 A
Ago	4	0.69 ± 0.27 B	0.71 ± 0.39 B	0.66 ± 0.38 B	0.98 ± 0.30 A	0.92 ± 0.23 A	0.86 ± 0.28 A
Sept	5	0.60 ± 0.13 b	0.80 ± 0.27 a	0.63 ± 0.18 b	0.67 ± 0.15	0.61 ± 0.10	0.60 ± 0.11
Oct	2	1.33 ± 0.47	1.64 ± 0.83	1.55 ± 0.52	1.69 ± 0.45	1.66 ± 0.36	1.60 ± 0.39
Nov	3	0.89 ± 0.31	0.91 ± 0.20	1.02 ± 0.23	0.81 ± 0.20	0.79 ± 0.18	0.91 ± 0.32
Dec	5	0.75 ± 0.40	0.67 ± 0.24	0.67 ± 0.21	0.68 ± 0.24	0.71 ± 0.25	0.72 ± 0.29

Data are means ± standard deviations with s = sampling events for each month. Different capital letters denote significant differences between forests within the same treatment (control, medium- or high-fertilization), while small letters indicate significant differences between treatments within the same forest (LME models; p-value < 0.1).

Figure 5.2. Average values of (a) soil temperature and (b) soil moisture in the experimental plots ($n = 12$) in the 45 measurement campaigns over the study period (June 2014 – May 2017). Mean soil respiration (Rs) measured in (c) the Scots pine stand and (d) the Pyrenean oak stand from control, medium- and high-fertilization plots ($n = 4$). Standard deviations are omitted to improve the clarity of the figure. Arrows indicate days of nitrogen fertilization. SP: spring, SU: summer, AU: autumn and WI: winter.

Figure 5.3. Nitrogen fertilization effects on cumulative soil respiration (R_s) throughout the study period (June 2014 – May 2017) in each forest and treatment (linear model, $p < 0.05$, $n = 4$). Mean R_s before and 24 h after the nitrogen fertilizer spreading in (b) Scots pine forest and (c) Pyrenean oak forest. Different capital letters denote significant differences between the moment of R_s measurement (before and 24 hours after the nitrogen fertilizer spreading), while small letters indicate significant differences between treatments (control, medium- and high-fertilization; GLM models, $p < 0.05$, $n = 48$). Values represent means $\pm$ standard errors.

The EOC showed different responses to N addition in the pine and oak stands (Figure 5.4c, d). In the first 5 cm of the pine soil, the EOC decreased with N addition whereas it remained stable in the oak soil. In the subsurface layer, the EOC only differed significantly between forests, but not between treatments. Extractable PO_4^{3-} was low ($3.46 \pm 1.51 \text{ mg kg}^{-1} \text{ dw}$) and rather similar between forests and treatments in the toplayer, although we found significantly higher PO_4^{3-} concentrations in the pine than in the oak soils in the subsurface layer (3.34 ± 1.79 and 1.92 ± 0.86 , respectively; Table S5.2). Ammonium was the dominant inorganic-N form in soils (Table S5.2), the $\text{NO}_3\text{--N}:\text{NH}_4\text{--N}$ ratio was on average 0.12 ± 0.04 in control plots from pine and 0.18 ± 0.03 in control plots from oak forests. Overall, soil NH_4^+ and NO_3^- were higher in oak than in pine forests, but only the NO_3^- showed a significant response to the treatments. In all cases, the high-N fertilization significantly increased soil NO_3^- concentrations (Figure 5.4e, f). Soil pH was more acidic in the pine than in the oak forest, and decreased with N addition in both forests (Figure 5.4g, h). The K^+ concentrations in topsoil were higher in oak than in pine stands and sensible to N additions (Table S5.2). In both forests, K^+ concentrations were higher under medium-fertilization compared to high-fertilization, whereas control plots showed intermediate values (Figure 5.4i, j).

Figure 5.4. Responses of soil properties (Cmic = microbial biomass carbon, EOC = extractable organic carbon, NO₃⁻ = nitrate concentration, pH = pH determined in KCl and K = potassium concentration) to N fertilization treatments at the two sampling depths (0–5 cm and 5–10 cm) in the pine and oak forests. Data are means ± standard deviations. Different capital letters denote significant differences between forests within the same treatment (control, medium- and high-fertilization), while small letters indicate significant differences between treatments within the same forest (LME models, n = 12).

Controlling factors of soil respiration

The datasets collected in each forest provided a good fit to the initial hypothesized model as indicated by the non-significant χ^2 values and the goodness of fit indices (RMSE < 0.05, NFI and GFI > 0.9; Browne and Cudeck, 1993, Figure 5.5a, b). The SEMs were able to explain the 51 % and 58 % of the variation in R_s in the Scots pine and the Pyrenean oak forests, respectively. The predictor variables soil moisture and soil temperature explained the greatest amount of variability in R_s , and had stronger standardized effects in the Pyrenean oak forest than in the Scots pine forest. Overall, the SEMs revealed a strong effect of N fertilization on the NO₃⁻ concentration, but not on the NH₄⁺ concentration (Table S5.3). Soil NH₄⁺ concentrations negatively influenced R_s (Table 5.2). The direct effect of N addition on soil pH was only

NFI = 0.98; GFI = 0.98; RMSEA = <0.001;
 $\chi^2 = 17.10$, $p = 0.52$; $df = 18$; $n = 144$

NFI = 0.97; GFI = 0.98; RMSEA = <0.001;
 $\chi^2 = 15.34$, $p = 0.64$; $df = 18$; $n = 144$

Figure 5.5. Overall structural equation models for the Scots pine stand a) and the Pyrenean oak stand b). Continuous paths depict causal significant relationships ($p < 0.05$), with their standardized regression weights indicated. Discontinuous paths represent non-significant relationships. Arrow widths are proportional to estimated regression weights. Squared multiple correlations (R^2) of dependent variables are also shown. Direct and indirect effects of tested variables on R_s in each forest are shown in Table 5.2. Goodness of fit statistics for each model are: NFI = Bentler and Bonnett's normed fit index, GFI = goodness of fit index, RMSEA = root mean square error approximation. See Figure 5.1 for variable abbreviations in the rectangles.

significant in the oak forest, although in both forests N addition indirectly affected soil pH through the modulation of NO_3^- (Table S5.3). Soil pH in turn exerted a negative effect on R_s (Table 5.2). Since there was a more acidic soil pH value with N fertilization, soil pH at the end resulted in a positive contribution to R_s . Microbial biomass C did not directly altered R_s in any of the forests, even if it was strongly reduced by the N addition. There was a remarkable opposite direct effect of EOC on R_s between forests. In the soil under pine, the microbial biomass C exerted a small indirect effect over R_s through the EOC reduction. Overall, the EOC increased with soil acidification. Interestingly, the EOC also experienced a strong decrease with N addition but only in the pine stand. Thus, EOC had a net positive effect on R_s that was direct in the oak stand but indirect in the pine stand through the effect of N fertilization.

The SEM multi-group analysis showed three remarkable differences between forests (Table 5.3). Firstly, soil temperature explained a greater proportion of the variation in R_s in the oak stand. Secondly, the paths from fertilization to EOC and then from EOC to R_s differed in direction and magnitude between forests. Thirdly, the release of several significant interconnections between the inorganic N forms and the pools of C (Stock C and EOC) also improved significantly the model fit in the multiple comparisons. The release of the equality constraint in the paths directly relating soil pH and soil moisture with R_s did not significantly improve the goodness of fit, probably due to a partition of their effects mediated by EOC and soil temperature, respectively.

Table 5.2. Standardized effects of tested variables on Rs in the overall structural equation models for the Scots pine forest a) and the Pyrenean oak forests b).

	a) Overall model for the Scots pine stand			b) Overall model for the Pyrenean oak stand		
	Direct effects	Indirect effects	Total effects	Direct effects	Indirect effects	Total effects
NO ₃ ⁻	-	0.073 **	0.073 **	-	-0.015 n.s.	0.015 n.s.
NH ₄ ⁺	-0.165 *	-0.098 **	-0.264 **	-0.138 *	-0.022 n.s.	-0.161 *
Cmic	-0.107 n.s.	0.037 *	-0.070 n.s.	0.008 n.s.	-0.016 n.s.	-0.008 n.s.
pH	-0.216 **	0.043 *	-0.173 **	-0.068 n.s.	-0.068 *	-0.136 *
EOC	-0.144 *	-	-0.144 *	0.256 **	-	0.256 **
SWC	1.015 **	-	1.015 **	1.304 **	-	1.306 **
ST	0.990 **	-	0.990 **	1.411 **	-	1.411 **
Stock C	-	-0.115 *	-0.115 *	-	-0.005 n.s.	-0.005 n.s.
Fertilized	-	0.174 ***	0.174 ***	-	0.050 n.s.	0.050 n.s.

Asterisks indicate levels of significance for the standardized path coefficients: *** p-value < 0.001, ** p-value < 0.01, * p-value < 0.05, • p-value < 0.1, n.s. non-significant. See Figure 5.1 for variable abbreviations.

Table 5.3. Multigroup comparisons of the path coefficients in nested models based on the forest type.

Specific parameter whose between-group equality constraint was released	ML χ^2	Δ ML χ^2	Probability of Δ ML χ^2
None	569.722		
Path from fertilization to Cmic	566.924	2.798	0.094
Path from fertilization to EOC	526.445	43.277	0.000
Path from fertilization to NH ₄ ⁺	569.658	0.064	0.800
Path from fertilization to pH	560.819	8.903	0.003
Path from fertilization to NO ₃ ⁻	563.170	6.552	0.011
Path from stock C to Cmic	568.073	1.649	0.199
Path from stock C to EOC	569.718	0.004	0.950
Path from stock C to NH ₄ ⁺	566.972	2.750	0.973
Path from stock C to pH	569.511	0.211	0.646
Path from stock C to NO₃⁻	475.238	94.484	0.000
Path from NH ₄ ⁺ to Rs	565.049	4.673	0.031
Path from NH ₄ ⁺ to Cmic	569.635	6.811	0.009
Path from NH₄⁺ to EOC	554.455	17.538	0.000
Path from NH ₄ ⁺ to pH	567.772	1.950	0.163
Path from NH₄⁺ to NO₃⁻	553.244	16.478	0.000
Path from NO ₃ ⁻ to Cmic	569.339	0.383	0.536
Path from NO ₃ ⁻ to EOC	569.716	0.006	0.938
Path from NO ₃ ⁻ to pH	568.287	1.435	0.231
Path from pH to Rs	562.911	6.811	0.009
Path from pH to EOC	567.173	2.549	0.110
Path from pH to Cmic	567.374	2.348	0.125
Path from EOC to Rs	552.184	17.538	0.000
Path from Cmic to Rs	568.173	1.549	0.213
Path from Cmic to Rs	568.295	1.427	0.232
Path from SWC to Rs	560.273	9.449	0.002
Path from ST to Rs	556.448	13.274	0.000
Covariance between SWC and ST	568.890	0.832	0.362
Variance of Rs	565.435	4.287	0.038
Variance of Cmic	551.650	18.072	0.000
Variance of EOC	554.682	15.040	0.000
Variance of pH	468.106	101.616	0.000
Variance of NO₃⁻	558.287	11.435	0.001
Variance of NH₄⁺	557.060	12.662	0.000
Error variance of stock C	431.539	138.183	0.000
Error variance of fertilization	569.722	0.000	1.000

The first row (None) specifies the maximum likelihood χ^2 estimates (ML χ^2) from the most constrained model, in which all free parameters are forced to be equal in both forests. The remaining rows below indicate the ML χ^2 resulting from the release of one constraint at a time. The right-most column shows the probability that the change in χ^2 estimates (Δ ML χ^2) with the release of that parameter improves the model significantly. The significance level of 95 % was adjusted to the Bonferroni corrected p-value threshold: $0.05/35 = 0.0014$. Significantly different path coefficients between forests are reported in bold. See Figure 5.1 for variable abbreviations.

Discussion

Soil respiration resilience to short-term N additions

In our study, we found that three years of N fertilization affected several soil properties. As expected, there was a direct positive relationship between the NO₃⁻ concentrations in soils and the N fertilization rates, which subsequently led to soil acidification and decreased microbial biomass C (Knorr et al., 2005; Riggs and Hobbie, 2016; Zhou et al., 2014). Unlike soil properties,

R_s was rather resilient to short-term N fertilization in both pine and oak forests. This is in contrast with the generally observed R_s decrease in most temperate forests exposed to N additions (Janssens et al., 2010). We consider that there are two main aspects responsible for the high stability of R_s observed in our study: the short period in which optimal conditions for biological activity take place under Mediterranean climate and the degree of soil N availability in these forest soils.

Contrary to what we hypothesized, there was not a significant reduction in the cumulative R_s throughout the 3 years of chronic N enrichment. The present results of monthly averaged R_s in both oak and pine stands supported evidences of a strong climate limitation regarding the R_s responses to N addition. The strong and inverse water and thermal seasonality of the Mediterranean climate has been stressed previously as an important constraint for soil CO_2 efflux in several ecosystems (Chang et al., 2014; Fernández-Alonso et al., 2018b; Lellei-Kovács et al., 2016; Rey et al., 2002). Thus, the R_s was sensitive to N additions only when adequate soil microclimate conditions for microbial and root activity occurred –i.e. high soil moisture and warm soil temperatures. This temporal pattern in R_s sensitivity to N additions suggests that seasonal variability of the Mediterranean climate might be a stronger driver of R_s than changes in soil N availability. This result agrees with previous research in semiarid and Mediterranean ecosystems where a lack of R_s response to N additions has also been observed during the dry season (Lo Cascio et al., 2017; Zhang et al., 2014). Additionally, the longer period in which R_s was sensible to N fertilization in the pine stand compared to the oak one –i.e. from February to May and in September for the pine, but solely in April at the oak forest– may reflect a stronger soil N limitation than in oak soils, in accordance with the dissimilar soil C:N ratios between both forests. Several studies have previously reported how the native N availability derived from soil stoichiometry influence different responses of R_s to N additions, inhibiting R_s in N richer sites and enhancing R_s in N poorer ecosystems (Lo Cascio et al., 2017; Pregitzer et al., 2008).

Our results showed the highest R_s increase in the medium-fertilization, with high-fertilization showing intermediate R_s values between control and medium-fertilization. Further, the positive R_s response to the individual application of medium-fertilization was another evidence of the higher pine soil sensitivity to N additions in comparison to the oak soil. The significant R_s pulse following regular application of N fertilizer is common among N limited stands whether they are hardwood or coniferous forests (Micks et al., 2004). Then, why might medium-fertilization have significantly increased R_s after N-fertilizer spreading only in pine stand? In our experiment, the N additions were accompanied by a 25 % reduction of microbial biomass in the top 10 cm of soil in the pine forest and 15 % reduction in the oak forest. Several microbial- and plant-mediated causes could explain the positive or absence R_s response following N additions together with the negative effects observed on microbial biomass.

A possible microbial-mediated mechanism explaining the observed R_s responses could be a decrease in the decomposer efficiency associated with changes in the soil microbial community composition (Burton et al., 2004). However, there were no evidences for a shift in the microbial composition at our forest soils since microbial biomass C to N ratios were rather stable in all fertilization treatments. The microbial biomass C:N ratio can be used as an indicator for the bacterial or fungal prevalence (De Vries and Shade, 2013). For instance, bacteria tend to have lower C to N biomass requirements and be more C inefficient than fungi (microbial biomass C to N $\approx$ 3–6 for bacteria and 5–15 for fungi according to Strickland and Rousk, 2010). Hence, a shift in the decomposer efficiency seems unlikely, given that microbial communities under pine stand continued to be predominantly slow growing microorganisms (oligotrophic) whereas under the oak stand continued to be fast growing microorganisms (copiotrophic) (Fernández-Alonso et al., 2018a).

Alternatively, the absence of N immobilization by microbes enable us to link the decreased microbial biomass following N additions with a reduced microbial respiration (Treseder, 2008) and thus with an impeded SOM decomposition in the long term (Riggs and Hobbie, 2016). Therefore, R_s might have increased through a plant-mediated response by which N enrichments enhance the photosynthetic rates and stimulate the rhizosphere respiration (Janssens et al., 2010; Liu and Greaver, 2010; Lu et al., 2011). Nitrogen fertilization have the potential to alleviate N limitation enabling trees and understory vegetation including grasses, mosses and lichens, to increase photosynthesis-related parameters comparing to control plots, such as the foliar N concentration and chlorophylls (Hyvönen et al., 2007; Ochoa-Hueso and Manrique, 2011; Pregitzer et al., 2008). Although we did not measure root exudates, results from a meta-analysis highlight that enhanced photosynthesis may transfer more labile root-derived C to the soil fuelling rhizosphere microbes and the decomposition of recalcitrant SOM by saprophytic microbes (Janssens et al., 2010). In the top 5 cm soils of N fertilized soils, we observed a trend towards increase in the root biomass that is consistent with a stimulated rhizosphere activity due to increased resource availability for both understory vegetation and decomposers. Since pine soils may be N limited to a greater extent than oak soils (Fernández-Alonso et al., 2018a), the stimulated photosynthesis could have been proportionally greater in the pine forest causing a stronger positive response of the rhizosphere activity. In our forests soils, the often mention shift in microbial enzymatic spectra in response to N additions (Janssens et al., 2010) do not seems a plausible underlying mechanism controlling R_s , since normally the reduced oxidative enzymes and increased hydrolytic enzymes are associated with positive impacts on EOC and negative impacts on R_s (Liu and Greaver, 2010; Zak et al., 2008).

Moreover, the displayed R_s decreasing tendency among N treatments (i.e. medium-fertilization $\geq$ high-fertilization $\geq$ control) is consistent with the decoupling between

photosynthesis and foliar N concentration reported under excess of N in other studies (Liu and Greaver, 2010; Ochoa-Hueso and Manrique, 2011), but also with potential adverse effects in systems approaching N saturation (Burton et al., 2004). In our experiment, the high-fertilization caused soil acidification and NO_3^- accumulation in the soil that have been related with toxicity effects on microbial and plant activity and thus R_s (Gundale et al., 2014; Zhang et al., 2014). Furthermore, excessive soil acidification leads to an increased leaching of base cations (Cusack et al., 2016; Li et al., 2018) as we detected a lowered K^+ concentrations in topsoils with the highest N loads. Therefore, the soil-mediated effects (i.e. soil acidification and base cations leaching) under the high-fertilization may cause intermediate R_s between control and medium- fertilization via restricted microbial and plant activities.

Overall, these results suggest a mechanism by which an indirectly stimulated rhizosphere activity after medium-fertilization offsets the negative effects over R_s of a declined soil microbial biomass. Since we do not have sufficient data to sustain this hypothesis, further work focusing on plant related responses is necessary to test this proposed underlying mechanism for the R_s resilience to anthropogenic N deposition in this Mediterranean ecotone forest.

Fertilization effects on soil carbon fluxes

The SEM multi-group approach revealed differences in C losses between forests, primarily highlighting: (1) that shifts on soil properties caused by N additions showed that oak R_s was indifferent to N fertilization whereas there was a net increase of pine R_s and (2) that there were different microclimate dependence of R_s in each forest.

Firstly, N fertilization could strongly alter R_s by changing the dynamics of soil EOC. In the pine SEM, we observed relationships between inorganic N forms and C pools affecting EOC concentration and thus R_s , which did not occur in oak SEM. The differences in native abiotic properties between forests (e.g. lower soil organic C content and less acidic soil pH in the oak stand compared to the pine stand) may have contributed to the low sensitivity exhibited by the paths in the oak SEM in response to N enrichment (Zhong et al., 2016).

The effect of N additions on EOC availability in soils depends on the balance between production processes, such as microbial decomposition, root exudation, litter leaching and desorption; and subsequent microbial decomposition processes of that EOC, which may be either immobilised in biomass or released as CO_2 efflux (Neff and Asner, 2001). However, soil acidification caused by N addition may also influence EOC mobility since the solubility of EOC is pH-dependent (Evans et al., 2008; Hagedorn et al., 2012; Monteith et al., 2007). Evans et al., (2008) compiled data from 12 studies and suggested that the response of EOC was dependent on whether the form of N added increased or decreased soil acidity, for instance the NH_4^+ salt addition tended to decrease EOC through reduced soil organic matter solubility linked to

increased soil solution acidity. This is consistent with the pine SEM, where the simulated N deposition with the addition of NH_4NO_3 strongly promoted nitrification and thus the accumulation of NO_3^- causing soil acidification (Hagedorn et al., 2012). The negative effect of N fertilization on EOC in the pine stand provides support for this soil pH/EOC solubility mechanism. It is also possible that, with the higher N availability at the pine oligotrophic soil, there was an N-induced increase in EOC production through root exudation coupled with the above mentioned enhanced plant photosynthesis and an stimulation of autotrophic respiration (Janssens et al., 2010; Liu and Greaver, 2010; Lu et al., 2011). Such an interpretation is sustained by the negative effect of pH on EOC and R_s in response to N fertilization.

We further found in the SEMs that N additions may promote EOC accumulation probably through the negative impact on microbial heterotrophic activity and thus R_s (Liu and Greaver, 2009; Zhou et al., 2014). Moreover, Waldrop and Zak (2006) suggested that the suppression of lignin-degrading enzymes by excess N increases the leaching of EOC enriched in recalcitrant compounds as intermediate decomposition products. Therefore, we suspect that increased EOC under N enrichment was due to decreases in microbial decomposition processes due to smaller microbial communities in soils coupled with repressed heterotrophic activities.

Overall, N addition could have a positive impact on EOC concentration in soil solution by decreasing microbial heterotrophic activity or increasing root exudation, and thereby affecting R_s , but also a negative impact by decreasing soil pH through a reduction in soil organic matter solubility. According to the SEMs, both mechanism seems to be balanced in the oak stand since fertilization had no effect on EOC whereas the second mechanism is likely the dominant in the pine soil. Further research is needed to distinguish which mechanism persists if chronic N enrichment continues in a longer run since the EOC is the major form in which C is cycled within soils (Evans et al., 2008).

Finally, soil temperature explained a greater proportion of R_s variation in the oak than in the pine SEM indicating a greater R_s temperature dependence on the oak growing season rather than on quarterly N fertilization. These SEM results were consistent with the significantly higher monthly R_s from May to August in the oak than in the pine stand, that precisely corresponded with the period of the year of maximum oak physiological activity (Salomón et al., 2016b, 2016c) and thereby maximum relative contribution of autotrophic respiration to total R_s (Chapter 4).

Conclusions

Our findings highlighted a high resilience of R_s to short-term N enrichment in this Mediterranean ecotone forest. However, in the pine stand with the medium-N fertilization, we

observed a significant pulse of R_s after the quarterly N applications and a strong seasonality in the stimulation of monthly R_s . We suggest that soil stoichiometry –oak stands are richer in soil N than pine stands–, seasonal variability of soil moisture and temperature –significant increases in R_s were only observed from February to May– and doses of N fertilizer –high-fertilization showed intermediate responses between those from control and medium-fertilization treatments– modulated the R_s response to N additions. Moreover, N addition caused soil acidification, changes in EOC concentrations and a substantial decrease in microbial biomass C suggesting that stronger effects in the belowground C cycle (increased sequestration of soil C) may occur if chronic N enrichment persists in a longer run.

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Supplementary information

Table S5.2. Results of linear mixed effects (LME) models showing the interaction effect between the factors F = Forest and T = Treatment on soil organic C and N contents, soil pH, P and K fertility of soils in the top layer (0-5 cm) and the subsurface layer (5-10 cm).

	Depth (cm)	Scots pine			Pyrenean oak			LME models		
		Control	Medium	High	Control	Medium	High	F	T	F × T
EOC (mg g ⁻¹ SOC)	0 - 5	4.5 ± 1.1	3.3 ± 0.9	3.1 ± 0.7	5.0 ± 1.0	5.0 ± 1.3	6.1 ± 2.1	***	n.s.	*
	5 - 10	5.6 ± 2.0	5.2 ± 1.4	6.1 ± 2.4	7.6 ± 1.9	9.1 ± 2.9	10.0 ± 3.2	***	n.s.	n.s.
Stock C (Mg ha ⁻¹)	0 - 5	24.3 ± 9.9	20.8 ± 4.7	29.3 ± 9.9	21.8 ± 3.2	22.3 ± 3.5	19.9 ± 2.2	*	n.s.	n.s.
	5 - 10	18.5 ± 3.8	18.5 ± 4.6	21.1 ± 10.4	13.3 ± 2.8	12.4 ± 2.9	11.7 ± 2.1	**	n.s.	n.s.
Stock N (Mg ha ⁻¹)	0 - 5	1.0 ± 0.4	0.9 ± 0.2	1.2 ± 0.4	1.6 ± 0.2	1.6 ± 0.3	1.5 ± 0.1	***	n.s.	n.s.
	5 - 10	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.4	0.8 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	n.s.	n.s.	n.s.
C:N	0 - 5	23.5 ± 2.1	22.6 ± 2.8	25.0 ± 1.8	13.7 ± 0.5	14.3 ± 0.9	12.9 ± 1.1	***	n.s.	n.s.
	5 - 10	20.1 ± 1.5	20.6 ± 3.2	22.0 ± 1.6	12.8 ± 1.0	13.7 ± 2.1	12.5 ± 2.1	***	n.s.	n.s.
PO ₄ ³⁻ (mg kg ⁻¹ dw)	0 - 5	3.2 ± 1.2	3.4 ± 1.8	3.5 ± 1.7	4.4 ± 1.3	3.4 ± 1.8	2.8 ± 0.7	n.s.	n.s.	n.s.
	5 - 10	3.2 ± 1.7	3.8 ± 2.2	3.0 ± 1.5	2.6 ± 0.9	1.6 ± 0.7	1.6 ± 0.6	**	n.s.	n.s.
K ⁺ (mg kg ⁻¹ dw)	0 - 5	226.7 ± 35.3	281.6 ± 57.7	216.1 ± 44.3	302.9 ± 86.6	312.8 ± 76.8	255.5 ± 56.5	*	*	n.s.
	5 - 10	211.2 ± 48.4	227.4 ± 43.5	193.4 ± 48.8	237.4 ± 18	233.9 ± 86.5	213.9 ± 63.4	n.s.	n.s.	n.s.
NO ₃ ⁻ (mg kg ⁻¹ dw)	0 - 5	2.2 ± 0.8	3.5 ± 1.5	19.4 ± 15.8	7.0 ± 2.2	14.0 ± 7.5	31.2 ± 15.5	**	**	n.s.
	5 - 10	2.4 ± 1.3	2.2 ± 0.8	5.7 ± 3.2	3.5 ± 2.1	3.4 ± 2.2	5.0 ± 3.3	n.s.	**	n.s.
NH ₄ ⁺ (mg kg ⁻¹ dw)	0 - 5	23.5 ± 6.6	21.4 ± 7.8	26.2 ± 14.5	37.4 ± 14.1	34.0 ± 9.9	41.6 ± 13.1	***	n.s.	n.s.
	5 - 10	14.3 ± 2.0	13.2 ± 5.5	12.1 ± 6.7	19.4 ± 8.3	17.4 ± 7.9	16.9 ± 7.6	**	n.s.	n.s.
pH	0 - 5	4.5 ± 0.1	4.5 ± 0.1	4.3 ± 0.1	5.1 ± 0.3	5.0 ± 0.2	4.6 ± 0.2	***	***	n.s.
	5 - 10	5.7 ± 0.2	5.6 ± 0.2	5.6 ± 0.3	5.9 ± 0.3	5.7 ± 0.2	5.6 ± 0.2	n.s.	*	n.s.

Data are means ± standard deviations (n = 12). Asterisks indicate levels of significance: *** p-value < 0.001, ** p-value < 0.01, * p-value < 0.05, • p-value < 0.1, n.s. non-significant. EOC = Extractable organic carbon.

Table S5.3. Standardized effects on the dependent variables other than R_s in the overall structural equations model for the Scots pine a) and Pyrenean oak forests b).

	a) Overall model for the Scots pine stand			b) Overall model for the Pyrenean oak stand		
	Direct effects	Indirect effects	Total effects	Direct effects	Indirect effects	Total effects
Cmic						
NO ₃ ⁻	0.092 n.s.	0.035 n.s.	0.127 *	0.352 **	-0.093 **	0.259 **
NH ₄ ⁺	-0.038 n.s.	0.027 n.s.	-0.011 n.s.	-0.286 **	0.076 *	-0.210 **
pH	-0.077 n.s.	-	-0.077 n.s.	0.416 **	-	0.416 **
Stock C	0.601 **	0.047 n.s.	0.648 **	0.193 n.s.	0.207 **	0.400 **
Fertilized	-0.395 **	0.057 *	-0.338 **	-0.354 ***	0.012 n.s.	-0.342 **
EOC						
NO ₃ ⁻	-0.008 n.s.	0.085 *	0.077 n.s.	-0.165 n.s.	0.040 n.s.	-0.125 n.s.
NH ₄ ⁺	0.319 **	-0.080 n.s.	0.238 **	-0.035 n.s.	-0.027 n.s.	-0.062 n.s.
Cmic	-0.254 **	-	-0.254 **	-0.063 n.s.	-	-0.063 n.s.
pH	-0.260 **	0.020 n.s.	-0.241 **	-0.254 **	-0.026 n.s.	-0.280 **
Stock C	0.514 **	0.087 n.s.	0.601 **	0.227 *	-0.146 **	0.081 n.s.
Fertilized	-0.665 **	0.158 **	-0.506 **	0.066 n.s.	0.038 n.s.	0.103 n.s.
pH						
NO ₃ ⁻	-0.452 **	-	-0.452 **	-0.222 **	-	-0.222 **
NH ₄ ⁺	0.547 **	-0.245 **	0.302 **	0.101 n.s.	-0.029 n.s.	0.072 n.s.
Stock C	-0.589 **	0.097 n.s.	-0.492 **	0.474 **	-0.006 n.s.	0.468 **
Fertilized	-0.129 n.s.	-0.167 ***	-0.296 **	-0.293 **	-0.116 **	-0.409 **
NO₃⁻						
NH ₄ ⁺	0.542 **	-	0.542 **	0.131 n.s.	-	0.131 n.s.
Stock C	0.050 n.s.	0.214 **	0.265 **	0.021 n.s.	-0.002 n.s.	0.019 n.s.
Fertilized	0.366 ***	-0.003 n.s.	0.363 **	0.527 **	0.001 n.s.	0.528 **
NH₄⁺						
Stock C	0.396 **	-	0.396 ***	-0.019 n.s.	-	-0.019 n.s.
Fertilized	-0.005 n.s.	-	-0.011 n.s.	0.011 n.s.	-	0.011 n.s.

Asterisks indicate levels of significance: *** p-value < 0.001, ** p-value < 0.01, * p-value < 0.05, n.s. non-significant. See Figure 5.1 for variable abbreviations.

Chapter 6

Tree species shape the assembly of microbial decomposer communities during oak leaf and pine needle litter decomposition

Abstract

Tree species influence terrestrial ecosystem dynamics through litter decomposition, which is a complex process dependent on litter intrinsic properties, environmental conditions and species-specific microbial decomposer communities. We examined how the global-change driven forest succession from Scots pine (*Pinus sylvestris* L.) to Pyrenean oak (*Quercus pyrenaica* Willd.) may influence litter decomposition in a Mediterranean ecotone forest. We performed a reciprocal transplant experiment using litterbags in two neighbouring pure forest stands of pine and oak to assess the decomposition of litter from either Scots pine (needles), Pyrenean oak (leaves) or an admixture 1:1 of both tree species (needles and leaves). We determined successive changes in litter mass loss, C and N contents and microbial community structure by the phospholipid fatty acid method (PLFA) over a two-year period. Overall, needles showed lower decomposition rates than leaves after two years of decomposition. There were litter-forest interaction effects on decomposition rates supporting the home-field advantage hypothesis exclusively for the leaves showing 18 % larger decomposition constant under oaks than under pines. Moreover, there were synergistic effects of mixing needles and leaves on decomposition constants that led native litter from litter admixtures to decay the fastest in each forest type. Our results evidenced that litter stoichiometry is the most important factor affecting mass loss and N dynamics in litter during the early stages of decay (ca. 1 year after decomposition began). However, the influence of forest type on the functional assembly of microbial communities, key driver of litter decomposition processes, showed an increasing importance with time. We found differential microbial colonisation patterns between forests suggesting a staggered functional adjustment of the microbial community driven firstly by litter stoichiometry, and then by the convergence of colonizing microbial communities toward soil microbes and soil organic matter characteristics. These specific feedbacks between tree species, soil microenvironment and microbial functionality have strong implications for litter decomposition and the biogeochemical cycling of C and N suggesting that the global change-driven forest succession from pine to oak may lead to a decrease of forest floor accumulation.

Keywords

Litterbag, litter mixtures, ecological stoichiometry, threshold elemental ratio, ecosystem ecology, plant-soil-microorganism interactions.

Highlights

- Needles showed lower decomposition rates than leaves after two years of decomposition.
- Leaves showed 18 % larger decomposition constants under oaks than under pines.
- There were synergistic effects of mixing needles and leaves on decomposition constants.
- Microclimate beneath oak may promote abiotic mechanisms of litter decay.
- Tree species mediated litter decomposition through litter stoichiometry and microbial colonization.

Introduction

Tree species influence the forest functioning and dynamics through their interactions with microbial communities and soil physicochemical characteristics (Aponte et al., 2011; Austin et al., 2014; Sheffer et al., 2015). Changes in the composition of plant species during vegetation succession may alter numerous ecological processes key for ecosystem services as is the litter decomposition in the nutrient cycling (Osono et al., 2014; Saura-Mas et al., 2012). Research on this topic draws a wide range of responses of the carbon (C) and nitrogen (N) fluxes to shifts in dominant tree species during litter decomposition that are normally associated with an interspecific variation in vegetation traits (e.g. woody deciduous or evergreen species) and subsequent quality of litter (Cornwell et al., 2008; Weedon et al., 2009). Nevertheless, the variation in litter decomposition may also be responsive to interaction effects between litter traits and other decomposition drivers that are still little known (Freschet et al., 2012).

Tree species vary in litter physical features, C and nutrient chemistry and stoichiometry contributing to differences in litter degradability and turnover rates (Barba et al., 2015). In addition to the intrinsic litter chemistry, tree species affect other multiple drivers of litter decomposition including micro-environmental conditions and species-specific variations in microbial community composition (Prescott and Grayston, 2013). Vegetation may modify the microclimate at the litter layer through the structure and dynamics of the canopy and the root system (Bardgett and Wardle, 2010; Yuan et al., 2012). Hence, species composition may indirectly control both biotic decomposition, by affecting microbial activities through changes in surface temperature and moisture regimes (Davidson and Janssens, 2006), and abiotic decomposition of litter, by altering photodegradation rates through different seasonal exposure to radiation (Austin and Vivanco, 2006). Moreover, the functional composition and activity of microbial decomposers in litter and upper soil layers is tied to other belowground properties

influenced by vegetation as the soil pH and soil C:N stoichiometry (Cleveland and Liptzin, 2007; Fierer et al., 2009; Sinsabaugh et al., 2008). The historical co-evolution of vegetation with a specific decomposer community leads to a high degree of specialization and thereby litter may decompose faster in an area dominated by its plant of origin than when is placed beneath another plant species, that is referred to as the home-field advantage (HFA, Austin et al., 2014; Ayres et al., 2009). However, the affinity between litter and microbial community for decomposition is context dependent since the magnitude and direction (null, positive and negative) of HFA may vary with environmental factors (e.g. microclimate and soil stoichiometry), species traits (litter quality) and litter mixture effects (Freschet et al., 2012; Sheffer et al., 2015; Veen et al., 2015). For instance, there are physiological linkages between the dynamic stoichiometries of decomposing litter and their successive shifts on microbial community structures owing to a selective process of the most resource-efficient decomposers (Austin et al., 2014; Kaiser et al., 2014). Accordingly, microbial communities may exhibit variations over the course of litter decay determined by the balance between C and N availability of the substrates and the C:N demand of microbial decomposers (Kaiser et al., 2014; Zechmeister-Boltenstern et al., 2015). As a result, it is important to understand how the colonisation of microbial communities occurs in litter and how differences in litter and soil stoichiometry in each forest type could regulate this process since it may have strong consequences on litter decomposition rates, C and N dynamics.

Species diversity often alters the mass loss of every single-species decaying alone and their concomitant nutrient cycling but there are not consistent predictable effects of litter mixing since it is highly species-specific (Prescott, 2002). In some studies additive and non-additive effects have been described –i.e. effects of individual species and species interaction, respectively–, and within the latter, synergistic or antagonistic interactions on decomposition rates of litter admixtures (Bonanomi et al., 2010; Gartner and Cardon, 2004). As a result, the effects of mixing the foliar litter differing in physical structure and chemistry composition (lignin concentration and stoichiometric ratios) such as conifers and broadleaf trees (Osono et al., 2014) may not be entirely predictable and hence comprehensive studies are needed in order to explain the mechanisms underpinning mixed litter decomposition in forest ecosystems (Sheffer et al., 2015).

In the Iberian Peninsula, global environmental shifts (climate and land-use changes) are driving the secondary forest succession of the Scots pine (*Pinus sylvestris* L.), which is experiencing die-off at its lowest altitudinal range as a result of increased evapotranspiration and water deficit, by the expanded range of the more drought tolerant Pyrenean oak (*Quercus pyrenainca* Willd.) (Díaz-Pinés et al., 2014; Moreno-Fernández et al., 2016; Spinoni et al., 2017; Stagge et al., 2017). The change in dominant tree species during this secondary succession may have large effects on ecosystem functioning since oaks and pines possess different functional

traits (Osono et al., 2014). In this study, we explored the differences in litter decomposition between the Scots pine and the Pyrenean oak in order to determine how important the global change-induced vegetation shift may be for the ecosystem biogeochemistry in an ecotonal area of the Guadarrama Mountains (Central Iberian Peninsula). This issue is of special importance in a context of change given that at this emplacement pine soils stored roughly the doubled C than oak soils (Díaz-Pinés et al., 2011b).

We conducted over two years a reciprocal transplant decomposition experiment using litterbags in neighbouring pure stands of Scots pine and Pyrenean oak developed on the same soil and climate conditions, but where the forests have been long enough to influence soil properties (Chapter 3; Díaz-Pinés et al., 2014; Fernández-Alonso et al., 2018). We explicitly assess the degree to which the nature of the litter and the forest site influence: i) the decay rates, ii) changes in C and N contents and iii) successional shifts in microbial community composition in degrading litter.

Materials and Methods

Study site

The litterbag experiment was performed in an ecotone area between the Scots pine forests (*Pinus sylvestris* L.) and Pyrenean oak forests (*Quercus pyrenaica* Willd) in the northern slopes of the Guadarrama mountain, Central System Range, Spain (40° 51' N, 4° 3' W, ETRS89; 1350 m a.s.l). The area presents continental Mediterranean mountain climate with mean annual temperature of 11.0 °C and mean annual precipitation of 621 mm for the study period 2014-2017 (data from the State Meteorological Agency, AEMET). Soils are acidic, develop on a siliceous bedrock and classified as Humic Cambisols (Soil Survey Staff, 2014). The legacy of past human activities determines the current composition and structure of the study ecotone forest (Díaz-Pinés et al., 2014). Scots pine stands have been subjected to forest management for timber production since the end of 19th century favouring the extension of their range at the expense of the Pyrenean oak in the lowermost limit (Canellas et al., 2000; Morales-Molino et al., 2017; Sánchez Palomares et al., 2008). Intensive grazing and centennial coppicing for firewood and charcoal production explain the coppice structure of Pyrenean oak stands that after the abandonment of their intensive use with the rural emigration in the 1970s are being converted into high forest (Alvarez et al., 2014; Salomón et al., 2016a). Hence, climate change coupled with management shifts are leading to a progressive increase in the area covered by mixed oak-pine stands in the ecotones of the Central Mountain Range (Moreno-Fernández et al., 2016).

Experimental design

Two monospecific forests were established in a specific pine-oak ecotone for the litterbag experiment that were located within 1400 m of each other: an even-aged pure Scots pine stand (80-year old trees and 64 m² ha⁻¹ basal area) and a pure Pyrenean oak coppice (60-year old stems and 17 m² ha⁻¹ basal area). The experimental area had southwestern exposition and uniform hill slopes of 11°. The forest floor had around 1-cm thickness in the pine stand (9.7 Mg C ha⁻¹) whereas formed a transient layer in the oak stand (1.3 Mg C ha⁻¹) (Díaz-Pinés et al., 2011b). We used the litterbag method to assess mass loss, C and N contents, and the evolution of the microbial community structure in decomposing litter throughout a 24-month period. The experiment involved litterbags with three types of litter (needles, leaves and mixed needle:leaf litter) left above the ground of the two forest stands.

During the late autumn 2013, senescent leaves and needles were directly collected by hand picking from the branches of randomly selected trees in their respective Pyrenean oak and Scots pine stands. In the following early autumn, green leaves and green needles were collected at the same site in order to assess how litter quality would be altered prior senescence. Collected senescent and green litter were oven-dried for two days at 50 °C to constant mass (Bärlocher, 2005). Litterbags (20 cm x 20 cm, top net of 1.5 × 2 mm fibreglass mesh, bottom net of 500 µm polyester mesh) were filled with 10 g (± 1 mg) of senescent litter and numbered with aluminium labels. The mesh size of the litterbags allowed for the bacterial and fungal colonization as well as micro- and meso-fauna decomposers (Brandford et al., 2002). In each forest stand, 0.5-ha squared area were divided in four plots (35 × 35 m, hereafter referred as plot replicates). In each plot, six triplets of litterbags (needle, leaves and mixed 1:1 needles and leaves) straitened with a polyester rope were pinned onto the surface of the forest floor and separated a mean distance of 5 m, making a total of 144 litterbags (2 forest types × 3 litter types × 4 plot replicates × 6 litter-type replicates for individual sampling). The litterbags were anchored with steel pegs to prevent their movement and to ensure a close contact with the forest floor. Litterbags were placed in the field in April 2014 and collected every 4 months during six sampling times. One triplet of litterbags per plot was randomly collected at each sampling date (24 litterbags), placed in ice-cold coolers and immediately transported to the laboratory.

Chemical and microbial analyses

Immediately after litterbag collection from the field, litter material was taken out of the litterbags, sorted out and weighed. Litter samples from mixed species litterbags were separated into needles and leaves by hand. Litter samples at time 0 –i.e. green and senescent litter– and decomposing litter collected from litterbags at 0.35, 0.69, 1.34, 1.74 and 2.01 after the onset of the experiment were split in three subsamples. One aliquot of litter samples was freeze-dried

(Christ Alpha 1-4 freeze-dryer, Bioblock Scientific, France), ground and kept at -20 °C until estimation of microbial community structure using phospholipid fatty acid (PLFA) analysis. Another aliquot was oven-dried for 48 hours at 50 °C and ground with a ball mill (Mikro-dismembrator S, B. Braun Biotech, Germany) for chemical analysis and the last aliquot was dried at 105 °C and weight in order to estimate sample moisture content, from which dry mass was derived (Berger and Berger, 2014).

Remaining mass and accumulated mass loss of litter at each sampling date was expressed as the percentage of original mass after correction for humidity. Carbon and N concentrations were determined using a LECO TruMac CNS analyser (LECO Corporation, USA) and expressed in mg g⁻¹ dry litter. The PLFAs signature of microbial communities in lyophilized litter were used to estimate main microbial groups (gram-positive, gram-negative, fungi, etc.). All analyses were made by duplicate. Total lipids were extracted from 1 g of litter using a single-phase chloroform:methanol:citrate buffer (Frostegård et al., 1991). The lipid extracts were then fractionated into neutral-, glyco- and phospholipids, and the latter were subjected to a mild alkaline methanolysis to obtain fatty acid methyl esters (FAMES) as described in Fernández-Alonso et al., (2018). FAMES were separated by gas chromatography (6890 GC, Agilent technologies, Wilmington, Delaware, USA) equipped with a HP-5ms capillary column and detected using a flame ionization detector. Noncanoic acid methyl ester was used as internal standard for FAME quantification (19:0, Sigma-Aldrich, Wilmington, DE, USA) and 26-component standard of bacterial acid methyl esters (BAME mix, Supelco, Bellefonte, PA, USA) was used as the external standard for FAME identification. Terminally branched PLFA i14:0, i15:0, a15:0, i16:0, a16:0, i17:0, a17:0 were used as biomarkers for estimating the gram-positive bacteria (Frostegård and Bååth, 1996; Potthoff et al., 2006), and monounsaturated 16:1 ω 9, 16:1 ω 11, 17:1 ω 9, 18:1 ω 11 and cyclopropyl saturated PLFA cy17:0, cy18:0, cy19:0 were used for estimating gram-negative bacteria (Bach et al., 2010; Koranda et al., 2013; Schindlbacher et al., 2011). Bacteria were calculated as the sum of gram-positive and gram-negative bacterial biomarkers. Saprophytic fungi were estimated using the polyunsaturated peaks 18:3 ω 6,9,12c and 18:2 ω 9,12c (DeGroot et al., 2005; Wang et al., 2015). From these microbial groups, the fungal to bacterial ratio (F:B ratio) and the gram-positive to gram-negative (GP:GN ratio) were calculated. The F:B and GP:GN ratios account for fundamental functional differences between microbes and thereby serve as a proxy of microbial community life strategies distinguishing between slower growing K-strategists –often fungal and gram-positive bacteria– and fast-growing r-strategists –often bacteria and more specifically gram-negative bacteria– (De Vries and Shade, 2013; Fierer et al., 2007; Kaiser et al., 2014; Keiser et al., 2011).

Data analyses

We used two variables the decomposition rate and the remaining mass to investigate dynamics of mass loss in litter decomposition. Decomposition rate was computed according to Olson (1963):

$$-\ln\left(\frac{W_t}{W_0}\right) = k \cdot t \quad (\text{Eq. 1})$$

where W_t is the remaining dry mass (g) of each litter sample at the sampled time t expressed in years, W_0 is the initial dry mass (g) of the litter and k is the decomposition rate (year^{-1}).

We conducted a generalized linear mixed (GLM) model with gamma error distribution and the logarithmic link function in the analysis of the interaction effect between forest type and litter type on $\left(\frac{W_t}{W_0}\right)$, including plot and time as the random effects (*nlme* package, Pinheiro et al., (2017)). The modelled slopes for each combination forest $\times$ litter represent the decomposition rates (Eq. 1). The remaining mass of litter was analysed by a linear mixed effect (LME) model containing the threefold interaction between the fixed factors forest types, litter types and time, and also including plot as a random factor. Since there were not overall interaction effects between forests and litter on the remaining mass, additional linear models were conducted for testing separately the effect of the litter or the forest for a given collection time, as well as, the effect of collection dates within each forest or litter types.

Differences in the C and N concentrations of initial green and senescent litter were assessed using linear models. Moreover, linear relationships between the C and N concentrations of decomposing litter and the accumulated mass loss (%) with variables averaged per sampling day ($n = 4$), were examined including the interaction effect of forest and litter types. Since no significant interaction was found between forest types and litter, we inspected in separated linear models the effect of the forest types and litter types on C and N concentrations. Temporal patterns of the C:N ratio in decomposing litter were analysed using a LME model including the interaction effect between forest type, litter type and time, as well as the plot as random factor. Since the interaction between forest and litter was significant, we analysed in additional linear models for a given collection time and forest the effect of litter, and for a given collection time and litter the effect of forest on the C:N ratio, as well as the effect of time within each litter and forest types.

The F:B ratio and GP:GN ratio obtained from PLFA analysis were used as explicative variables to explore the effects of litter and forest types on the microbial community assembly in decomposing litter. Individual scatter plots were made for each collection date with both F:B and GP:GN ratios z-standardized and linear models were used to test the interaction effects between forest and litter types on the first axis and second axis of the plots.

In all mixed models, the Wald test was used to identify the terms that were not significant. The variance explained by fixed effects only and the variance explained by both fixed and random effects (marginal and conditional R^2 , respectively (Nakagawa and Schielzeth, 2013)) were calculated as a goodness-of-fit measure of GLM and LME models (*MuMIn* package, (Barton, 2018); *piecewiseSEM* package, (Lefcheck, 2016)). Post-hoc analyses (*lsmeans* package, (Lenth, 2016)) were performed with Bonferroni-corrected p-values for multiple comparisons in models containing the significant interaction effect between forest and litter types. The regression assumptions of normality and homogeneity of variance were assessed by plotting the quantile-quantile residual plot and the standardized residual versus fitted values of each model. All analyses and figures (*ggplot2* package, Wickham, (2009)) were performed in the statistical platform R version 3.4.0. (R Core Team, 2017).

Results

Litter decomposition rates

The GLM model accounted for 83 % of the observed variability in k , although most of it was due to the effect of the time that was included as a random effect (Table S6.1). The decomposition rate of the different litter types varied between pine and oak forests. Oak leaves (either alone or mixed with needles) decomposed faster under the Pyrenean oak stand than under the Scots pine stand (Figure 6.1). In contrast, needles (either alone or with leaves) litterbags decomposed with a similar k in both forest types. There was an effect of mixing pine and oak litter in the decomposition rate that was supported by the highest k showed by the autochthonous litter from mixed litterbags in each forest type in comparison with the transplanted litter from the single species litterbag (Figure 6.1). Hence, needle litter from mixed species litterbag decomposed faster than leaf litter from single species litterbag in the pine stand, with the other two litter types showing intermediate values. Similarly, leaf litter from mixed species litterbag decomposed faster than needle litter from single species litterbag in the oak stand, with again the other two litter types showing intermediate values.

Figure 6.1. Decomposition constants (k , year⁻¹) of the litter types (Nd: needles, Lv: leaves, MNd: needles from mixed litterbags, MLv: leaves from mixed litterbags) in the Scots pine and Pyrenean oak forests. Upper case letters denote significant differences between forest types, while lower case letters indicate significant differences between litter types (GLM model, $p < 0.05$). Values are means $\pm$ standard errors ($n = 4$).

There was not a significant threefold interaction effect between forests, litter and time on the remaining mass ($F = 1.52$, $p = 0.21$) and hence the LME model was simplified (Table S6.2). The remaining mass of litter decreased over time and its temporal evolution varied between litter types (Table S6.2), although no significant differences were found in their temporal patterns between forest types ($F = 2.53$, $p = 0.11$). In both forests, temporal dynamics of the biomass loss of all litter types were quite similar over the 1.3 years after the onset of the experiment. After this period, the needles and leaves from mixed species litterbags showed an accelerated loss of biomass (Figure 6.2a) so that, after two years, needles and leaves from the mixed litterbags showed lower remaining mass than the needle litter from single species litterbags (Figure 6.2a). Overall, the mass loss rates of litter varied between forests: the remaining mass was consistently lower in the Pyrenean oak stand than in the Scots pine stand (Figure 6.2b, Table S6.2). The remaining mass significantly decreased in each sampling date except for the 8-month period comprising the 1.03 – 1.74 years after starting the litterbag experiment.

Figure 6.2. Temporal evolution of the remaining mass of decomposing litter for each a) litter type and b) forest type. Upper case letters denote significant differences between collection dates within litter (a) or within forests (b), while small letters indicate significant differences between litter types (a) or between forest types (b) in each collection date (LME models, $p < 0.05$). Values are means $\pm$ standard errors ($n = 8$ in panel a, $n = 16$ in panel b). Nd: needles, Lv: leaves, MNd: needles from mixed litterbags, MLv: leaves from mixed litterbags, Ps: *Pinus sylvestris* and Qp: *Quercus pyrenaica*.

Changes in carbon and nitrogen contents

Chemical properties of green (collected in late spring 2014) and senescent litter (collected in late autumn 2013) at the onset of the experiment are summarized in Table 6.1. Green needles had significantly higher C:N ratio than green leaves. The N contents varied widely between green leaves and senescent leaves from oak but this change did not occurred in the corresponding needles from pines. Consequently, leaf litter showed larger C:N ratio than needle litter. Senescent needles were characterized by significantly higher concentrations of C and N than senescent leaves.

Table 6.1. Concentrations (mg g^{-1} dry mass) of C and N in green and senescent needles from Scots pine and leaves from Pyrenean oak.

	Needles		Leaves	
Green				
C	520.4 ± 0.1	a	492.4 ± 0.1	b
N	14.4 ± 0.1	b	17.6 ± 0.1	a
C:N ratio	33.9 ± 0.1	a	26.1 ± 0.0	b
Senescent				
C	535.8 ± 0.4	a	493.6 ± 0.4	b
N	14.8 ± 0.1	a	9.7 ± 0.2	b
C:N ratio	36.1 ± 0.1	b	50.9 ± 0.2	a

Values are means $\pm$ standard errors ($n = 4$). Lower case letters denote significant differences between litter types.

The mass of C in decomposing litter (% of initial C mass) decreased throughout the decomposition experiment (Figure 6.3), following similar patterns to the total remaining mass of litter (Figure 6.2). Remaining mass of C in needles decreased similarly in both Scots pine and Pyrenean oak forest during litter decay (Figure 6.3a, c) whereas the remaining mass of C in leaves showed a strongest decrease in the Pyrenean oak forest (Figure 6.3b, d). The absolute amount of N in decaying litter increase in the early stages of decomposition and then drastically showed N mobilization (Figure 6.4). The net increase of N was greater and lasted longer in leaves than in needles (up to 127 % of the initial N mass after 1.74 years and up to 109 % after 0.69 years in leaves and needles, respectively). Moreover, decaying litter in Scots pine forest showed a stronger immobilization of N than litter decomposing in Pyrenean oak forest especially in litter from single species litterbags.

Figure 6.3. Changes in the absolute amount of carbon in decomposing a) needles from single species litterbag, and b) leaves from single species litterbag, c) needles from mixed species litterbag and d) leaves from mixed species litterbag, expressed as % of original content. Line types represent different forest types (Ps, *Pinus sylvestris*; Qp, *Quercus pyrenaica*).

Figure 6.4. Changes in the absolute amount of nitrogen in decomposing a) needles from single species litterbag, and b) leaves from single species litterbag, c) needles from mixed species litterbag and d) leaves from mixed species litterbag, expressed as % of original content. Line types represent different forest types (Ps, *Pinus sylvestris*; Qp, *Quercus pyrenaica*).

There were no significant interaction effects between the forest types and the litter types in the evolution of C and N concentrations in decomposing litter over two years (Table S6.3). Overall, litter from single or mixed species litterbags showed similar changes in C and N concentrations as litter mass was lost, and only differences were found between tree species (Figure 6.5a and c). The C concentrations in decomposing needles remained relatively constant during two years of decomposition (Figure 6.5a). In contrast, C concentration in leaves decrease linearly with the accumulated mass loss. Moreover, the changes in C concentration in decomposing litter differed between forest types, with a higher rate of decrease in the Pyrenean oak than in the Scots pine forests (Figure 6.5b). The N concentrations in the remaining mass of needles and leaves increased over time with similar rates (Figure 6.5c). There was a significant effect of the forest type in the slopes of the regression equations for N concentrations showing greater increments in the pine than in the oak forests (Figure 6.5d).

Figure 6.5. Changes in C and N concentrations of decomposing litter as a function of accumulated total mass loss. Panels a) and c) show differences between litter types (Nd, needles; Lv, leaves; MNd, needles from mixed litterbags; MLv, leaves from mixed litterbags) whereas panels b) and d) show differences between forest types (Ps, *Pinus sylvestris*; Qp, *Quercus pyrenaica*). Grey lines represent the regression equations (linear models, $p < 0.05$).

The litter C:N ratio was not affected by the threefold interaction effect between forest types, litter types and time ($F = 0.316$, $p = 0.813$) but its temporal evolution differed between litter and forest types (Table S6.4). In the Scots pine stand, the leaves maintained higher C:N ratios than needles throughout the whole study period independently if they were from single or mixed species litterbags (Figure 6.6a). In most of the sampling dates the leaves from mixed species litterbags showed significantly lower C:N ratios in the Pyrenean oak than in the Scots pine forests (Figure 6.6a and b). Hence, the leaves from mixed litterbags after two years of decomposition showed the lowest C:N ratio at the Pyrenean oak stand (Figure 6.6b).

Figure 6.6. Changes in C:N ratio of litter during decomposition in the a) Scots pine forest and b) Pyrenean oak forest. Small letters denote significant differences between litter types for a given collection time in each forest, while capital letters indicate significant differences between forest types for a given collection time in each litter type (LME models, $p < 0.05$).

Changes in microbial community structure

The resident phyllosphere communities in starting senescent litter showed different structures between needles and leaves (Figure 6.7a). The differences in autochthonous microbial communities were mainly due to a significantly higher GP:GN ratio in needles than in leaves since the F:B ratio was rather similar in both litters (Table S6.5a). The initial effect of the litter types in the GP:GN ratio progressively diminished over the decomposition experiment as can be seen in the reduced distance in the y-axis between needles (triangles) and leaves (circles) (Figure 6.7c-g) until it completely disappeared after two years (Figure 6.7h, Table S6.5h). The forest type also influenced the GP:GN ratio in decomposing litter: litter at the Pyrenean oak stand (red symbols) showed significantly higher GP:GN ratio than those at the Scots pine stand (green symbols) over the period of between 8 and 16 months (Figure 6.7d-f). Moreover, the effect of the forest type on the GP:GN ratio diluted in the 20 month (Figure 6.7g, Table S6.5g) and reversed at the month 24 (Figure 6.7h). There was a steady effect of the litter type on the F:B ratio of microbial communities in decomposing litter that appeared after 8 months and remained throughout the whole study period by which needle litter had higher F:B ratios than leaf litter (Figure 6.7d-h). The effect of the litter type on the F:B ratio was stronger in the pine than in the oak forest as evidenced the interaction effect between forest and litter types in the 12 and 20 month of decomposition (Table S6.5e and g). The effect of the forest type on the litter F:B ratio changed substantially over the course of the decomposition.

Figure 6.7. Scatter plots of the standardized variables fungal to bacterial ratios (F:B ratio) and gram-negative to gram-positive bacterial ratio (GP:GN ratio) in a) senescent needle and leaf litter ($t = 0$), b) decomposing litter from all collections and c-h) decomposing litter of individual collections. Bold symbols denote litter from single species litterbags (triangles Nd, needles; circles Lv, leaves) whereas empty symbols represent litter from mixed species litterbags (triangles MNd, needles from mixed litterbags; circles MLv, leaves from mixed litterbags). Colours indicate forest types (Qp in red, *Pyrenean oak*; and Ps in green, *Pinus sylvestris*). Points represent mean scores and error bars depict $\pm$ standard error ($n = 4$).

Discussion

Our results showed higher remaining mass of needles than leaves after two years of experiment, which is in line with our studies indicating larger C stock in the forest floor (Díaz-Pinés et al., 2011b) and lower decomposability of soil-C (Chapter 4) from the Scots pine stand than from the Pyrenean oak stand in Valsain. However, a faster decomposition of the leaves were not a rule of thumb in the experiment given the synergistic effects that occurred on decay rates within litter mixtures. Therefore, the different litter chemistry between tree species was not the only factor controlling the C and N dynamics during decomposition. Indeed, the influence of the forest type in the microclimate and functional specificity of colonizer microbes in litter seemed to play an important role in the studied litter decomposition processes.

Litter decomposition

There were litter-forest interaction effects on decomposition rates supporting the HFA hypothesis exclusively for the leaves: k for needle litter was similar in both forests, whereas leaf litter showed 18 % larger k in the oak stand than corresponding k in the pine stand. Moreover, we identified synergistic effects between needles and leaves on the decomposition constants within litter admixtures in accordance with most of the experiments reviewed by Gartner and Cardon (2004). This finding led native litter from litter admixtures to decay the fastest in each forest type (on average autochthonous litter decomposing in mixed species litterbags showed 16 % greater k than foreign litter in single species litterbags). These decomposition patterns could likely be attributed to: (1) indirect facilitation on native microbial communities by surrounding environment of each tree-species since microbial assemblies in litter differentiated over time (Zechmeister-Boltenstern et al., 2015); (2) more efficient utilization of those litter with which decomposers have historically co-evolved (Austin et al., 2014; Ayres et al., 2009); and (3) increased decay efficiency caused by nutrient transfers between litter types of different quality (Berger and Berger, 2012; Gartner and Cardon, 2004).

One of the mechanisms why HFA may be masked in needles is that soil microclimate created beneath the deciduous oak could enhance the decay of both litter types through a promoted abiotic decomposition (mainly thermal and photochemical degradation mechanisms). In previous studies carried out in same forest stands, we found colder temperatures during winter and higher temperatures during spring in soils under oak compared to soils under pine (Table 4.2). We interpret that differences in seasonal soil temperatures between forest stands are the result of the buffering effects of the canopy to extreme temperatures at the ground surface (Prescott, 2002). In winter, pine canopy may provide greater insulation of soil against the thermal variability in the atmosphere (Breshears et al., 1998) than the lower leaf area index of oak forests after leaf shedding

(Curiel Yuste et al., 2004), and hence reduce the intensity of freeze-thaw events responsible for degradation of litter physical structure through the disruption of recalcitrant components such as lignin (Wu et al., 2010). Moreover, the higher solar irradiance reaching the soil due to the absence of solar interception by the oak canopy during the spring may promote the observed warmer temperatures in the soil and enhance litter photodegradation, which is a mechanism that has been found to greatly contribute to litter decomposition in Mediterranean region (Gliksman et al., 2017). Taken together, these abiotic mechanisms can likely increase the subsequent microbial accessibility to easily biodegradable constituents that were formerly blocked in lignin of secondary cell walls of plant litter ('photopriming', Austin et al., 2016) and produce the greater mass loss found in the decomposing litter at the oak stand. Consequently, the microclimate facilitation of abiotic and biotic decay mechanisms of all litter types in the oak stand may explain the unexpected similar decay rates of the needles in both forests. However, new studies are needed to explicitly verify the indirect effect of the forest type through soil microclimate on decomposition rates of needle and leaf litter in the studied ecotone.

Nitrogen dynamics and microbial colonization

The N dynamics in litter were characterized by two stages. First, there was a net N immobilization phase due to the incorporation of N into decaying litter that was greater and lasted longer in leaves than in needles, which was then followed by N mobilization phase. The chemical background of the immobilized N is still little known although it has been suggested that the N is imported to the litter by microbial biomass (e.g. aminoacids and fungal melanins) during early stages of decomposition and then is complexed and immobilized during the progressive formation of lignin-like recalcitrant substances (Gallardo and Merino, 1992; Kraus et al., 2003). Therefore, the different N immobilization patterns observed between forests –i.e. litter decomposing in pine stands generally immobilized greater amounts of N– may reflect their diverging trends in microbial succession registered over time that were in turn ascribed to soil characteristics. The absolute N increment in decaying litter from forest ecosystems is determined by the amount and chemistry of recalcitrant compounds in initial litter as well as by their initial C:N ratio (Aber and Melillo, 1982; Gallardo and Merino, 1992). In this experiment, green leaves had significantly lower C:N ratio than green needles but this relationship was reversed in senescent litter because leaves experienced an intense translocation of N before their senescence. Escudero et al. (1992) estimated a resorption of ca. 47 % of the N in leaf litter of Pyrenean oak before senesce, which is consistent to the values obtained in this study for leaves, and ca. 50 % of N resorption in Scots pine needles. However, we found similar values in the C:N ratios of green and senescent needles. This could be explained because dead needles stayed attached to the branches for long before being shed, as suggested Berg and Tamm (1991) for needles from *Picea abies*, and therefore were

in an advanced stage of decomposition in the moment when they were hand-picked from pines for the litterbag experiment. Thus, the total N content of needle litter at the onset of the experiment were likely already tied to the lignin fraction as a consequence of the activity of resident phyllosphere communities and were not reflecting an actual amount of N available for microbes as suggested Osono and Takeda (2005). Therefore, our results showing oak leaves immobilizing N more than pine needles were consistent with previous reports linking foliar litter with low initial N content and high concentration of recalcitrant compounds to net increases in litter-N, as well as with the N suppressing effect on mass-loss rates of both lignin and litter in the already partly decomposed needles (Berg, 2000; Gallardo and Merino, 1992; Osono et al., 2014).

As litter decomposition proceeded, the C:N ratio of needles and leaves decreased progressively which has been normally linked to a reduction in litter decomposability likely through the degradation of soluble substances and unshielded holocellulose and the relative enrichment in recalcitrant compounds such as lignin (Berg, 2000; Vivanco and Austin, 2008) until a threshold value was reached. Then, decomposers started nutrient mineralization producing the release of N that in previous studies has been associated to the degradation of lignin (Berg and McClaugherty, 1989; Bonanomi et al., 2010; Osono and Takeda, 2004). At this second stage of decomposition, the decay rates of the litter are retarded and probably constrained by the degradation rate of lignin (Berg, 2000); this situation seemed to occur predominately in needles, where the C concentration in remaining mass was steady during the whole experiment, rather than in leaves, where C concentration progressively decreased as decomposition proceeded. It should be also noted that the described two-stages of litter decomposition are probably occurring simultaneously, but one or the other dominate at a given time. Consequently, net N mobilization began in decaying leaves after 1.74 years of decomposition whereas this occurred earlier in needles (ca. 0.69 years) when litter C:N ratio attained values between 25 – 30, which is coincident with the critical ratio demonstrated by a review of N dynamics on 47 litter types in temperate and boreal forests (Osono and Takeda, 2004). Our results suggest that the temporal evolution of needle and leaf stoichiometries affected the interactions between microbial functional groups and produced an early differentiation (ca. 1 year) of heterotrophic microbial communities during litter colonization. Overall, we were able to distinguish how initial phyllosphere communities in needles changed toward a greater dominance of K-strategist microorganisms, meaning gram-positive bacteria and fungal groups, and toward a higher abundance of r-strategist microorganisms in leaves, meaning bacterial groups, and within that, the gram-negative bacteria (De Vries and Shade, 2013; Fierer et al., 2007; Keiser et al., 2011). Observed successional dynamics in predominated life strategies of microbial communities are in accordance with the ecological stoichiometric theory, which may feedback in C and N recycling and thus alter litter decomposition rates in a way that is not predictable (Kaiser et al., 2014; Zechmeister-Boltenstern

et al., 2015). Therefore, the specific assembly of microbial communities for each litter type and decomposition forest site may be an explanation for the differences in HFA observed here for needles and leaves aside from the contrasting abiotic degradation linked to the forest type.

Besides, our results led to distinguish how litter stoichiometry and microbial community structure and function converged during litter decomposition toward soil organic matter (SOM) stoichiometry and soil microbes in each forest (Zechmeister-Boltenstern et al., 2015). This finding was consistent with the specific development of the colonizing microbial communities observed over time by Aneja et al. (2006) for litter types and decomposition sites from deciduous (beech) and coniferous (spruce) trees. We distinguished an early differentiation in the F:B ratio of colonizing microbial communities probably related to initial litter chemistry, which was subsequently complemented by a later differentiation in the GP:GN ratio likely linked to differences in microbial succession between forests. Initially, there were no differences in the F:B ratio of resident phyllosphere communities between senescent needles and leaves that were picked directly from the tree canopy. However, the stoichiometric imbalance between litter and biomass of phyllosphere microbes created a natural selection in the microbial community favouring colonization of certain life strategies that were more efficient at acquiring limited-N in each litter type (Zechmeister-Boltenstern et al., 2015). Thus, when degrading needles reached their threshold elemental ratio a preferential fungal over bacterial metabolism became increasingly evident in the microbial community of needles as decomposition proceeded (after ~ 1 year), which is consistent with the known ability of fungi for efficiently scavenging of N from recalcitrant litter (Osono and Takeda, 2005; Rodríguez et al., 2017; Zechmeister-Boltenstern et al., 2015). In a more advanced stage of decomposition (after ~ 2 years), the structure of microbial communities in decaying litter differed in the GP:GN ratios that could be related to an increasing importance of microbial remains (Kaiser et al., 2014) and physiology (Manzoni et al., 2012) in the formation of SOM. The soil beneath pines presents SOM with higher C:N ratio compared to oak soils (22.9 and 13.4 in the pine and in the oak forests, respectively; Díaz-Pinés et al., 2011; Fernández-Alonso et al., 2018). Hence, our results allow to link colonizing microbial communities after two years of litter decomposition with the specific soil microbial communities in each forest type. The N-poorer soil beneath pines is associated with greater dominance of gram-positive bacteria whereas the higher abundance of gram-negative beneath oaks is connected to the higher N availability in soils (Creamer et al., 2015; Fernández-Alonso et al., 2018a; Fierer et al., 2003; Waldrop and Firestone, 2004).

Conclusions

Our results evidence that litter stoichiometry is the most important factor affecting mass loss and N dynamics in litter during the early stages of decay (ca. 1 year after decomposition began). However, the effects of forest type by pre-determining decomposition conditions showed an increasing importance with time. Tree species influenced soil microclimate and the functional assembly of microbial communities, both key drivers of litter decomposition processes. We found differential microbial colonisation patterns between forests suggesting a staggered functional adjustment of the microbial community driven firstly by litter stoichiometry, and then by the convergence of colonizing microbial communities toward soil microbes and SOM characteristics. Such specific feedbacks between tree species, soil microenvironment and microbial functionality have strong implications for litter decomposition and biogeochemical C and N cycles and hence could be responsible for the HFA found exclusively for the oak heterotrophic microbes when decomposing leaves and for the synergistic effects between litter types in admixtures. Our findings indicate that the global change-driven forest succession from pine to oak may partly lead to a decrease of the forest floor accumulation since leaves showed lower remaining mass than needles after two years of decomposition.

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Supplementary information

Table S6.1. Parameter estimates of the generalized linear mixed model with gamma error distribution and logarithmic link function testing for the interaction effects between forests (Ps, *Pinus sylvestris*; Qp, *Quercus pyrenaica*) and litter types (Nd, needles; Lv, leaves; MNd, needles from mixed species litterbags and MLv, leaves from mixed species litterbags) on the decomposition constant (k , year⁻¹, Figure 6.1).

Terms	GLMM R ²	d.f.	F value	Wald test		Estimate	SE	t-value	Sign.
				χ^2	p-value				
marginal R ²	0.03								
conditional R ²	0.83								
Intercept		1		9.77	**	-0.312	0.100	-3.125	**
Forest		1	9.00	0.01	0.92				
Ps						-	-	-	-
Qp						-0.003	0.025	-0.103	0.918
Litter		3	3.66	9.97	*				
Nd						-	-	-	-
Lv						0.019	0.025	0.763	0.445
MNd						-0.056	0.025	-2.258	*
MLv						-0.006	0.025	-0.246	0.806
Forest × Litter		3	2.55	9.26	*				
Qp × Nd						-	-	-	-
Qp × Lv						-0.072	0.035	-2.051	*
Qp × MNd						-0.001	0.035	-0.043	0.966
Qp × MLv						-0.080	0.035	-2.283	*

Marginal R² represents the variance explained by fixed effects only. Conditional R² represents the variance explained by both fixed and random effects. d.f.: degrees of freedom, SE: standard error, Sign.: significance codes (** p < 0.01, * p < 0.05).

Table S6.2. Parameter estimates of the linear mixed model testing for the differences on the remaining mass as a function of forests (Ps, *Pinus sylvestris*; Qp, *Quercus pyrenaica*), litter (Nd, needles; Lv, leaves; MNd, needles from mixed litterbags; MLv, leaves from mixed litterbags) and time.

Terms	LMM R ²	d.f.	F value	Wald test		Estimate	SE	t-value	Sign.
				χ^2	p-value				
marginal R ²	0.77								
conditional R ²	0.77								
Intercept		1	24002.43	1874.54	***	95.311	2.201	43.296	***
Forest		1	8.16	8.16	**				
Ps						-	-	-	-
Qp						-2.661	0.931	-2.857	*
Litter		3	2.56	2.13	0.00				
Nd						-	-	-	-
Lv						2.576	3.043	0.847	0.398
MNd						4.173	3.043	1.372	0.172
MLv						3.392	3.043	1.115	0.266
Time		1	631.12	108.78	***	-16.934	1.624	10.430	***
Litter × Time		3	2.78	8.33	*				
Nd × Time						-	-	-	-
Lv × Time						-2.773	2.296	-1.208	0.229
MNd × Time						-6.204	2.296	-2.702	**
MLv × Time						-4.866	2.296	-2.119	*

Marginal R² represents the variance explained by fixed effects only. Conditional R² represents the variance explained by both fixed and random effects. d.f.: degrees of freedom, SE: standard error, Sign.: significance codes (*** p < 0.001, ** p < 0.05, * p < 0.05).

Table S6.3. Results of the linear models testing for the interaction effects of accumulated mass loss (AML, %), forest and litter on the concentration of C and N in decomposing litter.

Response variable		[C] (mg g ⁻¹)		[N] (mg g ⁻¹)	
Variables	d.f.	F-value	p-value	F-value	p-value
AML	1	33.22	***	538.34	***
Forest	1	15.86	***	9.14	**
Litter	3	182.84	***	143.04	***
Forest × AML	1	11.00	**	4.34	*
Litter × AML	3	7.33	***	1.41	0.25
Forest × Litter	3	0.49	0.69	1.22	0.31
Forest × Litter × AML	3	0.99	0.41	0.51	0.68

Significance codes: *** $p < 0.001$, ** $p < 0.05$, * $p < 0.05$.

Table S6.4. Parameter estimates of the linear mixed model testing for the differences on the C to N ratio in decomposing litter as a function of forests (Ps, *Pinus sylvestris*; Qp, *Quercus pyrenaica*), litter (Nd, needles; Lv, leaves; MNd, needles from mixed litterbags; MLv, leaves from mixed litterbags) and time.

Terms	LMEM R ²	d.f.	F value	Wald test		Estimate	SE	t-value	Sign.
				χ^2	p-value				
marginal R ²	0.84								
conditional R ²	0.84								
			29092	1447.4					
Intercept		1	.96	8	***	33.402	0.878	38.046	***
Forest		1	3.78	0.66	0.42				
Ps						-	-	-	-
Qp						0.567	0.698	0.812	0.448
Litter		3	56.36	183.80	***				
Nd						-	-	-	-
Lv						12.465	1.242	10.040	***
MNd						1.193	1.242	0.961	0.338
MLv						12.473	1.242	10.046	***
Time		1	736	81	***	-5.477	0.608	-9.008	***
Forest × Litter		3	5	15	**				
Qp × Nd						-	-	-	-
Qp × Lv						-1.543	0.987	-1.564	0.120
Qp × MNd						-0.067	0.987	-0.068	0.946
Qp × MLv						-3.368	0.987	-3.414	***
Litter × Time		3	25.35	76.04	***				
Nd × Time						-	-	-	-
Lv × Time						-5.121	0.860	-5.956	***
MNd × Time						-0.254	0.860	-0.295	0.768
MLv × Time						-5.699	0.860	-6.628	***

Marginal R² represents the variance explained by fixed effects only. Conditional R² represents the variance explained by both fixed and random effects. d.f.: degrees of freedom, SE: standard error, Sign.: significance codes (*** p < 0.001, ** p < 0.05, * p < 0.05).

Table S6.5. Results of linear models showing the interaction effect between forest types and litter types on the standardized variables fungal to bacterial ratio (F:B ratio) and gram-positive to gram-negative ratio (GP:GN ratio) from soil microbial communities measured as PLFAs ($n = 4$) in each collection day (Figure 6.7).

	Forest		Litter		Forest $\times$ Litter	
	F-value	p-value	F-value	p-value	F-value	p-value
a) t = 0 - Senescent litter						
F:B ratio	-	-	2.43	0.17	-	-
GP:GN ratio	-	-	452.65	***	-	-
c) t = 4 months - August 2014						
F:B ratio	4.87	*	0.19	0.90	1.11	0.36
GP:GN ratio	0.01	0.93	60.35	***	1.28	0.30
d) t = 8 months - December 2014						
F:B ratio	5.26	*	6.56	**	0.66	0.58
GP:GN ratio	4.85	*	12.59	***	0.32	0.81
e) t = 12 months - April 2015						
F:B ratio	5.04	*	4.79	*	2.67	.
GP:GN ratio	5.01	*	22.50	***	0.68	0.58
f) t = 16 months - August 2015						
F:B ratio	0.61	0.45	6.06	**	0.58	0.64
GP:GN ratio	11.06	**	6.16	**	0.54	0.66
g) t = 20 months - December 2015						
F:B ratio	11.92	**	20.51	***	5.70	**
GP:GN ratio	0.00	0.97	5.54	**	2.18	0.13
h) t = 20 months - December 2015						
F:B ratio	16.10	**	8.27	**	1.03	0.40
GP:GN ratio	67.34	***	3.00	.	1.38	0.29

Chapter 7

Changes in litter chemistry associated with global change-driven forest succession resulted in time-decoupled responses of soil carbon and nitrogen cycles

Abstract

Global change-driven forest succession may modify key soil processes with potentially important impacts over carbon (C) and nutrient cycling. We studied how changes in litter throughout the replacement of *Pinus sylvestris* by *Quercus pyrenaica* influence the structure and functioning of soil microbial communities and the capacity of soils to sequester C and retain nitrogen (N). We designed a microcosm experiment to simulate the chronological sequence from pine to oak forest conversion in Central Spain, using mixtures of senescent litter (oak leaves, pine needles and an equal mixture of needles:leaves) and soils (from pure oak, mixed and pure pine stands). We investigated changing patterns of soil-C and N contents, microbial community structure (PLFA) and greenhouse gas fluxes (CO₂, CH₄, N₂O) across the chronosequence. The succession from pine to oak forest was associated with substantial changes in microbial community structure and functioning. Soil-C sink capacity was reduced, although soil-N availability was enhanced. We further show how effects of secondary succession on the C cycle were mismatched with N dynamics in response to two chronologically decoupled facts. First, there was an acceleration in soil organic matter (SOM) turnover after microbial –especially bacterial– growth ceased to be so intensely inhibited by needle litter (ecotone soils), resulting in lower fungal to bacterial ratios; and second, N mineralization was stimulated once pine-derived SOM was no longer present in soils (pure oak forest soils), resulting in further acceleration of SOM turnover, suppression of CH₄ consumption and an increase in gram-negative bacteria. Our findings suggest that different sensitivities of key mechanisms (SOM decomposition, N mineralization, CH₄ consumption) to factors associated with succession (e.g. recalcitrance of pine SOM and allelopathic effects over bacteria) could have significant impacts on soil microbial ecology, C and nutrient cycling.

Keywords:

Greenhouse gas fluxes, carbon sequestration, carbon-use efficiency, nitrogen availability, microbial community structure, chronological sequence.

Highlights

- Forest succession from pine to oak results in decoupled soil nutrient dynamics.
- The different sensitivities of microbial functional groups trigger this decoupling.
- The vegetation shift produces changes in microbial community structure and ecology.
- Successional changes led to a significant loss of the soil carbon sink capacity.
- There is a substantial gain in available mineral nitrogen in oak soils.

Graphical abstract

Introduction

We are currently facing successional changes in forests responsible for essential shifts in the functional diversity of terrestrial ecosystems on a large scale (Ruiz-Benito et al., 2017). This is partly a consequence of the altitudinal migration of dominant tree species driven by the ongoing global change (Allen et al., 2010; Camarero and Gutiérrez, 2004; Sanz-Elorza et al., 2003). On the Iberian Peninsula, a gradual replacement of Scots pine (*Pinus sylvestris* L.) by several species from the genus *Quercus* sp. (Carnicer et al., 2014; Díaz-Pinés et al., 2014; Galiano et al., 2010) may occur as a result of both the predicted changes in temperature and precipitation and changes in land use and forestry (Alvarez et al., 2014; Hereş et al., 2012; Martínez-Vilalta and Piñol, 2002). This succession from temperate needle-leaved forests to temperate broad-leaved forest leads to an abrupt change in ecosystem functional traits (Ruiz-Benito et al., 2017) that will be linked to further changes in soil functioning. However, few studies have addressed how this ecological succession modulates the time and the direction of changes in soil greenhouse gas (GHG) fluxes, carbon (C) sequestration and nitrogen (N) cycling.

A change from needle-leaved forest to broad-leaved forest alters important quantitative and qualitative traits of plant litter inputs to the soil (e.g. litterfall rates, C:N ratios and lignin content), and thus affects soil organic matter (SOM) decomposition dynamics (Delgado-Baquerizo et al., 2015; Knapp et al., 2011) and biogenic production of GHGs –methane (CH₄), nitrous oxide (N₂O) and carbon dioxide (CO₂) (Heimann and Reichstein, 2008). Hence, shifts in the plant cover may affect factors directly controlling SOM decomposition, responsible for at least half the CO₂ emitted from soils (Bond-Lamberty et al., 2004a). Changes of the input of fresh litter to the soil may also directly affect various mechanisms (e.g. nutrient limitation, co-metabolism) responsible for stimulating SOM decomposition –the so called “priming effect” (Blagodatskaya and Kuzyakov, 2008; Creamer et al., 2015). According to the inherent quality of the broad-leaved litter, we may predict an acceleration of the turnover rate of the bulk SOM, but our understanding of other potential triggers and the timing of this response throughout the successional process is still limited. Another aspect that usually regulates SOM decomposition rate is the nutrient level (Berg, 2000). Conifers generally slow down the recycle of nutrients whereas deciduous trees require faster nutrient cycling to generate new foliage each year (Curiel Yuste et al., 2005). Thus, the encroachment of deciduous late successional trees may also have important consequences for coupled forest C and N cycling that currently are difficult to predict (Nave et al., 2011).

Regarding soil biota, global change-driven shifts in plant cover may also disrupt important and stable ecological interactions selected by nature in soils, since soil microbiota are physiologically adapted to the particular conditions occurring within an ecosystem as a result of

historical selection processes (Keiser et al., 2011; Strickland et al., 2009b). Microbial communities would therefore be more efficient decomposers of the plant-derived organic matter with which these communities have historically co-evolved, described as the “home-field advantage theory” (HFA) (Barba et al., 2015; Veen et al., 2015). However, the enormous functional redundancy of these communities may counteract this HFA, since many taxa are able to perform equivalent functions at the community level (Allison and Martiny, 2008; Curiel Yuste et al., 2014; Wohl et al., 2004). The HFA of soil microbial communities in relation to litter decomposition is not fully understood (Ayres et al., 2009) and could play a critical role in destabilising SOM in current large-scale scenarios of species substitution driven by global-change.

The above-mentioned aspects evidence that to predict how soils will respond to changes in forest functional traits we need to understand how the origin of SOM and the structure of soil microbial community relates to soil functioning. This study aims to disentangle how changes in the chemical composition of the litter input to soils (deciduous leaves vs conifer needles) affect the ecology of soil microbial communities and the key microbial functions controlling soil-C and N dynamics. The question of how potential regulating factors hasten decomposition in soils and hence influence GHG fluxes in response to the encroachment of broad-leaved species in coniferous forests is highly significant, since Scots pine forest soils store roughly double the C and N stocks of oak soils in Mediterranean temperate forests (Díaz-Pinés et al., 2011b). As environmental variability in the field may mask the effects of litter quality on decay processes (Vesterdal, 1999), we followed a full-factorial microcosm approach by mixing three types of forest soils with three types of senescent litter collected within a Mediterranean ecotone transect between Pyrenean oak (*Quercus pyrenaica* Willd.) and Scots pine forests in the Valsaín mountains (Central Spain). These microcosms were used to recreate the chronological sequence of the vegetation change. After a 54-day incubation period, we examined the changes across the chronosequence on microbial community structure, total C (TOC) and N contents (TN) and their more accessible pools (extractable organic carbon (EOC) and total dissolved nitrogen (TDN)), and the production of soil GHGs (CO₂, CH₄ and N₂O).

Materials and methods

Study site

The experimental site is located in a representative ecotone area between Pyrenean oak and Scots pine forests in Valsaín, on the northern slopes of the Guadarrama range in Central Spain (40° 51' N, 4° 3' W, ETRS89, ~1350 m.a.s.l.). In these mountains, pine forests occupy higher

altitudes (1300-1900 m) than oak forests (400-1300 m). The current climate change and the abandonment of the traditional use of Pyrenean oak coppices during the 1970s have contributed to a gradual altitudinal expansion of oak trees into the former pure Scots pine forest (Alvarez et al., 2014). At this location, three different stands were identified along a 1400 m transect, ranging from a pure Pyrenean oak forest to a pure Scots pine forest, with a mixed Scots pine-Pyrenean oak forest in the ecotone between both pure stands. The study area presents a continental Mediterranean mountain climate with high year-round fluctuations in precipitation and temperatures. Summers are warm with three/four months of drought, whereas winters are cold and may have a thin snow cover lasting up to several weeks. For the period 2014-2017, the mean annual precipitation was 621.4 ± 64.8 mm and the maximum and minimum mean annual temperatures were 16.5 ± 0.4 °C and 5.4 ± 0.1 °C respectively (data collected from a weather station run by the State Meteorological Agency situated 9 km away, http://www.aemet.es/es/serviciosclimaticos/vigilancia_clima). Soils are Cambisols (Soil Survey Staff, 2014) with a granite bedrock sandy loam texture. At the study site, the pH (4.0 - 5.5 in KCl, slightly more acidic in pine stands) and the accumulation of C in topsoil (0 - 50 cm: 40, 80 and 95 Mg C ha⁻¹ in Pyrenean oak, mixed and Scots pine forest respectively) are strongly influenced by tree species. For more information on the general and silvicultural characteristics of the study site, see Díaz-Pinés et al. (2014).

Soil and litter sampling

Soil sampling was done before the autumn rainfall period in September 2015 at a gravimetric field moisture content of about 7 %. In each forest type, five plots measuring 4 m² were randomly established with a minimum separation distance of 125 m. In each plot, five soil samples were collected from a depth of 0 - 5 cm using a core sampler (5 cm in diameter) after removing the plant litter layer (undecomposed litter that forms a transient layer in the oak forest but has ~1 cm thickness in pine forest). The 25 soil subsamples collected in each forest were hand mixed and homogenized to obtain three composite forest soil samples that were shipped on ice to the laboratory in Vienna. Samples were sieved through a 4-mm mesh and stored at 4 °C for no longer than a week until used in the incubation experiment.

Litter samples were collected before needle and leaf abscission in the late autumn of the previous litterfall period. Litter sampling was done by hand, picking only senescent needles and leaves from randomly selected representative trees in both pure oak and pine forests. Given that the litter collected had no previous contact with soils, we assumed it had not yet been inoculated by soil microorganisms. The collected litter was mixed to form a single and homogeneous sample per tree species. Bulk needle and leaf litter were oven-dried at 45 °C to constant weight, and then

cut into 1-cm length pieces to standardise the samples and aid their decomposition during incubation.

Experimental setup

Twelve microcosms (4 litter amendments $\times$ 3 soil inoculums) were used to assess whether substantial changes occurred in the structure and physiology of soil microbial communities when exposed to a non-native litter. Microcosms were built with field-moist soil inoculums from the three forests identified in the study site (pure Pyrenean oak forest, SO; mixed Scots pine-Pyrenean oak forest, SM; and pure Scots pine forest, SP) mixed with four litter amendments (oak leaves, Lv; mixed 1:1 needles-leaves, Mx; pine needles, Nd; and control with no litter, Soil-only). A total of 36 microcosms were used in the experiment following a full-factorial design with three replicates (12 microcosms $\times$ 3 laboratory replications). A subset of microcosms (SP+Nd > SP+Mx > SM+Nd > SM+Mx > SM+Lv > SO+Mx > SO+Lv) represented the different sequential habitats of the oak altitudinal expansion into the pure pine forest.

The microcosms consisted of stainless steel cylinders (187.3 cm³) filled with an amount of soil consistent with the mean soil bulk density in each forest stand (Díaz-Pinés et al., 2011b) and mixed with 2 g of litter, which is the actual field quantity in the litter layer. In addition, nine microcosms with litter-only (3 litter types $\times$ 3 laboratory replications) were used as controls to assess possible background respiration rates. The laboratory incubation procedure (45 microcosms in total) was carried out with the soil moisture content standardized to 50 % water-filled pore space. All microcosms were weighed periodically to determine moisture losses and, if necessary, sterile deionized water was added 24 hours prior to sampling events to maintain the moisture constant. Microcosms were incubated over a 54-day period in temperature-controlled incubators maintained in darkness at 20 °C (maximum mean monthly temperature in soils at 10-cm depth for the period 2014-2017, data collected with DS1922L Thermochron, iButtonLink).

Soil and litter analysis

Three aliquots were taken per original bulk litter sample to correct for oven dry mass, and another three aliquots previously ground with a ball mill (Mikro-dismembrator S, B. Braun Biotech, Germany) to determine TOC and TN concentrations using a LECO TruMac CNS analyser (LECO Corporation, USA). Field moisture content was determined from the fresh soil samples (< 4 mm). The content of TN and TOC, EOC and TDN were analysed in triplicate in soil inoculums (< 2 mm) before and after the incubation experiment (days 0 and 54). EOC and TDN were extracted with 0.5 M K₂SO₄ at a sample:extract ratio of 1:4 (w:v), and thereafter determined using an analyser (TOC-L Analyser coupled with TN unit, Shimadzu). Mean concentrations (n = 3) of TOC and TN were expressed in mg g⁻¹, and EOC and TDN in µg g⁻¹ of dry weight material.

Changes in EOC and TDN in the microcosms with incubation were calculated as the percentage variation from the initial concentration in soils (day 0).

Automatic measurements of CO₂ efflux from microcosms

CO₂ efflux rates from microcosms were determined using two fully automated measuring devices (adopted according to Schaufler et al (2010)). Each one was directly connected to a WMA-4 infrared gas analyser (PP-System, Massachusetts, USA) that monitored 16 chambers (15 microcosm chambers, 1 reference chamber). Twelve measurement periods of 60 hours were used in two separate batches to monitor the CO₂ efflux rates from the 45 microcosms. The system was operated as an open-flow-through method using a computer program to control the sequence and opening time of each chamber valve and data acquisition. The reference chamber was monitored after the CO₂ efflux of each microcosm had been measured. The air inside each chamber was sucked out to the analysers at a constant flow of 1 L min⁻¹. The incubator was flushed with compressed filtered ambient air (1 L min⁻¹) to avoid accumulation of CO₂. Concentrations of soil CO₂ were used in calculations when steady state was reached (after 2.5 min). The soil efflux rates in mg CO₂-C g⁻¹ dry weight day⁻¹ were calculated on the basis of the cylinder surface area, air flow rate, difference in gas concentration between the microcosm chambers and the reference chambers, and the amount of soil contained in each microcosm (Schindlbacher et al., 2004). Cumulative respired CO₂ at the end of the incubation period was averaged per microcosm (n = 3) and expressed in mg CO₂-C g⁻¹ C.

CH₄ and N₂O gas sampling from microcosms

The closed chamber method was used to quantify the N₂O and CH₄ fluxes 3, 6, 12, 25, 39 and 53 days after incubation. At each sampling event, four gas samples were taken 0, 10, 20 and 30 min after the chamber closure using a gas-tight syringe through a rubber septum installed in the top of the chamber. Before removing a 12 ml gas sample, the gas inside the chamber was gently pumped up and down three times with the syringe to guarantee complete homogenization (Berger et al., 2010). Gas samples were transferred into pre-evacuated glass vials (10 ml) sealed with aluminium crimp caps and butyl rubber septum. Each time a gas sample was taken, the collected gas volume was replaced with 12 ml of ambient air to avoid under-pressure, and hence an extraction of gas from the soil pores. The N₂O and CH₄ concentrations in soil and ambient air gas samples were determined using a gas chromatograph (6890 GC, Agilent technologies, USA) connected to a headspace sampler (G1888, Agilent technologies, USA) and equipped with a CarbonPLOT capillary column and two detectors. CH₄ was quantified using a flame ionization detector (FID), and N₂O was determined with a ⁶³Ni-electron capture detector. Three different standard mixes of CH₄ (1, 2 and 4 ppm) and N₂O (0.5, 1, 2 ppm) in N₂-gas were used for

calibration (Linde Gas, Austria). The specifications of the gas chromatography and detection limits are reported in Schaufler et al. (2010). The decrease in gas concentration in the chamber headspace due to the collection of the samples was corrected prior to the calculations. The hourly CH₄ and N₂O fluxes in the microcosms were calculated as the linear increase in gas concentrations over the chamber closing time. All fluxes with a coefficient of determination lower than 0.7 were discarded (Leitner et al., 2016). Additional calculations were made to convert efflux rates from a volumetric basis to a mass basis according to the ideal gas law (Fernández-Alonso et al., 2017). Cumulative CH₄ uptake and N₂O fluxes at the end of the incubation period were averaged per microcosm (n = 3) and expressed in $\mu\text{g CH}_4\text{-C g}^{-1}\text{ C}$ and $\mu\text{g N}_2\text{O-N g}^{-1}\text{ N}$ respectively.

Microbial community structure

The microbial community of each sample was analysed in duplicate using the Phospholipid Fatty Acids profile (PLFA) procedure. Before the incubation experiment, three aliquots of each previously ground litter were analysed, besides of field-moist soil inoculums. Soils from the microcosms in the 54-day incubation experiment were also analysed after carefully removing the litter. Briefly, samples were extracted from 1 g of litter and 1.5 g of soil using a modification of the chloroform extraction method (Bligh and Dyer, 1959; Frostegård et al., 1991). Total lipids were extracted with a mixture (1:2:0.8 in volume) of chloroform:methanol:citrate buffer (0.15 M, pH = 4). The organic phase containing the lipids was fractionated using disposable silica gel columns (ISOLUTE® SI, Biotage, Sweden) into neutral-, glyco- and phospholipids using chloroform, acetone and methanol as eluents respectively. The phospholipid extract was evaporated to dryness and subjected to a mild alkaline methanolysis reaction (15 min at 37° C) with methanol:toluene (1:1), 0.2 M methanolic KOH and 1 M acetic acid to obtain fatty acid methyl esters (FAMES). Noncanoic acid methyl ester was then added as an internal standard for FAME quantification (19:0, Sigma-Aldrich, USA). FAMES were recovered with hexane:chloroform (4:1, v:v), again evaporated and re-dissolved in isooctane. FAMES were determined using a gas chromatograph (6890 GC, Agilent technologies) equipped with a HP-5ms capillary column and detected with a FID. A 26-component standard of bacterial acid methyl esters (BAME mix, Supelco, USA) was used as the external standard for FAME identification.

Terminally branched PLFA i14:0, i15:0, a15:0, i16:0, a16:0, i17:0, a17:0 were used as biomarkers for estimating the gram-positive bacteria (Frostegård and Bååth, 1996; Potthoff et al., 2006), and monounsaturated 16:1 ω 9, 16:1 ω 11, 17:1 ω 9, 18:1 ω 11 and cyclopropyl saturated PLFA cy17:0, cy18:0, cy19:0 were used for quantifying gram-negative bacteria (Bach et al., 2010; Koranda et al., 2013; Schindlbacher et al., 2011). Total bacterial biomass was calculated as the sum of gram-positive and gram-negative bacterial biomarkers. The gram-positive to gram-negative bacterial (GP:GN) ratio was also calculated. Saprophytic fungi were estimated using the

polyunsaturated peaks 18:3 ω 6,9,12c and 18:2 ω 9,12c (DeGroot et al., 2005; Wang et al., 2015). The fungal to bacterial ratio (F:B) was calculated by dividing the saprophytic fungi by the total bacterial biomass. Actinomycetes were estimated according to the methyl-branched PLFA 10Me16:0, 10Me17:0 and 10Me18:0 (Zelles, 1999). Total PLFA microbial biomass (nmol g⁻¹ dw) was calculated as the sum of all the above-mentioned biomarkers and averaged per sample (n = 3). The relative abundance (%) of each functional group was determined over the total microbial biomass in each sample. Changes in the microbial groups with incubation were expressed as the percentage variation from the initial concentration in soils (day 0).

The metabolic quotient (qCO₂), defined as the ratio between respired CO₂ and microbial biomass (Anderson and Domsch, 1993), was calculated for each microcosm. The mean heterotrophic respiration throughout the incubation period was used as the respiration rate (μ g CO₂-C g⁻¹ dw h⁻¹, n = 217), and the total PLFA (nmol PLFA g⁻¹ dw) as an estimate of microbial biomass (Balser and Firestone, 2005; Willers et al., 2015). The qCO₂ measurement (μ g CO₂-C nmol⁻¹ PLFA h⁻¹) allows direct comparison of soil microbial activities from microcosms with different treatments as a proxy of C-use efficiency (Creamer et al., 2015).

Data analysis

Differences in nutrient concentrations and PLFA microbial groups in original 'Litter types' (Lv, Nd) and 'Soil inoculums' (SO, SM, SP) were tested using linear mixed effect models (LME) with 'Sample' as a random factor to account for within-sample correlation in the model (R, *nlme* package, Pinheiro et al. (2016)). LME models with 'Microcosms' as a random factor were used to test the interaction effect between 'Litter amendments' (Lv, Mx, Nd and Soil-only at days 0 and 54) and 'Soil inoculums' in nutrient pools, cumulative GHGs and microbial parameters. The models were simplified when the interaction effects were non-significant. Residuals were checked for normality and homogeneity of variances using the Shapiro–Wilk (*stats* package, R Core Team (2016)) and Levene test (*car* package, Fox and Weisber (2011)) respectively. A generalized linear mixed (GLM) model with gamma probability distribution was used with cumulative respired CO₂-C in microcosms to satisfy the assumptions. Pairwise comparisons using Tukey's honestly significant differences of least-square means (*lsmeans* package, Lenth (2016)) were performed to identify differences between factor levels. The non-parametric Kruskal-Wallis test (K-W) (*stats* package, R Core Team (2016)) was used to examine differences between 'Microcosms' when there was a lack of residual normality and homogeneity, using Dunn's Test for pairwise comparison of mean rank sums (*PMCR* package, Pohlert (2015)). Correlations and linear regressions were carried out between variables from incubated microcosms (*Hmisc* package, Harrel (2016)) using Pearson's product-moment correlation coefficient (r) when normality and homoscedasticity of regression residuals were met, otherwise

applying Spearman's rank coefficient (ρ). Constrained ordination methods were used to explore the relationship between nutrient pools and microbial composition in soils. Data were homogeneous and linear (length of the first axis in detrended correspondence analysis shorter than 4 standard deviation units (Lepš and Šmilauer, 2003)), so a redundancy analysis (RDA) was performed with nutrient pools as the environmental matrix, and the relative abundance of microbial groups (PLFA, %) as the response matrix. The significance level was 0.05 for all the analyses. Statistical analyses were conducted in R version 3.4.0. (R Core Team, 2017).

Results

Nutrient concentrations

The initial nutrient concentrations varied significantly between litter types and different soil inoculums (Table 7.1). Needle litter had significantly higher TOC and TN contents than leaf litter. The C:N ratio of leaves was much higher than the needle litter C:N ratio. However, this trend in the C:N ratio between litter types was reversed in the soils, and was higher in pine than in oak and mixed forest soils. The TOC content in soils decreased significantly along the ecotonal gradient from pine to oak forests. The lowest TN content was found in oak soil, the highest EOC content was observed in pine soil, while TDN content was quite similar among soils.

Table 7.1. Main nutrient concentrations of original material.

Type of original material	TOC (mg g ⁻¹ dw)	TN (mg g ⁻¹ dw)	C:N ratio	EOC (μg g ⁻¹ dw)	TDN (μg g ⁻¹ dw)
Litter					
Lv	487.9 ± 0.4 b	7.6 ± 0.1 b	64.3 ± 0.8 a	-	-
Nd	536.5 ± 0.7 a	14.1 ± 0.2 a	38.1 ± 0.5 b	-	-
Soil					
SO	33.6 ± 1.3 C	2.5 ± 0.1 B	13.4 ± 0.1 B	164.3 ± 3.8 B	29.6 ± 0.7
SM	42.2 ± 0.6 B	3.0 ± 0.1 A	14.1 ± 0.2 B	159.4 ± 2.9 B	28.2 ± 0.4
SP	65.3 ± 2.1 A	2.9 ± 0.1 A	22.9 ± 0.2 A	213.6 ± 3.6 A	28.3 ± 0.5

Values represent means ± standard errors (n = 3). Different capital letters denote significant differences between 'Soil inoculums' (SO: oak forest, SM: mixed forest, SP: pine forest), while small letters indicate significant differences between 'Litter types' (Lv: leaves, Nd: needles) (LME, $p < 0.01$).

When examining the incubation effects (after 54 days) in soil nutrient concentrations from microcosms, significant differences were found only for labile pools of C and N in soils (EOC, TDN) compared to initial concentrations (day 0) in soils. The percentage variation in EOC and TDN contents with regard to original soils are shown in Figure 7.1. The EOC decreased in all treatments, although there were significant differences only between soil inoculums (Figure

7.1a). The reduction in EOC was higher in pine than in mixed forest soil, and intermediate in oak soil. However, there was a significant interaction effect between litter amendments and soil inoculums in the percentage change in TDN (Figure 7.1b). Massive TDN desorption thus occurred in soil-only microcosms; almost no effect was found in the TDN content of litter-amended microcosms from oak forests; and there was a significant reduction in TDN in litter-amended microcosms from mixed and pine forests. A comparison of the two extreme habitats in the chronosequence of the vegetation succession (i.e. SP+Nd and SO+Lv) reveals a decrease in C pools (TOC and in EOC Figure 7.5d and g, respectively) but an increase in the TDN pool (Figure 7.5h). The middle successional stage (SM+Mx) played an intermediate role with regard to soil nutrient dynamics, and led to the highest stored amount of TN (Figure 7.5d-h).

Figure 7.1. Changes in nutrient concentrations (%) from microcosm-derived soils (SO: oak forest, SM: mixed forest, SP: pine forest) after the 54-day incubation period, a) extractable organic carbon (EOC) and b) total dissolved nitrogen (TDN), expressed as the percentage variation from the initial concentrations (mg or $\mu\text{g g}^{-1}$ dw) in each soil (day 0). Bars represent means $\pm$ standard errors ($n = 3$). Capital letters indicate significant differences between ‘Soil inoculums’, while small letters denote significant differences between ‘Litter amendments’ (LME, *** p-value < 0.001, * p-value < 0.05).

Greenhouse gas fluxes

In litter-only microcosms, the respired GHG fluxes were below the detection limits throughout the incubation experiment (data not shown), while the total GHGs emitted with the different litter amendments (including soil-only microcosms) varied among soil inoculums (Figure 7.2). In soil-only microcosms, there was a noticeable influence of the forest type on the decomposability of soil-C owing to the dissimilar cumulative respired-C registered at the end of the experiment (Figure 7.2a). After 54 days, the respired-C was higher in the oak forest than in the pine forest, and intermediate in the mixed forest (99.1 ± 2.7 , 61.7 ± 3.3 and 89.2 ± 2.3 mg $\text{CO}_2\text{-C g}^{-1}$ C respectively). The amendment of different litter types to soil inoculums also had a dissimilar effect on microbial heterotrophic respiration (Figure 7.2a): the cumulative respired-C in microcosms with oak soil was on average 7.8 % higher with leaf litter than with needle litter.

In microcosms with pine soil, the amendment of mixed or needle litter produced an increment up to 24.7 % of respired-C with regard to leaf litter. However, there was no difference in microbial respired-C between litter amendments in microcosms with mixed forest soil. When considering all the microcosms together, the cumulative respired-C was negatively correlated with TOC and N pools in soils (Table 7.2).

Figure 7.2. a) Cumulative CO₂-C (mg CO₂-C g⁻¹ C) emissions, b) cumulative CH₄-C (μg CH₄-C g⁻¹ C) uptake, and c) cumulative N₂O-N (μg N₂O-N g⁻¹ N) efflux from microcosm during the 54-day incubation period. Bars represent means ± standard errors (n = 3). Capital letters indicate significant differences between ‘Soil inoculums’, while small letters denote significant differences between ‘Litter amendments’ (***) p-value < 0.001). Soils from oak forest (SO), mixed forest (SM) and pine forest (SP).

All microcosms acted as net sinks in terms of CH₄ fluxes (Figure 7.2b). In soil-only microcosms, cumulative CH₄-C uptake was somewhat stable among soil inoculums (on average $-33.9 \pm 2.6 \mu\text{g CH}_4\text{-C g}^{-1} \text{C}$), although litter amendments affected their sink capacity differently. Overall, litter amendments diminished CH₄-C uptake in oak soil relative to their soil-only microcosm (Figure 7.2b), although the opposite occurred in mixed forest soil (on average 1.2 times higher uptake with litter amendments) and pine soil (0.3, 0.9 and 1.6 times higher uptake with leaves, needles and mixed litter, respectively). The TDN in soils reduced the CH₄-C uptake capacity in the ecotone as supported by the correlation coefficient in Table 7.2.

All microcosms functioned as net sources of N₂O (Figure 7.2c). In soil-only microcosms, the highest cumulative N₂O efflux occurred in the mixed forest soil ($190.9 \pm 20.6 \mu\text{g N}_2\text{O-N g}^{-1} \text{N}$). Oak and pine soils emitted on average $121.7 \pm 10.1 \mu\text{g N}_2\text{O-N g}^{-1} \text{N}$. Different litter amendments changed total N₂O emissions considerably, with needle litter maximizing the N₂O efflux in oak soil but minimising it in mixed and pine forest soils. The cumulative N₂O-N fluxes correlated positively with the soil TDN pool when all microcosms were considered together (Table 7.2).

Table 7.2. Correlation coefficients (r: Pearson's product-moment correlation, ρ : Spearman's rank correlation) between either cumulative GHG fluxes or properties of the microbial communities and nutrient pools from microcosm-derived soils.

	TOC (mg g ⁻¹ dw soil)		TN (mg g ⁻¹ dw soil)		C:N		EOC (μg g ⁻¹ dw soil)		TDN (μg g ⁻¹ dw soil)	
	Coef. cor.	p-value	Coef. cor.	p-value	Coef. cor.	p-value	Coef. cor.	p-value	Coef. cor.	p-value
Cumulative GHG fluxes										
CO ₂ -C (mg g ⁻¹ C)	r = -0.54	< 0.001	r = -0.45	< 0.01	ρ = -0.50	< 0.01	r = -0.20	n.s.	r = -0.42	< 0.05
CH ₄ -C (μg g ⁻¹ C)	ρ = -0.48	< 0.001	r = -0.48	< 0.01	ρ = -0.49	< 0.001	ρ = -0.01	n.s.	ρ = 0.61	< 0.001
N ₂ O-N (μg g ⁻¹ N)	ρ = 0.08	n.s.	r = 0.04	n.s.	ρ = -0.01	n.s.	ρ = -0.01	n.s.	r = 0.36	< 0.05
Microbial properties										
qCO ₂ (μg CO ₂ -C nmol ⁻¹ h ⁻¹)	r = -0.12	n.s.	r = -0.55	< 0.001	r = 0.02	n.s.	ρ = 0.15	n.s.	ρ = -0.20	n.s.
F:B ratio	r = 0.61	< 0.001	r = -0.33	< 0.05	r = 0.71	< 0.001	r = 0.70	< 0.001	ρ = -0.27	< 0.05
GP:GN ratio	ρ = 0.04	n.s.	r = -0.06	n.s.	ρ = 0.11	n.s.	ρ = 0.06	n.s.	ρ = -0.35	< 0.05
PLFA mic (nmol g ⁻¹ dw)	ρ = 0.44	< 0.01	r = 0.34	n.s.	ρ = 0.50	< 0.01	ρ = 0.28	n.s.	r = -0.82	< 0.001

Significant correlation coefficients are reported in bold (n.s.: non-significant). Microbial parameters are metabolic quotient (qCO₂), fungal to bacterial ratio (F:B), gram-positive to gram-negative bacterial ratio (GP:GN) and total microbial biomass (PLFA mic).

GHG fluxes throughout the chronosequence of the vegetation succession showed a tendency towards an increase in respired CO₂-C (Figure 7.5a) and a decrease in CH₄-C uptake (Figure 7.5b), whereas no clear pattern was found for N₂O-N efflux (Figure 7.5c).

Microbial community structure and activity

The total microbial biomass in the original litter was significantly higher in leaves than in needles (Table 7.3). Microbial group concentrations were also different between litter types, with leaf litter dominated by gram-negative bacteria and needle litter by both gram-positive and saprophytic fungi. However, total microbial biomass and microbial community composition were quite similar among forest soils at the onset of the experiment (Table 7.3). Differences were only found in the concentrations of saprophytic fungi among original soil inoculums, leading to the highest F:B ratio in the pine forest soil (Table 7.3). The microbial groups grew in all microcosms after the incubation period (54 days) with the exception of saprophytic fungi in soil-only microcosms from the oak forest (Figure 7.3). The type of soil inoculum and litter amendments had a different effect on the growth of microbial groups in the microcosms (Figure 7.3), consequently altering the original microbial community structure of soils. The addition of needle litter reduced the relative abundance of gram-negative bacteria in comparison with soil-only microcosms (Table 7.5). Only the amendment of needle litter increased the abundance of gram-positive bacteria in soils, leading to the highest GP:GN ratio (Table 7.5). Non-significant differences between litter or soil types were found in the abundance of actinomycetes (Table 7.4). The F:B ratio decreased in all incubated microcosms. Regardless of the litter type added, the highest relative abundance of total bacteria, which were mostly gram-negative, occurred in both mixed and oak forest soils (Table 7.5), while saprophytic fungi were dominant in pine forest soils. The F:B ratio was higher in pine soils than in mixed and oak soils (Table 7.5), and therefore correlated positively with C pools and negatively with N pools (Table 7.2). The GP:GN ratio was negatively linked with TDN in soils (Table 7.2). Overall, the microbial biomass growth induced by the different litter amendments varied significantly between soils (Figure 7.3, Table 7.5) and was linked to higher TOC and lower TDN contents (Table 7.2). Although the soils from mixed and pine forests supported greater microbial communities than oak soils, it is noteworthy that the vegetation succession involved higher biomass of decomposing microbes per unit of soil organic-C in the last sequential habitats of the chronosequence (Figure 7.5m).

Table 7.3. Microbial group concentrations (PLFAs), total microbial biomass (PLFA mic), fungal to bacterial ratio (F:B) and gram-positive to gram-negative bacterial ratio (GP:GN) in the original material: litter (Lv: leaves, Nd: needles) and soil (SO: oak forest, SM: mixed forest, SP: pine forest).

Type of original material	GP bacteria (nmol g ⁻¹ dw)	GN bacteria (nmol g ⁻¹ dw)	Sapro. fungi (nmol g ⁻¹ dw)	Actinomycetes (nmol g ⁻¹ dw)	PLFA mic (nmol g ⁻¹ dw)	F:B ratio	GP:GN ratio
Litter							
Lv	0.04 ± 0.01 b	3.75 ± 0.10 a	0.34 ± 0.02 b	0.03 ± 0.00 b	4.16 ± 0.11 a	0.09 ± 0.00 b	0.01 ± 0.01 b
Nd	0.36 ± 0.01 a	0.47 ± 0.02 b	1.19 ± 0.10 a	0.09 ± 0.01 a	2.10 ± 0.12 b	1.43 ± 0.10 a	0.77 ± 0.01 a
Soil							
SO	0.19 ± 0.03	0.34 ± 0.05	0.07 ± 0.00 B	0.07 ± 0.02	0.67 ± 0.10	0.13 ± 0.01 B	0.55 ± 0.03
SM	0.24 ± 0.02	0.43 ± 0.06	0.08 ± 0.01 B	0.12 ± 0.02	0.86 ± 0.11	0.12 ± 0.01 B	0.57 ± 0.02
SP	0.21 ± 0.06	0.37 ± 0.05	0.11 ± 0.01 A	0.11 ± 0.02	0.79 ± 0.12	0.20 ± 0.02 A	0.55 ± 0.06

Values represent means ± standard errors (n = 3). Different capital letters denote significant differences between 'Soil inoculums', while small letters indicate significant differences between 'Litter types' (LME, p < 0.05). The microbial groups are gram-positive bacteria (GP), gram-negative bacteria (GN), saprophytic fungi (Sapro. fungi) and actinomycetes.

Figure 7.3. Changes in microbial group concentrations (PLFAs, %) throughout the 54-day incubation period in a) oak forest soil, b) mixed forest soil, and c) pine forest soil from microcosms, expressed as the percentage variation from the initial concentrations ($\text{nmol g}^{-1} \text{ dw}$) in each soil (day 0). Bars represent means $\pm$ standard errors ($n = 3$). Capital letters indicate significant differences between ‘Soil inoculums’, while small letters denote significant differences between ‘Litter amendments’ for the respective soil (LME, $p < 0.05$). The microbial groups are gram-positive bacteria (GP), gram-negative bacteria (GN), saprophytic fungi (Sapro. fungi) and actinomycetes.

Table 7.4. Results of LME models showing the interaction effect between ‘Soil inoculums’ and ‘Litter amendments’ on parameters from soil microbial communities measured as PLFAs ($n = 3$, $p < 0.05$).

Variables	Soil inoculums		Litter amendments		Soil inoculums x Litter amendments	
	F	p	F	p	F	p
GP (%)	1.0	ns	4.5	**	0.7	ns
GN (%)	5.7	**	4.9	**	1.2	ns
Sapro. fungi (%)	36.5	***	17.7	***	1.3	ns
Actinomycetes (%)	0.8	ns	1.1	ns	1.1	ns
Bacterial group (%)	17.0	***	5.1	**	1.8	ns
PLFA mic (nmol g ⁻¹ dw)	29.9	***	55.2	***	1.7	ns
F:B ratio	35.9	***	17.2	***	1.9	ns
GP:GN ratio	0.5	ns	4.6	**	0.7	ns
R _H (μg CO ₂ -C g ⁻¹ dw h ⁻¹)	51.6	***	280.4	***	8.7	***
qCO ₂ (μg CO ₂ -C nmol ⁻¹ PLFA h ⁻¹)	21.2	***	28.4	***	2.6	*

Microbial parameters are gram-positive bacteria (GP), gram-negative bacteria (GN), total microbial biomass (PLFA mic), fungal to bacterial ratio (F:B), gram-positive to gram-negative bacterial ratio (GP:GN), mean heterotrophic respiration (R_H) and metabolic quotient (qCO₂). Asterisks indicate levels of significance: *** p-value < 0.001, ** p-value < 0.01, * p-value < 0.05, n.s. non-significant.

Table 7.5. Parameters from the soil microbial community measured as PLFAs (relative abundances, total microbial biomass and ratios).

Soil inoculum*	Day*	Litter amendment**	GP bacteria (%)	GN bacteria (%)	Sapro. fungi (%)	Actinomycetes (%)	Bacterial group (%)	PLFA mic (nmol g ⁻¹ dw)	F:B ratio	GP:GN ratio
SO	-	-	29.2 ± 0.8	49.8 ± 0.6 A	8.1 ± 0.4 B	12.9 ± 0.5	79.0 ± 0.2 A	1.18 ± 0.10B	0.10 ± 0.01B	0.59 ± 0.02
SM	-	-	30.3 ± 0.7	49.2 ± 0.5 AB	7.1 ± 0.3 B	13.5 ± 0.2	79.5 ± 0.2 A	1.63 ± 0.12A	0.09 ± 0.00B	0.62 ± 0.02
SP	-	-	28.9 ± 0.9	47.5 ± 0.6 B	10.3 ± 0.6 A	13.4 ± 0.3	76.3 ± 0.3 B	1.62 ± 0.13A	0.14 ± 0.01A	0.61 ± 0.03
-	0	-	27.1 ± 1.2 b	49.0 ± 0.8 ab	11.0 ± 0.9 a	12.9 ± 0.9	76.1 ± 0.5 b	0.78 ± 0.06c	0.15 ± 0.01a	0.56 ± 0.03b
-	54	Soil-only	28.1 ± 0.7 b	50.7 ± 0.7 a	7.1 ± 0.5 b	14.0 ± 0.1	78.8 ± 0.2 a	1.28 ± 0.12b	0.09 ± 0.01b	0.56 ± 0.02b
-		Lv	29.3 ± 1.1 ab	49.6 ± 0.9 ab	8.2 ± 0.4 b	12.9 ± 0.2	78.9 ± 0.2 a	1.71 ± 0.13a	0.10 ± 0.01b	0.60 ± 0.03ab
-		Mx	30.6 ± 0.7 ab	48.0 ± 0.8 ab	8.2 ± 0.5 b	13.3 ± 0.3	78.5 ± 0.2 a	1.90 ± 0.08a	0.11 ± 0.01b	0.64 ± 0.02ab
-		Nd	32.2 ± 0.7 a	46.9 ± 0.5 b	7.8 ± 0.5 b	13.1 ± 0.1	79.0 ± 0.2 a	1.72 ± 0.08a	0.10 ± 0.01b	0.69 ± 0.02a

Values represent means ± standard errors (*n = 15, **n = 9, lack of significance of the interaction effects, see Table 7.4). Different capital letters denote significant differences between 'Soil inoculums' (SO: oak forest, SM: mixed forest, SP: pine forest), while small letters indicate significant differences between 'Litter amendments' (Lv: leaves, Mx: mixed, Nd: needles).

The RDA explains 40.3 % of the variance between soil nutrient pools and soil microbial communities on the two sampling dates (Figure 7.4). The first two constrained axes were statistically significant ($p < 0.001$, permutation test) and explained 21.1 % and 17.6 % of the variance in RDA1 and RDA2, respectively. The relative abundance of saprophytic fungi was positively related to the C pools in soils (EOC, C:N and TOC), while actinomycetes and gram-negative bacteria were linked to higher N pools in soils (TN, TDN) (Figure 7.4a). The abundance of gram-positive bacteria was negatively correlated with TDN. Soil inoculum scores (Figure 7.4b) showed differences in soil microbial structures with the incubation experiment. In soil-only microcosms, the increment in TDN favoured gram-negative bacteria while the decrease in EOC reduced the abundance of fungal groups in all soils (Figure 7.4b). In litter-amended microcosms, the reduction in EOC and especially in TDN pools provoked a shift in soil microbial communities that favoured mainly gram-positive bacteria over saprophytic fungi in all soils. When comparing litter types, the addition of leaf litter favoured gram-negative bacteria in comparison to needle litter that favoured gram-positive bacteria, this effect was especially significant in the oak soil. In the chronosequence of the vegetation succession (Figure 7.5j-l), these shifts in the structure of soil microbial communities led to a decrease in soil F:B ratios and higher relative abundance of gram-negative bacteria.

The effect of litter amendments on the C-use efficiency varied between soils, as there was a significant interaction between both factors in the model (Table 7.4). In soil-only microcosms, the $q\text{CO}_2$ was significantly higher in the oak forest than in the pine and mixed forests (Table 7.6). The $q\text{CO}_2$ generally increased with litter amendments. Concerning soils, the highest $q\text{CO}_2$ with leaf litter occurred in oak soils, with needle litter in both oak and pine soils, whereas the $q\text{CO}_2$ was somewhat stable with mixed litter in all soil types (Table 7.6). Taking all the microcosms as a whole, the $q\text{CO}_2$ was negatively correlated with the TN content in soils (Table 7.2).

Figure 7.4. Redundancy analysis (RDA) of microbial community structure (PLFAs) and nutrient pools in soils. a) Biplot with microbial groups (shapes) and nutrient pools (arrows) as explanatory parameters. b) Score plot of soils (SO: oak forest, SM: mixed forest, SP: pine forest) according to the sampling time point (day 0 and day 54) and litter amendments (Lv: leaves, Mx: mixed, Nd: needles). Vertical and horizontal bars correspond to standard errors in RDA2 and RDA1 axes, respectively ($n = 3$). The microbial groups are actinomycetes, gram-negative bacteria (GN), gram-positive bacteria (GP) and saprophytic fungi (Sapro. fungi).

Table 7.6. Mean rate of heterotrophic respiration (R_H) throughout the incubation period and the metabolic quotients (qCO_2) of microcosms.

Soil inoculum	Litter amendment	R_H ($\mu g CO_2-C g^{-1} dw h^{-1}$)		qCO_2 ($\mu g CO_2-C nmol^{-1} PLFA h^{-1}$)	
SO	Soil-only	2.68 $\pm$ 0.08	A b	3.22 $\pm$ 0.24	A b
	Lv	7.02 $\pm$ 0.20	ABa	5.47 $\pm$ 0.28	A a
	Mx	7.06 $\pm$ 0.23	B a	4.21 $\pm$ 0.23	A ab
	Nd	6.88 $\pm$ 0.29	B a	4.87 $\pm$ 0.10	A ab
SM	Soil-only	2.98 $\pm$ 0.07	A b	2.01 $\pm$ 0.16	B b
	Lv	6.34 $\pm$ 0.21	B a	3.18 $\pm$ 0.13	B ab
	Mx	6.61 $\pm$ 0.21	B a	3.43 $\pm$ 0.12	A a
	Nd	6.80 $\pm$ 0.28	B a	3.65 $\pm$ 0.17	B a
SP	Soil-only	3.22 $\pm$ 0.13	A c	2.14 $\pm$ 0.14	B b
	Lv	7.23 $\pm$ 0.22	A b	4.10 $\pm$ 0.71	B a
	Mx	8.98 $\pm$ 0.26	A a	4.38 $\pm$ 0.45	A a
	Nd	9.47 $\pm$ 0.40	A a	5.08 $\pm$ 0.28	A a

Values represent means $\pm$ standard errors ($n = 3$). Different capital letters denote significant differences between 'Soil inoculums', while small letters indicate significant differences between 'Litter amendments' (LME, $p < 0.05$). Soils from oak forest (SO), mixed forest (SM) and pine forest (SP). Amendments with leaf (Lv), mixed (Mx) and needle (Nd) litter.

Figure 7.5. Variations in cumulative GHG fluxes (a-c), soil nutrient pools (d-h), metabolic quotient (i), relative abundance of microbial groups (j-l) and total microbial biomass (PLFAs) (m) in the chronosequence of the vegetation succession. Points represent means $\pm$ standard errors ($n = 3$). Different letters indicate significant differences between 'Sequential habitats'. Soils from oak forest (SO), mixed forest (SM) and pine forest (SP). Amendments with leaf (Lv), mixed (Mx) and needle (Nd) litter.

Discussion

Our findings show that global change-induced vegetation shifts from Scots pine to Pyrenean oak could potentially affect the ecology and functioning of the bulk soil with serious long-term consequences for the soil-C sink capacity of these Mediterranean ecotone systems. These ecosystem changes are the result of the widely-known influence of tree species composition on both soil chemical (nutrient pools, C:N ratios, quantity and chemistry of SOM) and biological properties (microbial composition and physiology), which have been thoroughly studied and described in the past (Alvarez et al., 2014; Díaz-Pinés et al., 2011b; Papen et al., 2005; Six et al., 2006; Wang et al., 2015). However, few studies have been designed to conduct an in-depth exploration of the fundamental soil processes and the essential physiological strategies of soil microbial communities that would potentially determine the fate of soil-C and -N during the transitional stages of a vegetation succession. A chronosequence approach allows us to examine the mechanisms driving the functioning of bulk soil during the forest succession, identifying the potential sensitivity of C and N pools and their relation with key microbial functional groups at time scales (e.g. years, decades and centuries) at which important changes could be expected to occur on ecosystem processes. While our results show the potential importance of a climate-change induced shift in litter chemistry over soil pools and functions, they should be carefully interpreted when extrapolated to rhizosphere field conditions where other confounding environmental factors not accounted in our laboratory experiment (e.g. microclimate, soil properties and/or rhizodeposition) might also modulate the observed responses (Paterson, 2003).

Although the C:N ratio of senescent leaves in our experiment was higher than the C:N ratio of senescent needles, the pine forest soil had the highest C:N ratio. This could be explained by differences in the length of time N is retained in the plant biomass in pine and oak stands. Scots pine immobilizes more N in aboveground biomass than oak due to the greater longevity (around 4 years) of the needles on the trees compared to oak leaves (Escudero et al., 1992). In addition to these greater quantities of N stored in aboveground pine biomass, the high proportion of recalcitrant compounds (e.g. tannins and polyphenols) in Scots pine needles (Lin et al., 2013; Pardo et al., 1997) could be associated with the mechanisms of N immobilization in decomposing litter. Polyphenolic compounds may form complexes with proteins during litter decay, constituting recalcitrant humic substances that hinder the microbial capacity to decompose SOM while inhibiting N mineralization (Gallardo and Merino, 1992; Kraus et al., 2003). In our experiment, the forest composition therefore indirectly controls biogeochemical cycles through the influence of litter chemistry on soil nutrient availability and stoichiometric gradients. This was highlighted by the differences in the cumulative respired-C per unit of organic-C, the TDN pools and the metabolic quotients among soil types in soil-only microcosms.

Regardless of the soil origin, litter amendments caused the metabolic activation of soil microorganisms, which was reflected in the stimulation of microbial growth (enhanced microbial biomass), changes in the composition of the microbial communities, and substantial increases in the respired-C per unit of organic-C. This major rise in cumulative respired-C in litter-amended microcosms compared to soil-only microcosms could be assumed to be a stimulation of the decomposing capacity of microbes (Blagodatskaya and Kuzyakov, 2008; Wang et al., 2015). The litter types also had a differential effect on heterotrophic respiration across soils, fully supporting the HFA hypothesis as stated earlier by other authors (Barba et al., 2015; Cleveland et al., 2014; Keiser et al., 2011; Strickland et al., 2009b). Litter stimulated the decomposition processes more under their “native” habitat than under “non-native” habitats. Interestingly, the respired-C in mixed forest soils was similar regardless of the litter origin, which may indicate a broad decomposing functionality of these soil microbial communities.

The effects of laboratory-simulated global-change driven-succession from Scots pine to Pyrenean oak revealed time-decoupled responses of soil-C and -N dynamics in the long run. We show here how metabolic paths and pools associated with C cycling (CO_2 efflux, EOC and TOC contents) revealed earlier responses to the broadleaf encroachment on the former pure pine forest than the metabolic paths associated with N cycling. This decoupling of C and N cycles was closely associated with shifts in microbial community structure and functioning over the successional stages of soils. Our results also clearly show how significant changes in pivotal soil processes controlling C and N cycling only emerged when alterations in chemical composition of SOM occurred in soils, regardless of the different litter quality added in the incubation experiment.

In the first stage of the succession, there was only an increase in the turnover rate of SOM (higher cumulative respired-C) when SOM of oak origin was mixed with SOM of pine origin (i.e. in mixed forest soils), resulting in a net loss of soil-C (TOC and EOC depletion). This early acceleration of C cycling in response to succession may be the result of complex interactions between biological, physical and chemical soil processes. On the one hand, the greater dominance of fungal groups in pine soils is consistent with an oligotrophic life-strategy and the known ability of fungal enzymes to decay recalcitrant compounds (Schneider et al., 2012; Stockmann et al., 2013) mainly under N constraining conditions (Heimann and Reichstein, 2008). These fungal-dominated communities under pines may also have contributed to increasing soil-C storage through the production of chemically-resistant fungal by-products and soil aggregation, as fungi are thought to bind soil particles and SOM (Six et al., 2006; Smith et al., 2014). On the other hand, the better quality of SOM from pine and oak trees as a substrate in the mixed forest favoured an increase in the microbial biomass per unit of soil organic-C, which was responsible for enhanced catabolic activity (rise in microbial heterotrophic respiration per unit of SOM). The early stages of oak encroachment led to a considerable decrease in the F:B ratio. The increased

bacterial abundance in mixed forest soils suggests that bacterial growth might have been constrained beneath the Scots pine cover, probably due to the low soil C:N ratio, and because bacteria are often less tolerant to the toxicity of tannins than fungi (Ushio et al., 2013). This unfavourable condition for bacterial growth in pine forests was more markedly evidenced in the high $q\text{CO}_2$ of the pure pine forest (i.e. with needle litter addition), compared to the $q\text{CO}_2$ of bacterial-dominated communities in the mixed forest. A shift in microbial composition towards lower F:B ratios with forest succession might therefore be the result of a reduction in chemically stabilised soil-C, an increase in substrate quality (lower soil C:N ratios) and a decrease in allelopathic compounds that inhibit bacterial growth.

At an advanced successional stage of the chronosequence (i.e. in oak forest soils), once the influence of pine had disappeared from the SOM, biogeochemical pathways were stimulated leading to more available N forms in soils, as evidenced by the substantial increase in soil TDN. This rise in available N forms was not necessarily associated with an increase in total N content or the greater bacterial presence compared to fungi. Other factors such as the small but consistent decrease observed in the soil C:N ratio and/or the further dilution of the allelopathic effect of pine SOM on bacterial growth may have triggered an increase in activity and the contribution of key microbial functional groups involved in the N cycle, such as diazotrophs (N-fixers) and/or autotrophic nitrifying bacteria. The late increase in gram-negative bacteria under pure oak soils, to which most nitrifiers (Zechmeister-Boltenstern et al., 2010) and a large portion of diazotrophic bacteria belong (Chowdhury et al., 2007), may partially support this idea. This is also consistent with the copiotrophic strategy developed by gram-negative bacteria (De Vries and Shade, 2013; Goberna et al., 2014; Kaiser et al., 2014), associated with high rates of microbial heterotrophic activity and low C-use efficiency (and hence high $q\text{CO}_2$: Allison et al., 2010; Fierer et al., 2007). This stimulation of the biogeochemical paths leading to mineral-N forms was further linked with the observed suppression of CH_4 oxidation processes at these late successional stages, since it is generally accepted that the presence of mineral N in soils diminishes the activity of CH_4 -consuming bacteria (Bodelier and Laanbroek 2004). Some proposed inhibition mechanisms reported in the literature include the toxic inhibition produced by the products of ammonium oxidation (e.g. hydroxylamine and nitrite), and the competitive inhibition of ammonia for the active site of the methane monooxygenase enzyme responsible for the CH_4 oxidation step to methanol and water (He et al., 2016).

However, the absence of a significant pattern of N_2O emissions from soils throughout the successional process may indicate a lower sensitivity of this GHG to vegetation change than in the case of CH_4 and CO_2 fluxes. Since N_2O is the product of denitrification, a process stimulated under anaerobic conditions (Butterbach-Bahl et al., 2013), and microcosm moisture was standardized at a 50 % water-filled pore space, we cannot exclude the possibility that the lack of

differences in soil N₂O emissions along the successional forest stages responded to non-optimal soil conditions for denitrification. Future studies under Scots pine and Pyrenean oak forests are therefore required to explore the potential effects of this global-change-induced vegetation shift with an increasing level of soil moisture.

Conclusions

We here identified changes in litter chemistry along the succession from pure Scots pine to pure Pyrenean oak forests that led to a significant loss of the soil-C sink capacity and a significant gain in biologically available forms of soil mineral N. We also observed a decoupling between C and N dynamics throughout the successional process, mainly driven by the differential sensitivity of key metabolic pathways to the presence of pine SOM and its influence on the ecology and physiology of soil microbial communities (oligotrophic in pine forest versus more functionally diverse in oak forest). Only when oak SOM was mixed with pine SOM (time scales of years) did we find an increase in soil heterotrophic respiration with a shift towards lower F:B ratios. Soil processes leading to increases of available N (via e.g. mineralization, nitrification) were only stimulated when pine SOM was totally eliminated from the system (time scales of decades-centuries), resulting in a copiotrophic soil environment (enhanced nutrient availability for growth) dominated by gram-negative bacteria, with accelerated SOM turnover rates (high CO₂ production), suppressed CH₄ consumption and decreased soil-C accumulation. Although these findings indicate a reduced capacity of these Mediterranean forest soils to sequester C, the evolution towards soil conditions with high nutrient availability may have a positive effect on plant growth leading to an increase in C sequestration in aboveground compartments.

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Chapter 8

General discussion

The main objective of this PhD thesis was to study the functionality of the plant-soil-microorganism system in the ecotone between Scots pine and Pyrenean oak forests considering the implications for the belowground biogeochemical cycles of C and N. Along this research, we addressed the effects on soil ecological dynamics of two main factors of global change (1) the vegetation shift of Scots pine by the more drought tolerant Pyrenean oak, and (2) the increased atmospheric N deposition. In this chapter, results of the PhD thesis are synthetically discussed.

Soil CO₂ efflux in the Scots pine – Pyrenean oak ecotone forest

For a better understanding of the role of tree species in the soil C cycling, we continuously measured soil CO₂ efflux by partitioning R_s in its heterotrophic and autotrophic components and assessing their microclimate and tree species dependence on the one hand (**Chapter 4**), and identifying the influence of soil properties under increased atmospheric N deposition on the other side (**Chapter 5**).

Biotic and abiotic factors controlling soil respiration components

Our research in **Chapter 4** is among the few studies that have examined the effects of tree species on the microclimate dependence of the heterotrophic and autotrophic soil respiration in drought-prone regions (Barba et al., 2016; Rey et al., 2002; Tedeschi et al., 2006). Our results indicated that soil temperature was the best predictor of R_s components, but we found a continuous dependence of root and microbial metabolic activities on SWC. Other studies carried out in Mediterranean ecosystems determine thresholds of soil moisture at which R_s is solely controlled by SWC rather than by soil temperature (Chang et al., 2014; Lellei-Kovács et al., 2016), but the empirical evidence found here highlights the need to incorporate soil temperature-SWC covariation in order to improve model predictions of R_s in this Mediterranean ecotone forest.

Our modelling approach using repeated measures to estimate daily, seasonal and annual rates of R_s components allowed us to find seasonal dynamics of soil metabolic activity that were clearly different between Pyrenean oak and Scots pine stands (Figure 4.5). The greatest seasonal differences in soil respiratory patterns between tree species were found for the R_A , which was the minority component attaining at most 30 % of R_s . We attributed the R_A patterns throughout the year to differences in leaf strategies influencing root phenological patterns (Curiel Yuste et al., 2004) although other influencing factors related to plant traits and physiological processes cannot be ruled out (Grossiord et al., 2012; Poyatos et al., 2005; Ubierna et al., 2009). Our results

confirmed that pine R_A was more sensitive to the soil water deficit during summer than oak R_A being the latter more sensitive to low winter temperatures (Figure 4.3).

Once R_A contribution to R_S was subtracted, we detected the strong and inverse water and thermal seasonality as constraints for microbial decomposing activity independently of the forest type. Although annual R_H rates were the highest in the Scots pine stand, probably driven by the larger litterfall rates and soil C stocks (Díaz-Pinés et al., 2011a, 2011b), the apparent turnover rates of soil organic C in the oak stand was almost twice as fast as in the pine stand. This faster apparent turnover rate of soil organic C derived from pine in comparison with the corresponding from oak was consistent with the results obtained under controlled conditions in the experiment using microcosms under laboratory conditions (**Chapter 7**) and with the higher remaining mass of needle litter after two years of decay in the litterbag experiment (**Chapter 6**).

Nitrogen deposition effects on soil respiration and related properties

It is well known that N deposition exerts a fertilization effect on forest ecosystems stimulating plant growth (De Vries et al., 2009; Pregitzer et al., 2008), but the effects on soil C efflux are still uncertain (Jandl et al., 2007). Our results showed that R_S in this Mediterranean ecotone forest was highly resilient to three years of N additions despite fertilization treatments caused significant changes in soil chemical and biological processes –e.g. N additions caused soil acidification, variations in EOC concentrations and decreases in microbial biomass carbon (**Chapter 5**). Interestingly, we were able to find a higher sensitivity to the N enrichment of the soil system in the pine stand than in the oak stand as evidenced by the significant pulse of R_S after the applications of the medium-N fertilization. However, the stimulation of R_S by N additions were conditioned by the strong soil microclimate seasonality that constrains both microbial and root metabolic activity (**Chapter 4**). Although we have not directly assessed the effect of N additions on root respiration, we detected a tendency toward a higher presence of root biomass in fertilized plots that is consistent with the 23 % increment in total root production due to N addition showed in a meta-analysis developed by Xia and Wan (2008). Therefore, we expected that the increase in R_S following N additions in the pine stand may be caused by positive impact on autotrophic respiration since this pine soil is N limited to a higher extent than the oak soil (**Chapter 7**).

The SEMs showed that the net effect of N fertilization on R_S could be explained through complex direct and indirect relationships between biotic and abiotic factors of the soil system that were more relevant in the pine stand. We found that soil acidification through nitrification processes driven by soil N enrichment decreased the leaching of EOC, which may be linked to a reduced mobility of organic compounds in the pine soil (Evans et al., 2008; Hagedorn et al., 2012). In contrast, the negative response of microbial biomass to N fertilization may involve lower

heterotrophic activity favouring the EOC accumulation (Liu and Greaver, 2010; Waldrop and Zak, 2006) and offsetting the effects of the soil pH/EOC solubility mechanism. On the other hand, the multigroup comparison analysis highlighted that oak R_s showed a greater dependence on soil temperature and thereby on oak growing season (**Chapter 4**) than on quarterly N fertilization.

It may therefore be concluded that soil stoichiometry –oak soil is more copiotrophic than pine soil (**Chapter 7**)–, seasonal variability of soil moisture and temperature –soil metabolic activity was constrained by the Mediterranean seasonality as was demonstrated in **Chapter 4**– and doses of N fertilizer –high-fertilization showed intermediate responses between those from control and medium-fertilization treatments **Chapter 5**– modulated the R_s response to N additions.

Global change-driven forest succession: the linkage between green and brown worlds

This thesis demonstrates how different the soil functioning is beneath Scots pines and Pyrenean oaks in the ecotonal area between both species and the potential consequences of a vegetation change on soil C and N cycles. We elucidated the mechanisms underlying C transfer between aboveground autotrophs and belowground heterotrophs –the so-called green and brown worlds (Zechmeister-Boltenstern et al., 2015)– by studying microbial community structure, heterotrophic activity, GHG emissions and N dynamics in two different experiments. First, we studied the influence of tree species on the decomposition processes of litter in the forest floor using a litterbag experiment in pure stands of pine and oak (**Chapter 6**). Once the interactions between heterotrophic microbial communities and their chemical environment (i.e. forest types and litter types) had been analysed, the changes on C and N cycles throughout the sequential habitats of the secondary vegetation succession were investigated using microcosms that were built with mineral soil inoculums mixed with litter (**Chapter 7**).

Litter stoichiometry was the most important factor that affected mass loss and N dynamics during the early stages of the litter decay, but the interaction dynamics between microbial functional groups and the forest type showed an increasing importance with time (**Chapter 6**). This idea came from the early differentiation that colonizer microbial communities showed in litter. A stoichiometric perspective of the microbial community assembly during litter decomposition may facilitate the interpretation of our results (see Figure 8.1).

Figure 8.1. Proposed effects of resource stoichiometry and tree species on microbial community structure and function. Major concepts of ecological stoichiometry and important microbial features are indicated (Kaiser et al., 2014; Mooshammer et al., 2014; Zechmeister-Boltenstern et al., 2015): threshold element ratio (TER), carbon use efficiency (CUE) and nitrogen use efficiency (NUE). Microbial community structure in response to resource stoichiometry: gram-positive to gram-negative bacterial ratio (GP:GN), fungal to bacterial ratio (F:B).

At the beginning of the experiment, heterotrophic microbial communities of leaves and needles were similar in both forests but began to differentiate early during a phase of high litter decay. In this first stage of decomposition, litter present high C:N ratios and therefore takes place the incorporation of N into decaying litter (Gallardo and Merino, 1992; Kraus et al., 2003). The N immobilization was greater in leaves than in needles and also lasted longer. This result is in line with reports linking foliar litter with low initial N content to net increases in litter-N (Berg, 2000; Osono et al., 2014).

Then, needles reached the threshold elemental ratio earlier in the decomposition process, as was evidenced by the N mobilization phase. This fact is normally associated with the degradation of lignin and retarded decay rates, which likely could explain the lower decomposition rates shown by needles than by leaves in our experiment (Berg and McClaugherty, 1989; Bonanomi et al., 2010; Osono and Takeda, 2004). In this moment, source-microorganism interactions led to natural selection in the microbial community favouring colonization of certain life strategies that were more efficient at acquiring the limited-N in the needle. Thus, a preferential fungal over bacterial metabolism became increasingly evident in needles as decomposition proceeded (after ~ 1 year), which is in line with the known ability of fungi for efficiently scavenging of N from recalcitrant litter (Osono and Takeda, 2005; Rodríguez et al., 2017; Zechmeister-Boltenstern et al., 2015). In a more advanced stage of decomposition (after ~ 2 years), there was a late differentiation of the microbial community assemblies between forests

according to GP:GN ratios that could be related to an increasing importance of microbial remains (Kaiser et al., 2014) and physiology (Manzoni et al., 2012) in the formation of SOM. In summary, we found differential microbial colonisation patterns between forests suggesting a staggered functional adjustment of the microbial community driven firstly by litter stoichiometry, and then by the convergence of colonizing microbial communities toward soil microbes and SOM characteristics. At the end of the experiment, the functional adjustment of microbial communities favoured organisms K-strategies in decomposing litter at the pine forest and r-strategies at the oak forests.

Since the stoichiometric differentiation of microbial decomposer communities regulates C and N fluxes (Kaiser et al., 2014; Zechmeister-Boltenstern et al., 2015), it is likely that has played a role on the decomposition constants shown by the litter. We observed HFA only for leaves (decomposition rates of leaves were 18 % larger in oak than in pine forests) and synergistic effects of mixing needles and leaves (native litter in admixtures decomposed on average 16 % more than foreign litter decaying alone). As a result of the lower decay rate of needle litter compared with leaf litter, we can suggest that at identical litterfall inputs pure pine forests in the ecotone contribute to greater forest floor accumulation than pure oak forests.

In line with the results of the litter decomposition experiment in (**Chapter 6**), microcosms recreating the global change-driven vegetation succession from pine to oak also highlighted close linkages between the origin of SOM and the shifts in the ecology and physiology of microbial communities in the mineral soil (oligotrophic in pine forest vs. copiotrophic in oak forest, see **Chapter 7**). These linkages resulted in time-decoupled responses of soil C and N cycles throughout the forest secondary succession (see Figure 7.5). In the first stage of the succession, we found an acceleration on the C turnover with a shift towards lower F:B ratios in the mixed forest soil in comparison to pure pine soils. Soil processes leading to N recycling acceleration were stimulated later in the pure oak soil when pine SOM was totally eliminated from the system, resulting in an alleviation of microbial N limitation for growth, a greater abundance of gram-negative bacteria, an even more accelerated SOM turnover rates.

Methodological remarks

Our experimental design was conceptualised as a case study on a representative contact area between Scots pine and Pyrenean oak. Previous experience on those areas indicated to us that the only possibility of taking real replicates of the forest types would be picking up sites separated from each other some dozens of km (20 to 100 km; Díaz-Pinés et al., 2011b). Such distances do not allow for an experimental design involving repeated measures over a three-year

period in permanent plots. Therefore, we decided to focus on one location and perform a robust replicated design at its level, at the cost of limiting our findings in terms of extrapolation of results at regional or global scales. However, we firmly believe that key findings obtained in our study case (e.g. R_S dependence of soil temperature-SWC covariation, strong R_H seasonality and species-specific response of R_A in **Chapter 4**; R_S resilience to N additions in **Chapter 5**; consistent faster turnover rate of pine derived-C in **Chapter 4**, **Chapter 6** and **Chapter 7**; plant-microbial links driving biogeochemical cycles in **Chapter 6** and **Chapter 7**) are translatable to other Scots pine-Pyrenean oak ecotone forests in the Guadarrama Range, and provide important insights about the consequences that could arise following shifts in forest composition in Mediterranean conditions.

New challenges for the forestry sector: including soil C sequestration into forest management planning and decision-making

This thesis addressed different aspects of the C cycling ever since pine needles and oak leaves are shed from the tree, until their complete decomposition occurs in the soil and the C is released again as CO_2 to the atmosphere. We focused on the role of tree species on C and N turnover rates from the perspective of soil ecological processes. This understanding is useful for the decision-making about sustainable forest management strategies since tree species selection can strongly influence soil C sequestration (Jandl et al., 2007).

Under the current scenario of global change, forest managers are increasingly including timber and non-timber production objectives such as the mitigation of global warming in forest management planning (Diaz-Balteiro and Romero, 2008; Ruiz-Peinado et al., 2014). In most cases, C sequestration criteria only incorporate the C stored in living tree biomass since can be easily estimated using allometric equations applied to forest inventories (Ruiz-Peinado et al., 2012, 2011) whereas the soil C sink capacity is often ignored, because presents high spatial variability and slow accumulation rate (Jandl et al., 2007; Rodeghiero et al., 2011).

In order to make a reliable assessment of the C sequestration objective in Mediterranean pine forests, recent studies have assessed the way in which various management aspects such as thinning regime and length of rotation period affect the dynamics of different C pools including the forest floor and mineral soil (Moreno-Fernández et al., 2015; Ruiz-Peinado et al., 2014, 2013). These studies indicated a strong influence of forest management actions on the C stored in the living tree biomass, although the temporal evolution of soil C stocks remained unaffected. These studies showing little or no effect of forest management on soil C storage also reported different sensitivities between soil C pools, showing C storage in mineral soil to be more resistant than the forest floor C storage (Johnson and Curtis, 2001; Nave et al., 2010; Peichl and Arain, 2006). The smaller amount of C stored in forest floors compared to mineral soil C pools and their shorter

residence times suggest that changes in forest floor C stock may have only modest effects on total soil C storage and may also require a short recovery period after the silvicultural interventions (Nave et al., 2010).

Key findings of this thesis suggest that species composition of the forest stand could have a stronger impact in the rate of C accumulation and its distribution within the soil profile than the management aspects referred above. The balance between C input and soil C release can be significantly affected by tree species selection between Scots pine and Pyrenean oak because affects three major mechanisms for soil C and N cycling: the chemical quality of litter inputs (litter and soil stoichiometry), the site conditions (soil microclimate and N availability) and microbial colonization of certain life strategies. Hence, the presence of pines slows down the C release in forest floor (**Chapter 6**) and mineral soil (**Chapter 4** and **Chapter 7**) but the existence of oaks improves soil fertility through higher availability of mineral N (**Chapter 7**), which is likely to enhance forest productivity and the subsequent C input to soil.

Chapter 9

Conclusions

On the basis of the results obtained in this PhD thesis, the following general conclusions can be drawn:

- Tree species condition soil functioning in the ecotone through specific relationships in the plant-soil-microorganism system that were reflected in the seasonal dynamics of R_s components, responses of soil properties and R_s to short-term N additions, turnover rates of litter and soil-C, microbial assemblies of certain life strategies, greenhouse gas emissions and soil N availability.
- The strong and inverse water and thermal seasonality of the Mediterranean climate constrains both microbial decomposition and root respiration and thereby it is necessary for appropriate modelling soil CO_2 efflux in this climatic region to consider the soil temperature-SWC covariation.
- Soil respiration components showed tree species-specific responses to soil microclimate conditions, being particularly remarkable the differences in seasonal patterns of R_A between tree species. Pine R_A was more sensitive to the soil water depletion throughout the summer than oak R_A being the latter more sensitive to low winter temperatures.
- The turnover rates of soil-C, measured as heterotrophic respiration or foliar litter decomposition, in the Scots pine forest were consistently lower in all the experiments than the values found for the Pyrenean oak forest. This fact contributes to a larger C sink capacity at similar C input rates into both topsoil and forest floor beneath the Scots pine than beneath the Pyrenean oak.
- Under field conditions the differences in C turnover between tree species increased regarding the observed under laboratory conditions. This suggests that changes in soil microbial community composition and activity over the year, the presence of C pools of varying decomposability and physical protection and Mediterranean climate constraints, among other factors, affect differently the heterotrophic activity of microbial communities in pine and oak forests.
- Soil respiration in the ecotone was resilient to three-year of N additions simulating the forecasted increased in atmospheric N deposition. The soil stoichiometry, seasonal climate constraints for biological activity and the doses of N fertilizer were factors that modulated the R_s response of these soil forests to N additions. Because of these factors, the pine soil showed higher vulnerability than the more copiotrophic oak soil to the N enrichment.
- Nitrogen additions caused soil acidification, leaching of potassium base cation and a substantial decrease in soil microbial biomass that may imply stronger effects in the C cycle

if chronic anthropogenic N deposition persists in a longer run than the observed in this short-term N addition experiment.

- Tree species directly affected the mass loss rates and N dynamics of decaying litter through the different litter chemistry between oak leaves and pine needles but also indirectly by affecting microbial colonisation of decaying litter.
- Tree species influenced the succession of decomposer communities during oak leaf and pine needle litter decomposition according to microbial life-history traits (K-strategist vs. r-strategist). We distinguished two-phases in the successional changes of microbial communities in decaying litter. First, the functional adjustment of microbial communities led to higher F:B ratio in needles than in leaves. In a more advanced stage of decomposition, the microbial community assemblies differed between forests showing higher GP:GN ratio in the pine stand than in the oak stand.
- There is a decoupling between soil C and N dynamics throughout the forest secondary succession from pine to oak mainly driven by the SOM chemistry and its interaction with the ecology and physiology of soil microbial communities (oligotrophic in pine forest vs. copiotrophic in oak forest). In the first stage of the succession, we found an acceleration on the C turnover with a shift towards lower F:B ratios in the mixed forest soil in comparison to pure pine soils. Soil processes leading to N recycling acceleration were stimulated later in the pure oak soil when pine SOM was totally eliminated from the system, resulting in an alleviation of microbial N limitation for growth, a greater abundance of gram-negative bacteria, an even more accelerated SOM turnover rates.

Chapter 10

References

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Chapter 11

Annexes

Experimental plots

Pyrenean oak forest

Legend

Other elements

- Center
- + iButton sensors
- Forest stand outer boundary

Treatments

- Control
- Root-exclusion
- Medium-fertilization
- High-fertilization

Coordinates of the center
UTM ETRS89 H30
X 409,939 m
Y 4,524,266 m

Scots pine forest

Legend	
Other elements	Treatments
● Center	■ Control
⊕ iButton sensors	■ Root-exclusion
□ Forest stand outer boundary	■ Medium-fertilization
	■ High-fertilization

Coordinates of the center
UTM ETRS89 H30
X 409,819 m
Y 4,523,045 m

Scientific production derived from this PhD Thesis

Peer-reviewed research articles:

Fernández-Alonso, M.J., Díaz-Pinés, E., Ortiz, C., Rubio A., 2018. Disentangling the effects of tree species and microclimate on heterotrophic and autotrophic soil respiration in a Mediterranean ecotone forest. *Forest Ecology and Management* 430, 533-544. <https://doi.org/10.1016/j.foreco.2018.08.046>

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Rubio, A., **Fernández-Alonso, M.J.**, Díaz-Pinés, E. Mecanismos del suelo implicados en las emisiones de dióxido de carbono en respuesta a un enriquecimiento en nitrógeno de los sistemas forestales. In: VI Workshop REMEDIA, 11-12 of April 2018, Granada, Spain.

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Fernández-Alonso, M.J., Kitzler, B., Ortiz Oñate, C., Rubio, A. Influencia del sitio y de la hojarasca foliar del Pino silvestre y del Melojo en el proceso de descomposición y sus microorganismos asociados. In: Congreso REMEDINAL, 21-22 of June 2016, Madrid, Spain.

Fernández-Alonso, M.J., Ortiz, C.; Rubio, A. Decoding soils CO₂ efflux in forest ecosystems. A study case in Valsaín (Spain). (Poster). In: IV Workshop REMEDIA, 23-25 of March 2015, Madrid, Spain.

Awards:

Award for the best scientific poster of the Agroforestry Session at the First PhD Students Symposium hold by the Technical University of Madrid on 25th October 2017.

Award for the best oral communication titled “La disponibilidad de nutrientes del suelo determina la respuesta microbiana a la adición de hojarasca en el ecotono Pino-Roble” at the IV Young Researchers Conference hold by the ETSI de Montes, Forestal y del Medio Natural on 24th March 2017.

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