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**SOIL CARBON DYNAMICS IN SCOTS PINE
FORESTS AT THE SISTEMA CENTRAL OF SPAIN
(SIERRA DE GUADARRAMA)**

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**SOIL CARBON DYNAMICS IN SCOTS PINE FORESTS AT THE SISTEMA
CENTRAL OF SPAIN (SIERRA DE GUADARRAMA)**

**DINAMICA DEL CARBONO DEL SUELO EN BOSQUES DE PINO
SILVESTRE DEL SISTEMA CENTRAL DE ESPAÑA (SIERRA DE
GUADARRAMA)**

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ABBREVIATIONS

BD: bulk density
C: carbon
dbh: diameter at breast height
FF: forest floor
FO: forest
GHG: greenhouse gas
glmm: generalized linear mixed model
LMA: large macroaggregates
MaOM: mineral-associated organic matter
MAP: mean annual precipitation
MAT: mean annual temperature
MGD: mean geometric diameter
Mi: microaggregates
MOS: Materia orgánica del suelo
MWD: mean weight diameter
N: nitrogen
NAGase: N-acetil- β -glucosaminidase
OC: organic carbon
OM: organic matter
PLFAs: phospholipide fatty acids
POM: particulate organic matter
RDA: redundancy analysis
Rs: soil respiration
sdw: soil dry weight
SEM: structural equation modelling
SL: shrubland
SMa: small macroaggregates
SOC: soil organic carbon
SOM: soil organic matter

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RESUMEN

Los suelos forestales son clave en el cambio global por el carbono (C) que acumulan y por su capacidad de sumidero de C. Además de factores abióticos como el clima y las propiedades del suelo, la capacidad de los suelos para almacenar C está influenciada por el uso del suelo, los cambios en el uso del suelo y la ordenación forestal, que en última instancia pueden modificar las condiciones microclimáticas, la calidad y cantidad de los residuos vegetales que se incorporan al suelo, así como la comunidad microbiana asociada. En este contexto, la tesis doctoral se centra en el estudio de la dinámica del C del suelo en bosques mediterráneos de pino silvestre y de la influencia que el manejo forestal tiene en él. Para alcanzar estos objetivos, se ha cuantificado el contenido de C del suelo, la protección que sufre la materia orgánica del suelo (MOS) y la respiración del suelo (R_s), así como la comunidad microbiana asociada. Además, esta tesis pone de relevancia el comportamiento diferenciado de la dinámica del C en los sistemas mediterráneos comparado con los sistemas templados. Se han utilizado dos escalas de trabajo. Primero, un enfoque regional en dos zonas climáticas diferenciadas (Sistema Central de España y Alpes Occidentales de Austria) para estudiar los efectos de la forestación de pastizales de montaña con especies de coníferas (pino silvestre en la región mediterránea y picea de Noruega y pino cembro en la región templada) en la MOS y la abundancia de isotópica de C. En segundo lugar, se utilizaron dos enfoques locales para estudiar: (i) los efectos del manejo forestal en la R_s a lo largo del turno de corta en dos bosques mediterráneos de pino silvestre y, (ii) los efectos de la forestación de áreas de pastizal-matorral en la agregación de la MOS del suelo y la comunidad microbiana en un ecotono mediterráneo de pino silvestre y matorral.

La forestación aumentó la relación C:N del suelo en ambas regiones, lo que en España fue causado por una disminución significativa de la concentración de N. Las tasas de mineralización no fueron influenciadas por la aforestación. La forestación causó un enriquecimiento de ^{13}C en el suelo. La altitud resultó ser un buen indicador de las condiciones climáticas indicando que el aumento de la altitud en las montañas mediterráneas alivia la limitación del agua, favoreciendo tanto el crecimiento de las plantas como la descomposición de la MOS. Por el contrario, la altitud en las montañas de la región templada se asociaron con severas limitaciones de temperatura, limitando los

procesos de transformación de la MOS. A pesar del impacto de la forestación en los procesos biogeoquímicos del suelo, la concentración de C se vio marginalmente afectada, concluyendo que 40 años después de la forestación de pastizales con bosques de coníferas no se mejoró el secuestro de C en el suelo mineral.

El estudio de larga duración realizado en dos bosques de pino silvestre mostró que la humedad del suelo es la variable abiótica más importante que regula la R_s en los bosques mediterráneos. Se encontró un umbral de humedad del suelo (12%) por debajo del cual la R_s fue dependiente de la humedad del suelo y por encima del mismo, la R_s fue dependiente de la temperatura del suelo. Se encontró una clara interacción entre las variables microclimáticas en todas las edades y manejos. Por otra parte, la gestión forestal moderada dio lugar a una mayor R_s en las edades maduras y jóvenes comparando con respecto a la gestión más intensa. Este hecho fue resultado de una mayor densidad de árboles y mayores stocks de C en el horizonte orgánico y los primeros 10 cm del suelo mineral bajo la gestión. La clase de edad tuvo un efecto moderado sobre la R_s . Sin embargo, interactuó con el manejo forestal modulando la respuesta de R_s a la temperatura y la humedad del suelo.

Independientemente del uso del suelo, los macroagregados pequeños y los microagregados fueron los más abundantes y comprendieron aproximadamente el 50% y el 20% de la masa del suelo, respectivamente. Además, la concentración de C en los Mi fue mayor en todos los usos estudiados. La forestación resultó en un aumento del diámetro medio ponderado de los agregados. Los macroagregados pequeños presentaron mayor concentración de C y N asociados a la materia orgánica particulada que los macroagregados grandes. La concentración de C y N de la materia orgánica asociada a las partículas minerales disminuyó significativamente con el aumento del tamaño de agregado. La forestación resultó en una reducción de las bacterias gram + y de la actividad β -glucosidasa, pero en un aumento en la relación hongo:bacteria.

En resumen, la tesis ha incrementado el conocimiento del ciclo de C de los suelos forestales de pino silvestre en la región mediterránea, con especial atención en los cambios de la comunidad microbiana del suelo, la distribución de agregados del suelo y la dinámica del C del suelo con la altitud tras la forestación, así como la influencia del manejo forestal en la R_s y su dependencia a la humedad y temperatura del suelo.

ABSTRACT

Forest soils are keystones in the actual framework of global change due to their large carbon (C) storage and their C sink capacity. In addition to abiotic factors such as climate and soil properties, capacity of soils to store C is strongly driven by land use, land use changes and forest management which ultimately may modified quality and quantity of vegetation inputs to the soil and soil microbial community structure. In this context, this PhD thesis has been focussed in the study of the soil C dynamics in Scots pine forests from Mediterranean areas and the influence that forest management has on it. To reach this objective, quantification of soil C content, soil organic matter (SOM) protection and soil respiration (Rs), as well as characterization the soil microbial community structure has been carried out. Furthermore, this thesis looks for putting into relevance the different behaviour of C dynamics in Mediterranean systems compared to temperate ones. Two scales of work have been used. Firstly, a regional approach in two contrasting climate areas (Sistema Central of Spain and Western Austrian Alps) to study the effects of mountain grassland afforestation with conifer species (Scots pine in Mediterranean region and Norway spruce and Stone pine in temperate region) on SOM cycling and C isotope abundance. Secondly, two local approaches were used to study (i) the effects of forest management on Rs by a long-term experiment in two Mediterranean Scot pine forest chronosequences and (ii) the effects of afforestation of former grassland-shrubland areas on SOM aggregation and microbial community structure in a Mediterranean Scots pine-shrubland ecotone.

Afforestation consistently increased soil C:N ratio in both regions, which in Spain was caused by a significant decrease in N concentration. Mineralization rates, however, were not influenced by the vegetation change. Afforested areas in Mediterranean mountains showed a consistent soil ^{13}C enrichment. The role of altitude as regulator in climatic conditions was clear and strongly suggests that in Mediterranean mountain grasslands increases in altitude alleviates water limitation, favoring both plant growth and SOM decomposition, and ultimately accelerating C cycling. In contrast, temperate grassland areas at high altitudes were associated with severe temperature limitations, which constrained SOM transformation processes. Despite afforestation impact on soil biogeochemical processes, C concentrations were marginally affected concluding that 40

years after afforestation of former grasslands with coniferous forests C sequestration in the mineral soil was not enhanced.

The long-term study in Scots pine forests strongly suggests that soil moisture is the most important abiotic feature regulating R_s in Mediterranean forests. A soil moisture threshold of 12% was found identifying two scenarios of R_s response: below the threshold R_s was dependent of soil moisture and above the threshold R_s dependency on soil temperature was proved. Clear interaction between microclimatic features was found in all the stand ages and forests. Furthermore, moderate forest management resulted in greater R_s in both mature and young stands comparing with the more intense management. This is the result of the higher tree density and greater soil C stocks in both forest floor and topsoil 10 cm in the moderate managed forest. Stand age had almost no effect on R_s but interacted with forest management modulating the response of R_s to soil temperature and soil moisture interaction.

Regardless of the land use studied, small macroaggregates (SMa) and microaggregates (Mi) were the most abundant and comprised around 50% and 20% of the soil mass, respectively. Besides, C concentration in Mi was higher in all the uses studied. Afforestation resulted in an increase of Ma content that led to greater mean weight diameter (MWD) comparing to the shrubland soil use. Small macroaggregates hold greater C and N concentration in particulate organic matter (POM) than large macroaggregates (LMa). Regarding mineral associated organic matter C (MaOM-C) and N (MaOM-N) concentration, significant decrease with increasing aggregate-size class was found. Afforestation resulted in lower gram + bacteria, as well as β -glucosidase activity but in higher fungi:bacteria ratio.

In summary, the thesis has increased the knowledge of soil C cycle in Scots pine forest of Mediterranean regions with regards to changes in soil microbial community structure, soil aggregates distribution and SOC dynamic with altitude by afforestation and the influence of forest management on R_s as well as its dependency on soil moisture and soil temperature.

INTRODUCTION

Carbon dioxide and its actual framework

Carbon dioxide (CO_2) is the fifth most abundant gas in the atmosphere preceded by nitrogen (N_2), oxygen (O_2), argon (Ar) and water vapour (H_2O). It is released to the atmosphere through human activities such as deforestation and burning fossil fuels, as well as natural processes such as respiration and volcanic eruptions. Although its abundance in the atmosphere is rather low compared to N_2 or O_2 , its importance resides in the fact that it is the most important gas of the so-called greenhouse effect by its radiative forcing (Solomon et al. 2007). Greenhouse effect is the process by which clouds and greenhouse gases (GHG) absorb the long-wavelengths radiated by the ocean and land surface and re-radiate to the Earth.

Concentration of CO_2 in the atmosphere has increased since the pre-industrial era (Figure I.1), driven largely by economic and population growth, and is now greater than 400 ppm (Dlugokencky and Tans 2017), being higher than ever in the last 800,000 years (IPCC 2014). The main contributors to increasing atmospheric CO_2 abundance are (1) fossil fuel combustion and oxidation and cement production (Tans 2009) and (2) land use change (Le Quéré et al. 2016). The increase in CO_2 has also been followed by other GHG as methane (CH_4) and nitrous oxide (N_2O) (Figure I.1). Together, they have influenced the terrestrial climate by favouring the increment in surface temperature, the raise in the sea level, the decrease in snow covers, the reduction in precipitation events but also to the increase in the number of extreme meteorological events (Hartmann et al. 2013).

Figure I.1 Atmospheric concentrations of the greenhouse gases carbon dioxide (CO_2 , green), methane (CH_4 , orange) and nitrous oxide (N_2O , red). Source: (IPCC 2014).

Worldwide concerns by the increase in the GHG concentration in the atmosphere led to the creation of the Intergovernmental Panel on Climate Change (IPCC) in 1988 by the

United Nation Environment Programme and the World Meteorological Organization. In the third conference of the United Nations Framework Convention on Climate Change, the Kyoto protocol was established which obliges to approximately 40 developed countries to limit or reduce GHG emissions. Although first commitment period has recently ended, fossil fuel and industry activities emissions have been increased for more than 50% from 1990, reaching 9.9 Gt C y⁻¹ at 2015 (Le Quéré et al. 2016). By contrast, emissions associated to land-use change have been slightly reduced from 1.5 Pg C y⁻¹ by 1990 to 1.3 Gt C y⁻¹ in the 2015 (Le Quéré et al. 2016).

In this scenario of increasing emissions of CO₂ to the atmosphere, the role of forest ecosystems has been put into relevance since they are estimated to absorb about 30% of fossil fuel CO₂ emissions with a sink capacity of 1.1 Pg C y⁻¹ (Pan et al. 2011). For these facts, forest ecosystems are keystone in the global C balance and its management has been seen as a mitigation tool in the climate change strategies (Millar et al. 2007).

Carbon cycle in forest ecosystems

Global C cycle is the result of a series of reservoirs of C in the Earth System, which are connected by exchange fluxes of C. According to the size of the exchange fluxes and the speed of the reservoir turnover, two different domains may be differentiated: (1) C in the atmosphere, the ocean, surface ocean sediments and on land in vegetation, soils and freshwaters which are characterized by large exchange fluxes and relatively ‘fast’ reservoir turnovers; and (2) C in rocks and sediments with turnover time of more than thousand of years and exchanges of C with the fast domain through volcanic emissions of CO₂, chemical weathering, erosion and sediment formation on the sea floor. Thus, importance of fast turnover reservoirs has received the attention of the scientific community to define strategies to mitigate the huge emissions due to fossil fuel combustion and oxidation, to cement production and to land use change.

Terrestrial ecosystems are the second higher reservoir of C with around 1,900-3,050 Pg C allocated in living biomass (450-650 Pg C, (Prentice et al. 2001)) and in dead organic matter (OM) in litter and soil (1,500-2,400 Pg C, (Batjes 1996)). Carbon enters terrestrial ecosystems by photosynthesis (123±8 Pg C yr⁻¹, (Beer et al. 2010)), process by which CO₂ is removed from the atmosphere and fixed as C into plants. Part of this C is cycled through plant tissues, litter and soil C and can be released back into the atmosphere

by autotrophic (plant) and heterotrophic (soil microbial and animal) respiration and additional disturbance processes (e.g., sporadic fires) (Figure I.2) on a very wide range of time scales (seconds to millennia).

Figure I.2 Carbon cycle diagram in terrestrial ecosystems. Source: Trumbore (2006)

From terrestrial ecosystems, forests have been recognized as important players in the global C cycle by (1) their storage capacity of roughly 860 Pg C and (2) their net sink capacity of 1.1 Pg C y^{-1} (Pan et al. 2011). In forests, the C is stored mostly in soil (44% to 1-m depth) and in live above and belowground biomass (42%), but also in deadwood (8%), and in litter (5%) (Pan et al. 2011). However, the role of forests as C sink or source depends on the balance between C fixation and C emission. Carbon fixation is driven by the photosynthesis of plants and C emissions are due to heterotrophic and autotrophic respiration.

Forest soils and the carbon cycle

Soils are important pools of C with approximately 2,344 Pg C is stored in the top three meters of soil, 1,500 Pg C stored in the first meter of soil and about 615 Gt stored in the top 20 cm (Batjes 1996; Jobbágy and Jackson 2000; Guo and Gifford 2002). From this

figure, C contained by forest soils suppose around 70% of the C contained in soils (Six et al. 2002). In Spain, forest soils have been found to store $\approx 1,200$ Tg C in the first meter (Chiti et al. 2012) with the 55% contained in conifer forest, the 25% contained in broadleaf forest and and 20% in evergreen broadleaf forests. Regarding the distribution along the soil profile, Chiti et al. (2012) found that more than 55% of the total C was stored in the first 30 cm and the rest accumulated in the 30 to 100 cm. This relies in the importance that topsoil has on the C cycle.

Stabilization of SOM

Organic C in soils is mainly stored in soil organic matter (SOM). The amount of SOM is the result of inputs and output which are influenced by multitude of factors -e.g. climate, vegetation type, nutrient availability, disturbance, land use, and forest management practices. Soil organic matter is a dynamic entity formed by a continuum of organic compounds. The different turnover rates, pool sizes and stabilization mechanisms allow their differentiation into functional pools (Elliott et al. 1996; von Lützow et al. 2007).

Soil aggregation processes are responsible of the stabilization of SOM and consist of all mechanisms involved in the binding of soil particles into stable aggregates, which are the basic unit of soil structure. Soil aggregates control the dynamic of SOM, nutrient cycling and soil biodiversity (Oades 1984). Aggregates protect SOM via the formation of physical barriers which separate substrate and microbial biomass, thus preventing enzymatic and microbial decomposition processes (Six et al. 2004). Because of this protection, soil aggregation plays a key role in the C dynamics. Multitude of fractionation procedures have been designed depending in the resulted pools (Six et al. 2002; von Lützow et al. 2007; Yang et al. 2009).

Physical fractionation methods rely in that association of soil particles and their spatial arrangement play a key role in SOM dynamics, because limits the bioaccessibility from microorganism to decompose the SOM. This methods involve the application of various degrees of disaggregating treatments (dry and wet sieving, slaking), dispersion (ultrasonic vibration in water), density separation and sedimentation (Elliott and Cambardella 1991; Christensen 2001). Aggregates-SOM interactions result in an aggregate hierarchy which was postulated by Oades (1984), in which roots and hyphae are expected to hold

macroaggregates together giving place for a nucleus where microaggregate formation can take place. Although it has been seen that aggregate hierarchy is also influenced by the biochemical recalcitrance of the organic compounds (Rovira et al. 2010), mineral protection is still the main process defining aggregation in soils. Soil aggregation should be examined as the result of the interaction of many driving factors including soil management, inorganic binding agents, soil micro- and macro-fauna, roots and environmental variables (Six et al. 2004). In addition, it is known that land use influences the role that aggregates play in the protection of organic C (Gartzia-Bengoetxea et al. 2009; Wei et al. 2013a; Zhang et al. 2013; Wei et al. 2013b; Guo et al. 2015).

Soil respiration

Soil respiration (R_s) is the main process by which C is returned to the atmosphere. Such an importance supposes that even minor changes in the conditions that modulate R_s may imply great losses of C to the atmosphere. Soil respiration is the result of two main components: the CO_2 released by living plant leaves, stems and roots -defined as autotrophic respiration-, and the CO_2 released during decomposition of nonliving organic matter by microorganisms -defined as heterotrophic respiration (Trumbore 2006; Kuzyakov 2006).

Soil respiration is affected by both abiotic and biotic factors (Reichstein and Beer 2008). Most of the scientific effort so far has been mainly focused on disentangling the effect of abiotic variables, namely soil temperature and soil moisture, on R_s (Davidson and Janssens 2006; Moyano et al. 2012; Sierra et al. 2015). Soil respiration strongly depends on temperature because temperature influences most of the biological and physicochemical processes in the soil. Soil organic matter decomposition sensitivity to temperature have been broadly reported from laboratory (Lloyd and Taylor 1994; Fang and Moncrieff 2001) and from field studies in many climates and ecosystems (Peng et al. 2009; Lellei-Kovács et al. 2016). However, the temperature sensitivity of SOM respiration is not constant along the whole range of temperatures and findings have indicated to be much higher at lower than at higher temperatures (Schindlbacher et al. 2010; Liang et al. 2015). The dependency has been explained by multiple shapes (Froment 1972; Rochette et al. 1991; Lloyd and Taylor 1994; Fang and Moncrieff 2001). However, continuous R_s dependency on soil temperature has not been to hold true when

water limitations occurs (Lellei-Kovács et al. 2016). Soil water content influences R_s by its effect on soil organic matter decomposition (Moyano et al. 2012), root respiration (Maier and Kress 2000), and microbial activity (Curiel Yuste et al. 2007). Mediterranean region is characterized for having drought periods where soil temperature influence on R_s may be overruled by soil moisture because of its direct effect on R_s (Asensio et al. 2007) but also by its effect on modulating R_s dependency on soil temperature (Rey et al. 2002; Almagro et al. 2009). However, much less attention has been paid to explore other factors influencing R_s , such as forest structure and management (Saiz et al. 2006a; Ma et al. 2014).

Forest management as a mitigation tool

In the last decades, foresters have found a new paradigm in which values and objectives of forest management have been redefined to a multi-resource forest management in which not only timber production is considered (Bengston 1994). Increase in GHG concentration in the atmosphere and the subsequent global change associated (IPCC 2014) have led to development of new strategies for GHG mitigation. The actual C storage from forest ecosystems -more than two times the C in the atmosphere- and their gross C uptake (≈ 4 to 6.2 Pg C y^{-1} (Birdsey and Pan 2015)) has made that scientific community proposes them as a reliable tool in the mitigation of global change (Malmsheimer et al. 2008; Puettmann et al. 2009). Forests management strategies in the future should not only be focused on increasing timber production, but also on promoting CO_2 sequestration to ameliorate global warming (Millar et al. 2007) by improving management practices (Jandl et al. 2007; Schelhaas et al. 2015).

Forest management practices has been seen to directly affect R_s by the effect of stand age (Saiz et al. 2006a; Ma et al. 2014) (Saiz et al. 2006a; Ma et al. 2014), thinning regime (Olajuyigbe et al. 2012), harvesting (Shabaga et al. 2015) and its associated soil disturbance and site improvement for planting (Strömgren et al. 2016). This magement practices influence the C allocated in soils (Jandl et al. 2007), the quantity of litter inputs to the soil (Peltoniemi et al. 2004; Moreno-Fernández et al. 2015), the root density (Klopatek 2002; Claus and George 2005), the soil microbial community (Holden and Treseder 2013) and the microclimatic features (Gray et al. 2002) which ultimately affect

Rs. In addition, forest management has been seen to affect the response of Rs to soil temperature (Ma et al. 2014) and soil moisture.

Forest management can influence not only the already stablished forests but also promote the increase in forest surface. Afforestation, understood as the conversion of treeless areas into forests (referred as *afforestation*) has been identified as a possible mitigation tool (Jandl et al. 2007; IPCC 2014) by increasing the C stored in vegetation (Powlson et al. 1998; Smith et al. 2014b). However, total effect of afforestation at the ecosystem level also depends on the net effect on SOC (Poeplau et al. 2011), since even minor changes in the equilibrium between soil C inputs and outputs may imply huge emissions into the atmosphere. Thus, the aim of the research should be disentangle this issues in order to provide useful tools for forest managers and policy makers, to contribute to the mitigation of global change (Rubio et al. 2011).

OBJECTIVES

The general objective of this PhD thesis was to characterize the soil C dynamics of Mediterranean Scots pine forests from its main altitudinal range to the climatic treeline, where forest encroachment into grassland-shrublands occurs. To achieve this goal, the following specific objectives were set-up:

- i. To determine the effects on SOM dynamics caused by the afforestation of grasslands with coniferous species, and the interaction with climatic conditions.
- ii. To quantify the R_s of Mediterranean Scots pine forests and its dependency to both soil temperature and soil moisture. Furthermore, quantitatively assess the effects of forest management, stand structure and soil features on R_s .
- iii. To quantify the effects on SOM aggregation and soil microbial community structure and activity after afforestation of former grass-shrubland areas with Scots pines.

CHAPTER 1:

Soil organic matter dynamics after afforestation of mountain grasslands in both a Mediterranean and a temperate climate

1.1. Introduction

Soils store twice as much organic carbon (C) as the atmosphere (Ciais et al. 2013), and represent the largest reservoir of C in terrestrial ecosystems. In this context, land use and land-use changes are key factors in the balance between soil organic C (SOC) stocks and atmospheric CO₂ (Houghton et al. 2012). Afforestation is one of the major land-use changes currently taking place in Europe, mainly driven by the promotion of afforestation by environmental policies for containing rural depopulation (MacDonald et al. 2000; Marey-Pérez and Rodríguez-Vicente 2009). Afforestation may contribute to mitigating atmospheric CO₂ concentration due to the increase in C stored in the vegetation (Powlson et al. 1998; Smith et al. 2014b), but its total effect at the ecosystem level also depends on the net effect on SOC (Poeplau et al. 2011), since even minor changes in the equilibrium between soil C inputs and outputs may imply huge emissions into the atmosphere.

Vegetation shifts may affect above- and belowground C dynamics in different ways. Whereas an encroachment of woody species in grasslands usually increases aboveground C pools (Risch et al. 2008; Pérez-Cruzado et al. 2014), its effect on belowground C is still unclear. The capacity of soils to store organic C is simultaneously driven by environmental and biotic factors, which together with the dynamics between soil organic matter (SOM), mineral surfaces and aggregates, modulate rates of SOM mineralisation and degrees of protection (Lehmann and Kleber 2015). There have been reports of increases (Liao et al. 2006; Wang et al. 2013a), losses (Berthrong et al. 2009; Hiltbrunner et al. 2013; Guidi et al. 2014b) and no changes (McCarron et al. 2003; Risch et al. 2008; Poeplau and Don 2013) in SOC stocks in mineral soils following the afforestation of former grasslands. This is likely the result of the huge variation in site-specific characteristics such as climate, vegetation type and composition, plant traits (e.g. roots) and soil (Post and Kwon 2000; Jackson et al. 2002; Poeplau et al. 2011). The search for common patterns of SOC changes following afforestation is today a pressing issue. Forest expansion with coniferous species has been associated with changes in above- and belowground litter quality and quantity (Pérez-Cruzado et al. 2014), which represents the substrate for microbial decomposition in the soil. The invasion of grass-dominated ecosystems by woody plants has been related to the slower decomposition rates of the litter originated from trees than from pasture lands (Hiltbrunner et al. 2013), as a result of

the higher lignin content of conifer-litter (Berg 2000). However recent approaches suggest that the chemical quality of C inputs plays a secondary role compared to environmental conditions, biotic controls and, stabilisation processes (Lehmann and Kleber 2015). Further, the observed decrease in total microbial biomass under forest soils (Macdonald et al. 2009) may lead to lower SOM decomposition rates than pasture lands (Ross et al. 2002; Hiltbrunner et al. 2013). Finally, afforestation with conifers may also imply a reduction in soil nitrogen (N) contents and exchangeable cations caused by the greater plant demand due to the increased biomass production compared to grasslands (Berthrong et al. 2009).

Afforestation may cause changes in the mineralisation rates of more accessible and easily decomposable SOM fractions, whereas more protected fractions may be better preserved. A good approach for quantifying the easily decomposable SOM pool is by measuring SOM mineralisation rates using laboratory incubations (Paul et al. 2001). Studies of natural isotopic abundance in the soil have also been applied to enhance the understanding of the processes involved in SOM decomposition. This research uses the fractionation processes involved in the biological mineralisation of vegetation residues – known as *kinetic fractionation*– which occur when soil microbes discriminate isotopically during the mineralisation of organic matter and release ^{13}C -depleted CO_2 , enriching the remaining substrate (Mariotti et al. 1981; Högberg et al. 2005). A higher abundance of heavy stable isotopes in the soil has been associated with enhanced SOM decomposition (Tiunov 2007; Werth and Kuzyakov 2010). The isotopic signature of N in SOM has also been found to be positively correlated to the degree of SOM mineralisation (Nadelhoffer et al. 1996; Liao et al. 2006). Unlike the dynamic of soil C stable isotopes, several of the soil N transformation processes –nitrification, denitrification and ammonia volatilisation– discriminate in favour of the lightest isotope (Högberg 1997); consequently ^{15}N variations after afforestation may be related to N losses (Högberg and Johansson 1993), open N cycle (Vitousek et al. 1989) and soil N saturation (Emmett et al. 1998). Changes in microbial decomposition processes may also be related to the presence of a canopy structure due to afforestation, modifying microclimatic conditions by lengthening the periods of snow cover (Köck et al. 2002) that buffer harsh winter conditions (Kammer et al. 2009), softening annual soil temperatures (Hiltbrunner et al. 2013), and varying soil moisture regimes due to interception (Le Maitre et al. 1999). All these changes in

microbial decomposition conditions may ultimately be reflected in the isotopic composition of the SOM after afforestation.

In recent decades, afforestation of mountain grasslands has occurred on a global scale (Jackson et al. 2002; Berthrong et al. 2009). This is particularly the case in Spanish (Poyatos et al. 2003; Camarero and Gutiérrez 2004) and Austrian mountain regions (Dullinger et al. 2004; Tasser et al. 2007), following the intensification of livestock production and economic constraints which have driven smallholders' farmers in mountainous regions out of business, together with the afforestation programmes promoted by European governments. The growing body of information on the effect of forest expansion on SOM has produced contrasting and highly site-dependent results (Guo and Gifford 2002). The aim of this work is to determine common patterns caused by afforestation of grasslands on SOM features in two European mountainous areas with contrasting climates (Mediterranean and temperate). We sampled soil from 17 paired sites, all containing grassland and afforested plots. We determined bulk C and N concentrations and their stable isotope composition from soil and vegetation, and estimated SOC decomposition rates under standardised conditions in the laboratory for six months. We hypothesised that afforestation leads to changes (1) in SOC and total N concentrations, (2) in SOC mineralisation rates, and (3) in the signatures of soil ^{13}C and ^{15}N in the soil; and (4) that these changes are climate-dependent.

1.2. Materials and methods

1.2.1. Site description and experimental design

Two different mountain regions were selected: the Sistema Central range in Spain, and the Central Alps in Austria (Figure 1.1). The Sistema Central range is characterised by a sub-Mediterranean climate (Walter 1985) with warm dry summers and high year-round temperature fluctuations, although areas of high elevation are snow-covered throughout the winter (García Romero and Muñoz Jiménez 2010). The Central Alps region has a temperate mountain climate (Walter 1985) with cool summers, frequent rainfall and long periods of snow cover.

Figure 1.1 Geographical distribution of both Spanish and Austrian sites.

Sites were set up in locations where afforestation of former grasslands had occurred at least 40 years prior to soil sampling, and grasslands were still present in the vicinity and under the same soil type and position within the landscape. This time span is sufficient to assure that the short-term effects caused directly by forest operations on SOM dynamics have already passed (Poeplau and Don 2013), allowing us to evaluate changes due to the vegetation shift in the longer run. In most cases, afforestation was man-made and conducted between 1950 and 1970 in small patches of private forest, with the aim of increasing forest cover share and wood production while keeping grasslands present to some extent. Ten sites were selected in Spain and seven in Austria (Figure 1.1) (Annex Table 1.1). Three replicated paired plots were established on each site, separated by 25–50 m. Each paired plot (20 m x 20 m) consisted of an afforested plot with a closed tree canopy and an adjacent grassland plot. Spanish sites were located on granite and gneiss bedrock (Annex Table 1.1) with soil pH values of the mineral A-horizon between 4.0 and 5.8. Soil texture ranged from loamy sand to sandy clay loam (Annex Table 1.1) and soils were classified as Cambisols and Umbrisols (Annex Table 1.1, IUSS Working Group

WRB (2014)). The tree species in the Spanish forests was Scots pine (*Pinus sylvestris* L.), and grasslands were mainly formed by *Festuca curvifolia* (Lag. ex Lange) and *Nardus stricta* L. Grazing in the Spanish grasslands is almost non-existent and occurs only in summer –fewer than 0.25 livestock units per ha. Austrian sites were located on different soil substrates (Annex Table 1.1) with A-horizon pH ranging from 3.4 to 6.4. The textures were sandy loam and sandy clay (Annex Table 1.1); soils were classified as Podzols, Cambisols and Leptosols (Annex Table 1.1, IUSS Working Group WRB (2014)). Austrian afforested plots included Norway spruce (*Picea abies* (L.) Karst), European larch (*Larix decidua* Mill.) and Swiss pine (*Pinus cembra* L.); dominant grassland species were *Carex sempervirens* Vill. and *Nardus stricta* L. The Austrian grasslands are moderately grazed by cattle –0.5-1 livestock units per ha– for a period of 6-10 weeks during summer. The main features of the study sites are shown in Annex Table 1.1.

1.2.2. Soil and plant material sampling

We sampled soil material from the mineral A-horizon from all the plots. We used genetic horizons instead of fixed soil depth sampling, as the genetic horizon system offers better comparability of the processes involved in SOC dynamics (Grüneberg et al. 2010), and the A-horizon stores most of the soil C (Jobbágy and Jackson 2000; Chiti et al. 2012). Mean A-horizon depth was 24.7 ± 9.8 and 23.3 ± 6.8 cm for forests and grasslands in Spain respectively, and 11.4 ± 4.0 and 10.6 ± 4.3 cm for forests and grasslands in Austria. Organic layer was present in afforested plots only, and was not considered in this study; therefore, it was completely removed before soil sampling. A composite soil sample was taken from the A-horizon from six randomly distributed spots in each vegetation plot. A small soil pit (20 cm x 20 cm x 30 cm) was dug at each spot, and a soil sub-sample was taken from the entire depth of the A-horizon with a garden shovel. Soil samples were transferred to the laboratory, roots were removed by hand and the soil was sieved (2 mm). Fresh material from the standing vegetation (needles from coniferous species and leaves from herbaceous species) was collected in each plot in the soil sampling location in Spain, and a composite sample for each site in Austria.

1.2.3. Estimation of potential SOC mineralisation efflux

CO₂ efflux from soil samples was monitored in the laboratory as a measure of mineralised C. 20 g fresh weight of homogenised A-horizon soil from each plot (n= 102)

was placed inside 150 ml glass bottles. The soil samples were set at 60% of water holding capacity and pre-incubated during two weeks at 10 °C in a thermostatic cabinet (Lovibond®, Amesbury, United Kingdom). This pre-incubation period permitted the equilibration of soil samples and avoided abrupt CO₂ production pulses following soil disturbance caused by soil sampling and sieving (Schindlbacher et al. 2010). After the equilibration phase, the temperature inside the cabinet was increased to 20 °C and maintained throughout the whole duration of the experiment in order to achieve maximum respiration rates and highlight differences between the sampling sites (Schinner et al. 1993).

The bottles with the soil samples were kept continuously inside the incubation cabinets and covered with Parafilm®. This allowed outgassing of CO₂ from the soil atmosphere with no significant desiccation of the soil samples. An air circulation system prevented CO₂ from accumulating in the headspace, which could otherwise interfere in the soil-headspace CO₂ diffusion process. The moisture levels of the soil samples were maintained by periodic addition of a standard rain mixture (Schierl 1991). To determine the soil-headspace CO₂ efflux, a non-dispersive infrared sensor (MSH-P-CO₂, Dymament Ltd., Derbyshire, UK) was placed inside each bottle and closed tightly. The sensor produced a voltage readout every 10 seconds that was transformed into CO₂ concentrations. Following the non-flow-through non-steady-state technique (Livingston and Hutchinson 1995), the change in CO₂ concentration over time was used to infer the soil CO₂ production rate (mg CO₂-C h⁻¹ kg⁻¹ soil dry weight (sdw)). The sensors were calibrated weekly with four different calibration mixtures (400, 580, 1000 and 2000 ppm CO₂, AirLiquide, Germany).

SOC mineralisation rates were monitored periodically for six months, with a higher temporal resolution at the beginning of the experiment, which was then lowered as the SOC mineralisation rate started to level off. Cumulative SOC mineralisation effluxes for the whole incubation period were estimated by linear interpolation of consecutive measurements. An estimation of SOC decomposability (mg CO₂-C g⁻¹ SOC, Díaz-Pinés et al. (2014)) was obtained by normalising total CO₂ by SOC content in the soil.

1.2.4. C and N concentration and stable isotope analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$)

Soil ($n = 102$) and plant ($n = 74$) samples were oven-dried ($40\text{ }^{\circ}\text{C}$) and ground ($< 100\text{ }\mu\text{m}$) prior to analysis. Samples with pH higher than 6 were acid-treated to check for carbonate presence, and no traces of carbonates were found for any of the sites. SOC and total N concentration and C and N isotopic composition of plant and soil samples were determined using continuous-flow isotope ratio mass spectrometry in a Costech ECS 4010 Elemental Analyzer (Costech Analytical Technologies, Inc., Valencia, CA, USA) fitted with a zero-blank auto-sampler coupled via a ConFloIII to a ThermoFinnigan DeltaPlus-XL (Thermo Scientific, Waltham, MA, USA). Isotopic compositions were expressed in δ notation, representing the ‰ variation from the standard reference material:

$$\delta (\text{‰}) = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \cdot 1000$$

where R is the ratio $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. The standards used were Vienna Pee Dee Belemnite and atmospheric air- N_2 respectively. Precision (in terms of standard deviation) in internal standards for elemental C and N abundances were over $\pm 0.3\text{ ‰}$ and 0.1 ‰ respectively.

The enrichment of heavy isotopes in soils compared to plant was used as an indicator of isotopic discrimination caused by SOM decomposition (Michener and Lajtha 2007).

$$\Delta = \frac{\delta_v - \delta_s}{1 + \frac{\delta_s}{1000}}$$

where v refers to the plant material and s to the soil. We considered that values from fresh leaves could avoid biases due to possible discrimination that may already be occurring in the leaf fall caused by partial decomposition when the leaves are on the forest floor (Garten et al. 2000); we also assumed that isotopic fractionation due to translocation of nutrients prior to leaf senescence and fall was marginal (Balesdent et al. 1993). A negative Δ value indicates enrichment in the isotopic composition of the SOM compared to the vegetation.

We also calculated the difference in the composition of the $\delta^{13}\text{C}$ stable isotope between afforested and grassland soils in each paired plot as a proxy for the change in the isotopic composition due to forest encroachment:

$$\Delta_{Af-G} = \text{Afforested soil } \delta^{13}\text{C} - \text{Grassland soil } \delta^{13}\text{C}$$

1.2.5. Statistical analyses

The soil variables studied were tested for differences between land-use types (afforested, grassland) at the regional scale using general linear mixed models (*nlme* package, Pinheiro et al. (2016)), with vegetation as a fixed factor and site as a random factor, to include within-site correlations in the model. Normality and homogeneity of the variance of the residuals were checked by the Shapiro-Wilk (*stats* package, R Core Team (2015)) and Levene test (*car* package, Fox and Weisberg (2011)) respectively. Correlations and linear regression were performed between the variables of interest and the site altitude (*stats* package, R Core Team (2015)). If normality of residuals was met, Pearson product-moment correlation coefficient was used (r); otherwise, correlations were explored with Spearman's rank correlation coefficient (ρ). Significance level was 0.05 for all tests. Results are expressed as mean $\pm$ one standard deviation. All statistical analyses were carried out in R (version 3.2.3, R Core Team (2015)).

1.3. Results

1.3.1. Relationships between altitude, mean annual temperature and mean annual precipitation

In Spain, altitude showed a close negative correlation ($R^2_{\text{adj}}=0.98$) with mean annual temperature (MAT), and a positive correlation ($R^2_{\text{adj}}=0.35$) with mean annual precipitation (MAP) (Figure 1.2). In Austria, the same negative trend was found between altitude and MAT ($R^2_{\text{adj}}=0.11$); but a negative correlation was found between MAP and altitude ($R^2_{\text{adj}}=0.26$) (Figure 1.2).

Figure 1.2 Relationship between mean annual temperature (MAT) and mean annual precipitation (MAP) with altitude for Austrian and Spanish sites.

1.3.2. SOC concentration, total N concentration and soil C:N ratio

There was no significant effect of afforestation on the SOC concentration in Spanish plots (76 ± 34 and 75 ± 28 g C kg⁻¹ for afforested and grassland respectively) or in Austrian plots (111 ± 46 g kg⁻¹ for afforested and 91 ± 29 g kg⁻¹ for grassland) (Figure 1.3a). Total N in grassland plots was significantly higher than in afforested ones (5.8 ± 2.2 vs. 4.6 ± 2.5 g N kg⁻¹; $p < 0.01$) (Figure 1.3b) in the Spanish sites. In Austria, total N concentration was similar in grassland and afforested plots (6.1 ± 2.1 vs. 5.7 ± 1.9 g N kg⁻¹) (Figure 1.3b). Soil C:N ratio was significantly higher in afforested plots (18.2 ± 5.7) than in grassland plots (13.0 ± 1.3) in the Spanish sites ($p < 0.001$, Figure 1.3c). Austrian sites showed a similar trend (19.6 ± 5.1 vs 15.5 ± 3.3 , $p < 0.01$) (Figure 1.3c). Supplementary Table 1.1 shows the soil features of individual sites. Weak positive relationships between altitude and SOC concentrations ($R^2_{adj} = 0.13$; $p < 0.05$), and total N ($R^2_{adj} = 0.13$; $p < 0.05$) were found in Spanish grasslands. Negative relationships were observed in Austrian grasslands between altitude and SOC concentration ($R^2_{adj} = 0.39$; $p < 0.01$) and total N ($R^2_{adj} = 0.69$; $p < 0.001$). In Austria, altitude was positively correlated with C:N ratio in both the grassland ($R^2_{adj} = 0.64$; $p < 0.001$) and the afforested sites ($R^2_{adj} = 0.26$; $p < 0.01$). No significant correlations between MAT or MAP and SOC, N and C:N were found across the dataset for both grassland and afforested areas in Spain and Austria.

Figure 1.3 Mean values (± 1 standard deviation) of soil organic carbon (SOC) concentration (a), soil total nitrogen (N) concentration (b) and C:N ratio (c) in the Sistema Central in Spain and the Central Alps in Austria. Different letters denote significant differences ($p < 0.05$) between grasslands and afforested plots in each region.

1.3.3. Potential SOC mineralisation efflux

On average, SOC mineralisation efflux rates in Spain were 0.18 ± 0.12 mg CO₂-C h⁻¹ kg⁻¹ sdw for afforested plots, and 0.14 ± 0.10 mg CO₂-C h⁻¹ kg⁻¹ sdw for grassland plots (n.s.). The mean SOC mineralisation efflux rates in Austrian afforested and grassland plots were 0.35 ± 0.21 and 0.33 ± 0.26 mg CO₂-C h⁻¹ kg⁻¹ sdw respectively. Despite some individual significant differences in SOC mineralisation efflux rates at the site level (Supplementary Table 1.2), there was no significant effect of afforestation on SOC mineralisation efflux.

Mineralised C throughout the whole incubation period (Figure 1.4) was not significantly different between afforested and grassland plots in Spain (724 ± 371 and 586 ± 308 mg CO₂-C kg⁻¹ sdw for afforested and grassland respectively) and Austria (1408 ± 644 and 1316 ± 795 mg CO₂-C kg⁻¹ sdw for forests and grasslands respectively). By the end of the incubation period, Austrian soils had emitted approximately twice as much CO₂ as Spanish soils.

Figure 1.4 Mean values (± 1 standard error, $N=30$ for Spain and $N=18$ for Austria) of cumulative soil CO_2 production throughout the whole incubation period by afforested and grassland soils in both regions (Sistema Central, Spain; and Central Alps, Austria).

The values of cumulative SOC decomposability throughout the whole incubation period were 11.3 ± 7.0 and 8.4 ± 4.0 $\text{mg CO}_2\text{-C g}^{-1}$ SOC for Spanish afforested and grassland plots respectively ($p > 0.05$). Decomposability was significantly correlated with MAT ($\rho = 0.57$; $p < 0.01$), MAP ($\rho = -0.75$; $p < 0.001$), and with C:N ratio ($\rho = 0.60$; $p < 0.01$), but not with C or N concentrations. In the Austrian soils, these values were 14.3 ± 7.0 and 14.4 ± 6.7 $\text{mg CO}_2\text{-C g}^{-1}$ SOC for afforested and grassland plots respectively ($p > 0.05$). However, in Austria there was no correlation between cumulative SOC decomposability and any other variable.

1.3.4. Stable isotopic composition in soil and plant material

In Spain, mean $\delta^{13}\text{C}$ values were more negative in pine needles than in grasses (-28.2 ± 1.4 vs. -27.3 ± 0.7 ‰ respectively). In Austrian sites, there were not differences between afforested and grassland plots (-29.3 ± 0.9 ‰ vs. -28.4 ± 0.7 ‰ for needles and grasses respectively). Mean soil $\delta^{13}\text{C}$ was significantly less negative in afforested than in grassland plots in Spanish sites (Figure 1.5) but not in Austrian sites. Mean soil $\delta^{15}\text{N}$

values were significantly higher ($p < 0.001$) in grasslands than in afforested plots in Spain (5.3 ± 1.5 and 3.3 ± 1.0) (Figure 1.5) but not in Austria (2.7 ± 1.4 ‰ and 2.5 ± 1.1 ‰).

Figure 1.5 Mean values (± 1 standard deviation) of soil $\delta^{13}\text{C}$ (a) and soil $\delta^{15}\text{N}$ (b) in the Sistema Central in Spain and the Central Alps in Austria. Different letters denote significant differences ($p < 0.05$) between afforested and grassland plots in each region.

Soil $\delta^{13}\text{C}$ showed a significant positive relationship with altitude in Spain in both afforested and grassland plots (Figure 1.6). In Austria, soil $\delta^{13}\text{C}$ only showed a significant positive relationship with altitude in grasslands (Figure 1.6). Plant material $\delta^{13}\text{C}$ was significantly correlated with altitude, positively in Spanish grasslands and afforested plots and in Austrian grasslands, but negatively in Austrian afforested plots (Figure 1.6). In Spain, soil $\delta^{13}\text{C}$ was correlated with MAP ($\rho = 0.59$; $p < 0.01$) and MAT ($r = -0.71$; $p < 0.001$); and plant $\delta^{13}\text{C}$ correlated to MAT ($\rho = -0.47$; $p < 0.05$) in Spain. Plant material $\delta^{13}\text{C}$ was positively correlated with the $\delta^{13}\text{C}$ in mineral soil in Spanish and Austrian grasslands, and in Austrian afforested plots (Figure 1.7).

Figure 1.6 Relationship between altitude and $\delta^{13}\text{C}$. Black symbols represent soil $\delta^{13}\text{C}$ and grey symbols represent plant material $\delta^{13}\text{C}$. Data refer to mean values ± 1 standard deviation.

Figure 1.7 Relationship between soil $\delta^{13}\text{C}$ and plant material $\delta^{13}\text{C}$. Data refer to mean values ± 1 standard deviation.

Mean $\Delta^{13}\text{C}$ values were significantly less negative in grasslands than in afforested plots (-1.4 ± 0.5 vs. -2.8 ± 1.4 ‰ Spain and -1.9 ± 0.6 vs. -2.8 ± 0.5 ‰ Austria). Mean $\Delta^{15}\text{N}$ values in Spain showed significant differences ($p < 0.01$) between afforested (-5.3 ± 1.3 ‰) and grassland plots (-4.3 ± 2.3 ‰). $\Delta^{15}\text{N}$ values in Austria were highly variable between sites and no significant differences were found between afforested (-5.1 ± 2.0 ‰) and grassland plots (-6.0 ± 2.8 ‰). Lower altitudes led to an enrichment of soil $\delta^{13}\text{C}$ following

afforestation, whereas high altitude led to depletion, as shown in the relationship between $\Delta_{\text{Af-G}}$ and altitude (Figure 1.8).

Figure 1.8 Effect of altitude on the change in isotopic composition of soil $\delta^{13}\text{C}$ between afforested and grassland plots. Spanish sites are shown in black and Austrian sites in grey. Data refer to mean values ± 1 standard deviation.

1.4. Discussion

Our experimental approach focused on the effect of the afforestation of mountain grasslands on SOM dynamics at the regional level. The study sites encompassed a wide range of environmental conditions, including differences in altitude, species composition and –in the case of Austria– bedrock material (Annex Table 1.1). It was therefore within our expectations that the variability between the sites would influence SOM dynamics in addition to the afforestation itself (Guo and Gifford 2002; von Lützow et al. 2006; Schindlbacher et al. 2010). However, our aim was to identify common patterns in SOM

changes due to afforestation, which would outweigh differences due to specific site conditions.

In agreement with previous findings (Risch et al. 2008; Wang et al. 2013b), we observed no consistent effect of converting grassland to forest on mineral SOC concentrations. SOC content is the net result of C inputs and outputs in the system, both of which may be altered following afforestation. Changes in C inputs are mainly caused by alterations in the quantity of above- and belowground litterfall (Pérez-Cruzado et al. 2012), whereas C losses due to mineralisation tend to be affected by litterfall quality, microclimatic conditions and the associated microbial community (Aerts 1997; Macdonald et al. 2009; Cleveland et al. 2014; Nazaries et al. 2015) which in combination may ultimately modify SOC mineralisation processes. Our results showed a (non-significant) trend of higher C concentrations in Austrian forests, likely due to higher C inputs in a closed-canopy forest compared to grasslands. Together with the presence of an organic layer in the forest (storing additional 10-30 Mg C ha⁻¹, unpublished data), this suggests a higher C storage rate in the topsoil following the change in land use from grassland to forest. Afforestation very consistently increased soil C:N ratios in both climate areas; this generalisation has not been confirmed at a regional scale so far and – to our knowledge – was only observed locally (Berthrong et al. 2009). Litter from coniferous species has a wide C:N ratio and a chemically complex structure (Berg 2000; Thuille and Schulze 2006; Pérez-Cruzado et al. 2014), usually leading to low relative N availability for microbial nutrition and a high biochemical recalcitrance of SOC. Enhanced N demand by trees (Smal and Olszewska 2008) compared to pasture may have decreased soil N concentrations. Overall, our hypothesis that afforestation implies changes in SOC and N concentrations was consistently confirmed with regard to N concentrations, whereas SOC results were somehow less clear.

Oppositely to our expectation that SOC mineralisation rates change after afforestation, we found no effect, as previously reported elsewhere (Hiltbrunner et al. 2013), and only a marginally higher decomposability of SOC at forest sites. Further, effects of climatic features on decomposability were observed at the Spanish sites, only. Our experimental approach used standardised conditions in the laboratory for the mineralisation of SOC, in pursuit of a mechanistic understanding, and as a result may have lost some power of extrapolation and representativeness, as suggested from the observed decoupling between

decomposition rates and SOC concentrations. We cannot therefore rule out the possibility that the heterotrophic soil CO₂ efflux under field conditions may differ between afforested and grassland plots due to altered soil temperature and snow-cover patterns caused by canopy effects (Köck et al. 2002; Kammer et al. 2009) and changed soil moisture regimes (Le Maitre et al. 1999). The markedly higher CO₂ emissions and decomposition rates observed in Austrian soils may be due to temperature constraints on the site, which preserve higher amounts of labile C in the Alpine temperate region compared to Mediterranean mountain areas.

The natural abundance of stable isotopes has been used to estimate the extent to which SOM dynamics are affected by different land uses (e.g. Michener and Lajtha 2007), and there is usually a correspondence between plant and soil isotopic signatures (Bird et al. 1994; Boutton et al. 1998). Regarding our hypothesis that afforestation leads to changes in the soil isotopic signature, we observed changes in soil $\delta^{13}\text{C}$ values following afforestation in most of the sites (Supplementary Table 1.3), suggesting a gradual replacement of grass-origin SOC by tree-origin SOC (Hiltbrunner et al. 2013), although the shift is also interactively affected by climate conditions. The subtle differences between ^{13}C signatures in grasses and needles prevents a clear differentiation, as would be the case with a clear transition from C3 to C4 plants. It is also likely that a substantial proportion of the SOC in the afforested plots is still from grass origins, and only a small fraction of coniferous litter has actually contributed to the new SOC pool 40 years after afforestation (Richter et al. 1999).

The relative depletion of ^{15}N in Mediterranean soils after afforestation may be related to recent organic matter inputs to the soil (Liao et al. 2006) and a progressive biogeochemical degradation (Tiunov 2007) of N-forms in grasslands, leading to a loss of ^{15}N -depleted forms (e.g. Pörtl et al. 2007). The identification of the specific biogeochemical processes responsible for the different $\delta^{15}\text{N}$ is highly speculative for two main reasons: i) the multiple processes discriminating against ^{15}N at different rates (Michener and Lajtha 2007); and ii) the fact that we only analysed the $\delta^{15}\text{N}$ of the bulk soil, strongly limiting our explanatory power (Pörtl et al. 2007; Craine et al. 2015). High soil $\delta^{15}\text{N}$ values such as those found in grasslands have been associated with a high availability of N (Martinelli et al. 1999) and open N cycles (Eshetu 2004; Awiti et al.

2008). This is similar to the pattern seen when Spanish and Austrian soil $\delta^{15}\text{N}$ values were compared.

We had hypothesized that climate would affect SOM changes following afforestation. Contrary to our expectations, MAT or MAP had a poor explanatory power with regard to the effects of land use change on soil features. On the contrary, altitude was found to be a key factor influencing SOM dynamics. Altitude jointly modulates temperature and precipitation in each geographic region, and these are important parameters in the production of above- and belowground organic matter (Hitz et al. 2001), its decomposition (Rodeghiero and Cescatti 2005) and its final accumulation (Post et al. 1982). Increasing altitude led to a significant rise in SOC and total N concentrations in Spanish grasslands (estimated as $5 \text{ g C kg}^{-1} 100 \text{ m}^{-1}$ and $0.4 \text{ g N kg}^{-1} 100 \text{ m}^{-1}$), but a significant decrease in Austrian grasslands (estimated as $9 \text{ g C kg}^{-1} 100 \text{ m}^{-1}$ and $0.8 \text{ g N kg}^{-1} 100 \text{ m}^{-1}$). This contrasting effect of altitude on C and N concentrations has already been identified, including both increases (Leifeld et al. 2009; Schindlbacher et al. 2010) and decreases (Garcia-Pausas et al. 2007; Gutiérrez-Girón et al. 2015). High-altitude areas in Spain were associated to high MAP, and were therefore less water-limited during the growing season compared to low-lying areas, which had less total precipitation and higher evapotranspiration rates. This therefore produces a gradient of higher summer plant productivity in Mediterranean mountains as the altitude increases. Microbial decomposition at high altitudes is also constrained by low temperatures outside the growing period (Simmons et al. 1996), which explains the greater C and N contents found in the highest Mediterranean areas studied. Opposing patterns have been detected in Mediterranean areas but at altitudes well above those in our study (Gutiérrez-Girón et al. 2015). In the Alpine region, the complex topography and regional effects on climate were probably responsible for the negative relationship between altitude and MAP. Summer drought is not a crucial limiting factor in temperate mountain regions, and plant productivity is mainly regulated by temperature, leading to shorter growing seasons at high altitudes (Djukic et al. 2010). This therefore produces a downward gradient of plant productivity in temperate mountains as the altitude increases.

As expected, altitude had a clear effect on C isotopic signatures. It is well documented that increasing altitude diminishes the ratio of internal to external partial pressure of CO_2 in leaves, which in turn causes lower plant ^{13}C discrimination (e.g. Körner et al. 1991).

Low temperatures in high altitudinal areas may also influence plant ^{13}C discrimination due to physiological effects on the plants that cause an increase in leaf thickness and higher carboxylation efficiency (Körner 1989; Körner et al. 1991). Nonetheless, the investigation of the variety of factors (e.g. water availability, light radiation, physiological and metabolic processes) that interactively determine isotopic composition in plant tissues (Brüggemann et al. 2011) is beyond the scope of this work, which is primarily focused in the mechanisms that cause changes in the stable isotopic signature of the soil. The change in soil $\delta^{13}\text{C}$ after afforestation is determined by the rate of incorporation of litter-C from the trees into the soil, the isotopic composition of the tree litter, and the change in the discrimination during the mineralisation of this C debris (Liao et al. 2006; Werth and Kuzyakov 2010). Enhanced mineralisation rates in afforestation would lead to higher discrimination and thus to a positive change in soil $\delta^{13}\text{C}$ due to afforestation, provided all other parameters remain unchanged. Figure 1.8 shows that the SOC becomes (slightly) ^{13}C enriched after afforestation at lower altitudes, and is isotopically depleted at higher altitudes. This suggests that SOC mineralisation –inferred from greater soil $\delta^{13}\text{C}$ – is higher in forests at low altitudes and in pastures at high altitudes. It can therefore be speculated that the mechanisms of C replacement and stabilisation after afforestation may vary with climate conditions (as defined by altitude), with the watershed point located at lower altitudes in the Alpine region (ca. 1775 m a.s.l.) than in Mediterranean mountains (ca. 1900 m a.s.l.). Our contention relies on very small (but highly significant) differences in $\delta^{13}\text{C}$ and on a number of assumptions; further, we were not able to corroborate this pattern with our decomposability results, or even SOC concentrations. Nevertheless, the relationship between the soil isotopic pattern and elevation offers a promising working hypothesis whereby biogeochemical pathways may be differently affected by land-use change depending on altitude and its associated climatic conditions. These thresholds suggest the idea of an optimal altitude where the plant communities in the study and their associated microorganisms undergo a change in their habitat conditions –mainly climate-related– which impedes normal development, although they may potentially be found above or below these altitudes. In Spain, the threshold for mineralisation and stabilisation is in line with the current altitudinal treeline for Scots pine in the Sistema Central (Cañellas et al. 2000), and we therefore recommend further studies to address this issue.

1.5. Conclusions

Afforestation of former grassland areas in Mediterranean and Alpine mountains consistently led to a greater soil C:N ratio, suggesting changes in the SOM quality of the topsoil, although potential mineralisation was not influenced by afforestation. Soil isotopic signatures were more markedly influenced by afforestation in the Mediterranean mountains. Increasing altitude likely stimulated biogeochemical processes in the Mediterranean sites due to the alleviation of water constraints; conversely, temperate sites showed indicators of functioning constraints at higher altitudes due to low temperatures. Since the effects of afforestation on total SOC concentrations were negligible, we conclude that afforestation of mountain grasslands does not enhance C sequestration in the mineral soil, in both Mediterranean and temperate areas, for at least the first 40 years after the land use change. However, more research is required to test whether this trend persists over time and whether expected climatic changes will affect the SOM dynamics after afforestation.

CHAPTER 2:

Forest management effects on soil respiration in Scots pine forests of Sistema Central of Spain

2.1. Introduction

Forest ecosystems have been recognized as important players in the global carbon (C) cycle by their storage capacity of ≈ 860 Pg C (Pan et al. 2011) and their gross C uptake of 4 to 6.2 Pg C year⁻¹ (Birdsey and Pan 2015). Nevertheless, the role of forests as C sink or source depends on the balance between C fixation and C emission. Carbon fixation is driven by photosynthesis of plants and C emissions are due to heterotrophic and autotrophic respiration. The combination of root respiration and their associated mycorrhizal fungi and of heterotrophic respiration is defined as soil respiration (R_s) and supposes between 30 and 80% of the total forest ecosystem respiration depending on the localized site and climatic conditions (Janssens et al. 2001; Davidson and Janssens 2006). Thus, in the last decades where climate change has generated many concerns, R_s have been seen as key stone for its role in the C cycle (Schlesinger and Andrews 2000) and scientific community has been focussed in the understanding of factors controlling R_s , and how these factors affect CO₂ emissions from soils. However, Mediterranean ecosystems have received less attention (Raich and Schlesinger, 1992; Raich and Potter, 1995; Rey et al., 2002), and are currently under-represented in research networks (Reichstein et al., 2003; Valentini, 2003).

Soil respiration is affected by both abiotic and biotic factors (Reichstein and Beer 2008). Most of the scientific effort so far has been mainly focused on disentangling the effect of abiotic variables, namely soil temperature and soil moisture, on R_s (Davidson and Janssens 2006; Moyano et al. 2012; Sierra et al. 2015). Soil respiration strongly depends on temperature because temperature influences most of the biological and physicochemical processes in the soil. This relationship has been reported broadly from laboratory (Lloyd and Taylor 1994; Fang and Moncrieff 2001), field studies in many climates and ecosystems (Peng et al. 2009; Lellei-Kovács et al. 2016) and with multiple shapes (Froment 1972; Rochette et al. 1991; Lloyd and Taylor 1994; Fang and Moncrieff 2001). Soil water content influences R_s by its effect on soil organic matter decomposition (Moyano et al. 2012), root respiration (Maier and Kress 2000), and microbial activity (Curiel Yuste et al. 2007). In Mediterranean climates, soil water content has been seen to play a key role not only by its direct effect on R_s (Asensio et al. 2007) but also by its effect on modulating R_s dependency on soil temperature (Almagro et al. 2009). However,

much less attention has been paid to explore other factors influencing R_s , such as forest structure (Saiz et al. 2006a; Ma et al. 2014).

Forests management strategies in the future should not only be focused on increasing timber production, but also on promoting CO_2 sequestration to ameliorate global warming (Millar et al. 2007). Forest management practices has been seen to directly affect R_s by the effect of stand age (Saiz et al. 2006a; Ma et al. 2014), thinning regime (Olajuyigbe et al. 2012), harvesting (Shabaga et al. 2015) and its associated soil disturbance and site improvement for planting (Strömgren et al. 2016). This influence is the result of the effects that forest management practices have on the C allocated in soils (Jandl et al. 2007), the quantity of litter inputs to the soil (Peltoniemi et al. 2004; Moreno-Fernández et al. 2015), the root density (Klopatek 2002; Claus and George 2005), the soil microbial community (Holden and Treseder 2013) and the microclimatic features (Gray et al. 2002) which ultimately affect R_s . In addition, forest management has been seen to affect the response of R_s to soil temperature (Ma et al. 2014) and soil moisture .

The aims of this study were (i) to investigate seasonal variations in R_s , soil temperature and soil moisture along the rotation period in two Scots pine forests with different management practices, (ii) to determine the relative importance of soil temperature and soil moisture in predicting R_s , and (iii) to disentangle the direct and indirect interrelationship of microclimatic, stand structure and soil variables and their effects on R_s in Mediterranean mountain forests. We hypothesized that R_s changes along the stand age and forest management due to the variation in stand structure which modifies microclimatic conditions and soil C stocks. In addition, due to the Mediterranean conditions of our experimental plots, we expect that R_s may be explained by soil temperature but also markedly by soil moisture presenting a moisture threshold which controls the R_s dependency to soil temperature.

2.2. Material and methods

2.2.1. Study site

Two managed monospecific Scots pine forests located in the northern-facing slopes of the Sierra de Guadarrama in the Central mountain range of Spain were selected to conduct the study: Pinar de Navafría (hereafter Navafría; $41^{\circ}2'N$, $3^{\circ}51'W$) and Pinar de Valsaín

(hereafter Valsaín; 40°48'N, 4°2'W). In both forests, elevation ranged from 1,200 to 2,200 m.a.s.l. Parent materials are gneiss and granites. Soils are acidic (Table 2.1) and classified as Humic Cambisols (IUSS Working Group WRB 2014). Navafría is characterized by a mean annual rainfall of about 1,100 mm and mean annual temperature of around 7.3 °C. Valsaín is characterized by a mean annual rainfall of about 900 mm and a mean annual temperature of around 8.5 °C. Average site quality index in both forests is approximately 23 m at 100 years (Rojo and Montero 1996). Management plans on both forests have been implemented since more than 100 years ago. Navafría has a uniform shelterwood system with a more intense thinning regime (>30 % basal area), 2 or 3 regeneration fellings over a 20-year regeneration period and mechanical soil preparation to promote seedling establishment (García-López and García 1994). Valsaín has a group shelterwood system with less intense thinning regime (20-30% basal area), and regeneration is carried out by opening small gaps (≈ 0.1 – 0.2 ha) in the regeneration area over a 40 year regeneration period (Montes et al. 2005). Navafría has a 100-year rotation period while Valsaín has 120-year rotation period.

2.2.2. Experimental design

A chronosequence was implemented in each of the forests. Three forest stands (≈ 0.5 ha) with different ages within the rotation period were served as experimental plots in both forests. Thus, a young stage stand (hereafter referred as 'young'; 0-20 and 10-30 years, for Valsain and Navafría respectively), a middle stage stand (hereafter referred as 'middle-aged'; 40-60 and 70-90 years, for Valsain and Navafría respectively) and a mature stage stand (hereafter referred as 'mature'; 100-120 and 90-110 years, for Valsain and Navafría respectively) were investigated in each forest. Table 2.1 summarizes the vegetation and soil main characteristics of the different stands. No understory was present in any of the stands selected for the study.

Table 2.1 Measured stand level and average soil characteristics for the uppermost 30 cm of the different stand ages at Navafría and Valsaín forests. BA, basal area; H_m, mean tree height; Dbh, mean diameter at breast height; N, tree density; BD, Bulk density; FE, fine earth content (size < 2 mm).

Feature	Navafría			Valsaín		
	Young	Middle-aged	Mature	Young	Middle-aged	Mature
Elevation (m asl)	1,650	1,530	1,700	1,340	1,410	1,340
<i>Stand features</i>						
N _{≥7.5} (trees·ha ⁻¹)	3,264	243	243	1,701	1,632	313
N _{<7.5} (trees·ha ⁻¹)	938	-	-	3,264	-	1,111
Dbh _{≥7.5} (cm)	13.4	49.0	46.2	13.3	21.7	37.0
Dbh _{<7.5} (cm)	3.1	-	-	3.7	-	3.0
Hm _{≥7.5} (m)	10	21.1	23.7	10.8	19.1	24.6
Hm _{<7.5} (m)	8.3	-	-	4.5	-	3.0
BA (m ² ·ha ⁻¹)	45.7	46.0	42.7	31.6	64.8	54.5
<i>Soil features</i>						
pH _{H2O}	5.1	5.6	5.1	5.4	5.3	5.6
BD (Mg soil·m ⁻³)	0.83	0.81	0.92	0.84	0.87	0.84
FE (%)	43.7	60.3	55	69.2	56.6	45.5
Sand (% of FE)	54	64	64	59	59	65
Silt (% of FE)	38	32	29	35	30	30
Clay (% of FE)	8	4	7	6	11	5
Texture	Sandy loam	Sandy loam	Sandy loam	Sandy loam	Sandy loam	Sandy loam

2.2.3. Soil CO₂ effluxes measurements

Soil respiration was measured from July 2009 to November 2012 every three weeks during the snow-free season from early April to late November. Four frames were installed in May 2009 in every stand ages of the chronosequences. The frames were distributed in two fenced areas of 150 m² separated by about 20 m from each other and centred in the middle of the stand ages plot to avoid potential edge effects. Soil CO₂ efflux was measured using the manual closed dynamic chamber approach (Bain et al. 2005), consisting in a self-made chamber attached to an infrared gas analyser (WMA-4, PP-Systems, Amesbury, MA, USA) by Teflon tubes of 2.5 mm inner diameter. Outlet gas from the IRGA was returned to the chamber to avoid vacuum effects. The manual chambers consisted in a single-wall rigid polyethylene light-dome (volume: 39 l), fixed to an aluminium frame of 0.82x0.82x0.05 m by a compressible PTFE seal at the bottom of the chamber and by means of 4 elastic ropes. The frames were inserted around 3 cm in

the mineral soil. To avoid overheating inside the chamber during the deployed time, the chamber was covered by aluminium foil. A small opening to the atmosphere with a stainless-steel needle of 5 cm long was inserted in the chamber to eliminate pressure differences between the inside and outside of the chamber headspace which could affect CO₂ diffusion gradients between the soil and the atmosphere (Hutchinson and Livingston 2001). The chamber was closed for 300 seconds and headspace CO₂ concentration recorded every 30 seconds. Soil CO₂ efflux was calculated from the linear headspace CO₂ concentration increase over the last 270 seconds of chamber closure, considering the chamber volume and the soil area, and expressed as $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. Measurements were carried out between 9:00 to 18:00 hours. To avoid time of the day effects on the R_s , care was taken to randomize the stand to measure every time. The infrared gas analyser was calibrated once every season, using 700 ppm standard CO₂ gas under ambient air pressure. While soil CO₂ effluxes were monitored, air temperature inside the chamber and topsoil (0-10 cm) temperature and volumetric moisture were recorded. Temperature was recorded with a Termistor Vertix 5989M probe (Herter Instruments, Barcelona) and soil moisture was measured with a time-domain reflectometer TDR 100 (Field Scout, Spectrum Technologies Inc, USA). All values of R_s were over the minimum detectable flux estimated for our procedure (Parkin et al. 2012), which was estimated to be $0.06 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$.

2.2.4. Ancillary soil and stand measurements

At the end of the observational period, different soil and stand features were measured in every stand ages of both forest (Table 2.1). Soil features were measured from the soil contained inside of every frame where R_s had been measured. Forest floor (FF) was sampled by collecting the organic layer contained in two squares of 0.2 m x 0.2 m distributed in the surface of each frame and measuring the height of the layer in every corner of the square and then averaged. After removing the FF, the first 30 cm of the mineral soil were sampled by 10-cm depth increments. Three sub-samples were taken from each frame resulting in four composite soil samples from each ages stand. In addition, one intact soil sample for soil bulk density calculation was collected from each frame and depth, by using stainless steel cutting rings of 10-cm high by 5-cm in diameter. Both FF and mineral soil samples were oven-dried at 50 °C until constant weight, ground to 250 μm and later analysed for C and N concentration in a LECO CNS 2000 analyser

(LECO Corporation, St. Joseph, MI, USA). Soil texture of the composite samples was measured by the pipette method (Gee and Bauder 1986). In every ages stand, two square plots of 150 m² each were randomly selected to measure both tree height by using a Height-Sonar-based Vertex III hypsometer and the T3 transponder (Haglöf, Sweden) and tree diameter at breast height (dbh) by using a calliper (Table 2.1).

2.2.5. Statistical analysis

Soil C and N stocks (Mg ha⁻¹) from the mineral soil were calculated with the following equation:

$$\text{Mineral soil Stock}_{[OC, N]} = BD \times D \times FE \times [OC, N] \times 10,$$

where BD is the bulk density of the soil (Mg soil·m⁻³), D is the thickness of the soil sample (meters), FE is the fine earth content (diameter < 2 mm) in mass (g FE·g soil⁻¹), $[OC, N]$ are the content of OC or N (g·kg FE⁻¹), respectively. Carbon and N stocks of the FF were calculated with the following equation:

$$\text{FF Stock}_{[OC, N]} = W \times [OC, N] \times \frac{1}{100},$$

where W is the weight of the FF per unit of area (g·m⁻²) and $[OC, N]$ are the content of OC or N (g·g⁻¹), respectively.

In order to investigate the effect of ageses and forest management on the soil respiration, soil temperature and soil moisture, we used the generalized linear mixed model (glmm) framework (lme4 package; Bates et al. (2015)), with the interaction of forest and ages stand as fixed effects and the frame from which measurements came from as random effect since measurements were repeated over the same frame throughout the study. Data was grouped in three seasons: spring, summer and autumn (days of the year 80-171, 172-263, 263-33, respectively). Relationship between R_s and soil temperature and soil moisture were investigated by fitting the following equations by linear regression methods (stat package; R Core Team (2015)) for every stand age of the forest studied:

$$R_s = \beta_0 + \beta_1 \cdot \text{Soil temperature (eq. 1),}$$

$$R_s = \beta_2 + \beta_3 \cdot \text{Soil moisture (eq. 2),}$$

$$R_s = \beta_4 + \beta_5 \cdot \text{Soil moisture} + \beta_6 \cdot \text{Soil moisture}^2 \text{ (eq. 3),}$$

being $\beta_0, \beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6$ the estimated parameters by the ordinary least square method. Data used to fit these equations was the result of averaging the measures of the four frames of each stand ages. However, when using both soil temperature and soil moisture to explain R_s , its interaction resulted in a nonlinear response being necessary to use generalized additive models (mgcv package; Wood (2011)). Tensor product smooths with cubic regression spline basis (Wood 2006) were used to represent the nonlinear covariate effect of the interaction between soil temperature and soil moisture over R_s in each level of the interaction between stand ages and forest. Random effects of each frame were introduced in the generalized additive model as a smooth that produces the parametric interaction of the predictors, and penalizes the corresponding coefficients with a multiple of the identity matrix (Wood 2008). General assumptions of normality and heteroscedasticity of the linear models were checked by inspecting the Q-Q plots and the model residual *versus* response values. When assumptions were not reached by using gaussian distribution of residuals, gamma shape was adequate to fulfil them. Fitting quality for linear equations and generalized additive model was assessed by measuring the adjusted coefficient of determination (R_{adj}^2).

Welch's heteroscedastic F test (Welch 1951) was used to test if differences in soil C stocks between stand ageses, both between and within forest (onewaytests package; Dag et al. (2017)).

Structural Equation modelling (SEM) is an advanced and robust multivariate statistical method that allows for hypotheses testing of complex path-relation networks (Grace 2006). SEM was used to test the direct and indirect effects that forest structure, soil microclimatic and soil features (Table 2.2) have on R_s . Microclimatic variables (soil temperature and soil moisture) together with R_s were evaluated as means of every year of the period studied. Forest site was codified as a binary variable -0 for Navafría and 1 for Valsaín-, with the objective to quantify the effects of forest management. Stand ages was coded as a numeric variable ranging from 1 for young stands to 3 for mature ones. SEM model was fitted following the instructions presented in Grace (2006) and Iriondo et al. (2003). Model estimation was based on maximum likelihood and the model fit adequacy was estimated using the model chi-square and its associated p-values.

Table 2. 2 Variables used to test their effects on soil respiration during the fitting process of the structural equation model. FF, forest floor.

Features category	Variables
Forest	Forest site, stand ages, tree density, basal area, mean tree height
Soil	Stock of C _{FF} , Stock of C ₀₋₁₀ , Stock of C ₁₀₋₂₀ , Stock of C ₂₀₋₃₀ , Stock of N _{FF} , Stock of N ₀₋₁₀ , Stock of N ₁₀₋₂₀ , Stock of N ₂₀₋₃₀ , clay content, silt content, sand content
Microclimatic	Soil temperature, soil moisture

All the results have been expressed as mean $\pm$ one standard deviation if not stated otherwise.

2.3. Results

2.3.1. Soil temperature and soil moisture

Strong seasonal variability was found for soil temperature and soil moisture in both Navafría and Valsaín forests (Figure 2.1). Soil temperature was significantly higher in summer than in spring and in autumn across age classes and forests (Table 2.3). Age effects on soil temperature were only found in Valsaín for summer where higher values were recorded in the mature stand compared with the middle-aged one (Table 2.3). Differences on soil temperature between forests were found in mature and young stands for all seasons studied (Table 2.3).

In average, soil moisture showed significant higher values in spring than in autumn and in summer for both forests and all the studied ages (Table 2.3). Age effects on soil moisture were found in Valsaín where young stand showed significantly greater values than mature one in summer and autumn (Table 2.3).

Soil temperature and soil moisture showed opposed seasonal trends (Figure 2.1), which resulted in significant negative correlation between soil temperature and soil moisture (Figure 2.2; Table 2.4), reflecting the general pattern of the Mediterranean climate.

Figure 2.1 Soil temperature (a-b), soil volumetric moisture (c-d) and soil respiration (e-f) of three ageses measured in Valsaín (a, c, e) and Navafría forest (b, d, f). Values are mean $\pm$ standard error of the mean ($n=4$). Measuring period was from July 2009 to November 2012. Grey areas indicate snow cover periods.

Figure 2.2 Relationship between soil temperature and soil moisture in spring (a, d, g), summer (b, e, g) and autumn (c, f, i) for young (a, b, c), middle-aged (d, e, f) and mature stands (g, h, i). Continuous line for Valsaín and dotted line for Navafría represent significant ($p < 0.05$) linear models fitted to the data. Circles represent data from Valsaín and triangles data from Navafría. Pearson moment correlation values of every relationship are shown in Table 2.4.

2.3.2. Soil respiration

Seasonal variability was also detected in R_s in both Navafría and Valsaín forest (Figure 2.1). In Navafría, the greatest R_s was measured in summer for all the stand ages (Table 2.3). Mature stands showed differences between forest and those were found in all the seasons, being greater the R_s in Valsaín than in Navafría (Table 2.3). Only spring and autumn R_s from young stands reached significant differences between forests, with greater R_s in Valsaín than in Navafría (Table 2.3).

Table 2.3 Seasonal mean soil respiration, soil temperature and soil volumetric moisture of the three stand ages studied in Valsaín and Navafría forests. Values are mean $\pm$ standard deviation. Different lowercase letters denote significant differences ($p < 0.05$) between stand ages within the same forest and season. Capital letters show significant differences ($p < 0.05$) between seasons within the same stand age and forest. Asterisks show significant differences ($p < 0.05$) between forest stand ages within the same season.

Forest	Ages	Soil respiration ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)						Soil temperature ($^{\circ}\text{C}$)						Soil moisture ($\text{m}^3 \cdot \text{m}^{-3}$)			
		Spring		Summer		Autumn		Spring		Summer		Autumn		Spring	Summer		Autumn
Navafría	Young	0.64 $\pm$ 0.25	B*	0.93 $\pm$ 0.40	A	0.58 $\pm$ 0.29	Bab*	5.5 $\pm$ 3.0	C*	12.7 $\pm$ 2.6	A*	8.0 $\pm$ 3.1	B*	0.25 $\pm$ 0.08	A	0.09 $\pm$ 0.08	B
	Middle-aged	0.84 $\pm$ 0.33	AB	0.95 $\pm$ 0.45	A	0.62 $\pm$ 0.44	Ba	6.4 $\pm$ 2.7	C	13.3 $\pm$ 2.4	A	9.2 $\pm$ 2.8	B	0.20 $\pm$ 0.06	A	0.07 $\pm$ 0.07	B
	Old	0.57 $\pm$ 0.37	B*	0.80 $\pm$ 0.43	A*	0.47 $\pm$ 0.25	Bb*	5.6 $\pm$ 3.3	C*	14.3 $\pm$ 2.8	A*	8.0 $\pm$ 3.6	B*	0.19 $\pm$ 0.09	A	0.06 $\pm$ 0.06	C
Valsaín	Young	1.05 $\pm$ 0.38	*	1.08 $\pm$ 0.42		0.83 $\pm$ 0.33	*	8.6 $\pm$ 2.9	B*	14.7 $\pm$ 1.9	Aab*	9.9 $\pm$ 3.0	B*	0.25 $\pm$ 0.05	A	0.09 $\pm$ 0.07	Ba
	Middle-aged	1.07 $\pm$ 0.41		1.01 $\pm$ 0.40		0.83 $\pm$ 0.33		8.1 $\pm$ 2.5	B	13.7 $\pm$ 2.0	Ab	9.8 $\pm$ 2.9	B	0.19 $\pm$ 0.05	A	0.07 $\pm$ 0.07	Bab
	Old	1.26 $\pm$ 0.56	*	1.10 $\pm$ 0.59	*	0.88 $\pm$ 0.49	*	10.3 $\pm$ 3.0	B*	16.1 $\pm$ 1.7	Aa*	10.8 $\pm$ 3.2	B*	0.17 $\pm$ 0.05	A	0.05 $\pm$ 0.05	Bb

Table 2.4 Pearson moment correlation values between soil temperature and soil moisture along the different seasons for all the stand ages and forest studied.

Forest	Ages	Pearson correlation between soil temperature and soil moisture					
		Spring		Summer		Autumn	
		r	p-value	r	p-value	r	p-value
Navafría	Young	-0.64	>0.05	-0.71	<0.005	-0.93	<0.005
	Middle	-0.58	>0.05	-0.74	<0.005	-0.87	<0.001
	Old	-0.52	>0.05	-0.68	<0.01	-0.80	<0.001
Valsaín	Young	-0.38	>0.05	-0.77	<0.001	-0.96	<0.001
	Middle	-0.17	>0.05	-0.72	<0.005	-0.87	<0.001
	Old	-0.21	>0.05	-0.77	<0.001	-0.93	<0.001

2.3.3. Soil temperature and soil moisture as soil respiration predictors

Neither soil temperature nor soil moisture did show significant relationship with soil respiration when using all data obtained during the experiment (data not shown). Exploratory data analysis showed that soil moisture mediated the R_s dependency on soil temperature and soil moisture by modulating the microclimatic variable that better explained R_s . The soil moisture threshold which maximized the explication power of each equation was 12% (Figure 2.3). Above the threshold R_s was better explained by soil temperature with between 37 to 60% of the total variance explained by the equation 1 (Table 2.5). Below the threshold, R_s was moisture dependent and equations 2 and 3 could explain between 35 to 62% of the variance except for the middle-aged stand at Valsaín where no significant explanation was achieved (Table 2.5).

Table 2.5 Fitted parameters of the linear equations used to explain soil respiration dependencies on soil temperature and soil moisture along the stand ages and forests. Bold numbers indicate parameters significantly different from 0 ($p < 0.05$). Mean values $\pm$ standard error.

Site	Ages	$R_S = \beta_0 + \beta_1 \cdot \text{Soil temperature}$				$R_S = \beta_2 + \beta_3 \cdot \text{Soil moisture}$				$R_S = \beta_4 + \beta_5 \cdot \text{Soil moisture} + \beta_6 \cdot \text{Soil moisture}^2$					
		β_0	β_1	N	$R^2_{\text{adj.}}$	β_2	β_3	N	$R^2_{\text{adj.}}$	β_4	β_5	β_6	N	$R^2_{\text{adj.}}$	
Navafría	Young	0.32±0.13	0.07±0.02	20	0.40					-0.24±0.22	0.34±0.09	-0.02±0.01	18	0.62	
	Middle-aged	0.37±0.19	0.08±0.03	15	0.37					-0.10±0.25	0.29±0.11	-0.02±0.01	23	0.37	
	Old	0.20±0.10	0.07±0.01	17	0.60					-0.03±0.19	0.28±0.08	-0.02±0.01	21	0.35	
Valsaín	Young	0.41±0.16	0.08±0.02	17	0.54	0.44±0.13	0.07±0.02	21	0.42						
	Middle-aged	0.30±0.24	0.09±0.03	12	0.41	0.75±0.16	0.03±0.03	26	0.02						
	Old	0.31±0.30	0.11±0.03	14	0.53					0.08±0.18	0.31±0.07	-0.02±0.01	24	0.49	

As either soil moisture and soil temperature explained separately the R_s , their interaction effect on R_s was studied. The gam explained more than 50% of the variance ($R_{adj}^2 = 0.51$) by using different tensor product splines for each combination of forest and stand ages (Table 2.6). No great differences in the shape were present along the stand ages of Valsaín although mature stand presented steepest effect by the interaction of both soil temperature and soil moisture (Figure 2.4). In Navafría, middle-aged stand showed that soil temperature and soil moisture interaction exerted a differentiated pattern compared with young and mature stands since (Figure 2.4).

Table 2.6 Fitted parameters of the generalized additive model for modelling the interaction of soil temperature and soil moisture along the different stand ages and forest. df, degree of freedom of parametric terms; edf, estimated degree of freedom of smooth terms; te, tensor product spline; s, smooth spline for random effects.

Terms	df/edf	F-value	p-value
<i>Parametric Terms</i>			
Forest	1	-1.433	>0.05
Stand Age	1	-1.533	>0.05
Forest*Stand Age	1	2.834	<0.005
<i>Smooth terms</i>			
te(Soil temperature, Soil moisture)	11.0	15.713	<0.001
te(Soil temperature, Soil moisture)*Valsaín*young stand	3.8	2.593	<0.05
te(Soil temperature, Soil moisture)* Valsaín *middle-aged stand	6.4	3.555	<0.001
te(Soil temperature, Soil moisture)*Valsaín*mature stand	0.0	0.020	>0.05
te(Soil temperature, Soil moisture)*Navafría*young stand	4.8	2.346	<0.05
te(Soil temperature, Soil moisture)* Navafría *middle-aged stand	7.2	2.946	<0.005
te(Soil temperature, Soil moisture)*Navafría*mature stand	4.0	5.310	<0.001
s(frame) random effect	15.4	7.617	<0.001

Figure 2.3 Relationships between soil respiration and soil temperature and soil moisture for the different stand ages and forests. Each point is the mean of 4 measurement points made per sampling day.

Figure 2.4 Tensor product splines of the interaction of soil temperature and soil moisture from the different stand ages and forests. Soil respiration, soil temperature and soil moisture are in $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, $^{\circ}\text{C}$ and $\text{m}^3 \cdot \text{m}^{-3}$, respectively.

2.3.4. Effects of microclimatic, soil and stand level features on soil respiration

The SEM obtained by the interrelationship of stand level, soil and microclimatic features (Table 2.7) explained more than 50% of the R_s variance (Figure 2.5). Direct, indirect and total effects of the variables are summarized in Table 2.7. Increases in soil moisture, soil temperature, C stock of the FF and basal area were strongly associated with increases in R_s , indicating that R_s could be well-explained by these variables. Soil moisture, C stock of the topsoil 10 cm and the forest were the variables with the greatest total effect on R_s (Table 2.7).

In addition, it was found from the SEM that, both C stock from FF and from 0-10 cm mineral soil can well be predicted with 55% and 73% of the variation explained (Figure 2.5). The features with the highest explanation power were forest site and basal area for C stock of the FF and forest site and C stock of the FF for the C stock of the 0-10 cm mineral soil (Table 2.7).

Table 2.7 Total direct and indirect effects in the structural model. Effects were calculated using standardized path coefficients.

Variable	Type of effects		
	Direct	Indirect	Total
Soil respiration			
Forest site	-	0.46	0.46
Stand ages	-	-0.12	-0.12
Basal area	0.28	-0.04	0.24
Stock C_{FF}	-	0.10	0.10
Stock C_{0-10}	0.25	0.29	0.54
Soil temperature	0.28	-0.37	-0.09
Soil moisture	0.64	-	0.64
Stock C_{FF}			
Forest site	0.43	-	0.43
Basal area	0.51	-	0.51
Stock C_{0-10}			
Forest site	0.76	0.08	0.84
Stand ages	-0.19	-	-0.19
Basal area	-0.17	0.09	-0.07
Stock C_{FF}	0.18	-	0.18
Soil temperature			
Stand ages	0.25	-	0.25

Variable	Type of effects		
	Direct	Indirect	Total
Soil moisture			
Forest site	-	0.38	0.38
Stand ages	-	-0.23	-0.23
Basal area	-	-0.03	-0.03
Stock C _{FF}	-	0.08	0.08
Stock C ₀₋₁₀	0.46	-	0.46
Soil temperature	-0.58	-	-0.58

Figure 2.5 Structural equation model representing relationships among microclimatic, stand level and soil variables. Positive effects are indicated by solid lines and negative effects by dashed lines, with standardized estimated regression weights indicated. Squared multiple correlation estimates are indicated for each endogenous variable. T_s, mean soil temperature; M_s, mean soil moisture; R_s, mean soil respiration; Stock C_{FF}, C stock of forest floor; Stock C₀₋₁₀, C stock of first 10 cm of mineral soil. Arrow widths are proportional to estimated regression weights. Only significant ($p > 0.05$) paths are shown. Model fit parameters: sample=96, normed fit index=0.99, comparative fit index=1.00, $\chi^2_{df=10} = 5.23$, $P=0.88$

2.4. Discussion

Soil respiration values measured during our experiment were in the range of previous ones found for Mediterranean Scots pine forests (Inclán et al. 2008). Seasonal trends in R_s were found in both Valsaín and Navafría forests during the study period (Figure 2.1). This variability has been reported previously for a wide type of forests (Rey et al. 2002; Saiz et al. 2006b; Inclán et al. 2008; Chang et al. 2014) and characterizes drought-stressed regions as the Mediterranean one.

2.4.1. Effect of microclimatic variables on soil respiration

Soil temperature and soil moisture exhibited opposite markedly seasonal patterns along the study period (Figure 2.1). Both abiotic variables covaried negatively in summer and autumn but only negative trends were found in spring when soil moisture was highest.

Soil temperature effect on R_s has been widely modelled with multitude of shape functions (Lloyd and Taylor 1994; Kirschbaum 1995; Sierra et al. 2015). Our results were better fitted by a linear relationship (Fang and Moncrieff 2001). Continuous R_s dependence on soil temperature has been seen to hold true mainly in mesic ecosystems where soil moisture is not a limiting factor (Lloyd and Taylor 1994). However, Mediterranean region is characterized for having drought periods where soil temperature influence on R_s may be overruled by soil moisture effects on R_s (Rey et al. 2002; Almagro et al. 2009). In our study, seasonal variation of R_s followed soil temperature only during part of spring and autumn as previously reported for other Mediterranean ecosystems (Rey et al. 2002; Almagro et al. 2009) and soil moisture during the rest of the experimental time. Soil moisture thresholds at which temperature sensitivity of R_s is reduced have been found in several studies from different ecosystems (Fang and Moncrieff 2001; Palmroth et al. 2005; Gaumont-Guay et al. 2006; Jassal et al. 2008; Wang et al. 2014; Lellei-Kovács et al. 2016). This inflection point in our experimental site was located on a soil moisture level of $0.12 \text{ m}^3 \cdot \text{m}^{-3}$. This threshold established two scenarios of R_s dependencies. Above the threshold, R_s was positively temperature-dependent since these conditions are not soil moisture limited. However, the threshold values in soil moisture seems to be site specific as the factors limiting water uptake by plants and microbes may differ by ecosystem. Even in the same climate region, different soil moisture thresholds have been found in previous studies. For example, Almagro et al. (2009) investigated how soil moisture modulated the sensitivity of R_s in different

ecosystems of the Mediterranean region and found that the threshold value of soil moisture was $0.10 \text{ m}^3 \cdot \text{m}^{-3}$. However, Rey et al. (2002) found in a Mediterranean oak forest that soil temperature accounted for 85% of the variation of R_s when soil moisture was above 20%. Furthermore, Chang et al. (2014) found in a Mediterranean riparian forest that soil moisture threshold was around 17 %.

Previous works have seen that interaction effects between soil moisture and soil temperature improve R_s explanation (Lellei-Kovács et al. 2011). In our study, interaction between soil moisture and soil temperature allowed to increase variance explanation accounting for more than the half of the total R_s variance. Our results highlight the tight relationship that microclimatic variables such as soil temperature and soil moisture present in the Mediterranean scots pine forests studied as it has seen previously for other Mediterranean forest (Rey et al. 2002; Almagro et al. 2009). The different shapes in the fitted surfaces along forest and stand ageses put into relevance the importance to quantify effects of both microclimatic variables since future predictions in terms of increasing temperatures and reduction of precipitation for Mediterranean region (IPCC 2014) may result in clearly different increments or reductions of the mean R_s (Figure 2.4).

2.4.2. Forest management effects on soil respiration

Forest management effects over R_s were found in the Mediterranean scots pine forest studied. Moderate mangament from Valsaín resulted in higher mean R_s values at mature and young stands during the study period. These differences may be motivated by three reasons: (i) the greater C stock of the topsoil 10 cm present in Valsaín that may imply more substrate availability for microbes which decompose the organic matter producing CO_2 . This is in accordance with significant relationship between soil C stocks and R_s found in the area (Inclán et al. 2008). (ii) The higher tree density and basal area in Valsaín's stands compared with Navafría's ones which ultimately might result in higher root density (Vanninen and Makela 1999) and consequently in a greater autotrophic respiration which can account for more than the half of the total soil respiration (Högberg et al. 2001), but also in higher root exudates that serve as labile substrate for microorganism; finally, (iii) the above mentioned higher soil temperature that may result in greater CO_2 rates.

2.4.3. Stand age effects on soil respiration, soil temperature and soil moisture

Stand age effects on microclimatic variables were only found in Valsaín. Mature stand showed higher soil temperatures and at the same time lower soil moisture content than the young one. The lowest tree density presented in the mature stand favours direct exposure of soil surface to insolation resulting higher soil temperatures (Gray et al. 2002; Masyagina et al. 2010). However, the finding of lower moisture levels with stand age and thus with lower tree density is opposite to the findings reported by Gray et al. (2002) who found positive relationships between soil moisture content and forest gap sizes. Nevertheless, findings of Gray et al. (2002) were achieved in a forest ecosystems with almost twice of the precipitation than our experimental plots. Thus, we believe that in our study the stand age effects on microclimatic conditions was most probably motivated by the lowest tree density and therefore the greatest insolation received by the soil which ultimately favour a higher evaporation.

Although forest age has been seen to influence on the magnitude of a forest as carbon sink or source has been already pointed out (Law et al. 2003), our results did not show significant direct relationship of stand ages on R_s being in accordance with previous findings from Ma et al. (2014) who found that stand age did not affect total R_s .

This may be the result of the high spatial variability (as it was stated by the high SD after averaging the four frames measured).

2.4.4. Separating direct and indirect factors influencing soil respiration

Structural equation modelling has been used previously to found not only direct but also indirect effects of biotic and abiotic variables over R_s (Geng et al. 2012). Our results point out the importance to make such an analysis to disentangle the extent to which these factors may constrain direct or indirectly the R_s in Mediterranean forests. This approach suggests that, in the one hand, forest management not only directly influence R_s as stated previously, but also exerted a positive indirect effect on R_s . Thus, our results show that in the Mediterranean forests studied, the less intense forest management, may suppose an increase in the soil CO_2 effluxes due to its tight association with the C stock of the topsoil 10 cm. In the other hand, SEM analysis allowed us to find that stand ages exerted a reduced total negative effect on R_s , by an indirect relationship through soil C stock of topsoil 10 cm and soil temperature, showing that increase in stand age may be related

with decreasing R_s , being in accordance with findings from Saiz et al. (2006a) in a Sitka spruce chronosequence where R_s decreased with stand age.

2.5. Conclusions

Our results strongly suggest that soil moisture is the most important abiotic feature regulating R_s in Mediterranean forests. Furthermore, in contraposition to temperate forests, R_s was not continuously explained by soil temperature. This fact mainly responds to the soil moisture limitations that Mediterranean forests suffer during the summer and early autumn. Thus, a soil moisture threshold of 12% was found which identified two scenarios of R_s response: below the threshold, R_s was dependent of soil moisture; and above the threshold, R_s dependencies on soil temperature were proved. Interaction between microclimatic features was clear in all the stand ages and forests.

Moderate forest management of Valsaín resulted in greater R_s in both mature and young stand-ages comparing with the more intense management of Navafría. This is the result of the fact that moderate management was associated to the higher tree density and the greater soil C stocks in both forest floor and topsoil 10 cm. Stand ages had almost no effect on R_s . However, interactively with forest management modulated the response of R_s to soil temperature and soil moisture interaction.

The fitted model obtained by the interaction of soil moisture and soil temperature along the different stand ages and forest management intensities suppose a valuable tool for accurately assessing the R_s in these Mediterranean Scots pine forests.

CHAPTER 3:

Afforestation effects on soil aggregation and soil microbial community structure in a Mediterranean Scots pine- shrubland ecotone

3.1. Introduction

Soils act as a large reservoir of organic carbon (OC) and their store capacity is strongly driven by quality and quantity of vegetation inputs, soil properties, climate and soil microbial community, which ultimately modulate soil organic C (SOC) mineralization processes (Lehmann and Kleber 2015). In this context, land use and land use changes are key issues in the balance of SOC stocks due to their influence in the C inflows and outflows from soils (Guo and Gifford 2002), but also to their influence in the different agents involved in SOC accumulation.

Encroachment of forests is taking place globally in the last decades, due to both global warming and land cultivation abandonment (Smal and Olszewska 2008). This phenomena is of special importance in alpine areas, where grasslands initially evolve into shrub-covered ecosystems, before trees occupy the former grassland areas (Chauchard et al. 2010; Leonelli et al. 2011). This shift of the vegetation land cover will enhance quality and quantity of litter inputs and thus variations in the C cycling as well as in CO₂ sequestration by increasing C storage in forest biomass are expected (Kammer et al. 2009). At the same time, forest advance will alter the amount and the residence time of OC in the soil (Kuzyakov 2006) through the quality and quantity of the plant litter inputs provided to the soil and its associated soil microbial community structure and function (Curiel Yuste et al. 2012). In addition to biotic drivers, forest advance will also lead to a more favourable microclimate with deeper snow layers –result of the insulation effect of the forest canopy– and warmer soils in winter (Körner 1998). Winter soil snow cover may reduce the episodes of freezing and subsequent thaw cycles of soils which although do not directly affect C and nitrogen (N) dynamics, but first and foremost soil structure which is a key factor for soil stability (Kværnø and Øygarden 2006), but also of soil organic matter (SOM) dynamics (Hagedorn et al. 2010). Although SOM decomposition in cold areas and its interaction with the living soil has received much scientific attention in the last two decades (Heimann and Reichstein 2008), changes in soil aggregation process could also have a dramatic influence on threat to mountain soils as a result of land use change.

Soil aggregation processes consist of all mechanisms involved in the binding of soil particles into stable aggregates, which are the basic unit of soil structure. Soil aggregates controls the dynamics of SOM, nutrient cycling and soil biodiversity (Oades 1984).

Aggregates protect SOM via the formation of physical barriers which separate substrate and microbial biomass, thus preventing enzymatic and microbial decomposition processes (Six et al. 2004). Because of this protection, soil aggregation plays an important role in the C dynamics. Different studies have looked at ways in which tillage practices (Zhang et al. 2013; Guo et al. 2015), deforestation (Wei et al. 2013b) or afforestation (Gartzia-Bengoetxea et al. 2009; Wei et al. 2013a) influenced the role that aggregates play in the protection of organic C. Conversion from agricultural land to forest can increase the amount of SOC due to the accumulation of C in macroaggregates and its redistribution from fine particles to coarser fractions (Wei et al. 2013a). Furthermore, Gartzia-Bengoetxea et al. (2009) pointed out that macroaggregates may play an important role in aggregate dynamics in mature forests. Soil aggregation should be examined as the result of the interaction of many driving factors including soil management, inorganic binding agents, soil micro- and macro-fauna, roots and environmental variables (Six et al. 2004).

It is well known that soil microorganisms have a crucial role in providing ecosystem services through their effects on the development of soil structure by binding soil particles and organic matter to create aggregates (Six et al. 2004). Microorganisms have two main ways to act in soil aggregation: i) using fungal hyphae to get a mechanical union of soil particles and ii) via the exudation of by products to get the union of primary particles (Garcia-Franco et al. 2015). This has led to the idea that fungi are more important in soil aggregation than bacteria (De Gryze et al. 2005). In this sense, some studies have found decreased fungal abundance (Chiu et al. 2006) and fungal/bacterial ratio (Briar et al. 2011) with decreasing particle size. After afforestation of shrubland, Garcia-Franco et al. (2015) suggested that the increase in SOC pools linked to changes in microbial activity and fungal community structure promoted the formation of macroaggregates. An increase in soil fungal/bacterial ratio is therefore expected to lead to greater soil C accumulation (Smith et al. 2014a).

The present study aims to investigate the effects of a pine forest encroachment on an alpine grassland-shrubland area on i) soil microbial community structure and activity and ii) the storage and potential stabilization of SOC. Such data could prove very useful in C accounting, especially in quantifying SOC stocks changes due to natural or even anthropogenically driven vegetation shifts. We sampled soil from three paired sites, all containing shrubland, shrubland-pine forest and pine forest plots. We determined the soil

aggregate-size class distribution and the SOC and N concentrations and their stable isotope composition from soil, SOM fractionation and soil microbial community structure. The specific objectives were to evaluate (1) changes in soil aggregation, (2) changes in OC and total N of aggregate-size classes and (3) changes in the soil microbial community among land uses. Our first working hypothesis was that macroaggregates are more abundant in forest and their aggregate mean size is higher than in grassland-shrubland soils and regardless soil use, aggregate-associated SOC increases with aggregate-size. We also hypothesised that microbial community structure and activity respond to different soil use.

3.2. Materials and Methods

3.2.1. Study area

The study area is placed on the northern face of the Sierra de Guadarrama in the Sistema Central range of Spain (40° 59' N; 3° 49' W) with an altitude between 1900 and 2000 m a.s.l. The Sistema Central range is characterised by a sub-Mediterranean climate (Walter 1985) with warm dry summers, high year-round temperature fluctuations and high elevation areas snow-covered throughout the winter (García Romero and Muñoz Jiménez 2010). The mean annual temperature is 5.2 °C, the mean temperature of the minimum in coldest month is -3.8 °C, the mean temperature of maximum in the hottest month is 21.7 °C and the mean annual precipitation is 1609 mm (Sánchez Palomares et al. 1999). Soils were classified as Dystric Cambisol (IUSS Working Group WRB 2014) on granite bedrock. Soil characteristics are shown in Table 3.1.

Table 3.1 Main soil properties for the topsoil (0-5 cm) in the three land uses studied. SL, Shrubland; SL-FO, Shrubland-Forest; FO, Forest.

Land use	pH		EC	RF (%)	Texture (%)			BD (Mg·m ⁻³)	Textural class
	H ₂ O	KCl			Sand	Silt	Clay		
SL	4.8	3.6	36.9	35.0	70.5	18.1	11.4	0.98 ± 0.11	Sandy Loam
SH-FO	4.8	3.6	41.5	39.1	69.3	16.1	14.6	0.77 ± 0.03	Sandy Loam
FO	4.7	3.5	39.3	38.0	72.9	11.4	15.7	0.78 ± 0.11	Sandy Loam

RF: rock fragments (>2 mm); BD: bulk density

3.2.2. Experimental setup

Our study was carried out in an ecotone area between the alpine grassland/shrubland and the scots pine forest. The ecotonal area was identified by making use of aerial orthophotographs from different years (1965's vs. 2009's photo). Afforestation had occurred at least 40 years prior to soil sampling, and grasslands were still present in the vicinity and under the same soil type and position within the landscape. This time span is sufficient to assure that the short-term effects caused directly by forest operations on SOM dynamics have already passed (Poeplau and Don 2013), allowing us to evaluate changes due to vegetation shifts in the longer run. Grazing is almost non-existent and occurs only in summer—fewer than 0.25 livestock units per ha. Three different land uses were identified in the ecotone: a grassland/shrubland area (denoted as SL) composed mainly by patches of *Cytisus oromediterraneus* Rivas Martínez et al. and *Juniperus communis* L., with *Festuca curvifolia* Lag. ex Lange and *Luzula lactea* (Link.) E. Mey occupying the areas between patches but also with very sparse *Pinus sylvestris* L.; the shrubland/sparse scot pine forest area (denoted as SL-FO) composed by patches of *C. oromediterraneus*, *J. communi* and *P. sylvestris* L. with both low tree density (560 trees·ha⁻¹) and low mean height (3.5 m); and the scot pine forest area (denoted as FO) composed by and irregular stand structure of *P. sylvestris* with a tree density of 1200 trees·ha⁻¹ and mean height of 8.9 m. Three replicated paired plots (20 x 150 m) were established and separated by 5 m covering a total area of about 70 x 150 m. Each paired plot contained three subplots (20 x 50 m) with the three land uses identified.

3.2.3. Soil sampling and storage

In June 2012, three composite soil samples —made by five random subsamples each— from the top mineral soil (0-5 cm) were collected from every land use in each paired plot. Prior to soil sampling, forest floor if present was removed. Immediately after sampling, samples were cooled and transferred to the laboratory where roots were removed manually. In addition, one intact soil sample for bulk density determination was taken from every land use in each paired plot. Both soil and bulk density were sampled by using stainless steel cylinders 5.0 cm height and 5.0 cm in diameter.

In the laboratory, soil samples were divided into two groups depending on the analysis to carry on. The first group of samples was sieved (2 mm) and stored at -20 °C in plastic bags until analysed for PLFA's (Stenberg et al. 1998) and enzymatic activity. The second

group of samples was stored in laboratory conditions extended in trays until the aggregate-size analyse was done. Soil cores for bulk density were oven-dried at 105 °C until constant weight that was reached (after 48 h).

3.2.4. Aggregate-size class analysis

Aggregate separation was made by dry-sieving as presented in Nie et al. (2014). Field moisture content of all samples was less than 6.0% by weight, allowing limited mechanical stress and aggregate breakdown during sample manipulation. Recognisable plant residues and stones were removed prior to pass the samples through a 6-mm mesh sieve. Aggregate fractions were obtained by sieving 100 g of soil through nested sieves (2, 0.25 and 0.053 mm) mounted on a mechanical sieve shaker OASS 203 (Orto Alresa, Madrid, Spain). The resulting aggregate-size classes were: large macroaggregates (LMA, 6-2 mm); small macroaggregates (SMa, 2-0.250 mm); microaggregates (Mi, 0.250-0.053 mm) and silt + clay size particles (s+c, < 0.053 mm). Three-minute sieving intervals were found to be enough to separate the aggregate-size classes while minimizing aggregate abrasion.

To assess changes in soil structure by afforestation, we made use of two aggregation indices from Kemper and Rosenau (1986), the mean weight diameter (MWD) and the mean geometric diameter (MGD):

$$MWD (mm) = \sum_{i=1}^n x_i \times w_i,$$

$$MGD (mm) = \exp \left[\frac{\sum_{i=1}^n w_i \times \ln x_i}{\sum_{i=1}^n w_i} \right],$$

where w_i is the weight fraction ($\text{g} \cdot 100\text{g}^{-1}$) in each aggregate class and x_i is the mean diameter of each aggregate size class (mm).

3.2.5. Soil organic matter fractionation

Fractionation of SOM into particulate organic matter (POM) and mineral-associated organic matter (MaOM) in the aggregates of varied sizes was made. POM was defined as the SOM of size greater than 53 μm and MaOM as the SOM of size lower than 53 μm , and were determined by physical dispersion of the soil samples using a calibrated ultrasonic probe-type with an output energy of 250 J ml^{-1} , which was determined to be enough to completely disperse all the samples and not enough to significantly redistribute organic matter (Schmidt et al. 1999). Briefly, 30 g of each aggregate-size class (except

s+c particles) were added to 150 mL of distilled water and allowed to stand for 12 h prior to ultrasonic dispersion. Afterwards, aggregate dispersion was carried out with an Analog 450 probe-type sonicator (Branson Sonifier, Branson Sonic Power Co., Danbury, Conn.,) with a flat tip of 125 mm diameter immersed 15 mm into the suspension. The suspension was continuously stirred to facilitate the ultrasonic effect and cooled in a water bath to avoid temperature raising and thus adverse effects on SOM (Kaiser and Asefaw Berhe 2014). The dispersed suspension was then wet-sieved over the corresponding sieves -i.e. depending on the aggregate-class that came from-, until the rinsing water was clear. Organic material >2 mm floating on the water was removed from large macroaggregates as it does not belong to POM (Six et al. 1998) and discarded from the study since it was not present in all the samples (data not shown). The mixture of sand and POM, and the MaOM were dried in an oven dried at 55 °C for 48 h and C and N content were estimated with a LECO CNS 2000 analyser (LECO Corporation, St. Joseph, MI, USA) after the mixture was ground to pass a 250 µm sieve. In order to compare C and N concentration of both POM and MaOM, all values were expressed in a sand-free basis (g·kg⁻¹ sand-free aggregate; Cambardella and Elliott (1992)). Mass recovery after fractionation was on average 97.5±2.6%.

3.2.6. Soil C and N concentration and C stable isotope abundance in aggregate-size classes

Composed samples from every subplot of each land use inside the paired-plot were made. Prior to isotope analyses samples were oven-dried (40 °C) and ground (< 100 µm). Total N, OC concentration and stable isotope composition (δ¹³C) on aggregate-size classes were determined using continuous-flow isotope ratio mass spectrometry in a Costech ECS 4010 Elemental Analyzer (Costech Analytical Technologies, Inc., Valencia, CA, USA) fitted with a zero-blank auto-sampler coupled via a ConFloIII to a ThermoFinnigan DeltaPlus-XL (Thermo Scientific, Waltham, MA, USA). Isotopic compositions were expressed in δ notation, representing the ‰ variation from the standard reference material:

$$\delta (\text{‰}) = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \cdot 1000$$

where R is the ratio ¹³C/¹²C. The standards used were Vienna Pee Dee Belemnite. Precision (in terms of standard deviation) of internal standards for elemental C was ± 0.3 ‰.

3.2.7. Enzyme activity analysis

The potential extracellular activities of two important enzymes involved in soil C cycling through the microbial degradation of both polysaccharides, cellulose (β -glucosidase) and chitin (*N*-acetyl- β -glucosaminidase), were analysed. The enzyme β -glucosidase was measured based on the procedure from Hoffmann and Dedeken (1964) modified by Strobl and Traunmüller (1996). Briefly, 5 g of sieved (2 mm) bulk soil at 60% of field capacity were incubated at 37 °C with 5 ml of β -glucosido-saligenin used as substrate and 10 ml of 2 M acetate buffer (pH de 6.2) for three hours. Afterwards, samples were filtered through Whatman filter paper (no. 42) and their absorbance at 578 nm were measured in a spectrophotometer (Shimadzu UV-1203) and β -glucosidase activity expressed as $\mu\text{g saligenin g}^{-1} \text{ dw soil } 3 \text{ h}^{-1}$.

The enzyme *N*-acetyl- β -glucosaminidase (NAGase) was measured based on the method proposed by Parham and Deng (2000). Briefly, 1 g of sieved (2 mm) bulk soil at 60% of filled capacity was incubated at 37 °C with 1 ml of 10 mM of *p*-nitrophenyl-*N*-acetyl- β -*D*-glucosaminide solution and 4 ml of 0.1 M acetate buffer for one hour. After the incubation, 1 ml of 0.5 M CaCl_2 and 4 ml of 0.5 M NaOH were added to stop the reaction and samples were filtered through Whatman filter paper (no. 42) after being mixed. The NAGase activity was determined as the colour intensity of the filtrate, measured with a spectrophotometer (Shimadzu UV-1203) at 405 nm and expressed as $\mu\text{g saligenin g}^{-1} \text{ dw soil h}^{-1}$.

3.2.8. Phospholipid fatty acids analysis and soil microbial biomass

Phospholipid fatty acids (PLFAs) from sieved (2 mm) bulk soil samples were measured by a modified procedure from Frostegård et al. (1991) described in Koranda et al. (2011). Briefly, fresh soil samples were extracted with a mixture of methanol:chloroform:citrate buffer mixture (2:1:0.8, v/v/v), afterwards neutral- and glycolipids were removed making use of silica acid columns ISOLUTE ® SI Column (Biotage AB, Uppsala, Sweden) and PLFA were diluted in methanol. After the extract was evaporated to dryness, PLFA were subjected to mild alkaline methanolysis. Dried fatty acid methyl esters were re-dissolved in isooctane and concentrations were determined by a 6890N gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a split splitless inlet on a HP-5MS capillary column (50 m×0.20 mm×0.33 μm) and detected with a flame ionisation detector. A mixture of bacterial acid

methyl esters (BAME mix, Supelco, Vienna, Austria) was used as qualitative standard and concentrations of single fatty acids methyl esters were calculated using the internal standard (19:0) peak as a reference (19:0, Sigma-Aldrich, USA). Standard nomenclature was used to designate PLFAs, i.e. A:B ω C, where A denotes the number of atoms of C on the fatty acid chain, B denotes the number of points of double bonds, and C denotes the position of the first double bond starting at the aliphatic (or omega, ω) end of the molecule. The different PLFAs were classified by functional groups (Table 3.2).

Table 3.2 Functional groups of different PLFAs found in the soil samples.

Functional group	PLFA group	Specific PLFA markers	Reference
<i>Fungal groups</i>			
Saprophytic fungi	Polyunsaturated PLFAs	18:3 ω 6 ^a , 18:2 ω 9,12 ^{b,c}	Potthoff et al. (2006) ^a Ratledge and Wilkinson (1988) ^b Yu et al. (2009) ^c
Arbuscular Mycorrhizae Fungi (AMF)		16:1 ω 5 ^c	Olsson et al. (1995, 1999) ^c
<i>Bacterial groups</i>			
Gram-positive (Gram +) bacteria	saturated PLFAs	i14:0 ^d , i15:0 ^d , a15:0 ^d , i16:0 ^d , a16:0 ^d , i17:0 ^d , a17:0 ^d , 17:0 10Me ^d	Bach et al. (2010) ^d
Gram-negative (Gram –) bacteria	cyclopropyl and monounsaturated PLFAs	16:1 ω 9 ^f , 16:1 ω 11 ^d , 17:1 ω 9 ^f , 18:1 ω 11 ^f , cy18:0 ^g , cy19:0 ω 9 ^h , cy17:0(9/10) ^h	Bach et al. (2010) ^d Schindlbacher et al. (2011) ^f Koranda et al. (2013) ^g Dennis et al. (2013) ^h Wild et al. (2014) ⁱ
F/B ratio	Multiple groups	Fungi groups /Bacterial groups	
Actinomycetes	10Me-PLFAs	16:0 10Me ^d , 18:0 10Me ^d	Bach et al. (2010) ^d
Protozoan		20:0	Schindlbacher et al. (2011) ^f

3.2.9. Data analysis

Stocks of soil OC or N (Mg ha⁻¹) were calculated with the following equation:

$$Stock_{[OC, N]} = BD \times D \times FE \times [OC, N] \times 10,$$

where BD is the bulk density of the soil (Mg soil·m⁻³), D is the thickness of the soil sample (meters), FE is the fine earth content in mass (Mg FE·Mg soil⁻¹), $[OC, N]$ are the content of OC or N (kg·Mg FE⁻¹), respectively. The stocks of OC and N in every aggregate class were calculated with:

$$Stock_{[OC, N]i} = BD \times D \times FE \times A_i \times [OC, N]_i \times 10,$$

where A_i is the content of the aggregate i in the soil matrix, and $[OC, N]_i$ is the OC or N content of the i fraction.

All statistical analyses and graphical representation were carried out in R software (version 3.2.3, R Core Team (2015)). All data were tested for differences between land use types (SL, SL-FO, FO) making use of generalized linear mixed models (lme4 package, Bates et al. (2015)) with gamma distribution error. For data on bulk soil (SOC, total N, C:N and soil microbial community) land use was used as fixed factor and plot was introduced as random factor to include within-site correlations in the model. Within aggregate-size classes analysis were carried out with also aggregate-size class as fixed factor in the model. Tukey post-hoc multiple comparison test was done (multcomp package, Hothorn et al. (2016)) to achieve for the differences between land use types after generalized linear mixed models. Normality and homogeneity of the variance of the residuals were checked by the Shapiro–Wilk (stats package, R Core Team (2015)) and Levene test (car package, Fox and Weisberg (2011)), respectively. Non-parametric Kruskal-Wallis test (stats package, R Core Team (2015)) was used to assess differences in soil $\delta^{13}\text{C}$ between land uses due to the lack of normality and homogeneity assumptions. Significance level was set up at $p < 0.05$ for all tests, unless noted otherwise in the text.

Redundancy analysis (RDA, vegan package, Oksanen et al. (2017)) was performed on data of microbial functional groups concentration ($\text{nmol g}^{-1} \text{ dw soil}$) and specific variables of enzyme activity and aggregate properties for determining the relationships between soil microbial community and environmental conditions. Preliminary to the RDA analysis, a detrended correspondence analysis was done to check that the gradient length was lower than 4 standard deviation units; thus, linear responses are expected and RDA is suited for being used (Lepš and Šmilauer 2003). Due to the difference in magnitude and units, species and environmental data were centred and scaled to unit variance. Forward selection procedure, based on a Monte Carlo permutation with 1000 iterations, was used to determine the most significant discriminating variables on the composition of individual PLFAs, and the significant variables ($p < 0.05$) were used in the final analyses. All results are expressed as mean ± 1 standard deviation.

3.3. Results

3.3.1. OC and N concentrations and stocks

Topsoil OC concentration ranged from 35.8 to 40.4 mg g^{-1} (Table 3.3) with no differences between the land use types. Total N concentration in topsoil ranged from 2.1

to 3.1 mg g^{-1} with greater concentrations in SL than in the FO (Table 3.3). The C:N ratio in the topsoil showed the following gradient: FO > SL-FO > SL (Table 3.3).

Topsoil C stocks did not differ between the land use types (Table 3.3) (12.4 to 8.4 Mg C ha^{-1}). Significant higher topsoil N stock was found under the SL vegetation ($0.9 \pm 0.2 \text{ Mg N ha}^{-1}$) compared to the SL-FO ($0.6 \pm 0.1 \text{ Mg N ha}^{-1}$) and FO ($0.5 \pm 0.2 \text{ Mg N ha}^{-1}$).

Table 3.3 Soil organic carbon (SOC) and total nitrogen (N) concentrations and stocks and C:N ratio in the topsoil (0-5 cm) of the investigated land use types. SL, Shrubland; SL-FO, Shrubland-Forest; FO, Forest.

Land use	SOC (g kg^{-1})	Total N (g kg^{-1})	C:N ratio	SOC stock (Mg ha^{-1})	Total N stock (Mg ha^{-1})
SL	40.4 ± 9.9	3.1 ± 0.6 a	13.1 ± 0.6 c	12.4 ± 3.1	0.9 ± 0.2 a
SL-FO	36.0 ± 4.5	2.5 ± 0.3 ab	14.3 ± 0.8 b	8.4 ± 1.5	0.6 ± 0.1 b
FO	35.8 ± 7.3	2.1 ± 0.4 b	17.2 ± 1.4 a	8.8 ± 3.3	0.5 ± 0.2 b

3.3.2. Aggregate-size class distribution

Small macroaggregates represented > 50 % of the soil mass; Mi represented about 20 %, while LMa and silt+clay fractions represented 10 and 5 % of the soil mass, respectively (Figure 3.1).

The mass of LMa was significantly higher in FO soils than in soils of the other two soils. Forest and SL-FO soils presented significantly larger amounts of SMA than SL soil. Forest soil had lower mass of Mi ($p < 0.01$) than SL-FO or SL soils. .

Forest soils showed significantly higher MWD ($1.34 \pm 0.11 \text{ mm}$) than the soils under the rest of uses ($1.20 \pm 0.07 \text{ mm}$ and $1.15 \pm 0.10 \text{ mm}$ for SL and SL-FO soils, respectively). Furthermore, FO soils also showed significantly greater MGD ($0.74 \pm 0.08 \text{ mm}$) than soils from SL ($0.61 \pm 0.06 \text{ mm}$) and SL-FO ($0.61 \pm 0.05 \text{ mm}$).

Figure 3.1 Mass distribution ($\text{g } 100 \text{ g}^{-1}$ soil) among the different aggregate-size classes. Different lowercase letters denote significant differences ($p < 0.05$) among land uses at each aggregate-size class and capital letters show significant differences ($p < 0.05$) among different aggregate-size classes in each land use. Bars represent mean values $\pm$ standard deviation. LMa=large macroaggregates; SMa=small macroaggregates; Mi=microaggregates; s+c=silt and clay size particles.

3.3.3. Aggregate-associated organic carbon, nitrogen, C:N ratio and $\delta^{13}\text{C}$

Regardless of the land use type, the OC and N concentrations associated to the aggregate-size classes followed a common pattern: $s+c > \text{Mi} > \text{SMa} > \text{LMa}$ (Figure 3.2a, 2b). Forest soil had several-fold higher OC concentration in LMa than SL and SL-FO soils (Figure 3.2a). Forest and SL-FO soils had lower OC concentration associated to s+c class than SL soil (Figure 3.2a).

Aggregate-associated N concentration was significantly different between land uses in the Mi and s+c classes ($p < 0.01$). Both N concentrations associated to Mi and s+c classes in FO soil were significantly lower than in SL soil (Figure 3.2b). In addition, N concentration associated to s+c class in SL-FO soil was significantly different from SL but also from FO soil (Figure 3.2b).

Figure 3.2 Concentration (g·kg⁻¹) of a) associated organic carbon (OC), b) associated nitrogen (N), as well as c) the C:N ratio among the different aggregate-size classes in the ecotone studied. Different lowercase letters denote significant differences ($p < 0.05$) among land uses at each aggregate-size class and capital letters show significant differences ($p < 0.05$) among different aggregate classes in each land use. Bars represent mean values $\pm$ standard deviation. LMa=large macroaggregates; SMa=small macroaggregates; Mi=microaggregates; s+c=silt and clay size particles.

Aggregate-associated C:N ratio was significantly different across land uses in all the aggregate-size class (Figure 3.2c) and FO soils presented the highest values. In FO soils

a decreasing tendency on aggregate-associated C:N ratio was found according to the decreasing aggregate-size classes ($p < 0.001$).

Neither C nor N concentrations in POM (POM-C and POM-N, respectively) showed significant differences across land use types (Figure 3.3a and c). However, both the POM-C and POM-N concentrations in SMa were significantly higher than in LMa and in Mi in all land use types.

Figure 3.3 Concentration (g substance·kg⁻¹ sand-free aggregate) of a) particulate organic carbon (POM-C), b) mineral associated organic carbon (MaOM-C), c) particulate organic nitrogen (POM-N), and d) mineral associated organic nitrogen (MaOM-N), as well as carbon to nitrogen ratios in e) particulate organic matter (POM-C:N) and e) mineral associated organic matter (MaOM-C:N) across the different aggregate-size class in the ecotone studied. Mean values $\pm$ standard deviation are shown. Different lowercase letters denote significant differences ($p < 0.05$) among land uses at each aggregate-size class and capital letters show significant differences ($p < 0.05$) among different aggregate-size classes in each land use type. LMa, large macroaggregates; SMa, small macroaggregates; Mi, microaggregates; s+c, silt and clay size particles.

Carbon and N concentrations in MaOM (MaOM-C and MaOM-N, respectively) showed significant differences between land use types in LMa, thereby a significant gradient appeared from soils under FO cover to soils under SL cover (Figure 3.3b and d). Furthermore, in Mi classes the MaOM-N showed higher concentration in soils under SL and SL-FO than under FO soil (Figure 3.3d). Contrary to POM, both the MaOM-C and MaOM-N significantly decreased with the increasing aggregate-size class (Figure 3.3b).

Overall in the aggregate-size classes, the C:N ratios between C and N concentration in POM and MaOM were greater in soils under FO cover than in soils under SL or SL-FO (Figure 3.3e and f), except for the POM-C:N in LMa that was not significant.

The same patterns of topsoil OC and N stocks were found across land uses and aggregated-size classes (Figure 3.4a and b). The OC and N stocks associated to LMa were significantly greater in soils under FO than in soils under SL or under SL-FO, while OC and N stocks associated to Mi and s+c were significantly greater in soils under SL vegetation than in soils under SL-FO or FO (Figure 3.4a and b). Regarding the aggregate-size classes, both OC and N stocks were greater in Mi than in LMa, whereas SMa and s+c had intermediate values.

Figure 3.4 Topsoil stocks (Mg·ha⁻¹) of a) organic carbon (OC) and b) nitrogen (N) among the different aggregate-size class in the ecotone studied. Bars represent mean values $\pm$ standard deviation. Mean values $\pm$ standard deviation is shown. Different lowercase letters denote significant differences ($p < 0.05$) among land uses at each aggregate-size class and capital letters show significant differences ($p < 0.05$) among different aggregate-size classes in each land use type. LMa, large macroaggregates; SMa, small macroaggregates; Mi, microaggregates; s+c, silt and clay size particles.

The $\delta^{13}\text{C}$ content in the different aggregate-size class ranged from -25.9 ± 0.2 to -24.9 ± 0.1 ‰ (Table 3.4). $\delta^{13}\text{C}$ content in both Mi and s+c classes presented significant

differences with greater isotopic content from SL to FO with SL-FO in between (Table 3.4).

Table 3.4 Contents of $\delta^{13}\text{C}$ (‰) among the different aggregate-size class in the ecotone studied. Values are means $\pm$ standard deviation. Different letters denote significant differences ($p < 0.05$) among land uses. SL, Shrubland; SL-FO, Shrubland-Forest; FO, Forest; LMa, large macroaggregates; SMa, small macroaggregates; Mi, microaggregates; s+c, silt and clay size particles.

Land use	Aggregate class (mm)					
	LMa	SMa	Mi	s+c		
SL	n.d.	-25.1 $\pm$ 0.2	-24.9 $\pm$ 0.0	a	-24.9 $\pm$ 0.1	a
SL-FO	n.d.	-25.5 $\pm$ 0.4	-25.2 $\pm$ 0.1	ab	-25.3 $\pm$ 0.1	ab
FO	-25.6 $\pm$ 0.3	-25.7 $\pm$ 0.4	-25.9 $\pm$ 0.2	b	-25.8 $\pm$ 0.2	b

n.d., non-determined value due to the absence of a reliable value

3.3.4. Soil microbial community structure and enzyme activities

Gram + bacteria were significantly ($p < 0.01$) more abundant in SL soils. Shrubland-Forest had greater concentrations than FO soils (Table 3.5). Protozoan abundance was significantly greater in FO than in the other two land uses ($p < 0.001$; Table 3.5). Total bacteria presented only significant differences between SL and FO soil. Total soil PLFAs content under SL were significantly greater than in those under SL-FO and FO (Table 3.5).

Enzyme activity of β -glucosidase was significantly greater in SL than in FO soils. However, no differences were found in NAGase activity between soil land uses (Table 3.5).

The RDA ordination analysis explained more than 60% of the variance in the relationship between aggregate properties and β -glucosidase activity and the concentration of microbial functional groups in the different ecotone soils. The first two constrained axes were highly significant ($p < 0.001$) and accounted for more than 55% of the total variance (34.8% and 21.7% for axis 1 and 2, respectively). Gram + bacteria were positively related to MaOM-N concentration in both SMa and Mi aggregates, OC in the s+c aggregate-size class and the activity of β -glucosidase but clearly opposed to the POM-C concentration in the LMa aggregates. Gram – bacteria were positively associated to greater content of POM-C in LMa (Figure 3.5a). Forest soil were associated with the abundance of protozoa community. RDA showed that FO sites had greater MWD and C concentration in the OM of the LMa aggregates. Shrubland sites were associated to

greater SOC concentration, MaOM-N concentration in both SMa and Mi size classes and greater β -glucosidase activity (Figure 3.5b).

Figure 3.5 Redundancy analysis (RDA) ordination biplot of a) the concentration of soil microbial community types and both soil aggregate variables and β -glucosidase activity; b) the distribution of the sites and both soil aggregate variables and β -glucosidase activity. Sap. Fungi, saprophytic fungi; Gram +, Gram-positive bacteria; Gram –, Gram-negative bacteria; AMF, arbuscular mycorrhiza fungi; SOC, soil organic carbon.

Table 3.5 Concentration ($\text{nmol} \cdot \text{g}^{-1}$ soil dw) of total PLFAs, gram-positive bacteria (Gram +), gram-negative bacteria (Gram –), arbuscular mycorrhiza (AMF), saprophytic fungi (Saprophytic), protozoan, fungal/bacteria ratio (F:B) and β -glucosidase (μg saligenin g^{-1} dw soil 3 h^{-1}) and NAGase activity (μg saligenin g^{-1} dw soil h^{-1}). Values are means $\pm$ standard deviation. Different letters denote significant differences ($p < 0.05$) among land uses.

Land use	Total PLFA	Total bacteria	Gram +	Gram –	AMF	Saprophytic fungi	Actinomycetes	Protozoan	F:B	β -glucosidase	NAGase			
SL	389.3±69.1a	269.2±47.3a	220.0±44.8a	50.4±13.1	2.5±0.9	9.6±2.1	17.9±7.0	3.1±1.1	b	0.04±0.01	b	298.2±30.1	a	552.5±212.9
SL-FO	303.0±35.3b	180.4±15.6ab	133.8±16.1b	46.7±10.1	2.2±0.7	9.0±2.6	14.9±3.3	4.0±0.5	b	0.05±0.02	ab	258.0±35.9	ab	587.7±182.3
FO	283.3±57.5b	140.8±30.5b	80.7±22.0 c	60.2±13.4	2.6±0.9	9.7±3.4	16.0±3.7	7.2±1.3	a	0.07±0.02	a	222.0±41.2	b	613.8±190.6

3.4. Discussion

Previous studies have shown that in forest soil, most OC is occluded with macroaggregates (Caravaca et al. 2004; Wei et al. 2013a) resulting in a physical protection of this from microbial attack and mineralization (Wu et al. 2012). As we hypothesize, the forest use showed an increase in both large macroaggregates and small macroaggregates amounts that led to greater MWD comparing to the SL soil use. In this sense, our finding is in accordance with the results from Guidi et al. (2014) who found a positive relationship between the forest land use and the aggregate-size in a grassland-forest succession stage in European Alps. The change in soil structure under forest use was also accompanied by an increase in OC concentrations associated to large macroaggregates and higher soil C:N ratio, probably due to an increase in the litter inputs to the soil (Archer et al. 2001) and the fact that litter from coniferous species has a wide C:N ratio and a chemically complex structure (Berg 2000; Pérez-Cruzado et al. 2014). This reinforce the idea previously reported that differences in annual organic matter inputs (Gartzia-Bengoetxea et al. 2009) or litter quality (Gracia-Franco et al., 2015) may contribute to a different aggregates stabilization.

Opposite to our expectations, we found that aggregate-associated OC and N concentrations did not increase with aggregate-size in any of the studied uses and, on the contrary, the highest concentrations were within the smallest aggregate-size classes. This finding does not match with the aggregate hierarchy concept in which microaggregates are bound together into macroaggregates by transient and temporary binding agents (Tisdall and Oades 1982). Although this aggregate hierarchy, as well as the important role of organic matter in soil aggregation has usually been reported for temperate soils, no difference between aggregate-associated OC concentrations was found for mature forests by Gartzia-Bengoetxea et al. (2009) either. These authors concluded, in a similar way as Oades (1984), that aggregate formation was probably directly related to particulate organic matter (POM) in the studied soil forests. Thus, new microaggregates are formed around POM inside macroaggregates (Gale et al. 2000) when these pieces of OM become colonized by microorganisms and encrusted by mineral materials (Oades 1984). The decrease in the aggregate-associated C:N ratio with the decrease in aggregate-size classes found in forest soils may indicate that forest soils do meet more markedly the aggregate hierarchy (Oades 1984; Elliott 1986). Small macroaggregates were the most abundant

aggregate-size class in all the land uses studied but also where highest concentration of POM-C was found, reinforcing the importance of POM in the macroaggregate formation (Six et al. 1998; Pikul et al. 2007; Gartzia-Bengoetxea et al. 2009; Meyer et al. 2012). This finding also points out the key role that small macroaggregates plays in the protection of newly SOM in the soils studied as indicated by the significantly greater amount of POM in this aggregate-size class. Though no differences were found in SOC stocks in bulk soils in the different uses, microaggregate fraction showed significant higher stocks of OC comparing with the rest of the size fractions because of high OC concentrations and specifically the higher concentrations of mineral associated OC, as smaller particles have higher surface for the stabilization of OM.

An increased vegetation cover type could sustain a higher microbial activity due to differences in root exudates release (Renella et al. 2007). Indeed, soils, like natural habitats, have a maximum carrying capacity which results from the interaction between the soil structure and aggregates, nutrient accessibility (Jones and Griffiths 1967) and ecological interactions between plants and microbes. The effect of land use on diverse groups of PLFA markers was driven by a lower PLFA abundance in forest soils. This fact may be the result of a higher physical protection from microbial attack of occluded OC in forest macroaggregates, as forest soils showed an increase in macroaggregates and in large macroaggregate-associated OC concentrations against shrubland soils. We did find distinct groups of bacteria dominating different soil uses. Specifically, gram-positive bacteria were more abundant than gram-negative bacteria in shrubland soil compared to the Forest soil. These differences are consistent with general understanding of gram-positive and gram-negative substrate preference and the higher C:N ratio observed in Forest soil. Gram-positive bacteria preferentially use old and microbial-processed SOM, whereas gram-negative bacteria are thought to favor fresher plant inputs as C source (Fierer et al. 2003; Potthast et al. 2012; Smith et al. 2014a). Our results support this fact since gram-positive bacteria were related with greater contents of more evolved C and N forms -i.e. OC associated to microaggregates and MaOM-N in both small macroaggregates and microaggregates-, but also suggest that shrubland soil is depleted in easily-decomposable substrate. In this sense, POM-C, generally considered a labile C form (Cambardella and Elliott 1992; Gregorich et al. 2006), was not different between land uses but the relationship found in the redundancy analysis between forest sites and

the POM-C concentration in large macroaggregates indicates that soils under forest vegetation receive greater freshly and less decomposed OM inputs. On the other, shrubland showed the higher OC stock within the smallest fractions, if microaggregates are formed around POM inside macroaggregates in this use, the OM would be more microbial-processed and as a consequence, preferred by gram-positive bacteria as it was mentioned above.

β -glucosidase activity is involved in C cycling as it catalyses the last step of cellulose degradation that produces glucose, which is an important energy source in soil (Sotomayor-Ramírez et al. 2009). The higher activity of β -glucosidase in shrubland soil compared with forest soil agrees with the higher total PLFAs, which indicate a higher capacity of the soil microorganisms to metabolize carbohydrates in this soil. The natural abundance of stable isotopes has been used to estimate the extent to which SOM dynamics are affected by different land uses (Michener and Lajtha 2007; Ortiz et al. 2016). The $\delta^{13}\text{C}$ results under different land uses in the smallest sizes fractions seem to agree with this higher capacity to metabolize carbohydrates in the shrubland soil against the forest soil, thus higher $\delta^{13}\text{C}$ values mean more evolved organic matter.

The significantly higher F/B ratio observed in forest soil than in shrubland one indicates a higher dominance of fungi compared to bacteria in this soil use. Fungal-dominated communities have implications for SOC cycling and its storage as fungal biomass, residues and secondary metabolites have been demonstrated to have slower rates of decomposition relative to bacterial biomass and residues (Bailey et al. 2002; Six et al. 2006; Smith et al. 2014a). Moreover, fungi play an important role in soil aggregation since the extracellular polysaccharide that their mycelium produces acts as cement between soil particles (Oades and Waters 1991). An increase in F/B ratio is therefore expected to lead to a greater soil C aggregation and, eventually, to higher C accumulation. This fact is observed in the accumulation of OC in LMa in forest soil, agreeing with the higher C:N ratio in this soil, probably due to the higher C:N ratio of the inputs but also because fungi have a higher C:N ratio than bacteria. These results are also consistent with the finding that forest had the highest values of macroaggregates. The aggregate-size class and their pores associated play determinant role in the links between organisms in a soil food web (Elliott and Coleman 1988). In this sense, it is well established that fungi predominate in macroaggregates, while bacteria predominate in microaggregate because

fungi's hyphae could not penetrate small pores of soil microaggregates (De Gryze et al. 2005). Accordingly, the greater abundance of protozoa found in forest soils could also be related with the lower bacteria abundance due to their predator activity (Elliott et al. 1980).

3.5. Conclusions

Regardless of the land use studied, small macroaggregates and microaggregates were the most abundant and comprised around 50% and 20% of the soil mass, respectively. Besides, C concentration in Mi was higher in all the uses studied. Afforestation resulted in an increase of macroaggregates content that led to greater mean weight diameter comparing to the shrubland soil use. It appears that small macroaggregates (2-0.250 mm) may play an important role in aggregate dynamics in all the studied soils and because a higher concentration of POM-C was found in this fractions size, an effort should be considered to conserve this pool in order to maintain long-term functioning of the ecosystems. On the other hand, although no differences were found in SOC stocks in bulk soils, microaggregate fraction showed significant higher stocks of OC comparing with the rest of the size fractions. This last fact was more marked in shrubland soil, where the higher OC stock in microaggregates comparing with the rest of uses, suggests that this soil is depleted in easily-decomposable substrate but at the same time soil microorganisms have a higher capacity to metabolize carbohydrates as it was indicated by the higher β -glucosidase activity. Both findings should be considered for further forest advance since together with the greater fungi:bacteria ratio found in forest soils, clear changes in soil microbial community and aggregate size may be expected if afforestation continues.

CONCLUSIONS

Afforestation of former grassland areas with Scots pine in Mediterranean conditions have been found to lead to a greater soil C:N ratio, suggesting changes in the SOM quality of the topsoil. This result, however, was not followed by different potential mineralisation as it would be expected after afforestation. In contrast to our expectation, afforestation with Scots pine forest negligible affects total SOC concentrations, concluding that afforestation of Mediterranean mountain grasslands does not enhance C sequestration in the topsoil mineral soil, at least the first 40 years after the land use change. Nevertheless, natural abundance of isotopic C was markedly influenced by afforestation of Mediterranean mountains, the higher $\delta^{13}\text{C}$ in forest SOC is most probable the result of an increase in processed litter inputs from forest floor. As a remarkable finding, increasing altitude likely stimulated biogeochemical processes in the Mediterranean sites due to the alleviation of water constraints. However, different response was found with higher SOC mineralisation in forests at low altitudes and in pastures at high altitudes. Therefore, mechanisms of C replacement and stabilisation after afforestation may vary with climate conditions (as defined by altitude) with the watershed point located 1900 m a.s.l.

The long-term experiment of R_s measure from Scots pine forests allowed us to clearly identify soil moisture as the most important abiotic feature regulating R_s in Mediterranean forests. The results showed that, in contraposition to temperate forests, R_s was not continuously explained by soil temperature. This fact mainly responds to the soil moisture limitations that Mediterranean forests suffer during the summer and early autumn. Thus, a soil moisture threshold of 12% was found which identified two scenarios of R_s response: below the threshold, R_s was dependent of soil moisture; and above the threshold, R_s dependency on soil temperature was proved. Moderate forest management of Valsaín resulted in greater R_s in both mature and young stand-ages comparing with the more intense management of Navafría. This is the result of the fact that moderate management was associated to the higher tree density and the greater soil C stocks in both forest floor and topsoil 10 cm. Stand age had slight effects on R_s but its interaction with forest management modulated the response of R_s to soil temperature and soil moisture. The model fitted by the interaction of soil moisture and soil temperature along the different stand ages and forest management intensities supposes a valuable tool for accurately assessing the R_s in these Mediterranean Scots pine forests.

Afforestation of former grassland areas with Scots pine in Mediterranean conditions did not result in changes of the aggregate dynamics. It appears that small macroaggregates (2-0.250 mm) may play a key role in aggregate dynamics in the Mediterranean studied soils and because a higher concentration of POM-C was found in this fraction size, an effort should be considered to conserve this pool in order to maintain long-term functioning of the ecosystems. On the other hand, although no differences were found in SOC stocks in bulk soils, microaggregate fraction showed significant higher stocks of OC comparing with the rest of the size fractions. Soils under shrubland vegetation presented higher SOC stock in microaggregate fraction compared with forest soils. This fact is the result of a higher concentration of soil microorganisms (as stated by the greater total PLFA found in this soils) which in addition showed a higher capacity to metabolize carbohydrates as it was indicated by the higher β -glucosidase activity. Thus, together with the greater fungi:bacteria ratio found in forest soils, clear changes in soil microbial community may be expected if afforestation continues.

Finally, it can be concluded that afforestation of former grasslands with Scots pines will not imply great changes in terms of C dynamics but still reduction of SOC mineralization with altitude, increase of SOC protected by large macroaggregates and a higher ratio of fungi:bacteria may be expected. Furthermore, it has been seen that a moderate intensity of forest management increases R_s in the early and late stage of the Scots pine rotation period but also increases the soil C stocks up to 10 cm depth (including forest floor) which supposes an important finding in the actual global change scenario. Regarding microclimatic variables, soil moisture was found a key feature in the future scenarios of climate change since not only drives R_s during part of the year but also because of the limited soil temperature dependence of R_s . All these findings are important to take into account for forest managers when reviewing forest plans.

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ANNEXES

Chapter 1.1:

Table 1.1 Summary of pH, soil texture, soil organic carbon (SOC) concentration, soil nitrogen (TN) concentration and soil and plant C:N ratio of the investigated paired-plots in the Sistema Central sites in Spain (ES) and in the Central Alps sites in Austria (AT). Data refers to mean $\pm$ standard deviation

SITE	pH		Soil texture*	SOC (g kg ⁻¹ dry weight)				TN (g kg ⁻¹ dry weight)				Soil C:N ratio				Plant C:N ratio			
	Afforested	Grassland		Afforested		Grassland		Afforested		Grassland		Afforested		Grassland		Afforested		Grassland	
ES1	4.5 $\pm$ 0.2	4.9 $\pm$ 0.0	sa l	55 $\pm$ 14	a	91 $\pm$ 12	b	4.1 $\pm$ 0.9	a	7.8 $\pm$ 0.9	b	13.3 $\pm$ 0.6	b	11.6 $\pm$ 0.3	a	24.2 $\pm$ 0.7	a	37.1 $\pm$ 6.2	b
ES2	4.4 $\pm$ 0.2	4.7 $\pm$ 0.1	sa l	102 $\pm$ 40		70 $\pm$ 22		5.2 $\pm$ 1.6		5.2 $\pm$ 1.4		19.0 $\pm$ 1.9	b	13.4 $\pm$ 0.4	a	29.9 $\pm$ 2.2	a	44.5 $\pm$ 9.6	b
ES3	4.0 $\pm$ 0.3	4.3 $\pm$ 0.2	sa l	128 $\pm$ 4		103 $\pm$ 16		9.6 $\pm$ 0.5		7.8 $\pm$ 1.6		13.4 $\pm$ 0.5		13.2 $\pm$ 0.5		33.5 $\pm$ 2.3	a	55.7 $\pm$ 6.1	b
ES4	4.7 $\pm$ 0.1	4.9 $\pm$ 0.0	sa l	71 $\pm$ 15		71 $\pm$ 15		4.7 $\pm$ 1.0		5.1 $\pm$ 1.0		15.1 $\pm$ 0.2		13.8 $\pm$ 1.1		34.9 $\pm$ 2.8	a	47.1 $\pm$ 2.8	b
ES5	4.7 $\pm$ 0.1	5.0 $\pm$ 0.2	sa l	80 $\pm$ 24		107 $\pm$ 18		5.0 $\pm$ 2.0		8.1 $\pm$ 1.4		16.6 $\pm$ 2.7	b	13.2 $\pm$ 0.8	a	35.6 $\pm$ 2.5		35.4 $\pm$ 2.8	
ES6	4.3 $\pm$ 0.1	4.8 $\pm$ 0.0	sa l	90 $\pm$ 11		73 $\pm$ 22		4.9 $\pm$ 1.2		6.1 $\pm$ 1.9		18.9 $\pm$ 2.9	b	12.0 $\pm$ 0.1	a	29.6 $\pm$ 2.9		29.8 $\pm$ 1.0	
ES7	4.4 $\pm$ 0.4	5.4 $\pm$ 0.1	l sa	87 $\pm$ 13	b	61 $\pm$ 7	a	5.7 $\pm$ 0.4		5.7 $\pm$ 0.6		15.3 $\pm$ 1.1	b	10.7 $\pm$ 0.2	a	28.9 $\pm$ 2.3	a	46.3 $\pm$ 11.0	b
ES8	5.7 $\pm$ 0.1	5.8 $\pm$ 0.1	sa l	86 $\pm$ 4		49 $\pm$ 35		4.9 $\pm$ 0.2		3.5 $\pm$ 2.2		17.8 $\pm$ 0.1	b	13.5 $\pm$ 1.4	a	37.4 $\pm$ 2.3		51.0 $\pm$ 21.7	
ES9	5.0 $\pm$ 0.1	5.2 $\pm$ 0.1	l sa	28 $\pm$ 9		28 $\pm$ 6		0.9 $\pm$ 0.2	a	2.0 $\pm$ 0.5	b	31.3 $\pm$ 4.8	b	14.2 $\pm$ 0.9	a	38.7 $\pm$ 1.5	a	59.0 $\pm$ 9.6	b
ES10	5.0 $\pm$ 0.2	5.0 $\pm$ 0.2	sa l	29 $\pm$ 16	a	95 $\pm$ 9	b	1.5 $\pm$ 1.2	a	6.5 $\pm$ 0.7	b	21.4 $\pm$ 6.9		14.7 $\pm$ 0.3		34.2 $\pm$ 8.7		35.4 $\pm$ 3.7	
AT1	3.4 $\pm$ 0.4	3.5 $\pm$ 0.1	sa l	99 $\pm$ 40		117 $\pm$ 19		4.8 $\pm$ 1.7		6.9 $\pm$ 1.1		20.8 $\pm$ 1.6	b	16.9 $\pm$ 0.9	a	26.1 $\pm$ 0.0		38.3 $\pm$ 0.0	
AT2	4.7 $\pm$ 0.5	4.4 $\pm$ 0.2	n.d.	75 $\pm$ 14		74 $\pm$ 14		4.9 $\pm$ 1.3		6.4 $\pm$ 0.8		15.6 $\pm$ 1.8	b	11.6 $\pm$ 1.0	a	24.4 $\pm$ 0.0		24.4 $\pm$ 0.0	
AT3	6.0 $\pm$ 0.9	6.4 $\pm$ 0.5	n.d.	151 $\pm$ 11	b	104 $\pm$ 18	a	8.5 $\pm$ 1.2		8.3 $\pm$ 0.7		17.9 $\pm$ 1.8	b	12.5 $\pm$ 1.0	a	33.0 $\pm$ 0.0		16.2 $\pm$ 0.0	
AT4	4.4 $\pm$ 0.6	4.1 $\pm$ 0.1	n.d.	86 $\pm$ 12	a	125 $\pm$ 10	b	5.2 $\pm$ 0.7		7.3 $\pm$ 0.5		16.6 $\pm$ 0.3		17.2 $\pm$ 0.4		33.3 $\pm$ 0.0		36.3 $\pm$ 0.0	
AT5	3.6 $\pm$ 0.3	4.3 $\pm$ 0.2	n.d.	153 $\pm$ 56	b	50 $\pm$ 9	a	5.9 $\pm$ 2.5		2.9 $\pm$ 0.5		26.3 $\pm$ 1.8	b	17.4 $\pm$ 0.5	a	26.3 $\pm$ 0.0		43.0 $\pm$ 0.0	
AT6	3.4 $\pm$ 0.3	4.1 $\pm$ 0.7	n.d.	137 $\pm$ 68		65 $\pm$ 20		5.3 $\pm$ 3.0		3.0 $\pm$ 1.1		26.6 $\pm$ 2.2		22.2 $\pm$ 1.0		45.9 $\pm$ 0.0		50.2 $\pm$ 0.0	
AT7	4.7 $\pm$ 0.5	5.7 $\pm$ 0.4	n.d.	73 $\pm$ 3	a	91 $\pm$ 4	b	5.6 $\pm$ 0.4	a	6.9 $\pm$ 0.3	b	13.2 $\pm$ 0.7		13.2 $\pm$ 0.2		31.4 $\pm$ 0.0		26.7 $\pm$ 0.0	

* sa l = sandy loam, sa c l = sandy clay loam, l sa = loamy sand, sa c = sandy clay, n.d. = no data available; Different letters indicate significant differences between paired-plots in each site at confidence level $p < 0.05$

Table 1.2 Summary of soil organic carbon (SOC) mineralization efflux rates, cumulative soil CO₂ production and cumulative SOC decomposability of the investigated paired-plots in the Sistema Central sites in Spain (ES) and in the Central Alps sites in Austria (AT). Data refers to mean $\pm$ standard deviation

SITE	SOM mineralization efflux rate (mg CO ₂ -C kg ⁻¹ h ⁻¹)		Cumulative soil CO ₂ production (mg CO ₂ -C kg ⁻¹ dry soil)		Cumulative SOC decomposability (mg CO ₂ -C g ⁻¹ C)	
	Afforested	Grassland	Afforested	Grassland	Afforested	Grassland
ES1	0.07 $\pm$ 0.03	0.08 $\pm$ 0.02	274 $\pm$ 109	352 $\pm$ 92	5.4 $\pm$ 2.7	3.8 $\pm$ 0.9
ES2	0.25 $\pm$ 0.14	0.13 $\pm$ 0.03	1041 $\pm$ 578	533 $\pm$ 148	9.8 $\pm$ 3.5	7.8 $\pm$ 1.4
ES3	0.17 $\pm$ 0.01	0.15 $\pm$ 0.01	690 $\pm$ 3	626 $\pm$ 79	5.4 $\pm$ 0.2	6.1 $\pm$ 0.7
ES4	0.14 $\pm$ 0.05	0.11 $\pm$ 0.02	575 $\pm$ 205	468 $\pm$ 80	8.7 $\pm$ 4.4	7.0 $\pm$ 2.7
ES5	0.14 $\pm$ 0.01	0.13 $\pm$ 0.01	564 $\pm$ 29	560 $\pm$ 55	7.5 $\pm$ 2.4	5.3 $\pm$ 1.0
ES6	0.24 $\pm$ 0.05	0.29 $\pm$ 0.06	982 $\pm$ 223	1174 $\pm$ 202	10.8 $\pm$ 1.5	16.6 $\pm$ 3.1
ES7	0.17 $\pm$ 0.08	0.12 $\pm$ 0.05	673 $\pm$ 356	493 $\pm$ 183	7.5 $\pm$ 3.0	7.9 $\pm$ 2.4
ES8	0.34 $\pm$ 0.02	0.09 $\pm$ 0.01	1348 $\pm$ 146	b 383 $\pm$ 55	a 15.6 $\pm$ 1.2	10.3 $\pm$ 5.6
ES9	0.17 $\pm$ 0.05	0.05 $\pm$ 0.01	650 $\pm$ 239	228 $\pm$ 40	24.4 $\pm$ 8.9	8.2 $\pm$ 1.3
ES10	0.11 $\pm$ 0.03	0.26 $\pm$ 0.05	439 $\pm$ 110	a 1041 $\pm$ 217	b 17.9 $\pm$ 7.3	11.0 $\pm$ 2.1
AT1	0.34 $\pm$ 0.20	0.26 $\pm$ 0.12	1388 $\pm$ 789	1025 $\pm$ 450	13.3 $\pm$ 2.5	8.7 $\pm$ 3.0
AT2	0.40 $\pm$ 0.20	0.24 $\pm$ 0.04	1541 $\pm$ 821	1003 $\pm$ 204	21.6 $\pm$ 12.9	14.1 $\pm$ 4.8
AT3	0.35 $\pm$ 0.04	0.26 $\pm$ 0.12	1413 $\pm$ 203	1080 $\pm$ 540	9.3 $\pm$ 0.8	10.8 $\pm$ 5.8
AT4	0.51 $\pm$ 0.22	0.21 $\pm$ 0.09	2097 $\pm$ 908	849 $\pm$ 345	15.9 $\pm$ 9.5	17.2 $\pm$ 6.5
AT5	0.25 $\pm$ 0.05	0.66 $\pm$ 0.26	1043 $\pm$ 209	2667 $\pm$ 1001	12.5 $\pm$ 3.5	21.6 $\pm$ 8.9
AT6	0.40 $\pm$ 0.27	0.89 $\pm$ 0.55	1717 $\pm$ 1181	3535 $\pm$ 2008	12.7 $\pm$ 6.7	52.1 $\pm$ 15.2
AT7	0.22 $\pm$ 0.07	0.31 $\pm$ 0.13	964 $\pm$ 287	1272 $\pm$ 545	13.1 $\pm$ 3.7	13.9 $\pm$ 6.0

Different letters indicate significant differences between paired-plots in each site at confidence level $p < 0.05$

Table 1.3 Summary of soil carbon isotopic concentration ($\delta^{13}\text{C}$), soil nitrogen isotopic concentration ($\delta^{15}\text{N}$), plant material carbon concentration ($\delta^{13}\text{C}$), isotopic carbon discrimination ($\Delta^{13}\text{C}$) and isotopic nitrogen discrimination ($\Delta^{15}\text{N}$) of the investigated paired-plots in the Sistema Central sites in Spain (ES) and in the Central Alps sites in Austria (AT). Data refers to mean $\pm$ standard deviation

SITE	Soil $\delta^{13}\text{C}$ (‰)			Plant material $\delta^{13}\text{C}$ (‰)		$\Delta^{13}\text{C}$ (‰)		$\Delta_{\text{Af-G}}$ (‰)	Soil $\delta^{15}\text{N}$ (‰)			$\Delta^{15}\text{N}$ (‰)				
	Afforested	Grassland		Afforested	Grassland		Afforested		Grassland		Afforested	Grassland				
ES1	-24.8±0.1	b -25.2±0.2	a	-27.1±1.0	-26.9±0.2	-2.4±1.1	-1.8±0.0	0.4±0.2	4.2±0.4	a 5.9±0.1	b	-5.8±0.3	-4.3±0.8			
ES2	-25.6±0.7	-24.7±0.2		-28.1±0.9	b -26.2±0.2	a	-2.6±1.5	-1.6±0.1	-0.9±0.9	2.8±1.2	3.8±0.4	-4.1±2.3	-5.2±0.5			
ES3	-25.3±0.1	-25.6±0.4		-27.4±0.6	-26.6±0.3		-2.1±0.8	-1.0±0.1	0.3±0.2	3.7±0.3	4.5±0.8	-5.7±0.5	-5.7±0.7			
ES4	-25.2±0.1	-25.3±0.2		-27.1±1.5	-26.9±0.2		-1.9±1.5	-1.7±0.1	0.1±0.3	4.9±0.8	6.0±0.3	-6.1±0.8	-4.6±1.6			
ES5	-25.0±0.2	b -25.8±0.3	a	-28.3±0.7	-27.1±0.2	-3.4±0.9	a -1.4±0.5	b	-0.7±0.4	3.5±0.7	a 7.5±0.4	b	-5.7±0.4	-3.6±1.1		
ES6	-25.9±0.2	b -26.5±0.1	a	-28.9±0.8	b -27.6±0.1	a	-3.2±1.0	-1.1±0.1	0.7±0.2	2.2±0.8	a 6.4±0.4	b	-3.6±0.8	a -0.2±0.5	b	
ES7	-25.9±0.3	-26.5±0.4		-27.6±0.4	-28.3±0.1		-1.7±0.7	-1.8±0.4	0.6±0.6	2.7±0.4	a 4.8±0.9	b	-4.5±0.9	-3.9±2.4		
ES8	-25.7±0.1	b -26.6±0.1	a	-30.1±0.6	b -28.0±0.0	a	-4.5±0.5	a -1.5±0.1	b	0.8±0.3	3.3±0.2	6.3±2.4	-6.2±0.4	-8.1±2.1		
ES9	-25.4±0.0	b -26.2±0.3	a	-26.9±1.1	-27.1±0.9		-1.5±1.1	-0.9±0.8	0.9±0.3	2.2±0.5	3.3±0.6		-4.3±0.4	-2.3±1.0		
ES10	-25.6±0.5	-26.2±0.1		-30.5±0.8	b -28.3±0.2	a	-5.0±1.1	a -2.1±0.3	b	0.7±0.4	3.6±0.5	4.1±0.7	-6.6±1.7	-5.2±0.5		
AT1	-26.8±0.5	-26.3±0.3		-29.5	-27.8		-2.7±0.5	a -1.5±0.3	b	-0.5±0.7	4.2±1.2	4.1±1.1	-3.3±1.2	b -6.3±1.1	a	
AT2	-26.9±0.2	a -26.3±0.3	b	-29.7	-27.8		-2.8±0.2	a -1.5±0.3	b	-0.6±0.4	2.0±0.7	2.5±0.7	-6.4±0.7	-5.0±0.7		
AT3	-25.1±0.5	b -26.2±0.1	a	-27.6	-28.7		-2.6±0.5	-2.5±0.1		1.1±0.5	0.6±0.5	1.4±0.4	-6.3±0.5	a -3.6±0.4	b	
AT4	-27.1±0.2	-27.2±0.3		-30.3	-27.6		-3.6±0.3	a -1.6±0.3	b	0.0±0.4	3.4±0.3	2.7±0.4	-3.3±0.6	b -11.1±0.4	a	
AT5	-26.8±0.2	a -26.0±0.2	b	-30.3	-28.5		-3.3±0.3	a -1.4±0.3	b	-0.7±0.4	2.5±0.6	2.9±0.4	-4.7±0.3	a -3.5±0.4	b	
AT6	-26.9±0.2	-26.5±0.7		-29.4	-29.5		-2.5±0.2	-3.0±0.7		-0.3±0.5	3.4±2.0	2.7±0.6	-8.2±2.0	-9.3±0.6		
AT7	-26.6±0.2	b -27.0±0.0	a	-28.7	-29.4		-2.1±0.2	-2.4±0.1		0.3±0.2	2.7±0.3	b 1.1±0.0	a	-3.5±0.3	b -4.6±0.0	a

Different letters indicate significant differences between paired-plots in each site at confidence level $p < 0.05$