


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

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Abstract We studied the effect of mountain grassland afforestation with conifer trees (*Pinus sylvestris*, *Picea abies* and *Pinus cembra*) on soil organic matter (SOM) cycling and carbon (C) isotopic composition in two contrasting climate areas using a regional approach. Seventeen paired sites (each containing at least 40 years prior afforested and grassland plots) were investigated in the mountains of Central Spain and Western Austria. Topsoil CO₂ effluxes were monitored under standardized conditions for six months as a proxy for soil organic carbon (SOC) mineralisation. The bulk C and nitrogen (N) concentrations and their isotopic composition in the soil and in the plants were assessed. The soil C:N ratio was consistently greater after afforestation in both regions, which in Spain was caused by a significant decrease in N concentration. No consistent effect was

found on mineralisation rates due to vegetation change. Afforestation produced a more consistent soil ¹³C enrichment in the Spanish than in the Austrian sites. Our work strongly suggests that increasing altitude in Mediterranean mountain grasslands alleviates water limitation, favouring 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. In spite of the impact of afforestation on soil biogeochemical processes, C concentrations were marginally affected. We therefore conclude that grassland conversion to coniferous forests does not enhanced C sequestration in the mineral soil, for at least 40 years after land-use change.

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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. 2014), 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 below-ground 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. 2014) 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 ¹³C-depleted CO₂, 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, ¹⁵N 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.

Materials and methods

Site description and experimental design

Two different mountain regions were selected: the Sistema Central range in Spain, and the Central Alps in Austria (Supplementary Fig. 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.

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 (Supplementary Fig. 1) (Table 1). Three replicated paired plots were established on each site, separated by 25–50 m. Each paired plot (20 × 20 m) consisted of an afforested plot with a closed tree canopy and an adjacent grassland plot. Spanish sites were located on granite

Fig. 1 Mean values (± 1 SD) of soil organic carbon (SOC) concentration (a), Soil total nitrogen (N) concentration (b), 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

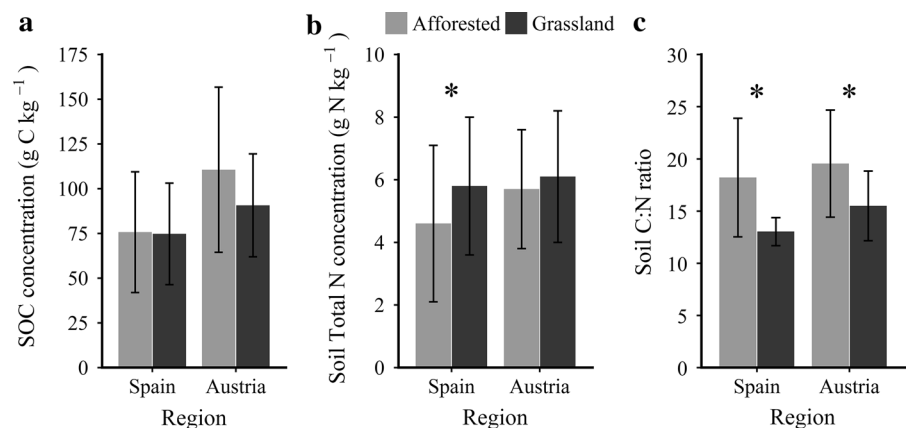


Table 1 Geographical coordinates, main climatic (MAP mean annual precipitation; MAT mean annual temperature) and physiographic features, soil classification (IUSS Working Group WRB 2014) and species composition of the study sites in the Sistema Central in Spain (ES) and the Central Alps in Austria (AT)

Site	Longitude	Latitude	MAP ^a (mm)	MAT ^a (°C)	Altitude (m a.s.l.)	Bedrock	Soil classification		Slope (°)		Species composition	
							Forest	Grassland	Forest	Grassland	Forest	Grassland
ES1	3°44'W	41°03'N	1352	5.1	1840	Granite, gneiss	Umbrisol		20	6.5	Pisy	Fecu
ES2	3°49'W	40°59'N	1539	3.8	2010	Granite, gneiss	Cambisol		12	12	Pisy	Fecu
ES3	3°49'W	40°49'N	1240	5.5	1780	Granite, gneiss	Umbrisol		18	15	Pisy	Defl, Feib
ES4	3°52'W	40°50'N	1158	6.7	1655	Granite, gneiss	Umbrisol		9.5	1	Pisy	Fecu
ES5	3°56'W	40°49'N	1246	5.5	1780	Granite, gneiss	Umbrisol		11	4	Pisy	Nast, Fecu
ES6	4°10'W	40°37'N	795	7.3	1640	Granite, gneiss	Cambisol		11	5.9	Pisy	Nast, Feib
ES7	4°57'W	40°18'N	1174	10.1	1320	Granite, gneiss	Umbrisol		24	11.5	Pisy	Nast, Feib
ES8	4°59'W	40°23'N	1075	10.9	1250	Granite, gneiss	Umbrisol		7	8	Pisy	Nast
ES9	5°02'W	40°27'N	1067	8.6	1530	Granite, gneiss	Umbrisol		3	4	Pisy	Feib
ES10	5°11'W	40°19'N	944	8.5	1445	Granite, gneiss	Cambisol		5.5	6.5	Pisy	Nast
AT1	11°47'E	47°19'N	1032	2.9	1779	Dolomites, greywacke-slates, quartzitic phyllites	Podzol		39	39	Piab, Lade, Pice	Nast
AT2	11°35'E	47°06'N	865	2.5	1896	Schist, phyllites	Cambisol		24	24	Piab, Lade	Nast
AT3	11°21'E	47°17'N	1012	3.8	1618	Carbonated Breccia	Leptosol		35	35	Piab, Lade	Case
AT4	11°04'E	47°12'N	1043	3.5	1735	Siliceous	Cambisol		25	25	Piab, Lade	Case, Nast
AT5	11°02'E	46°52'N	940	1.9	2120	Siliceous	Podzol		45	45	Pice	Nast
AT6	10°51'E	46°57'N	905	2.4	2050	Siliceous	Podzol		45	45	Pice	Nast
AT7	10°34'E	47°18'N	1341	4.3	1527	Dolomites	Leptosol		42	42	Piab, Lade	Case

^a Climatic features (MAP and MAT) were modelled following Hasenauer et al. (2003) in the Austrian sites and Sánchez-Palomares et al. (1999) in the Spanish sites. Species codes: Pisy, *Pinus sylvestris*; Piab, *Picea abies*; Lade, *Larix decidua*; Pice, *Pinus cembra*; Nast, *Nardus stricta*; Fecu, *Festuca curvifolia*; Feib, *Festuca iberica*; Defl, *Deschampsia flexuosa*; Case, *Carex sempervirens*

and gneiss bedrock (Table 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 (Supplementary Table 1) and soils were classified as Cambisols and Umbrisols (Table 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 (Table 1) with A-horizon pH ranging from 3.4 to 6.4. The textures were sandy loam and sandy clay (Supplementary Table 1); soils were classified as Podzols, Cambisols and Leptosols (Table 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 to 1 livestock units per ha—for a period of 6–10 weeks during summer. The main features of the study sites are shown in Table 1.

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 ($20 \times 20 \times 30$ 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.

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, Dynament Ltd., Derbyshire, UK) was placed inside each bottle and closed tightly. The sensor produced a voltage readout every 10 s 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 [$\text{mg CO}_2\text{-C h}^{-1} \text{kg}^{-1}$ 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 ($\text{mg CO}_2\text{-C g}^{-1}$ SOC, Díaz-Pinés et al. 2014) was obtained by normalising total CO_2 by SOC content in the soil.

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°C) and ground ($<100\ \mu\text{m}$) 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:

$$\delta(\text{‰}) = \left[\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right] \times 1000$$

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 ± 0.3 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:

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

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 ± 1 SD. All statistical analyses were carried out in R [version 3.2.3, R Core Team (2015)].

Results

Relationships between altitude, mean annual temperature and mean annual precipitation

In Spain, altitude showed a close negative correlation ($R_{\text{adj}}^2 = 0.98$) with mean annual temperature (MAT), and a positive correlation ($R_{\text{adj}}^2 = 0.35$) with mean annual precipitation (MAP) (Supplementary Fig. 2). In Austria, the same negative trend was found between altitude and MAT ($R_{\text{adj}}^2 = 0.11$); but a negative correlation was found between MAP and altitude ($R_{\text{adj}}^2 = 0.26$) (Supplementary Fig. 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) (Fig. 1a). 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; $p < 0.01$) (Fig. 1b) 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) (Fig. 1b). 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 ($p < 0.001$, Fig. 1c). Austrian sites showed a similar trend (19.6 ± 5.1 vs 15.5 ± 3.3 , $p < 0.01$) (Fig. 1c). Supplementary Table 1 shows the soil features of individual sites. Weak positive relationships between altitude and SOC concentrations ($R_{\text{adj}}^2 = 0.13$; $p < 0.05$), and total N ($R_{\text{adj}}^2 = 0.13$; $p < 0.05$) were found in Spanish grasslands. Negative relationships were observed in Austrian grasslands between altitude and SOC concentration ($R_{\text{adj}}^2 = 0.39$; $p < 0.01$) and total N ($R_{\text{adj}}^2 = 0.69$; $p < 0.001$). In Austria, altitude was positively correlated with C:N ratio in both the grassland ($R_{\text{adj}}^2 = 0.64$; $p < 0.001$) and the afforested sites ($R_{\text{adj}}^2 = 0.26$; $p < 0.01$). 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.

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 2), there was no significant effect of afforestation on SOC mineralisation efflux.

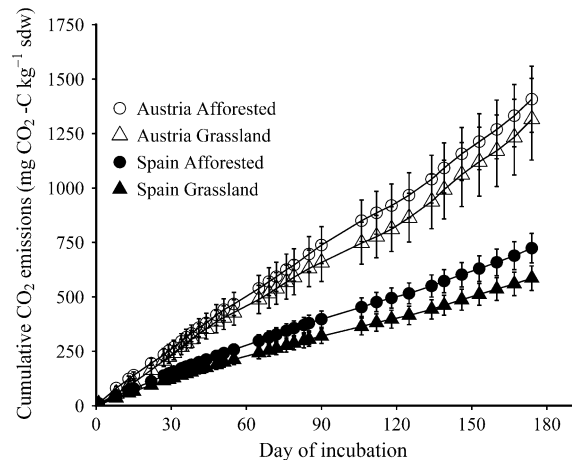


Fig. 2 Mean values (± 1 SE, $N = 30$ for Spain and $N = 18$ for Austria) of cumulative soil CO₂ production throughout the whole incubation period by afforested and grassland soils in both regions (Sistema Central, Spain; and Central Alps, Austria)

Mineralised C throughout the whole incubation period (Fig. 2) 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.

The values of cumulative SOC decomposability throughout the whole incubation period were 11.3 ± 7.0 and 8.4 ± 4.0 mg CO₂-C g⁻¹ SOC for Spanish afforested and grassland plots respectively (n.s.). 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 mg CO₂-C g⁻¹ SOC for afforested and grassland plots respectively (n.s.). However, in Austria there was no correlation between cumulative SOC decomposability and any other variable.

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. $-27.3 \pm 0.7\text{‰}$ respectively). In Austrian sites there

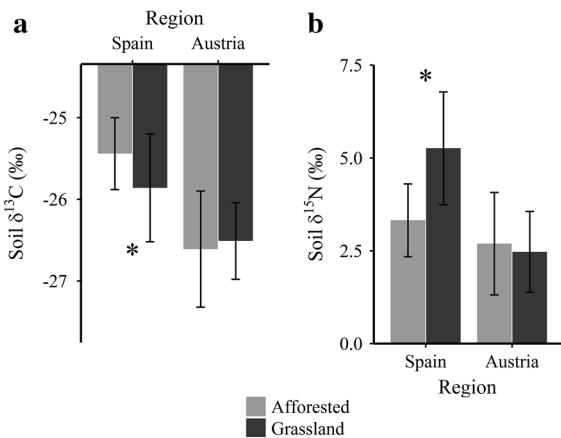


Fig. 3 Mean values (± 1 SD) of soil $\delta^{13}\text{C}$ (a), soil $\delta^{15}\text{N}$ (b) in the Sistema Central in Spain and the Central Alps in Austria. Different letters denote significant differences ($p < 0.05$) between afforested and grassland plots in each region

were not differences between afforested and grassland plots (-29.3 ± 0.9 vs. $-28.4 \pm 0.7\text{‰}$ for needles and grasses respectively). Mean soil $\delta^{13}\text{C}$ was significantly less negative ($p < 0.05$) in afforested than in grassland plots in Spanish sites (Fig. 3) 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) (Fig. 3) but not in Austria (2.7 ± 1.4 and $2.5 \pm 1.1\text{‰}$).

Soil $\delta^{13}\text{C}$ showed a significant positive relationship with altitude in Spain in both afforested and grassland plots (Fig. 4). In Austria, soil $\delta^{13}\text{C}$ only showed a significant positive relationship with altitude in grasslands (Fig. 4). 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 (Fig. 4). 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 (Fig. 5).

Mean $\Delta^{13}\text{C}$ values were significantly less negative in grasslands than in afforested plots (-1.4 ± 0.5 vs. $-2.8 \pm 1.4\text{‰}$ Spain and -1.9 ± 0.6 vs. $-2.8 \pm 0.5\text{‰}$ Austria). Mean $\Delta^{15}\text{N}$ values in Spain showed significant differences ($p < 0.01$) between afforested ($-5.3 \pm 1.3\text{‰}$) and grassland plots ($-4.3 \pm 2.3\text{‰}$). $\Delta^{15}\text{N}$ values in Austria were highly variable between sites and no significant differences were found between afforested ($-5.1 \pm 2.0\text{‰}$) and grassland plots ($-6.0 \pm 2.8\text{‰}$). 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 (Fig. 6).

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

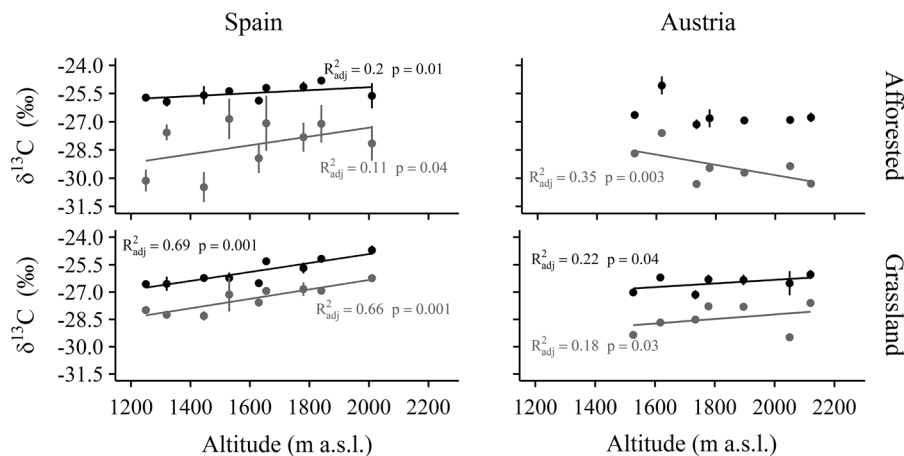


Fig. 4 Relationship between altitude and $\delta^{13}\text{C}$. Black symbols represent soil $\delta^{13}\text{C}$ and grey symbols represent plant material $\delta^{13}\text{C}$. Data refer to mean values ± 1 SD

Fig. 5 Relationship between soil $\delta^{13}\text{C}$ and plant material $\delta^{13}\text{C}$. Data refer to mean values ± 1 SD

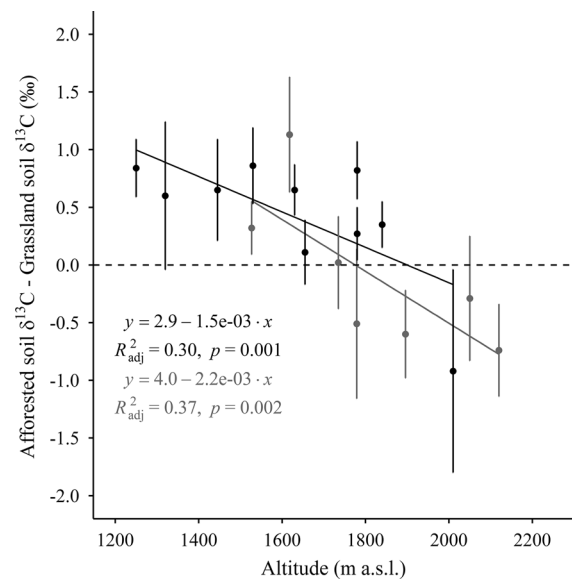
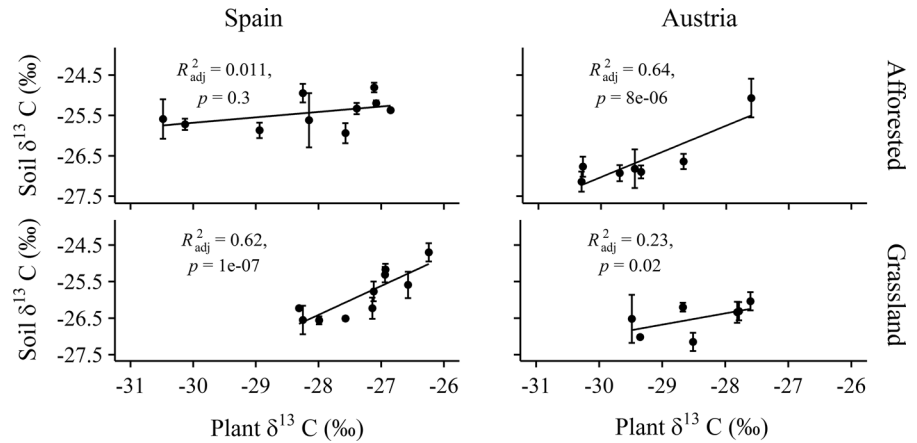


Fig. 6 Effect of altitude on the change in isotopic composition of soil $\delta^{13}\text{C}$ between afforested and grassland plots. Spanish sites are shown in black and Austrian sites in grey. Data refer to mean values ± 1 SD

material (Table 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). With regard to 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 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 below-ground 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 g C kg⁻¹ 100 m⁻¹ and 0.4 g N kg⁻¹ 100 m⁻¹), but a significant decrease in Austrian grasslands (estimated as 9 g C kg⁻¹ 100 m⁻¹ and 0.8 g N kg⁻¹ 100 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 (García-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 CO₂ in leaves, which in turn causes lower plant ¹³C discrimination (e.g. Körner et al. 1991). Low temperatures in high altitudinal areas may also influence plant ¹³C 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 δ¹³C 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 δ¹³C due to afforestation, provided all other parameters remain unchanged. Figure 6 shows that the SOC becomes (slightly) ¹³C enriched after afforestation at lower altitudes, and is isotopically depleted at higher altitudes. This suggests that SOC mineralisation—inferred from greater soil δ¹³C—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 δ¹³C 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.

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 constrains 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.

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