

Anthropogenic disturbance of natural forest vegetation on calcareous soils alters soil organic matter composition and natural abundance of ^{13}C and ^{15}N in density fractions

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Abstract In the last century, many calcareous soils in Castilla León (northwestern Spain) have been transformed from natural *Quercus ilex* forest to cropped land. Reforestation with *Pinus halepensis* has been taking place during the past 40 years. In order to obtain a better understanding of how these disturbances affect ecosystem functioning, we studied the quantity and quality of soil organic matter (SOM) in natural forest ecosystems, cropland and Pinus plantations. Density fractionation combined with ultrasonic dispersion enables separation and study of SOM fractions: free organic matter (OM), OM occluded into aggregates and OM stabilized in organo-mineral complexes, considered on the basis of the type of physical protection provided. We separated SOM density fractions and determined the concentrations of C and N, C/N ratios and the natural isotopic abundance ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values). Transformation of Quercus forest to cropland resulted in major losses of SOC and N, as expected. However, subsequent reforestation with Pinus resulted in good recovery of the original SOC and soil N pools. This indicates the potential for enhanced C storage in agricultural soils by their reversion to a forested state. Study of the density fractions and their ^{13}C and ^{15}N signatures enabled better understanding of the high stability of OM in calcareous soils, and analysis of $\delta^{13}\text{C}$ variations throughout the profile also enabled

identification of past C3/C4 vegetation change. Despite the different OC contents of soils under different land use, OM stabilization mechanisms were not significantly different. In calcareous soils, accumulation of SOC and N is mainly due to organo-mineral associations, resulting in physico-chemical stabilization against further decomposition.

Keywords Land use change · Density fractions · $\delta^{13}\text{C}$ · $\delta^{15}\text{N}$

Introduction

The increase in the concentration of atmospheric carbon dioxide during the past century has drawn attention to the relation between the capacity of ecosystems to act as C sinks, changes in land use and climate change (Briones et al. 2006). Soil organic matter (SOM) is recognized as an important factor in C-driven climate change (Sanderman et al. 2003). Because of this, SOM dynamics and the capacity of soils to accumulate and stabilize organic carbon in response to different land use changes have received much attention in recent years (Rovira and Vallejo 2003; Llorente and Turrión 2010; Wick et al. 2009).

Anthropogenic disturbance of ecosystems leads to changes in the quantity and quality of SOM (Hobbs 1999). Comparisons between cultivated and uncultivated soils have demonstrated a reduction in SOM content with cultivation (Mann 1986). However, when arable land is converted to permanent vegetation, the SOM content increases gradually because of greater inputs of organic matter (Haynes and Beare 1996).

Studies of the effects of land use change on the SOM in calcareous soils are scarce. However, calcareous soils are of particular interest because of the high stability of their

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organic matter (García et al. 1997; Llorente et al. 2008) and because they represent approximately 12% of the world soil resources (FAO 1996).

SOM is a complex mixture of material from various sources and may exist in the soil profile as decomposed or stabilized matter, or in any form intermediate between these two extremes. Physical fractionation methods such as density fractionation have been proposed for the study of SOM dynamics (Christensen 1992), because this method yields distinct organic matter (OM) fractions that differ in dynamics, structure and function (Golchin et al. 1994; Six et al. 2001). Many authors have used density fractionation of SOM to represent different pools of SOM with different turnover rates (e.g. Swanston et al. 2002; Helfrich et al. 2006; Llorente and Turrión 2010) and to identify mechanisms that control changes in C and N pools (Jolivet et al. 2003). Density fractionation enables us to identify labile, active fractions of SOM, which may respond much faster to management changes than the total SOM content, and also passive fractions, which are more closely associated with long-term SOM dynamics (Barrios et al. 1996). SOM density fractionation following ultrasonic dispersion enables separation and study of three different pools according to the different degree of physical protection provided (Sohi et al. 2001). The main fractions are (1) “free” particulate OM (FPF), isolated before ultrasonic breakdown of stable aggregates, (2) OM occluded within aggregates (OF), isolated after breakdown of aggregates by ultrasonic dispersion, and (3) the organo-mineral fraction (OMF) recovered as the residual (heavy) material.

A relatively inexpensive analytical method such as stable isotopic analysis is useful for assessing chemical shifts in SOM (Glaser 2005). The principle of using the stable isotope techniques is based on the fact that the abundance of heavy and light isotopes (e.g. ^{13}C and ^{12}C , respectively) varies due to isotopic discrimination of the heavy isotope in SOM compounds during biological and/or physical processes (Andreux et al. 1990). Krull and Skjemstad (2003) demonstrated that isotope fractionation is highly influenced by soil chemistry, mineralogy and type of organic matter input. The application of stable carbon isotope ($\delta^{13}\text{C}$) analysis (and to a lesser degree $\delta^{15}\text{N}$) to SOM studies has increasingly been used to estimate soil C and N turnover (e.g. Balesdent and Mariotti 1988; Bernoux et al. 1998; Glaser 2005; Sevink et al. 2005) and to assess the degree of SOM decomposition (e.g. Wedin et al. 1995; Connin et al. 2001). Although many studies have addressed the isotopic composition of density fractions (Shang and Tiessen 2000; Roscoe et al. 2001; Crow et al. 2006), the mechanisms of N fractionation associated with organic matter decomposition are still unclear (Liao et al. 2006). Stable isotope data are useful for assessing C and N turnover in SOM density fractions. Because the light fraction from density

fractionation primarily represents labile material of relatively recent origin, we would expect this fraction to reflect ^{13}C and ^{15}N values that are closer to current vegetation (Compton and Boone 2000; Accoe et al. 2002). The heavy fraction, representing older, more humified and amorphous organic compounds, displays relatively enriched $\delta^{13}\text{C}$ values (Ehleringer et al. 2000).

The objective of the present study was to investigate organic matter dynamics in calcareous moor soils under different types of land use-native forest (*Quercus ilex*), forest plantation (*Pinus halepensis*) and agricultural land (cropped with cereals) using density fractionation of SOM and stable C and N isotopic approach.

More specifically, the aims of the study were (a) to assess the effect of land use and depth on OM abundance and on the ^{13}C and ^{15}N isotope composition of calcareous soils; (b) to study the roles of different density fractions in C and N stabilization in calcareous soils; (c) to determine the ^{13}C and ^{15}N isotope composition of density fractions.

Materials and methods

Site description

The study was carried out in the region of Castilla y León (northwestern Spain), UTM: 30T 384465 E 4639001 N. The mean annual rainfall in the region is below 420 mm, under a xeric moisture regime, and the mean annual temperature is 12.3°C. The altitude of the moor is between 800 and 900 m, with gentle slopes (< 7%). The soils are Xerepts, which are quite similar by their physicochemical parameters but differ in their land use history. The native vegetation in the area is holm-oak wood (*Quercus ilex* subsp. *ballota*). In the ninetieth century, most of the forest was converted into agricultural land, but since the 1950s, reforestation with *Pinus halepensis* has been carried out on abandoned agricultural land. Agricultural land is currently cropped with cereals (usually barley). The average basal area of the *Quercus ilex* forest is 13 m² ha⁻¹ with an average density of 3,200 trees ha⁻¹. The average basal area of the *Pinus* plantation is 50 m² ha⁻¹, with an average density of 1,184 trees ha⁻¹.

General characteristics of the samples are shown in Table 1.

Sampling procedures

A land use map of the calcareous moor of Castilla y León was elaborated with a GIS (ArcGis 9.0 for Windows) The map was used to select the sampling plots on the basis of the following criteria: (a) *Quercus ilex* forest (QF), cropped land (CL) and *Pinus halepensis* plantations (PP) in adjacent

Table 1 Basic properties of the soils under study (0–30 cm)

Area	Land use	Tree cover	Texture	CaCO ₃ (%)	SOC (%)	N (%)	C/N	pH
CERRATO	Cropped land	No	Clay loam	19.9	2.78	0.25	11.0	8.1
CERRATO	Quercus forest	No	Clay loam	23.4	2.98	0.29	10.4	8.2
CERRATO	Quercus forest	Yes	Clay loam	20.9	5.42	0.41	13.1	7.9
CERRATO	Pinus plantation	Yes	Clay loam	18.4	5.45	0.38	14.1	7.9
AMPUDIA	Cropped land	No	Clay loam	10.1	1.59	0.18	8.6	8.1
AMPUDIA	Quercus forest	No	Clay loam	15.4	2.54	0.26	9.8	8.1
AMPUDIA	Quercus forest	Yes	Clay loam	13.4	5.87	0.46	12.7	8.1
AMPUDIA	Pinus plantation	Yes	Clay loam	22.2	3.47	0.21	21.0	8.2
MONTE VIEJO	Cropped land	No	Clay loam	59.8	1.56	0.26	10.6	8.3
MONTE VIEJO	Quercus forest	No	Clay loam	26.5	3.75	0.34	10.1	8.2
MONTE VIEJO	Quercus forest	Yes	Clay loam	31.1	6.51	0.50	11.9	7.9
MONTE VIEJO	Pinus plantation	Yes	Clay loam	42.0	3.70	0.36	10.9	8.1

Mean values for 4 samples

areas; (b) minimal area of each type of land use, 1 ha, and (c) establishment of each type of land use during at least 40 years. Three plots were selected for each type of land use, with four representative profiles for each type of land use. A total of 48 profiles were thus sampled at 0–10 cm, 10–20 cm and 20–30 cm depth intervals. Visible plant residues and roots were removed, and soil was air-dried, sieved (< 2 mm) and stored at room temperature until analysis.

Litter from soil under each land use type was sampled, oven-dried at 50°C and ground.

Ultrasonic equipment

We used a Branson 450 W Sonicator, equipped with a titanium probe. The probe depth was fixed in 15 mm. The sonicator was calibrated by determining the real power output calorimetrically (North 1976). The probe output energy was calculated from:

$$P = (m_w c_w + C_{\text{cont}}) \Delta T t^{-1} + H t,$$

where P is the calculated power (W), m_w is the mass of water (g), c_w is the specific heat of water (4.18 J g⁻¹ K⁻¹), C_{cont} is the heat capacity of the container (J K⁻¹), ΔT is the temperature change (K), t is the sonication time (s) and H is the heat loss (J s⁻¹). The heat capacity of the glass beaker (C_{cont}) was determined using the method of mixtures (Morra et al. 1991), according to:

$$C_{\text{cont}} = m_1 c_w \left((T_1 - T_2) (T_3 - T_2)^{-1} \right) - m_2 c_w,$$

where C_{cont} and c_w are as above, m_1 is the mass (g) of an amount of water heated to T_1 (K), which is added to the beaker that already contained an amount of water m_2 (g) at room temperature (K). The final equilibrium temperature (K) of the water in the beaker is T_3 . The wanted energy

output was 300 J ml⁻¹, and the corresponding time was calculated from:

$$t = m_s E P^{-1},$$

where t is the sonication time (s), m_s is the mass of soil (g), E is the energy (J g⁻¹), P is the power output (W).

Density fractionation of soil

A density fractionation procedure was applied to the topsoils of the 48 profiles. The method follows the concepts of Golchin et al. (1994) who differentiated three degrees of physical protection of OM: FPF, non-protected; OF, occluded within aggregates—extractable by sonication (protected); and OMF retained in the dense residue after sonication (the most protected).

Briefly, 5 g of soil sample was placed in small centrifuge bottles (50 ml capacity), and 35 ml of NaI 1.8 g ml⁻¹ was added. The bottles were shaken gently and the floating material, considered as the FPF, was then recovered by centrifugation at 8,000 g for 30 min at 18°C and filtered over a vacuum filter by washing with deionized water. The recovered NaI was added to the residue remaining in the bottle. The bottle was placed in an ice bath and sonicated at 300 J ml⁻¹ with a probe-type ultrasonic disintegrator (Branson 450 W). The floating material, considered as the OF, was recovered by centrifugation and washed in the same way as the FPF. The remaining material, considered as the OMF, was washed with deionized water. All fractions were dried at 40°C and ground in a mortar.

Physical and chemical analyses

For soil characterization, texture, pH, total N, total C, carbonate and organic C, and C/N were determined. Total

concentrations of soil C and N were determined in an automated C/N analyser (CHN-2000, Leco). Organic carbon was calculated as the difference between total and carbonate carbon. Soil total calcium carbonates were determined by use of 1 M HCl titrated with 0.5 M NaOH (FAO 2007). Dried soil samples were crushed and treated with 1 N HCl to remove any inorganic carbonate (acid pre-treatment has no effect on $\delta^{13}\text{C}$ of soil organic matter (Nordt et al. 1994).

Natural abundances of ^{13}C and ^{15}N values in soils, plant material, and SOM density fractions were measured and dried (at 45°C for 1 week), ground and weighed into tin capsules for continuous flow isotope ratio mass spectrometry (BayCEER—Labor für Isotopen-Biogeochemie, Bayreuth, Germany).

Isotope ratios were determined with an elemental analyzer coupled to an isotope ratio mass spectrometer (Carlo Erba CN 2500, Italy coupled with DeltaPLUS Isotope MS via ConFlo III Interface, Thermo Finnigan, Bremen, Germany). Standard gases were calibrated in relation to international standards (CO_2 in PeeDee belemnite) by use of reference substances (NBS 16–20) for carbon isotope ratios, supplied by the International Atomic Energy Agency (IAEA), Vienna.

Natural abundances of ^{13}C and ^{15}N were expressed in δ units, by reference to the international standards, according to equations (1) and (2), respectively:

$$\delta^{13}\text{C}_{\text{‰}} = 10^3 \times \frac{\left(\frac{^{13}\text{C}}{^{12}\text{C}}\right)_{\text{samp.}} - \left(\frac{^{13}\text{C}}{^{12}\text{C}}\right)_{\text{stand.}}}{\left(\frac{^{13}\text{C}}{^{12}\text{C}}\right)_{\text{stand.}}}; \quad (1)$$

$$\delta^{15}\text{N}_{\text{‰}} = 10^3 \times \frac{\left(\frac{^{15}\text{N}}{^{16}\text{N}}\right)_{\text{samp.}} - \left(\frac{^{15}\text{N}}{^{16}\text{N}}\right)_{\text{stand.}}}{\left(\frac{^{15}\text{N}}{^{16}\text{N}}\right)_{\text{stand.}}}; \quad (2)$$

Statistical analyses

Analysis of variance (ANOVA) was used (1) to compare SOC and N among land use types and depths; (2) to compare the proportion of the SOC and total N that is represented by each density fraction, the C concentration in each density fraction, and C/N ratio, overall or for a given land use type; (3) to compare the effect of land use and depth on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in whole soil, and (4) to compare the isotope composition of density fractions, overall for given land uses. In cases of significant F -statistics ($P < 0.05$), differences between means were tested with the Tukey procedure for multiple comparisons. Residuals were tested for homoscedasticity and normality with Levene's and Kolmogorov–Smirnov tests, respectively. All statistical analyses were performed with the Systat 14.0 Statistical Software Package (SPSS for Windows).

Results

C and N contents of whole soil

Land use change from a natural *Quercus ilex* forest to cropped land (100 years ago) resulted in a significant ($P < 0.05$) net loss of 47% of SOC and 41% of total N in the upper 30 cm of soil. The subsequent reforestation of the studied calcareous soils with *Pinus halepensis* has resulted in recovery of 90% of the lost SOC and 70% of total N 40 years after establishment of the plantation (Fig. 1). This is probably due to the higher input of fresh organic matter at the soil surface under forest. Total N and SOC contents were not significantly different throughout the soil profile in cropped soils (Fig. 1). This is explained by the low input of organic matter and the effect of tillage to 30 cm depth, which homogenizes soil properties throughout the profile. Furthermore, the SOC and total N contents varied significantly among land uses, in the order: *Quercus* forest > *Pinus* plantation > cropped land. The organic C and total N contents were the lowest in the cropped land (Fig. 1).

The C/N ratio varied between 8.6 and 21.0 and did not differ significantly among different types of land use or among depths (data not shown).

C and N distribution in SOM fractions

The recovery of organic C after fractionation ranged from 96 to 105% (average, 100%). Recoveries higher than 100% may be due to incomplete removal of NaI. Losses of OC during fractionation occurred due to loss of material during manipulation of the sample at the various stages. The recovery of N ranged from 70 to 101% (mean, 86%). The greater loss of N than of OC may be due to leaching of soluble N-rich compounds.

The FPF and OF obtained by density fractionation were visually distinct. The FPF comprises recognizable plant material, whereas the intra-aggregate was amorphous dark material.

The concentrations of N for any given fraction were similar for all land use types. The concentration of OC was significantly higher in OF and FPF under *Pinus* plantation than in cropped land (Figs. 2, 3). Comparison of OC and total concentration of N among density fractions revealed significantly lower values in OMF, as the latter was dominated by minerals. The highest total concentrations of N and OC were observed in the OF. With regard to the contribution of organic C of individual SOM fractions to TOC, most of the OC was associated with OMF, which accounted for 43 to 85% of the TOC, and the lowest portion was represented by OF, which ranged from 2 to 6% (Fig. 2).

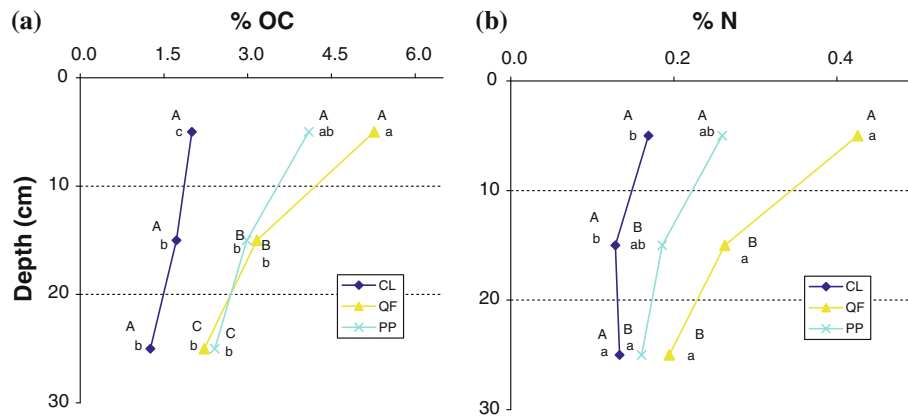


Fig. 1 Average contents of OC and N in soils under different types of land use and at different depth. Differences between means were tested by the Tukey procedure for multiple comparisons. Depth indicated with the same *capital letter* are not significantly different

The relative distribution of total N among the fractions showed that most of the N was located in the OMF, which accounted for 43 to 88% of total N, and the lowest portion was represented by OF, which ranged from 1 to 3% of total N. Comparison of the N contribution of each fraction among different types of land use revealed that FPF and OMF were sensitive to land use change (Fig. 3, %N/TN soil).

The OC/N ratio, for any given type of land use, was always lower in the OMF than in OF and FPF, but was only significant for soils under forest cover (Fig. 2). There were no clear differences among types of land use, although for any given fraction the C/N ratio in soils under pine forest appeared to be higher than in the other soils.

Natural abundance of ^{13}C and ^{15}N in whole soils

Progressive enrichment of $\delta^{13}\text{C}$ was observed with depth in the soil profile for all types of land use, with increases ranging from 2 to 4‰ from topsoil to 30 cm depth (Table 2). The same pattern of $\delta^{15}\text{N}$ enrichment of SOM with depth in the soil profile was observed; increases in $\delta^{15}\text{N}$ ranged from 1.1 to 4.4‰ from topsoil to 30 cm depth (Table 2).

Comparison of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values among different types of land use revealed significant differences ($P < 0.05$) only for the topsoil. Topsoils under *Quercus ilex* cover displayed the lowest $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Table 2, Fig. 4), associated with the lower isotope signature of the corresponding litter (Table 3).

The $\delta^{13}\text{C}$ values of *Quercus ilex* forest plant materials ranged from -26.2 to -26.8 ‰, i.e. slightly lower than the $\delta^{13}\text{C}$ values in *Pinus halepensis* plantation plant materials (-25.9 ‰) and cropped land plant materials (-24.6 ‰). The $\delta^{15}\text{N}$ values for plant material increased in the order

($P < 0.05$) for a given land use (*Depth*land use interaction*). Land uses indicated with the same *lower case letter* are not significantly different ($P < 0.05$) for a given depth (*Land use*depth interaction*). CL Cropped land; QF Native *Quercus* forest; PP *Pinus* plantation

Pinus (-4.3 ‰) < Quercus (-3.9 ‰ to -3.4 ‰) < crops (1.5‰) (Table 3).

Natural abundance of ^{13}C and ^{15}N in SOM fractions

The $\delta^{13}\text{C}$ values varied by up to 13‰ among density fractions. The OMF was enriched relative to OF and FPF. Significant differences ($P < 0.05$) were found in $\delta^{13}\text{C}$ values in the OMF, in the order: PP > CL > QF (Fig. 5). The $\delta^{15}\text{N}$ values also displayed enrichment, of up to 4.9‰ among density fractions, of the heavy fraction in relation to OF and FPF (Fig. 5). The $\delta^{15}\text{N}$ values in the FPF fraction differed significantly ($P \leq 0.05$) in the order: CL > QF > PP (Fig. 5).

Discussion

C and N contents in whole soil

Land use change from a natural *Quercus ilex* forest to cropped land in the past 100 years resulted in an average net loss of 47% of SOC and 41% of total N. This loss is consistent with the findings of Burke et al. (1989) who reported 50% SOC loss for land use transformation from grassland to cropped land in US soils, and those of other authors who reported that cultivation generally leads to decreases in SOM content (e.g. Glaser et al. 2000; La Scala et al. 2008). Soil tillage induces soil C loss by acceleration of organic C oxidation, which results in the release of large amounts of CO_2 to the atmosphere (Ellert and Janzen 1999; Prior et al. 2000). Another tillage-related factor that contributes to soil C losses is soil aggregate disruption, which exposes once-protected organic matter to decomposition (De Gryze et al. 2006; Grandy and Robertson 2007).

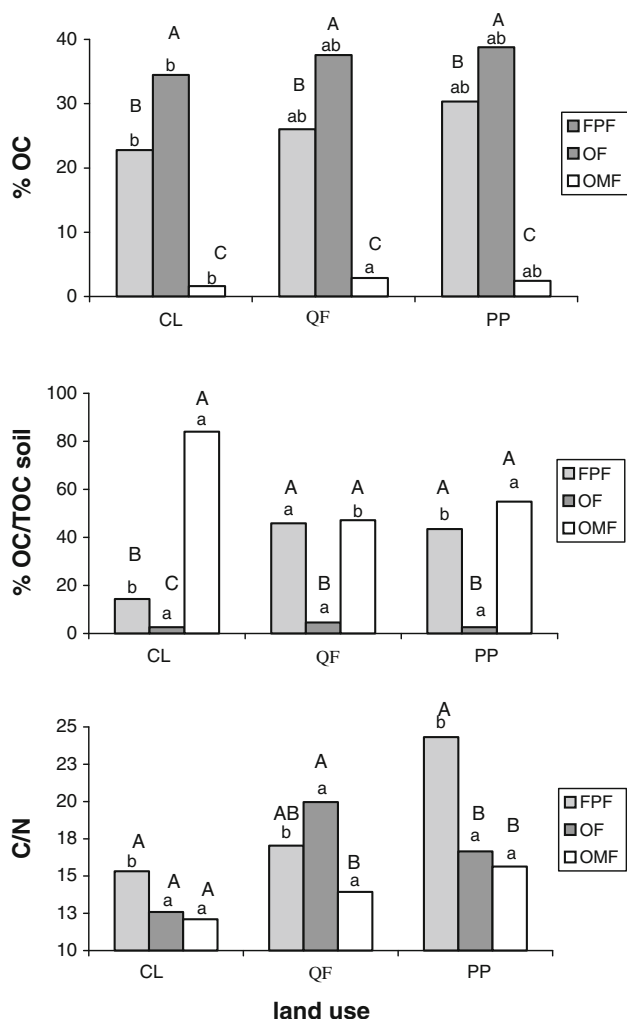


Fig. 2 Average contents of OC, OC/TOC in soil and C/N ratio in density fractions for the different types of land use compared. Differences between means were tested by the Tukey procedure for multiple comparisons. Fractions indicated with the same *capital letter* are not significantly different ($P < 0.05$) for a given land use (*Fractions*use interaction*). Land uses indicated with the same *lower case letter* are not significantly different ($P < 0.05$) for a given fraction (*Use*fraction interaction*). CL Cropped land; QF Native *Quercus* forest; PP *Pinus* plantation; FPF free particulate fraction; OF occluded fraction; OMF organo-mineral fraction

The subsequent reforestation of the studied calcareous soils with *Pinus halepensis* results in recovery of 90% of the lost SOC and 70% of total N after 40 years of the plantation (Fig. 1). This is probably due to the higher input of fresh organic matter at the soil surface under forest land and indicates potential storage of OC in the reforested soils.

C and N distribution in SOM fractions

Comparison of OC and N concentration among density fractions revealed significantly lower values in OMF than

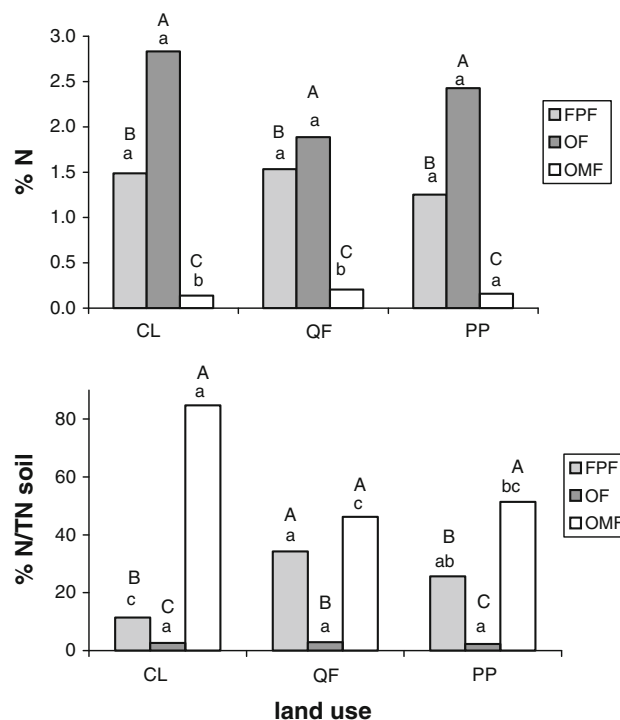


Fig. 3 Average content of total N and % N/TON in soil in density fractions of soils for the different types of land use compared. Differences between means were tested by the Tukey procedure for multiple comparisons. Fractions indicated with the same *capital letter* are not significantly different ($P < 0.05$) for a given land use (*Fractions*use interaction*). Land uses indicated with the same *lower case letter* are not significantly different ($P < 0.05$) for a given fraction (*Use*fraction interaction*). CL Cropped land; QF Native *Quercus* forest; PP *Pinus* plantation; FPF free particulate fraction; OF occluded fraction; OMF organo-mineral fraction

in the other fractions, as the latter was dominated by minerals (Christensen 1992; Golchin et al. 1995). The highest concentrations of N and OC corresponded to the OF, in accordance with studies documenting a positive influence of aggregation on the accumulation of N and OC (e.g. Six et al. 2000). This accumulation is due to the physical protection of OM by aggregates attributed to compartmentalization of substrate and microbial mass (Six et al. 2002).

Regarding the contribution of different SOM fractions to soil TOC content, most of the OC was associated with OMF. The present results are similar to those reported by Baisden et al. (2002), who found about 69–86% of TOC in the mineral-associated fraction in Californian soils, and also similar to those reported by Golchin et al. (1995), who found that the heavy fraction accounted for about 30–90% of TOC in soils from temperate climates. OM was mainly located in the organo-mineral complex, resulting in physicochemical stabilization against further decomposition.

However, comparison of OC concentration (g C g^{-1} fraction) among density fractions revealed the lowest

Table 2 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in whole soil samples

Depth (cm)	$\delta^{13}\text{C}$ (X \pm sd)			$\delta^{15}\text{N}$ (X \pm sd)		
	0–10	10–20	20–30	0–10	10–20	20–30
CERRATO						
CL	-17.36 \pm 1.54	-17.59 \pm 1.35	-14.37 \pm 2.31	3.88 \pm 0.19	5.13 \pm 0.82	5.14 \pm 1.27
QF	-20.97 \pm 1.44	-16.39 \pm 1.12	-17.72 \pm 6.13	0.53 \pm 0.57	4.35 \pm 0.10	5.47 \pm 0.15
PP	-20.04 \pm 2.71	-18.17 \pm 1.91	-19.03 \pm 4.77	1.97 \pm 1.07	5.09 \pm 0.19	5.10 \pm 1.51
AMPUDIA						
CL	-17.23 \pm 1.58	-16.80 \pm 1.67	-12.56 \pm 0.34	4.60 \pm 0.22	5.13 \pm 0.29	6.55 \pm 0.13
QF	-25.71 \pm 0.75	-21.87 \pm 0.97	-18.12 \pm 0.94	2.84 \pm 0.32	5.65 \pm 0.36	6.63 \pm 0.21
PP	-16.19 \pm 1.92	-13.94 \pm 2.34	-12.32 \pm 2.14	3.18 \pm 0.92	5.75 \pm 0.57	6.01 \pm 0.84
MONTE VIEJO						
CL	-11.80 \pm 0.62	-13.86 \pm 4.65	-11.90 \pm 1.15	5.10 \pm 0.29	5.52 \pm 0.27	5.86 \pm 0.27
QF	-19.97 \pm 3.88	-17.19 \pm 2.79	-16.29 \pm 2.79	2.40 \pm 1.13	4.87 \pm 1.78	6.21 \pm 0.94
PP	-16.60 \pm 1.49	-14.53 \pm 0.62	-16.41 \pm 6.14	2.59 \pm 1.74	5.01 \pm 0.70	5.38 \pm 0.41
Analysis of variance						
Use		***			n.s.	
Depth		*			***	
Use*Depth		**			***	

X \pm sd: Mean value and standard deviation for 4 samples

CL cropped land; QF native *Quercus* forest; PP *Pinus* plantation

For ANOVA analysis, values of $P > 0.05$: n.s., $P < 0.05$: *, $P < 0.01$: ** and $P < 0.001$: *** are reported

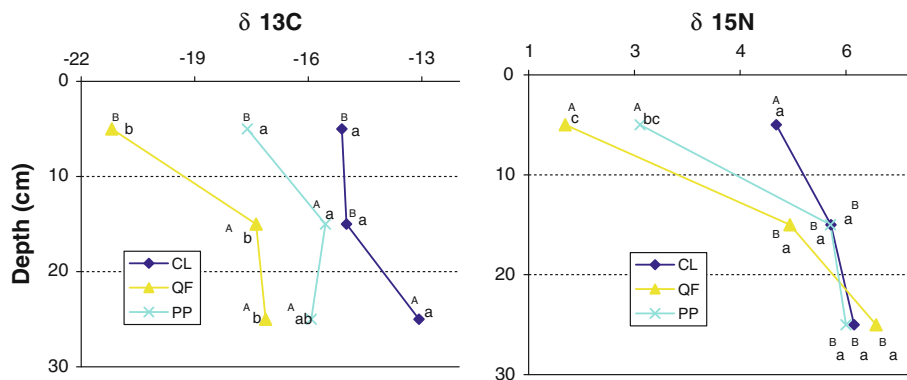


Fig. 4 Average $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in soils sampled under different types of land use and at different depths. Differences between means were tested by the Tukey procedure for multiple comparisons. Depth indicated with the same capital letter are not significantly different

($P < 0.05$) for a given land use (*Depth*land use interaction*). Land uses indicated with the same lower case letter are not significantly different ($P < 0.05$) for a given depth (*Land use*depth interaction*). CL Cropped land; QF Native *Quercus* forest; PP *Pinus* plantation

Table 3 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in plant material samples

	$\delta^{13}\text{C}$ (X \pm sd)		$\delta^{15}\text{N}$ (X \pm sd)	
	Mean	sd	Mean	sd
CL	-24.56	± 1.47	1.50	± 1.09
QF	-26.78	± 0.55	-3.92	± 0.12
PP	-25.89	± 0.58	-4.28	± 1.01

X \pm sd: Mean value and standard deviation for 4 samples

CL cropped land; QF native *Quercus* forest; PP: *Pinus* plantation

values in OMF, as the latter was dominated by minerals, as suggested by Golchin et al. (1995), and the highest values of OC concentration were found in OF. This high

concentration has been suggested to be due to the physical protection of OM by aggregates attributed to compartmentalization of substrate and microbial mass (Killham et al. 1993).

Most of the N was located in the OMF, in accordance with the results reported by Billings (2006).

The OC/N ratio, for any given type of land use, was always lower in the OMF. These results, which indicate a higher degree of decomposition in mineral-associated SOM, are consistent with those reported by Rovira and Vallejo (2003) for soils over calcareous material and under *Quercus rotundifolia*. The higher values of the OC/N ratio

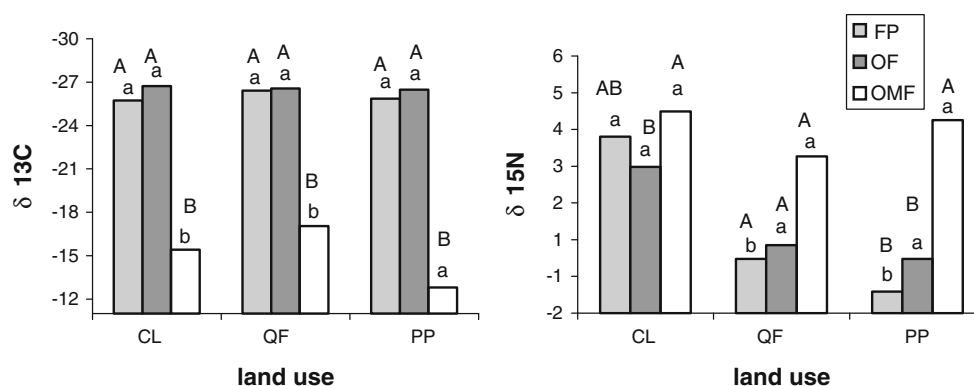


Fig. 5 Average $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in density fractions for the different types of land use compared. Differences between means were tested by the Tukey procedure for multiple comparisons. Fractions indicated with the same *capital letter* are not significantly different ($P < 0.05$) for a given land use (*Fractions*use interaction*). Land uses indicated

with the same *lower case letter* are not significantly different ($P < 0.05$) for a given fraction (*Use*fraction interaction*). CL Cropped land; QF Native *Quercus* forest; PP *Pinus* plantation; FPF free particulate fraction; OF occluded fraction; OMF organo-mineral fraction

for the soils under pine forest are consistent with the findings of different studies of litter composition, such as that by Traversa et al. (2008), who compared the C/N ratio of the litter under *Pinus halepensis* and *Quercus ilex*.

Natural abundance of ^{13}C and ^{15}N in whole soils

The utility of $\delta^{13}\text{C}$ of SOM is based on the systematic isotopic variation between C3 (trees, shrubs and cool season grasses) and C4 plants (warm season grasses). The $\delta^{13}\text{C}$ value of SOM in the upper soil profile (0–10 cm) for a given site is similar to that of the vegetation at the site (Bekele and Hudnall 2003). Isotopic signals from antecedent vegetation persist deeper in the soil profile (Boutton, 1996). Thus, changes in the relative proportions of C3 and C4 plants can be detected by a measured difference between the isotopic composition of the current plant community and that of the SOM at various depths. The studied calcareous soils are commonly mixed with gypsy outcrops that supports a shrub steppe vegetation dominated by *Salsola vermiculata*, *Kochia postrata* and *Camphorosma monspeliaca*, all of them Chenopodiaceae and, therefore, C4 plants. Over those calcareous soils, plants of Crassulaceae family, C4 plants, are also presents nowadays (Jiménez et al. 2006) and probably were abundant in the past.

Progressive enrichment of $\delta^{13}\text{C}$ was observed with depth in the soil profile for all types of land use, with increases ranging from 2 to 4‰ from topsoil to 30 cm depth. As decomposition of fresh plant litter progresses and the decomposition products become incorporated into the soil profile, the ^{13}C isotopic signature normally increases, by about 2–4‰, with increasing soil depth (Nadelhoffer and Fry 1988, Handley and Scrimgeour 1997; Boutton et al. 1998; Buchmann et al. 1998; Glaser 2005); this was also the case in the present study.

Hypotheses proposed to explain this pattern include (1) changes in the abundance of atmospheric $\delta^{13}\text{CO}_2$ since the Industrial Revolution, (2) preferential feeding by microbes on isotopically light material, (3) metabolic fractionation during decomposition, (4) long-term changes in plant water-use efficiency, among others (Balesdent et al. 1993; Ehleringer et al. 2000).

The abundances of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in fresh plant litter were lower than in the bulk soil. These results are consistent with those reported by other authors, e.g. Nadelhoffer and Fry (1988), Accoe et al. (2003) and Bird et al. (2003). The results of a litter bag experiment led Connin et al. (2001) to suggest that mechanisms responsible for isotopic discrimination are, at least partly, characteristic of earlier decay stages. Recent studies suggest that soil microorganisms may alter the isotopic signature of SOM during decomposition, through mechanisms such as metabolic discrimination, selective consumption of substrates and preferential use of intramolecular position within substrates (Schmidt and Gleixner 1998; Hobbie and Werner 2004). As decomposition of fresh plant litter progresses and the decomposition products become incorporated into the soil profile, the ^{13}C isotopic signature normally increases, by about 2–4‰, with increasing soil depth (Handley and Scrimgeour 1997; Boutton et al. 1998; Buchmann et al. 1998; Glaser 2005); this was also the case in the present study.

The same pattern of $\delta^{15}\text{N}$ enrichment of SOM with depth in the soil profile was observed. In whole soil, $\delta^{15}\text{N}$ is related to the degree of decomposition (Koba et al. 1998). The $\delta^{15}\text{N}$ profiles may reflect ^{15}N fractionation by microbial decomposers (Silfer et al. 1992) and increasing substrate age (Tiessen et al. 1984). However, patterns of $\delta^{15}\text{N}$ enrichment in soil profiles are not consistent across locations. Shearer et al. (1978) reported higher mean $\delta^{15}\text{N}$ values in soil samples collected near the surface than in

those from underlying horizons, across a range of cultivated and undisturbed sites. Similar trends have been reported by Riga et al. (1970) for agricultural soils. In other cases, little variation in $\delta^{15}\text{N}$ with depth has been observed (Rennie et al. 1975).

Natural abundance of ^{13}C and ^{15}N in SOM fractions

It is important to note that any technique that requires repeated soil washing and decanting such as the density fractionation method may result in loss of organic compounds (Gavinelli et al. 1995), with resulting impacts on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of the fractions. However, use of stable isotopes yielded some insight into the nature of density fractions.

The light fraction obtained by density fractionation primarily represents labile material of relatively recent origin, and we would expect this fraction to reflect ^{13}C and ^{15}N values similar to current vegetation (Compton and Boone 2000; Accoe et al. 2002).

The OMF was enriched in ^{13}C relative to OF and FPF in accordance with the findings of other authors (e.g. Six et al. 2001; Fernández et al. 2003; Crow et al. 2006). Average variations in $\delta^{13}\text{C}$ values of 10‰ between OMF and the other fractions were observed (Fig. 5). These variations in $\delta^{13}\text{C}$ values may be explained by past input of plant material from different types of plant (i.e. C3 compared with C4). Generally, the heavy fraction, which represents older, more humified and amorphous organic compounds, is relatively enriched in $\delta^{13}\text{C}$ (Ehleringer et al. 2000). More labile C pools are isotopically lighter than less labile pools and are utilized first by the microbial community (Crow et al. 2006). However, microbial enrichment of ^{13}C never exceeds 4‰, so that a different origin of plant material is more likely for these very large differences, which suggests that C4 plant material predominated in the past throughout the study region.

Relative enrichment of $\delta^{15}\text{N}$ was also observed in the heavy fraction in relation to OF and FPF (Fig. 5). The relative enrichment by $\delta^{15}\text{N}$ in the heavy fraction is consistent with the generally elevated $\delta^{15}\text{N}$ signatures observed in older SOM, which has been subjected to significant microbial processing (Billings et al. 2002). Liao et al. (2006) suggested that greater abundance of $\delta^{15}\text{N}$ in a fraction reflect a greater degree of humification. Kramer et al. (2003) found that $\delta^{15}\text{N}$ values increased in separated density fractions with increasing aliphaticity, which reflects microbial processing. Other researchers have shown that microbial processing results in accumulation of ^{15}N (e.g. Piccolo et al. 1996).

Conclusions

Historical transformation of *Quercus* forest to cropped lands in calcareous soils resulted in major losses of OC and

N, as expected. However, subsequent reforestation with *Pinus* throughout the past 40 years has resulted in good recovery of the original SOC and soil N. This shows a major potential for enhanced soil C storage in agricultural soils by their reversion to a forested state. The present results further reveal that in calcareous soils, accumulation of OC and N is mainly due to organo-mineral associations, resulting in physicochemical stabilization against further decomposition, which guarantees long-term C sequestration. The calcareous soils under study showed typical enrichment in ^{13}C and ^{15}N with depth, which is explained by isotopic fractionation during decomposition in relation to the increasing age of the SOM with depth. The same applies to the enrichment of ^{13}C and ^{15}N in OMF, which corresponds to a more humified OM. However, the results of the study revealed the possible past presence of C4 type vegetation in the study area.

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