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Changes in soil organic matter in a forestry chronosequence monitored by thermal analysis and calorimetry

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Cambios de la materia orgánica del suelo en una cronosecuencia forestal monitorizada mediante análisis térmico y calorimetría

Alterações na matéria orgânica do solo numa cronosequência florestal monitorizada por análise térmica e calorimetria

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ABSTRACT

Modelling soil organic matter dynamics requires reproducible and accurate data from several methods that follow such evolution based on changes in soil organic matter properties. The objective of this study is to investigate changes in the chemical, thermal and biological properties of soil organic matter after afforestation using emerging methods such as thermal analysis, isothermal calorimetry, and ^{13}C CPMAS NMR. These methods were applied to a chronosequence of soils where large losses of carbon have occurred in the 29 years since afforestation. Results show that over this period the soil organic matter becomes more aromatic, resulting in increased thermal stability and decreased microbial activity. Over longer time frames, between 29 and 40 years after afforestation, soil organic matter increased, mainly in the aliphatic and carbohydrate fractions with enhanced thermal stability and consequent metabolic changes from microbial adaptation to the new organic matter.

RESUMEN

Para entender la evolución de la materia orgánica y poder modelizarla es necesario el uso de distintos métodos que permitan seguir esa evolución en función de sus propiedades. Uno de los objetivos de este estudio es investigar los cambios que se producen en la composición química y en las propiedades térmicas y biológicas de la materia orgánica en suelos repoblados utilizando técnicas emergentes en este tipo de estudios, como los análisis térmicos, la calorimetría y la resonancia magnética nuclear. Se han aplicado estos métodos a una cronosecuencia en la que se ha producido importantes pérdidas de carbono tras el cambio de uso del suelo de agrícola a forestal. Los resultados indican que tras el cambio de uso y durante el período de pérdida de carbono, la materia orgánica evoluciona hacia un estado más aromático caracterizado por una mayor estabilidad térmica y una disminución de las velocidades de degradación microbiana. A partir de los 29 años tras la repoblación comienza la ganancia de carbono. Los resultados muestran que la materia orgánica acumulada tras el período de pérdida es más rica en compuestos alifáticos y carbohidratos que la original y que dichos cambios también se acompañan de un aumento de la estabilidad térmica y cambios biológicos que indican adaptaciones metabólicas de la población microbiana al carbono secuestrado. La aplicación de los métodos propuestos proporciona un conocimiento más profundo sobre la incidencia de los cambios de las propiedades de la materia orgánica sobre su degradación biológica que afecta al concepto de la capacidad de secuestro que deriva de los estudios basados exclusivamente en datos cuantitativos de la cantidad de carbono.

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RESUMO

Para compreender a evolução da matéria orgânica e poder aplicar modelos é necessário o recurso a diferentes métodos que permitam acompanhar essa evolução em função das suas propriedades. Um dos objetivos deste estudo é investigar as alterações que ocorrem na composição química e nas propriedades térmicas e biológicas da matéria orgânica dos solos após florestação, utilizando nestes estudos técnicas emergentes como a análise térmica, a calorimetria e a ressonância magnética nuclear. Estes métodos foram aplicados a uma cronosequência onde ocorreram importantes perdas de carbono na transição de solo agrícola para florestal. Os resultados mostram que a mudança de uso e durante o período em que ocorrem as perdas de carbono, a matéria orgânica evolui até um estado mais aromático caracterizado por uma maior estabilidade térmica e uma diminuição das velocidades de degradação microbiana. A partir dos 29 anos após a florestação começam os ganhos de carbono. Os resultados mostram que a materia orgánica acumulada após o período de perda é mais rica en compostos alifáticos e hidratos de carbono que a original e que as referidas alterações também são acompanhadas de um aumento da estabilidade térmica e alterações biológicas que indicam adaptações metabólicas da população microbiana ao carbono sequestrado. A aplicação dos métodos propostos proporciona um conhecimento mais profundo da incidência das alterações das propriedades da materia orgánica e sua degradação biológica que afeta o conceito da capacidade de sequestro que resulta de estudos baseados exclusivamente em dados quantitativos da quantidade de carbono.

KEY WORDS
Afforestation, soil basal metabolism, heat rate, calorespirometric ratios

PALABRAS CLAVE
Repoblación, metabolismo basal del suelo, tasa de calor, razones calorrespirométricas

PALAVRAS-CHAVE
Florestação, metabolismo basal do solo, taxa de calor, razões calorrespirométricas

1. Introduction

Land use change is one of the main practices contributing to rapid losses of soil carbon, C in the form of CO_2. About one fifth of global anthropogenic CO_2 emissions (Van der Werf et al. 2009) are transferred from the soil to the atmosphere as a consequence of changes in land use. Identification of changes in soil organic matter (SOM) over time after a land use change is therefore of great interest for understanding SOM stability and turnover. Conversion of arable land to forest land increases C sequestration in biomass, but the impact on soil is less clear. Little is known about the time-dependant changes in SOM chemical properties linked to SOM degradation (Cerli et al. 2008; Huang et al. 2011), this being a reason to introduce new methods that can assess SOM evolution based on SOM composition and/or on different SOM properties rather than in quantitative terms.

Cross Polarization-Magic Angle Spinning (^{13}C-CPMAS) nuclear magnetic resonance (NMR) provides data on the chemical structure of SOM. It is a type of NMR spectroscopy using a solid sample that is spinning very fast at the magic angle (54.74 degrees with respect to the direction of the magnetic field). Cross polarization is combined with the magic angle spinning to enhance the signal from weakly coupled nuclei such as ^{13}C. It is used to reveal the chemical nature of the carbon constituting the SOM (Knicker 2011) but it is time consuming and prohibitively expensive. Measuring SOM dynamics requires simple, accurate and reproducible methods that can be used with large numbers of samples to obtain data on both the chemical structure and biological activity of organic matter associated with soil samples. In recent years, differential scanning calorimetry (DSC) and thermogravimetry (TG) have been used to meet the increasing demand for rapid and more reproducible assessment of SOM quality and stability (Barros et al. 2007; Plante et al. 2009). DSC and TG measure thermal stability, which is postulated to be linked to SOM chemical composition. Thermal analysis may therefore be useful for inferring shifts in SOM qualities that affect

microbial metabolism. However, few studies have attempted to relate the thermal properties of SOM to the biological properties (Marinari et al. 2010; Plante et al. 2011; Siewert et al. 2012).

Metabolic rates of SOM decomposition can be measured by isothermal calorimetry, a simple, sensitive and reliable procedure that reduces the problems derived from the lengthy handling practices used with more traditional techniques (Sparling 1983; Albers et al. 1995; Critter et al. 2004). Isothermal calorimetry measures the metabolic heat rate that provides a more global quantification of total metabolism in soil, since CO_2 is limited to respiration processes usually involving heterotrophic metabolism and oxidation of carbohydrates. Degradation of substrates that are more reduced than carbohydrates, such as aliphatic or aromatic C, may not release CO_2 but instead release heat during early degradation reactions. As a result, CO_2-production can assess the degradation of SOM fractions considered labile, while the heat production rate can measure the degradation of the SOM continuum. Isothermal calorimetry can also be used for simultaneous measurement of the basal metabolic rate, expressed as both the heat rate (Φ) and soil respiration rate (RCO_2) (Barros et al. 2010; Barros et al. 2011) and provides the calorespirometric ratio (Φ/RCO_2), which gives information about the redox state of substrates being metabolized by the microorganisms, and the efficiency of conversion of substrate carbon into living cells in processes associated with microbial biomass gain (Hansen et al. 2004; Wadsö et al. 2004; Barros et al. 2010). This parameter has been successfully used in plant and insect studies (Summers et al. 2009; Acar et al. 2004), but determination of the Φ/RCO_2 ratio has only recently been reported for basal metabolism in soils (Barros et al. 2011). The latter study showed that measurement of this ratio can provide some insight into the nature of the organic substrates being degraded, and may contribute to knowledge about SOM turnover, thus avoiding some of the difficulties in establishing the biological stability of SOM exclusively through respirometric CO_2 measurements (Plante et al. 2011).

In this study, all of these methodologies are applied to a chronosequence of forest stands established in former pasture land in which the SOM has been greatly depleted (Pérez-Cruzado et al. 2012a, 2012b). The proposed methods measure changes in the chemical structure and degradability of SOM in the chronosequence, and yield a more complete assessment of SOM changes due to soil management than those based exclusively on C quantitation data.

2. Material and Methods

2.1. Stand selection and sampling

The study was carried out in NW Spain (Lugo) on former pasture that has been under low intensive management for many years but afforested with *Pinus radiata* at different times. Site preparation for forest establishment consisted of ripping with no fertilization. No tillage or weed control was carried out in the plantations. Additional information about stand characteristics and experimental design can be found in Pérez-Cruzado et al. (2012a) and Pérez-Cruzado et al. (2013).

Representative whole rotation length was achieved through consideration of *Pinus radiata* stands in different stages of rotation for these species in the region: establishment (3 yr), young stages immediately after canopy closure (13 yr), mature plantations (29 yr), and end of rotation (40 yr). Stands representative of the dynamic process under study were selected by choosing stands at each development stage where the value of the C best matches (confidence interval of 95%) the global tendency observed through non-parametric statistical analysis for the 40 pairwise plots previously studied by Pérez-Cruzado et al. (2012a).

The 20-year-average annual rainfall is 900 mm and the average annual temperature is 13.5 ºC. The wettest month is November, with

an average rainfall of 139 mm, and the driest August, with 45 mm. The lowest mean monthly temperature, 9.5 ºC, occurs in February, and the highest, 19.1 ºC, in August. The soil was developed from schist, and is classified as Alumi-humic Umbrisol (IUSS Working Group WRB 2006). The soil has a sandy loam texture and is moderately well drained. The soil humidity and temperature regimes are Udic (mean period with partial drought, 1 month) and Mesic (mean frost-free period, 10 months) respectively.

Soil collected from 0-10 cm was used for the study. The sampling procedure is explained in detail in a previous article (Pérez-Cruzado et al. 2013). The soil samples and the chronosequence for this paper were selected representing a general trend of C evolution observed in previous works involving a network of 120 paired plots of 120 pastures adjacent to 120 afforested plantations under different tree species. A 20 m diameter plot was established within each stand at a distance of more than 30 m from the edges to avoid the edge effect. Five samples per plot were taken from between tree rows to minimize any disturbance from the site preparation. The forest plots were the same as established for C determinations in aboveground biomass.

2.2. General soil properties

The pH of the soil (measured with dry, 2 mm sieved soil) was measured in 0.1 M KCl (soil: solution ratio 1:2.5) with a glass electrode. Total C and N (measured in ground samples) were determined with a LECO Elemental Analyzer. Previous analysis of organic C showed it constitutes 94 to 98% of the total C in this study (data not shown). At each sampling plot, five soil cores were collected with a 100 cm^3 metal cylinder and oven-dried at 105 ºC to determine bulk density in Mg ha^{-1} (Blake and Hartge 1986).

2.3. ^{13}C-CPMAS NMR analysis

^{13}C-CPMAS NMR reveals the contribution of different C types to SOM, and gives further insight into the interpretation of SOM thermal properties. It was applied to the samples showing the greatest differences in thermal properties: the pasture sample used as reference, the soil with the minimum C content after afforestation (29 yr stand), and the sample representing the end of the rotation (40 yr stand). Samples studied by ^{13}C-CPMAS were treated with HF following the procedure reported by Duguy and Rovira (2010). 1D ^{13}C-^{1}H CPMAS NMR experiments were performed at 298 K in a 17.6 T Varian Inova-750 spectrometer equipped with a T3 solid probe (Agilent, Inc, USA) operated at a 750 MHz proton frequency and 188.6 MHz ^{13}C frequency. Samples were prepared in 3.2 mm rotors with an effective sample capacity of 22 µL, with approximately 30 mg of powdered sample. Carbon chemical shifts were referred to the carbon methylene signal of solid adamantane at 28.92 ppm that was also used to calibrate the 1D CPMAS experiments. The inter-scan delay was set at 0.5 s, the number of scans was 100000 and the MAS rate was 20 kHz. Heteronuclear decoupling during acquisition of the FID was performed with Spinal-64 at a proton field strength of 70 kHz. The cross polarization time was 1 ms. During cross polarization, the field strength of the proton pulse was held constant at 75 kHz, and that of the ^{13}C pulse was linearly ramped with a 20 kHz ramp near the matching sideband. Other relevant experimental conditions used for the CP-MAS experiment were the contact time of 1 ms and the pre-scan delay of 0.5 s, a compromise between sufficient sensitivity to obtain quantitative signal integrals and a reasonable measurement time.

The ^{13}C spectra obtained at 188.6 MHz can be advantageous over the usual spectra of soils acquired at lower ^{13}C frequencies since it affords a substantial gain in signal resolution and sensitivity per milligram of sample. However, the large magnetic field can result in a certain amount of spinning side bands that affect the results. In our experience with ^{13}C CPMAS spectra obtained at the conditions described above with pure organic compounds, the spinning side bands are quite small. These only appear for carbonylic groups and usually account at most for 1-2% of the integral of the central peak.

The solid-state ^{13}C-CPMAS NMR spectra were manually phased, baseline corrected and integrated with MestreNova software (Mestrelab Research Inc). For integration, the spectra were divided into four regions representing different chemical environments of a ^{13}C nucleus: alkyl C (0-45 ppm), O-alkyl C (45-110 ppm), aromatic C (110-160 ppm), and carbonyl C (160-210 ppm) (Knicker and Lüdemann 1995; Leifield and Kögel-Knabner 2005). The alkyl/O-alkyl C ratio, i.e. the ratio of alkyl C region intensity (0-45 ppm) to O-alkyl C region intensity (45-110 ppm), was calculated as suggested by Baldock and Preston (1995). This ratio is considered an indicator of the extent of decomposition and/ or of substrate quality for microbes.

The aromaticity was calculated from **equation 1**:

$$Aromaticity\ (\%) = \left(\frac{AromaticC}{AromaticC + AlkylC + O - alkylC} \right) x100 \quad (1)$$

as proposed by Hatcher et al. (1981). This equation can also be used to assess the extent of SOM humification, under the assumption that SOM becomes aromatic during decomposition (Dai et al. 2001).

2.4. Thermal analysis

Differential scanning calorimetry (DSC model Q100, TA Instruments) was applied in order to obtain SOM thermal properties and monitor the evolution of those properties in the soil samples with respect to the original reference soil. For DSC analyses, soil samples were dried and gently ground in an agate mortar. Analyses were conducted at a heating rate of 10 ºC min^{-1} from 20 to 600 ºC under a flux of dry air of 50 ml/ min as described by Dell´Abate (Dell'Abate et al. 2000). Aliquots of soil (10 mg) were placed in open aluminium pans in all measurements. Temperature and heat calibrations were done by melting indium. Integrated areas and peak heights of the exothermic DSC events were measured from a linear baseline drawn between 160 and 600 ºC, avoiding the small endothermic peak at 575 ºC caused by a solid-solid phase change in quartz and peaks due to dehydroxylation of clay minerals. Results are expressed on a dry weight basis in kJ per gram of soil, and the exothermic heat (Q) was also normalized to the C content of the soil samples and expressed in kJ/g C.

T50 values, defined as the temperature at which half of the exothermic energy is released, were calculated as suggested by Rovira et al. (2008) to check the relation with SOM properties. Origin Pro Lab software was used for deconvolution of DSC curves, and the area of each component peak was expressed as a fraction of the total DSC curve area. Temperatures at the maxima of the deconvoluted peaks were also determined. Significance of the deconvolution was determined by the Chi-Square goodness of fit test.

2.5. Isothermal calorimetric measurements

Soil basal metabolism was monitored in a TAM 2277 calorimeter (TA Instruments), a heat conduction calorimeter with 3 calorimeter channels. Each channel has two calorimeter ampoules: one for the sample and the other for a reference sample. The calorimeter was statically calibrated for the soil measurements at an amplifier setting of 300 microwatts (µW) full scale. For calorimetric analysis, soil samples were sieved (2 mm) and stored at 4 ºC in polyethylene bags. Prior to calorimetric experiments, the samples were adjusted to 60% of water holding capacity (WHC) and incubated for approximately 48 hours at the temperature of the calorimetric measurements, 25 ºC, as suggested in previous articles (Harris et al. 2012).

Each soil sample was prepared for calorimetric measurements after equilibration at 25 ºC by weighing 1.5 g into each of three 4-mL stainless steel ampoules; the open ampoules were then left together with a vial containing water in a sealed polyethylene bag for 48 hours. This treatment allows the soil metabolism to equilibrate to the measurement temperature (25 ºC) and allows the samples to reach

vapour equilibrium. A small vial containing 0.2 mL of 0.4M NaOH was then placed in one of the sample calorimetric ampoules. The three sample calorimetric ampoules were then closed and placed in the calorimeter at the same time, together with the reference ampoules filled with silica sand in order to maintain inert conditions in the reference ampoule.

The calorimetric channels with only soil measure the basal metabolic heat production rate, Φ, continuously in microwatts, μW or μJ/s, while the channel with NaOH measures the soil basal metabolic heat production rate plus the heat produced by the reaction between metabolic CO_2 and NaOH:

$$2NaOH + CO_2 = Na_2CO_3 + H_2O \quad (2)$$

The enthalpy change, ΔH, of the CO_2 reaction with the NaOH is -108.5 kJ mol^{-1} at this concentration of NaOH (Criddle et al. 1991; Russell et al. 2006). At the end of the experiment, the NaOH vial was removed and the sample resealed and replaced in the calorimeter to check the basal metabolic heat rate of the soil sample containing the NaOH against the basal metabolic heat rate recorded in the other two soil samples. The procedure is explained in detail in previous articles (Barros et al. 2010; Barros et al. 2011).

The metabolic heat production rate, Φ , the rate of CO_2 production, RCO_2, and the ratio of metabolic heat production rate to CO_2 production rate, Φ / RCO_2, were calculated from the tabulated Φ data by averaging the Φ values from the two channels with only soil and subtracting the values from the Φ measured from the soil sample with NaOH:

$$\phi_{(s+NaOH)} - \phi_{(s)} = \phi_{(CO_2)} \quad (3)$$

where $\Phi_{(s\ +\ NaOH)}$ is the heat production rate in microwatts per gram of soil, $\mu W g^{-1}$, in the calorimetric channel with the soil and the NaOH vial, and $\Phi_{(s)}$ is the heat production rate in the calorimetric channel with the soil only. The difference yields the heat production rate of the reaction between the NaOH and the CO_2, $\Phi_{(CO2)}$. The following quotient yields the rate of CO_2 released by the soil basal respiration, RCO_2, given in picomols of CO_2 per gram of soil and per second, picomol CO_2 $g^{-1}s^{-1}$:

$$\frac{\phi_{CO_2}}{\Delta H} = RCO_2 \quad (4)$$

where ΔH is the enthalpy of the reaction between the CO_2 and the NaOH.

The calorespirometric ratio is then determined by the following equation:

$$\frac{\phi_s}{RCO_2} = \phi / RCO_2 \quad (5)$$

and given in kilojoules per mol of CO_2, kJ mol^{-1} CO_2.

This quotient is related to the redox state of the substrate being metabolized by the microorganisms (Hansen et al. 2004; Wadsö et al. 2004; Barros et al. 2010). The RCO_2 and Φ are divided by the active biomass to give the metabolic quotient, qCO_2, and the heat to biomass ratio, qmic. Both rates were also normalized to the C content of the samples. Soil active microbial biomass, C_{mic}, is calculated by the recently adapted Sparling's method (Sparling 1983; Sesto-Cabral and Sigstad 2011) and given in micrograms of microbial biomass C per gram of soil, μg C_{mic} g^{-1}, and related to the soil C content to give the ratio of microorganisms to soil carbon, C_{mic}/C. All these indices were calculated on a dry weight basis.

2.6. Statistical analysis

Correlations among the indices determined in this paper were calculated by Pearson´s correlation coefficient using Origin Pro Lab Software. Three replicates from each soil sample were used in elemental analysis, DSC measurements, and isothermal calorimetric measurements. Uncertainties are based on the average and standard deviation of the three replicates. A reproducibility of 10% is assumed for the ^{13}C-CPMAS results (Knicker 2011). Effects of afforestation are explored by mean contrasts.

3. Results

3.1. Evolution of soil chemical elemental properties (Table 1)

The soil of the original pasture was characterized by high C content. Afforestation led to large losses in C (70% of the original C) during the first 29 years. This chronosequence only showed partial C gains at the end of the rotation compared to that of the pasture precursor. The C losses taking place over 29 years was accompanied by decreases in the C/N ratio and pH. The organic surface layer steadily increased during the entire chronosequence. The C/N ratio decreased during the C lost period but increased at the end of the rotation reflecting the partial recovery of C into the soil. The pH started to decrease 3 years after afforestation, reaching the minimum value at the end of the rotation.

Table 1. Soil elemental properties. Average n = 3 ± SD

Forest age (yr)	Soil pH	C (%)	N (%)	C/N	Organic layer (Mg ha^{-1})
0	5.8±0.8	12.4±0.2	0.88±0.06	14.2±0.9	0.00
3	5.8±0.7	11.0±0.8	0.83±0.02	13.3±1.3	0.31
13	5.6±0.1	6.5±0.1	0.47±0,03	13.8±1.1	30.96
29	5.3±0.9	3.7±0.1	0.35±0.04	10.5±1.4	56.22
40	4.6±0.2	6.7±0.1	0.46±0.04	14.7±1.5	69.32

3.2. Changes in soil organic matter composition through the rotation (Table 2)

The ^{13}C-CPMAS NMR spectra (Figure 1) were dominated by the O-alkyl C (45-110 ppm) and alkyl C (0-45 ppm) regions, with less abundant aromatic C (110-160 ppm) and carbonyl C (160-210 ppm). The O-alkyl C percentage increased with time after afforestation, while the carbonyl C decreased. At the end of the rotation (40 yr), the partial C recovery was mainly in alkyl C and O-alkyl C. The alkyl C/O-alkyl C ratio (A/O-A) was lower in the 29 and 40 yr samples than in the pasture reference, indicating evolution of SOM in the forest samples to a lower degree of degradation than in the pasture. The aromaticity of C increased in the 29 yr old stand compared with the pasture reference, but the alkyl and O-alkyl C gain between 29 and 40 yr caused a decrease in aromaticity in the 40 yr sample compared with that of the pasture reference.

Table 2. Relative contribution (%) of signal intensity of chemical shifts in the solid-state ^{13}C-CPMAS spectra, as well as the ratio of alkyl C to O-alkyl C (A/O-A) and percent of aromaticity

Forest age (yr)	Alkyl-C	O-alkyl-C	Aromatic	Carbonyl-C	A/O-A	Aromaticity
0	27	45	14	14	0.600	16
29	21	51	21	7	0.411	23
40	25	56	14	5	0.446	15

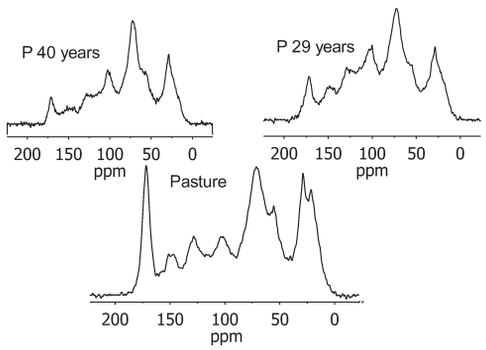

Figure 1. ^{13}C CPMAS spectra of soil samples given in ppm. The spectra P 29 years represents the SOM 29 years after afforestation. The spectra P 40 years is yielded by the SOM 40 years after afforestation.

3.3. Evolution of biological properties

Measurements of soil microbial biomass (C_{mic}) and soil basal metabolic heat (Φ) and CO_2 (RCO_2) rates showed that biological properties changed during the rotation. Soil microbial biomass (C_{mic}, **Figure 2a**) decreased throughout the chronosequence. The decrease in C_{mic} is

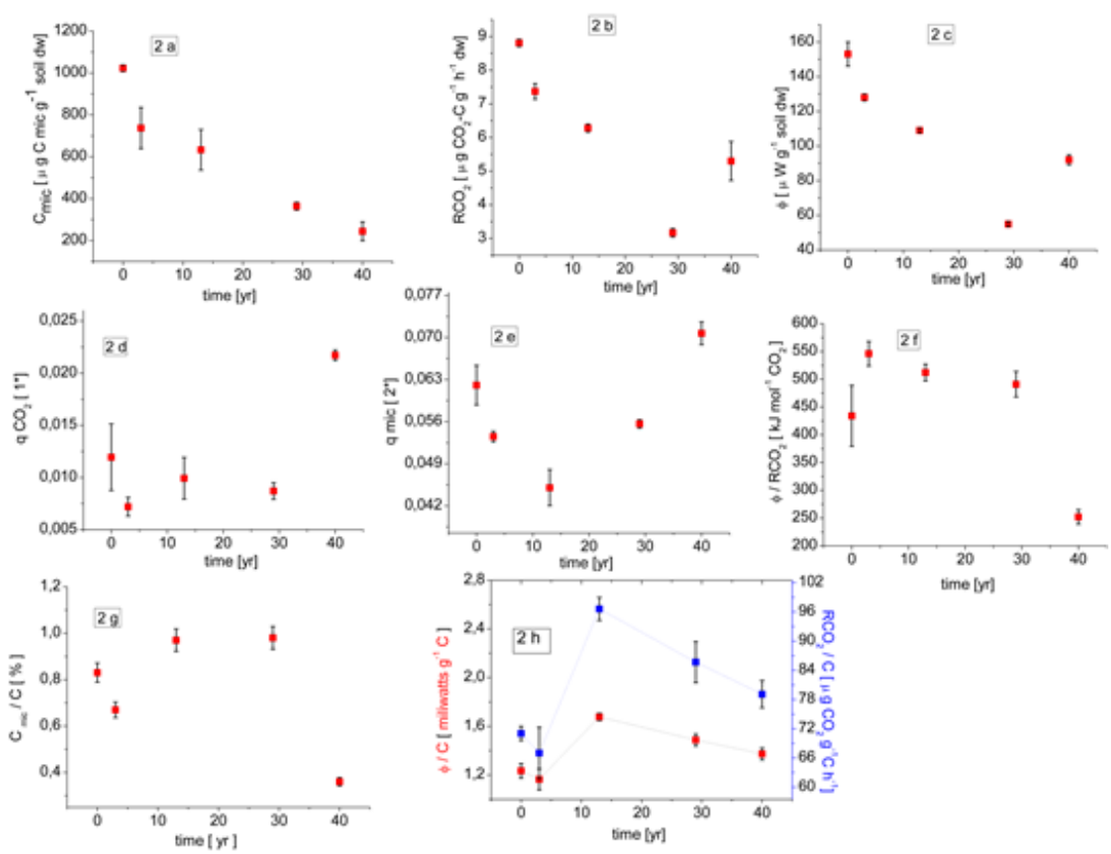

Figure 2. Evolution of the soil microbial data calculated in this work along the chronosequence. Figures: **2a**, soil microbial biomass; **2b**, CO_2 rate per gram of soil; **2c**, heat rate per gram of soil; **2d**, metabolic quotient given in μg CO_2-C μg^{-1} C_{mic} h^{-1} [1*]; **2e**, heat rate to microbial biomass ratio in μW μg^{-1} C_{mic} [2*]; **2f**, calorespirometric ratio; **2g**, microbial biomass to soil C ratio; **2h**, CO_2 and heat rates normalized to the C content of samples.

correlated with C and N depletion along the chronosequence (Table 3), except the C gain at the end of the rotation (40 yr) that was not accompanied by a proportional increase in C_{mic}. RCO_2 and Φ (Figures 2b and 2c) were both correlated with C and N content (Table 3), including the increase between 29 and 40 years.

The ratio of C_{mic} to the C content (C_{mic}/C) (Figure 2g) showed that the amount of C in biomass increased from around 75% for the early periods to nearly 100% at the intermediate period and then decreased to about 35% at 40 yr. The metabolic quotient, qCO_2 was approximately constant up to 29 yr (Figure 2d) and then increased at 40 yr. The heat to biomass ratio, qmic (Figure 2e), decreased during the first 13 yr after which it increased. Both of these indices of metabolic efficiency were greater in the 40 yr forest sample than the pasture reference. Figure 2h shows Φ and RCO_2 normalized to the C content. Both rates were higher after afforestation than that of the pasture. The observed evolution showed similar biological stabilization in the 3 yr sample to that in the pasture, followed by a remarkable increase of microbial SOM degradation rates in the sample representing the 13 yr stand. After that period there was a trend of these rates decreasing with time. Φ and RCO_2 rates lead to differing calorespirometric ratios (Φ/RCO_2, Figure 2f). The calorespirometric ratio increased from 430 kJ/mol CO_2 before afforestation to 550 kJ/mol CO_2 shortly after afforestation, declined slowly to 480 kJ/mol CO_2 at 29 yr, then declined to a final value of 250 kJ/mol CO_2 in the 40 yr sample. This may be interpreted as a shift in oxidation state of the substrate being degraded.

Table 3. Correlations found

		Φ	RCO_2	Q
C	Pearson coeff.	0.928*	0.957**	0.957*
N	Pearson coeff.	0.958**	0.915*	0.970**
C_{mic}	Pearson coeff.	0.963**	0.874*	0.957*
Φ	Pearson coeff.	-	0.892*	0.992***
RCO_2	Pearson coeff.	-	-	0.939*

Pearson coeff.: Pearson´s correlation coefficient. Statistical signification: * 0.05; ** 0.01, *** 0.0001.

3.4. Thermal properties of bulk soil samples (Table 4)

Figure 3 shows the DSC curves obtained for soil samples were all bimodal and varied in area along the chronosequence. T50 values (Table 4) increased at 29 and 40 years indicating SOM evolution to a more thermally stable state than in the pasture and at earlier times in the rotation. None of both T50 and Q_C reflected changes in SOM thermal properties during the C lost period (3yr and 13 yr samples). The integration of DSC curves gave the total heat (Q) in kJ per gram of soil (Table 4). Q was correlated with the soil C content, N content, C_{mic}, and the heat and CO_2 rates from microbial metabolism (Table 3). Normalizing Q to soil C gives the values of Q_C in Table 4. The Q_C value at 40 yr is remarkably lower than the Q_C values from the other samples. Deconvolution of the DSC curves into two peaks yielded R^2 = 0.99 values in all samples with Chi-Square values < 1.0 in all cases (Figure 4) indicating significant fits to Gaussian curves. The temperatures of the peaks in Figure 4 were not particularly informative, but the contribution of each peak to total DSC area (PA1 and PA2)

changed along the chronosequence. The ratio, PA1/PA2, was correlated with the aromaticity of SOM as determined by ^{13}C-CPMAS NMR ($r = -0.99$, $p < 0.0005$). Although it is important to take into account the low number of points when interpreting the obtained correlations, all of them were significant at the 0.05 level.

Table 4. DSC quantitative data

Forest age (years)	T 50	PA1 (%)	PA2 (%)	PA1/PA2	Q_c (kJ g^{-1} C)	Q (k Jg^{-1})
0	354 ± 1	54.2	45.8	1.18	24 ± 1	2.93 ± 0.09
3	352 ± 1	62.0	38.0	1.63	23 ± 3	2.47 ± 0.06
13	354 ± 3	65.2	34.8	1.87	25 ± 1	1.65 ± 0.03
29	363 ± 2	39.2	60.8	0.64	27 ± 1	0.98 ± 0.01
40	373 ± 1	61.4	38.6	1.59	16 ± 1	1.10 ± 0.01

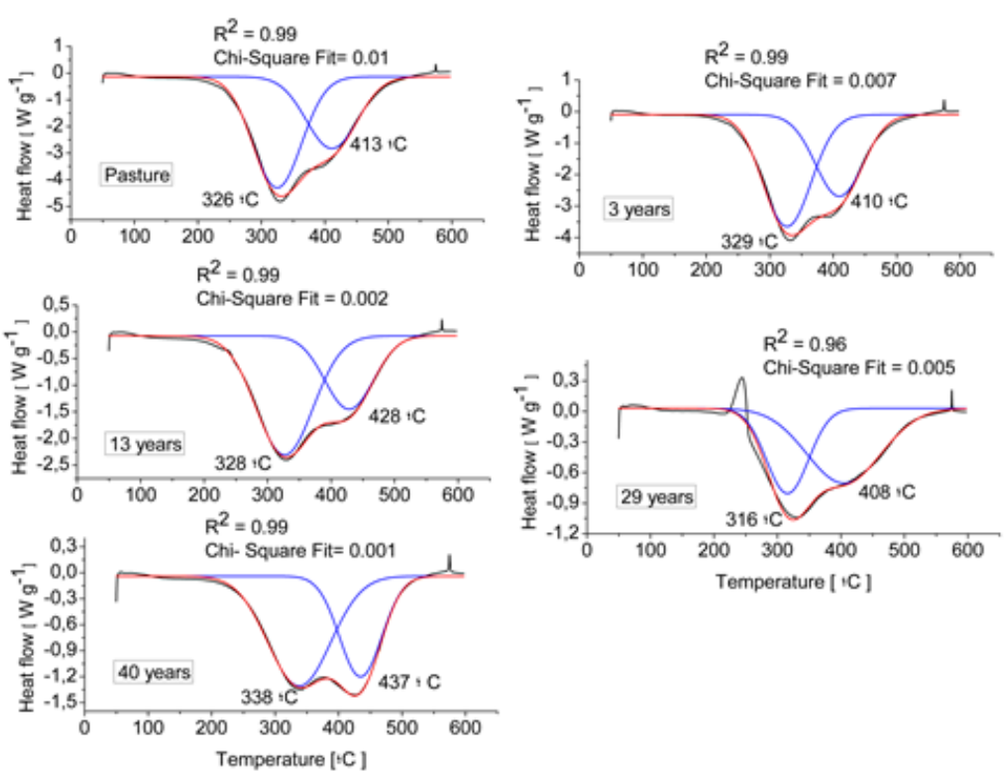

Figure 4. Results for the two peaks fit to DSC curves to monitor the variation of the peak height of both peaks in the chronosequence. Red lines represent the theoretical Gaussian curve fit. Black lines are the original DSC curves. Blue lines are the deconvoluted peaks. Each plot shows the correlation coefficient and the Chi-Square fit values of the curves adjustment, together with the temperatures of the deconvoluted peaks.

4. Discussion

4.1. Chemical and Thermal SOM properties

Afforestation causes qualitative and quantitative changes in the chemical structure of SOM in the soil. With increasing time after afforestation, the ^{13}C-CPMAS data show an increasing relative contribution of the O-alkyl-C corresponding to cellulose, and a depletion of the relative contribution of carbonyl C. The contribution of carbonyl is usually attributed to humic substances (Dai et al. 2001) suggesting evolution of SOM to a less humified state in the afforested stands in agreement with the A/O-A ratios. Lower A/O-A ratios are attributed to a lower degree of degradation of SOM by soil microbes (Baldock and Preston 1995). SOM in the stand with the minimum C (29 yr) is more aromatic than in the rest of the stands. The increased aromaticity can be attributed to the fact that terrestrial residues become more reduced as decomposition proceeds (Baldock et al. 2004). Decreased aromaticity at the end of the rotation can be explained by a higher contribution of external C inputs in agreement with the evolution of the organic litter shown in **Table 1**.

The areas of the DSC curves in **Figures 3** and **4** were correlated with the total carbon content. Therefore if DSC curves are given on mass basis as in **Figure 3**, the observed evolution reflects the C quantity and the SOM nature. If heat from combustion is normalized to the C content, a value should be obtained which is attributable to the nature of the SOM through the Q_C determined in this work. Results here indicated that the heat of combustion is not completely captured by the DSC. For comparison with the Q_C values in **Table 4**, the heats of combustion of alkyls, aromatics and carbohydrates are approximately 57, 47, and 38 kJ g^{-1}C, respectively. Considering the composition of the SOM, the obtained Q_C data are far from these values. Another factor influencing Q_C are endothermic reactions appearing in soils with lower C percentages, caused by dehydroxylation of clays, as occurred in sample 29 yr, indicating the presence of gibbsite in that soil (Fernández et al. 2012). Therefore, the Q_C values cannot be exactly related to the SOM chemical nature in this work.

The first combustion peak in all DSC curves obtained here is commonly assigned to more labile substrates, mainly cellulose (Baffi et al. 2007) while reactions at temperatures higher than 400 ºC are attributed to more recalcitrant and/or refractory C, such as aromatic substrates and lignin (Plante et al. 2009; Mamleev et al. 2009). Under these generally accepted assumptions, deconvoluted peak areas may provide additional information about the SOM. In spite of the fact that they do not necessarily represent the reactions taken place during the heating processes, deconvolution permits us to theoretically quantify the contribution of the energy derived from the more or less labile SOM substrates to the total DSC curve, and indirectly to the total SOM. It is observed in **Figure 3** that although the temperatures of the combustion peaks are relatively insensitive to changes in the SOM in the younger stands, the relative areas of the deconvoluted peaks changed remarkably through the chronosequence (**Table 4**). The PA1/PA2 ratio increases at 3 and 13 years after afforestation, due to increased contribution of PA1 during that period; the ratio goes through a minimum at 29 yr, in agreement with the higher aromaticity of this sample, and increases again at 40 yr, reflecting the gain in cellulose given by NMR. The PA1/PA2 ratio was also strongly influenced by the aromaticity of samples as determined by ^{13}C CPMAS. Therefore, deconvolution of DSC curves may give additional information to track the evolution of SOM due to afforestation.

4.2. Soil Biological properties

Afforestation causes C losses that take place in parallel with the depletion of microbial biomass and a proportional decrease in metabolic rates during the first 29 years after afforestation in this chronosequence. The C content and metabolic rates given per gram of soil are partially recovered at the end of the rotation (40 yr), but are lower than those in the pasture reference. This effect is widely discussed in literature. The C depletion is attributed to the increasing growth of aerobic decomposers that contribute to the rate of SOM mineralization after afforestation

(Paul et al. 2002; Byrne and Farrell 2005). The lower metabolic rates in forest lands compared to pastures in humid temperate regions and soils with a high C content are explained by their litter characteristics. Accumulation of C coincides with the occurrence of plant species producing compounds with antimicrobial effects that are typical in pine. This explains the low microbial biomass seen here at the end of the rotation, together with the observed decrease in the pH typical of a pine forest too (Chen et al. 2008).

The evolution of microbial metabolic data in Figure 2g shows that C_{mic}/C ratios are higher in the forest stands than in the pasture during most of the C loss period after afforestation, indicating that C mineralization takes place through a microbial metabolism that is more efficient than that in the pasture. Similar results are obtained when the heat rate and RCO_2 values are normalized to the C content. Decreases in C_{mic}/C, Φ/C and RCO_2/C indicate increasing recalcitrance of SOM (Plante et al. 2011), but during the SOM loss period, the values are higher than in the pasture reference. This indicates a lower biological stabilization of SOM in the 13, 29 and 40 yr stands compared to the pasture and 3 yr stands, which may favor the observed C losses. However, if compared to the highest Φ/C and RCO_2/C values, there is a clear trend of SOM increasing the biological stabilization 13 years after afforestation in this chronosequence.

The concomitant measurement of CO_2 and heat rate yields the calorespirometric ratio (Φ/RCO_2) of the microbial metabolism. The Φ/RCO_2 values through the chronosequence indicate changes in the type of compounds being metabolized. Φ/RCO_2 values higher than 455 kJ mol^{-1} CO_2 are attributed to degradation of substrates more reduced than carbohydrates, such as alkyl and aromatic C compounds. Values of this parameter between 455 and 240 kJ mol^{-1} CO_2 are assigned to respiration of carbohydrates; the value depends on the microbial metabolic efficiency (Hansen et al. 2004), corroborated here by the strong correlation found between Φ/RCO_2 and qCO_2. The high Φ/RCO2 values found in the forest stands during the first 29 years of the SOM loss period suggest microbial degradation of substrates that are more reduced than carbohydrates, compatible with the evolution of PA1 and PA2 in **Table 4**. The contribution of PA2 to total DSC curve area decreases with time, indicating that both the labile and most recalcitrant SOM fractions are degraded simultaneously. These data are consistent with those reported by Dorodnikov et al. (2007), who found a faster turnover of recalcitrant C compounds than expected. Although most studies on SOM dynamics after afforestation report losses of easily decomposable cellulose and hemicelluloses as well as preservation of recalcitrant organic compounds (Cerli et al. 2008), recent studies have demonstrated that degradation of the more recalcitrant SOM occurs in surface horizons, as suggested here (Fierer et al. 2003; Von Lützow et al. 2006).

Except for decreased microbial biomass, C_{mic}, the 40 yr sample does not continue the trends seen in the other four samples. The slight enhancement of qmic, the heat yield of microbial metabolism, may signal the beginning of the change in the trends from the 29 yr sample. Higher dissipation of heat per unit of biomass is associated with a less efficient metabolism (Kimura and Takahashi 1985; Von Stockar et al. 1993) that can be attributed to the increased aromaticity and thermal stability in this sample. The different chemical and thermal properties of SOM in the 40 yr sample is likely due to the different source of organic matter, i.e. pine versus pasture. At the end of the rotation, there is a partial C recovery and a proportional enhancement of heat and respiration rates, which suggest accumulation of biodegradable forms of SOM compatible with the increased contribution of O-Alkyl C given by NMR. The Φ/RCO_2 value in this soil is remarkably low compared with the rest of the samples, indicating microbial metabolism that is mainly based on carbohydrate respiration, but under a low efficient metabolism, compatible with the higher qCO_2 in this sample. The higher contribution of carbohydrates also accounts for the thermal properties, i.e. an increased ratio of PA1 to PA2 and a decrease in Q_C, but cannot explain the increment in the T50 value. Therefore the 40 yr sample shows accumulation of labile SOM degraded through a less efficient

metabolism. This evolution can only be explained by the development of SOM physical protection mechanisms in the afforested sites, as a consequence of the observed microbial activity and the decay in the pH. The initially observed enhancement of the microbial degradation rates of SOM, given per unit of C, favours the C lost but at the same time contributes to the formation of aggregates by the interaction of metabolic products of the microbial action with clays (Schulz et al. 2013; Bronick and Lal 2005). The DSC demonstrated the existence of gibbsite in the soil mineral composition of these samples that may contribute to the formation of macroaggregates protecting SOM to microbial degradation. Microbial metabolism is also responsible for organomineral interactions with Al and Fe oxides, which are very common in acid soils like the ones in this work. The stabilization of SOM by these organomineral interactions is increasingly recognized as the major long term mechanism influencing SOM decomposability and is considered responsible for enhancement of SOM thermal stability determined by DSC, since these interactions tend to increase the temperature of the exothermic reaction of SOM combustion during thermal analysis (Peltre et al. 2014). These mechanisms explain the observed T50 increment in the oldest stands and the higher biological stabilization of these samples when compared to that in the younger sites. The depletion of the calorespirometric ratio in the 40 yr sample and the decay in the efficiency of the microbial metabolism could be attributed to less availability of carbohydrates due to the physical protection.

Results here reinforce the important role of the soil microbial population on organic matter turnover, described by other authors as a function of microbial ecology (Kleber et al. 2011). Providing more indicators of soil microbial degradation, such as the heat rate due to microbial metabolism together with the CO_2 rate, as done here, can contribute to improving the understanding of relations between SOM microbial population and SOM structure. Results showed here suggest that biological stabilization of SOM involves features not indicated by the chemical characteristics as demonstrated by other authors (Bronick and Lal 2005). Therefore, tracking the degradability of SOM caused by land use change requires direct measurement of degradation and mineralization rates independently of the SOM chemical properties, together with an assessment of the thermal characteristics which describe it.

5. Conclusions

Afforestation causes changes in SOM properties that determine the rate of C mineralization. Carbon initially present from the pasture vegetation is steadily lost for about three decades before carbon input from pine begins to contribute to SOM.

SOM evolves to a more recalcitrant chemical state during the SOM lost period, characterized by higher aromaticity and higher thermal stability than the initial SOM. Carbon loss involves metabolism of both labile and recalcitrant fractions of SOM. This dynamic, together with increased contribution of forest litter as time since afforestation increases, is responsible for an inflexion in the C evolution that favours C accumulation at the end of the rotation in this chronosequence.

The microbial metabolic response indicates that microbial adaptation to the C gained is necessary and is still taking place in these samples at the end of the rotation. SOM chemical and thermal properties also indicate that afforestation with *Pinus radiata* favours accumulation of carbohydrates and thermally stable SOM that is less biologically stable at the end of the rotation than that in the pasture, but more biologically stable than in the younger stands.

Thermal properties and calorimetric data of microbial activity seem to be sensitive enough to also detect the stabilization of SOM attached to mechanisms of physical protection.

These findings demonstrate the importance of studies focussing on changes of SOM structure and degradability in order to determine the mechanisms and factors affecting C sequestration in soils.

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