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Characterization of a Mediterranean litter by ^{13}C CPMAS NMR: relationships between litter depth, enzyme activities and temperature

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Summary

Organic matter mineralization of forest litter is catalysed by the action of different extracellular enzymes produced by microorganisms. Coupling enzyme activities with data on the general macromolecular structure of organic matter, provided by cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance (^{13}C CPMAS NMR), allows researchers new insights into organic matter degradation processes. In this paper, the effect of the temperature of incubation on the degradation processes was evaluated in three distinct layers (OhLn, OhLv and OhLf) of an evergreen oak litter (*Quercus ilex* L.), located in the Mediterranean area of south-eastern France. We studied degradation phenomena by a combination of ^{13}C CPMAS NMR and microbiological analysis. In order to determine the microbial activity of litter layers, three enzyme activities (laccase, cellulase and butyrate esterase) were measured in a 6-month mesocosm study. Results showed an increase in the alkyl C to *O*-alkyl-C ratio and an increase of the phenolic C and carboxyl C regions, indicating a preferential degradation of polysaccharides. The aromaticity also increased with litter depth and degradation, and humification processes were more elevated at 30°C. ANOVA showed significant effects ($P < 0.001$) of increased temperature, depth and time of degradation on microbiological variables. Further information is needed about the variations in temperature and temperature-litter response and soil functions to link fundamental understanding of carbon stabilization, climate change and global C cycling.

Introduction

Currently, the application of the solid-state ^{13}C nuclear magnetic resonance spectroscopy with cross-polarization magic angle spinning (^{13}C CPMAS NMR) technique is widely accepted to characterize the chemical composition of soil organic matter (SOM). This technique allows identification of resonances of the different components of SOM samples and follows their degradation processes under a wide range of variables. The ^{13}C CPMAS NMR technique has been successfully used to study processes of wood pulping (Haw *et al*., 1984), lignin composition of forest humus layers (Kögel, 1986), forest fire and plant cover effects on soil chemical composition (Golchin *et al*., 1997), woody debris (Preston *et al*., 1998) and to characterize changes in the chemical composition of decomposing organic material in marine environments (Baldock *et al*., 2004), soil (Forte *et al*., 2006), litter (Lorenz *et al*., 2000) and compost (Chen *et al*., 1989; Marche *et al*., 2003) samples. The ^{13}C CPMAS NMR method differs from other techniques that characterize chemical composition of SOM (i.e. extractive techniques with strong acid and alkaline solutions), because CPMAS NMR is non-destructive, which allows more accurate correlations between chemical and biological data.

The mineralization of forest organic matter is catalysed by the action of different extracellular enzymes produced by microorganisms. This process acts in the different biogeochemical cycles by releasing nutrients that can be easily assimilated by plants and

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soil organisms. In addition, some enzymes are produced according to 'economic rules': enzyme production increases when simple nutriments are scarce and complex nutriments are abundant. The response is mediated by inductive or constitutive enzymes (Allison & Vitousek, 2005). As a consequence, enzyme dynamics during decomposition of organic matter are widely studied in litter (Gallardo & Merino, 1993; Criquet *et al*., 1999, 2000; Fioretto *et al*., 2000). Because these processes are essential in soil enzymology and forest ecosystem functioning, they are important indicators of microbial biodiversity and soil quality (Taylor *et al*., 2002). With the development of powerful NMR and enzymology techniques, we are able to find new insights into forest matter degradation. Correlating ^{13}C CPMAS NMR data with those acquired by enzymatic and microbial techniques will highlight more precisely the impact of numerous factors when studying organic matter mineralization. Currently, among the different environmental factors, there is a particular interest in studying the relation between degradation processes and temperature, because of the consequences of global climate change on forest organic matter recycling. Seasonal temperature and moisture are known to be major factors that affect microbial activities and decay rates of litters (Fioretto *et al*., 2000; Criquet *et al*., 2002) under the Mediterranean climate. In the context of global climate change, the aim of this study was to investigate the effect of increasing temperature on the chemical and biological patterns of Mediterranean litter degradation, using evergreen oak as a model. In this work, mesocosms were used and kept under constant moisture (60% WHC) in order to avoid moisture fluctuations that make difficult the study of the effects of other factors on litter degradation processes (Criquet *et al*., 2002, 2004). Based on annual mean, maximal and minimal temperatures recorded in the sampling area over 30 years (Loisel, 1976; Criquet *et al*., 2002; Nèble, 2005), three different temperatures were selected for this study: 4, 15 and 30°C. Temperature effects may be particularly pronounced under a Mediterranean climate due to the fact that many of the soils of this zone have been progressively degraded (Pascual *et al*., 1998). The vegetation of the Mediterranean bioclimate is characterized by sclerophylous tree species, of which evergreen oak (*Quercus ilex* L.) is one of the most abundant. In this work, we aim to study the effect of temperature (4, 15 and 30°C) on the relationships between microbiological processes and the dynamics of transformation of carbon pools in three distinct litter layers (OhLn, OhLv and OhLf). For this purpose, we combined ^{13}C CPMAS NMR spectroscopy with microbiological techniques (i.e. enzyme activities) during a 6-month mesocosm experiment.

Methods

Litter

Evergreen oak litter (*Quercus ilex* L.) was collected in August 2005 from a 1000 m^2 dense copse at 'La Gardiole de Rians', Var Departement (43°34′27″N, 5°42′10″E), France. Three litter layers from the Oh horizon were collected: the OhLn layer (upper layer), formed by leaves over a period of less than 1 year, non-degraded and non-compressed; the OhLv layer, formed by leaves still recognizable despite decay, non-compressed; and finally the OhLf layer, formed by non-recognizable leaves and often compressed in lumps. All samples were air dried at room temperature and stored until their utilization; 1 g of litter was weighed and dried overnight at 105°C in an oven to calculate the dry weight.

Litter mesocosm

Each mesocosm was carried out in a 30-litre polyethylene rectangular tank (40-cm long × 30-cm wide × 25-cm deep). Three litter layers were placed into the mesocosm, which was previously filled with 2 kg of soil, collected under the litter layers of the same sampling plot. Each layer was separated by a plastic grid (mesh 0.5 cm^2) to avoid mixing layers during the sampling procedure. Each layer was placed according to its location on the forest floor; OhLf followed by OhLv and OhLn, respectively. All mesocosms were covered with a transparent and perforated polyethylene film, to allow gas exchange, and a total of seven mesocosms was prepared for each temperature (4, 15 and 30°C). Mesocosms were kept moist throughout the experiment by adding the amount of distilled and sterilized water to reach 60% of the water-holding capacity of the litter.

^{13}C CPMAS NMR procedure

The cross-polarization magic angle spinning ^{13}C nuclear magnetic resonance (^{13}C CPMAS NMR) data were obtained with a Bruker Advance DSX 400 MHz spectrometer using a commercial two channel Bruker probe head (Bruker BioSpin, MRI & NMR divisions, Billerica, MA). Samples were analysed at two different times: 0 and 180 days. A composite ($n = 4$) and representative litter sample was prepared for each layer, each temperature and each date; 300 mg of dried and ground composite sample (particle size < 0.3 mm, Cyclotec™ 1093 sample mill, Foss Analytical, Nanterre) was placed in a 7-mm zirconium rotor and spun at the Magic-angle at 6 kHz. The ^{13}C CPMAS NMR technique was performed with a ramped ^{1}H pulse during a contact time of 1 ms and with ^{1}H decoupling during the acquisition time to improve the resolution. Recording 16K transients with a recycle delay of 3 s represented standard conditions to obtain a good signal-to-noise ratio. The ^{13}C chemical shifts were referenced to tetramethylsilane and calibrated with the glycine carbonyl signal, set at 176.5 p.p.m. All measurements were made at room temperature. Chemical shift ranges of ^{13}C CPMAS NMR spectra of litter are assigned to the following dominant forms: alkyl C (0–45 p.p.m.), *O*-alkyl C (45–110 p.p.m.); methoxyl C (50–60 p.p.m.); aromatic C (110–160 p.p.m.), phenolic C (140–160 p.p.m.), and carboxyl C (160–190 p.p.m.). Deconvolution of the NMR spectra was

performed using the DmFit software (Massiot, 2002). The degree of humification was calculated according to Kögel (1986) using the alkyl C to *O*-alkyl-C ratio with the respective regions of the spectra. The average chain length of the methylenic chains was estimated by the alkyl-C to carboxyl-C ratio (Knicker *et al*., 2000). Finally, the lignin content was estimated according to Haw *et al*. (1984).

Enzyme assays

Unless otherwise indicated, all enzyme activities were measured from extracts obtained according to Criquet *et al*. (1999) and reactions were followed spectrophotometrically (Uvikon 860, Kontron Instruments, Montigny Le Bretonneux, France). Laccase activity in the extracts was measured by means of syringaldazine as substrate (Harkin & Obst, 1973; Criquet *et al*., 1999). For kinetic measurements, 200 µl of enzyme extract, 790 µl of 0.1 M phosphate buffer (pH 5.7) and 10 µl of a 5 mM syringaldazine solution were mixed. The oxidation rates of syringaldazine to quinone were followed at 525 nm ($\varepsilon = 65\,000$ M^{-1} cm^{-1}) at 30°C. Initial velocities were determined from the linear parts of the curves. The results are expressed in units defined as µmoles of quinone formed from syringaldazine $minute^{-1}$ (U) g^{-1} of dry litter (U g^{-1} DW). For cellulase activity measurements, 100 µl of enzyme extract and 900 µl of 50 mM acetate buffer (pH 6.0) with 1% of CMC (carboxymethylcellulose) were mixed and incubated at 50°C for 1 hour. After the incubation time, the sugars released from the hydrolysis of CMC were measured by using the colorimetric method of Somogyi-Nelson (Nelson, 1944; Somogyi, 1952). Cellulase activity was expressed in µmoles of glucose released $minute^{-1}$ (U) g^{-1} of dry weight litter (U g^{-1} DW). Butyrate esterase activity was measured by using *p*-Nitrophenyl Butyrate (*p*NPB) as substrate. For kinetic measurements, 200 µl of enzyme extract, 690 µl of 50 mM phosphate buffer (pH 6.5) and 10 µl of a 100 mM *p*NPB solution were mixed and incubated at 30°C. Kinetics of *p*-NP release were followed at 412 nm ($\varepsilon =$ 277 M^{-1} cm^{-1}), and initial velocity was determined from the linear part of the curve. The results are expressed in units defined as µmoles of *p*-NP $minute^{-1}$ (U) g^{-1} of dry weight litter (U g^{-1} DW).

Statistical analysis

A multivariate analysis of variance (MANOVA) was performed using STATISTICA 6.1 (StatSoft, Tulsa, Oklahoma, USA. http://www.statsoft.com) to determine significant effects of temperature, depth and incubation time on litter variables (enzyme activities). The means of enzyme activities were compared using a *t*-test, with a significance level of $P < 0.05$. A multivariate ordination method, principal component analysis (PCA), was also performed to analyse the NMR data set and to show relationships between ^{13}C CPMAS NMR data and enzymes activities.

Results

Temperature effects on litter ^{13}C CPMAS NMR spectra

Initial litter. Figure 1 shows ^{13}C CPMAS NMR spectra of the three evergreen oak litter layers studied before and after incubation at 4, 15 and 30°C over a period of 0 to 180 days. Signals of the spectra have been assigned by using references obtained from previous studies about soil organic matter (Huang *et al*., 1988; Lorenz *et al*., 2000; Kögel-Knabner, 2002). Figure 1 shows that the spectra are dominated by signals from the *O*-alkyl-C region (45–110 p.p.m.), characteristic of polysaccharides. The intensity of this region decreased with litter depth compared with material at 0 hours (Table 1). Spectra are also characterized by signals from the alkyl C region (0–45 p.p.m.) characteristic of CH_3-groups of lipids, waxes and cutins (Quideau *et al*., 2000; Dignac *et al*., 2002) and whose intensity in the initial samples increased with litter depth (from 16.2 to 22.46%) (Table 1). Other important regions, whose intensities in the initial samples increased with litter depth, are methoxyl, phenolic, aromatic and carboxyl (Table 1). With respect to litter quality index, aromaticity, lignin content and humification index increased with litter depth

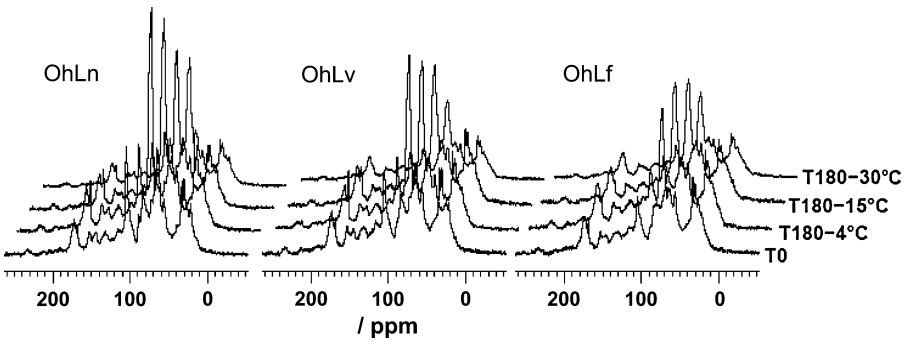

Figure 1 Solid-state ^{13}C CPMAS NMR-spectra of different oak litter layers evaluated at three temperatures (4, 15 and 30°C). Figure shows spectra at two different times of degradation.

Table 1 Litter organic matter composition from the mesocosm experiments. Integration values for the major C-types in the solid-state ^{13}C CPMAS NMR-spectra

		Carboxyl C			Aromatic C			Phenolic C			Methoxyl C			*O*-alkyl C			Alkyl C			Aromaticity			Lignin			Alkyl C:Carboxyl C			Alkyl C:*O*-Alkyl-C		
Depth	Days	4°C	15°C	30°C	4°C	15°C	30°C	4°C	15°C	30°C	4°C	15°C	30°C	4°C	15°C	30°C	4°C	15°C	30°C	4°C	15°C	30°C	4°C	15°C	30°C	4°C	15°C	30°C	4°C	15°C	30°C
OhLn	0	6.16	6.16	6.16	7.05	7.05	7.05	2.5	2.5	2.5	4.29	4.29	4.29	52.66	52.66	52.66	16.2	16.2	16.2	10.18	10.17	10.18	12.15	12.14	12.14	2.63	2.63	2.63	0.25	0.25	0.25
	180	8.58	8.83	10.4	8.57	9.22	10,00	2.71	2.83	3.45	5.24	6.23	6.11	43.67	41.81	38.61	21.25	21.23	22.69	12.33	13.21	15,00	14.89	16.04	18.4	2.48	2.4	2.18	0.39	0.41	0.5
	Variation/%	**39.28**	**43.34**	**68.83**	**21.56**	**30.78**	**41.84**	**8.4**	**13.2**	**38,00**	**22.14**	**45.22**	**42.42**	**−17.07**	**−20.60**	**−26.68**	**31.17**	**31.04**	**40.06**												
OhLv	0	8.66	8.66	8.66	10.15	10.15	10.15	3.2	3.2	3.2	6.11	6.11	6.11	41.15	41.15	41.15	21.1	21.1	21.1	14.62	14.61	14.62	17.88	17.88	17.88	2.44	2.44	2.44	0.42	0.42	0.42
	180	11.74	9.1	10.58	10.49	9.66	11.32	3.49	2.99	3.39	5.32	6.77	7.3	39.57	38.74	36.33	19.75	24.42	23.52	15.83	13.91	16.45	19.51	16.95	20.33	1.68	2.68	2.22	0.4	0.52	0.54
	Variation/%	**35.56**	**5.08**	**22.17**	**3.34**	**−4.82**	**11.52**	**9.06**	**−6.56**	**5.93**	**−12.92**	**10.80**	**19.47**	**−3.83**	**−5.85**	**−11.71**	**−6.39**	**15.73**	**11.46**													
OhLf	0	9.88	9.88	9.88	10.66	10.66	10.66	3.15	3.15	3.15	6.84	6.84	6.84	38.74	38.74	38.74	22.46	22.46	22.46	15.32	15.32	15.32	18.82	18.82	18.82	2.27	2.27	2.27	0.48	0.48	0.48
	180	11.83	9.87	10.25	12.18	10.6	10,93	3.44	3.14	3.23	6.46	7.04	7.05	36.56	36.32	37.04	21.33	25.39	23.31	17.71	15.22	15.78	22.06	18.69	19.43	1.8	2.57	2.27	0.48	0.58	0.52
	Variation/%	**19.73**	**−0,1**	**3.74**	**14.25**	**−0.56**	**2.53**	**9.2**	**−0.31**	**2.53**	**−5.55**	**2.92**	**3.07**	**−5.62**	**−6.24**	**−4.38**	**−5.03**	**13.04**	**3.78**													

Variations in major C types are given with respect to the initial values (day 0). Index variations were not calculated.

(Table 1). Finally, the average length of methylene chains decreased with depth in the initial litter samples (Table 1). In order to reduce the dimensionality of data, a PCA was performed. Figure 2 shows the results of PCA established with the chemical and enzyme variables of initial (0 day) and final (180 day) samples. The first principal component (PC 1) accounting for 57.4% of the variance, appeared representative of the chemical shifts observed in the different layers as well as during the incubation time. This PC allowed discrimination of samples rich in carbohydrates from samples rich in recalcitrant compounds. Thus samples of the upper layer (OhLn) were located at the negative extremity of the first axis (PC 1) characterized by its richness in *O*-alkyl C. On the contrary, OhLv (middle layer) and OhLf (deepest layer) samples were gradually located toward the opposite extremity of the PC 1, characterized by an increase in the recalcitrant compounds. Thus, the PC 1 translates the chemical modifications that can be observed during the course of degradation of evergreen oak leaves over diachronic or synchronic processes. Initial and final values of enzyme activities were also integrated in the PCA. The location of these enzymatic variables on the factorial map indicates that the richness in the chemical compounds was related to the corresponding degrading enzymes. Indeed, cellulase activity was located towards *O*-alkyl C, whereas butyrate esterase and laccase vectors were oriented toward the more recalcitrant compounds. Dynamics of enzyme activities over the incubation experiment are detailed in the enzyme activities sub-section.

Upper layer (OhLn). As we can see in the PCA, OhLn samples are quite separate from others samples (OhLv and OhLf). PCA shows that major modifications were found at 30°C after 180 days of degradation, OhLn becoming more quickly humified at this temperature. Based on the initial litter, the *O*-alkyl C region showed the more important variations as it decreased by 26.68, 20.60 and 17.07% at 30, 15 and 4°C, respectively (Figure 1, Table 1). In general, the higher temperature induced increases in the carboxylic C, methoxyl C, aromatic C, alkyl C and phenolic C groups (Table 1). At the same time, lignin content, aromaticity and humification index were greatest after 180 days of degradation at 30°C (Table 1). The average length of methylenic chains (alkyl C to carboxyl C ratio; Knicker *et al.*, 2000) showed that short chains dominated at 30°C after 6 months of degradation (Table 1).

Middle layer (OhLv). The OhLv layer showed similar chemical modifications to the upper layer. However, when considering PCA, the amplitude of chemical shifts along the PC 1was less than in the upper layer. As we observed in the upper layer, decrease in the *O*-alkyl C region was also faster in the OhLv with increasing temperature (Table 1, Figure 2). All the other regions increased at all temperatures (Table 1), with some exceptions for aromatic C and phenolic C, which decreased at

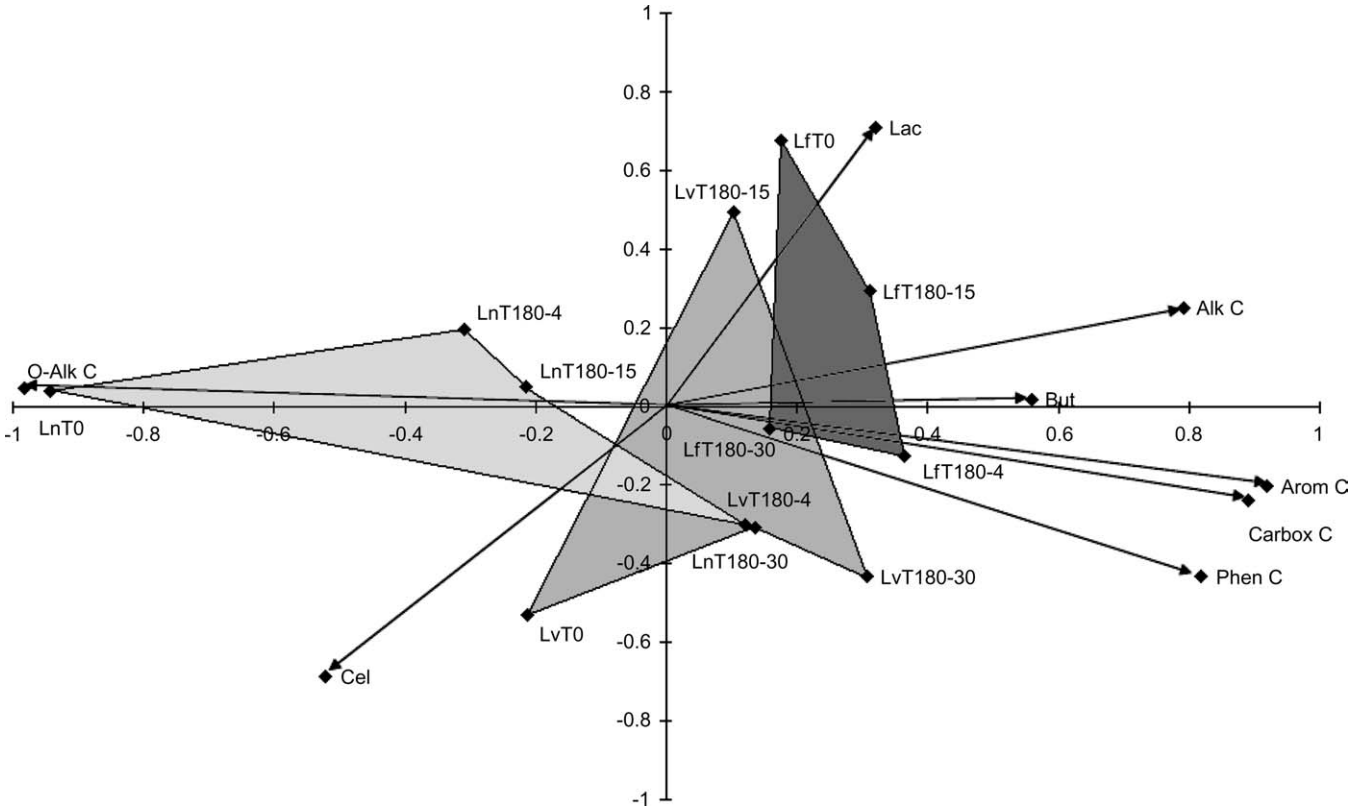

Figure 2 Enzymatic and chemical variables from the three litter layers plotted in the first two principal components. Data show the three initial litter layers (OhLn, OhLv and OhLf) and degradation time (T), after 180 days of incubation at three temperatures (4, 15 and 30°C). Chemical variables are: *O*-alkyl-C; Alkyl-C, (Alk C); aromatic-C, (Arom C); carboxyl-C, (Carbox C); and phenolic-C, (Phen C). Enzymes variables are: laccase, (Lac); cellulase, (Cel); and butyrate esterase (But).

15°C, as well as methoxyl C and alkyl C, which decreased at 4°C. As a result of this degradation, a marked increase in the alkyl C intensities was observed at the end of incubation time at 15°C and 30°C, the OhLv layer becoming as recalcitrant as the OhLf layer (Figure 2). Moreover, the average length of methylenic chains (alkyl C to carboxyl C ratio) showed that long chains dominated throughout the 6 months of degradation (Table 1). On the other hand, in samples incubated at 4°C, marked losses of intensity were observed in methoxyl C and alkyl C groups, whereas carboxyl C, aromatic C and phenolic C groups increased after 6 months of degradation (Table 1).

Deepest layer (OhLf). The deepest layer appeared as the more recalcitrant layer at the beginning of incubation, because of its large lignin content (18.82%), great aromaticity (15.32) and large humification index (0.48), (Table 1). As a consequence, PCA showed little evolution of the OhLf layer along the PC 1 over the incubation time and for the different incubation temperatures. The most important variation was, however, observed for the carboxyl C and the aromatic C regions, which increased at 4°C by 19.73 and 14.25%, respectively, and for alkyl C, which increased by 13.04% at 15°C (Table 1).

Enzyme activities

To analyse the effect of depth, time and temperature on enzyme activities, a multifactorial analysis of variance test (MANOVA) was performed on the whole data set. Results of the MANOVA showed that all these factors had significant effects ($P < 0.001$) on enzyme activities measured during the incubation experiment (Table 2). Levene tests were also performed on the exploratory variables (enzymes) and indicated parametric distributions for the three enzymes measured (data not shown). Thereafter, Student's *t*-test was performed individually on each enzyme and each sub-layer to discriminate significant effects of temperature.

Cellulase activity. Figure 3 shows cellulase activities measured from 0 to 180 days of degradation at 4, 15 and 30°C. After 5 days, a decrease of cellulase activity was observed in the OhLn and OhLf layers (Figure 3a,b), probably due to leaching of these enzymes during the initial moistening of these litter layers. As shown in Figure 3, marked spatial and temporal variations were observed thereafter during the course of the incubation experiments. The greatest cellulase activity was observed after 15 days of incubation at 30°C, in both OhLn and OhLv layers. After that, cellulase activity gradually decreased until 90 days and

Table 2 Multi-factorial analysis of variance (MANOVA)

Effect	d.f.	Cellulase SS	Cellulase MC	Cellulase *F*	Cellulase *P*	Laccase SS	Laccase MS	Laccase *F*	Laccase *P*	Butyrate E SS	Butyrate E MS	Butyrate E *F*	Butyrate E *P*
Intercept	1	5.018	5.018	12821.40	0.00	0.0287	0.0287	4545.53	0.00	16.263	16.2639	12862.09	0.00
Time	6	0.274	0.456	116.73	0.00	0.0026	0.0004	70.228	0.0	2.428	0.4047	320.11	0.00
Depth	2	0.758	0.379	968.56	0.00	0.0042	0.0021	332.25	0.00	0.7325	0.3662	289.67	0.00
Temperature	2	0.621	0.310	794.05	0.00	0.0005	0.0002	43.51	0.00	0.6046	0.3023	239.09	0.00
Time × depth	12	0.138	0.011	29.59	0.00	0.0124	0.001	163.4	0.00	0.5572	0.0464	36.72	0.00
Time × temperature	12	0.442	0.036	94.16	0.00	0.0026	0.0002	35.44	0.00	0.770	0.0641	50.75	0.00
Temperature × depth	4	0.176	0.044	112.55	0.00	0.0006	0.0001	24.6	0.00	0.5960	0.1490	117.85	0.00
Time × depth × temperature	24	0.197	0.006	21.04	0.00	0.0026	0.0001	17.668	0.00	1.4118	0.0588	46.52	0.00
Error	189	0.073	0.000			0.0011	0.000006			0.2389	0.0012		
Total	251	2.682				0.0270				7.3401			

d.f., degree of freedom; MS, mean of squares; SS, sum of squares.
Levels of significance are indicated for $P < 0.001$.

tended to stabilize. In the upper layer (OhLn), significant differences were found between 30°C and the two other temperatures. No differences in temperature effect on cellulase activity were observed between the two lowest temperatures evaluated. Similar patterns in the evolution of cellulase activity were observed in the OhLv layer, with the difference that cellulase activity was significantly greater at 4°C than at 15°C throughout the experiment. The activity of cellulases was less in the OhLf layer than in the other two layers (Table 2). Moreover, cellulase activity in the OhLf layer was significantly affected by temperature, incubation at 30°C giving the greatest cellulase activities. However, the cellulase dynamics at 30°C were different in the OhLf layer as no increase in activity was observed within the first month of the experiment.

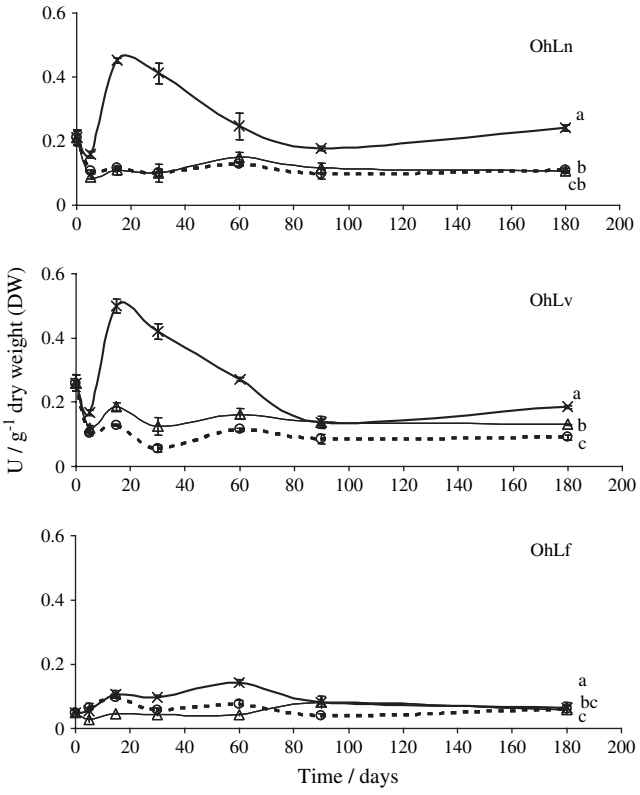

Figure 3 Variation in the cellulases activities measured during 180 days of decomposition of three layers (OhLn, OhLv and OhLf) of leaf litter at three temperatures: 30°C (crosses), 15°C (open circles) and 4°C (open triangles). Mean values of six replicates ($n = 6$). Bars are standard errors. Means with the same letter are not significantly different using a *t*-test ($P < 0.05$).

Laccase activity. Figure 4 shows laccase activities measured from 0 to 180 days of degradation at 4, 15 and 30°C. Laccase activity responded differently with respect to temperature, depth and incubation time (Table 2). In the upper layer (OhLn), significant differences were found between all the temperatures evaluated and the greatest activity was found after 15 days of degradation at 30°C (Figure 4a). Laccase activities showed similar fluctuations at 4C and 15°C but were significantly different in their intensities. Our results showed also that by the end of incubation no further laccase activity was detected at 15°C (Figure 4b). In the OhLv layer, no significant differences were detected over the experimental time between laccase activities at 30 and 4°C (Figure 4b). However, adverse dynamics in laccase activities were observed in this layer with regard to temperature. Indeed, at 30°C, laccase activity increased markedly over the first 60 days and thereafter decreased; the inverse dynamic occurred at 4°C. The OhLf layer showed similar dynamics with regard to the OhLv layer, but with some differences, especially at the beginning and at the end of the experiment. Indeed, as for the cellulases, initial activities quickly decreased, probably because of the same leaching phenomenon described above. As described in the OhLn layer, the OhLf litter also had no more laccase activity by the end of experiment at 30°C (Figure 4c).

Butyrate esterase (BE) activity. BE activity showed different dynamics with respect to temperature and litter depth. Only in the upper layer (OhLn) incubated at 30°C, did BE activity rise linearly with time, reaching its optimum value at 180 days

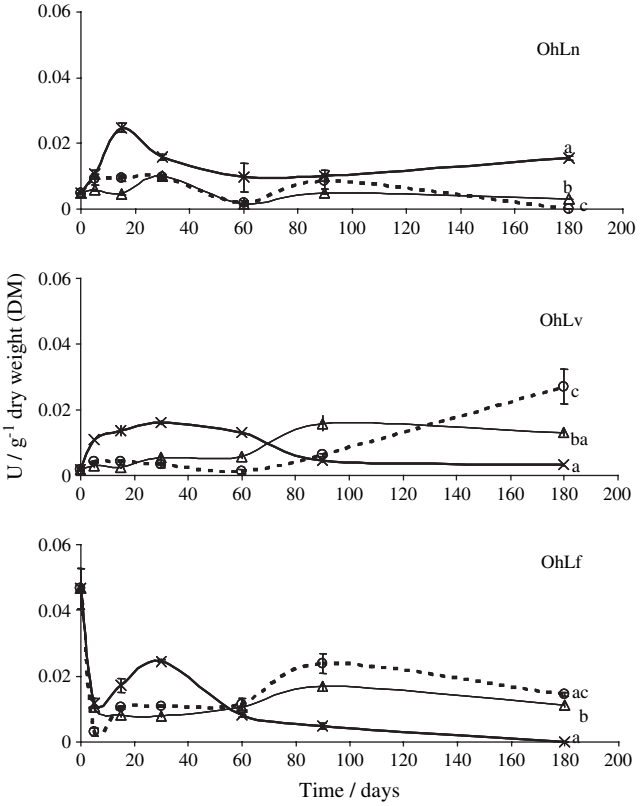

Figure 4 Variation in the laccase activities measured during 180 days of decomposition of three layers (OhLn, OhLv and OhLf) of leaf litter at three temperatures: 30°C (crosses), 15°C (open circles) and 4°C (open triangles). Mean values of six replicates ($n = 6$). Bars are standard errors. Means with the same letter are not significantly different using a *t*-test ($P < 0.05$).

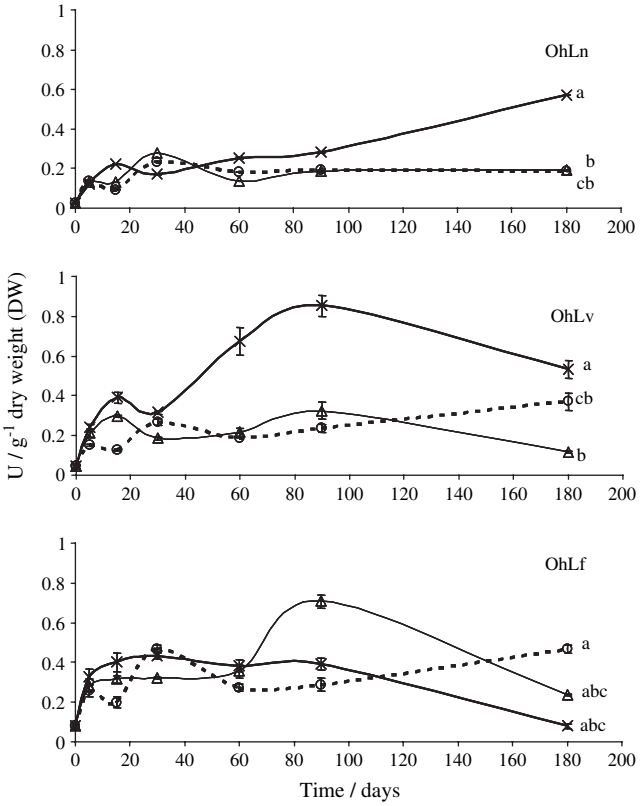

Figure 5 Variation in the butyrate esterase activities measured during 180 days of decomposition of three layers (OhLn, OhLv and OhLf) of leaf litter at three temperatures: 30°C (crosses), 15°C (open circles) and 4°C (open triangles). Mean values of six replicates ($n = 6$). Bars are standard errors. Means with the same letter are not significantly different using a *t*-test ($P < 0.05$).

(Figure 5a). BE activities showed similar fluctuations at 15°C and at 4°C but were significantly different in their intensities (Figure 5a). The OhLv layer had the more BE active (Figure 5a). Our results showed that the BE activity was significantly greater at 30°C and that it peaked after 90 days of incubation. The dynamics of BE activity were the same at 4°C but considerably and significantly reduced in intensity. At 15°C two shoulders of activity were observed; one after 5 days of degradation, and the other after 30 days of degradation (Figure 5b). No significant difference was detected for the OhLv litter incubated at 4 or 15°C. In the deepest layer (OhLf), no significant differences ($P < 0.05$) in BE activity were found between all the temperatures evaluated.

Discussion

Effect of temperature on chemical composition of litter layers

All our ^{13}C CPMAS NMR spectra showed similar patterns for all investigated regions and results are consistent with other NMR studies (Lorenz *et al*., 2000; Kavdir *et al*., 2005). The major chemical group in litter samples was the *O*-Alkyl-C compounds, indicating a great potential of samples for microbial degradation (Kavdir *et al*., 2005). Our results show a progressive decrease of these polymers with litter depth, incubation time and temperature, 30°C being the temperature inducing the faster degradation rate. Almendros *et al.* (2000) also followed the chemical transformations of a composted evergreen oak litter (*Quercus ilex* ssp. *ballota Samp*.) and they found only a 4% decrease of the *O*-alkyl region after 168 days of incubation at 28°C. After incubation at 30°C, the results of our study show a decrease of the *O*-alkyl-C region by 26.68, 11.71 and 4.38% in the OhLn, OhLv and OhLf layers, respectively. This difference may be due to the experimental protocols used in both studies. Indeed, the evergreen oak litter layers of our study were incubated under oxic conditions. Almendros *et al*. (2000) used piles of crushed leaves for their incubation. This may have favoured, in spite of mixing every 14 days, anoxic conditions, thus resulting in a slower degradation of litter material. Baldock *et al*. (1992) proposed a model describing the decomposition of plant materials in mineral

soils. They suggested three well-defined stages: (i) the first source of carbon used by the microbial community is the polysaccharides. As a consequence, the *O*-alkyl region of spectra decreases and other polymers like lignin are preserved, shown by an increase in the aromatic NMR signals, (ii) Degradation of polysaccharides is followed by a partial degradation of aromatic C, and a decrease in aromatic signals is seen, and (iii) Finally, as a consequence of selective degradation by soil microorganisms, accumulation of more recalcitrant groups is expected, and alkyl C groups increase their NMR signals (Baldock *et al*., 1992; Quideau *et al*., 2000). Just as these authors suggested, preferential degradation of polysaccharides was also observed in our results, and particularly in the upper layer samples. It appeared clearly that degradation rates of polysaccharides were faster in layers showing the highest carbohydrate contents, and that temperature can considerably affect these rates. Indeed, within these layers, the higher degradation rates of polysaccharides were clearly related to the increasing temperature. Based on our results, litter organic matter degradation was affected by the quality of litter layer and by the temperature. Hence, temperature can affect differently the degradation of litter with regard to the litter layer and chemical group concerned. As mentioned above, high temperatures promoted polysaccharide degradation. On the contrary, the degradation of recalcitrant C groups such as methoxyl C or alkyl C, was favoured by lower temperatures. The results of our work show that the second group to be degraded varied according to temperature and litter depth. Thus, methoxyl C compounds were degraded at 4°C and phenolic C groups were degraded at 15°C, both in the middle layer. These results indicate that degradation processes can be complex with regards to temperature fluctuations and the stage of OM decomposition. They probably originate from shifts in microbial populations and activities, as we also observed important modifications in functional diversity of microorganisms (BioLog™) according to temperature variation (data not shown). Previous studies (e.g. Baldock *et al*., 1992; Almendros *et al*., 2000; Kavdir *et al*., 2005) mentioned that, during the humification process, progressive aromatization as well as an increase in alkyl carbons can be expected. In our work, an accumulation of alkyl C groups was observed at 15 and 30°C for the three layers evaluated. The increase of alkyl carbons may originate from microbial biomass produced during degradation or from by-products released during the degradation of cutin (Quideau *et al*., 2005), which is an abundant polymer found in evergreen oak leaves. This polymer is composed of interesterified hydroxyl and hydroxyl-epoxy fatty acids attached to the outer epidermal layer of cells of leaves, and this insoluble polyester is embedded in a complex mixture of insoluble lipids and waxes (Kolattukudy, 2001). Hence, progressive degradation of polymers like cutin produces short aliphatic chains, which probably explain the increase in alkyl-C signals observed in the different layers of the evergreen oak litter.

Litter depth effect

Andersson *et al*. (2004) discussed a possible depth distribution of enzymes. Our results about cellulases activity support this hypothesis: the deeper the layer the less the activity. The greatest cellulase activities were observed in the OhLn and in the OhLv layers during degradation at 30°C. Moreover, this is in agreement with signal sets obtained between 45 and 110 p.p.m., which show the effect of cellulase enzymes on the degradation of polysaccharides. Major C-resources may stimulate more active C-mineralizing enzymes or induce new isoforms. We reported previously (Alarcón-Gutiérrez *et al*., 2006) that cellulase isoforms were more numerous in both upper and middle layers than in the deepest one, thereby supporting the fact that new isoforms are produced when enzyme substrates are abundant. Thus, due to the fact that soil enzymes can be constitutively produced (Koroljova-Skorobogatko *et al*., 1998), results may indicate that, in the layer more recalcitrant to degradation (OhLf), the poor cellulase activity originates from constitutive enzymes produced at a basal level instead of inducible enzymes. Samples have more cellulase activity when they are rich in *O*-alkyl-C groups. On the contrary, other enzymes, like laccase and butyrate esterase, are more active in samples rich in recalcitrant compounds. Laccases are enzymatic proteins involved in the transformation of polyphenolic compounds (Criquet *et al*., 1999). They participate in humus formation by generating radicals that react with each other to form dimers, oligomers and finally aromatic polymers (Claus, 2004). Hence, in addition to the degradation of polysaccharides, the products of reactions catalysed by laccases may increase the signal of aromatic or phenolic regions of the NMR spectra. Our results show a marked increase in aromatic signals at 4°C in the deepest layer and, at the same time, an increase in laccase activity. The butyrate esterase activity followed a similar biological pattern as laccases. Their activity also increased with the enrichment of litter layer in alkyl-C recalcitrant compounds. Thus, it finally appears that complex substrates, generated during the course of litter degradation, seem to induce the production of extracellular enzymes (Allison & Vitousek, 2005). Moreover, as suggested by Bundt *et al*. (2001) and Von Lützow *et al*. (2006), the location of OM in soil profiles may affect its availability to decomposers and consequently modifies the structure and the activity of microbial communities.

Conclusions

We found that:

1 Litter organic matter composition analysed by solid-state ^{13}C CPMAS NMR followed spatial and temporal variations. The main C-types varied according to depth and temperature, and these variations showed relationships with the different enzymatic pools investigated (i.e. laccase, cellulase and butyrate esterase).

2 The progressive accumulation of recalcitrant compounds, during the decomposition processes, affects litter organic matter quality and enzyme activities. As enzyme pools and OM quality were modified, it can be expected that the microbial structure community has also evolved with temperature.
3 The microbial structure population should be investigated using molecular approaches and be coupled to the NMR spectra of litter.
4 Overall, we conclude that increasing global temperatures will contribute to speeding up organic litter degradation, by affecting soil enzyme functioning. However, rather more information is needed about the variations in temperature and temperature response functions in litter and soil. Thus, fundamental understanding of carbon stabilization could be linked to climate change and global C cycling.

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References

Alarcón-Gutiérrez, E., Couchaud, B., Ziarelli, F., Floch, C., Albrecht, R., Le Petit, J. *et al*. 2006. Estudio en mesocosmos del efecto del nitrógeno inorgánico [$(NH_4)_2SO_4$] sobre las actividades enzimáticas de una hojarasca de encino (*Quercus ilex* L.). In *Proceedings of the Second International Meeting on Environmental Biotechnology and Engineering September 26th–29th. Mexico City*, pp. 17–19. Instituto Politecnico Nacional Press, Mexico City.

Allison, S.D. & Vitousek, P.M. 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biology & Biochemistry*, **37,** 937–944.

Almendros, G., Dorado, J., Gonzalez-Vila, F.J., Blanco, M.J. & Lankes, U. 2000. 13C NMR assessment of decomposition patterns during composting of forest and shrub biomass. *Soil Biology & Biochemistry*, **32,** 793–804.

Andersson, M., Kjoller, A. & Struwe, S. 2004. Microbial enzyme activities in leaf litter, humus and mineral soil layers of European forests. *Soil Biology & Biochemistry*, **36,** 1527–1537.

Baldock, J.A., Oades, J.M., Waters, A.G., Peng, X., Vassallo, A.M. & Wilson, M.A. 1992. Aspects of the chemical structure of soil organic materials as revealed by solid-state ^{13}C NMR spectroscopy. *Biogeochemistry*, **16,** 1–42.

Baldock, J.A., Masiello, C.A., Gelinas, Y. & Hedges, J.I. 2004. Cycling and composition of organic matter in terrestrial and marine ecosystems. *Marine Chemistry*, **92,** 39–64.

Bundt, M., Widmer, F., Pesaro, M., Zeyer, J. & Blaser, P. 2001. Preferential flow paths: biological 'hot spots' in soils. *Soil Biology & Biochemistry*, **33,** 729–738.

Chen, Y., Inbar, Y., Hadar, Y. & Malcolm, R.L. 1989. Chemical properties and solid-state CPMAS 13C-NMR of composted organic matter. *Science of The Total Environment*, **81–82,** 201–208.

Claus, H. 2004. Laccases: structure, reactions, distribution. *Micron*, **35,** 93–96.

Criquet, S., Tagger, S., Vogt, G., Iacazio, G. & Le Petit, J. 1999. Laccase activity of forest litter. *Soil Biology & Biochemistry*, **31,** 1239–1244.

Criquet, S., Farnet, A.M., Tagger, S. & Le Petit, J. 2000. Annual variations of phenoloxidase activities in an evergreen oak litter: influence of certain biotic and abiotic factors. *Soil Biology & Biochemistry*, **32,** 1505–1513.

Criquet, S., Tagger, S., Vogt, G. & Le Petit, J. 2002. Endoglucanase and [beta]-glycosidase activities in an evergreen oak litter: annual variation and regulating factors. *Soil Biology & Biochemistry*, **34,** 1111–1120.

Criquet, S., Ferre, E., Farnet, A.M. & Le Petit, J. 2004. Annual dynamics of phosphatase activities in an evergreen oak litter: influence of biotic and abiotic factors. *Soil Biology & Biochemistry*, **36,** 1111–1118.

Dignac, M.-F., Knicker, H., & Kögel-Knabner, I. 2002. Effect of N content and soil texture on the decomposition of organic matter in forest soils as revealed by solid-state CPMAS NMR spectroscopy. *Organic Geochemistry*, **33,** 1715–1726.

Fioretto, A., Papa, S., Curcio, E., Sorrentino, G. & Fuggi, A. 2000. Enzyme dynamics on decomposing leaf litter of *Cistus incanus* and *Myrtus communis* in a Mediterranean ecosystem. *Soil Biology & Biochemistry*, **32,** 1847–1855.

Forte, C., Piazzi, A., Pizzanelli, S. & Certini, G. 2006. CP MAS 13C spectral editing and relative quantitation of a soil sample. *Solid State Nuclear Magnetic Resonance*, **30,** 81–88.

Gallardo, A. & Merino, J. 1993. Leaf decomposition in 2 Mediterranean ecosystems of southwest Spain – influence of substrate quality. *Ecology*, **74,** 152–161.

Golchin, A., Clarke, P., Baldock, J.A., Higashi, T., Skjemstad, J.O. & Oades, J.M. 1997. The effects of vegetation and burning on the chemical composition of soil organic matter in a volcanic ash soil as shown by 13C NMR spectroscopy. I. Whole soil and humic acid fraction. *Geoderma*, **76,** 155–174.

Harkin, J.M. & Obst, J.R. 1973. Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia*, **29,** 381–387.

Haw, J.F., Maciel, J.G.E. & Schroeder, H.A. 1984. Carbon-13 nuclear magnetic resonance spectrometric study of wood and wood pulping with cross polarization and magic-angle spinning. *Analytical Chemistry*, **56,** 1323–1329.

Huang, Y., Stankiewicz, B.A., Eglinton, G., Snape, C.E., Evans, B., Latter, P.M. *et al*. 1998. Monitoring biomacromolecular degradation of *Calluna vulgaris* in a 23 year field experiment using solid state 13C-NMR and pyrolysis-GC/MS. *Soil Biology & Biochemistry*, **30,** 1517–1528.

Kavdir, Y., Ekinci, H., Yuksel, O. & Mermut, A.R. 2005. Soil aggregate stability and 13C CP/MAS-NMR assessment of organic matter in soils influenced by forest wildfires in Canakkale, Turkey. *Geoderma*, **129,** 219–229.

Knicker, H., Schmidt, M.W.I. & Kögel-Knabner, I. 2000. Nature of organic nitrogen in fine particle size separates of sandy soils of highly industrialized areas as revealed by NMR spectroscopy. *Soil Biology & Biochemistry*, **32,** 241–252.

Kögel, I. 1986. Estimation and decomposition pattern of the lignin component in forest humus layers. *Soil Biology & Biochemistry*, **18,** 589–594.

Kögel-Knabner, I. 2002. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biology & Biochemistry*, **34,** 139–162.

Kolattukudy, P.E. 2001. Polyesters in higher plants. *Advances In Biochemical Engineering and Biotechnology*, **71,** 1–49.

Koroljova-Skorobogatko, O.V., Stepanova, E.V., Gavrilova, V.P., Morozova, O.V., Lubimova, N.V., Dzchafarova, A.N. *et al*. 1998. Purification and characterization of the constitutive form of laccase from the basidiomycete Coriolus hirsutus and effect of inducers on laccase synthesis. *Biotechnology and Applied Biochemistry*, **28,** 47–54.

Loisel, R. 1976. *La végétation de l'étage méditerranéen dans le sud-est continental français*. State Doctorate Thesis, Université d'Aix-Marseille III, France.

Lorenz, K., Preston, C.M., Raspe, S., Morrison, I.K. & Feger, K.H. 2000. Litter decomposition and humus characteristics in Canadian and German spruce ecosystems: information from tannin analysis and 13C CPMAS NMR. *Soil Biology & Biochemistry*, **32,** 779–792.

Marche, T., Schnitzer, M., Dinel, H., Pare, T., Champagne, P., Schulten, H.R. *et al*. 2003. Chemical changes during composting of a paper mill sludge-hardwood sawdust mixture. *Geoderma*, **116,** 345–356.

Massiot, D., Fayon, F., Capron, M., King, I., Le Calve, S., Alonso, B. *et al*. 2002. Modelling one- and two-dimensional solid-state NMR spectra. *Magnetic Resonance in Chemistry*, **40,** 70–76.

Nèble, S. 2005. *Effets de l'épandage de boues de station d'épuration sur l'évolution des caractéristiques chimiques, microbiologiques et enzymatiques d'une litière de chêne-liège (Quercus suber* L.*) en milieu sylvo-pastoral*. PhD Thesis, University Aix-Marseille III, France.

Nelson, N. 1944. A photometric adaptation of Somogyi method for determination of glucose. *Journal of Biological Chemistry*, **153,** 375–380.

Pascual, J.A., Hernández, T., García, C. & Ayuso, M. 1998. Enzymatic activities in an arid soil amended with urban organic wastes: laboratory experiment. *Bioresource Technology*, **64,** 131–138.

Preston, C.M., Trofymow, J.A., Niu, J. & Fyfe, C.A. 1998. 13CPMAS-NMR spectroscopy and chemical analysis of coarse woody debris in coastal forests of Vancouver Island. *Forest Ecology and Management*, **111,** 51–68.

Quideau, S.A., Anderson, M.A., Graham, R.C., Chadwick, O.A. & Trumbore, S.E. 2000. Soil organic matter processes: characterization by 13C NMR and 14C measurements. *Forest Ecology and Management*, **138,** 19–27.

Quideau, S.A., Graham, R.C., Oh, S.W., Hendrix, P.F. & Wasylishen, R.E. 2005. Leaf litter decomposition in a chaparral ecosystem, Southern California. *Soil Biology & Biochemistry*, **37,** 1988–1998.

Somogyi, M. 1952. Notes on sugar determination. *Journal of Biological Chemistry*, **195,** 19–23.

Taylor, J.P., Wilson, B., Mills, M.S. & Burns, R.G. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biology & Biochemistry*, **34,** 387–401.

Von Lützow, M., Kögel-Knabner, I., Ekschmitt, K., Matzner, E., Guggenberger, G., Marschner, B. *et al*. 2006. Stabilization of organic matter in temperate soils: mechanisms and their relevance under different soil conditions – a review. *European Journal of Soil Science*, **57,** 426–445.

