Pedobiologia **52** (2009) 387—399

Available online at www.sciencedirect.com

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Pedobiologia

www.elsevier.de/pedobi

Spatial variations of chemical composition, microbial functional diversity, and enzyme activities in a Mediterranean litter (*Quercus ilex* L.) profile ☆

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Received 17 October 2008; received in revised form 14 January 2009; accepted 16 January 2009

KEYWORDS
^{13}C CPMAS NMR;
Chemical litter composition;
Soil depth;
Biolog^{TM}

Summary

Litter decomposition on the forest floor is an essential process in soil nutrient cycles and formation. These processes are controlled by abiotic factors such as climate and chemical litter quality, and by biotic factors such as microbial community diversity and activity. The aim of this study was to investigate the importance of litter depth with respect to (i) chemical litter quality as evaluated by solid-state ^{13}C NMR, (ii) enzyme activities, and (iii) microbial functional diversity in four different litter layers (OLn, OLv, OF, and OH). A Mediterranean soil profile under an evergreen oak (*Quercus ilex* L.) forest was used as a model. The recalcitrant OM fraction, corresponding to the deepest layer, showed low enzyme activities. Peroxidases and fluorescein diacetate hydrolases (FDA) were more active in the OLn layer and probably originated largely from plants. High cellulase activity in the OLn and the

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0031-4056/$ - see front matter
doi:10.1016/j.pedobi.2009.01.002

OLv layers, which are rich in polysaccharides, corresponded with the high content of *O*-alkyl carbon compounds. Following polysaccharide degradation, laccases and lipases were much more evident in the intermediate layers. This spatial variation in nutrient demand reflected a preferential degradation of the specific plant polymers. Phosphatases were more active along the three upper layers and probably reflected a P limitation during litter degradation. Alkaline/acid (AcPAlP/AcP) ratio increased in the deepest layer, suggesting an increased participation of bacteria AlP in phosphatase pools. Results of Biolog™ also indicated spatial variations in microbial functionality. Indeed, FF plates showed the highest functional diversity in the uppermost layer, while ECO plate functional diversity was highest in the intermediate layers. Finally, our results indicated that microbial activity and functional diversity of micro-organisms change with litter depth on a very small scale and vary with chemical organic matter (OM) composition. Thus, the observed increases in the biological variables studied were determined by the evolution of OM chemical structures, the nature and availability in C nutrients, and they ultimately resulted in a progressive accumulation of recalcitrant compounds.

Introduction

Soil organic matter (SOM) transformation is a key process in the global carbon cycle. Indeed, SOM represents the largest terrestrial C pool since it is about three times the C amount of both plant and animal biomass (Kögel-Knabner et al. 2008). Plant litter, together with the input from plant roots, derived from net primary production, represents the main source of organic matter (OM) in forest soils (Litton et al. 2003; Lützow et al. 2006). On this point, Rapp (1969) mentioned that, in evergreen oak (*Quercus ilex* L.) forests, the litter inputs to soil were estimated to be 3.84–7.0 and 2.45–2.72 t ha^{-1} yr^{-1} when only foliage material is considered. Organic matter stabilization in soils is strongly influenced by processes occurring in litter, especially C mineralization and C storage (Oades 1988). The dynamic equilibrium between these two processes is of particular importance, since any change in the size and the turnover rate of SOM pools may potentially alter the atmospheric CO_2 concentration and consequently the global climate (Kögel-Knabner et al. 2005). In France, as well as in other countries of the European Union, research programs on this topic have increased significantly. In this context, fundamental data need to be collected in order to understand the complex relationships between physico-chemical and biological variables involved in C transformation of litter layers. Indeed, such understanding is a preliminary and indispensable stage to other studies which aim to predict C dynamics in soils as a function of global climate change. This fundamental knowledge is especially important in the case of the Mediterranean area, because the typical humus forms found in this region are related to summer aridity and the sclerophyllous nature of the vegetation, and will probably start to appear in many other areas of the biosphere as a consequence of climate change. Thus, one of our objectives was to expand current knowledge about the complex interactions between litter biota and litter physico-chemical characteristics such as OM quality. With this aim, Mediterranean evergreen oak litter (*Q. ilex* L.) was chosen as a model, since this species forms characteristic forest climax populations widely distributed throughout the western Mediterranean area (Rapp 1969). In a previous paper (Tagger et al. 2008), our studies have focused on the qualitative micro-morphological characterization of the interactions between fungi and both meso- and microfauna during the course of degradation of this litter. Our results have shown that this Mediterranean litter has a very slow decomposition rate, resulting in thick, complex, and structured humus with several sub-layers OL (OLn & OLv), OF, and OH (Green et al. 1993); the last one characterizing amphimull humus widely found under Mediterranean bioclimate.

In the present study, we focused on micro-organisms and their functions involved in the transformations of OM of the same litter model. Indeed, soil micro-organisms are known to play essential roles in carbon and nutrient cycles of forest ecosystems due to their action on SOM decomposition (Fitter et al. 2005; Friedel et al. 2006). Moreover, the structure and the activity of microbial communities at a specific site are also known to be affected by, among other things, the location of OM in a soil profile (Bundt et al. 2001; Lützow et al. 2006). Thus, the decomposition

process is influenced by litter abundance and litter quality (Booth et al. 2005; Gusewell and Freeman 2005; Ruan et al. 2005) as well as moisture and temperature conditions (Sinsabaugh et al. 1994; Jandl et al. 2007). As a consequence, the location of OM in a soil profile may affect its availability to decomposers and consequently modifies the structure and the activity of microbial communities (Sinsabaugh et al. 1991; Bundt et al. 2001; Lützow et al. 2006). A recent study of Šnajdr et al. (2008) pointed out that variations in microbial functions (i.e. enzyme activities), microbial biomass, and community composition with respect to a forest soil profile (2 horizons) occurred effectively at a small scale. However, in their study, SOM was not analyzed, and thus, no data were available about the relationships between microbial functions and SOM quality. Moreover, to our knowledge, data that link NMR characterization of SOM to microbial functions are still very scarce.

Solid-state ^{13}C CPMAS NMR spectroscopy is one of the most powerful non-destructive methods for structural studies (Bank et al. 1996; Terashima et al. 1997). Therefore, another objective of our study was to investigate, at the scale of soil horizons, the importance of litter depth with regards to (i) litter OM quality as evaluated by a non-destructive solid-state ^{13}C NMR technique, (ii) relationships between litter OM quality and enzyme activities (i.e. alkaline (AlP) and acid (AcP) phosphatases, laccases, peroxidases, cellulases, lipases, and (FDA)), and microbial functional diversity, using Biolog™ ECO and FF plates.

Materials and methods

Litter

Evergreen oak litter (*Q. ilex* L.) was collected in February 2005 from a 1000 m^2 dense coppice at 'La Gardiole de Rians' (43°34′27″N, 5°42′10″E), Var Department, France, in a Mediterranean-type bioclimate. Litter samples were collected randomly and independently from five profiles measuring 5 cm × 30 cm × 30 cm (depth, length, and width). In each profile, four litter layers from the O horizon were distinguished (Green et al. 1993; Tagger et al. 2008): the OLn layer (uppermost layer, 0–2 cm above the soil surface), formed by leaves over a period of less than 1 yr, non-degraded and non-compressed; the OLv layer (2–3 cm above the soil surface) formed by leaves still recognizable despite decay but non-compressed; the OF layer (3–4 cm from the soil surface), formed by fragmented leaves and often compressed in lumps, and finally the OH layer (4–5 cm above the soil surface), formed by fine organic matter. All samples were air-dried at room temperature and stored at 4 °C over 70 h before their use. To determine dry weight, 1 g of litter was weighed and dried overnight at 105°.

^{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 Avance DSX 400 MHz spectrometer, using a commercial two-channel, 4-mm Bruker probe head. A composite ($n = 5$) and representative litter sample was blended for each layer by placing 100 mg of dried and ground sample (particle size <0.3 mm, Cyclotec™ 1093, Foss Co., France) in a 4-mm zirconium rotor and spun at magic angle at 10 kHz. ^{13}C CPMAS NMR was performed with a ramped ^{1}H pulse during a contact time of 2 ms to obtain the best signal-to-noise ratio. ^{1}H decoupling was performed during the acquisition to improve the resolution. Recording 20 K 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 by tetramethylsilane and calibrated with glycine carbonyl signal, set at 176.05 ppm. All measurements were monitored at room temperature. Spectra chemical shift range of litter was characterized by the following dominant peaks: alkyl C (0–45 ppm), *O*-alkyl C (45–110 ppm); methoxyl C (50–60 ppm); aromatic C (110–140 ppm), phenolic C (140–160 ppm), and carboxyl C (160–190 ppm). Deconvolution of the NMR spectra was performed using the software DmFit (Massiot et al. 2002). The intensity of each C region was expressed as a percentage of the total C. The degree of humification was calculated according to Baldock et al. (1997) using the alkyl-C to *O*-alkyl-C ratio with the respective peaks of the spectra. The alkyl-C to carboxyl-C ratio (Knicker et al. 2000), relating the respective areas (A) was calculated as alkyl C/carboxyl C $= (0-45\,\text{ppm})\,/\,A(160-190\,\text{ppm})$. The aromaticity index (AI) was calculated as $\text{AI} = 100(A(110-160\,\text{ppm})/A(0-160\,\text{ppm}))$ (Lorenz et al. 2006).

Finally, the lignin content was estimated using the following equation established by Haw et al. (1984): $\%\ \text{lignin} = (100\%)((183/9.92)I'\text{lig}/(183/9.92)I'\text{lig} + (162/6)I'\text{carb})$. The weighting factor in this equation is the ratio of formula weights to the number of carbon atoms in the average formulas assumed for the repeat units in the lignin and

carbohydrate fractions (for details, see Haw et al. 1984).

Enzyme assays

Unless otherwise indicated, all enzyme activities were measured in triplicate from extracts obtained from each layer according to Criquet et al. (1999). Laccase activity in the extract was measured using syringaldazine as a substrate (Harkin and Obst 1973; Criquet et al. 1999). For enzyme measurement, 200 μl of enzyme extract, 790 μl of 0.1 M phosphate buffer (pH 5.7) and 10 μl of a 5 mM syringaldazine–methanol solution were mixed. The oxidation rate of syringaldazine (525 nm, $\varepsilon = 65\,000$ $M^{-1}\,cm^{-1}$) to quinone was monitored at 30 °C. The results are expressed in units defined as μmol of quinone formed from syringaldazine $min^{-1}\,U\,g^{-1}$ of dry weight ($U\,g^{-1}$ DW). Cellulase activity in the extract was measured using carboxymethylcellulose (CMC) as substrate, according to Criquet (2002). For cellulase activity measurement, 100 μl of enzyme extract and 900 μl of 50 mM acetate buffer (pH 6.0) with 1% CMC were mixed and incubated at 50 °C for 1 h. After the incubation, the sugars released from the hydrolysis of CMC were determined spectrophotometrically by measuring absorbance at 520 nm (Uvikon 860, Kontron Instruments, Montigny Le Bretonneux, France), using the colorimetric method of Somogyi–Nelson (Somogyi 1952; Nelson 1994). A calibration curve was established with glucose (0–100 $\mu g\,ml^{-1}$) and the activity of the cellulases was expressed in μmol of glucose released $min^{-1}\,U\,g^{-1}$ of dry weight ($U\,g^{-1}$ DW). Lipase activity was measured using *p*-nitrophenyl butyrate (*p*-NPB) as substrate according to Margesin et al. (2002). For enzyme measurement, 200 μl of enzyme extract, 800 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. The *p*-nitrophenol (*p*-NP) release was monitored spectrophometrically (Uvikon 860, Kontron Instruments, Montigny Le Bretonneux, France) at 412 nm ($\varepsilon = 277\,M^{-1}\,cm^{-1}$). The results were expressed in units defined as μmol of *p*-NP $min^{-1}\,U\,g^{-1}$ of dry weight ($U\,g^{-1}$ DW). The FDA activity was estimated according to Schnürer and Rosswall (1982) with the following modifications: 200 μl of enzyme extract and 800 μl of 50 mM phosphate buffer (pH 7.6) with 7 μl of FDA (Sigma–Aldrich Chemical Co. Ltd.) (2 $mg\,ml^{-1}$ acetone AR grade) were added to obtain a final concentration of 14 $\mu g\,ml^{-1}$. Assay samples were incubated without agitation for 30 min at 37 °C. The reaction was stopped by adding 1 ml of acetone/water solution (1:1 v/v) to 1 ml of the reaction solution; the fluorescein produced was measured at 490 nm (Uvikon 860, Kontron Instruments, Montigny Le Bretonneux, France). Results were expressed in μmol of fluorescein released $min^{-1}\,U\,g^{-1}$ of dry weight ($U\,g^{-1}$ DW). Peroxidase activity was measured using 2,7-diaminofluorene (DAF) as a substrate (Criquet et al., 2001). For enzyme measurement, 200 μl of enzyme extract, 800 μl of 10 mM phosphate buffer (pH 5.7), 10 μl of a 5 mM DAF solution, and 10 μl of H_2O_2 1 mM were mixed and incubated at 30 °C. DAF oxidation was monitored spectrophotometrically (Uvikon 860, Kontron Instruments, Montigny Le Bretonneux, France) at 600 nm ($\varepsilon = 10\,228\,M^{-1}\,cm^{-1}$). The results were expressed in units defined as μmoles of oxidized substrate $min^{-1}\,U\,g^{-1}$ of dry weight ($U\,g^{-1}$ DW).

Two types of phosphatase activity were measured: acid phosphomonoesterase and alkaline phosphomonoesterase (Gianfreda et al. 2005). Since the origin of AcP can be from plants and microbial communities (Kramer and Green 2000), and AlP originates mainly from microbial communities (Dick et al. 1983; Jungk 1991), the AlP to AcP ratio can be used as a microbial biomarker (Dick et al. 2000). Methods were based on the measurement, at 412 nm, of *p*-nitrophenol released during the incubation of litter with *p*-nitrophenyl phosphate mono-ester. Acid phosphatase activity was monitored according to Tabatabai and Bremner (1969). In total, 100 μl of enzyme extract, 900 μl of 10 mM acetate buffer (pH 5), and 10 μl of 15 mM *p*-nitrophenyl phosphate were mixed and incubated 1 h at 37 °C. Alkaline phosphatase activity was monitored according to the same procedure except that NaOH–glycine 10 mM (pH 9) was used for the incubation. A *p*-NP calibration curve (0–20 $mg\,l^{-1}$) was generated under the same conditions as the assay enzymes. Results were expressed in units defined as μmoles of *p*-NP released $min^{-1}\,U\,g^{-1}$ of dry litter ($U\,g^{-1}$ dry litter).

Microbial functional diversity

We analyzed the heterotrophic litter microbial communities (metabolic diversity) using the Biolog™ method reported by Garland (1996). This technique is based on tetrazolium dye reduction as an indicator of sole-carbon-source utilization community-level method. Biolog™ ECO plates (for bacteria) and Biolog™ FF plates (for fungi) have been used to apply this technique to ecological studies to estimate metabolic potential of microbial communities (Stefanowicz, 2006). One gram of freshly ground litter was suspended in

10 ml of sodium pyrophosphate (0.1%) solution and shaken for 20 min. Sample suspensions were allowed to settle for 10 min, then decanted and diluted to 1/100 with a NaCl solution (0.8%). Each well of Biolog plates was inoculated with 150 µl of sample supernatant. Three analytical replicates were performed for each layer. All samples were incubated at 25 °C and the optical density (OD) was read for each plate at 8-h intervals over a period of 150 h. The average well colour developments (AWCD) of the different replicates were calculated according to Garland (1996) where AWCD equals the sum of the difference between the OD of control (no substrate) and substrate wells divided by 31 (number of substrate wells in ECO plates) or 95 (number of substrate wells in FF plates). AWCD at 65 h (maximum slope) was also used in statistical analyses. At the end of the incubation, the number of negative wells was recorded in order to determine the potential of microbial communities to degrade some substrates.

Statistical data analysis

A significant effect of depth on enzyme activities was evaluated using a one-way ANOVA analysis ($p<0.05$). Post-hoc comparisons (LSD test) were used to compare the enzyme activities and the Biolog™ results at different depths at an alpha level of 0.05. A two-way ANOVA was used to evaluate any significant effect ($p<0.001$) of depth and time on Biolog™ ECO and FF plates. The Kolmogorov–Smirnov test was used to test significant ($p<0.05$) differences between distributions of Biolog™ ECO and FF results. Principal components analysis (PCA) with Pearson's correlation was employed to look at relationships between enzyme activities and NMR data for all litter layers. ANOVA analyses and post-hoc tests were done using Statistica Vs 6 (StatSoft, Maison-Alfort, France), while PCA and Kolmogorov–Smirnov analyses were conducted using XLSTAT Vs 7.1 (Addinsoft, Paris, France).

Results

Figure 1 shows ^{13}C CPMAS NMR spectra of the four evergreen oak litter layers studied. Signals of the spectra have been assigned using references obtained from previous studies about soil organic matter (Baldock et al. 1997; Huang et al. 1998). For all spectra, the alkyl-C, *O*-alkyl-C, and carboxyl-C regions dominated over the aromatic and methoxyl intensities (Figure 1). The spectra were dominated by signals from the *O*-alkyl-C region (45–110 ppm), characteristic of polysaccharides, which decreased in intensity with litter depth from 45% to 31% of the total carbon (Table 1). Spectra were also characterized by signals in the alkyl-C region (0–45 ppm). Intensity of this region increased with litter depth, from 17.75% to 26.84% of the total C (Table 1). This region is known to be characteristic of methylene structures of lipids, waxes, and cutins (Quideau et al. 2000; Dignac et al. 2002), but can also derive from short-chain acids or amino acids (Dick et al. 2005). Moreover, in Figure 1, it can also be observed that the signals in the alkyl region are split into two. The clear signal or shoulder at 22 ppm is known to reflect a considerable amount

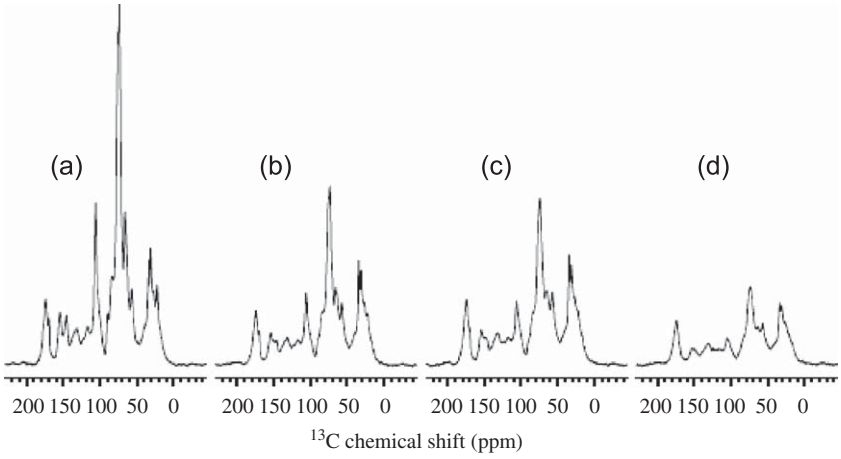

Figure 1. ^{13}C CPMAS NMR spectra of litter (0–5 cm depth) samples: (a) OLn layer, (b) OLv layer (c) OF layer, and (d) OH layer. Spectra chemical shift range of litter was characterized by the following dominant peaks: alkyl C (0–45 ppm), *O*-alkyl C (45–110 ppm), methoxyl C (50–60 ppm), aromatic C (110–140 ppm), phenolic C (140–160 ppm), and carboxyl C (160–190 ppm).

Table 1. Relative intensities (%) derived from the ^{13}C CPMAS NMR spectra of the litter layers.

Depth	Region of spectra (ppm)					
	Alkyl C 0–45	Methoxyl 45–60	*O*-alkyl C 45–110	Aromatic C 110–140	Phenolic C 140–160	Carboxyl C 160–190
OLn	17.75	3.94	45.44	6.84	8.03	6.78
OLv	23.52	5.78	37.72	8.27	7.46	8.91
OF	23.46	6.19	35.27	9.94	8.03	10.52
OH	26.84	6.22	31.03	9.99	7.24	13.6

Table 2. Various ratios, lignin content (%), and aromaticities (%) derived from the ^{13}C CPMAS NMR spectra of different litter layers.

Depth	Ratios			%	
	Alkyl C/*O*-Alkyl-C	Alkyl C/carboxyl C	*O*-alkyl C/aromatic C	Lignin	Aromaticity
OhLn	0.3	2.6	6.6	19.7	16.0
OhLv	0.5	2.6	4.6	21.5	17.3
OhLf	0.6	2.2	3.6	25.4	20.1
OhLh	0.7	2.0	3.1	25.2	19.9

of acetyl C in leaf litters (Lemma et al. 2007). Other important regions were methoxyl, aromatic and carboxyl signals (Figure 1). Their intensities also increased with litter depth (Table 1) showing a gradual increase in recalcitrance. On the other hand, the intensity of phenolic regions decreased slightly from 8.0% to 7.2% from the upper to the lowest layers (Table 1). Lignin content, aromaticity index, and humification index (alkyl-C to *O*-alkyl-C ratio) increased with litter depth (Table 2). In contrast, *O*-alkyl-C to aromatic-C ratio decreased with depth. Finally, the alkyl-C to carboxyl-C ratio decreased from 2.6 to 2 with depth (Table 1). Such low values probably indicated the dominance of short-chain acids rather than long-chain paraffinic structures (Dick et al. 2005), and that these short chains are accumulating with soil depth.

On the other hand, enzyme activities showed important and a significant depth ($p<0.001$) effect (Figure 2). Within the phosphatase pool (Figures 2A and B), alkaline and acid phosphatase activities ranged from 8.7×10^{-3} to 18.5×10^{-3} U g^{-1} DW and from 11×10^{-3} to 45×10^{-3} U g^{-1} DW, respectively. The AlP/AcP ratio (Figure 2C) was similar for the OLn, OLv, and OF layers and ranged from 0.3 to 0.42. This ratio also significantly increased in the deepest OH horizon. Similar patterns in AlP and AcP activity were observed in the litter profile (Figures 2A and B). Indeed, the first three upper layers (OLn, OLv, and OF) showed higher activities ($p<0.05$) than the deepest one (OH) for both AlP and AcP. When considering these three layers, AcP activity did not show significant differences ($p>0.05$), whereas AlP fluctuated much more within these layers.

Cellulase activity ranged from 21×10^{-3} to 75×10^{-3} U g^{-1} DW (Figure 2D). Results showed two activity levels – a high activity level for the OLn and the OLv layers, and a low activity level for the OF and the OH layers. Significant ($p<0.05$) differences were detected between these two levels of activity. The OLn and OLv litter layers exhibited high activity, however, the highest ($p<0.05$) cellulase activity was obtained for the OLv layer.

Laccase activity ranged from 0.34×10^{-3} to 3×10^{-3} U g^{-1} DW (Figure 2E). As for cellulase activity, two activity levels were observed with laccase: a low activity level including OLn and OH layers and a high activity level including OLv and OF layers. Significant differences ($p<0.05$) were obtained between these two levels but no differences ($p>0.05$) were observed within them.

Higher peroxidase activity (Figure 2F) was observed in the OLn layer, showing a value 120-fold higher than in the OH layer where peroxidase activity only reached 3.4×10^{-3} U g^{-1} DW. Insignificant differences ($p>0.05$) were observed between OLv, OF, and OH layers.

Lipase activity showed similar patterns to laccase (Figure 2G). Two levels of activity were observed: low, including OLn and OLh layers, and ranging from 34×10^{-3} to 48×10^{-3} U g^{-1} DW, and high, including

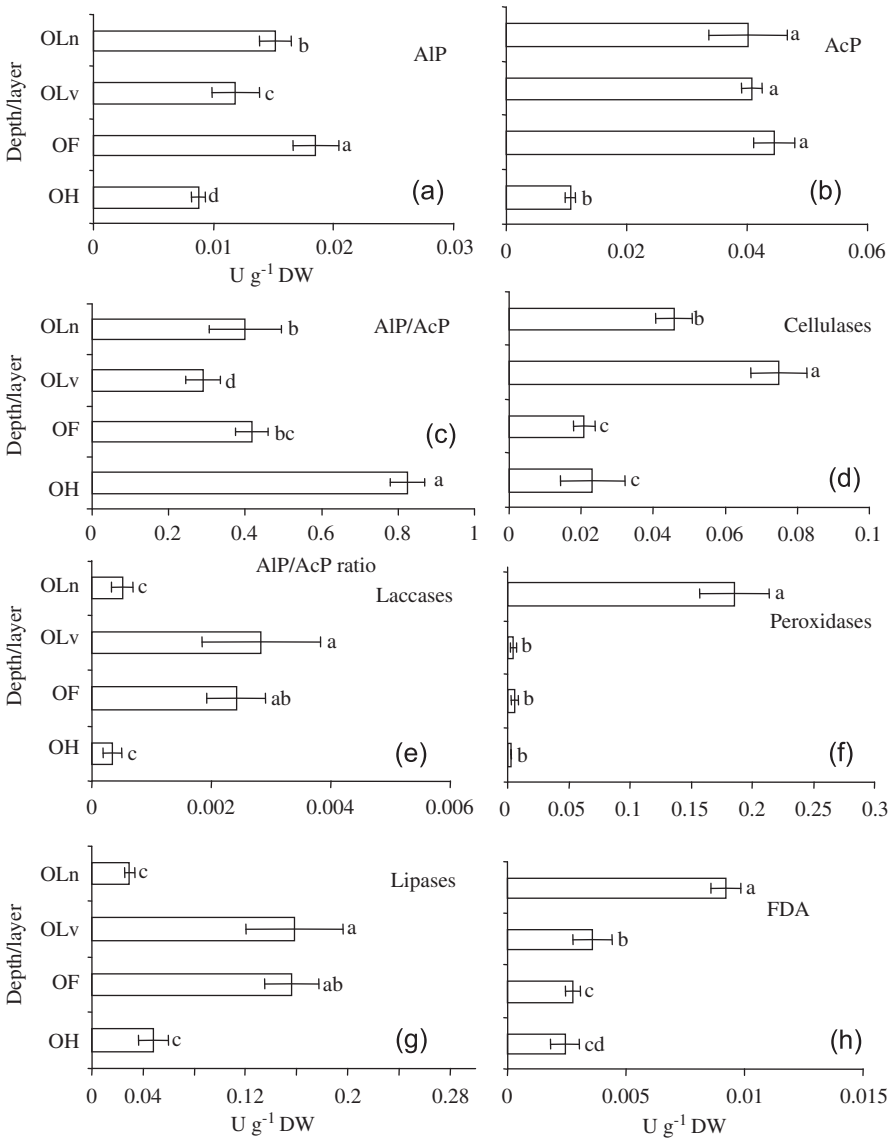

Figure 2. Enzyme activities of different litter layers including (a) alkaline phosphatase, (b) acid phosphatase, (c) AlP/AcP: Alkaline to acid phosphatase ratio, (d) cellulose, (e) laccase, (f) peroxidise, (g) lipase, and (h) fluorescein diacetate hydrolase (FDA). Values represent means ($n = 5$) and standard deviations (in brackets). Means within a graph followed by the same letter are not significantly different from each other ($p < 0.05$).

OLv and OLf litter layers, and ranging from 0.15 to 0.16 U g^{-1} DW. Significant differences ($p < 0.05$) were observed between these layers.

The FDA activity ranged from 0.37 U g^{-1} DW, in the uppermost (OLn) layer, to 2.4×10^{-3} U g^{-1} DW in the deepest (OH) layer (Figure 2H). A significant difference ($p < 0.05$) was observed between the three upper layers (OLn, OLv, and OF), but no significant difference ($p > 0.05$) was observed between the OF and OH layers (Figure 2G).

Figure 3 shows the average well colour development over 150 h of OD detection. The Biolog™ values increased rapidly during the first 65 h of incubation. After that time, maximum AWCD development was obtained in OF layer for ECO and in OLn layer for FF plates. In both cases, OH layer showed low activity throughout the incubation (Figure 3). In order to discriminate the functional diversity of micro-organisms in the different layers, ANOVA was performed using data corresponding to the maximum slope (i.e. after 65 h). ANOVA showed significant effect ($p < 0.001$) when evaluating litter depth and incubation time effects on the AWCD development. In details, post-hoc comparisons (LSD test) showed a significant difference ($p < 0.05$) only for the OH layer when

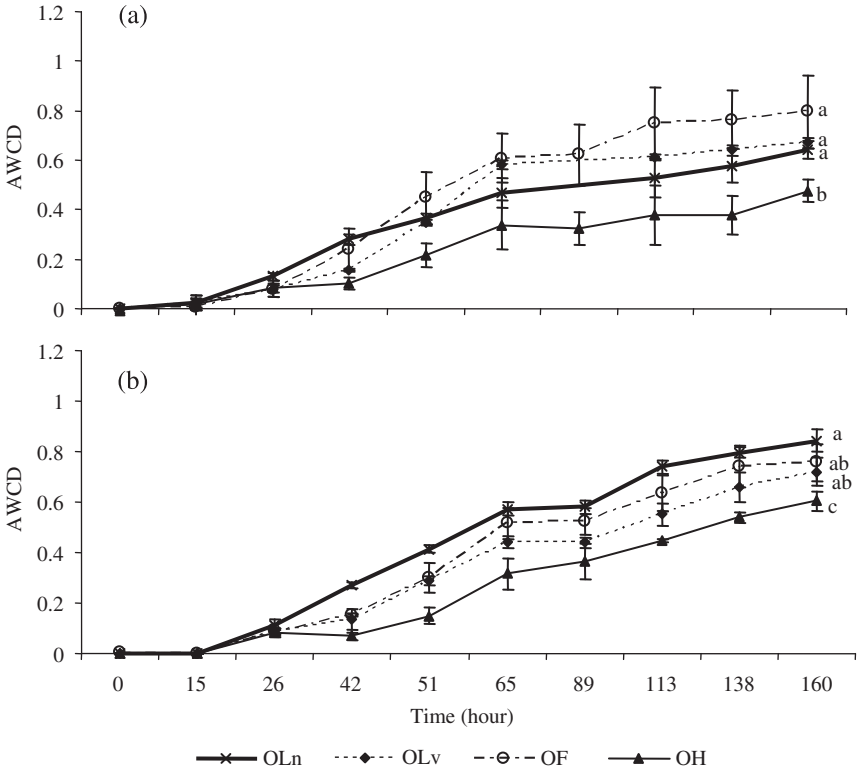

Figure 3. Curves results from the Biolog™ plates. Average well colour development (AWCD) with time for ECO and FF plates in different litter layers. Values represent means ($n = 5$) and standard deviations (in brackets). Mean curves followed by the same letter are not significantly different from each other ($p < 0.05$).

Table 3. AWCD at 65 h and percentage of negative wells estimated from Biolog™ microplates.

	Litter depth			
	OLn	OLv	OF	OH
AWCD at 65 h in ECO plates*	0.467 (0.06)^{bd}	0.582 (0.01)^{b}	0.608 (0.1)^{bc}	0.339 (0.1)^{a}
AWCD at 65 h in FF plates*	0.573 (0.02)^{bd}	0.44 (0.02)^{b}	0.515 (0.06)^{c}	0.315 (0.06)^{a}
% Of negative wells in ECO plates**	1.33 (0.58)^{ab}	4.0 (2.0)^{a}	3.0 (1.7)^{a}	6.7 (2.3)^{b}
% Of negative wells in FF plates**	7.3 (9.29)^{ab}	10.33 (3.05)^{d}	7.6 (5.5)^{abc}	27.3 (6.11)^{d}

Values represent means ($n = 3$) and standard deviations (in parentheses). Means within a row followed by the same letter are not significantly different from each other ($p < 0.05$).
*AWCD $= \Sigma(OD_i - OD_{A1})/95$ (for FF) or 31 (for ECO) with $OD_i = OD_{490\,nm}$ for FF or $OD_{595\,nm}$ for ECO. $OD_{A1} = OD_{595\,nm}$ of control well A1 in ECO plates, and $OD_{490\,nm}$ of control well A1 in FF plates.
**Positive well OD > 0.1.

evaluating the effect of depth layer on AWCD of ECO plates (Figure 3A). Moreover, the lowest (1.33%) and the highest (6.7%) percentage of negative wells were detected in OLn and OH layers, respectively (Table 3). When evaluating functional diversity of FF plates, post-hoc analysis showed significant differences in AWCD ($p < 0.05$) between the first upper layers and OH layer (Figure 3B, Table 3). Additionally, fungi AWCD of OLv differed significantly from the OLn but not from the OF layer, but no significant difference was observed between AWCD of OLn and OF layers. After incubation, the percentage of negative wells was recorded in each plate. In FF plates, the deepest layer, OH, showed the highest number of non-assimilated substrates (27.3%, Table 3). In contrast, the fairly degraded OLn layer showed the highest metabolic diversity with an average percent of non-assimilated substrates (negative wells) of 7.3%.

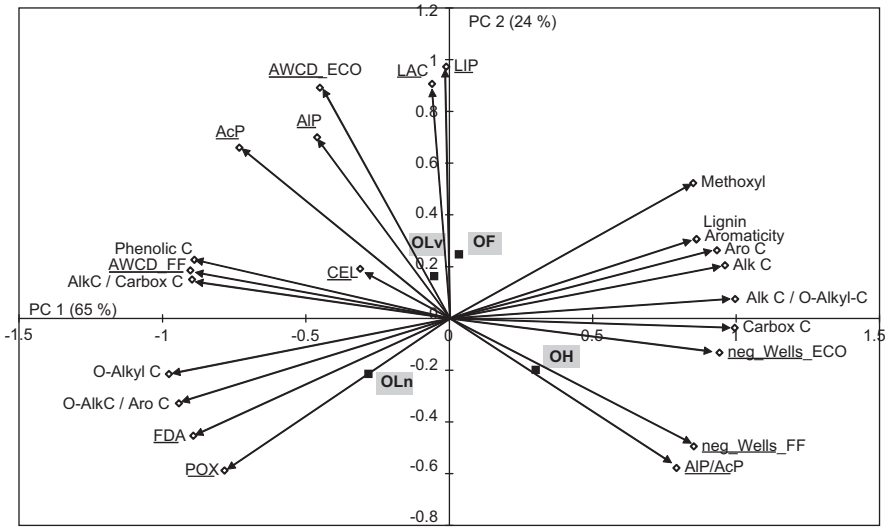

Figure 4. Two dimension scatter from the PCA of different chemical and biological parameters. Data show the variables (AWCD for ECO and FF plates, percent of negative wells, enzyme and RMN results) and the different litter layers (OLn, OLv, OF, and OH). Underlined characters are biological variables.

Figure 4 shows the results of PCA established with Biolog™, enzyme activities and ^{13}C NMR variables. PC 1 (65%) and PC 2 (24%) together accounted for 89% of the variance. The different layers were located on the 1st factorial map.

Discussion

The aim of our study was to add to existing knowledge about the interactions between biotic and abiotic factors during the degradation of a Mediterranean evergreen oak litter. If data about the relationships between soil OM and microbial parameters are numerous in the literature, those that link OM quality with microbial functions and are determined with non-destructive methods (e.g. solid-state NMR) are still very scarce, especially at the scale of litter horizons. Important variations in these parameters were observed in the first 5 cm of the litter profile, corresponding to four different litter layers. All litter ^{13}C CPMAS NMR spectra, showed the highest intensities in the *O*-alkyl-C and alkyl-C regions. The *O*-alkyl-C region, including major polysaccharides structures, was more important than other regions in the uppermost layer (OLn) and in other layers, showing richness in easily available carbohydrates for micro-organisms. Different litter layers showed a progressive accumulation of recalcitrant molecules, the deepest layer being the horizon with the highest contents in carboxyl C, aromatic C, methoxyl C, and alkyl C.

During the course of degradation and transformation of OM, several increases were observed in the different enzyme pools (Figure 2) and in functional diversity of micro-organisms (Figure 3) along the litter profile. These increases in biological variables were determined by the evolution of OM chemical structures, the nature and availability of C nutrients, and they ultimately resulted in a progressive accumulation of recalcitrant compounds (Tables 1 and 2). At the beginning of the degradation process, the OLn layer appeared to be located at the negative end of the 1st axis (Figure 4), characterized by a poorly degraded OM (richness in *O*-alkyl C) and by high levels of activity of several enzymes such as FDA hydrolases and peroxidises, which more likely originated from plant than microbial matter. In contrast, the deepest layer was placed at the right hand of the first component, showing an increase in recalcitrant compounds. The tendency towards aromaticity (Almendros et al. 2000) and the presence lignin content (Sosanwo et al. 1995) is generally indicative of an increase in recalcitrant OM in the deepest layer.

The tendency towards increasing contents of recalcitrant C compounds and a decrease in the *O*-alkyl-C region in the litter profile was also observed by Almendros et al. (2000), who studied the degradation patterns of leaves from 12 forests and shrub species of the Mediterranean ecosystems. Helfrich et al. (2006) found a gradual increase in recalcitrance with increasing litter depth when evaluating maize field, grass and forest

samples. A rise in alkyl-C to *O*-alkyl-C ratios, obtained along the litter profile, indicates an increase in the degradation status of litter chemical compounds (Baldock et al. 1997) with litter depth. High contents of alkyl-C carbon compounds were detected in our study. These have also been detected in other studies. They have mainly been ascribed to waxes and lipids by Dignac et al. (2002), and to cutin and suberin polymers by Winkler et al. (2005). Knicker et al. (2000) also suggested that during humification, paraffinic compounds are enriched relative to carbohydrates. However, results from our study showed a considerable amount of acetyl C, characterized by the 22 ppm signal, as well as a low alkyl-C to carboxyl-C ratio (i.e. around 2). Thus, and according to recent literature data (Dick et al. 2005), it seems more likely that these results reflect the dominance of short-chain acids, originating from the transformation of lipid or proteinaceous structures, rather than from long paraffinic structures. Additionally, and considering the variation of the alkyl-C to carboxyl-C ratio in the different litter layers, it can be concluded that these short chains probably accumulate during the course of degradation of litter (i.e. litter depth).

It was suggested (Kourtev et al. 2002) that some litter chemical characteristics such as recalcitrance and aromaticity, may determine its enzymatic potential, and that some enzyme activities such as of laccase and AcP, are the expression of the soil community to metabolic requirements and nutrient availability (Caldwell 2005). In this work, the majority of litter enzymes showed high activities in layers with intermediate degree of degradation. However, peroxidase and FDA activities were considerably higher in the OLn layer. This can be explained by the plant origin of these enzymes (Criquet et al., 2001), or by the occurrence of interfering substances in the OLn layer such as nucleophilic amino acids that can enhance FDA activity (Wanandy et al. 2005; Alarcón-Gutiérrez et al. 2008).

High cellulase activity in the OLn and OLv layers corresponded with horizons enriched in polysaccharides (i.e. with high contents in *O*-alkyl carbon compounds). On the contrary, low cellulase activities were detected in layers with more recalcitrant OM (OF and OH). Thus, the different patterns of enzyme activities observed represented the metabolic functions expressed by litter micro-organisms in relation with OM chemical composition, as has been suggested in other studies (Baldock et al. 1997; Gallo et al. 2005). Hence, it appeared that polysaccharides were preferentially degraded during the first step of degradation and that the catabolism of more recalcitrant compounds such as lignin, lipids, cutin, or waxes occurred mainly in underlying layers. As a consequence, enzymes implicated in the transformation of these kinds of compounds were much more expressed by micro-organisms in OLv and OF layers. Finally, the most humified fraction, corresponding to the most recalcitrant OM, was characterized by the lowest activity level as well as the lowest functional diversity of micro-organisms. This was illustrated by the higher functional diversity of fungi (FF plates) observed in the OLn layer and the higher diversity of bacteria (ECO plates) in intermediate layers. ECO plates did not develop fungi while marked growth of fungi mycelium was observed in FF plates. As mentioned by Heuer and Smalla (1997) and Dobranic and Zak (1999), the ECO plates method cannot detect fungi because fungi do not reduce the tetrazolium violet form used in these plates. Thus, it is possible that AWCD results point to higher functional diversity of fungi in the OLn layer compared to higher diversity of bacteria in intermediate layers. These results are in agreement with other studies (Saito 1956; Jensen 1974; Berthelin and Toutain 1979; Osono 2005; Torres et al. 2005), which have suggested that fungi were the primary colonizers and that bacteria were more numerous in fermentative litter layers. Additionally, enzyme activities were linked with colour development (AWCD), reflecting the physiological state of microbial cells (Preston-Mafham et al. 2002). Enzyme and metabolic results suggest that some ongoing allocation of enzyme production appears to be essential. Indeed, the enzyme synthesis could be integrated into a single, overall micro-organism maintenance cost (Sinsabaugh and Moorhead 1994; Schimel and Weintraub 2003) and, in our work, this seems to be the case for the deepest layer where enzyme and microbial activities are still at basal levels. Indeed, the recalcitrance of OM limits carbon availability to soil microbes, which, in turn, become energy limited (Flanagan and Van Cleve 1983). However, there are others factors constraining microbial activities in litter such as moisture conditions and nitrogen availability (Nèble et al., 2007). Gradual humification results in reduced availability of resources, and therefore, increased caloric requirements (Lahdesmaki and Piispanen 1988). Consequently, even if an important quantity of carbons was present in the deepest layer, it was unavailable due to their recalcitrance.

The AlP/Acp ratio decreased with increasing litter depth, indicating a preferential plant and/or fungi origin of AcP in the uppermost layer. This observation was in contrast to what was observed in the deepest layer, where the proportion of AlP

increased considerably and where A1P may have originated from bacteria (Nakas et al. 1987; Criquet et al. 2004). Moreover, high phosphatase activity in the upper layers, in combination with high C demand from micro-organisms, may reflect a phosphorus limitation (Criquet et al. 2007), which could affect litter degradation rates (Qualls and Richardson 2000). This phenomenon is well known and can be explained by the regulation by phosphates ions of *PHO* genes involved in the synthesis of phosphatases (Oshima et al. 1996).

Our study clearly showed that physico-chemical and biological interactions of litter must be considered in the study of litter stabilization. These interactions could be important when evaluating microbial and enzyme allocation in litter profiles, as well as in the investigation of the effect of exogenous perturbations on functional ecology and OM recycling of forest ecosystems. Chemical carbon alteration of organic matter was detectable with ^{13}C CPMAS NMR while the biological state of litter was measurable with the physiological profile community level (CLPP) and enzyme activities. Thus, consistent degradation of *O*-alkyl-C groups was linked with high cellulose activity and microbial activities. In contrast, persistent groups such as alkyl-C were linked with high lipase activity, and always in deepest layers. Of course, this complexity in the spatial variations limits the extrapolation of specific results to different types of litter; however, similarities in chemical organic matter composition between litters could be due to similar patterns in microbial functional diversity and enzyme activities.

Acknowledgements

This work was supported by the SFERE-CONACyT program (France-México), by the Dirección General de Relaciones Internacionales de la Secretaría de Educación Pública (DGRI-SEP) from México, and by the Institut de Recherche pour le Développement (IRD-DSF scholarship) from France.

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