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# Secondary metabolites of *Pinus halepensis* alter decomposer organisms and litter decomposition during afforestation of abandoned agricultural zones

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## Summary

**1.** Over a century of agricultural abandonment across the Mediterranean region has favoured the installation of the pioneer expansionist species Aleppo pine (*Pinus halepensis* Miller). This species synthesizes a wide range of secondary metabolites that are partially released during needle decomposition, and which can thus affect the 'brown food chain'. Litter decomposition is a key process connecting ecosystem structure and function, and involving microbial and faunal components.

**2.** The goal of this study was to determine the effect of chemical compounds from Aleppo pine needles on the litter decomposition process along a gradient of Mediterranean forest secondary succession. Using *in situ* litterbags, we compared the dynamics of decomposers, particularly the relative contributions of fungal and mesofauna biomass to litter mass loss (calculations based on the measured decomposer biomass, published fungal growth efficiency and mesofauna feeding rate), against the dynamics of secondary metabolites associated with decomposed needles in three successional stages (early, middle and late, i.e. pinewoods that were aged 10, 30 and over 60 years old).

**3.** Our first key finding was that fungi accounted for the largest portion of overall litter mass loss (60–79%) and detritivorous mesofauna contributed to 8–12%. In the early stage of succession, fungal biomass after 6 months of decomposition was lower than in middle and late stages, and may be responsible for the delay in litter colonization by mesofauna. We linked this result to a clearly longer residence time for phenolic compounds in young pine forest, leading to an overall slowdown in the decomposition process.

**4. Synthesis.** Litter phenolic content emerged as a key functional trait for predicting litter decomposition, delaying the colonization of litter by decomposers in Mediterranean forest ecosystems. Another key finding is that the relative contributions of fungi and detritivores to needle mass loss were different between the successional stages. From the food-web perspective, the organic matter available for higher trophic levels thus remains unchanged beyond 30 years after pine colonization.

**Key-words:** Aleppo pine, carbon budget, determinants of plant community diversity and structure, functional diversity, litter decomposition, Mediterranean region, mesofauna, micro-organisms, plant secondary metabolites, secondary succession

## Introduction

Anthropogenic pressure is a world-wide threat to ecosystems. Climate change, changing land use and overexploitation of

natural resources affect the way ecosystems work through a series of modifications, including changes in plant communities (Vitousek *et al.* 1997; Chapin *et al.* 2000; Diaz & Cabido 2001). The characteristics of the Mediterranean area (long-standing anthropogenic pressure, significant current human activity and broad biodiversity) make it one of the world's regions most threatened by current changes, warranting its recent classification as a 'biome in crisis' (Hoekstra *et al.*

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Correction added on 13th February, after first online publication: error in Y-axis of Fig.2 corrected.

2005). The Mediterranean region is therefore a key model system for studying the effects of global change such as afforestation of abandoned agricultural areas, on key ecosystem processes (Lavorel *et al.* 1998).

Since the early 19th century, the northern margins of the Mediterranean basin have undergone strong rural depopulation (Barbéro *et al.* 1990; Debussche & Lepart 1992). Abandoned agricultural lands are soon to be naturally colonized by pioneer plant species through processes of secondary succession; with forest cover increasing fivefold over the last 150 years (Fernandez, Bousquet-Mélou & Prévosto 2013a). Among these pioneer species, *Pinus halepensis* Miller is classed as an expansionist species (Barbero & Quezel 1989) that has spread to dominate forests in this area (Acherar, Lepart & Debussche 1984; Debussche & Lepart 1992; Maestre & Cortina 2004), thereby threatening the existing mosaic of forests, shrublands and pastures of high patrimonial value (Quézel & Médail 2003).

The different stages of successional dynamics in Mediterranean forest ecosystems are well described (Lepart & Escarré 1983; Quézel & Médail 2003), but the functional mechanisms determining these successional dynamics remain poorly understood. Allelopathic mechanisms are recognized as one of the drivers in the successional replacement of plant species (Rice 1984; Pellissier 1993; Reigosa, Sanchez-Moreiras & Gonzalez 1999; Mallik 2003; Fernandez *et al.* 2008). Allelopathy is a major driver of many biotic interactions (Rice 1984; Fernandez *et al.* 2006, 2013b) owing to the huge diversity of secondary metabolites that are produced by plants (e.g. terpenoids and phenolic compounds), particularly Mediterranean species. Aleppo pine is known to be a major producer of secondary metabolites with allelopathic and autotoxic capacities (Robles *et al.* 2003; Maestre & Cortina 2004; Ormeño, Fernandez & Mévy 2007). The ecological role of these compounds includes defence against predators, pathogens and competing organisms, and tolerance towards some abiotic factors (changes in temperature, pollution episodes and drought; Robles *et al.* 2003; Yazaki 2006; Dicke & Baldwin 2010). Allelopathic chemicals are released into the environment through root exudation, volatilization, leaching from plant parts into the soil and leaf litter decay (Rice 1984). The litter decomposition process involves soil decomposers (including soil fauna, fungi and bacteria) that are specialized in degrading different compounds, depending on their chemical nature (Cortez *et al.* 1996). However, many secondary metabolites are difficult to degrade and can prove toxic for certain decomposers (Kainulainen & Holopainen 2002; Kainulainen, Holopainen & Holopainen 2003), making them key regulators of nutrient cycling (Souto, Gonzalez & Reigosa 1994; Poinsot-Balaguer 1996; Hättenschwiler & Vitousek 2000; Kraus, Dahlgren & Zasoski 2003; Ormeño *et al.* 2006).

Studying the effects of secondary metabolites on the decomposition process has remained a major challenge because of the very broad diversity of secondary metabolites and the impact of these compounds on the brown food chain. The few studies that have focused on this impact on plant-soil interactions through decomposer activities and the litter decomposition process have used both direct and indirect

methods. Direct approaches, such as those generally used in laboratory studies, have looked at the effects of some secondary metabolites added to microcosms and mainly concern the impact of tannins (Schimel *et al.* 1996; Bradley, Titus & Preston 2000; Hättenschwiler & Vitousek 2000; Fierer *et al.* 2001; Schofield, Mbugua & Pell 2001; Kraus, Dahlgren & Zasoski 2003) or monoterpenoids (White 1986, 1991, 1994; Bremner & McCarty 1988) on fungi and bacteria (respiration, mineralization of nitrogen) and enzyme activities. Approaches such as these are particularly helpful for elucidating the role of specific compounds on specific functions. Indirect approaches, such as those used in field studies, focus on the correlation between litter chemistry and the decomposition process. However, these studies are restricted to the relation between the concentration of tannins or other phenolic compounds and litter decomposition rate, or microbial activity. Also, there are no *in situ* studies dealing with the impact of secondary metabolites on decomposer organisms. In this study, we sought to address this lack by correlating the fate of the two main families of secondary metabolites, phenolic compounds and terpenoids, and the relative contribution of the main decomposers to litter mass loss.

The aim of this study was to use a mechanistic approach to determine how, through the litter, secondary compounds of Aleppo pine needles evolve during succession, change the relative importance of decomposers associated with needle decomposition, and so modify the quality of organic matter available for higher trophic levels.

Specific objectives were to compare the decomposition process as a whole in three successional stages of Aleppo pine ('early', 'middle' and 'late') through (i) a complete picture of litter chemical composition: C, N and the two main families of secondary metabolites, namely phenolic compounds and terpenoids; (ii) the pattern and magnitude of needle colonization, the fungal and bacterial catabolic diversity, and the quantitative contribution to decomposition based on decomposer carbon biomass measurements; and (iii) the interrelation between decomposers and litter quality.

The main questions were (i) which PSM type, terpenoids or phenolics predominantly influence the decomposition process, given their known allelopathic or toxic potential on organisms (Fernandez *et al.* 2006, 2008, 2013b); (ii) what is the relative importance of the main decomposer functional groups for the decomposition process and (iii) whether the pattern and magnitude of PSM and decomposers change from one successional stage to another and so modify decomposition efficiency.

We hypothesised that during secondary succession, leaf litter decomposition would be enhanced in later successional stages, as soil communities adapt better to local resources, particularly litter secondary metabolites (Mayer 2008).

## Materials and methods

### STUDY SITE

The study was conducted in the Mediterranean region of France, along the southern slopes of the Luberon massif in the Luberon

Regional Nature Park. The climate is typically Mediterranean, with a marked summer drought, most precipitation generally occurring during the relatively cold winter season (Quézel & Barbero 1982). The mean annual rainfall was 773 mm and mean temperatures for January and July were 6.6 and 23.1 °C, respectively (Meteo-France data, 2007–2009). We selected nine sampling sites on the basis of similar global index (climatic and topo-edaphic conditions) using the model from Ripert & Vennetier (2002). In order to track the decomposition process during secondary succession, three successional stages of *P. halepensis* were selected: (i) abandoned agricultural land colonized by young-dispersal *P. halepensis* (about 10 years old), referred to as the 'early-stage' plots; (ii) monospecific forest stands of medium-aged *P. halepensis* (about 30 years old), recently closed canopy with sparse understorey, referred to as the 'middle-stage' plots and (iii) mature forests of older *P. halepensis* (> 60 years old) with well-developed late successional species (pine-oak transition forest), referred to as the 'late-stage' plots.

The sites that were studied form a secondary post-culture chronosequence (Gondard *et al.* 2003), with deep agricultural silty-clayey soils (> 1 m) classified as Calcisols in the IUSS World Reference Base (IUSS, Working Group WRB 2006), in flat terrain with high fertility. For each successional stage, we randomly selected three sites and five trees per site.

#### COLLECTION OF MATERIAL AND EXPERIMENTAL DESIGN

Needles from all three successional stages (three sites per successional stage) were collected during August, when abscission peaks, from five trees per site. By shaking trees, we helped the abscission of needles whose senescence was complete, and we collected these fallen needles on a plastic sheet placed on the ground below the trees to prevent their contamination by soil. After homogenization of material collected per successional stage, needles (5-g dry mass equivalent) were then placed in 2-mm mesh litter bags (10 × 15 cm) to allow colonization by soil mesofauna and microbes and to exclude macrofauna (Swift, Heal & Anderson 1979). The initial size of the needles was large enough to prevent loss through the net mesh, but some fragments could be lost after fauna activity.

In each site (three early-stage sites, three middle-stage sites and three late-stage sites), eight litter bags filled with needles corresponding to the site were placed around each of the five trees. In the middle of December, we placed litter bags equidistant from each other around trees at 30–130 cm from the trunk, where accumulation of litter on soil was maximum. The sampling was randomized except for one pair of adjacent litter bags, one bag being used for chemical and microbial measurements and the other for mesofauna measurement. After 6, 12, 18, 24, 30 and 36 months, three pairs of litter bags were retrieved from around three trees randomly chosen at each site. Sampling dates were set to follow spring and autumn rainfall periods, variable depending on the year, but generally mid-June and early or mid-December. For the sampling at 36 months, 27 supplementary bags were retrieved (three per sampling site) to have enough material for biochemical analysis. A total of 351 litter bags were thus used in the experiment.

#### LITTER BAG PROCESSING

The experiment monitored microbial catabolic profiles, fungal biomass and mesofauna abundances associated with decomposed

needles. To link the activity of these organisms to changes in leaf litter chemistry, we also tracked the concentrations of nutrients (C, N) and secondary metabolites (phenolic compounds and terpenoids) in the needles.

For this purpose, half of the litter bags were used for mesofauna and nutrient analysis, and the other half for chemical and microbial analysis. Needle moisture content and decay rates were determined from the full set of litter bags used. At  $t_0$ , three litter samples per successional stage were processed as described below.

In order to prevent contamination of litter by soil, we wiped each needle thoroughly before analysis.

#### Litter decay rates and moisture content

Based on the dry mass data, we calculated the percentage of litter mass remaining (%MR) for each sample. For mesofauna samples, dry mass was determined after oven-drying at 60 °C for 3 days. For secondary metabolite measurements, we first freeze-dried the samples (Lyovac GT2®). Moisture content was calculated as ratio of dry mass to fresh mass, and litter mass loss was calculated as ratio of remaining dry mass to initial dry mass.

#### Mesofauna extraction

Mesofauna was extracted from fresh litter using the dry funnel method (Berlese 1905). Animals were stored in 70% alcohol, counted using a binocular scope and identified to genus for Collembola (Gisin 1960) and to order for Acari (Gamasida, Acaridida, Actinedida and Oribatida; Coineau 1974). Other invertebrates were separated according to taxa (e.g. Arachnida, Diplopoda, Chilopoda, Aranea and Hymenoptera). Samples from the last sampling date (36 months) were unusable owing to a technical problem.

#### Fungal biomass

Fungal biomass was determined by quantifying ergosterol, which is a fungal membrane constituent and thus a good indicator of living fungal biomass (Gessner & Chauvet 1993; Ruzicka *et al.* 2000). Samples were frozen and lyophilized to enable more efficient extraction of ergosterol (Gessner & Schmitt 1996).

Ergosterol was extracted from 50 mg of needles with 5 mL of an alcohol base (KOH/methanol 8 g L<sup>-1</sup>) for 30 min and purified by solid-phase extraction on a Waters® (Milford, MA, USA) Oasis HLB cartridge (Gessner & Schmitt 1996). The extract produced was purified and quantified by high-performance liquid chromatography (HPLC) on a Hewlett Packard series 1050 system running with HPLC-grade methanol at a flow rate of 1.5 mL min<sup>-1</sup>. Detection was performed at 282 nm, and the ergosterol peak was identified based on the retention time of an ergosterol standard.

#### Relative contribution of decomposers to needle decomposition: towards a carbon budget

The relative contribution of organisms to overall loss of needle mass was based on biomass measurements, expressed in% of initial needle carbon, corresponding to standing stocks. As described in Hieber & Gessner (2002), the overall biological decomposition of litter was assumed to be a function of detritivorous invertebrate feeding and microbial assimilation of litter constituents. We did not measure bacterial contribution to litter decomposition in our study because

bacterial biomass accounts for only a minor fraction (less than 4%) of total microbial biomass (i.e. fungal plus bacterial) (Baldy, Gessner & Chauvet 1995).

Fungal biomass was estimated from ergosterol content using a conversion factor of 250 (Montgomery *et al.* 2000). Fungal carbon was estimated by assuming 43% carbon in fungal dry mass (Baldy, Gessner & Chauvet 1995).

Litter mass loss due to fungal assimilation was related to the fraction of assimilated needle material converted to fungal biomass and to fungal CO<sub>2</sub>. The percentage of initial leaf carbon assimilated by fungi was then calculated by dividing total fungal net production (equivalent to the accumulated standing stock at the respective sampling date through the fungal biomass expressed in fungal carbon per initial needle carbon) by an average growth efficiency of 0.35 (i.e. 35% of leaf carbon is transformed into fungal carbon biomass and 65% into CO<sub>2</sub> from fungal respiration; Suberkropp 1991). The contribution of fungal assimilation to loss of leaf carbon was estimated by dividing fungal carbon assimilation by loss of needle carbon.

Collembola biomass was determined according to the length–dry mass regressions given by Petersen (Petersen 1975):  $\log Y$  (dry mass,  $\mu\text{g}$ ) =  $\log a + \log b \times \log X$  (body length, mm). Body length of Collembola was estimated for each species by average body length from the literature (Gisin 1960; Petersen 1975). Detritivore Acari biomass was determined by assuming an individual dry mass of 12.76  $\mu\text{g}$  (Borcard 1988).

For the carbon budget, we considered that mesofauna and needles contained the same percentage of carbon. Litter mass loss due to detritivorous feeding depends on relative consumption rate (feeding rate per unit body mass, assumed to be 15% in this study, based on data from a labelled litter experiment to determine percentage litter assimilation by soil fauna; Caner *et al.* 2004) and biomass of detritivores. The percentage of initial leaf carbon assimilated by detritivores was then calculated by dividing total detritivorous net production (equivalent to the accumulated standing stock at the respective sampling date through the detritivorous biomass expressed in detritivorous carbon per initial needle carbon) by 0.15 (feeding rate). The contribution of detritivorous assimilation to loss of leaf carbon was estimated by dividing detritivorous carbon assimilation by loss of needle carbon.

### Catabolic profiles

Microbial (fungal and bacterial) catabolic profiles were assessed using Biolog<sup>®</sup> EcoPlates (Biolog Inc., Hayward, CA, USA) for all sampling dates except the initial one, using a procedure adapted from Garland & Mills (1991). We purposely did not adjust inoculum concentration because we considered total microbial number to be an inherent characteristic of microbial communities in each sample. Briefly, 2.95 g (dry mass equiv.) of ground needles were shaken in 100 mL of a sterile 0.1% solution of tetrasodium pyrophosphate for 1 h to suspend the microbial communities. Each 96-well plate contains three replicate blocks of 31 individual carbon sources, which are considered to be equivalent to root exudates (Preston-Mafham, Boddy & Randerson 2002), with a water blank for each block of replicates. A 125- $\mu\text{L}$  aliquot of extract solution, diluted 1:110, was added to all 96 wells in each EcoPlate. The plates were incubated at 30 °C for 7 days, and absorbance was measured at 595 nm on a microplate reader (Meter-tech  $\Sigma$ 960, Avanteq, France). Different microbial communities can exhibit different patterns of substrate use, as revealed by colorimetric reaction.

### Chemical analysis

**C and N.** Litter samples were dried at 80 °C and milled (< 2 mm) for C and N determination on a Flash EA 1112 series C/N elemental analyser (ThermoScientific, Waltham, Massachusetts, USA).

### Plant secondary metabolites (PSM)

**Terpenoids.** Terpenoids were extracted from 500 mg of ground needles with 5 mL of cyclohexane, and 50  $\mu\text{L}$  of an internal standard (dodecane) was added. The samples were filtered and then analysed by gas-chromatography/mass-spectrometry (GC-MS) on an Agilent HP6890 system equipped with an MSD5973 Network mass detector, an ALS7673 automatic injector and an HP5-MS apolar column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ; J&W Agilent Technologies, Crawford Scientific, Lanarkshire, Scotland, UK). Qualitative analysis of the compounds was carried out in comparison against library mass of spectra and literature retention times (Adams 1989). Retention indices were determined relative to injected Wisconsin Diesel Range Hydrocarbons (Interchim, Montluçon, France) and confirmed by comparison against expected literature values (Adams 1989).

**Phenolic compounds.** Phenolic compounds were analysed using two complementary methods: colorimetric for quantifying total phenolic content and chromatographic for measuring concentrations of some dominant phenolics, in order to identify allelopathic compounds and to calculate the carbon content of each compound for the carbon allocation budget. Both methods used aqueous extracts (1 g litter in 20 mL distilled water).

**Colorimetric analysis**—The method used to extract total needle phenolic contents was based on the work of Peñuelas *et al.* (1996): 0.25 mL of filtered aqueous extract was mixed with 0.25 mL of Folin–Ciocalteu reagent (Folin & Denis 1915) and 0.5 mL of saturated aqueous Na<sub>2</sub>CO<sub>3</sub> to stabilize the colour reaction, after which 4 mL of distilled water was added to dilute the extract. After 1 h, the reaction was completed and measured at 765 nm on a UV/Vis spectrophotometer (Thermoscientific). Quantitative results were expressed with reference to gallic acid.

**Chromatographic analysis**—Analyses were performed on needle extracts using a method adapted from Fiamegos *et al.* (2004). We used a phenolic extraction–derivatization technique via phase transfer catalysis (PTC). The methylation–extraction method was optimized for phenolics, fatty acids and diacid, and tested for a small number of compounds (2 or 3) from each chemical family: fatty acids (palmitic and stearic acids), fatty diacids (succinic and azelaic acids), simple phenols (catechol and pyrogallol), acetophenones (acetovanillone and acetosyringone), phenolic acids (4-hydroxybenzoic, protocatechuic and gallic acid) and cinnamic acids (*p*-coumaric, caffeic and sinapic acids). These compounds were selected based on their occurrence in *P. halepensis* (qualitative analysis) or on their proneness to methylation under these conditions.

The methylation process meant that quantified derivatives probably included several allelochemicals.

Stock solutions (1 mg mL<sup>-1</sup>) of each compound were prepared by dissolving pure standards (25 mg) in deionized water (25 mL). Methanol/water (1:1 v/v) was used for less soluble compounds. Tetrahexylammonium bromide (THAB, 0.1 M; 217.3 mg in 5 mL) and the internal standard were dissolved in dichloromethane.

The procedure was carried out in 35 mL Pyrex tubes equipped with PTFE screw caps. Each individual extract (10 mL) was added with stirring to the following solution: KH<sub>2</sub>PO<sub>4</sub> buffer (500 µL of a 1 M solution; pH 8.0), tri-*n*-butylmethylphosphonium bound polymer (1.4 mmol Cl<sup>-</sup> g<sup>-1</sup> resin (50 mg), THAB in dichloromethane (100 µL of a 0.1 M solution), internal standard in dichloromethane (50 µL of a 100 µg mL<sup>-1</sup> solution), dichloromethane (850 µL) and methyl iodide (100 µL). Tubes were sealed and heated (80 °C) for 1 h to allow methylation. After cooling, NaCl was added, and the solutions were vigorously shaken. After separation, the organic layer was removed, filtered through a filter syringe (0.45 µm) and analysed by GC-MS. The same procedure was used to methylate standards for calibration curves (plotted by taking the ratio of external variable standard to internal constant standard).

#### STATISTICAL ANALYSIS

To compare needle decomposition rates between successional stages, needle mass loss data were fitted to the simple exponential model  $m_t = m_0 e^{-kt}$ , where  $m_t$  is needle mass remaining (g) at time  $t$  (years),  $m_0$  is initial needle mass (g), and  $k$  (year<sup>-1</sup>) is the exponential decomposition rate coefficient (Olson 1963). A multiple slope comparison was made to test for differences between the decomposition rates of the different successional stages after log transformation (STATGRAPHICS PLUS 5.1, StatPoint Technologies, Warrenton, Virginia, USA).

The experimental design included two fixed factors: time of decomposition process (from 0 to 36 months of decomposition, referred to as 'time') and successional stage of Aleppo pine (early stage, middle stage or late stage, referred to as 'successional stage'). For all measured variables, two-way ANOVAS (Crawley 2005) were used to test the effects of successional stage and sampling date, followed by Tukey tests for *post hoc* pairwise comparisons (Zar 1984). If any interaction occurred, we performed one-way ANOVA for each sampling date to test the effect of successional stage. Correlations among variables were explored using Pearson product-moment correlations. Normality and homoscedasticity of the data were first checked using Ryan-Joiner and Levene tests, respectively (Zar 1984). When conditions were not met, data were ln-transformed before performing statistical tests. All statistical tests used  $\alpha = 0.05$  to determine statistical significance. For Tukey tests, different letters denote significant differences with  $a < b < c$ . All these analyses were conducted using Minitab (release 13 for WINDOWS, Minitab SARL, Paris, France).

For the Biolog data, the average well colour development (AWCD) of each internal replicate block of 31 wells in each plate was calculated at each reading interval to determine the incubation time corresponding to AWCD = 0.4 absorbance units ( $t_{0.4}$ ). The absorbance value at  $t_{0.4}$  in each well was then normalized. Principal component analysis (PCA) was conducted using R software to reveal differences between substrate utilization profiles. As there were 31 dependent variables with possible collinearity, the appropriate parametric model would have been MANOVA. However, MANOVA is sensitive to deviations

from multivariate normality, so we used a more robust permutation-based procedure, PMANOVA (PRIMER software v6, Primer-E Ltd, UK; Pesarin 2001).

## Results

#### LITTER MASS LOSS AND MOISTURE CONTENT

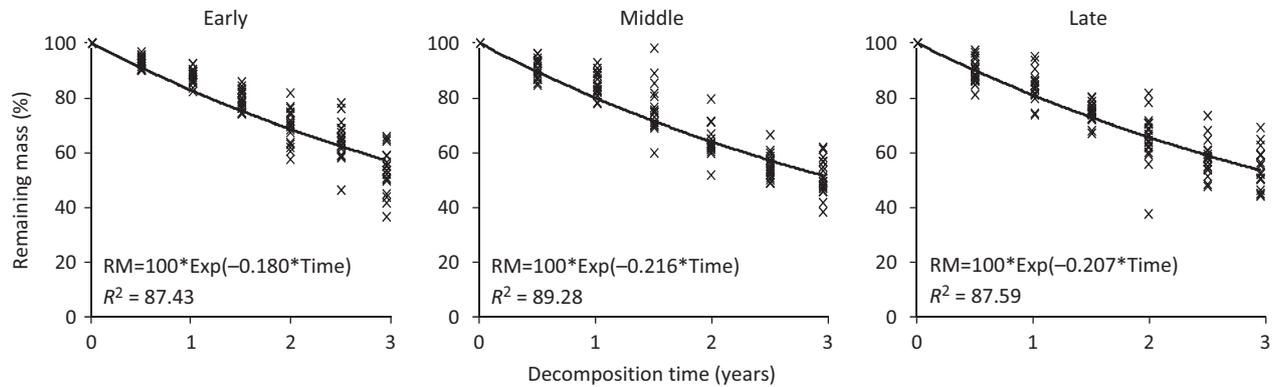
On average, 53% of the litter was decomposed after 3 years. Decomposition rate was slightly but significantly lower in early-stage plots than in middle- and late-stage plots (multiple-slopes comparison,  $P < 0.001$ ), at  $0.18 \pm 0.0087$  vs.  $0.22 \pm 0.0096$  and  $0.21 \pm 0.0094$  years<sup>-1</sup> (mean  $\pm$  SD), respectively (Fig. 1). The heterogeneity between litter bag mass losses had increased at the end of the experiment (Levene test,  $P < 0.001$ ). The between-stage difference in decomposition rates was mainly due to a higher mass remaining in early-stage plots at 6, 12, 18 and 30 months of decomposition (one-way ANOVA,  $P < 0.05$ ), whereas this difference was no longer significant at 36 months of decomposition.

Concerning moisture content, we generally observed lower values in early-stage plots than in the two others, except after 36 months of decomposition (one-way ANOVA,  $P < 0.05$ ). Litter moisture content was always lower in June than in October in early-stage plots, whereas no difference between seasons was observed for the other two stages, except at 24 months, for which moisture content was highest compared with all the other sampling dates (one-way ANOVA,  $P < 0.05$ ; Table 1).

#### BIOCHEMICAL CHARACTERISTICS

##### C and N

Carbon content decreased gradually with decomposition time, with highest C content at initial time compared with all the other sampling dates, followed by 6 and 12 months, then 18, 24 and 30 months, and finally the lowest observed after 36 months of decomposition (two-way ANOVA,  $P < 0.05$ ; Table 1). We also found a significant effect of successional stage on carbon litter content over the course of the decomposition process, with a higher carbon content in the early-stage plots than in the middle- and late-stage plots (two-way ANOVA,  $P < 0.05$ ; Table 1). Nitrogen litter content tended to be higher for middle- and late-stage plots at 24 and 30 months of decomposition, but without significant differences. Consequently, C/N ratio decreased over the course of the decomposition process, with higher levels at 0 and 6 months of decomposition, and lower levels at 30 and 36 months of decomposition (two-way ANOVA,  $P < 0.001$ ). There was also a significant difference in C/N ratio between the three successional stage plots, but only at 24 months of decomposition, with higher C/N ratio in early-stage plots than in middle- and late-stage plots (one-way ANOVA,  $P < 0.05$ ).



**Fig. 1.** Dynamics of remaining mass expressed as a relative fraction of initial mass for *Pinus halepensis* needle litter incubated in litter bags in early, middle- and late-stage plots. Curves represent the exponential model.

### Plant secondary metabolites (PSM)

**Phenolic compounds.** Phenolic index fell from 12.4 to 1.4 mg gallic acid  $\text{g}^{-1}$  of litter dry weight (mean of the three successional stage plots at the initial sampling date and after 36 months of decomposition, respectively; Fig. 2). Litter phenolic content was higher in the early-stage plot from the beginning to 12 months of decomposition compared with the other two successional stage plots (one-way ANOVA,  $P < 0.05$ ), in which 80% of phenolic compounds disappeared after the first 6 months of decomposition (ANOVA,  $P < 0.01$ ). Differences among stage plots were no longer significant after 18 months. Caffeic and *p*-coumaric acid litter contents followed the same dynamics, but the difference between stage plots remained 6 months longer (one-way ANOVA,  $P < 0.05$ ).

**Terpenoids.** Total terpenoids decreased from 13.7 at the initial time (late-stage plots) to 0.9 (early-stage plots)  $\text{mg g}^{-1}$  of litter dry weight at 36 months of decomposition.

During the first year of decomposition, we did not observe a decrease in total terpenoid litter concentrations except in late-stage plots where concentrations were significantly higher at 6 than at 12 months (one-way ANOVA,  $P < 0.05$ ; Table 1), but the three terpene families showed different dynamics. Monoterpenes decreased drastically (73%) in the first 6 months, except in early-stage plots (one-way ANOVA,  $P < 0.05$ ). Sesquiterpenes and diterpenes survived longer in litter, with a 90% and 59% decrease between 12 and 18 months of decomposition in the three stage plots, respectively (one-way ANOVA,  $P < 0.05$ ).

There were significantly smaller amounts of monoterpenes in the early-stage plots than in the other two stage plots for all the sampling dates, except at 12 months of decomposition, where the difference was not significant (one-way ANOVA,  $P < 0.05$ ). We also observed smaller amounts of sesquiterpenes and diterpenes in the early-stage plots at the beginning of the decomposition process, and these differences disappeared after 24 and 30 months for sesquiterpenes and diterpenes, respectively (one-way ANOVA,  $P < 0.05$ ).

### MICROBIAL ACTIVITY

In PCA, the first two axes explained 39% and 12% of variance, respectively (Fig. 3). The first axis mainly isolated the '30 months' timepoint, marking an increase in polymer use by the microbial community. The second axis differentiated the first 18 months of decomposition from the '24 months' and '36 months' timepoints, marking an increase in amino acid and carboxylic acid use and a decrease in carbohydrate use.

Of the 31 carbon sources of the Biolog plates, 18 showed marked changes in use by micro-organisms: microbial communities used mainly simple sugars at the beginning of the process, whereas carboxylic acids and amino acids were used from the second year onwards. Also, the micro-organisms managed to consume most polymeric substances at 30 months.

Forward selection and unrestricted Monte Carlo permutation tests indicated that the 'time' factor made statistically significant contributions ( $P < 0.01$ ) to explaining the variance in the microbial catabolic profiles data, but without a significant effect of successional stages.

### FUNGAL BIOMASS

Ergosterol litter content ranged from 0 (early-stage plots at the beginning of the decomposition process) to 0.576  $\text{mg g}^{-1}$  of litter (late-stage plots at 30 months) and was significantly lower in the early-stage plots than in the other 2 stage plots until 12 months of decomposition (one-way ANOVA,  $P < 0.001$ ). In addition, fungal biomass increased sharply during the first 6 months irrespective of the successional stage (Fig. 2).

Ergosterol content was significantly and negatively correlated with phenolic index, with a linear relationship between phenolic compound concentration and fungal biomass [ $P < 0.01$ ;  $r = -0.592$ , (ergosterol) =  $0.52 - 0.03$  (phenol)].

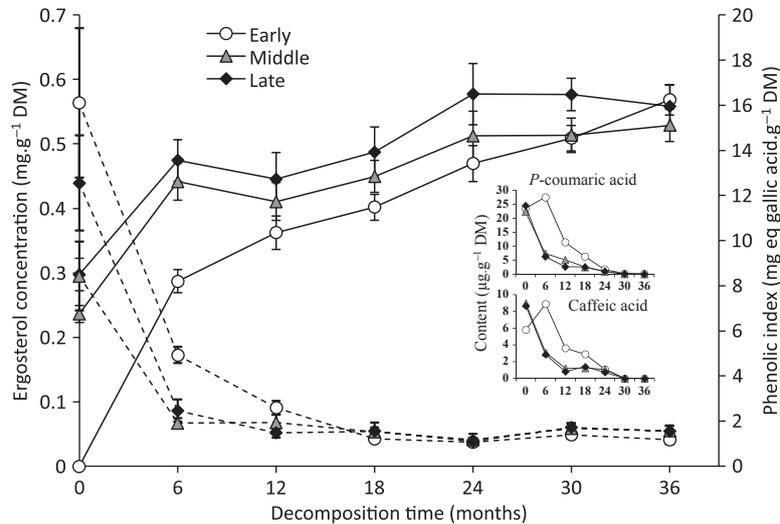
### MESOFAUNA

Mesofauna was dominated by Acari (proportion ranging between 52% and 82% of total abundance) and Collembola (proportion ranging between 9% and 45%) for all the successional stage plots. Collembola abundance was significantly lower in the early-stage plots at 6 and 18 months of decomposition (one-way

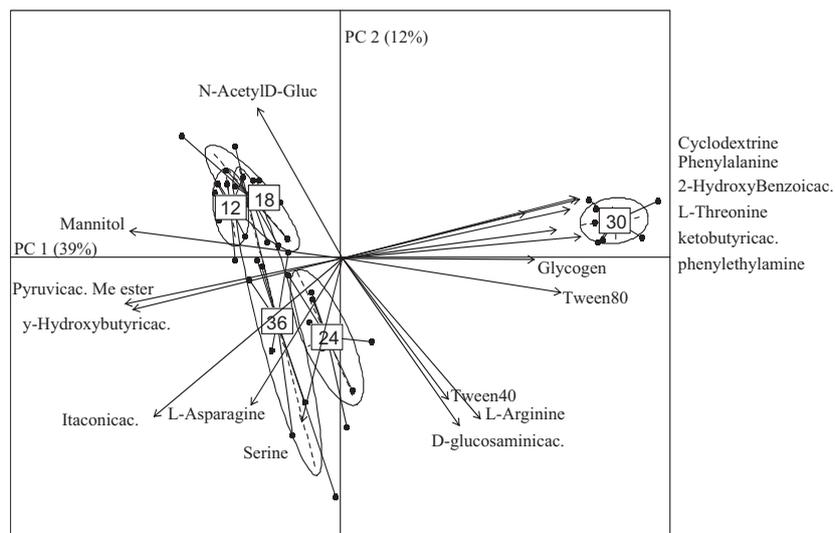
**Table 1.** Litter moisture content, carbon and nitrogen contents, C/N ratio, total terpenoids, and mono-, sesqui-, and diterpene contents (mg and  $\mu\text{g g}^{-1}$  litter dry weight, respectively)

Decomposition time (months)	Successional stages	Moisture content (%)	C (mg g <sup>-1</sup> DW)	N (mg g <sup>-1</sup> DW)	C/N	Terpene sum (mg g <sup>-1</sup> DW)	Monoterp (μg g <sup>-1</sup> DW)	Sesquiterp (μg g <sup>-1</sup> DW)	Diterp (μg g <sup>-1</sup> DW)
0	Early	–	528.6 (9.6)	5.2 (0.7)	105.4 (16.2)	7.6 (0.1)	<b>356.4 a (86.5)</b>	<b>3754.7 a (192)</b>	4298.8 (838.9)
	Middle	–	523.4 (1.2)	4.2 (0.3)	125.2 (8.7)	12.8 (0.6)	<b>1103.1 b (221.3)</b>	<b>6266.3 b (210.1)</b>	5578.2 (1091.6)
	Late	–	518.0 (8.7)	4.9 (0.3)	107.3 (6.4)	13.7 (0.5)	<b>1648.9 c (70.8)</b>	<b>6804.3 b (168.7)</b>	4828.2 (664.3)
6	Early	–	498.4 (6.7)	5.1 (0.3)	99.3 (5.4)	6.4 (1.0)	<b>216.5 a (62.1)</b>	<b>2893.8 a (554.9)</b>	<b>3171.4 a (532.7)</b>
	Middle	–	498.5 (4.2)	4.4 (0.1)	113.3 (2.8)	13.9 (0.8)	<b>465.5 b (34.9)</b>	<b>6367.1 b (223.6)</b>	<b>7049.9 b (749.2)</b>
	Late	–	490.3 (6.2)	4.6 (0.3)	110.1 (7.9)	12.5 (0.8)	<b>625.8 b (84.4)</b>	<b>6223.6 b (561.6)</b>	<b>5582.3 b (555.4)</b>
12	Early	35.8 (1.2)	507.4 (7.1)	5.6 (0.2)	92.1 (4.0)	6.8 (0.7)	186.9 (37.7)	<b>2830.2 a (375.6)</b>	<b>3773.7 a (379.3)</b>
	Middle	39.3 (2.4)	497.9 (4.2)	5.3 (0.2)	95.0 (4.0)	11.3 (0.8)	300.3 (24.3)	<b>4350.1 b (293.7)</b>	<b>6566.5 b (559.9)</b>
	Late	40.3 (2.9)	488.0 (2.2)	5.4 (0.4)	93.8 (6.6)	8.7 (0.7)	408.5 (116.5)	<b>3807 ab (287.9)</b>	<b>4474.1 a (557)</b>
18	Early	<b>14.7 a (2.3)</b>	505.2 (6.4)	6.3 (0.3)	82.2 (4.0)	1.5 (0.3)	<b>98.6 a (19.3)</b>	<b>143.9 a (33.9)</b>	<b>1235.8 a (276.5)</b>
	Middle	<b>33.7 b (4.7)</b>	470.9 (10.4)	6.2 (0.4)	77.4 (3.9)	3.0 (0.3)	<b>243.5 b (13.1)</b>	<b>447.2 b (4738)</b>	<b>2245.7 b (215.6)</b>
	Late	<b>32.8 b (5.3)</b>	481.5 (23.3)	6.3 (0.3)	77.4 (3.0)	3.4 (0.3)	<b>310.9 b (43.9)</b>	<b>570 b (60.7)</b>	<b>2494.7 b (243.8)</b>
24	Early	<b>59.9 a (1.1)</b>	499.7 (6.9)	6.9 (0.4)	<b>74.6 b (4.4)</b>	1.0 (0.2)	<b>134.5 a (19.8)</b>	91.6 (19.4)	<b>782.5 a (145.4)</b>
	Middle	<b>60.7 ab (1.2)</b>	469.7 (7.5)	7.9 (0.3)	<b>60.1 ab (1.9)</b>	1.7 (0.2)	<b>253.2 b (25.2)</b>	153 (26.3)	<b>1321.2 b (140.2)</b>
	Late	<b>64.7 b (1.6)</b>	479.4 (9.9)	7.7 (0.4)	<b>63.2 a (2.9)</b>	1.4 (0.2)	<b>241.4 b (30.4)</b>	182 (43.7)	<b>972.6 ab (128.5)</b>
30	Early	<b>25.4 a (2.5)</b>	492.4 (6.0)	8.5 (0.5)	59.9 (4.1)	1.7 (0.2)	<b>220.4 a (31.7)</b>	80.6 (15)	1328.6 (167.8)
	Middle	<b>41.5 b (4.2)</b>	473.0 (5.2)	9.6 (0.5)	50.3 (2.1)	1.7 (0.2)	<b>299.8 ab (23)</b>	72.4 (14.7)	1298.9 (157)
	Late	<b>38.9 b (4.7)</b>	482.3 (7.3)	9.6 (0.5)	51.1 (2.2)	2.0 (0.3)	<b>340.8 b (37.81)</b>	79.7 (20.9)	1577.5 (263.8)
36	Early	43.5 (4.0)	498.5 (8.4)	9.4 (0.7)	55.0 (3.6)	0.9 (0.3)	<b>152.8 a (31)</b>	58.5 (23.2)	687.2 (233.5)
	Middle	37.0 (5.6)	464.7 (8.9)	9.3 (0.3)	50.4 (1.6)	1.3 (0.2)	<b>326.3 b (34)</b>	57.6 (7.8)	884 (115.7)
	Late	44.1 (4.6)	467.7 (8.4)	9.2 (0.3)	51.4 (1.7)	1.9 (0.6)	<b>404.8 b (58.39)</b>	195.3 (121.8)	1282.9 (430.8)

Values are given as means (SD). Bold values with different letters denote significant differences between stages ( $P < 0.05$ , Tukey's *post hoc* test after one-way ANOVA).



**Fig. 2.** Dynamics of ergosterol litter content (solid lines) and phenolic index (dashed lines) over the course of decomposition (means and SD). Inset: change in two specific phenolic compound litter contents (caffeic and *p*-coumaric acid) during decomposition in the three successional stage plots. For readability, error bars are not given on the graph; see statistical analyses results in Results 'phenolic compounds'.



**Fig. 3.** Factorial map and correlation circle of the 18 most used C sources obtained by principal component analysis applied on data for colour development in the Biolog Ecoplate wells at standardized times. Numbers in boxes correspond to decomposition time in months.

ANOVA,  $P < 0.05$ ). The abundance of detritivorous mites was significantly lower in young pine forest litter only at 18 months of decomposition (one-way ANOVA,  $P < 0.05$ ), whereas abundances of other predators and other detritivores were lower only at 6 months (one-way ANOVA,  $P < 0.05$ ). After 2 years of decomposition, Collembola and Acari abundances had increased strongly in all successional stage plots (one-way ANOVA,  $P < 0.01$ ) (Fig. 4).

#### CARBON ALLOCATION BUDGET

Fungal carbon biomass (in terms of standing stocks, in% initial needle carbon) was tiny in senescent needles at the initial sampling date ( $t_0$ ) in the early-stage plots, but reached more than 2% and 3% of initial needle carbon in the middle- and

late-stage plots, respectively (Fig. 5). This difference persisted after 6 months of decomposition (one-way ANOVA,  $P < 0.05$ ), with a lower relative fungal biomass in the early-stage plots than the middle- and late-stage plots. At 30 months, this pattern had reversed, with higher fungal biomass in early-stage plots (one-way ANOVA,  $P < 0.05$ ). Biomass values peaked after 6 months regardless of stage, but then remained high until the end of the experiment in the early-stage plots, whereas they dropped after 12 months in the middle- and late-stage plots (one-way ANOVA,  $P < 0.05$ ).

Detritivorous biomass (in terms of standing stocks, in% initial needle carbon) was similar for all stages, but varied significantly over the course of the decomposition process, peaking at 24 and 30 months of incubation in the field (two-way ANOVA,  $P < 0.05$ ).

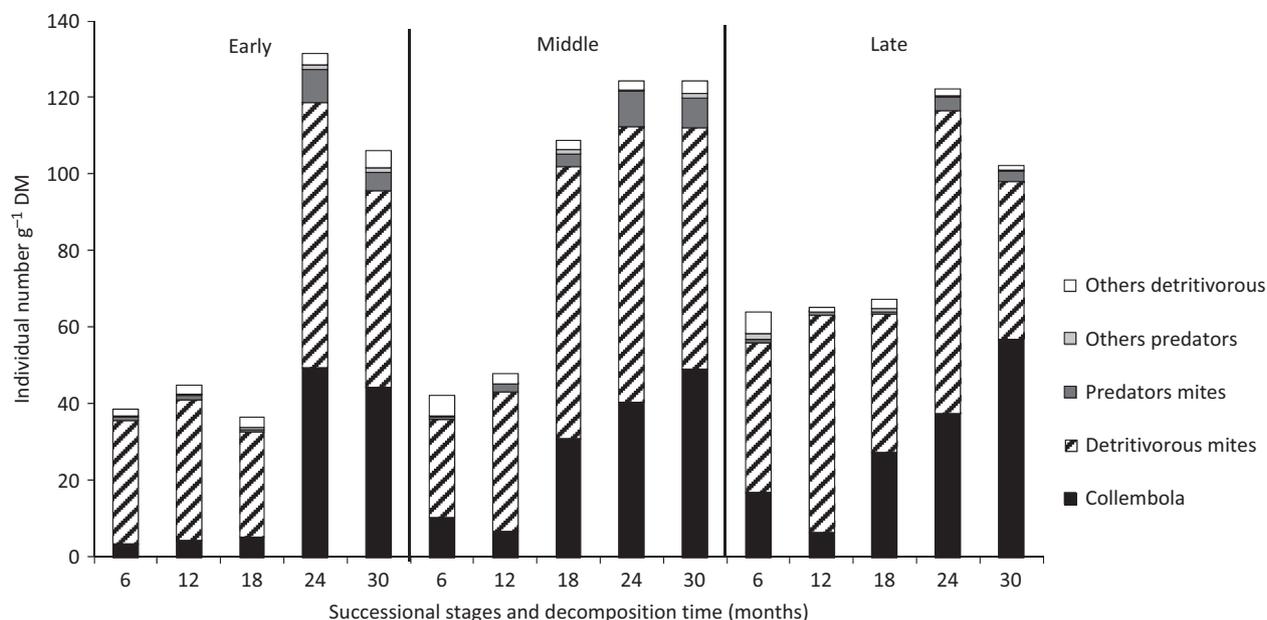


Fig. 4. Dynamics of abundance of different functional groups belonging to mesofauna.

Fungal biomass was higher than detritivore biomass at all timepoints and across successional stages, resulting in a fivefold to eightfold higher maximum contribution of fungi to needle mass loss compared with that of detritivores (Table 2). Fungal contributions were similar between stages, except at 30 months of decomposition, when they were greater in the early-stage plots than in the other two stage plots (one-way ANOVA,  $P < 0.05$ ). The detritivore contribution was significantly higher in the late-stage plots than in the early-stage plots (one-way ANOVA,  $P < 0.05$ ).

We examined litter PSM expressed in carbon to complete the carbon allocation budget. Terpenoid carbon (as a percentage of initial needle carbon) averaged about 200 times higher than phenolic carbon in litter, regardless of the successional stage (Fig. 5). Carbon litter PSM differed from one stage to another during the 12 first months for phenolic carbon, and during the first 18 months for terpenoid carbon, but then converged at later dates (two-way ANOVA,  $P < 0.01$ ; Fig. 5). Phenolic carbon decreased sharply during the first 6 months in the middle- and late-stage plots, but between 6 and 12 months in the early-stage plots (one-way ANOVA,  $P < 0.05$ ). Terpenoid carbon decreased linearly over the first 18 months in the late-stage plots, but decreased from 6 months onwards in the middle-stage plots (one-way ANOVA,  $P < 0.05$ ) and in two steps, between 0 and 6 months and between 12 and 18 months of decomposition, in the early-stage plots (one-way ANOVA,  $P < 0.05$ ).

## Discussion

The present study describes a mechanistic approach to the litter decomposition process in different successional stages of pinewoods under a Mediterranean climate, based on a thorough investigation of the interrelation between litter

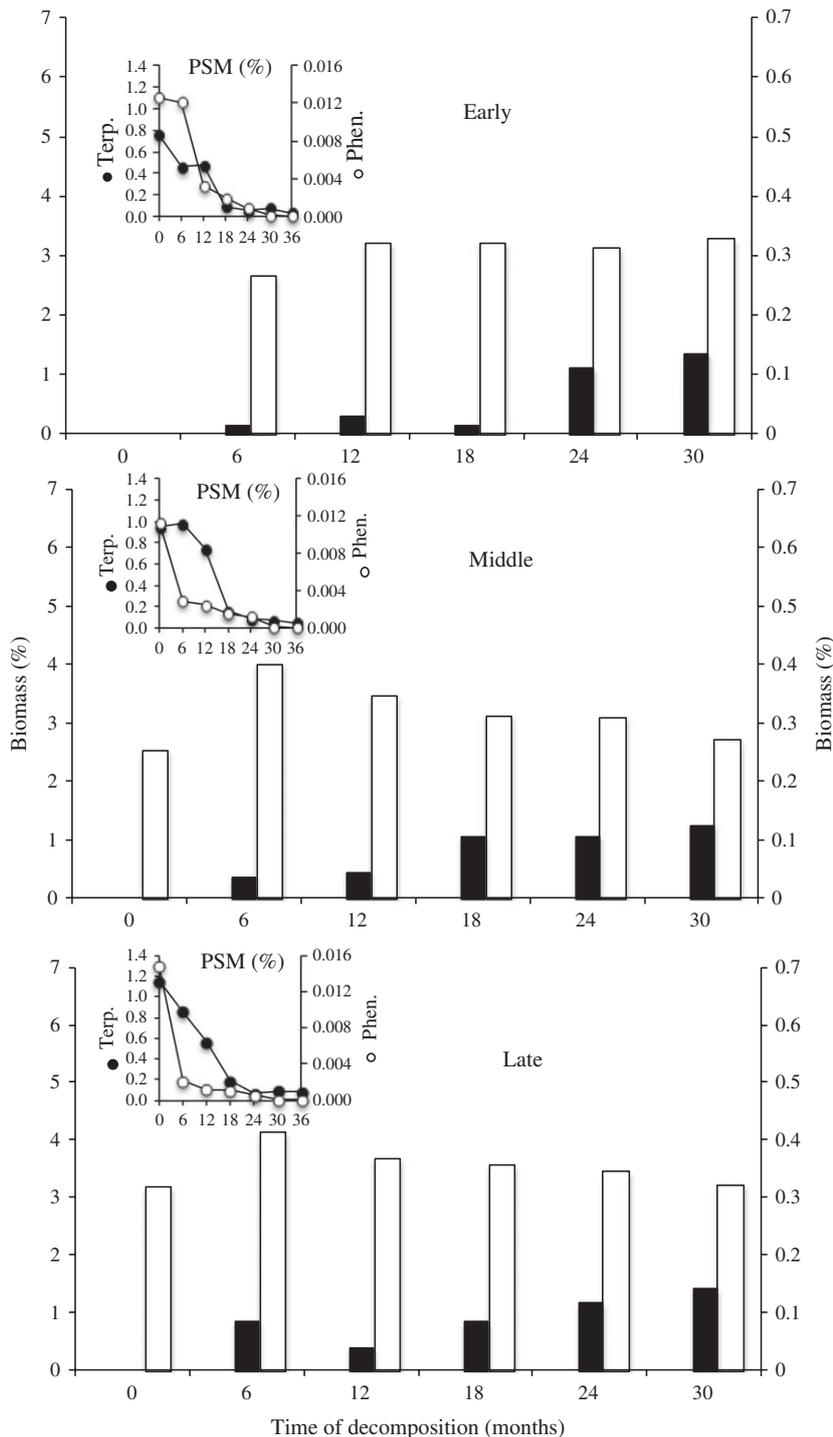
secondary metabolites and decomposer communities during the course of the process, which sheds light on the 'black box' of decomposition. The three main findings of the study are (i) the importance of phenolic compounds as drivers of the decomposition process; (ii) the predominance of fungi in the process and (iii) a lower decomposition rate in early successional stage due to high levels of phenolics in decomposed needles, which delay the colonization of litter by decomposers.

We now explore these findings and their implications below.

### DYNAMICS OF CHEMICAL LITTER COMPOSITION DURING DECOMPOSITION

Decomposition leads to a gradual change in litter quality (Joffre *et al.* 2001). At the initial time, the chemical composition of litter under the three ages of pinewood showed slight differences, but we observed different kinetics of change in chemical composition during the litter decay process between successional stages. These differences were more pronounced during the first year of decomposition and progressively faded with increasing length of decomposition.

Phenolic residence time was longer in litter decomposing under early-stage forest, litter content remaining stable during the first 6 months of decomposition in the early stage, whereas most litter phenolic compounds showed a strong 70% decrease over this same 6-month period in middle- and late-stage forest. This pattern was particularly marked for caffeic acid and *p*-coumaric acid, which like most cinnamic acids have been implicated in many biological interactions (Rice 1984; Batish *et al.* 2008), especially with the microbial compartment (Harrison, Bol & Bardgett 2008) through allelopathic mechanisms.



**Fig. 5.** Changes in the relative proportions of decomposer biomass during decomposition in the three successional stage plots (early, middle, late). White bars correspond to fungal biomass. Black bars correspond to detritivore biomass. For comparison, biomasses are expressed in % of initial needle carbon content. Insert: change in the PSM (plant secondary metabolites), for example terpenoids and phenolics, carbon litter contents during decomposition in the three successional stage plots. All values are expressed as % of initial needle carbon. For readability, error bars are not given on the graph; see statistical analyses results in Results 'carbon allocation budget'.

To explain the between-stage differences in litter phenolic residence times, besides biological degradation, it is also important to take into account external factors through the different characteristics of open (early stage) vs. closed (middle and late stages) canopy areas. In the open habitat, both soil and litter were particularly dry in the summer, even though all three stages had received similar rainfall during the experiment. As phenolics are mainly soluble compounds (Kuiters 1990), they may have been less efficiently leached out of young pine litter, and consequently persisted longer in the

needles during the first 6 months of decomposition. This prompts the conclusion that water stress in the Mediterranean region could extend the residence time of secondary metabolites, at least phenolic compounds, by the cumulative negative effects of low leaching and low biological decomposition in arid conditions.

Conversely, terpenoids were retained for longer periods of time in decomposed needles and were less abundant in young pine forest litter. Aleppo pine needle terpenoid content is known to increase in response to both interspecific and

**Table 2.** Relative contributions of fungi and detritivorous mesofauna decomposers to needle mass loss (as% of initial needle carbon) in the three successional stages (early, middle and late) after 6–12 and 30 months of needle breakdown

	Early	Middle	Late
Mesofauna biomass (%)	8 (30 months)	10 (30 months)	12 (30 months)
Fungal biomass (%)	60 (6–12 months)	79 (6 months)	67 (6 months)

Calculations were specified in Materials and methods, at the sampling date for which this contribution was maximal (30 months for detritivores and 6–12 months for fungi).

intraspecific competition (Ormeño *et al.* 2006). The open-habitat young pine forests studied here were characterized by less intraspecific competition than those in the middle or late stages, which could partly explain between-stage differences in needle terpenoid contents (Ormeño *et al.* 2006). Even though terpenoids are known to have strong allelopathic effect on microbial population (Adamczyk *et al.* 2013), the between-stage difference in terpenoids did not appear to significantly affect litter colonization by decomposers.

#### PATTERN AND MAGNITUDE OF DECOMPOSER FUNCTIONAL GROUPS AND THEIR INTERRELATIONS DURING DECOMPOSITION PROCESS: TOWARDS A CARBON BUDGET OF NEEDLE LITTER DECOMPOSITION

The most abundant groups of microarthropods associated with the litter were Oribatida and Collembola, as found in other studies (Petersen & Luxton 1982; Berg *et al.* 1998; Cortet & Poinso-Balaguer 1998). We observed that the abundances of these organisms only started increasing once the litter was colonized by micro-organisms, as they make the litter more palatable for invertebrates (Berg *et al.* 1998; Maraun *et al.* 2003). Ergosterol litter content was tiny at the beginning of the process, but then sharply increased to reach a plateau after 24 months of decomposition, reaching up to 580 µg of ergosterol per g of litter. There is a paucity of studies using ergosterol to quantify amounts of fungi associated with decomposed litter in terrestrial environments. Although we had no available reference for decomposed needles, our values fit the ergosterol values determined from shrub leaf litter under a Mediterranean climate (Ormeño *et al.* 2006), litter of four deciduous tree species in riparian and upland forests (Wu *et al.* 2011), and birch leaves decomposing under controlled conditions (Treplin & Zimmer 2012). Also, our values in decomposed pine needles under a Mediterranean climate are consistent with estimates from aquatic environments, where the ergosterol technique has a long history of use as a reliable measure of the role of fungi in the decomposition process (Gessner & Chauvet 1994; Baldy, Gessner & Chauvet 1995). All these values, higher than those obtained with other methods (e.g. hyphal length measurements), confirm that fungi are more important than expected for decomposition, even under a Mediterranean climate and in secondary compound-rich litter.

Concerning dynamics of microbial communities, we observed a succession of catabolic capacities during the decomposition process. Water-soluble compounds such as low-molecular-weight carbohydrates were used preferentially during the first 18 months. After this date, labile substrates should have been depleted, as the community began to use more recalcitrant compounds such as polymeric substances, amino acids and carboxylic acids. Kourtev, Ehrenfeld & Huang (2002) suggested that certain chemical characteristics of litter, such as recalcitrant C compounds and N availability, could determine its microbial enzymatic potential, *that is*, the response of the soil community to metabolic requirements and nutrient availability (Caldwell 2005). Our findings support the previously described phase dynamics of leaf mass loss in terrestrial environments (Chapin, Matson & Mooney 2002), where soluble compounds are lost at the beginning of the process, with the decomposition of the most recalcitrant compounds (e.g. lignin) controlling the end of the process. The present study also confirms that decomposers preferentially use labile substrates, but then go through a shift once these compounds are no longer available (Rinkes *et al.* 2011), with a succession of three guilds during decomposition (fast-growing opportunistic micro-organisms with high affinities for soluble substrates, modest-growth-rate specialized decomposers with high affinities for holocellulose and slow-growing miners that are specialist lignin decomposers), according to the Guild Decomposition Model (Moorhead & Sinsabaugh 2006).

Globally, our findings on decomposer dynamics confirm the positive relationship between fungi and microarthropods: Collembola and Oribatida are attracted to fungi, the growth of which is in turn stimulated by micrograzing (Krivtsov *et al.* 2004). Most species of microbiodetritivorous soil mesofauna are known to feed on fungi (for a review, see Maraun *et al.* 2003), and grazing can even induce microbial community changes (Anderson 1988; Wall & Moore 1999). Although mesofauna feeding on fungi do not contribute directly to decay, interactions between the two biological compartments could enhance decomposition (Rihani, Kiffer & Botton 1995) as we observed for the middle and late stages.

Finally, focusing on the relative contribution of the functional groups (fungi and microarthropods) to decomposition, our results demonstrate that the rate of needle breakdown is primarily controlled by fungi, fungal carbon biomass explaining up to around 69% of needle carbon loss within the first 12 months of decomposition. Mesofaunal carbon biomass, corresponding to detritivorous acari (Oribatida) and Collembola, represented only a maximum of 0.14% of initial needle carbon and explained only 10% of needle carbon loss. However, the contribution of detritivores to needle breakdown may have been underestimated, as (i) the carbon biomass considered did not include all of the detritivorous invertebrates (e.g. crustaceans, centipedes and insects); (ii) the percentage of feeding on decomposed needles by Acari and Collembola may have been underestimated (15% of the observed biomass was attributed to needle consumption) and (iii) CO<sub>2</sub> produced by invertebrate respiration could account for a large share of needle carbon transformation. Even

though invertebrate respiration can prove to be substantial, our results show the predominance of fungi in overall needle carbon loss through carbon biomass production. This result is consistent with values reported in soils, where faunal biomass represents less than 4% of microbial biomass across all biomes (Fierer *et al.* 2009). Soil invertebrates have relatively limited abilities to digest soil resources and are thus largely reliant on micro-organisms to assimilate what they need from soil organic resources (the 'Sleeping Beauty paradox'; Lavelle 1997).

Fungi thus play an eminent role in the needle mass loss, whereas soil mesofauna contribute only sparingly to the decomposition process. With our original approach, this being the first study reporting on the contribution of decomposers in a terrestrial environment based on biomass measurements, this finding challenges the widespread claim that soil fauna is the main driver of litter decomposition (Swift, Heal & Anderson 1979).

#### LITTER DECOMPOSITION IN THE THREE SUCCESSIONAL STAGES: LINK BETWEEN DECOMPOSERS AND LITTER QUALITY

Our study lends valuable insights into the importance of land use change for ecosystem functioning, through the chemical characteristics of the local resources for the soil brown food chain.

Phenolic content could explain decomposer dynamics, as it is directly linked to needle biochemical characteristics: ergosterol (fungal biomass) increased, while litter phenolics decreased in time, and there were more phenolics in early-stage plots and consequently fewer fungi. This negative correlation between phenolic concentrations and fungal biomass confirms that fungi are inhibited by certain phenolic compounds that were initially present in litter (Kainulainen, Holopainen & Holopainen 2003; Hättenschwiler, Tiunov & Scheu 2005; Ormeño *et al.* 2006), but it may also confirm the role of fungi in the degradation of secondary metabolites by exo-enzymatic activities (Criquet 1999; Kainulainen, Holopainen & Holopainen 2003), detoxifying litter with allelopathic capacities (Bonanomi *et al.* 2005) that inhibit mesofauna feeding activity (Gallardo & Merino 1993; Andrés 1999).

The observed lag in fungal colonization of young pine forest litter, combined with greater quantities of phenolics during the first year of decomposition, converge to keep the abundance of litter-associated mesofauna in check. For example, Collembola abundance peaked after 24 months in young pine forest, but after 18 months in the other two types of pine forest. Based on our carbon budget data, the maximum contribution of fungi was similar between stages, whereas the maximum contribution of detritivores was significantly lower in the early stage, regardless of the decomposition time. Taken together, our data suggest surprisingly that phenolics exert a stronger influence on the decomposer community than terpenoids, even though there was roughly 200 times more terpenoid carbon in the litter than phenolic carbon, terpenoids

representing the main source of PSM carbon for decomposers in this Mediterranean forested ecosystem. Phenolic compounds, which have already been shown to play an important role in other ecosystems (Loranger *et al.* 2002), may well be a relevant trait for explaining litter mass loss even in Mediterranean forest.

The differences between successional stages for the dynamics of chemical compounds and decomposers could explain the lower rate of decomposition observed for needles from young trees, the association between Collembola and fungi increasing the rate of leaf consumption, and thus the subsequent rate of needle decomposition (Rihani, Kiffer & Botton 1995). Whereas decay rates in middle- and late-stage plots are consistent with those reported in the literature in pine forest (Garcia-Pausas, Casals & Romanya 2004; Almagro & Martinez-Mena 2012), decay rate was lower in early-stage plots, corresponding to rates observed in drier environments (desert, Garcia-Pausas, Casals & Romanya 2004). Our study points out that decomposition under a Mediterranean climate is extremely slow, because of markedly dry summer periods, leading to a discontinuous process closely linked to water availability. By comparison, we found similar needle decomposition efficiency under a boreal climate (Kainulainen & Holopainen 2002) subjected to a long snowy period, but lower than in temperate (Polyakova & Billor 2007) and aquatic environments (Whiles & Wallace 1997).

In conclusion, the afforestation of abandoned agricultural land in the Mediterranean region by Aleppo pine promotes optimal soil communities of decomposers by selecting for organisms capable of breaking down recalcitrant material (Negrete-Yankelevich *et al.* 2008). We observed a more efficient needle decomposition in middle and late stages than in the early-stage plots, partly due to soil decomposers that were well adapted to local resources, particularly litter rich in phenolic compounds. Hence in pine forest, (i) litter phenolic content emerged as a key functional trait for predicting litter decomposition, delaying the colonization of litter by decomposers and thereby the decomposition process, and (ii) recovery following agricultural land abandonment takes 30 years after colonization (middle stage), longer than the 12 years observed by Van der Wal *et al.* (2006).

Another key finding based on our carbon budget is that the different relative contributions of fungi and detritivores to needle mass loss rate ultimately means that, from a food-web perspective, the organic matter available for higher trophic levels remains unchanged beyond 30 years after pine colonization.

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