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Effect of monospecific and mixed litters on bacterial communities' structure and functions under contrasting Mediterranean climate conditions

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A B S T R A C T

Global climate change is expected to contribute to intense droughts and heatwaves in the Mediterranean basin, motivating ecological research to explore the responses of litter microbial communities to more drastic climatic conditions. Here, we investigated how the functioning of microbial decomposition systems may be affected by litter source and mixing in two contrasting Mediterranean climate contexts: a sub-humid climate in France vs a semi-arid climate in Algeria. Studies included in situ incubation of litter bags of monospecific litters (*Pinus halepensis*, *Pistacia lentiscus*, and *Quercus ilex*) and binary mixtures (*Pinus/Pistacia*; *Pinus/Quercus*) of litters in France and Algeria. Following a 12-month incubation, the remaining litter was chemically characterised using elemental analyses and solid-state ¹³C NMR spectroscopy. Microbial properties evaluated included basal respiration (BR), active microbial biomass (MB), community-level physiological profiling (CLPP), and molecular fingerprinting (T-RFLP). In both countries, negative correlations were observed between MB and ratios of C/N and (aromatics + phenols)/N. Basal respiration was also negatively correlated to both ratios in France; however, the opposite trend was found in Algeria. The effect of litter mixing on MB, BR, and microbial functional diversity (catabolic diversity index ECO H') also differed between climates. The mixture of *Pinus* and *Quercus* litter led to limited heterotrophic CO₂ release in the Algerian arid context i.e. a synergistic effect on MB associated with an antagonistic effect on BR. Higher bacterial genetic diversity (Terminal Restriction Fragment TRF richness and evenness) was found in Algeria whereas higher functional diversity (catabolic diversity index ECO H') was found in France, suggesting that the higher genetic diversity potential in the arid climate context did not lead to functionally diverse bacterial communities. Our findings on the differing effect of litter chemical properties (aromatics + phenols)/N and C/N- on microbial functioning (BR and MB) in sub-humid and semi-arid climates indicate that the impact of litter chemistry (litter source; litter admixtures) on C cycling is climate-dependent.

1. Introduction

Microbial communities, i.e. bacteria and fungi, are important players in organic matter decomposition, and the amounts of carbon stored or released from soils depend strongly on their activities (Bardgett et al., 2014; Manzoni et al., 2012; McGuire and Treseder, 2010). Since soil microorganisms can affect biogeochemical feedbacks from climate change, the environmental drivers that govern microbial decomposers' functioning should be worth investigating (Bardgett et al., 2014; Todd-Brown et al., 2012).

Climate conditions, mainly temperature and moisture, are known to be major drivers of microbial decomposer communities and thus of the dynamics of organic matter decomposition (Setiawan et al., 2016; Zimmer, 2002). In Mediterranean ecosystems, moisture limitation is considered the most constraining climate factor, due to increasing temperatures and longer periods of summer drought (Pons and Quézel, 1998). A recent study showed that warming rates in the Mediterranean basin exceed worldwide trends: compared to pre-industrial temperatures, its mean annual temperature has already risen by 1.4 °C, versus an average worldwide increase of 1.1 °C (Cramer et al., 2018). In

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addition, more frequent heatwaves are expected for the future in this particularly vulnerable region (Cramer et al., 2018; Giorgi and Lionello, 2008). Under warmer conditions, litter decomposition should be enhanced due to stimulation of microbial decomposer enzymatic activities (Erhagen, 2013). For instance, Mao et al. (2018) described an increase in microbial respiration and litter decay rates with increasing temperatures in a boreal peatland in northeast China. On the other hand, drought inhibits microbial activities (Allison and Treseder, 2008), reduces microbial biomass (Sheik et al., 2011), and may induce shifts in microbial community composition (Allison et al., 2013; Singh et al., 2010).

The effect of climate factors on litter decomposition has been investigated through numerous studies conducted across different bioclimatic regions: cold biomes (Cornelissen et al., 2007); tropics (Powers et al., 2009); China (Zhou et al., 2008); Canada (Trofymow et al., 2002); USA (Harmon et al., 2009; Sheik et al., 2011); and Europe (Berg et al., 1993; McTiernan et al., 2003). These studies showed that there are temperature or moisture thresholds at which the dominant regulator of litter decomposition switches from one factor to another (Bradford et al., 2016; Prescott, 2010). The effect of climate parameters seems to be context-dependent: temperature is the main regulating factor in cold biomes and precipitation in warmer biomes (Adair et al., 2008; Prescott, 2010). Using a long-term in situ warming experiment in the United States, Sheik et al. (2011) found that under warmer conditions, the soil water budget regulates the abundance and diversity of microbial populations. Thus, the exacerbated drought conditions expected for the Mediterranean basin –where water deficit is already known as the main constraining climatic factor– could counteract the effects of higher temperatures (Prescott, 2010; Wohlfahrt et al., 2008).

This makes understanding how decomposer communities could respond to changing climate conditions a major issue for sustainable development in the Mediterranean (Cramer et al., 2018). Certain studies have taken the first step, such as Zouidi et al. (2019) who compared coniferous forest soils of semi-arid and arid zones in Algeria, and found that microbial biomass and basal respiration were lower in the arid zone as a consequence of its characteristic long drought periods. However, while there is a wealth of studies in temperate Northern European countries (Brunel et al., 2017; Cramer et al., 2018; Santonja et al., 2017a), few studies have addressed the most vulnerable zone, the more arid Southern Mediterranean countries (Cramer et al., 2018).

It is not only climate conditions, but also organic matter quality and quantity via litter inputs that have been found to control microbial decomposers (Setiawan et al., 2016; Zimmer, 2002). Generally, good-quality litters (low C:N and Lignin:N ratios) are expected to promote decomposer activities leading to faster decomposition than in litters of low quality (Berg, 2014; Setiawan et al., 2016). However, considering only monospecific litters may be a considerable oversimplification of decomposition complexity. *In natura*, litter falls and decomposes in mixtures, allowing physicochemical interactions (such as nutrient transfer) among the decomposing leaves (Cuchiatti et al., 2014; Handa et al., 2014; Perez et al., 2013). Due to these interactions, the overall effect of litter mixing on microbial decomposers may not be predictable from monospecific litters (Gartner and Cardon, 2004; Hättenschwiler et al., 2005). Litter mixing is assumed to promote microbial biomass and diversity due to diversification of nutrient resources (Cuchiatti et al., 2014; Meier and Bowman, 2008; Vos et al., 2013). Recent research suggests that it is the identity of the litter species within the mixture that affects the decomposition process more than litter diversity per se (Brunel et al., 2017; Cuchiatti et al., 2014; Prescott and Grayston, 2013; Santonja et al., 2017a, 2017b; Wu et al., 2013). Furthermore, since climate factors, litter quality and microbial decomposers are closely related, their interactions may induce different litter-mixing effects according to the environmental context (Gartner and Cardon, 2004). Although numerous studies have investigated the relative effects of climate and litter quality on litter decomposition (Bradford et al., 2016; Mao et al., 2018), few attempts have been made

in a single study to investigate how the effect of a given litter admixture may vary between different habitats or along environmental gradients. The interaction between climate and litter quality thus remains to be deciphered (Jonsson and Wardle, 2008; Lecerf et al., 2011).

The main objective of this study was to investigate how litter type (i.e. species identity and whether litters are monospecific or binary mixtures) impacts microbial decomposer communities under two contrasting Mediterranean basin climates differing in aridity (sub-humid climate in France and semi-arid climate in Algeria). The study was based on the following hypotheses:

1. At a local spatial scale, litter type (species identity and admixture or monospecific litters) may be a key determinant shaping microbial communities and their functioning.
2. At a broad Mediterranean-basin spatial scale, climate contrasts (sub-humid climate in France vs semi-arid climate in Algeria), may structure microbial communities' functioning and smooth the effect of litter type.
3. Mixing litters with contrasting functional traits (leafy/resinous) may enhance microbial diversity and functioning through non-additive synergistic effects, in accordance with the concept of niche complementarity and resource partitioning (Hättenschwiler et al., 2005); this may depend on species identity.

These three hypotheses were tested via a litter-bag experiment including three typical Mediterranean plant species: *Pinus halepensis* (a coniferous, resinous evergreen species), *Quercus ilex* (a leafy evergreen species), *Pistacia lentiscus* (a leafy evergreen species). Litter bags of monospecific and equitable binary mixtures of litters from *Pinus halepensis* and either from *Quercus ilex* or from *Pistacia lentiscus* were prepared and incubated in situ in their "home sites" in France and Algeria. After one year in the field, analysis was carried out on chemical properties using solid-state ^{13}C NMR spectroscopy, and on microbial community diversities (T-RFLP and Biolog), biomass (Substrate-induced respiration) and activities (Basal respiration) of litters.

2. Material and methods

2.1. Site description and litter sampling

Sampling sites were selected in two contrasting Mediterranean basin climate zones in France and Algeria:

- In France, sampling sites were located in the Massif of la Trevaresse, in public forestland called *Domaine départemental de La Quille* (N43°38'33.97" E5°25'58.36") near the village of Le Puy Sainte Reparade within the region Sud Provence-Alpes-Côte d'Azur (South-Eastern France) in the Bouches-du-Rhône department. Growing under the sub-humid bioclimatic stage (Quézel, 1979; Quézel and Médail, 2003), the forest stands are around 60 years old and belong to preserved environments, not altered by human activities (no silviculture) for at least 40 years (Brunel et al., 2017). They are composed of Aleppo pine (*Pinus halepensis*) -the dominant species- and holm oak (*Quercus ilex*) and downy oak (*Quercus pubescens*). Erected bromine (*Bromus erectus*), rosemary (*Rosmarinus officinalis*), and kermes oak (*Quercus coccifera*) are also present in this area (Pires and Pavon, 2018). The Trevaresse massif is part of a limestone geological region called *La Provence Calcaire* that is formed by a sedimentary cover dating from the secondary (Mesozoic) and tertiary (Cenozoic) eras, where carbonate, gypsum and dolomitic soils from the Triassic to the Cretaceous are widely represented. Soils here are often thin, poorly developed, very stony, with low water reserves (Curt and Marsteau, 1997) and frequently eroded, therefore particularly fragile and vulnerable (Pires and Pavon, 2018).
- In Algeria, sampling sites were located in the Massif of Jebel Sid Ahmed Zeggai (N34°46'33.71" E0°04'02.47") situated near the town

of Ain el Hadjar in the Wilaya of Saida (North-Western Algeria) and under the semi-arid bioclimatic stage (Zouidi et al., 2019). Declared a national forest in 1962, the stands are situated on brown limestone soils and dominated by Aleppo pines at least 60 years old (Zouidi et al., 2019). The accompanying species are lentisk (*Pistacia lentiscus* L.), juniper (*Juniperus oxycedrus* L.), holm oak (*Quercus ilex* L.) and alfa (*Stipa tenacissima* L.) (Zouidi et al., 2019). This study site is also a preserved environment without artificialisation, where no sylvicultural practices have been applied for at least 40 years. According to Lucas (1952), the territory of the wilaya of Saida consists essentially of limestone or dolomitic layers.

In each country, five sampling sites (400 m²) were chosen at least 2 km apart. The sites selected had similar pedoclimatic and topographic features: elevation (500–900 m), exposure (South), slope (10–15%) and soil type (Calcaric Leptosol according to IUSS Working Group WRB, 2006). For each site, maximum and minimum temperatures and relative air humidity were measured in situ by EL-USB 2.0 probe (Conrad) throughout the one-year field incubation period (April 2017–March 2018). Annual precipitation, mean annual temperature, and precipitation of driest and wettest months were also collected from Worldclim (Worldclim, 1970–2010, Hijmans et al., 2005) to highlight the cross-Mediterranean climate contrasts between France and Algeria (Table 1). While several numerical indices have been proposed to measure aridity (Stadler, 2005), annual precipitation remains the simplest aridity index used by the Intergovernmental Panel on Climate Change (IPCC, 2007; Maliva and Missimer, 2012): its 643 mm of annual precipitation in France vs 350 mm in Algeria confirms the latter's more arid conditions. This is also supported by higher mean annual temperature, a more extreme hot season, and lower relative air humidity in Algeria (Table 1).

In March 2017, for each species and for each site, twenty samples of litter were randomly collected over a 1000 m² area to obtain a composite sample. 2 kg of the litter of each species were sampled from the very upper layer of horizon O1 to ensure homogenous characteristics, and this composite sample was used to prepare the litter bags. Contaminating debris (e.g. leaves of other species, including branches and seeds) were removed carefully from each collection. The samples were stored at 4 °C for one week until litter-bag preparation.

2.2. Litter-bag preparation and experimental set-up

25 litter bags (5 sites × 5 litter types) were prepared per country. 30 g (dry weight, DW) of litter was placed in each 30·10 cm, 2 mm rigid

Table 1
Climatic parameters recorded in the field over the 12-month incubation in France and Algeria and Worldclim data.

	France	Algeria
Area location	N43°38'33.97" E5°25'58.36"	N34°46'33.71" E0°04'02.47"
Environmental data monitored in situ (April 2017–March 18)		
Minimal temperature of the coldest month (°C)	−6.5	−5.5
Maximal temperature of the hottest month (°C)	38	49
Annual mean temperature (°C)	15.15	17.94
Minimal relative humidity of air (the driest month)	25.5	5.5
Worldclim data		
Annual mean temperature (°C)	13.1	15.5
Annual precipitation (mm)	643	350
Precipitation of the driest month (mm)	16	8
Precipitation of the wettest month (mm)	85	45

nylon mesh bag. A total of 50 litter bags were prepared and placed in 2 other non-sampling sites for exposure at the end of March 2017. After 12 months in the field, the litter bags were collected. After drying, an aliquot of each sample was kept and ground prior to chemical analysis. All microbial analyses started immediately and were performed in the space of one week. For T-RFLP, samples were frozen and analysed one month later.

2.3. Litter chemical properties

Analysis of the chemical properties of litters from *P. halepensis*, *P. lentiscus*, *Quercus ilex* and the two admixtures was performed as follows. Total organic carbon (TOC) and nitrogen (N) contents were analysed by combustion in an Elemental Analyzer (Flash EA 1112 series ThermoScientific, Illkirch-Graffenstaden, France). Additionally, litter chemical properties were analysed by solid-state ¹³C NMR. Spectra were obtained on a Bruker Avance III HD −400 MHz NMR spectrometer operating at a ¹³C resonance frequency of 100.7 MHz and using a commercial Bruker double-channel probe. A single measurement was performed for each litter bag. About 100 mg of sample were placed in zirconium dioxide rotors of 4-mm outer diameter and spun at the Magic Angle Spinning (MAS) rate of 10 kHz. The Cross Polarization (CP) technique was applied with a ramped 1H-pulse starting at 100% power and decreasing to 50% during contact time (2 ms) to avoid Hartmann-Hahn mismatches. The experiments were performed at ambient temperature and 4 K scans were accumulated using a delay of 2.5 s, giving an experimental time of 3 h. The ¹³C chemical shifts were referenced to tetramethylsilane and calibrated with a glycine carbonyl signal, set at 176.5 ppm. The ¹³C NMR spectra were divided into 7 chemical shift regions according to Knicker and Lüdemann (1995): i.e. alkyl C (0–45 ppm), methoxyl C or N-alkyl C (45–60 ppm), O-alkyl C (60–90 ppm), di-O-Alkyl C (90–110 ppm), aromatic C-C/aromatic C–H (110–140 ppm), aromatic C–O (phenols) (140–160 ppm) and carboxyl/amide C (160–190 ppm). Deconvolution of each spectrum was performed on DmFit 2011 to determine the relative intensity of each selected region (Massiot et al., 2002). We focused on three types of carbon components: i) Alkyl C (in fatty acids, amino acids or paraffinic structures) assumed to derive mainly from recalcitrant waxes, ii) O-Alkyl C and di-O-Alkyl C assigned to labile carbohydrates and iii) aromatic C-C/aromatic C–H and aromatic C–O (Phenols) mainly linked to the recalcitrant fraction of organic matter, i.e. lignin and tannin compounds (Knicker, 2011).

2.4. Microbial basal respiration and substrate-induced respiration

Fresh litter (3 g DW) was placed in 117 mL glass jars immediately sealed with hermetic rubber septa, and incubated for 2 h at 23 °C. Thereafter, 1 mL of air was sampled in the headspace with a syringe and injected into a gas chromatograph (Chrompack CHROM 3 – CP 9001) to analyse CO₂ production. The gas chromatograph was equipped with a thermal conductivity detector and a packed column (Porapack). The carrier gas helium flow was regulated at 60 mL h^{−1}. Ambient CO₂ concentrations were subtracted from sampled CO₂ concentrations and the resulting values were adjusted at 22 °C according to Ideal Gas Laws using a Q₁₀ = 2. Substrate-induced respiration (SIR) rates were estimated using a procedure from Anderson and Domsch, 1978. Three grams DW equivalent of fresh litter were placed in 117 mL glass jars and amended with powdered glucose (1000 µg C g^{−1} soil), found to maximise the respiration rate in litter in a preliminary assay (data not shown). One milliliter of air was sampled in the headspace with a syringe and injected into a gas chromatograph to analyse CO₂ production for 1 h. Substrate-induced respiration was converted into microbial biomass (MB) using the relation established by Beare et al., 1990.

2.5. Bacterial Community Level Physiological Profiling (CLPP)

The potential of cultivable microbial communities to oxidise different C-substrates was determined via Biolog EcoPlate (Biolog, California, USA), using a procedure adapted from Garland and Mills (1991). Briefly, 4 g of dry litter were added to 100 mL of 0.1% sodium pyrophosphate sterile buffer in a 250 mL flask and shaken for 2 h (80 rpm). The litter suspension was diluted and standardised at optical density (OD) 595 nm = 0.03 with a sterile physiological solution (NaCl 0.85%). 125 µL of microbial suspension were used to inoculate each well. The plates were incubated at 25 °C for 5 days and microbial development was monitored by reading the absorbance at 590 using a TECAN® spectrophotometer. The absorbance value for each well was blanked against the control well and negative absorbance values were set to zero. The minimum OD for a positive well was fixed at 0.25 (Garland and Mills, 1991). OD after 48 h were chosen according to the exponential phase of growth curves for all plates, for further comparison. Catabolic diversity was assessed using Shannon's index:

$$H' = - \sum_{i=1}^N p_i \log_{10} p_i \quad (1)$$

where p_i is the ratio of color development of the i th well to the sum of color development of all positive wells.

2.6. Bacterial community genetic structure

The DNA was extracted from 0.25 g of litter, using NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer instructions and quantified using Quant-iT™ dsDNA High-Sensitivity Assay Kit (Invitrogen, Canada). Then, all DNA extracts were diluted to 0.5 ng/µL for subsequent analysis. Bacterial community structures were analysed using T-RFLP fingerprinting as described by Blaud et al. (2015). Briefly, bacterial 16S rRNA gene was amplified using primers FAM labelled 63F and 1389R. Biorad T100 thermal cycler was used for the amplification with the following programs: initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 90 s, followed by a final extension time at 72 °C for 10 min. Bacterial PCR products were digested with restriction enzyme *AluI* (Thermo Fisher). Restriction fragments were mixed with formamide containing 0.5% of LIZ500 internal size standard (Applied Biosystems) and denatured at 94 °C for 3 min. Samples were electrophoresed in an ABI 3730 PRISM® capillary DNA sequencer (Applied Biosystems). The T-RFLP profiles obtained with the sequencer were analysed using GeneMapper® v3.7 software (Applied Biosystems). Fragments between 50 and 500 bp and with peak height ≥ 50 fluorescence units were included in T-RFLP analysis. Fragments having a relative abundance of proportion $< 0.5\%$ were removed from the matrices.

Table 2

Litter chemical properties of the three types of litters (*Pinus halepensis*, *Pistacia lentiscus*, and *Quercus ilex*) collected from both countries (France and Algeria) at $t = 0$ (prior to litter bags' conception): Carbon content (%C), Nitrogen content (%N), C/N, chemical groups defined from ^{13}C NMR spectra.

	France			Algeria		
	<i>Pinus halepensis</i>	<i>Pistacia lentiscus</i>	<i>Quercus ilex</i>	<i>Pinus halepensis</i>	<i>Pistacia lentiscus</i>	<i>Quercus ilex</i>
Alkyl C	21.99	20.48	20.89	26.56	27.7	19.86
di-O-Alkyl C + O-Alkyl C	48.94	48.55	54.46	49.1	44.36	51.78
Methoxyl C	4.2	4.79	4.87	4.89	3.98	5.1
Carboxyl C	4.82	6.35	5.83	4.88	7.48	7.33
Aromatics + Phenols	20.05	19.83	13.96	14.57	9.91	15.93
(Aromatics + Phenols)/N	16.67	22.36	10.53	17.23	6.16	11.06
C (%)	46.97	43.48	38.49	51.06	47.37	42.66
N (%)	1.20	0.89	1.33	0.85	1.61	1.44
C/N	39.06	49.03	29.03	60.38	29.46	29.63

2.7. Statistical analyses

The normality and homogeneity of data variances were determined on the residuals from the regression model via the Shapiro-Wilk and Levene tests, respectively. Data were transformed to common logarithms, \log_{10} , when necessary to meet the requirements of normality and homogeneity of variance for ANOVA. Two-way ANOVA was used to determine whether, and to what extent, microbial properties (i.e. basal respiration, active microbial biomass via SIR, bacterial catabolic diversity index ECO H' and bacterial richness and evenness via genetic analysis) and chemical characteristics (using NMR data and C/N ratio) depended upon i) litter type, ii) geographical location (France or Algeria) and iii) their interactions. When a 2-way interaction was found, we separated data for each geographical location and type of litter using a one-way ANOVA. Statistically significant ($p < 0.05$) main effects and interactions were analysed further using Tukey HSD post hoc tests. The non-additivity or additive effect of litter admixture on the litter markers studied was assessed by calculating predicted averages based on results from the monospecific litters. The relative non-additive effect (NAE%) can be calculated using the ratio: [(observed-predicted) / predicted] * 100. Each NAE% obtained by litter admixture was tested by one-sided Student's *t*-test. A ratio differing significantly from 0 would indicate a non-additive (synergistic or antagonistic) effect of the mixture on microbial properties. The effects of geographical location and litter type on the catabolic and genetic structures of microbial communities were explored by performing Between-Class Analyses on catabolic (Biolog®) or genetic (TRFLP) profiles. Differences in catabolic or genetic profiles among litters were assessed through a Monte Carlo test (9999 permutations). Specific relationships between certain microbial markers (BR and MB) and litter chemical properties (^{13}C NMR C-groups, C/N) were assessed via Pearson's correlation coefficients. All statistical analyses were performed using XLSTAT-Pro 7.5 (Addinsoft, Paris, France), except for TRFLP profiling analyses, performed with R software (version 2.12.0 R Development Core Team, 2008).

3. Results

3.1. Litter chemistry

Litter chemical properties (solid-state ^{13}C NMR and C/N) at $t = 0$ (composite sample of litters from France and Algeria used for litter-bag preparation) are shown in Table 2. In France, ^{13}C NMR results showed variations in di-O-Alkyl C + O-Alkyl C content (linked to the labile carbohydrate fraction) depending on litter type: content was higher in *Quercus ilex* litters (54.46%) than in the two other litter types (48.94% and 48.55% respectively for *Pinus halepensis* and *Pistacia lentiscus* litters). In Algeria, the same trend was observed for the labile fraction of organic matter i.e. carbohydrates: *Quercus ilex* litters (51.78%) > *Pinus halepensis* litters (49.1%) > *Pistacia lentiscus* litters (44.36%).

Table 3

Two-way ANOVA on chemical properties from solid-state ^{13}C NMR data and elementary analyses (with F and p-values) according to factors: Country (France vs Algeria) and Litter (*Pinus halepensis* (Ph), *Pistacia lentiscus* (Pl), *Quercus ilex* (Qi), and the equitable admixtures of Ph/Pl and Ph/Qi). ns = non-significant, * < 0.05, ** < 0.01, *** < 0.001.

Effects	d.f.	(di-O-AlkylC+O-AlkylC)	Carboxyl C	Methoxyl C	Alkyl C	(Aromatics + Phenols)	(Aromatics + Phenols)/N	C/N
Country (C)	1	11.51 **	1.16 ns	5.06 *	102.61 ***	8.32 **	3.47 ns	20.47 ***
Litter (L)	4	14.54 ***	34.13 ***	19.16 ***	8.38 ***	5.67 **	36.80 ***	107.60 ***
C × L	4	10.24 ***	0.72 ns	4.25 **	14.01 ***	4.45 **	22.86 ***	87.82 ***

Moreover, in Algeria, Alkyl C content (mainly linked to recalcitrant waxes) was lowest in *Quercus ilex* litters (19.86%) and highest in *Pistacia lentiscus* litters (27.7%), whereas in France, only slight variations in Alkyl C content were observed among the three types of litter (Table 2). For both the ratio of (aromatics + phenols) to Nitrogen and the C/N ratio, which are used as indicators of litter recalcitrance, the trend among litters differed according to country. In France, the following trend was observed for both ratios at $t = 0$: *Pistacia lentiscus* (22.36) > *Pinus halepensis* (16.67) > *Quercus ilex* (10.53). In Algeria, the following trend was observed for both ratios at $t = 0$: *Pinus halepensis* (17.23) > *Quercus ilex* (11.06) > *Pistacia lentiscus* (6.16). Taken together, these observations suggest a general trend towards better carbon quality for litters of *Quercus ilex*.

After the 12-month incubation in the field, ANOVA analysis showed an interaction effect between both factors (country and litter type) on almost all the tested chemical markers (Table 3). The trends observed after the 12-month field-incubation were almost identical to those observed at $t = 0$, with a few exceptions: in Algeria, the (aromatics + Phenols)/N ratio trend was *Pinus halepensis* > *Quercus ilex* = *Pistacia lentiscus* (Fig. 2(b)).

3.2. Linking litter chemistry to microbial biomass and respiration

Results showed an interaction between the two factors (country and litter type) for most chemical (Table 3) and microbial (Table 4) markers.

In France, microbial biomass (MB), and basal respiration (BR), were strongly positively correlated ($r = 0.852$; $r^2 = 0.726$; $p < 0.0001$) and followed a similar trend: *Quercus ilex* > *Pinus/Quercus* > *Pinus halepensis* = *Pinus/Pistacia* > *Pistacia lentiscus* (Fig. 1(a) and (b)). For the mixed litters, an additive effect was observed in France on both microbial markers in the *Pinus/Quercus* admixture, but not for BR in the *Pinus/Pistacia* admixture, where a non-additive synergistic effect was observed ($p < 0.0001$). These results show that litter-mixing effects on BR in France differ according to the litter species (*Quercus* or *Pistacia*) mixed with *Pinus*. Moreover, a strong negative correlation was found between both microbial markers and i) C/N ratio ($r = -0.812$, $r^2 = 0.660$; $p < 0.0001$; $r = -0.883$, $r^2 = 0.780$; $p < 0.0001$ for MB and BR respectively) and ii) (Aromatics + Phenols)/N ratio ($r = -0.821$, $r^2 = 0.674$; $p < 0.0001$; $r = -0.808$, $r^2 = 0.653$; $p < 0.0001$ for MB and BR respectively). It should be noted that both chemical markers, i.e. C/N and (Aromatics + phenols)/N, showed an identical pattern in the monospecific litters (Fig. 2(a) and (b)): a lower value (better litter quality) for *Quercus ilex* and a higher value (poorer litter quality) for *Pistacia lentiscus* ($F = 146.249$, $p < 0.0001$;

Table 4

Two-way ANOVA on microbial properties (with F and p-values) according to factors: Country (France vs Algeria) and Litter (*Pinus halepensis* (Ph), *Pistacia lentiscus* (Pl), *Quercus ilex* (Qi), and the equitable admixtures of Ph/Pl and Ph/Qi). ECO H': bacterial catabolic index of diversity, AWCD: Average Well Color Development from Biolog data, T-RF: Terminal restriction fragment. ns = non-significant, * < 0.05, ** < 0.01, *** < 0.001.

Effects	d.f.	Basal respiration	Microbial biomass	ECO H'	ECO AWCD	T-RF richness	T-RF evenness
Country (C)	1	15.69 ***	9.19 **	11.973 **	6.92 *	16.312 ***	15.920 ***
Litter (L)	4	27.60 ***	37.09 ***	8.575 ***	14.07 ***	2.47 ns	2.35 ns
C × L	4	66.65 ***	20.53 ***	9.614 ***	9.66 ***	1.37 ns	2.39 ns

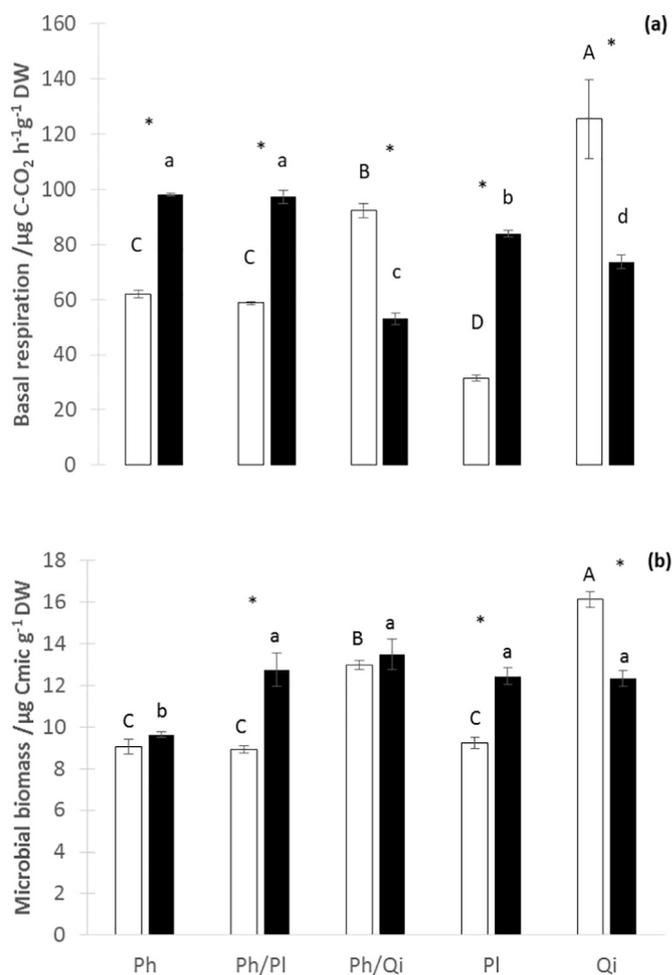


Fig. 1. Basal respiration (a) and active microbial biomass (b) in litters of *Pinus halepensis* (Ph), *Pistacia lentiscus* (Pl), *Quercus ilex* (Qi) or equitable admixtures (50/50) of Ph/Pl or Ph/Qi from France (white bars) and Algeria (black bars) after a 12-month incubation in the field ($n = 5$). Letters indicate significant differences between litters for a country (upper-case and lower-case for France and Algeria respectively) and stars indicate significant differences between countries for one type of litter.

$F = 59.211$, $p < 0.0001$ for C/N and (Aromatics + phenols)/N respectively). The similar trends and the strong positive correlation ($r = 0.949$; $r^2 = 0.900$; $p < 0.0001$) observed between ratios (C/N

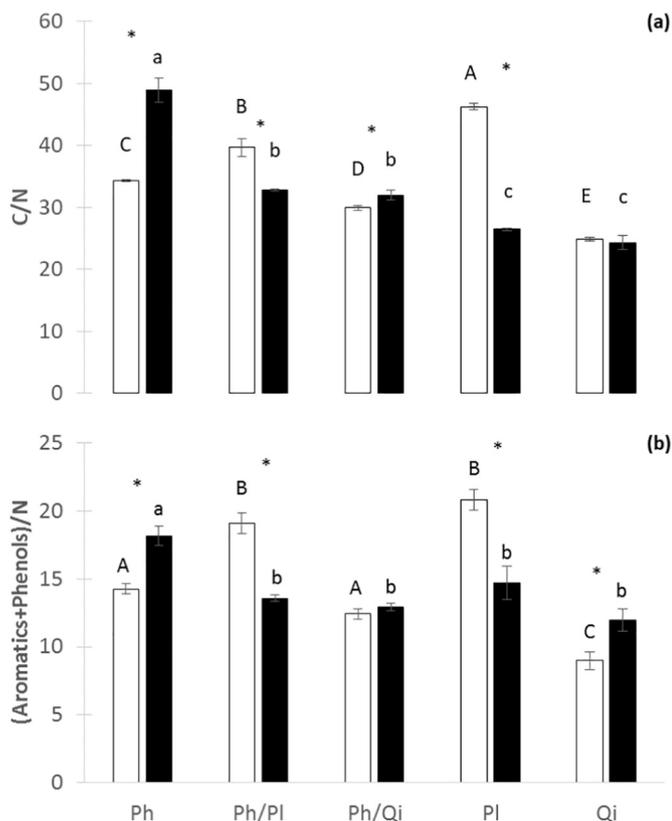


Fig. 2. C/N (a) and (Aromatics+Phenols)/N (b) in litters of *Pinus halepensis* (Ph), *Pistacia lentiscus* (Pl), *Quercus ilex* (Qi) or equitable admixtures (50/50) of Ph/Pl or Ph/Qi from France (white bars) and Algeria (black bars) after a 12-month incubation in the field ($n = 5$). Letters indicate significant differences between litters for a country (upper-case and lower-case for France and Algeria respectively) and stars indicate significant differences between countries for one type of litter.

and (Aromatics+Phenols)/N indicate that a large proportion of organic carbon content is linked to the recalcitrant aromatic fraction. In addition, the (di-O-AlkylC+O-AlkylC) content assigned to polysaccharides and the Alkyl C content assigned to fatty acids were almost identical whatever the litter type ($F = 3.382$, $p < 0.05$; $F = 5.693$, $p < 0.01$ for (di-O-AlkylC + O-AlkylC) and Alkyl C respectively). A low positive correlation was found between (di-O-AlkylC + O-AlkylC) content and BR and MB ($r = 0.397$, $r^2 = 0.158$, $p < 0.05$; $r = 0.644$, $r^2 = 0.415$, $p < 0.001$, for BR and MB respectively). No correlation was found between Alkyl C and any microbial markers (neither MB nor BR).

In Algeria, unlike France, MB and BR were negatively correlated ($r = -0.511$, $r^2 = 0.262$, $p < 0.01$). The following trend was observed for BR in Algeria (Fig. 1(a)): *Pinus halepensis* = *Pinus/Pistacia* > *Pistacia lentiscus* > *Quercus ilex* > *Quercus/Pinus*. MB did not vary with litter type, except for *Pinus halepensis* litters, which exhibited a far lower microbial biomass (Fig. 1(b)). Thus, higher basal respiration was obtained for litters of *Pinus halepensis* (higher (Aromatics+Phenols)/N and lower microbial biomass), while for the other litter types, different BR were obtained for similar recalcitrance and microbial biomass (Figs. 1 and 2). As in France, MB in Algeria was negatively correlated to (Aromatics+Phenols)/N ratio ($r = -0.631$, $r^2 = 0.399$, $p < 0.001$) and C/N ($r = -0.640$, $r^2 = 0.410$, $p < 0.001$), with *Pinus halepensis* litters exhibiting the highest (Aromatics+Phenols)/N and the lowest MB, while the other litter types had similar (Aromatics+Phenols)/N and MB. Our observations showed that MB was negatively linked to litter recalcitrance in both countries. Contrary to France, BR in Algeria was positively correlated to C/N ratio ($r = 0.499$, $r^2 = 0.202$,

$p < 0.05$) and (Aromatics+phenols)/N ratio ($r = 0.495$, $r^2 = 0.245$, $p < 0.05$). It should be noted that both ratios were also positively correlated in Algeria, as observed in France ($r = 0.729$, $r^2 = 0.532$, $p < 0.0001$), indicating that organic carbon content is linked to the recalcitrant fraction of organic matter in both countries. MB showed a negative correlation with (di-O-Alkyl C + O-Alkyl C) content ($r = -0.459$, $r^2 = 0.211$, $p < 0.05$) and no correlation was found between BR and this chemical marker. (di-O-Alkyl C + O-Alkyl C) content did not vary markedly between litter types, except for *Pistacia lentiscus* litters, which exhibited the lowest polysaccharides content ($F = 12.706$, $p < 0.0001$). Importantly, Alkyl C content was not correlated to any microbial marker, neither in France nor in Algeria.

For the litter admixtures, mainly non-additive effects were found in Algeria, unlike in France. The *Pinus/Pistacia* admixture led to a non-additive synergistic effect on microbial biomass and basal respiration ($p < 0.05$), while effects in the *Pinus/Quercus* admixture differed according to the microbial marker considered: non-additive synergistic effect on MB ($p < 0.05$) and non-additive antagonistic effect on BR ($p < 0.0001$).

3.3. Bacterial catabolic profiling

Between-Class Analyses (BCA) was performed on Biolog® profiles of bacterial communities from the different litter types for each country. For each BCA (Fig. 3), the two first principal components showed that 16.7% and 33.3% of the variance was explained by litter composition for the sub-humid climate (France) and semi-arid climate (Algeria), respectively. A Monte Carlo test showed significant differences among litters, with a higher level of significance in Algeria than in France.

In France (Fig. 3A), catabolic profiles of the three monospecific litters were discriminated along the first axis, while the admixtures of litters were separated from the monospecific litters on the second axis. Although litter catabolic structure varied according to litter type, the bacterial catabolic diversity index ECO H' did not show significant variations among litter types in France (Fig. 4).

In Algeria (Fig. 3B), catabolic profiles of mixed and monospecific litters of *Pinus halepensis* and *Pistacia lentiscus* differed from those of *Quercus ilex* on the first axis. The catabolic profile of *Pinus/Pistacia* was similar to those of *Pinus* and *Pistacia*, while that of *Pinus/Quercus* differed from those of *Pinus* and *Quercus* (Fig. 3B). ECO H' was higher in the *Pinus/Quercus* admixture (Fig. 4). In the monospecific litters, it was lower in *Pistacia lentiscus* litters than in the two others. Moreover, an additive effect was found for the *Pinus/Pistacia* admixture, whereas a non-additive synergistic effect was found for the *Pinus/Quercus* admixture ($p < 0.001$). Overall, the bacterial catabolic diversity index ECO H' was higher in litters from the French sub-humid climate than in those from the Algerian semi-arid climate ($F = 5.031$, $p < 0.05$).

3.4. Bacterial community genetic structure

Genetic characterisation of the bacterial communities, performed using T-RFLP, revealed higher richness and evenness in litters from Algeria than in those from France, whatever the litter type ($F = 13.946$, $p < 0.001$ for richness and $F = 12.147$, $p < 0.001$ for evenness; Fig. 5).

Between-Class Analyses (BCA) performed on T-RFLP profiles showed that 22.5 and 16.9% of the variance was explained by litter composition for France and Algeria, respectively (Fig. 6). A Monte Carlo test showed significant differences among litters, with a higher level of significance in France than Algeria.

BCA also showed that litter type shaped genetic profiles differently depending on the country. Under the French sub-humid climate, the genetic structures of the three monospecific litters were clearly discriminated from each other, while those of both litter admixtures were similar to that of *Pinus halepensis* (Fig. 6A). Under the Algerian semi-arid climate, the *Pinus halepensis* bacterial genetic structure was

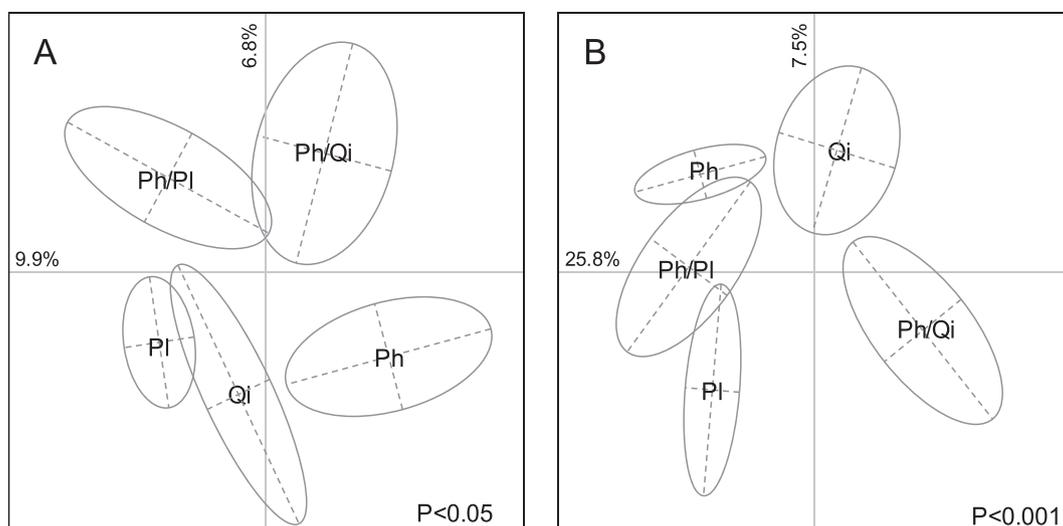


Fig. 3. Between-Class Analyses based on bacterial catabolic profiles (BILOG) of litters: *Pinus halepensis* (Ph), *Pistacia lentiscus* (PI), *Quercus ilex* (Qi) or equitable admixtures (50/50) of Ph/PI or Ph/Qi in France (A) and Algeria (B) after a 12-month incubation in the field. Letters represent the barycenter of the replicates ($n = 5$) for each treatment. The simulated p value from the Monte Carlo test is given in the lower right corner.

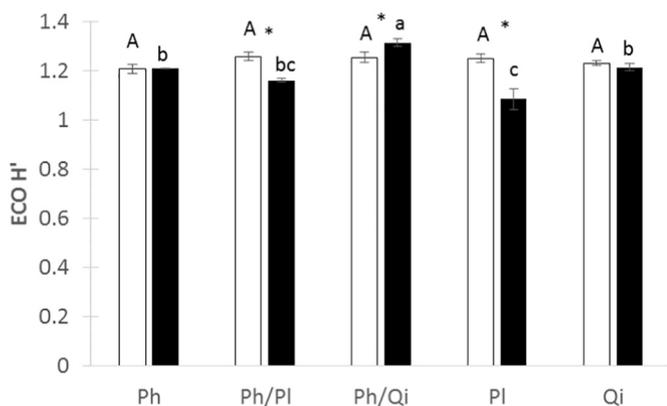


Fig. 4. Bacterial catabolic diversity index (ECO H') in litters of *Pinus halepensis* (Ph), *Pistacia lentiscus* (PI), *Quercus ilex* (Qi) or equitable admixtures (50/50) of Ph/PI or Ph/Qi from France (white bars) and Algeria (black bars) after a 12-month incubation in the field ($n = 5$). Letters indicate significant differences between litters for a country (upper-case and lower-case for France and Algeria respectively) and stars indicate significant differences between countries for one type of litter.

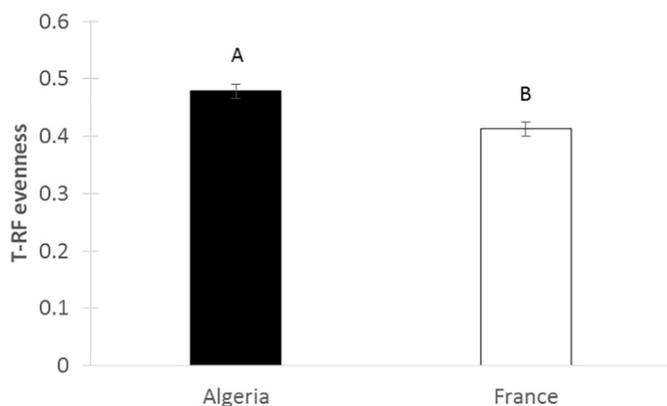


Fig. 5. Bacterial T-RF evenness in France (white bar) and Algeria (black bar) after a 12-month incubation in the field ($n = 25$). Letters indicate significant differences between countries.

discriminated from those of the two other monospecific litters, while the genetic profiles of both litter admixtures were equally similar to those of the two monospecific litters composing them (Fig. 6B). It should be noted that for both countries, the genetic structures of litter admixtures were more similar than indicated by Biolog® profiles.

4. Discussion

This study set out to explore how litter microbial community structure and functioning may be differently shaped by litter type (admixture vs monospecific; species identity) depending on contrasting Mediterranean climates: sub-humid in France (Northern Mediterranean) vs semi-arid in Algeria (Southern Mediterranean).

After one year in the field, major microbial markers (basal respiration and microbial biomass) were shaped by litter type i.e. litter chemical properties (mainly C/N and (aromatics + phenols)/N ratios) depending on climate. Under the French sub-humid climate, low C/N and (aromatics + phenols)/N strongly enhanced both microbial biomass and basal respiration. The crucial influence of litter chemical properties on decomposition dynamics has been well documented. The Lignin/N ratio is widely accepted as a particularly relevant “index” of litter quality and resistance to degradation, i.e. recalcitrance, which can be used for a better understanding of the litter-quality effect on decomposition (Aerts, 1997; Hobbie et al., 2006; Melillo et al., 1982; Perez et al., 2013; Trofymow et al., 2002). Litter recalcitrance exerts a negative control on decomposers' dynamics due to a lower amount of easily degradable organic matter and N limitation (Currie et al., 2009).

Under the Algerian semi-arid climate, unlike the trends observed in France, microbial biomass and basal respiration were differently correlated to litter chemical markers: conversely to MB, higher BR was associated with high litter recalcitrance (*Pinus halepensis* litter). Such observations may be consistent with the concept of microbial Carbon Use Efficiency “CUE” (Cotrufo et al., 2013; Manzoni et al., 2012). CUE defines how Carbon is partitioned between growth (microbial biomass) and respiration, i.e. how effectively microbial decomposers convert a substrate's carbon to biomass, compared to the amount of carbon lost in respiration (CO₂) (Manzoni et al., 2012). CUE is assumed to decrease with increased litter recalcitrance and nutrient limitation (Cotrufo et al., 2013; Manzoni et al., 2012; Six et al., 2006; Stewart et al., 2015): the additional energy required for investment in lignocellulolytic enzymes results in less carbon allocated for microbial biomass (Manzoni et al., 2012; Recous et al., 1995). In Algeria, the microbial communities

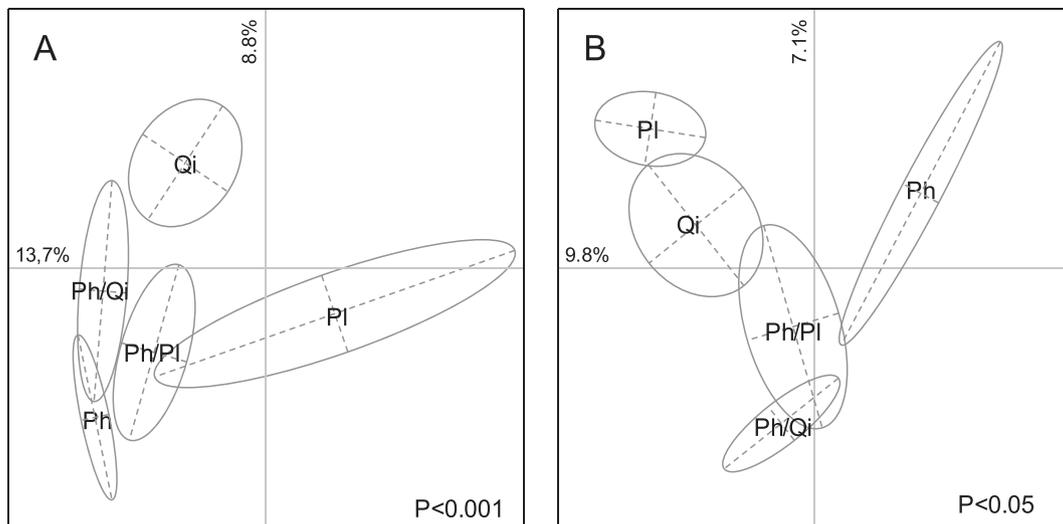


Fig. 6. Between-Class Analyses based on bacterial genetic profiles (TRFLP) of litters: *Pinus halepensis* (Ph), *Pistacia lentiscus* (Pl), *Quercus ilex* (Qi) or equitable admixtures (50/50) of Ph/Pl or Ph/Qi in France (A) and Algeria (B) after a 12-month incubation in the field. Letters represent the barycenter of the replicates ($n = 5$) for each treatment. The simulated p value from the Monte Carlo test is given in the lower right corner.

of *Quercus ilex* litters (lower C/N and (aromatics + phenols)/N) performed better in sustaining microbial biomass with limited C loss through respiration, whereas in *Pinus halepensis* litters (higher C/N and (aromatics + phenols)/N), microbial metabolism promoted CO₂ emissions. The stoichiometry of decomposing litter has also been proven to determine the amount of CO₂ released during decomposition: litters with a high C/N ratio promote the release of CO₂ as microorganisms –with a relatively fixed C/N ratio– try to alleviate resource stoichiometric imbalance by lowering their CUE (Mooshammer et al., 2014; Cleveland and Liptzin, 2007; Daufresne and Loreau, 2001; Manzoni et al., 2010, 2008). The results presented here should be taken with caution, since they involved laboratory methods providing potential MB and BR (under favourable temperature and moisture conditions) without performing direct CUE measurements, which are generally based on quantification of isotopically-labelled compound transformation (Geyer et al., 2019; Sinsabaugh et al., 2013). However, our results show how litter chemical properties may differently shape microbial physiological functioning depending on contrasting climates across the Mediterranean (sub-humid climate in France vs semi-arid climate in Algeria).

This study also aimed to investigate the effect of litter mixing on microbial decomposers using two different binary litter admixtures (*Pinus halepensis*/*Pistacia lentiscus* and *Pinus halepensis*/*Quercus ilex*). In France, additive effects were observed both for bacterial catabolic diversity and for MB whatever the litter admixture. On the other hand, basal respiration varied according to the species combined: mixing *Pinus* with *Pistacia* led to higher BR than expected from monospecific components, while an additive effect was found when *Pinus* was mixed with *Quercus*. Mixing relatively fast-decomposing *Quercus ilex* litters with slow-decomposing *Pinus halepensis* litters likely induced synergistic effects on microbial biomass and respiration due to the diversification of resources used by the decomposer community (Cuchietti et al., 2014; Hättenschwiler et al., 2005; Liu et al., 2016; Vos et al., 2013). In contrast, mixing two slow-decomposing litters, *Pinus* and *Pistacia*, enhanced basal respiration through synergism. Chapman and Newman (2010) suggested that synergistic interactions between microbial communities are favoured when chemically-similar litters are mixed, due to the substrate-specificity of the enzymes produced. Thus, less chemically-heterogeneous mixtures promote more efficient overall transformation of organic matter by more specialised microbial communities.

In Algeria, unlike in France, litter mixing favoured microbial biomass (non-additive synergistic effect) in both litter admixtures. This is

in agreement with other studies showing increased microbial biomass in mixed litters (Chapman et al., 2013; Chapman and Newman, 2010; Santonja et al., 2017a). For instance, Brunel et al. (2017) found that whatever the *Quercus* spp. (*Quercus ilex* or *Quercus pubescens*) mixed with *Pinus*, microbial biomass was increased in binary litter admixtures. Microbial communities are believed to colonise mixed litters more rapidly due to the greater diversity of niches and substrates (Chapman et al., 2013; Chapman and Newman, 2010). However, we found that the effect of litter admixtures on bacterial catabolic diversity depended on the species mixed with *Pinus*: the *Pinus/Quercus* admixture enhanced bacterial catabolic diversity through synergism, while the *Pinus/Pistacia* admixture had an additive effect. Thus, the litter admixture effect depends on species identity (Brunel et al., 2017). For instance, Santonja et al. (2017b) found that the effects of litter admixtures on microbial diversity and C release differed according to the presence of certain litter species: a strong positive effect on bacterial and fungal diversity was found when litter mixtures contained a *Quercus* spp. (*Quercus coccifera*).

Thus, under semi-arid or sub-humid climates, our study shows that mixing *Pinus* with *Quercus* litters enhances microbial biomass and catabolic diversity of microbial communities compared with monospecific *Pinus* litters. The ameliorative role of *Quercus* spp. in litter admixtures compared to monospecific litters of coniferous species has previously been documented in Mediterranean ecosystems, where it has been shown to promote microbial growth, abundance and functional biodiversity (Brunel et al., 2017; Santonja et al., 2017a, 2017b). Moreover, under Algerian semi-arid climate, our study suggests that the *Quercus/Pinus* admixture plays a positive role in limiting litter carbon release through heterotrophic respiration: this admixture promoted MB and mitigated BR compared to a *Pinus/Pistacia* admixture. This underlines the importance of vegetation assemblage in the efforts to limit C release under climate change (Cramer et al., 2018; Intergovernmental Panel on Climate Change, 2018).

The catabolic and genetic structures of litter microbial communities were clearly controlled by litter type in a climate-dependent way (sub-humid climate in France or semi-arid climate in Algeria). Pailler et al. (2014) previously highlighted the key role of “climate-plant-soil” interaction in shaping microbial catabolic profiles at a regional scale under two contrasting climate conditions, i.e. humid and sub-humid Mediterranean climates. In addition, here, we observed that the effect of litter admixture on microbial decomposer properties (i.e. catabolic structures, basal respiration, microbial biomass and diversity) differed

across the Mediterranean basin. Similarly, [Jonsson and Wardle \(2008\)](#) found that non-additive litter-mixing effects vary widely along environmental gradients. In our study, the litter admixtures in sub-humid and semi-arid climates showed distinct bacterial catabolic profiles but similar bacterial genetic profiles. This suggests that similar bacterial genetic structures do not necessarily lead to similar decomposer functioning highlighting the concept of functional redundancy among microbial communities.

Furthermore, the high Terminal Restriction Fragment (TRF) richness and evenness observed in Algeria were not associated with higher bacterial functional diversity (ECO H' index). It seems that the high genetic diversity potential found in the Algerian semi-arid climatic context did not lead to functionally diverse bacterial communities. Drought stress experienced under the Algerian semi-arid climate conditions may have caused a metabolism shift towards physiological maintenance, such as intracellular osmolyte production, which has high energy costs ([Kakumanu and Williams, 2014](#)). Consistent with our results, [Sherman and Steinberger \(2012\)](#) reported that microbial functional diversity (H') was driven by climate contrasts within a Mediterranean gradient, with higher functional diversity found under a more humid climate.

5. Conclusion

Here we found that microbial physiological functioning (basal respiration, microbial biomass) was explained by certain litter chemical properties (C/N and (aromatics + phenols)/N ratios, used as indexes of litter recalcitrance). However, this effect strongly varied according to the Mediterranean climate context (sub-humid climate in France vs semi-arid climate in Algeria), suggesting that litter chemistry (type of species, admixture of litters) impacts C cycling differently depending on the climate.

Furthermore, we showed that when the benefits of litter mixing for microbial decomposers are assessed, it is not only species identity but also climate context that should be considered. Our study reveals that mixing *Pinus halepensis* with *Quercus ilex* promotes microbial biomass, while limiting heterotrophic respiration in litters. In the light of the increasing calls for mitigation of carbon release, this may hold promise, especially in the arid southern Mediterranean climate. Further studies should consider complementary aspects such as carbon storage in plant biomass and carbon stabilisation in the organo-mineral horizons of soil, to determine whether mixed stands could play a role in the mitigation of climate change.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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