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Soil microbial properties in the Mediterranean

**Abstract**

This study explores how chemical and microbial properties of litters can be affected by coastal environments across the Mediterranean basin. A litterbag experiment including *Pinus halepensis* Mill. and *Pistacia lentiscus* L., collected from both inland and coastal areas was set up in France, Greece and Algeria. Control litterbags were left in their sampling sites and a transfer of litterbags from inland to coastal area was performed to test whether the effect of the specific constraints of coastal environments varies according to the country and the litter type. After 10 months, litter chemical composition (CP/MAS $^{13}$C-NMR) and microbial activities (cellulase, respiration, Biolog) and structure (TRFLP) were analysed. Coastal conditions led to various responses: i) litter aromaticity differed in the coastal zones depending on the country (high in Greek coastal area, low in Algerian coastal zone), ii) less functionally-diversified microbial communities were found in Greek coastal area compared to French and Algerian coasts, iii) genetic diversity and richness were strongly impacted after transfer to the coastal zone whatever the country. The type of litter shaped microbial communities: i) at a local scale i.e. in either coastal or inland area, catabolic profiles and cellulase activities varied with the plant species, ii) at a regional scale, the effect of coastal conditions differed with the plant species (basal respiration, Shannon-Weaver index, catabolic diversity H', cellulases and catabolic profiles). Thus, litter microbial properties differed in coastal environments across the Mediterranean basin and plant litter type plays a major role in microbial properties at large spatial scale.

**Key words**: extracellular enzyme activities, litter microbial communities, Mediterranean area, water potential stress.

## Introduction

Mediterranean ecosystems are subjected to various environmental pressures: a drastic climate characterized by severe summer drought, soils weakly developed and poor organic matter content (Sardans et al., 2008) and long-standing and intense human activities. Drastic pedoclimatic conditions are more intense in coastal environments where water-potential stress is reinforced by wind regime and combined to an additional stress *i.e.* osmotic stress due to sea-spray exposure (Boukhdoud et al., 2016, Qasemian et al., 2014). A reduced water availability in soils or litters induces a shift in microbial community diversities (Chowdhury et al., 2011) together with a lower basal respiration and extracellular enzyme production, which hinder organic matter decomposition (Yuan et al., 2007). Coastal ecosystems under such particular constraints may be more threatened by climate change that will notably be characterized by higher temperatures and longer periods of summer drought in the Mediterranean area (Gibelin and Déqué, 2003, Sanchez et al., 2004).

Climate in various biomes has been proven to be a major driver structuring decomposer communities and consequently regulating organic matter turn-over (Aerts, 1997, Wall et al., 2013, Zhou et al., 2008). However, some studies revealed that, local differences linked to plant species assemblages and thus to organic matter diversity and quality, have a stronger effect on the processes of decomposition than climate variations between continents (Cornwell et al., 2008). Zhang et al. (2008) found that litter quality is the most important direct regulator of litter properties at the global scale. Their results showed significant relationships between litter decomposition rates and the combination of climatic factors and litter quality (C/N and Total nutrients).

Thus, there is a urgent need to decipher how litter microbial communities may be affected by such climate modifications at various spatial scales and whether it may vary with the chemical quality of litter and pre-exposure to osmotic stress (coastal environments).This study aimed at

investigating how microbial functioning in litters from two Mediterranean plant species, commonly found in both Mediterranean coastal and inland areas and exhibiting different functional traits (*Pinus halepensis*, a coniferous species, and *Pistacia lentiscus*, an evergreen dicot species) may be driven by the specific conditions of coastal environments. Moreover, we used the contrasted climate conditions across the Mediterranean basin, comparing France, Greece and Algeria environments, to test the effect of a gradient of aridity on litter functioning. Most of litter decomposition experiments used litter from plants exposed artificially to global change factors such as warming, irrigation or elevated $CO_2$ (Suseela and Tharayil, 2018) but very few studies used field-scale transplantation experiments to investigate the *in situ* impacts of climate variations (Berger et al., 2015, Keiser and Bradford, 2017, Makkonen et al., 2012). Here, an original experimental design based on litterbag use was performed: litterbags of either *P. halepensis* or *P. lentiscus* litters were exposed in inland to coastal areas in the three selected countries to test the effect of a gradient of aridity on litter functioning and, within each country, a pool of litter bags was transferred from inland to coastal area to test the effect of coastal conditions on inland communities. Consequently, we aimed at addressing the following questions: i) are microbial communities and their functions shaped by coastal conditions and do more drastic climate conditions found across the Mediterranean basin strengthen these stresses linked to the coastal conditions? ii) for each country, what are the responses of microbial communities from inland areas when they are subjected to coastal area conditions and their specific stresses and are their responses plant-species dependent?

According to our hypotheses: i) in each country, coastal environmental conditions will play a key role defining a particular microbial functioning in the coastal area and ii) more arid climate conditions will act at a larger scale i.e. the Circum-Mediterranean region and will smoothen the effect of coastal conditions and of litter type on microbial properties. Thus, *in*

*fine*, this study will provide useful information about environmental factors driving microbial communities and their activities at different spatial scales, more precisely about the vulnerability of coastal areas.

## Materials and Methods

*Site description and litter sampling*

Sampling sites were selected in stands of *Pinus halepensis* (forest and matorral) in association with *Pistacia lentiscus* throughout a latitudinal gradient across the Mediterranean basin (Table 1). The inland sites (Table 1) were located in the Massif de l'Etoile in the periurban area of Marseille (France), in the forests of Argoli near Cleones (Peloponese, Greece) and in the forests of Traras near Ain Kebira (Wilaya of Tlemcem, Algeria), respectively. The coastal sites (Table 1) were located in the Parc National des Calanques of Marseille (France), near Monemvassia (Greece) and near Ghazaouet (Algeria). In each country, three sampling sites (400 $m^2$) were chosen in the coastal area (at less than 500 m from the coastal line) and three others in the inland area (at 20 km from the sea). The distance between sites, whether coastal or inland, is 2 km at least, far exceeding the spatial dependence of most microbiological properties in soil (Nannipieri et al, 2003). The selected sites had similar pedoclimatic and topographic features: 25–230 m in elevation, similar exposure (South), slope (10-15%) and soil type (Calcaric Leptosol according to IUSS Working Group WRB, 2006). For each site, the exact location (GPS), maximal and minimal temperatures and the relative humidity of air measured *in situ* by EL-USB 2.0 probe (Conrad) over a two-year period and annual precipitation, mean of annual temperature, precipitation of driest and wettest months (worldclim, 1970-2010, Hijmans et al., 2005) were collected (Table 1). At each site and for each plant species, in May 2014, twenty samples were randomly collected over a 400 $m^2$ area to obtain a composite sample (2 kg) to prepare the litter bags. The very upper layer of horizon

126 Ol of *P. lentiscus* and *P. halepensis* litters was sampled to ensure homogenous characteristics
127 of the litter collected. Contaminating debris (e.g. leaves of other species, including branches
128 and seeds) were removed carefully from each collection. The samples were homogenized and
129 stored at 4°C until litter bag preparation.
130
131 *Litterbag preparation and experimental set-up*
132 30 g (dry weight, DW) of *P. halepensis* or *P. lentiscus* litter were placed per litterbag (30×10
133 cm, 2 mm rigid nylon mesh). A total of 162 litterbags were prepared: 108 control litterbags (3
134 countries x 2 areas (coastal and inland) x 3 sites x 3 replicates x 2 types of litter) and to test
135 the effect of the coastal zone i.e. 54 litterbags were transferred from inland to coastal areas (3
136 countries x 3 sites x 3 replicates x 2 types of litter). Litterbags were then placed in 6 sites for
137 litter exposure (3 countries x 2 areas), independent from the sampling sites, in early June
138 2014: 18 control litterbags in each inland site of the three countries (9 litter bags for each
139 species), 18 control litterbags and 18 'transfer' litterbags in each coastal site of the three
140 countries. After 10 months in the field, litterbags were collected in March 2015. An aliquot of
141 each sample was kept after drying and ground prior to chemical analysis. For microbial
142 analyses, all the experiments started immediately and were performed over one week.
143
144 *Plant litter physico-chemical properties*
145 The physico-chemical properties of *P. halepensis* and *P. lentiscus* litters collected in coastal
146 and inland sites were determined as follows: as a preliminary, litters were characterized for
147 moisture, pH and electrical conductivity (EC). Total Organic Carbon (TOC) and Total
148 Nitrogen (TN) were measured using high temperature catalytic oxidation technique (Multi
149 N/C 2100, Analytik Jena, Germany). The sample was injected (50 µl) into the furnace filled
150 with a Pt preconditioned catalyst. The combustion was realized at 800 °C and the combustion

products were carried by high purity oxygen (Linde Gas) allowing detection of $CO_2$ by non-dispersive infrared (NDIR) and detection of NO by chemiluminescence (CLD).

After ten months in the field, TOC and TN of litters from litter bags were characterized as described above. Moreover, litter chemical properties were also analysed by solid-state $^{13}C$ NMR. Spectra were obtained on a Bruker Avance-400 MHz NMR spectrometer operating at a $^{13}C$ resonance frequency of 100.7 MHz and using a commercial Bruker double-bearing probe. About 400 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 Polarisation (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 20K scans were accumulated using a delay of 2.5s, for an experimental time of 2h. The $^{13}C$ chemical shifts were referenced to tetramethylsilane and calibrated with glycine carbonyl signal, set at 176.5 ppm. The $^{13}C$ NMR spectra were divided into 7 chemical shift regions according to Dignac et al. (2002): i.e. alkyl C (0-45 ppm), methoxyl C (45-60 ppm), O-alkyl C (60-90 ppm), di-O-alkyl C (90-110 ppm), aromatic C (110-140 ppm), phenolic C (140-160 ppm) and carboxyl 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). The aromaticity ratio (aromatic C/O alkyl C + alkyl C + aromatic C) was calculated according to Baldock et al. (1997).

*Microbial activities*

Cellulase activity was assayed using CarboxyMethylCellulose (CMC) 0.1% (w/v) in 8 mL of sodium acetate buffer (50 mM, pH 5) added to 1 g of litter (dry weight) incubated for 4h at 50°C. Glucose was quantified according to the Somogyi-Nelson method and absorption

was read at 870 nm (Farnet et al, 2010). Enzyme activities were expressed in µmoles of reaction products released per minute (U) per gram of dry soil (U $g^{-1}$ DS).

Basal respiration was measured to assess the ecophysiological state of litter microbial communities. Three g DW equivalent of fresh litter were placed in 117 ml glass jars. The glass jars were immediately sealed with hermetic rubber septa, and incubated for 4 hours at 25°C. After incubation, 1 ml of air was sampled in the head space with a syringe and injected into a gas chromatograph (Chrompack CHROM 3 – CP 9001) to analyse $CO_2$ 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 $CO_2$ concentrations were subtracted from sampled $CO_2$ concentrations and resulting values were adjusted at 22°C according to Ideal Gas Laws using a $Q_{10}$ = 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 maximize the respiration rate in litter in a preliminary assay (data not shown). One ml of air was sampled in the headspace with a syringe and injected into a gas chromatograph to analyse $CO_2$ production for 1 hour (see above). Substrate-induced respiration was converted into microbial biomass (MB) using the relation established by Beare et al. (1990).

The catabolic diversity of cultivable microbial communities was determined with Biolog EcoPlate™ (BIOLOG Inc., Hayward, CA) using a procedure adapted from Garland and Mills (1991). Briefly, 4 g DW equivalent of sub-sample were added to 50 ml of sterile 0.1 % Na-pyrophosphate solution (pH 7). The mixture was then shaken with an orbital shaker for 20 min and centrifuged at 500 g for 10 min at 4 °C to obtain a microbial suspension. Exactly 1 ml of supernatant was diluted into 99 ml of sterile saline solution (0.85% NaCl), hand-mixed for 30 sec. and left to stand for 10 min. We purposely did not adjust the inoculums in order to

obtain uniform cell density, instead considering the total microbial count as an inherent characteristic of the microbial communities of each mesocosm. A 125 µl aliquot of the diluted solution was added to each of 96 wells in a Biolog EcoPlate™. Plates were incubated at 25 °C and colour formation in each well was monitored at 595 nm using a TECAN® spectrophotometer. Measurements were performed three times a day until average well colour development (AWCD) exceeded a value of 1.0 standardized absorbance units (i.e. 3–5 day incubation). Microbial C-use intensity was assessed as the rate of average well colour development (AWCD) calculated as follows: AWCD =Σ ODi/31, where ODi is the optical density for each well in the mid-exponential growth phase (i.e. after 72h). Absorbance values for the wells with C sources were blanked against the control well. The incubation time resulting in an AWCD = 0.5 absorbance unit ($T_{0.5}$) was calculated for each sample and used to standardise the optical density of each C-containing well (Garland and Mills, 1991).

*Microbial genetic structure*

DNA was extracted from 0.25g of litter, using NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer instructions. The quality of DNA extractions was checked by electrophoresis on 1% agarose gels stained with GelRed (Molecular Probes, USA) using a Gel Doc image analyser (BioRad, USA). DNA concentration was quantified using Quant-iT$^{TM}$ dsDNA High-Sensitivity Assay Kit (Invitrogen, Canada). Then, all DNA extracts were then diluted to 0.5 ng / µL for subsequent analysis. Bacterial community structures were analyzed using T-RFLP footprinting 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 ºC for 2 min, followed by 30 cycles of 94 ºC for 30 s, 57 ºC for 45 s, and 72 ºC for 90 s, followed by a final extension time at 72 ºC for 10 min. Bacterial PCR

products (10 µl) were then digested with 10 U of the restriction enzyme *AluI* and 1× restriction enzyme buffer (Thermo Fisher) in a total volume of 15 µl at 37 ºC for 3 h. After a desalting step, 2µl of PCR products were mixed with formamide containing 0.5% of LIZ500 (T-RFLP) internal size standard (Applied Biosystems,) in a total volume of 12 µl and denatured at 94 ºC 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). The fragments between 50 and 500 bp and peaks height ≥ 50 fluorescence units were included in T-RFLP analysis. Fragments having a relative abundance of proportion < 0.5% were removed from the matrices.

*Statistical analyses*

The catabolic (BIOLOG) and genetic (TRFLP) diversities was calculated using Shannon's index (H'):

$$H' = -\sum_{i=1}^{N} pi \log_{10} pi$$

where $p_i$ is either the ratio of colour development of the $i^{th}$ well to the sum of colour development of all positive wells or the relative abundance of a given restriction fragment for BIOLOG or TRFLP techniques.

The normality and homogeneity of the variances of the data were determined on the residuals from the regression model with 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. For control litterbags, three-way ANOVA were used to determine whether, and to what extent, microbial properties (i.e. basal respiration, active microbial biomass via SIR, catabolic diversity H', cellulase activities and

bacteria richness and equitability via genetic analysis) and chemical characteristics (using NMR data and C/N ratio) depended upon i) litter species (*Pinus halepensis vs Pistacia lentiscus*) ii) the area conditions (coastland *vs* inland), iii) the geographical location (France, Greece and Algeria) and iv) their interactions. When a 2-way interaction was found between geographical location and context (coastal and inland) or plant litter species, we separated data for each modality of geographical location, context, transfer or plant litter species using a one-way ANOVA. Statistically significant ($P<0.05$) main effects and interactions were analysed further using the Tuckey HSD *post hoc* tests. Principal component analysis (PCA) was performed on covariance matrices from Biolog® to explore the effects of geographical location, condition and litter type on the catabolic structure of microbial communities and tested by PERMANOVA. PCA provided an ordination of the microbial C-substrate utilization profiles, which were plotted in one and two dimensions respectively, based on the scores of the first two principal components (PC). All statistical analyses when performed using R software (3.2.1, R Development Core team, 2015), levels of significance are indicated as $p \leq 0.05$.

## Results

### *Influence of the coastal environmental conditions on litter properties along a gradient of aridity in the Mediterranean basin*

Here, we explored whether environmental constraints, which can be enhanced in coastal environments (i.e. differences in salinity exposure, humidity, temperature, etc.), affected litter microbial functioning. Thus, first, it was important to demonstrate how these environmental characteristics actually varied between inland and coastal zones in the three countries.

These environmental conditions were indeed highly discriminated with respect to certain parameters and depending on the geographical location. Temperature and precipitation variations were strongly different between both coastal and inland areas. In France and Greece, maximal temperature and annual mean temperature were higher in the area close to the coastline, while precipitations of the driest and wettest months were lower. An opposite trend was observed in Algeria, with more intense arid conditions in the inland site. These contrasted climate conditions are particularly well described using annual precipitation as an index of aridity (Stadler, 2005). The climate gradient between the Northern and the Southern sides of the Mediterranean basin is clearly observed using this parameter and the differences in precipitations between coastal and inland areas are particularly strong in Greece and Algeria with opposite trends as described above. Moreover, values of both conductivity and chloride ion concentrations linked to salinity exposure via sea sprays, were higher in litters situated at the coastal areas in the three countries, showing that salinity stress was stronger in the areas close to the coastline. We also determined the litter chemical properties at t = 0 from the twelve composite samples of litters (2 species x 2 context (coastal vs inland) x 3 countries) used for litter bag preparation and results are shown in Table 2. Several global trends were observed. Whatever the country or the distance from the sea, pH was always lower in *Pinus halepensis* litter (from 4.7 to 6.24) than in *Pistacia lentiscus* litter (from 6.22 to 6.55). Moreover, in *P. halepensis* litter, total Organic Carbon amounts were always higher (from 2.67 to 4.1 g C/L *vs* 1.55 to 2.22 g C/L) and this trend was also observed for Total N amounts (from 3.49 to 5.12 g C/L *vs* 2.41 to 3.18 g C/L). $^{13}$C NMR results showed that the proportion of di-O-alkyl C + O-alkyl C was higher in *P. halepensis* litter and this was also observed for aromatic + phenolic % in most cases. Thus, litter physico-chemical characteristics depended on the type of species and not on the geographical location (coastal vs inland area, Northern vs Southern side of the Mediterranean).

298 Three way-ANOVA revealed significant interactions between the coastal conditions and the
299 country for most of the microbial markers considered: cellulase activities, catabolic diversity,
300 basal respiration, and microbial biomass (Table 3).
301 Cellulases varied with the three factors tested (Figure 1 a and c). However, these activities
302 followed a global trend whatever the species and the area (coastal or inland): Greece > France
303 = Algeria.
304 For microbial biomass (MB), the effects of coastal conditions varied with the plant species
305 and the country (Figure 1b and d): no differences of MB between coastal and inland areas
306 were observed in *P. lentiscus* litters whatever the country (Fig 1b), while in *P. halepensis*
307 litters, higher MB was found in Algeria inland area only (coastal x country interaction,
308 $F=8.88$, $p \leq 0.001$, Fig 1d).
309 Our results also highlighted variations in catabolic diversity H' (ECO) and basal respiration
310 depending on coastal x country interaction (Figure 2a and b). In coastal area, H' (ECO) was
311 lower in Greece where the more drastic climate conditions were found compared to the two
312 other countries and the opposite trend was observed for BR. Thus, microbial communities
313 with less-diversified catabolic potential led to a higher respiration in the Greek coast. In
314 inland area, conversely, H'(ECO) increased with the gradient of aridity
315 (Algeria>Greece>France), while BR was similar whatever the country (Figure 2a).
316 Thus, for RB, MB and H'(ECO), the coastal conditions modified such important microbial
317 markers depending on the plant species and the country across the Mediterranean basin.
318 These results have to be considered regarding the chemical properties of litters characterised
319 by solid-state NMR of $^{13}C$. Aromatic compounds were negatively correlated with MB ($r^2$=-
320 0.26, $p \leq 0.05$). These results are corroborated by the three-way ANOVA (Table 3): the amount
321 of aromatic compounds was lower in litter where MB was high (Algerian coast) and
322 conversely, aromatics were more abundant in litter where MB was low (Greek coast) (coastal

x country interaction F=1.98, $p \leq 0.01$). O-Alkyl C signal (assigned to polysaccharides) also varied depending on the coastal x country interaction (F=8.08, $p \leq 0.001$) and higher O-Alkyl C signal in *P. halepensis* litter was observed together with high MB.

The effects of the country, the area (coastal or inland) and the plant species were also tested on the catabolic structures of litter microbial communities. PERMANOVA showed significant differences of the catabolic structure of litters between the three countries (pseudo-F=5.23, $p \leq 0.001$) and between areas (pseudo-F=4.40, $p \leq 0.001$). Moreover, coastal conditions shaped microbial catabolic profiles depending on the country (pseudo-F=3.43, $p \leq 0.01$). For microbial genetic structure, the first two principal components (PCs) account for 33 % of the variation in the data; PC1 accounts for 20 % and PC2 for 13 %. PCA revealed significant differences between the three countries (pseudo-F=3.39, $p \leq 0.001$) and between areas (pseudo-F=9.32, $p \leq 0.001$), showing that geographical conditions shaped microbial genetic structure and that it differed depending on the coastal and inland areas. Figure 3a and b shows the results of the PCA for the four combinations of area (inland or coastal) and litter (based on either genetic or catabolic profiles) plotted on the first two principal components. For catabolic diversity, the first two principal components (PCs) account for 22 % of the variation in the data; PC1 accounts for 12 % and PC2 for 10 %. For the two plant species considered, catabolic and bacterial genetic structures changed depending on coastal and inland areas (Figure 3 a and b) indicating that the effect of coastal context was stronger than the effect of species (which would have homogenized catabolic profiles for one species). On the other hand, in either inland or coastal areas, both catabolic and genetic structures were different depending on the species showing that this is an important environmental driver at a local scale.

It is noteworthy that no significant effect of the factors tested was found on bacterial genetic richness or diversity assessed via H' index (data not shown).

*Effect of litter transfer from inland to coastal area on litter properties*

In order to understand how environmental conditions of coastal environments may structure microbial functioning, litters were transferred from inland to coastal area to test our initial hypothesis: environmental conditions acting at a regional scale (coastal *vs* inland) are preponderant over those acting at a local scale (litter chemical signature specific to a plant species).

First, chemical markers from NMR data showed that, for both litters, the Aromaticity ratio was different between transferred litters and litters from the coastal area (Table 4). Thus, the 'chemical environment' of microbial communities was not similar in transferred and coastal litters for both species and whatever the geographical location.

The effects of litter transfer (mainly negative) to the coastal zone observed on different microbial markers (Table 4) varied with the country and the species. For cellulases, variations after transfer to the coastal zone, were only found in Greece and differed with the species: i) in *P. halepensis* litters, cellulases decreased significantly (to reach a lower level compared to both coastal and inland activities), ii) in *P. lentiscus* litters, cellulases increased compared to the original area i.e. inland area (Figure 4 a and b). Thus, in Greece, exposure of litter microbial communities to environmental conditions specific to the coastal area (higher temperatures and more intense drought than in the two other countries as shown in Table 1) led to a modification in activities linked to the transformation of the labile fraction of organic matter and the results observed after this exposure to stress differed with the plant species.

Our results also revealed the strong negative effects of the coastal environmental conditions on MB and H'(ECO) depending on the geographical location (Table 4). This is in Algeria where litter transfer to coastal area strongly affected both microbial markers (data not shown), while no variations were observed in France and Greece after transfer for these markers.

PCA from Biolog data revealed a change in catabolic profiles due to litter transfer to the coastal zone (Figure 5a and b). PC1 and PC2 explained 12.7% and 10.7% of variance for *P. lentiscus* litter (Figure 5a) and 13.5% and 12.5% for *P. halepensis* litter (Figure 5b). PERMANOVA indicated effects of transfer to the coastal area on catabolic profiles in *Pinus* (pseudo-F=2.64, $p \leq 0.001$) and *P. lentiscus* (pseudo-F=2.22, $p \leq 0.001$) litters. These effects were significant for the three countries in the case of *P. lentiscus* litters and in Algeria only for *P. halepensis* litter. Interestingly, microbial catabolic structure after litter transfer in Algeria was similar to that from the coastal area for both *P. lentiscus and P. halepensis* litters (Figure 5a and b).

PCA based on T-RFLP data also revealed a change in genetic profiles due to litter transfer to the coastal zone (Figure 6a and b). PC1 and PC2 explained 9.1% and 4.9% of variance for *P. lentiscus* litter (Figure 6a) and 11.2% and 5.1% for *P. halepensis* litter (Figure 6b). PERMANOVA indicated significant effects of transfer to the coastal area on microbial communities in *P. halepensis* (pseudo-F=5.64, $p \leq 0.01$) and *P. lentiscus* (pseudo-F=7.41, $p \leq 0.01$) for the three countries.

## Discussion

### *Variations in litter microbial functioning across an aridity gradient in the Mediterranean basin depends on the distance from the sea*

Our study revealed that the effects of the coastal environment differed depending on the geographical location: more precisely, in Greece, coastal conditions led to a specific functioning with higher cellulase and basal respiration and a lower catabolic index of diversity. On the other hand, in coastal environments in France and Algeria, more catabolically-diversified communities were found (higher H' (ECO) index). Moreover, all these differences in microbial functioning were accompanied by different genetic structures in

coastal and inland areas of the three countries as described by PCA from T-RFLP data. This may be explained by contrasted climate conditions between coastal and inland environments in Northern *vs* Southern part of Mediterranean basin as described above. Summer drought is indeed more drastic in coastal area in Greece, while higher temperatures are found in the inland area in Algeria. Moreover, additional osmotic stress in the coastal area (as revealed by conductivity and chloride ion concentrations) has probably reinforced the stronger drought constraints found in Greek coastal area. Rath et al. (2015) indicated that additional salt stress to drying exerts a stronger effect on microbial growth and moreover a shift in microbial structure (assessed via PLFA) after salt treatment is induced. Our recent study (Kheir et al., 2019) also sustained such findings since osmotic stress, experienced by microbial communities from coastal environments, modified differently catabolic structure of litter microbial communities after drought stress. Rajaniemi and Allison (2009) also showed that microbial community composition and biomass can be strongly modified by abiotic stress such as salinity and provoked a shift towards a higher proportion of Gram-negative bacteria.

Here, our findings also underlined that the type of litter shaped microbial communities: i) at a local scale i.e. in either coastal or inland areas, certain microbial markers (catabolic profiles, cellulase activities) varied with the type of plant species, ii) at a regional scale, the effect of coastal conditions differed with the plant species (variations in BR, H'(ECO), cellulases and catabolic profiles). This goes in line with our previous study (Farnet Da Silva et al., 2016), which demonstrated that catabolic diversity varied with litter type under water potential stresses linked to coastal environments. Sherman and Steinberger (2012) studied catabolic potential of litter microbial communities across an aridity gradient in Israel, from the Mediterranean shore to the arid zone. They also found a shift in litter microbial catabolic structure explained by plant traits. Moreover, their study revealed that low organic matter input in the arid zone -because of low plant productivity- induced a diversification of resource

utilization. These findings are of importance since modification in catabolic potential of microbial communities may induce a shift in decomposition process since transformation of certain compounds such as lignin is sensitive to the structure of microbial communities (Mc Guire and Treseder, 2010).

*Modification of litter microbial functioning when microbial communities from inland area are subjected to coastal environmental conditions*

After transfer of litters from inland to coastal area, certain microbial markers, such as cellulase activities, were strongly impacted and the intensity of the effect varied with the plant species. Our initial hypothesis was based on the fact that coastal conditions may have smoothen the effect of litter type leading to homogeneous microbial responses to changes in environmental climate conditions whatever the plant species. Thus, our study underlined the importance of species traits in microbial responses to stress. The quantity and quality of organic matter shape the structure and composition of microbial communities and their resistance to stresses (Bradford et al., 2002). Mediterranean litters are described as particularly rich in secondary metabolites (terpenes, tannins, VOC …) and lignins (Di Castri, 1973, Rousk et al., 2010, Terradas and Savé, 1992). These compounds are known to modify microbial communities and their activities: they can be toxic for microbial cells (Popova et al., 2009, Adamczyk et al., 2015), interact with enzymes, leading to adverse effects on their catalytic potential (inhibition/precipitation of enzymes). Some tannins can modify the quantity and the nature of N sources available for microorganisms because of adsorption or enzymatic inhibition (Hättenshwiler and Vitousek, 2000). Thus, the importance of litter chemical signature on microbial community structure and functioning has to be taken into account when studying soil microbial vulnerability under environmental constraints. More particularly, the effect of litter admixture on microbial responses to coastal condition exposure

should be valuable to determine since the diversification of nutrient resources is known to enhance microbial diversity and consequently may favour resistance to water potential stress. These subtle variations in organic matter can indeed lead to additive or non-additive (synergistic or antagonistic) effects (Brunel et al., 2017) and various plant species exhibiting different functional traits should be included in such research (Prescott and Grayston, 2013). Previous studies have indeed shown that changes in plant species can actually modify both the biomass and structure of soil microbial communities (Laganière et al., 2009; Matos et al., 2010) and thus their responses to stress.

Coastal environmental conditions appeared to have induced a shift in microbial catabolic structure of both litters, mainly in Algeria. Interestingly, in *P. halepensis* litter, the catabolic profile resulting from transfer in the Greek coastal area differed from catabolic profiles of both inland and coastal areas (control and transfer area respectively). These results may partially explain the decrease in cellulase activities after transfer: the shift in catabolic diversity of microbial communities after transfer may have not sustained such activities. On the other hand, in *P. lentiscus* litter, the potential of cellulose transformation was identical in the transferred litter and in litter from the coastal area. Some studies have attempted to decipher the respective influence of either climate conditions or chemical signature of organic matter, using very contrasted bioclimates. Gholz et al (2000) and Perez et al. (2013) indicated that the effect of 'home field advantage' is particularly pregnant for microbial communities from litters with a high amount of cellulose and hemicellulose. The study of Makkonen et al. (2012) revealed utterly different results: transfers of 16 types of litters from four different biomes (subarctic, temperate, Mediterranean and tropical climate) led to a homogeneous dynamics of litter transformation. Moreover, the authors found that the complexity of decomposer communities including micro-, meso and macro fauna (using various mesh size of litterbags), does not accelerate decomposition rate and this is particularly true for the

Mediterranean biome. This means that decomposers from a wide range of bioclimates are not specialized in the transformation of 'autochthonous' organic matter. Opposite results were found in the study of Milcu and Manning (2011), which showed that a range of soil fauna size classes contributes to 'home field advantage' effect and that experimental bias (e.g. fine mesh sizes) impacts the contribution of soil fauna. It has to be underlined that our study also involved micro and mesofauna since 2 mm mesh-size litterbags were used. We found that the concept of 'home field advantage' applied to cellulose transformation depended on the litter type. For *P. halepensis*, this potential clearly depended on the coastal conditions since, when microbial communities from inland areas were transferred to the coastal zones, their potential to hydrolyze cellulose strongly decreased.

Transfer experiment of litters also showed that major microbial markers such as MB and the catabolic index of diversity, H' (ECO), were differently affected depending on the geographical location: after transfer, H' (ECO) decreased in Algeria and not in France or Greece, and MB decreased in Algeria. Thus, this revealed that the contrasted climate conditions across the Mediterranean Basin specialized microbial responses when facing a change in environmental conditions (transfer from inland to coastal area). Piélou Index of diversity based on T-RFLP also underlined that litter transfer led to a decrease in genetic diversity (whatever the geographical location or the litter type). This study indicates that coastal conditions have probably shaped a particular microbial functioning and that microorganisms from other environments have to adapt to these new specific conditions. However, more than the chronicity of the stress linked to sea-spray exposure, the effect of pulses of salinity increase (due to seasonal variations i.e. wind intensity, precipitation…) is probably a more challenging factor for microorganism resistance (Schimel et al., 2007). Our previous study (Qasemian et al., 2014) indeed revealed variations in conductivity in coastal environments over the year (higher conductivity in summer because of drought). As discussed

above, the recent study of Rath et al. (2017) evoked the importance of salinity in enhancing drought exposure impact on soil microorganisms.

Whatever the geographical location and the species, after transfer of litterbags, a shift in the genetic structure was observed and differed from those of both inland and coastal areas. These findings were observed after a 10-month exposure in the new habitat i.e. costal area and this revealed that these variations in environmental conditions are likely to shape differently microbial communities over long periods. This also showed that acclimation of litter microbial communities, in terms of diversity, to other environmental conditions was quite a long-term process. Pesaro et al. (2004) found that after a drought stress, functional properties such as respiration, are rapidly recovered, while the initial levels of diversity and biomass are not reached one month after stress. Schimel et al. (2007) suggested that, while physiological effects of water potential stresses are supposed to drive short-term microbial responses, the modifications in microbial community structure and composition are likely to occur over a longer period. Moreover, these authors assume that microbial diversity in soil should not be considered as a juxtaposition of 'functional groups' but results from the interaction between these functional groups (such as denitrifiers, lignocellulose decomposers…) and from their resistance to variations in environmental conditions and stresses of different types.

This study revealed that litter microbial communities from inland and coastal areas had different genetic and catabolic structures. Crits-Christoph et al., (2013) found that conductivity was one of the major drivers of soil microbial genetic diversity in arid environments. The study of Qasemian et al., (2014), who tested the effect of coastal conditions on microbial activities at a microlocal scale, also revealed modifications of microbial catabolic structure in litters depending on the distance from seashore. The understanding of area–species diversity relationships is of major importance since it allows prediction of the impact of environmental changes such as more intense climate stresses

(Decaëns, 2010). Moreover, the coastal conditions affected major microbial properties across a gradient of aridity in the Mediterranean basin depending on plant litter species, indicating the huge importance of litter chemical signature even at wide spatial scale. Since both microbial diversity and enzyme activities are of major importance in sustaining organic matter turn-over, our results contributed to understand better the relative importance of external filters to shed light on litter microbial functioning in changing environments.

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721
722 Data availability statement
723 The data that support the findings of this study are available from the corresponding author
724 upon reasonable request.

725 **Table 1** Location of the areas where litter bags were left in the three countries, environmental parameters monitored monthly *in situ* over a two-
726 year period and climate variables from worldclim data (Hijmans et al., 2005).

| | France | | Greece | | Algeria | |
|---|---|---|---|---|---|---|
| | Coastal | Inland | Coastal | Inland | Coastal | Inland |
| Area location | N43°12'34.08" E5°21'24.05" | N43°22’29.74’’ E5°25'44.66" | N36°40'21.22" E23°2'2.26" | N37°47'42.47" E22°44'22.81" | N35°06’33.05’’ E1°49’46.47’’ | N35°03’12.17’’ E1°43’37.78’’ |
| Environmental data monitored *in situ* (2014-16) | | | | | | |
| Minimal temperature of the coldest month (°C) | 2.5 | -1.5 | 3 | -2 | 3.5 | -1.5 |
| Maximal temperature of the hottest month (°C) | 41.5 | 40.5 | 50 | 43.5 | 44 | 47.5 |
| Minimal relative humidity of air (the driest month) | 30 | 15 | 16 | 24 | 28.5 | 18.5 |
| Worldclim data | | | | | | |
| Annual mean temperature (°C) | 14.1 | 13.1 | 18.2 | 15.7 | 14.4 | 20 |
| Annual precipitation (mm) | 602 | 643 | 543 | 666 | 360 | 77 |
| Precipitation of the driest month (mm) | 13 | 16 | 2 | 9 | 5 | 1 |
| Precipitation of the wettest month (mm) | 85 | 85 | 107 | 117 | 44 | 11 |

727

728

729 **Table 2** Chemical characteristics of the composite samples of litters which were used to prepare litter bags for each environmental condition

730 (coastal and inland areas) and geographical location (France, Greece, Algeria).

731

| | France | | | | Greece | | | | Algeria | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | coastal | | inland | | coastal | | inland | | coastal | | inland | |
| | *P h* | *P l* | *P h* | *P l* | *P h* | *P l* | *P h* | *P l* | *P h* | *P l* | *P h* | *P l* |
| TOC (mg C/kg) | 2851.9 | 1557.71 | 2951.67 | 1855.96 | 4104.09 | 1756.91 | 2647.36 | 2225.68 | 2556.62 | 1694.72 | 2675.63 | 1593.12 |
| Total N (mg N/kg) | 88.0 | 102.4 | 65.6 | 69.8 | 52.0 | 61.0 | 55.0 | 110.0 | 77.6 | 97.0 | 48.2 | 53.0 |
| $NH_4^+$ (mg N/kg) | 0.48 | 0.63 | 0.46 | 0.52 | 0.26 | 0.55 | 0.38 | 0.85 | 0.59 | 0.44 | 0.27 | 0.28 |
| pH | 6.16 | 6.55 | 6.22 | 5.76 | 5.88 | 6.27 | 5.7 | 6.19 | 5.55 | 6.46 | 6.15 | 6.54 |
| Conductivity (mS/cm) | 1557 | 2020 | 590 | 640 | 1634 | 982 | 701 | 641 | 954 | 830 | 630 | 690 |
| $Cl^-$ (%) | 2.80 | 3.62 | 1.04 | 1.13 | 2.92 | 1.75 | 1.25 | 1.40 | 1.70 | 1.47 | 1.11 | 1.22 |

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733

734 **Table 3** Results of the three-way ANOVA to test the effects of the plant species (*Pinus halepensis vs Pistacia lentiscus*), of the environmental
735 condition (coastal *vs* inland area), the geographical location (France, Greece and Algeria) and their interactions on the microbial and chemical
736 markers from control litter bags. BR: Basal Respiration, MB: Microbial biomass, H' (ECO): Shannon-Weaver index from catabolic profiles, R
737 (T-RFLP), richness from T-RFLP data, H' (T-RFLP): Shannon-Weaver index from T-RFLP.

738

| Control | Df | BR | MB | cellulase | H' | COOH | Aromatic | O-alkyl-C | Alkyl-C |
|---|---|---|---|---|---|---|---|---|---|
| Species | 1 | 4.82* | 1,23 | 40.51*** | 0,009 | 202.39*** | 3,06 | 23.01*** | 17.03*** |
| Context | 1 | 2,45 | 5.95* | 11.37** | 3,62 | 2,65 | 1,68 | 3,27 | 10.15** |
| Geographical location (GL) | 2 | 0,39 | 1,35 | 108.20*** | 13.31*** | 14.54*** | 0,8 | 1,74 | 2,27 |
| Species* GL | 1 | 0,02 | 7.32** | 7.73*** | 1,16 | 36.42*** | 33.12*** | 0,01 | 0,28 |
| Species* Context | 2 | 0,4928 | 4.49* | 16.66*** | 0,67 | 12.06*** | 6.43** | 7.13** | 6.76** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Context* GL | 2 | 9.37*** | 8.83*** | 4.90** | 12.51*** | 84.23*** | 1,98 | 8.08*** | 10.35*** |
| Species* Context*GL | 2 | 2,11 | 6.05** | 7.63*** | 7.34** | 31.85*** | 19.666*** | 15.56*** | 9.31*** |

| Coastal transfer | Df | BR | MB | cellulase | H' | COOH | Aromatic | O-alkyl-C | Alkyl-C |
|---|---|---|---|---|---|---|---|---|---|
| Species | 1 | 5.002* | 3,07 | 14.97*** | 1,73 | 390.33*** | 21.03*** | 39.57*** | 14.88*** |
| Context | 2 | 1,36 | 0,59 | 8.56*** | 1,8 | 5.12** | 1,67 | 6.53** | 14.47*** |
| Geographical location (GL) | 2 | 2,97 | 11.04*** | 94.10*** | 7.79*** | 24.02*** | 1,21 | 2,52 | 4.66* |
| Species* GL | 2 | 1,61 | 7.10** | 10.34*** | 0,48 | 67.63*** | 2,09 | 15.00*** | 26.17*** |
| Species* Context | 2 | 0,85 | 3.69* | 7.33*** | 1,69 | 27.15*** | 23.48*** | 0,66 | 2,52 |
| Context* GL | 4 | 5.82*** | 5.20*** | 9.52*** | 9.14*** | 10.28*** | 3.57** | 6.38*** | 5.72*** |
| Species* Context*GL | 4 | 3.62** | 3.28* | 10.84*** | 4.46** | 31.77*** | 11.04*** | 11.46*** | 7.24*** |

739 * P≤ 0.05 ; ** P≤ 0.01 ; *** P≤ 0.001.

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742 **Table 4** Results of the three-way ANOVA to test the effects of the plant species (*Pinus halepensis vs Pistacia lentiscus*), the transfer (control vs
743 transferred litters), the geographical location and their interactions on the microbial and chemical markers from control and transfer litter bags.
744 BR: Basal Respiration, MB, Microbial biomass, H' (ECO): Shannon-Weaver index from catabolic profiles, R (T-RFLP), richness from T-RFLP
745 data, H' (T-RFLP): Shannon-Weaver index from T-RFLP.

| | Df | BR | MB | Cellulase | H'(ECO) | R (T-RFLP) | H' (T-RFLP) | COOH | Aromatic | O-Alkyl-C | Alkyl-C |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | 1 | 5.002* | 3.07 | 14.97*** | 1.73 | 0.322 | 0.078 | 390.33*** | 21.03*** | 39.57*** | 14.88*** |
| Transfer | 2 | 1.36 | 0.59 | 8.56*** | 1.8 | 4.808** | 6.198** | 5.12** | 1.67* | 6.53** | 14.47*** |
| Geographical location (GL) | 2 | 2.97 | 11.04*** | 94.10*** | 7.79*** | 6.229** | 2.987* | 24.02*** | 1.21 | 2.52 | 4.66* |
| Species* GL | 2 | 1.61 | 7.10** | 10.34*** | 0.48 | 0.514 | 0.007 | 67.63*** | 2.09 | 15.00*** | 26.17*** |
| Species* Transfer | 2 | 0.85 | 3.69* | 7.33*** | 1.69 | 1.191 | 0.065 | 27.15*** | 23.48*** | 0.66 | 2.52 |
| Transfer * GL | 4 | 5.82*** | 5.20*** | 9.52*** | 9.14*** | 1.677 | 0.035 | 10.28*** | 3.57** | 6.38*** | 5.72*** |
| Species* Transfer * GL | 4 | 3.62** | 3.28* | 10.84*** | 4.46** | 0.411 | 0.062 | 31.77*** | 11.04*** | 11.46*** | 7.24*** |

746 * $p \leq 0.05$ ; ** $p \leq 0.01$ ; *** $p \leq 0.001$.

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**Figure 1 a and b** Cellulase (A and C) and microbial biomass (B and D) from control litter bags of *Pistacia lentiscus* (A and B) and *Pinus halepensis* (C and D) from coastal (▒) or inland (□) areas. Means and standard deviations (n=9). Different letters indicate significant differences between countries for coastal or inland areas (Tuckey HSD *post hoc* test, $p \leq 0.05$).

**Figure 2** Shannon-Weaver Index from catabolic diversity (A) and Basal Respiration (B) of control litter bags from either coastal (▒) or inland (▒) areas in Greece, France and Algeria. Means and standard deviations (n=18). Different lowercase or upper-case letters indicate significant differences between countries for coastal or inland areas, respectively (Tuckey HSD *post hoc* test, $p \leq 0.05$). Asterisks indicate differences (*t*-test) between inland and coastal area.

**Figure 3 a and b** PCA from bacteria T-RFLP fingerprints (A) and catabolic profiles from Biolog data (B) obtained from control litter bags and showing the effect of the type of litter (*Pinus= Pinus halepensis vs Pistacia = Pistacia lentiscus*) and of the condition (coastal *vs* inland) on both genetic and functional microbial structures. Centroids, representing the average of 21 replicates, are reported with standard deviations.

**Figure 4** Cellulase in litter bags from inland control (I), coastal control (C) and transfer from inland to coastal zones (I>C) in the three countries, Greece (▒), France (▒) and Algeria (▒) and for both plant species i.e. *Pinus halepensis* (A) and *Pistacia lentiscus* (B). Means and standard deviation (n=9). Different letters indicate significant differences between means of C, I and I>C for each geographical location (Tuckey HSD *post hoc* test, $p \leq 0.05$)

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775 **Figure 5 a and b** PCA from catabolic profiles from Biolog data for *Pistacia lentiscus* (A) and
776 *Pinus halepensis* (B) obtained from control and transfer litterbags showing the effect of litter
777 transfer from inland to coastal areas depending on the species and the geographical location
778 on catabolic microbial structures (white symbols: France, grey symbols: Greece, black
779 symbols: Algeria). Centroids, representing the average of nine replicates, are reported with
780 standard deviations.

781

782 **Figure 6 a and b** PCA from T-RFLP fingerprints for *Pistacia lentiscus* (A) and *Pinus*
783 *halepensis* (B) obtained from control and transfer litterbags showing the effect of litter
784 transfer from inland to coastal areas depending on the species and the geographical location
785 on microbial genetic structures (white symbols: France, grey symbols: Greece, black symbols:
786 Algeria). Centroids, representing the average of nine replicates, are reported with standard
787 deviations.

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799 Figure 1 a and b

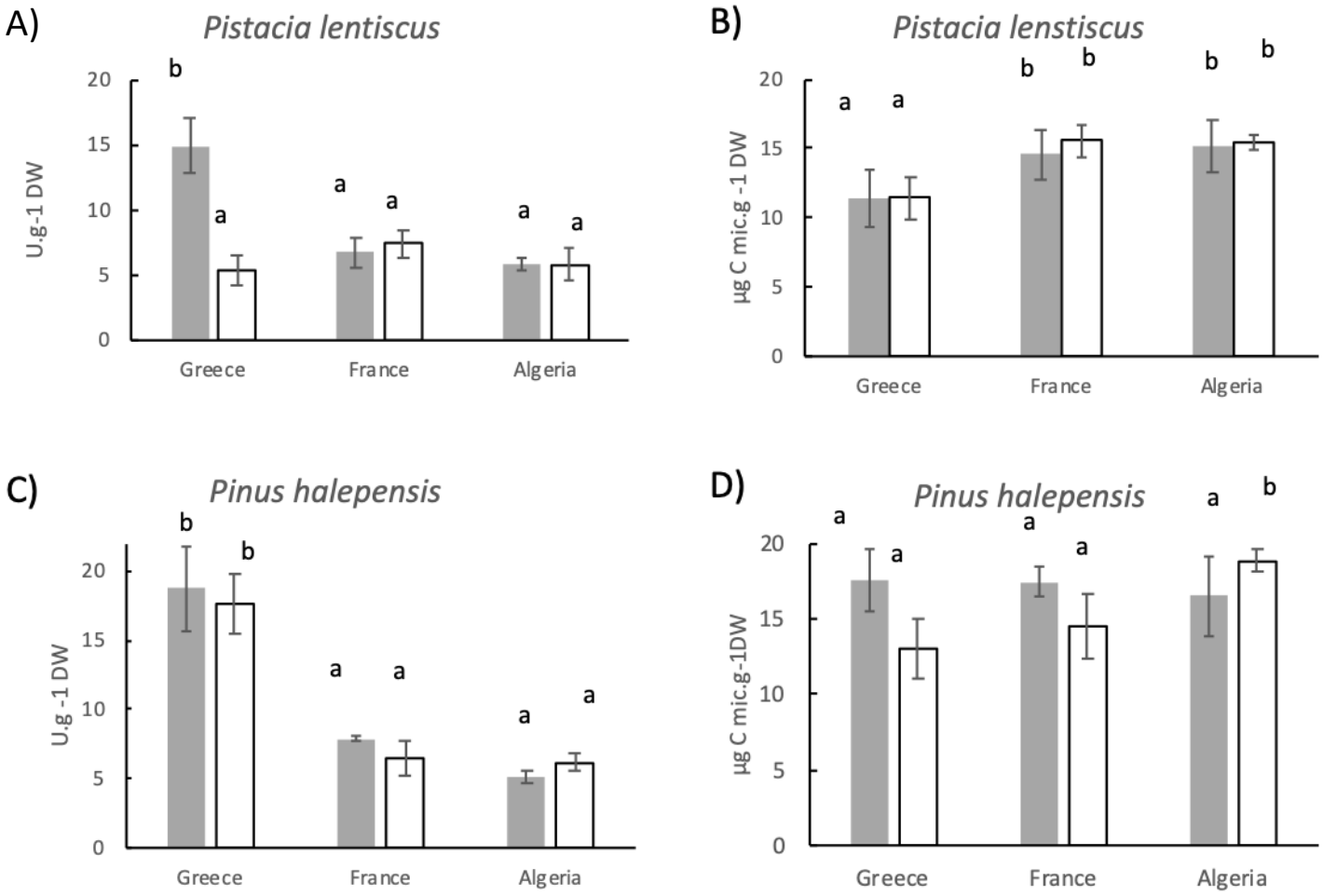


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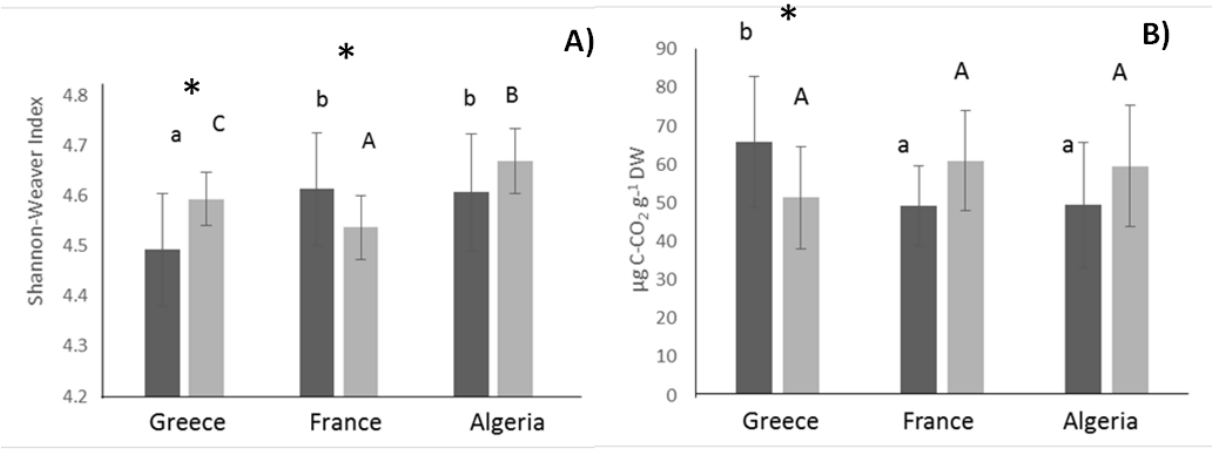
Figure 2


A)
Shannon-Weaver Index
4.8
4.7
4.6
4.5
4.4
4.3
4.2
*
a
C
*
b
A
b
B
Greece
France
Algeria
B)
µg C-CO2 g-1 DW
90
80
70
60
50
40
30
20
10
0
b
*
A
a
A
a
A
Greece
France
Algeria

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824 Figure 3 a and b

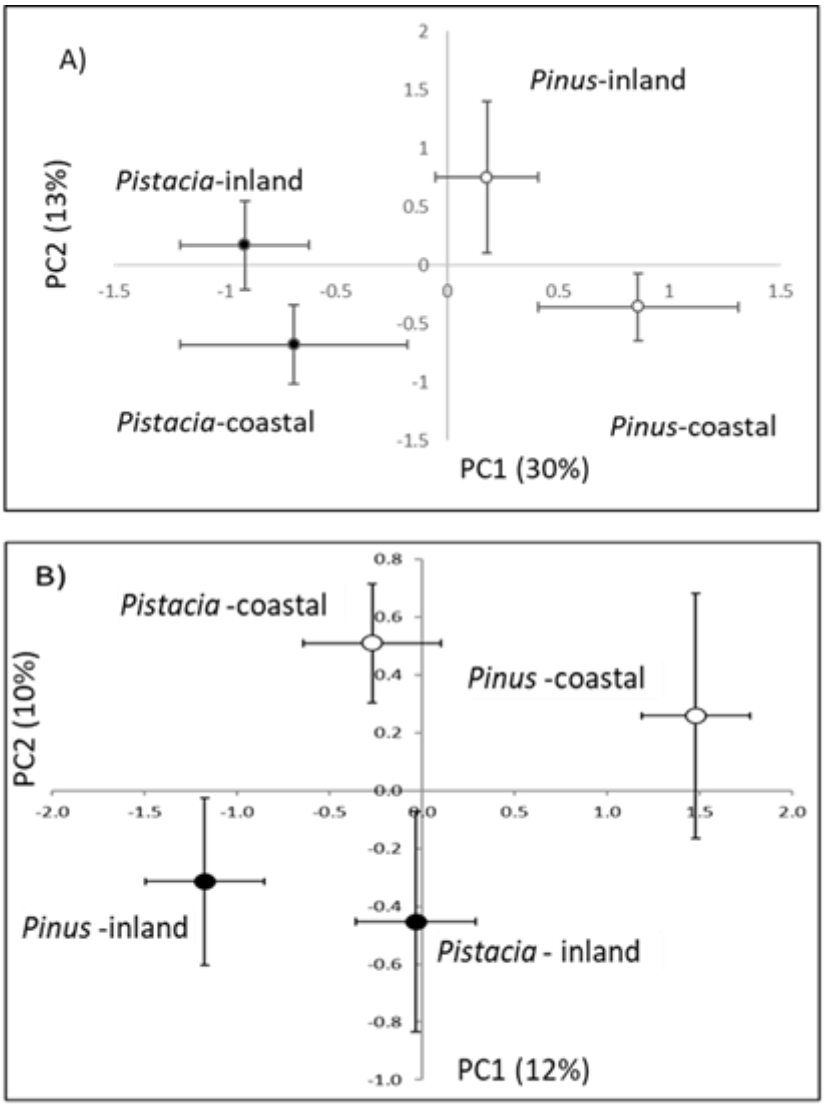


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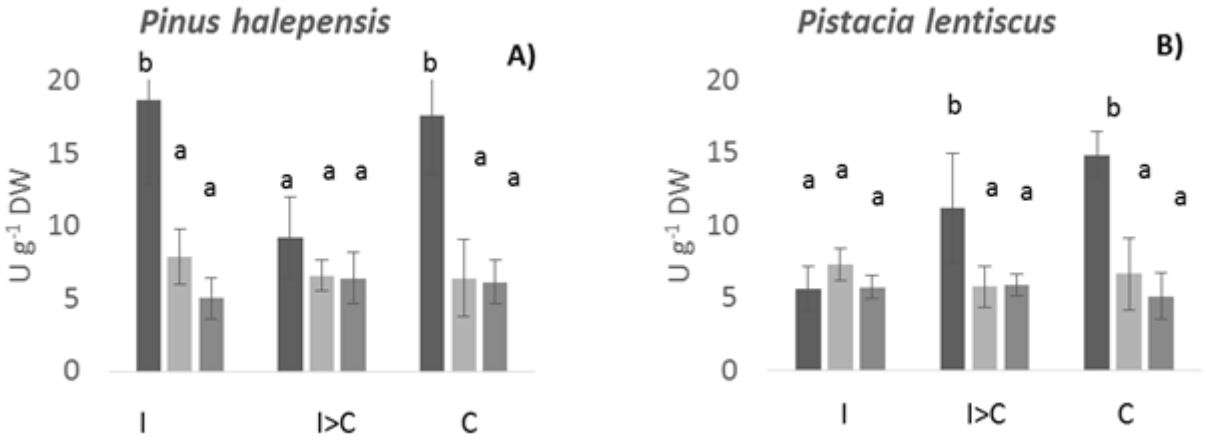
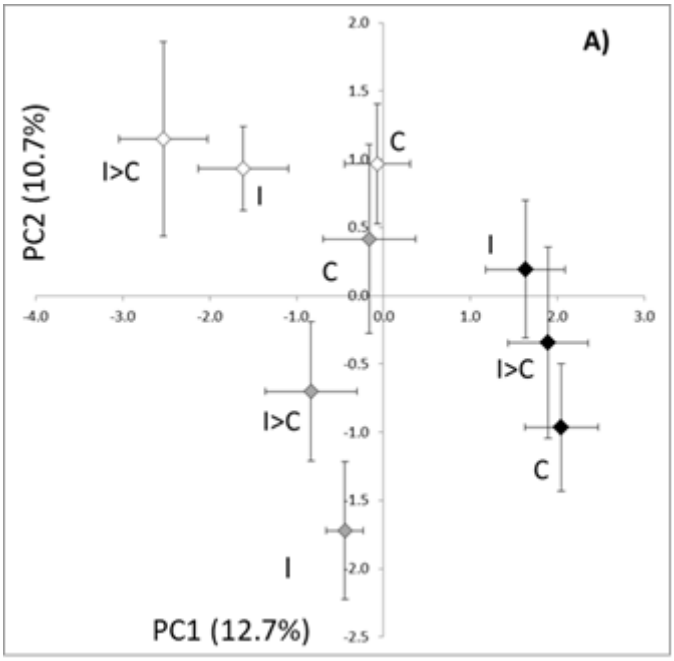
Figure 4 a and b



845

846 Figure 5 a and b



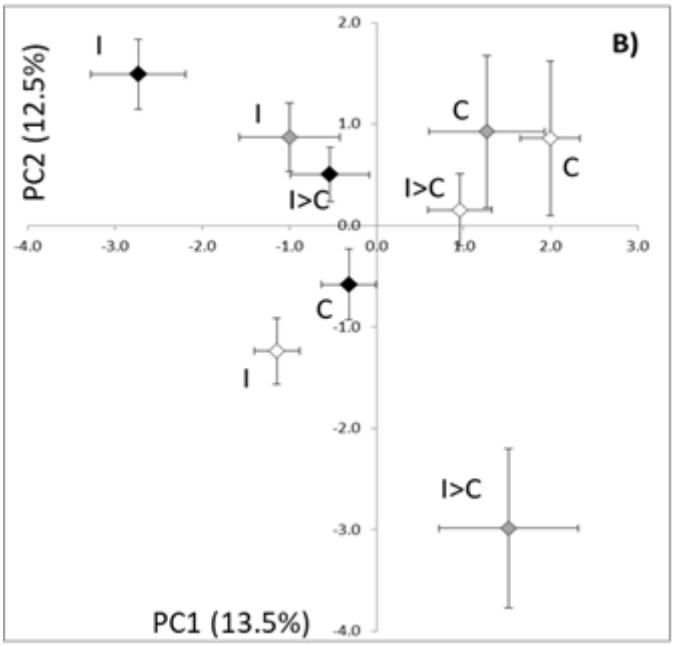




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Figure 6 a and b