Coastal environments shape chemical and microbial properties of forest litters in circum Mediterranean region

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2   **Mediterranean region**

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22

23   Soil microbial properties in the Mediterranean

24

25

26   **Abstract**

27   This study explores how chemical and microbial properties of litters can be affected by

28   coastal environments across the Mediterranean basin. A litterbag experiment including *Pinus*

29   *halepensis* Mill. and *Pistacia lentiscus* L., collected from both inland and coastal areas was set

30   up in France, Greece and Algeria. Control litterbags were left in their sampling sites and a

31   transfer of litterbags from inland to coastal area was performed to test whether the effect of

32   the specific constraints of coastal environments varies according to the country and the litter

33   type. After 10 months, litter chemical composition (CP/MAS ^{13}C-NMR) and microbial

34   activities (cellulase, respiration, Biolog) and structure (TRFLP) were analysed. Coastal

35   conditions led to various responses: i) litter aromaticity differed in the coastal zones

36   depending on the country (high in Greek coastal area, low in Algerian coastal zone), ii) less

37   functionally-diversified microbial communities were found in Greek coastal area compared to

38   French and Algerian coasts, iii) genetic diversity and richness were strongly impacted after

39   transfer to the coastal zone whatever the country. The type of litter shaped microbial

40   communities: i) at a local scale i.e. in either coastal or inland area, catabolic profiles and

41   cellulase activities varied with the plant species, ii) at a regional scale, the effect of coastal

42   conditions differed with the plant species (basal respiration, Shannon-Weaver index, catabolic

43   diversity H', cellulases and catabolic profiles). Thus, litter microbial properties differed in

44   coastal environments across the Mediterranean basin and plant litter type plays a major role in

45   microbial properties at large spatial scale.

46

47   **Key words**: extracellular enzyme activities, litter microbial communities, Mediterranean area,

48   water potential stress.

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50

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

76 investigating how microbial functioning in litters from two Mediterranean plant species,

77 commonly found in both Mediterranean coastal and inland areas and exhibiting different

78 functional traits (*Pinus halepensis,* a coniferous species, and *Pistacia lentiscus*, an evergreen

79 dicot species) may be driven by the specific conditions of coastal environments. Moreover,

80 we used the contrasted climate conditions across the Mediterranean basin, comparing France,

81 Greece and Algeria environments, to test the effect of a gradient of aridity on litter

82 functioning. Most of litter decomposition experiments used litter from plants exposed

83 artificially to global change factors such as warming, irrigation or elevated CO_2 (Suseela and

84 Tharayil, 2018) but very few studies used field-scale transplantation experiments to

85 investigate the *in situ* impacts of climate variations (Berger et al., 2015, Keiser and Bradford,

86 2017, Makkonen et al., 2012). Here, an original experimental design based on litterbag use

87 was performed: litterbags of either *P. halepensis* or *P. lentiscus* litters were exposed in inland

88 to coastal areas in the three selected countries to test the effect of a gradient of aridity on litter

89 functioning and, within each country, a pool of litter bags was transferred from inland to

90 coastal area to test the effect of coastal conditions on inland communities. Consequently, we

91 aimed at addressing the following questions: i) are microbial communities and their functions

92 shaped by coastal conditions and do more drastic climate conditions found across the

93 Mediterranean basin strengthen these stresses linked to the coastal conditions? ii) for each

94 country, what are the responses of microbial communities from inland areas when they are

95 subjected to coastal area conditions and their specific stresses and are their responses plant-

96 species dependent?

97 According to our hypotheses: i) in each country, coastal environmental conditions will play a

98 key role defining a particular microbial functioning in the coastal area and ii) more arid

99 climate conditions will act at a larger scale i.e. the Circum-Mediterranean region and will

100 smoothen the effect of coastal conditions and of litter type on microbial properties. Thus, *in*

101 *fine*, this study will provide useful information about environmental factors driving microbial

102 communities and their activities at different spatial scales, more precisely about the

103 vulnerability of coastal areas.

104

105 **Materials and Methods**

106 *Site description and litter sampling*

107 Sampling sites were selected in stands of *Pinus halepensis* (forest and matorral) in association

108 with *Pistacia lentiscus* throughout a latitudinal gradient across the Mediterranean basin (Table

109 1). The inland sites (Table 1) were located in the Massif de l'Etoile in the periurban area of

110 Marseille (France), in the forests of Argoli near Cleones (Peloponese, Greece) and in the

111 forests of Traras near Ain Kebira (Wilaya of Tlemcem, Algeria), respectively. The coastal

112 sites (Table 1) were located in the Parc National des Calanques of Marseille (France), near

113 Monemvassia (Greece) and near Ghazaouet (Algeria). In each country, three sampling sites

114 (400 m^2) were chosen in the coastal area (at less than 500 m from the coastal line) and three

115 others in the inland area (at 20 km from the sea). The distance between sites, whether coastal

116 or inland, is 2 km at least, far exceeding the spatial dependence of most microbiological

117 properties in soil (Nannipieri et al, 2003). The selected sites had similar pedoclimatic and

118 topographic features: 25–230 m in elevation, similar exposure (South), slope (10-15%) and

119 soil type (Calcaric Leptosol according to IUSS Working Group WRB, 2006). For each site,

120 the exact location (GPS), maximal and minimal temperatures and the relative humidity of air

121 measured *in situ* by EL-USB 2.0 probe (Conrad) over a two-year period and annual

122 precipitation, mean of annual temperature, precipitation of driest and wettest months

123 (worldclim, 1970-2010, Hijmans et al., 2005) were collected (Table 1). At each site and for

124 each plant species, in May 2014, twenty samples were randomly collected over a 400 m^2 area

125 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

151 products were carried by high purity oxygen (Linde Gas) allowing detection of CO_2 by non-

152 dispersive infrared (NDIR) and detection of NO by chemiluminescence (CLD).

153 After ten months in the field, TOC and TN of litters from litter bags were characterized as

154 described above. Moreover, litter chemical properties were also analysed by solid-state [13]C

155 NMR. Spectra were obtained on a Bruker Avance-400 MHz NMR spectrometer operating at a

156 ^{13}C resonance frequency of 100.7 MHz and using a commercial Bruker double-bearing probe.

157 About 400 mg of sample were placed in zirconium dioxide rotors of 4-mm outer diameter and

158 spun at the Magic Angle Spinning (MAS) rate of 10 kHz. The Cross Polarisation (CP)

159 technique was applied with a ramped 1H-pulse starting at 100 % power and decreasing to 50

160 % during contact time (2 ms) to avoid Hartmann-Hahn mismatches. The experiments were

161 performed at ambient temperature and 20K scans were accumulated using a delay of 2.5s, for

162 an experimental time of 2h. The ^{13}C chemical shifts were referenced to tetramethylsilane and

163 calibrated with glycine carbonyl signal, set at 176.5 ppm. The ^{13}C NMR spectra were divided

164 into 7 chemical shift regions according to Dignac et al. (2002): i.e. alkyl C (0-45 ppm),

165 methoxyl C (45-60 ppm), O-alkyl C (60-90 ppm), di-O-alkyl C (90-110 ppm), aromatic C

166 (110-140 ppm), phenolic C (140-160 ppm) and carboxyl C (160-190 ppm). Deconvolution of

167 each spectrum was performed on DmFit 2011 to determine the relative intensity of each

168 selected region (Massiot et al. 2002). The aromaticity ratio (aromatic C/O alkyl C + alkyl C +

169 aromatic C) was calculated according to Baldock et al. (1997).

170

171 *Microbial activities*

172 Cellulase activity was assayed using CarboxyMethylCellulose (CMC) 0.1% (w/v) in 8

173 mL of sodium acetate buffer (50 mM, pH 5) added to 1 g of litter (dry weight) incubated for

174 4h at 50°C. Glucose was quantified according to the Somogyi-Nelson method and absorption

175 was read at 870 nm (Farnet et al, 2010). Enzyme activities were expressed in µmoles of

176 reaction products released per minute (U) per gram of dry soil (U g^{-1} DS).

177   Basal respiration was measured to assess the ecophysiological state of litter microbial

178 communities. Three g DW equivalent of fresh litter were placed in 117 ml glass jars. The

179 glass jars were immediately sealed with hermetic rubber septa, and incubated for 4 hours at

180 25°C. After incubation, 1 ml of air was sampled in the head space with a syringe and injected

181 into a gas chromatograph (Chrompack CHROM 3 – CP 9001) to analyse CO_2 production. The

182 gas chromatograph was equipped with a thermal conductivity detector and a packed column

183 (Porapack). The carrier gas helium flow was regulated at 60 ml h^{-1}. Ambient CO_2

184 concentrations were subtracted from sampled CO_2 concentrations and resulting values were

185 adjusted at 22°C according to Ideal Gas Laws using a $Q_{10} = 2$. Substrate-induced respiration

186 (SIR) rates were estimated using a procedure from Anderson and Domsch (1978). Three

187 grams DW equivalent of fresh litter were placed in 117 ml glass jars and amended with

188 powdered glucose (1000 µg C g^{-1} soil) found to maximize the respiration rate in litter in a

189 preliminary assay (data not shown). One ml of air was sampled in the headspace with a

190 syringe and injected into a gas chromatograph to analyse CO_2 production for 1 hour (see

191 above). Substrate-induced respiration was converted into microbial biomass (MB) using the

192 relation established by Beare et al. (1990).

193   The catabolic diversity of cultivable microbial communities was determined with

194 Biolog EcoPlate™ (BIOLOG Inc., Hayward, CA) using a procedure adapted from Garland

195 and Mills (1991). Briefly, 4 g DW equivalent of sub-sample were added to 50 ml of sterile 0.1

196 % Na-pyrophosphate solution (pH 7). The mixture was then shaken with an orbital shaker for

197 20 min and centrifuged at 500 g for 10 min at 4 °C to obtain a microbial suspension. Exactly 1

198 ml of supernatant was diluted into 99 ml of sterile saline solution (0.85% NaCl), hand-mixed

199 for 30 sec. and left to stand for 10 min. We purposely did not adjust the inoculums in order to

200 obtain uniform cell density, instead considering the total microbial count as an inherent

201 characteristic of the microbial communities of each mesocosm. A 125 µl aliquot of the diluted

202 solution was added to each of 96 wells in a Biolog EcoPlate™. Plates were incubated at 25 °C

203 and colour formation in each well was monitored at 595 nm using a TECAN®

204 spectrophotometer. Measurements were performed three times a day until average well colour

205 development (AWCD) exceeded a value of 1.0 standardized absorbance units (i.e. 3–5 day

206 incubation). Microbial C-use intensity was assessed as the rate of average well colour

207 development (AWCD) calculated as follows: $AWCD = \Sigma\ OD_i/31$, where OD_i is the optical

208 density for each well in the mid-exponential growth phase (i.e. after 72h). Absorbance values

209 for the wells with C sources were blanked against the control well. The incubation time

210 resulting in an $AWCD = 0.5$ absorbance unit ($T_{0.5}$) was calculated for each sample and used to

211 standardise the optical density of each C-containing well (Garland and Mills, 1991).

212

213 *Microbial genetic structure*

214 DNA was extracted from 0.25g of litter, using NucleoSpin® Soil kit (Macherey-Nagel,

215 Düren, Germany) following the manufacturer instructions. The quality of DNA extractions

216 was checked by electrophoresis on 1% agarose gels stained with GelRed (Molecular Probes,

217 USA) using a Gel Doc image analyser (BioRad, USA). DNA concentration was quantified

218 using Quant-iT™ dsDNA High-Sensitivity Assay Kit (Invitrogen, Canada). Then, all DNA

219 extracts were then diluted to 0.5 ng / µL for subsequent analysis. Bacterial community

220 structures were analyzed using T-RFLP footprinting as described by Blaud et al. (2015).

221 Briefly, bacterial 16S rRNA gene was amplified using primers FAM labelled 63F and 1389R.

222 Biorad T100 thermal cycler was used for the amplification with the following programs:

223 initial denaturation at 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 30 s, 57 ºC for 45 s,

224 and 72 ºC for 90 s, followed by a final extension time at 72 ºC for 10 min. Bacterial PCR

225 products (10 µl) were then digested with 10 U of the restriction enzyme *AluI* and 1×

226 restriction enzyme buffer (Thermo Fisher) in a total volume of 15 µl at 37 ºC for 3 h. After a

227 desalting step, 2µl of PCR products were mixed with formamide containing 0.5% of LIZ500

228 (T-RFLP) internal size standard (Applied Biosystems,) in a total volume of 12 µl and

229 denatured at 94 ºC for 3 min. Samples were electrophoresed in an ABI 3730 PRISM®

230 capillary DNA sequencer (Applied Biosystems). The T-RFLP profiles obtained with the

231 sequencer were analysed using GeneMapper® v3.7 software (Applied Biosystems). The

232 fragments between 50 and 500 bp and peaks height ≥ 50 fluorescence units were included in

233 T-RFLP analysis. Fragments having a relative abundance of proportion < 0.5% were removed

234 from the matrices.

235

236 *Statistical analyses*

237 The catabolic (BIOLOG) and genetic (TRFLP) diversities was calculated using Shannon's

238 index (H'):

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

239

240 where p_i is either the ratio of colour development of the i [th] well to the sum of colour

241 development of all positive wells or the relative abundance of a given restriction fragment for

242 BIOLOG or TRFLP techniques.

243 The normality and homogeneity of the variances of the data were determined on the residuals

244 from the regression model with the Shapiro-Wilk and Levene tests, respectively. Data were

245 transformed to common logarithms, $\log_{10}$, when necessary to meet the requirements of

246 normality and homogeneity of variance for ANOVA. For control litterbags, three-way

247 ANOVA were used to determine whether, and to what extent, microbial properties (i.e. basal

248 respiration, active microbial biomass via SIR, catabolic diversity H', cellulase activities and

249 bacteria richness and equitability via genetic analysis) and chemical characteristics (using

250 NMR data and C/N ratio) depended upon i) litter species (*Pinus halepensis vs Pistacia*

251 *lentiscus*) ii) the area conditions (coastland *vs* inland), iii) the geographical location (France,

252 Greece and Algeria) and iv) their interactions. When a 2-way interaction was found between

253 geographical location and context (coastal and inland) or plant litter species, we separated

254 data for each modality of geographical location, context, transfer or plant litter species using a

255 one-way ANOVA. Statistically significant ($P<0.05$) main effects and interactions were

256 analysed further using the Tuckey HSD *post hoc* tests. Principal component analysis (PCA)

257 was performed on covariance matrices from Biolog® to explore the effects of geographical

258 location, condition and litter type on the catabolic structure of microbial communities and

259 tested by PERMANOVA. PCA provided an ordination of the microbial C-substrate utilization

260 profiles, which were plotted in one and two dimensions respectively, based on the scores of

261 the first two principal components (PC). All statistical analyses when performed using R

262 software (3.2.1, R Development Core team, 2015), levels of significance are indicated as $p \leq$

263 0.05.

264

265 **Results**

266

267 *Influence of the coastal environmental conditions on litter properties along a gradient of*

268 *aridity in the Mediterranean basin*

269 Here, we explored whether environmental constraints, which can be enhanced in coastal

270 environments (i.e. differences in salinity exposure, humidity, temperature, etc.), affected litter

271 microbial functioning. Thus, first, it was important to demonstrate how these environmental

272 characteristics actually varied between inland and coastal zones in the three countries.

273 These environmental conditions were indeed highly discriminated with respect to certain

274 parameters and depending on the geographical location. Temperature and precipitation

275 variations were strongly different between both coastal and inland areas. In France and

276 Greece, maximal temperature and annual mean temperature were higher in the area close to

277 the coastline, while precipitations of the driest and wettest months were lower. An opposite

278 trend was observed in Algeria, with more intense arid conditions in the inland site. These

279 contrasted climate conditions are particularly well described using annual precipitation as an

280 index of aridity (Stadler, 2005). The climate gradient between the Northern and the Southern

281 sides of the Mediterranean basin is clearly observed using this parameter and the differences

282 in precipitations between coastal and inland areas are particularly strong in Greece and

283 Algeria with opposite trends as described above. Moreover, values of both conductivity and

284 chloride ion concentrations linked to salinity exposure via sea sprays, were higher in litters

285 situated at the coastal areas in the three countries, showing that salinity stress was stronger in

286 the areas close to the coastline. We also determined the litter chemical properties at t = 0 from

287 the twelve composite samples of litters (2 species x 2 context (coastal vs inland) x 3

288 countries) used for litter bag preparation and results are shown in Table 2. Several global

289 trends were observed. Whatever the country or the distance from the sea, pH was always

290 lower in *Pinus halepensis* litter (from 4.7 to 6.24) than in *Pistacia lentiscus* litter (from 6.22 to

291 6.55). Moreover, in *P. halepensis* litter, total Organic Carbon amounts were always higher

292 (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

293 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

294 proportion of di-O-alkyl C + O-alkyl C was higher in *P. halepensis* litter and this was also

295 observed for aromatic + phenolic % in most cases. Thus, litter physico-chemical

296 characteristics depended on the type of species and not on the geographical location (coastal

297 vs inland area, Northern vs Southern side of the Mediterranean).

Three way-ANOVA revealed significant interactions between the coastal conditions and the country for most of the microbial markers considered: cellulase activities, catabolic diversity, basal respiration, and microbial biomass (Table 3).

Cellulases varied with the three factors tested (Figure 1 a and c). However, these activities followed a global trend whatever the species and the area (coastal or inland): Greece > France = Algeria.

For microbial biomass (MB), the effects of coastal conditions varied with the plant species and the country (Figure 1b and d): no differences of MB between coastal and inland areas were observed in *P. lentiscus* litters whatever the country (Fig 1b), while in *P. halepensis* litters, higher MB was found in Algeria inland area only (coastal x country interaction, F=8.88, p$\leq$ 0.001, Fig 1d).

Our results also highlighted variations in catabolic diversity H' (ECO) and basal respiration depending on coastal x country interaction (Figure 2a and b). In coastal area, H' (ECO) was lower in Greece where the more drastic climate conditions were found compared to the two other countries and the opposite trend was observed for BR. Thus, microbial communities with less-diversified catabolic potential led to a higher respiration in the Greek coast. In inland area, conversely, H'(ECO) increased with the gradient of aridity (Algeria>Greece>France), while BR was similar whatever the country (Figure 2a).

Thus, for RB, MB and H'(ECO), the coastal conditions modified such important microbial markers depending on the plant species and the country across the Mediterranean basin.

These results have to be considered regarding the chemical properties of litters characterised by solid-state NMR of ^{13}C. Aromatic compounds were negatively correlated with MB (r^2=-0.26, p$\leq$0.05). These results are corroborated by the three-way ANOVA (Table 3): the amount of aromatic compounds was lower in litter where MB was high (Algerian coast) and conversely, aromatics were more abundant in litter where MB was low (Greek coast) (coastal

323 x country interaction F=1.98, p≤0.01). O-Alkyl C signal (assigned to polysaccharides) also

324 varied depending on the coastal x country interaction (F=8.08, p≤0.001) and higher O-Alkyl

325 C signal in *P. halepensis* litter was observed together with high MB.

326 The effects of the country, the area (coastal or inland) and the plant species were also tested

327 on the catabolic structures of litter microbial communities. PERMANOVA showed significant

328 differences of the catabolic structure of litters between the three countries (pseudo-F=5.23,

329 p≤0.001) and between areas (pseudo-F=4.40, p≤0.001). Moreover, coastal conditions shaped

330 microbial catabolic profiles depending on the country (pseudo-F=3.43, p≤0.01). For microbial

331 genetic structure, the first two principal components (PCs) account for 33 % of the variation

332 in the data; PC1 accounts for 20 % and PC2 for 13 %. PCA revealed significant differences

333 between the three countries (pseudo-F=3.39, p≤0.001) and between areas (pseudo-F=9.32,

334 p≤0.001), showing that geographical conditions shaped microbial genetic structure and that it

335 differed depending on the coastal and inland areas. Figure 3a and b shows the results of the

336 PCA for the four combinations of area (inland or coastal) and litter (based on either genetic or

337 catabolic profiles) plotted on the first two principal components. For catabolic diversity, the

338 first two principal components (PCs) account for 22 % of the variation in the data; PC1

339 accounts for 12 % and PC2 for 10 %. For the two plant species considered, catabolic and

340 bacterial genetic structures changed depending on coastal and inland areas (Figure 3 a and b)

341 indicating that the effect of coastal context was stronger than the effect of species (which

342 would have homogenized catabolic profiles for one species). On the other hand, in either

343 inland or coastal areas, both catabolic and genetic structures were different depending on the

344 species showing that this is an important environmental driver at a local scale.

345 It is noteworthy that no significant effect of the factors tested was found on bacterial genetic

346 richness or diversity assessed via H' index (data not shown).

347

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

349 In order to understand how environmental conditions of coastal environments may structure

350 microbial functioning, litters were transferred from inland to coastal area to test our initial

351 hypothesis: environmental conditions acting at a regional scale (coastal *vs* inland) are

352 preponderant over those acting at a local scale (litter chemical signature specific to a plant

353 species).

354 First, chemical markers from NMR data showed that, for both litters, the Aromaticity ratio

355 was different between transferred litters and litters from the coastal area (Table 4). Thus, the

356 'chemical environment' of microbial communities was not similar in transferred and coastal

357 litters for both species and whatever the geographical location.

358 The effects of litter transfer (mainly negative) to the coastal zone observed on different

359 microbial markers (Table 4) varied with the country and the species. For cellulases, variations

360 after transfer to the coastal zone, were only found in Greece and differed with the species: i)

361 in *P. halepensis* litters, cellulases decreased significantly (to reach a lower level compared to

362 both coastal and inland activities), ii) in *P. lentiscus* litters, cellulases increased compared to

363 the original area i.e. inland area (Figure 4 a and b). Thus, in Greece, exposure of litter

364 microbial communities to environmental conditions specific to the coastal area (higher

365 temperatures and more intense drought than in the two other countries as shown in Table 1)

366 led to a modification in activities linked to the transformation of the labile fraction of organic

367 matter and the results observed after this exposure to stress differed with the plant species.

368 Our results also revealed the strong negative effects of the coastal environmental conditions

369 on MB and H'(ECO) depending on the geographical location (Table 4). This is in Algeria

370 where litter transfer to coastal area strongly affected both microbial markers (data not shown),

371 while no variations were observed in France and Greece after transfer for these markers.

372   PCA from Biolog data revealed a change in catabolic profiles due to litter transfer to the

373   coastal zone (Figure 5a and b). PC1 and PC2 explained 12.7% and 10.7% of variance for *P.*

374   *lentiscus* litter (Figure 5a) and 13.5% and 12.5% for *P. halepensis* litter (Figure 5b).

375   PERMANOVA indicated effects of transfer to the coastal area on catabolic profiles in *Pinus*

376   (pseudo-F=2.64, p$\leq$0.001) and *P. lentiscus* (pseudo-F=2.22, p$\leq$0.001) litters. These effects

377   were significant for the three countries in the case of *P. lentiscus* litters and in Algeria only

378   for *P. halepensis* litter. Interestingly, microbial catabolic structure after litter transfer in

379   Algeria was similar to that from the coastal area for both *P. lentiscus and P. halepensis* litters

380   (Figure 5a and b).

381   PCA based on T-RFLP data also revealed a change in genetic profiles due to litter transfer to

382   the coastal zone (Figure 6a and b). PC1 and PC2 explained 9.1% and 4.9% of variance for *P.*

383   *lentiscus* litter (Figure 6a) and 11.2% and 5.1% for *P. halepensis* litter (Figure 6b).

384   PERMANOVA indicated significant effects of transfer to the coastal area on microbial

385   communities in *P. halepensis* (pseudo-F=5.64, p$\leq$0.01) and *P. lentiscus* (pseudo-F=7.41,

386   p$\leq$0.01) for the three countries.

387

388   **Discussion**

389   *Variations in litter microbial functioning across an aridity gradient in the Mediterranean*

390   *basin depends on the distance from the sea*

391   Our study revealed that the effects of the coastal environment differed depending on the

392   geographical location: more precisely, in Greece, coastal conditions led to a specific

393   functioning with higher cellulase and basal respiration and a lower catabolic index of

394   diversity. On the other hand, in coastal environments in France and Algeria, more

395   catabolically-diversified communities were found (higher H' (ECO) index). Moreover, all

396   these differences in microbial functioning were accompanied by different genetic structures in

16

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

17

422 utilization. These findings are of importance since modification in catabolic potential of

423 microbial communities may induce a shift in decomposition process since transformation of

424 certain compounds such as lignin is sensitive to the structure of microbial communities (Mc

425 Guire and Treseder, 2010).

426

427 *Modification of litter microbial functioning when microbial communities from inland area are*

428 *subjected to coastal environmental conditions*

429 After transfer of litters from inland to coastal area, certain microbial markers, such as

430 cellulase activities, were strongly impacted and the intensity of the effect varied with the plant

431 species. Our initial hypothesis was based on the fact that coastal conditions may have

432 smoothen the effect of litter type leading to homogeneous microbial responses to changes in

433 environmental climate conditions whatever the plant species. Thus, our study underlined the

434 importance of species traits in microbial responses to stress. The quantity and quality of

435 organic matter shape the structure and composition of microbial communities and their

436 resistance to stresses (Bradford et al., 2002). Mediterranean litters are described as

437 particularly rich in secondary metabolites (terpenes, tannins, VOC …) and lignins (Di Castri,

438 1973, Rousk et al., 2010, Terradas and Savé, 1992). These compounds are known to modify

439 microbial communities and their activities: they can be toxic for microbial cells (Popova et

440 al., 2009, Adamczyk et al., 2015), interact with enzymes, leading to adverse effects on their

441 catalytic potential (inhibition/precipitation of enzymes). Some tannins can modify the quantity

442 and the nature of N sources available for microorganisms because of adsorption or enzymatic

443 inhibition (Hättenshwiler and Vitousek, 2000). Thus, the importance of litter chemical

444 signature on microbial community structure and functioning has to be taken into account

445 when studying soil microbial vulnerability under environmental constraints. More

446 particularly, the effect of litter admixture on microbial responses to coastal condition exposure

447   should be valuable to determine since the diversification of nutrient resources is known to

448   enhance microbial diversity and consequently may favour resistance to water potential stress.

449   These subtle variations in organic matter can indeed lead to additive or non-additive

450   (synergistic or antagonistic) effects (Brunel et al., 2017) and various plant species exhibiting

451   different functional traits should be included in such research (Prescott and Grayston, 2013).

452   Previous studies have indeed shown that changes in plant species can actually modify both the

453   biomass and structure of soil microbial communities (Laganière et al., 2009; Matos et al.,

454   2010) and thus their responses to stress.

455   Coastal environmental conditions appeared to have induced a shift in microbial catabolic

456   structure of both litters, mainly in Algeria. Interestingly, in *P. halepensis* litter, the catabolic

457   profile resulting from transfer in the Greek coastal area differed from catabolic profiles of

458   both inland and coastal areas (control and transfer area respectively). These results may

459   partially explain the decrease in cellulase activities after transfer: the shift in catabolic

460   diversity of microbial communities after transfer may have not sustained such activities. On

461   the other hand, in *P. lentiscus* litter, the potential of cellulose transformation was identical in

462   the transferred litter and in litter from the coastal area. Some studies have attempted to

463   decipher the respective influence of either climate conditions or chemical signature of organic

464   matter, using very contrasted bioclimates. Gholz et al (2000) and Perez et al. (2013) indicated

465   that the effect of 'home field advantage' is particularly pregnant for microbial communities

466   from litters with a high amount of cellulose and hemicellulose. The study of Makkonen et al.

467   (2012) revealed utterly different results: transfers of 16 types of litters from four different

468   biomes (subarctic, temperate, Mediterranean and tropical climate) led to a homogeneous

469   dynamics of litter transformation. Moreover, the authors found that the complexity of

470   decomposer communities including micro-, meso and macro fauna (using various mesh size

471   of litterbags), does not accelerate decomposition rate and this is particularly true for the

472 Mediterranean biome. This means that decomposers from a wide range of bioclimates are not

473 specialized in the transformation of 'autochthonous' organic matter. Opposite results were

474 found in the study of Milcu and Manning (2011), which showed that a range of soil fauna size

475 classes contributes to 'home field advantage' effect and that experimental bias (e.g. fine mesh

476 sizes) impacts the contribution of soil fauna. It has to be underlined that our study also

477 involved micro and mesofauna since 2 mm mesh-size litterbags were used. We found that the

478 concept of 'home field advantage' applied to cellulose transformation depended on the litter

479 type. For *P. halepensis*, this potential clearly depended on the coastal conditions since, when

480 microbial communities from inland areas were transferred to the coastal zones, their potential

481 to hydrolyze cellulose strongly decreased.

482 Transfer experiment of litters also showed that major microbial markers such as MB and the

483 catabolic index of diversity, H' (ECO), were differently affected depending on the

484 geographical location: after transfer, H' (ECO) decreased in Algeria and not in France or

485 Greece, and MB decreased in Algeria. Thus, this revealed that the contrasted climate

486 conditions across the Mediterranean Basin specialized microbial responses when facing a

487 change in environmental conditions (transfer from inland to coastal area). Piélou Index of

488 diversity based on T-RFLP also underlined that litter transfer led to a decrease in genetic

489 diversity (whatever the geographical location or the litter type). This study indicates that

490 coastal conditions have probably shaped a particular microbial functioning and that

491 microorganisms from other environments have to adapt to these new specific conditions.

492 However, more than the chronicity of the stress linked to sea-spray exposure, the effect of

493 pulses of salinity increase (due to seasonal variations i.e. wind intensity, precipitation…) is

494 probably a more challenging factor for microorganism resistance (Schimel et al., 2007). Our

495 previous study (Qasemian et al., 2014) indeed revealed variations in conductivity in coastal

496 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

522 (Decaëns, 2010). Moreover, the coastal conditions affected major microbial properties across

523 a gradient of aridity in the Mediterranean basin depending on plant litter species, indicating

524 the huge importance of litter chemical signature even at wide spatial scale. Since both

525 microbial diversity and enzyme activities are of major importance in sustaining organic matter

526 turn-over, our results contributed to understand better the relative importance of external

527 filters to shed light on litter microbial functioning in changing environments.

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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

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

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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 * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

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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

transferred litters), the geographical location and their interactions on the microbial and chemical markers from control and transfer litter bags.

BR: Basal Respiration, MB, Microbial biomass, H' (ECO): Shannon-Weaver index from catabolic profiles, R (T-RFLP), richness from T-RFLP

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≤0.05).

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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≤0.05). Asterisks indicate differences (*t*-test) between inland and coastal area.

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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.

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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≤0.05)

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

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

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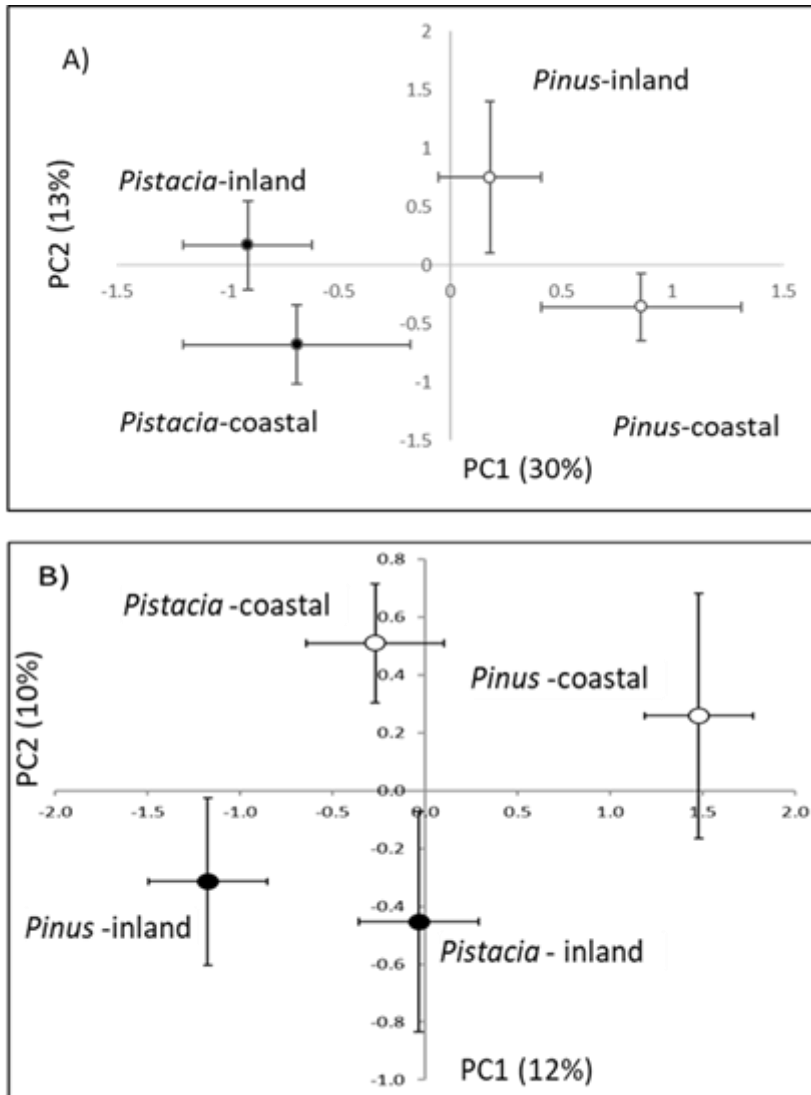
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824   Figure 3 a and b

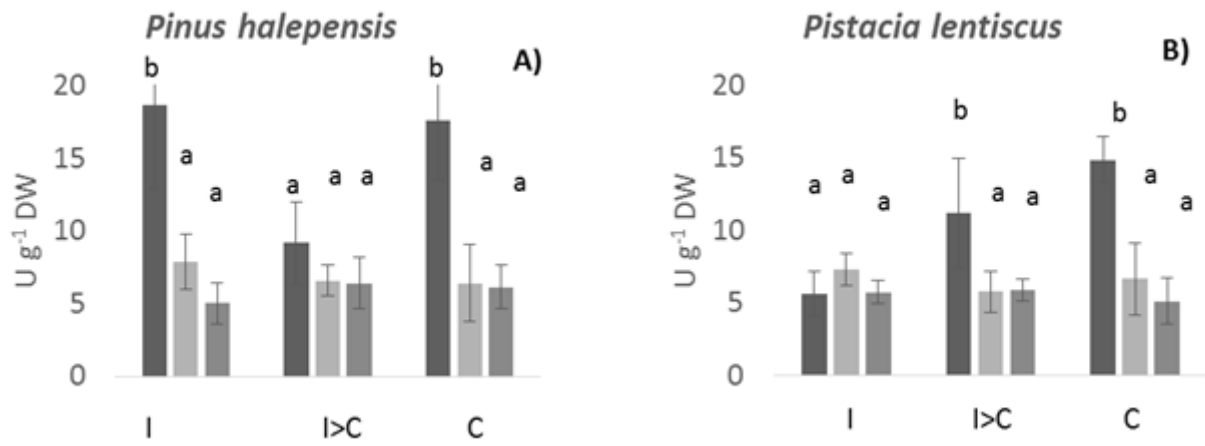825

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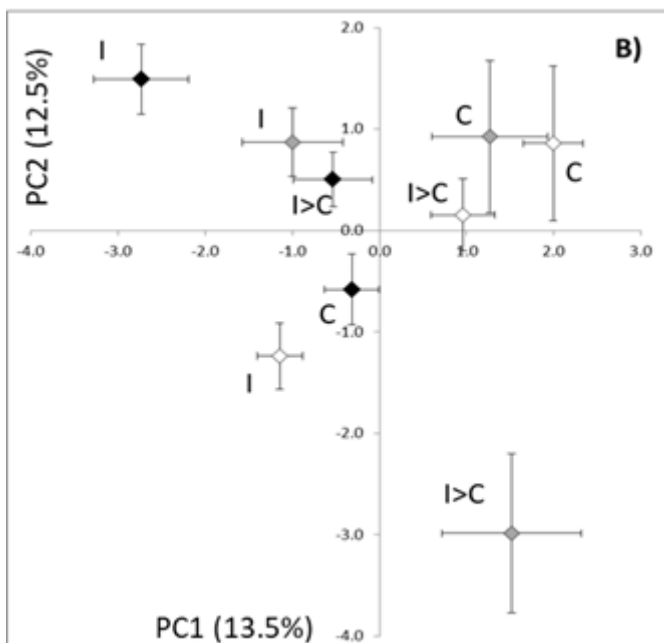
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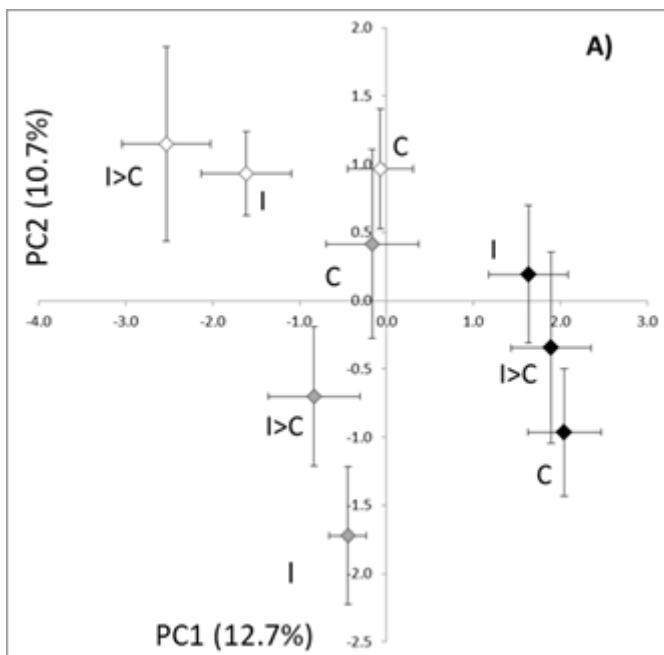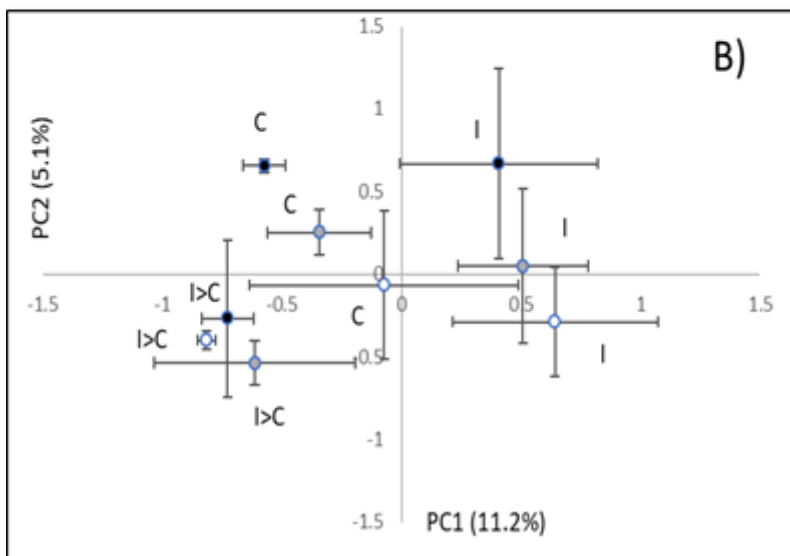
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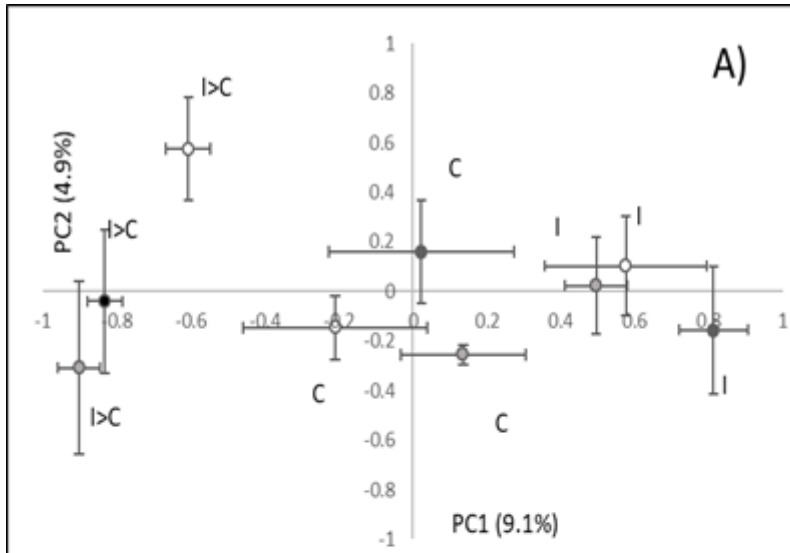
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