1 # Assessment of the diversity and abundance of the total and active fungal population and
2 its correlation with humification during two-phase olive mill waste (‘‘alperujo”)
3 composting

4

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21 **Abstract:**

22 Metagenomic and transcriptomic techniques applied to composting could
23 increase our understanding of the overall microbial ecology and could help us to
24 optimize operational conditions which are directly related with economic interest. In
25 this study, the fungal diversity and abundance of two-phase olive mill waste
26 (“alperujo”) composting was studied using Illumina MiSeq sequencing and quantitative
27 PCR, respectively. The results showed an increase of the fungal diversity during the
28 process, being Ascomycota the predominant phyla. *Penicillium* was the main genera
29 identified at mesophilic and maturation phases, and *Debaryomyces* and *Sarocladium* at
30 thermophilic phase, respectively. The fungal abundance was increased during
31 composting, which confirm their important role during thermophilic and maturation
32 phases. Some Basidiomycota showed an increased during the process, which showed a
33 positive correlation with the humification parameters. According to that, the genus
34 *Cystofilobasidium* could be used as a potential fungal biomarker to assess AL compost
35 maturation.

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40 **Keywords:**

41 Fungal community; Illumina MiSeq; Two-phase olive mill waste, qPCR,

1. Introduction

The agrofood and food processing industries generate an important amount of wastewater, by-products, sewage sludge and organic wastes loads, which can be reused as organic amendments after biological treatments (Morales et al., 2016). One example is the olive oil industry, which yearly generates millions of tons of “alperujo or alpeorujo” (AL) in the Mediterranean basin, an acidic and highly contaminant sludge, rich in phenolic compounds (Alburquerque et al., 2004). Composting has been shown as an efficient and low-cost technology for AL treatment and the composts produced are often used as organic amendments and fertilisers in agricultural soils (Muktadirul Bari Chowdhury, et al., 2013; Tortosa et al., 2012;Tortosa et al., 2014).

Composting is the biological process in which, the organic matter is aerobically degraded by the metabolic activity of the microbial population dwelling in the raw materials (de Bertoldi et al., 1983; Ryckeboer et al., 2003). In this controlled process, an extensive microbial succession occurs, mainly bacteria and fungi (López-González et al., 2015a, b). It is commonly accepted that bacterial population activity is more intensive during mesophilic and thermophilic phases compared to activities from fungi or actinomycetes, which are more active during maturation. This is due to the fast metabolism of bacteria, being more competitive than fungal populations, and their higher surface/volume ratio, diversity and generation times, which allow them to better adapt to the rapid substrate changes and conditions occurring during composting (Ryckeboer et al., 2003). It is well known that fungal populations play a pivotal role in organic matter degradation and carbon cycling, especially during maturation phase, when the humification occurs (de Bertoldi et al., 1983). Different studies have also

65 shown the capability of fungi to remove the phenols presents in AL and humify this by-
66 product, thanks to the presence of an enzymatic toolbox to remove lignocellulosic
67 substrates (Reina et al., 2017; Sampedro et al., 2004).

68 Recently, high-throughput sequencing technologies like Illumina MiSeq/HiSeq
69 or quantitative polymerase chain reaction (qPCR) have been applied to composting,
70 which are increasing our understanding of the overall microbial ecology of that process
71 (Antunes et al., 2016). Bacteria and fungi are the two most abundant groups of compost
72 microorganisms. However, the vast majority of the available studies in composting are
73 focused on bacteria, since fungal databases display limitations related to the number of
74 sequenced, well annotated fungal genomes and the absence of a universally accepted
75 DNA barcode (Gopal et al., 2017). Nevertheless, new data about fungal diversity and
76 abundance of different composting configurations, materials or bulking agents
77 (lignocellulosic, food and garden wastes, press mud, cattle manure with construction
78 and demolition wastes, cow manure, pumice or sewage sludge) have been provided
79 which could help to optimize operational conditions (de Gannes et al., 2013; Galitskaya
80 et al., 2017; Holman et al., 2016; Langarica-Fuentes et al. 2014; Neher et al., 2013;
81 Oliveira et al., 2016; Tian et al., 2017; Wang et al., 2018 a,b; Yu et al., 2015).

82 Only one report using high-throughput sequencing applied to olive mill waste
83 composting has been published. In Tortosa et al. (2017), we evaluated how bacterial
84 population was affected by the process and we found several genera that could be use as
85 potential biomarkers for maturation. On the other hand, no studies have been currently
86 published about fungal abundance using qPCR from the genomic DNA and the
87 retrotranscripted cDNA. Taking all these facts into account, the aims of this work were

88 to study i) how the diversity and the abundance of the total and metabolically active
89 fungal population evolved during AL composting using Illumina MiSeq sequencing and
90 qPCR technologies, and ii) their correlations among the physicochemical characteristics
91 of the process, with emphasis on finding fungal biomarkers of humification of the AL
92 compost.
93
94 **2. Materials and methods**

95 **2.1. Composting performance and compost sampling**

96 The AL composting procedure, physicochemical and bacterial evolution and
97 composts characterization used in this study were previously described by Tortosa et al,.
98 (2012 and 2017). Briefly, the AL was mixed with sheep manure in equal proportions of
99 fresh weight (1:1) and then, two trapezoidal piles of 10 t each were done as biological
100 replicates (M1 and M2). These piles were managed under the open system of windrow
101 piles using a backhoe loader. A total of seven mechanical turnings were made according
102 to the temperature and the biooxidative phase evolution, respectively. The moisture was
103 kept above 40 %, using an aspersion irrigation system and the composting process
104 lasted 22 weeks.

105 Mesophilic (1st week), thermophilic (7th week) and maturation phases (22nd
106 week) were sampled. For each phase, a composite sample of 1 kg were made by mixing
107 25-30 subsamples, which were randomly taken from several localizations of M1 and
108 M2 piles (different heights, lengths and depths, respectively). Composite samples were
109 transported into the lab within 4 hours after sampling using a portable fridge and were
110 kept at -80 ºC until genomic analysis. Three replicates per composting phase were

111 performed and individually extracted and analyzed. The nomenclature of these samples
112 was: Meso1-3 (Meso1, Meso2 and Meso3); Thermo1-3 (Thermo1, Thermo2 and
113 Thermo3); Matu1-3 (Matu1, Matu2 and Matu3) for mesophilic, thermophilic and
114 maturation phases, respectively.

115

116 **2.2. Genomic DNA, RNA extraction and cDNA retrotranscription**

117 Total genomic DNA extraction was carried out using a protocol previously used
118 (Tortosa et al., 2017). Briefly, frozen compost (-80 ºC) samples (± 5 g) were added into
119 a sterile porcelain mortar, homogenized by adding liquid nitrogen and ground using a
120 sterile pestle as it was recommended by Neher et al., (2013). After that, 250 mg of
121 compost samples were weighed and extracted using the commercial PowerSoil® DNA
122 isolation kit (MO BIO, Catalog No. 12888-100) after mechanical shaking using a
123 Mikro-Dismembrator S (Sartorius Stedim Biotech) for 30 s at 1600 rpm. Genomic
124 DNAs were resuspended in molecular biology grade water, electrophoresed in 1%
125 agarose gel (40 min at 80 mV), and visualized using GelRed® Nucleic Acid Gel Stain
126 (Biotium, Catalog No. #41003-1). Finally, genomic DNAs were quantified using a
127 Qubit™ 4 Fluorometer (Invitrogen, Catalog No. Q33226) and were kept at -20 ºC until
128 genomic analysis.

129 RNA extraction was performed using frozen compost samples (- 80ºC) and
130 homogenized and extracted as described above. The compost weights were previously
131 optimized according to their humic acids concentration: 1.5, 0.5 and 0.2 g (fresh weight)
132 for mesophilic, thermophilic and maturation samples, respectively. Commercial RNA
133 PowerSoil® Total RNA Isolation kit (MO BIO, Catalog No. 12866-25) was used

134 according to manufacter's instructions. The RNAs obtained were quantified using a
135 Nanodrop 1000 Spectrophotometer (Thermo Scientific) at 260 nm and were treated with
136 DNase I (RNase free) (Abion, Life technologies, Catalog No. AM2222) to remove co-
137 extracted DNA. The treatment was done according to manufacter's instructions and its
138 efficiency was checked by 18S rRNA gene qPCR amplification as is mentioned below.

139 After that, a reverse transcription was done using the PrimeScript TM RT
140 reagent Kit (Takara Bio INC, Catalog No. #RR037A) following the manufacter's
141 instructions. Equal RNA (~100 ng) quantity from for all compost samples was used
142 using the Random 6 mers option. Finally, cDNAs obtained were quantified using a
143 Qubit™ 4 Fluorometer (Invitrogen, Catalog No. Q33226) and were kept at -20 ºC until
144 analysis.

145

146 **2.3. Illumina MiSeq amplification, taxonomic assignment and diversity indices**

147 The internal transcribed spacer (ITS) region was amplified by Illumina MiSeq
148 sequencing technology, at the facilities of Life Sequencing S.L. (Valencia, Spain). The
149 ITS1-F-KYO2 (5'-CTHGGTCATTTAGAGGAASTAA-3') and ITS2-KYO1 (5'-
150 CTRYGTTCTTCATCGDT-3') primers and PCR conditions used were previously
151 described by Toju et al. (2012). Before amplification, genomic DNA samples were
152 purified using PowerClean ®DNA Clean-Up Kit (MO BIO, Catalog No. 12877-50) and
153 tested for PCR inhibition. A total of 18 amplicons were obtained and the raw sequences
154 were cured for quality (quality score of 20) and the Illumina primer's barcodes were
155 removed using PEARL software V.0.9.1 (available in
156 http://www.exelixis-lab.org/web/software/pear) and CUTADAPT V.1.8.1. (Available in

157 http://cutadapt.readthedocs.io/en/stable/), respectively. Sequences shorter than 200
158 nucleotides or unresolved nucleotides were eliminated, as well as chimeras.
159 For each amplicon, the taxonomical assignation and the relative abundances
160 were done as was described in Cole et al. (2014), using the Ribosomal Data Project
161 website. The RDP classifier tool according to UNITE Fungal ITS gene option at 80%
162 confidence Cut-off (RDP, release 11.5, http://pyro.cme.msu.edu) was employed, and the
163 raw sequences were aligned and clustered into operational taxonomic units (OTUs)
164 using the Infernal Alignment and the Complete Linkage Clustering RDP tool
165 (unsupervised method), respectively. In addition, Good's coverage index, Shannon-
166 Wiener (H') and Chao-1 diversity indices were calculated using the available RDP web
167 tools.
168
169 **2.4. Quantitative PCR (qPCR)**
170 The number of copies of 18S rRNA gene from genomic DNA and cDNA was
171 quantified by qPCR using FungiQuant quantitative real-time PCR assay (FungiQuant-F
172 5'-GSWCTATCCCCAKCACGA-3' and FungiQuant-R 5´-
173 GGRAAACTCACCAGGTCCAG-3') (Maza-Márquez et al., 2018). The qPCR was
174 performed in a Step One Plus™ Real-Time PCR system (Agilent Technologies) using
175 an Mx3000P System. The qPCR reactions were performed in triplicate using the iTaq
176 Universal SYBR Green Supermix (Sigma Aldrich, Catalog No. #172-5125) with a final
177 reaction volume of 25 µl. The following conditions were used for PCR amplification:
178 95°C for 3 min, 40 cycles consisting of 94 °C for 30 s, 62 °C for 30 s and 72°C for 45 s,
179 and 72 °C for 7 min.

180 Copy numbers of 18S rRNA genes were calculated using a standard curve with
181 serial tenfold dilution (10^{-1}-10^{-8}) from a linearised plasmid (pGEM-T Easy vector,
182 Promega, Catalog No. A1360) in which the target gene was inserted. Amplicons of 18S
183 rDNA were generated from the culture of *Candida albicans* strain ATCC 10231 (Maza-
184 Márquez et al., 2018). The PCR products were cloned using the TOPO® TA cloning®
185 system (Invitrogen, Catalog No.K457501), following the manufacturer's instructions.
186 The calibration curves showed a correlation coefficient r^2>0.99 and the efficiency of
187 PCR amplification was between 90-100% in all the assays. Melting curve was
188 constructed using increasing temperature from 60 ºC to 95 ºC. Verifications of each
189 primers size and single band after qPCR were performed by electrophoresis in agarose.
190 Results were expressed as the copy numbers of 18S rRNA gene from DNA or cDNA
191 per compost dry weight of (CDW) in grams (g).
192
193 **2.5. Statistical analysis**
194 The mean and the absolute error of the abundance of relative sequences were
195 estimated for Mesophilic, Thermophilic and Maturation phases, respectively. Also, the
196 Statistical Analysis of Taxonomical and Functional Profiles (STAMP) open-source
197 software v2.0.9 release (Parks et al., 2014) was used to calculate the one-way ANOVA
198 with Tukey-Kramer post-hoc test at $p < 0.05$, applying Storey's FDR for multiple
199 correction and eta-squared for sample size correction tests, respectively. These
200 statistical tests were done assuming normal distribution and homoscedasticity of the raw
201 data. According to user's guide recommendations, STAMP option of Principal
202 Component Analysis (PCA) and the unweighted pair-group method with arithmetic

203 average (UPGMA) plotted as a dendrogram were used to estimate the phyla distribution
204 among piles and composting phases respectively at 0.75 of threshold.
205 Finally, Pearson correlation coefficients were calculated using GNU-PSPP open-
206 source software v0.9.0 (available in https://www.gnu.org/software/pspp/) to estimate the
207 relationships between physico-chemical characteristics of AL composts, previously
208 published in Tortosa et al. (2017) and fungal community at the order level.
209
210 **2.6. Accession numbers**
211 Illumina sequences are available at the EMBL-EBI European Nucleotide
212 Archive Database (http://www.ebi.ac.uk/ena) under the accession numbers
213 ERS3048724-ERS3048741.
214
215 **3. Results and discussion**
216 **3.1. Sequencing analysis and diversity indices**
217 Clone libraries could provide an important estimation on microbial diversity
218 during composting processes (López-González et al., 2015 a, 2015b). However, high-
219 throughput sequencing technologies applied to composting are providing relevant
220 information on the microbiome of these processes. In this study, Illumina MiSeq was-
221 used to analyze the mycobiome in a real composting process during mesophilic,
222 thermophilic and maturation phases, respectively. The number of sequences obtained
223 before curing ranged in 73263-81375 for the composting libraries, being the total
224 unclassified sequences less than 0.5 % of the total sequences (Table 1). These values
225 agree with those described by Gu et al. (2017) and Wang et al. (2018a) for chicken

manure and cow manure composting, respectively. According to the taxonomical classification (Table 1), the identification percentage of the sequences at each composting phase was relevant (> 97 %), descending to 80 % at order level in the thermophilic phase.

Diversity indices like OTUs richness (R), the Good's coverage and the Shannon (H') and Chao-1 indices applied to high-throughput analyses have been widely used in order to assess the microbial evolution along the process. In the present study, OTUs Richness ranged between 1426-2320 and Good's coverage index showed values between 90.0-94.9% (Table 2). According to these values, it can be concluded that the sequences obtained were representative of the fungal community in the compost samples. The diversity expressed as Shannon-Wiener H' index, increased during the process, being at the thermophilic phase higher (H': 4.29) compared to the mesophilic (H': 2.77) or maturation (H': 3.14) phases. In addition, the Chao-1 index showed the highest value at the thermophilic phase (Chao-1: 3844) in agreement with the H' value. Results obtained by López-González et al. (2015b) found less fungal diversity associated to cooling or maturation stages. Also, Tian et al. (2017) showed an increase~~d~~ in Shannon and Simpson indices during composting of Chinese medicinal herbal residues. Meanwhile, Huhe et al. (2017) noted the opposite behaviour during composting cattle farm waste, an inverse parabolic diversity evolution within temperature and time. Despite this apparent discrepancy, it might be inferred that fungal diversity could be directly related to the temperature evolution during the process and the native fungal population of raw materials used for composting.

249 **3.2. Fungal diversity**

250 In general terms, Ascomycota was the predominant phylum along the process,
251 representing more than 90 % of the total sequences identified at mesophilic,
252 thermophilic and maturation phases (Fig. 1). Basidiomycota was also relevant during
253 thermophilic and maturation phases, accounting for 5.48 ± 1.19 and 9.73 ± 2.03 % of
254 the total sequences identified, respectively. Ascomycota is the largest phylum of fungi
255 and they occur in numerous terrestrial and aquatic ecosystems (Schoch et al., 2009).
256 This phylum is commonly found during composting processes and has been previously
257 described as the predominant phylum in olive mill waste composting (Ntougias et al.,
258 2013) and in other organic waste composting like Chinese medicinal herbal residues
259 (Tian et al., 2017) or cow manure (Wang et al., 2018a).

260 Figure 2 represents the evolution of the most relevant order indentified during
261 the process. Chaetothyriales, Diaporthales, Hypocreales and Saccharomycetales
262 (Ascomycota) (Fig. 2A); Agaricales and Atheliales (Basidiomycota) (Fig. 2B); and
263 Mucorales (Zycomycota) (Fig. 2C), significantly increased during thermophilic phase,
264 meanwhile Capnodiales and Eurotiales, belonging to Ascomycota decreased (Fig. 2A).
265 On the other hand, the basidiomycetous Cystofilobasidiales, Sporidiobolales and
266 Tremellales increased at the end of the process, especially during maturation (Fig. 2B).
267 According to genera classification (Table 3), *Penicillium, Davidiella* and
268 *Debaryomyces* were the principal genera identified at mesophilic phase, accounting for
269 93.68 ± 0.28, 2.63 ± 0.23 and 1.36 ± 0.30 % of the total sequences identified,
270 respectively. In the thermophilic phase, the most predominant genera were *Sarocladium*
271 and *Debaryomyces,* which statistically increased ($p > 0.05$) at 33.43 ± 1.42 and 24.55 ±

272 1.40% of the sequences.

273 Our results are in accordance with Cho et al. (2009), who also detected the
274 presence of the xerotolerant yeast *Debaryomyces hanseii* in pig manure and mushroom
275 cultural waste composting at the thermophilic composting phase. *Sarocladium* is known
276 as a plant pathogen, especially responsible for the sheath rot in rice. However, it is also
277 used as fungal pathogen control due to its antagonistic activity against other fungi by
278 producing cerulenin, a secondary metabolite which inhibits fatty acid and steroid
279 metabolism (Araújo et al., 2017). It is probable that *Sarocladium* was present in the
280 bedding of the sheep manure used for composting, but fortunately, it was not present in
281 mature composts indicating the sanitization capability of the composting process. Other
282 minority genera increased at this phase such as *Athelia* (4.09 ± 1.09 %), *Togninia* (2.17
283 ± 0.89 %), *Coprinellus* (1.16 ± 0.09 %) and *Mucor* (1.13 ± 0.12 %).

284 *Penicillium,* however, drastically decreased at this phase (2.69 ± 0.23 %),
285 probably displaced by thermotolerant species. In addition, an important percentage of
286 unclassified sequences were found (22.66 ± 2.86 %). *Mucor* and *Coprinellus* are well-
287 known thermotolerant species and the last one also involved in lignocellulose
288 degradation (Singh et al., 2009). At maturation phase, *Penicillium* shown absolute
289 predominance, with close to 80 % of the sequences identified, together with
290 *Debaryomyces, Cystofilobasidium* and *Rhodosporidium*, which represented 7.76 ± 1.11,
291 5.31 ± 1.96 and 3.61 ± 0.18 % of the sequences identified, respectively. *Penicillium* is a
292 well known inhabit fungi of composting processes since several representatives of this
293 genus can resist adverse conditions, including high temperatures and high pollutant
294 concentrations. This fungus is also typically isolated by using culture-dependent

295 technologies in lignocellulose-based composting (López-González et al., 2015b) and it
296 is able to transform lignocellulosic substrates thanks to the secretion of hydrolytic
297 enzymes. This fungus is widespread in olive mill wastes (Ntougias et al., 2013) and
298 composts from different origin (Ryckeboer at al., 2003), such as composts from manure
299 and wood wastes (Neher at al., 2013). Indeed, this mesophilic fungus seems to be a
300 promising tool to enhance composting process (Wang et al., 2011).
301
302 **3.3. Fungal abundance (qPCR)**
303 An increase in the number of copies of 18S rRNA gene from genomic DNA was
304 found during the process, which it was $2.74 \times 10^5 \pm 4.49 \times 10^4$, $4.90 \times 10^5 \pm 8.12 \times 10^4$
305 and $4.97 \times 10^5 \pm 1.53 \times 10^5$ CDW (g^{-1}) for mesophilic, thermophilic and maturation
306 phases, respectively (Fig. 3). This increase was statistically relevant ($p<0.05$) from
307 mesophilic to thermophilic phases, but not between thermophilic and maturation. For
308 cDNA, an increase in the number of 18S rRNA copies was also found during the
309 process, showing $5.90 \times 10^4 \pm 4.96 \times 10^3$, $1.19 \times 10^5 \pm 9.30 \times 10^3$ and $2.29 \times 10^5 \pm 8.30 \times$
310 10^3 CDW (g^{-1}) for mesophilic, thermophilic and maturation phases, respectively.
311 The amplification and quantification of the number of 18S rRNA gene presents
312 in genomic DNA and retrotranscript RNA (cDNA) by qPCR could represent a useful
313 tool to estimate the abundance of total and metabolically active fungal population,
314 respectively (Benítez et al., 2018). Until now, few studies have been published using
315 this technology applied to composting. Galitskaya et al. (2017) showed an increase in
316 the number of 18S rRNA copies amplified from genomic DNA (total fungal population)
317 during the cooling phase in municipal solid waste composting. On the other hand, Tian

318 et al. (2017) found a slight decrease in the total fungal abundance during the Chinese
319 medicinal herbal composting. In the present study, we found an increase in the
320 abundance of fungal population during the process, especially the metabolically active
321 population (cDNA) during maturation. It is generally accepted that the fungal
322 community is more active during cooling and maturation phases than during the
323 mesophilic or thermophilic phases (Ryckeboer et al., 2003). Specific environmental
324 conditions of temperature, pH or moisture during mesophilic or cooling phases can
325 favour fungal proliferation (de Bertoldi et al., 1983). Moreover, it is assumed that fungal
326 enzymatic activities like cellulolityc and ligninolytic could be increased along the
327 process when temperature decreases (de Bertoldi et al., 1983).
328
329 **3.4. Statistical analysis: Heatmaps, PCA and Pearson correlations**
330 Reproducibility in metagenomic and transcriptomic studies is an important
331 factor to take into account. Understanding microbial succession could help to optimize
332 operational conditions which are directly related with economic interest. In this study
333 the heatmap and the dendrogram of the identified sequences at order level (Fig. 4)
334 revealed that the amplicons were grouped properly into three differential clusters
335 according to each composting phases (mesophilic, thermophilic and maturation),
336 showing that the amplification and sequencing were satisfactory for reproducibility and
337 repeatability. The principal component analysis (PCA) plot explained more than 98 %
338 of the total variances of the sequences identified at order level (PC1) and also, DNA
339 libraries belonging to each composting phase were similarly grouped (Fig. 4). As it was
340 noted in the dendrogram plot, the PCA showed that mesophilic and maturation

341 amplicons were closed to each other compared to thermophilic counterparts that meant
342 that both composting phases showed similarities in the fungal diversity detected. These
343 data confirmed that the temperature is preferentially the main factor which affects the
344 fungal diversity and abundance during composting of lignocellulosic wastes like AL
345 (Toumela et al., 2000).

346 The Pearson correlation matrix showed that most of the sequences identified at
347 order level were significantly correlated ($r > 0.7$) with diversity indices (Shanon-
348 Wiener, H'; Chao-1 and Good) and also, with temperature evolution and cellulose
349 content during the process (Table 4). This behaviour was especially notable for orders
350 belonging to Ascomycota, being Capnodiales the order with more correlations with
351 Pearson coefficients (bigger that 0.7 within all parameters). On the other hand,
352 Basidiomycota preferentially showed significative correlations ($r > 0.7$) with parameters
353 related with the organic matter degradation and the humic substances formation. This
354 correlation was strongly notable during maturation, when Cysofilobasidiales,
355 Tremellales and Sporidiobolales were relatively more abundant. These orders showed a
356 negative correlations with organic matter (OM), hemicellulose, total organic carbon
357 (TOC), fat and water-soluble carbohydrates (WSCH) content, which were reduced
358 during the process. Meanwhile, humification degree (HD), the percentage of humic
359 acids (P_{AH}) and germination index (GI) were increased at the same time that
360 Cysofilobasidiales, Tremellales and Sporidiobolales relative abundance, showing
361 significative linear regressions correlation (R^2>0.85) (data not shown). Belonging to
362 Basidiomycota, the genera *Cystofilobasidium* is frequently found in composts from
363 different origin (Ryckeboer at al., 2003) like urban solid waste and sewage sludge

364 composting (de Bertoldi et al., 1983), and agricultural wastes composting (Yu et al.,
365 2015) showing an important cellulolytic and ligninolytic activities (Goud et al., 2001).
366 The organic matter of AL composts is characterized by its important lignocellulosic
367 fraction (Alburquerque et al., 2009). It is well known that an important organic matter
368 degradation linked to humic substances formation happened (Tortosa et al., 2012), being
369 an ideal environment for *Cystofilobasidium* growth and propagation. Despite that more
370 research is needed to confirm it, *Cystofilobasidium* could be considered as a fungal
371 biomarker to asses AL compost maturation.
372
373 **4. Conclusions**
374 The fungal diversity was increased during AL composting, being Ascomycota
375 the predominant phyla. *Penicillium* was the principal genera identified during the
376 process, especially at mesophilic and maturation phases, and thermotolerant species
377 such as *Debaryomyces* and *Sarocladium* dominated the thermophilic phase. The
378 abundance of the total and metabolically active fungal populations assessed by qPCR
379 was increased at the end of the process, which confirm the key role of fungi during
380 thermophilic and maturation phases. Some Basidiomycota were increased at the end and
381 positively correlated with humification parameters during composting, being
382 *Cystofilobasidium* a potential fungal biomarker to assess AL compost maturation.
383
384 **Conflict of Interest**
385 The authors declare that they have no conflict of interest.
386

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564 **Figure captions**

565 Figure 1. Evolution of relative abundance of phyla during mesophilic, thermophilic and
566 maturation phases, respectively. For each taxa, different letter indicates statistical
567 difference ($p < 0.05$).

568

569 Figure 2. Evolution of the main order identified during mesophilic, thermophilic and
570 maturation phases, respectively. For each taxa, different letter indicates statistical
571 difference ($p < 0.05$).

572

573 Figure 3. 18S rRNA gene copies from genomic (gDNA) and retrotranscripted (cDNA)
574 DNA during mesophilic, thermophilic and maturation phases, respectively. For each
575 sample, different letter indicates statistical difference ($p < 0.05$).

576

577 Figure 4. Order taxa heatmap (left) and principal component analysis (PCA, right)
578 during mesophilic (blue), thermophilic (orange) and maturation (green) phases,
579 respectively.

580 **Table captions**

581

582 Table 1. Taxa (N) and sequences (S) number obtained from Illumina-sequencing data at
583 mesophilic (Meso 1-3), thermophilic (Thermo 1-3) and maturation (Matu 1-3) phases,
584 respectively.

585

586 Table 2. OTUs Richness (R), Singletons, Good´s coverage (%) and diversity indices
587 Shannon-Wiener (H') and Chao-1 from Illumina-sequencing data at mesophilic (Meso
588 1-3), thermophilic (Thermo 1-3) and maturation (Matu 1-3) phases, respectively.

589

590 Table 3. Relative abundance (%) of the different fungal genera at mesophilic (Meso 1-
591 3), thermophilic (Thermo 1-3) and maturation (Matu 1-3) phases, respectively. Data are
592 shown as the mean value of three replicates ± standard deviation.

593

594 Table 4. Pearson correlation matrix (n=18) between composting parameters and Order
595 taxa identified during AL composting. Green folders represent parameters with
596 correlation coefficients > 0.70, respectively.

Figure 1

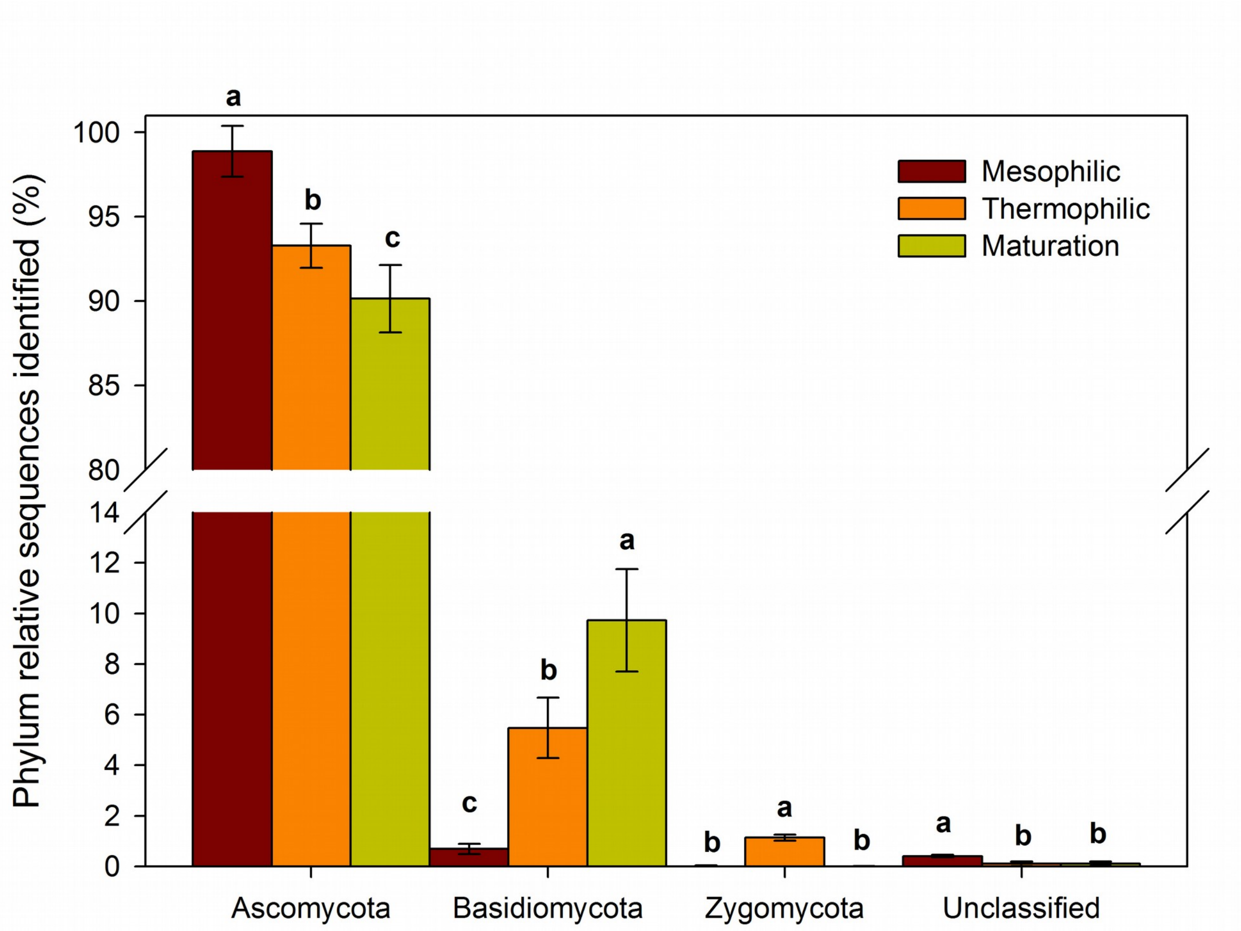


Figure 2

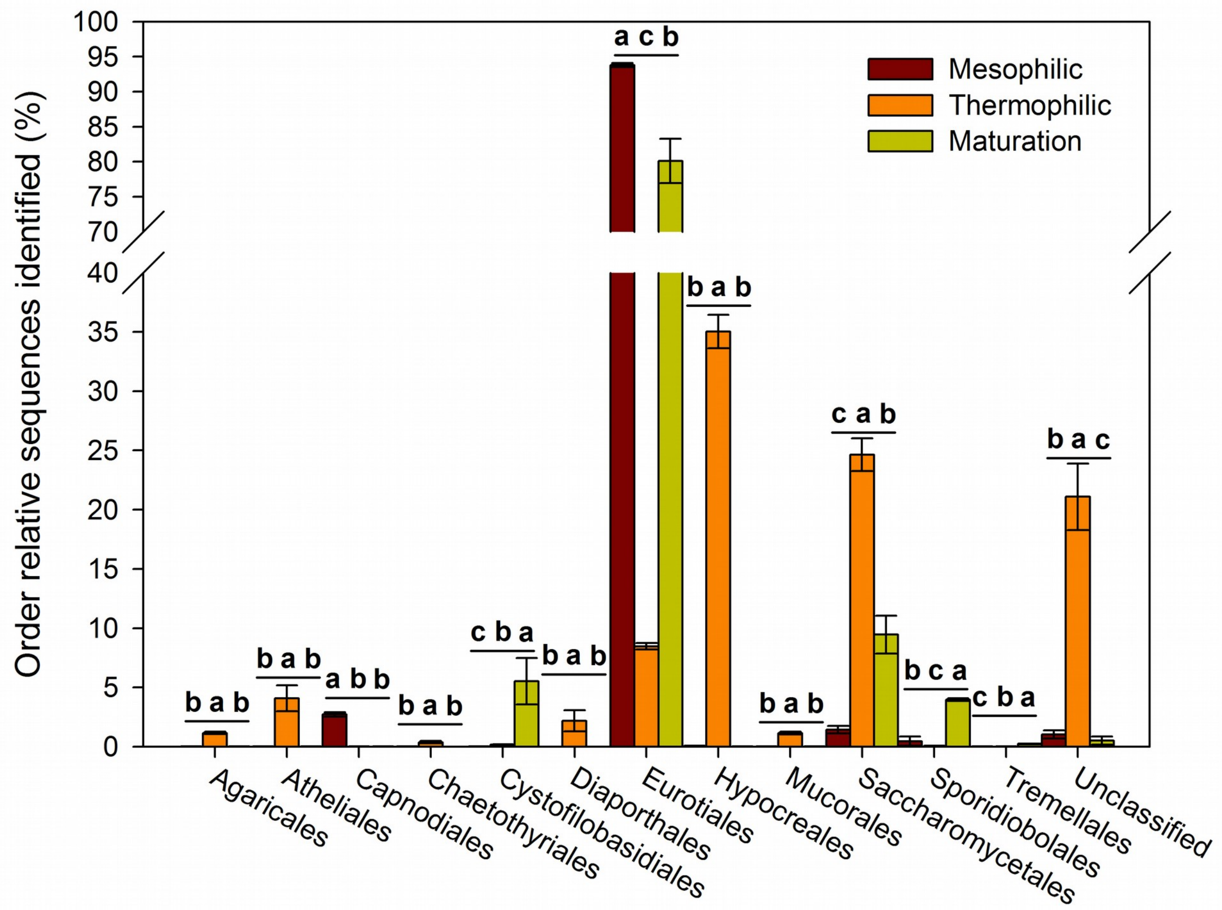
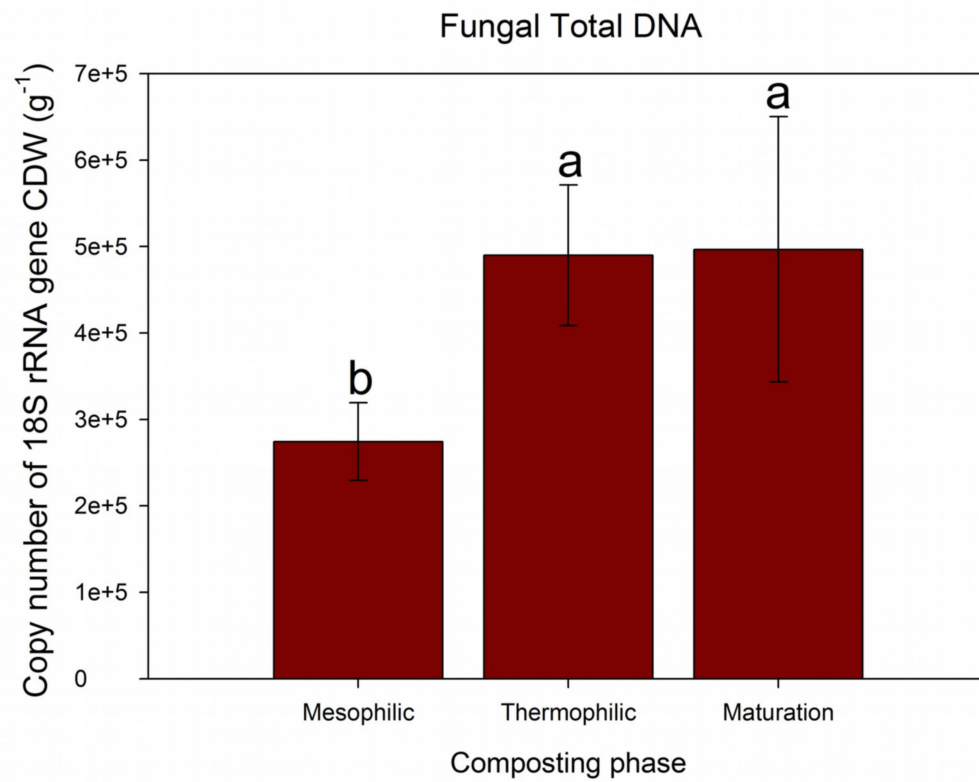


Figure 3



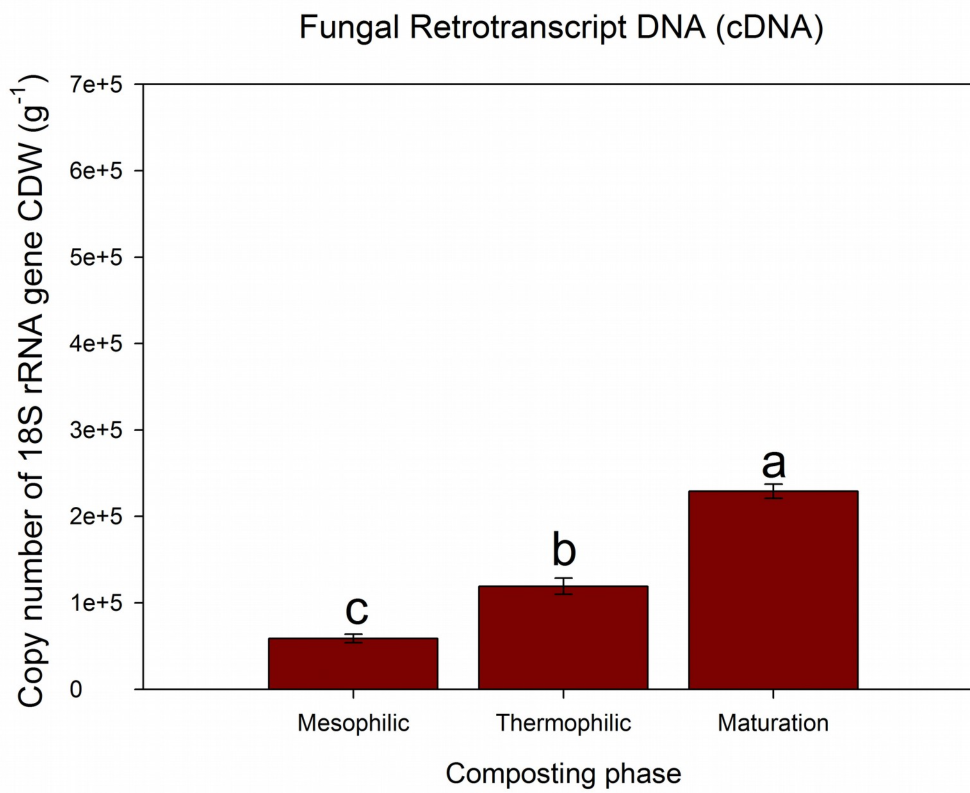


Figure 4

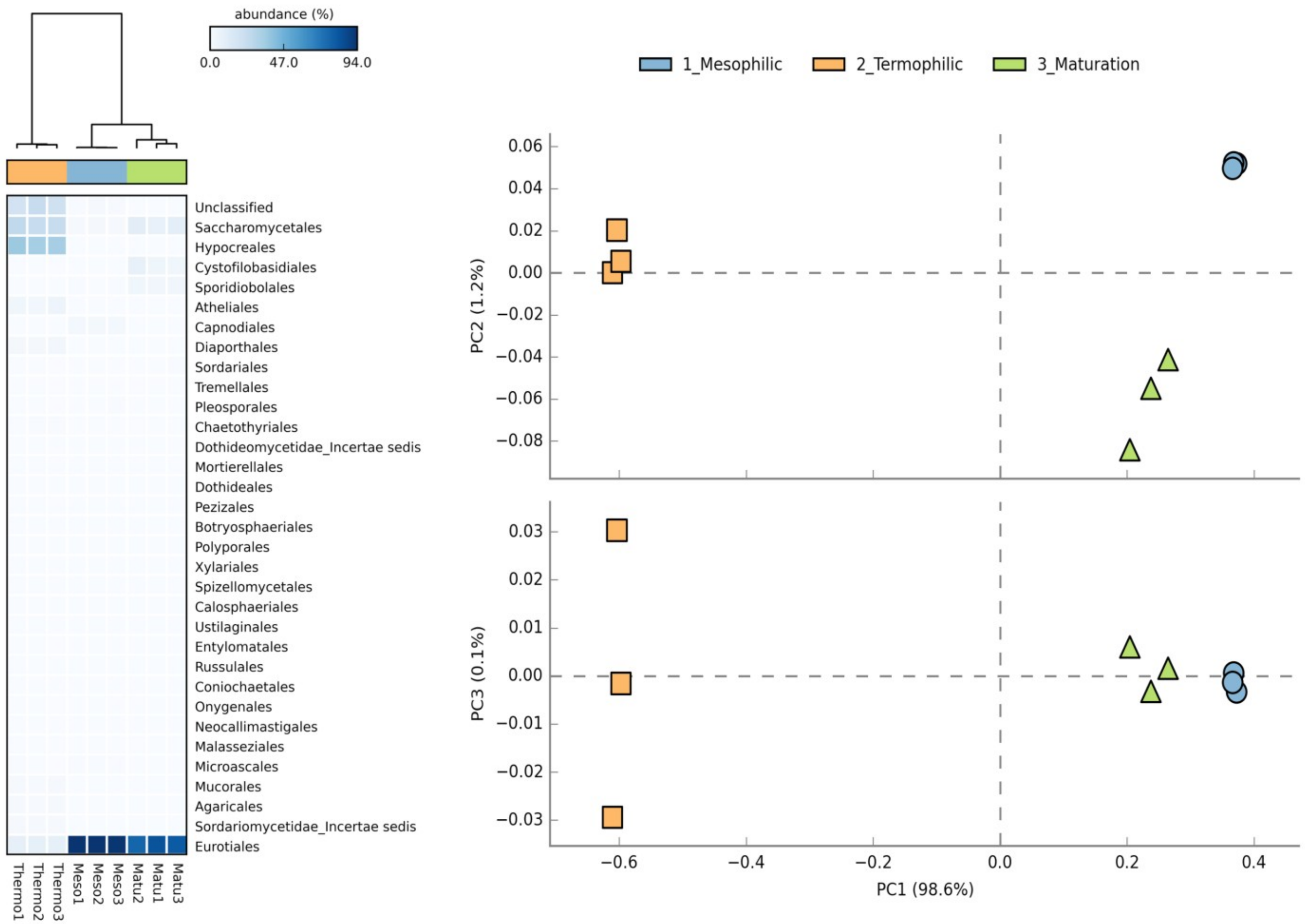


Table 1.

AL compost	Composting phase libraries					
	Meso1-3		Thermo1-3		Matu1-3	
	N	S	N	S	N	S
Phylum	4 ± 1	81041 ± 10299 (99.6%)	3 ± 0	77932 ± 10685 (99.9%)	4 ± 1	73179 ± 25204 (99.9%)
Class	11 ± 2	80962 ± 10308 (99.5%)	9 ± 0	76069 ± 11209 (97.5%)	10 ± 1	73158 ± 25193 (99.9%)
Order	19 ± 5	80723 ± 10501 (99.2%)	19 ± 1	61848 ± 9732 (79.3%)	18 ± 2	73133 ± 25187 (99.8%)
Family	23 ± 6	80763 ± 10303 (99.2%)	26 ± 1	61649 ± 9765 (79.0%)	23 ± 1	73128 ± 25185 (99.8%)
Genus	32 ± 7	79952 ± 10640 (98.2%)	37 ± 2	60452 ± 9557 (77.5%)	34 ± 3	73012 ± 25143 (99.7%)
Total sequences identified	81375 ± 10311 (100%)		78016 ± 10714 (100%)		73263 ± 25243 (100%)	
Total unclassified sequences	334 ± 49 (0.4%)		83 ± 34 (0.1%)		84 ± 47 (0.1%)	

Note: Values are expressed as the mean of the sequences from each library and ± its standard deviation. Numbers in brackets represent the percentage of identified sequences respect to the total identified sequences.

Table 2.

Composting phase	Richness (OTUs)	Singletons	Good's coverage (%)	Shannon-Weiner (H')	Chao-1
Meso1-3	1426a	4095a	94.9b	2.77a	2715a
Thermo1-3	2320b	7611c	90.2a	4.29c	3844b
Matu1-3	1437a	4346b	93.9b	3.14b	2558a

Note: Different lower-case letter among composting phases indicate statistical differences according to one-way ANOVA with Tukey-Kramer *post-hoc* test at $p < 0.05$. Good's coverage index was calculated as [(1-singletons/total number of sequences) x 100].

Table 3.

Phylum	Class	Order	Family	Genus	Meso 1-3	Thermo 1-3	Matu 1-3
					Relative abundance (%) Composting phase libraries		
Ascomycota	*Eurotiomycetes*	*Chaetothyriales*	*Herpotrichiellaceae*	*Exophiala*	-	0.38 ± 0.10	-
		Eurotiales	*Eurotiales_Incertae sedis*	*Thermomyces*	-	0.01 ± 0.00	-
			Trichocomaceae	*Eupenicillium*	-	-	0.31 ± 0.02
				Penicillium	93.68 ± 0.28	2.69 ± 0.23	79.64 ± 3.16
	Dothideomycetes	*Capnodiales*	*Mycosphaerellaceae*	*Davidiella*	2.63 ± 0.23	-	-
	Saccharomycetes	*Saccharomycetales*	*Pichiaceae*	*Yamadazyma*	-	0.04 ± 0.00	-
			Saccharomycetaceae	*Debaryomyces*	1.36 ± 0.30	24.55 ± 1.40	7.76 ± 1.11
				Saccharomyces	0.03 ± 0.01	-	0.14 ± 0.07
			Saccharomycetales_Incertae sedis	*Candida*	0.02 ± 0.01	0.02 ± 0.01	1.52 ± 0.84
	Sordariomycetes	*Diaporthales*	*Togniniaceae*	*Togninia*	-	2.17 ± 0.89	-
		Hypocreales	*Hypocreales_Incertae sedis*	*Acremonium*	0.03 ± 0.00	0.03 ± 0.01	-
				Sarocladium	0.02 ± 0.00	33.43 ± 1.42	0.01 ± 0.01
				Stachybotrys	-	0.14 ± 0.08	-
		Microascales	*Microascaceae*	*Graphium*	-	0.32 ± 0.07	-
				Microascus	0.52 ± 0.08	0.01 ± 0.01	0.14 ± 0.01
		Sordariales	*Lasiosphaeriaceae*	*Podospora*	-	-	0.28 ± 0.31
		Sordariomycetidae_Incertae sedis	*Plectosphaerellaceae*	*Plectosphaerella*	-	1.21 ± 0.18	-
Basidiomycota	*Agaricomycetes*	*Agaricales*	*Entolomataceae*	*Clitopilus*	0.01 ± 0.01	-	0.01 ± 0.00
			Psathyrellaceae	*Coprinellus*	-	1.16 ± 0.09	-
		Atheliales	*Atheliaceae*	*Athelia*	-	4.09 ± 1.09	-
	Microbotryomycetes	*Sporidiobolales*	*Sporidiobolales_Incertae sedis*	*Rhodosporidium*	0.26 ± 0.02	0.03 ± 0.01	3.61 ± 0.18
				Rhodotorula	0.41 ± 0.01	0.03 ± 0.01	0.35 ± 0.09
	Tremellomycetes	*Cystofilobasidiales*	*Cystofilobasidiaceae*	*Guehomyces*	-	0.08 ± 0.03	0.20 ± 0.14
				Cystofilobasidium	-	0.09 ± 0.02	5.31 ± 1.96
		Tremellales	*Tremellaceae*	*Cryptococcus*	-	-	0.22 ± 0.02
Zycomycota	*Mucoromycotina_Incertae sedis*	*Mucorales*	*Mucoraceae*	*Mucor*	0.01 ± 0.00	1.13 ± 0.12	-
				Unclassified	0.95 ± 0.44	22.66 ± 2.86	0.34 ± 0.02
				Sum of sequences (%)	99.93	94.27	99.87

Table 4.

Phylum	Order	Temp	pH	EC	OM	Lig	Cellu	Hemi	T_{OC}	T_N	CN	Fat	WSC	WSCH	HR	HD	P_{AH}	GI	Shanon	Chao1	Good	gDNA	cDNA
Ascomycota	Capnodiales	0,21*	-0,79**	0,56**	0,88**	-0,76**	0,43**	0,92**	0,62**	-0,21**	0,61**	0,90**	-0,01*	0,86**	-0,83**	-0,92**	-0,90**	-0,68**	-0,68**	-0,33**	0,66**	-0,78**	-0,37**
	Chaetothyriales	0,72**	-0,10*	0,42**	-0,04*	-0,15*	-0,81**	-0,15*	0,35*	0,71**	0,32*	-0,10*	0,48**	-0,01*	0,15*	0,14*	0,08*	-0,28*	0,94**	0,87**	-0,95**	0,32**	-0,19*
	Diaporthales	0,70**	-0,09*	0,41**	-0,02*	-0,15*	-0,81**	-0,15*	0,33*	0,63**	0,30*	-0,08*	0,72**	0,03*	0,15*	0,13*	0,07*	-0,27*	0,91**	0,90**	-0,88**	0,23*	-0,22*
	Dothideomycetidae_Incertae sedis	0,56*	-0,06*	0,33**	-0,01*	-0,14*	-0,69**	-0,14*	0,27*	0,41**	0,23*	-0,07*	0,60*	0,04*	0,12*	0,10*	0,05*	-0,22*	0,75**	0,70**	-0,63**	0,21*	-0,19*
	Eurotiales	-0,63*	-0,04*	-0,30*	0,18*	0,02*	0,82**	0,30*	-0,23**	-0,64**	-0,19*	0,25*	-0,37*	0,16*	-0,26*	-0,29*	-0,22*	0,15*	-0,99**	-0,83**	0,96**	-0,48**	0,12*
	Hypocreales	0,73**	-0,10*	0,44*	-0,03*	-0,17**	-0,84**	-0,16*	0,37**	0,69**	0,32*	-0,10*	0,39*	-0,01*	0,15**	0,15*	0,08*	-0,30*	0,97**	0,84**	-0,94**	0,38**	-0,18*
	Malasseziales	-0,27*	-0,23*	-0,08*	0,19*	-0,15**	0,68**	0,32*	-0,04*	-0,62**	0,13*	0,23*	-0,19*	0,16*	-0,30*	-0,31*	-0,27**	-0,04*	-0,59**	-0,25*	0,67**	0,01*	-0,49**
	Mortierellales	0,12*	-0,59**	0,43*	0,67**	-0,59**	0,38**	0,67**	0,47**	-0,14*	0,46*	0,64**	NS	0,61*	-0,59**	-0,69**	-0,68**	-0,51*	-0,51**	-0,15*	0,51**	-0,66**	-0,31*
	Pleosporales	0,65**	-0,53**	0,66**	0,49*	-0,59**	-0,30**	0,44**	0,64**	0,48**	0,62**	0,45**	0,22*	0,47*	-0,27*	-0,42**	-0,47*	-0,62**	0,34**	0,40**	-0,40**	-0,15*	-0,38**
	Saccharomycetales	0,46**	0,22*	0,10**	-0,37*	0,17*	-0,75**	-0,48**	0,03*	0,53**	0,02*	-0,43**	0,28*	-0,35*	0,40*	0,47**	0,40*	0,04*	0,99**	0,76**	-0,93**	0,62**	-0,04*
	Sordariales	-0,64**	0,52**	-0,70**	-0,62*	0,60**	0,49**	-0,50**	-0,69**	-0,59**	-0,51**	-0,58**	-0,28*	-0,64**	0,42**	0,51*	0,55*	0,66**	-0,18*	-0,15*	0,29**	0,62**	-0,08*
	Sordariomycetidae_Incertae sedis	0,74**	-0,10*	0,44*	-0,03*	-0,17**	-0,84**	-0,16*	0,37**	0,68**	0,32*	-0,09*	0,53**	0,01*	0,15*	0,14**	0,07*	-0,30*	0,97**	0,89**	-0,93**	0,32*	-0,20*
Basidiomycota	Agaricales	0,73**	-0,09*	0,44**	-0,03*	-0,16*	-0,85**	-0,16*	0,37**	0,68**	0,32**	-0,10*	0,47**	-0,01*	0,16*	0,15*	0,08*	-0,29*	0,97**	0,87**	-0,94**	0,35**	-0,19*
	Atheliales	0,72**	-0,09*	0,42*	-0,02*	-0,16*	-0,84**	-0,16*	0,35**	0,64**	0,31*	-0,09*	0,59**	0,02*	0,15*	0,14*	0,07*	-0,28*	0,95**	0,88**	-0,90**	0,30*	-0,21**
	Cystofilobasidiales	-0,88**	0,81**	-0,94**	-0,82**	0,88**	0,45**	-0,72**	-0,93**	-0,55**	-0,85**	-0,77*	-0,37*	-0,81**	0,54**	0,74**	0,78**	0,92**	-0,23**	-0,47**	0,27*	0,39**	0,51**
	Tremellales	-0,94**	0,91**	-0,99**	-0,84**	0,94**	0,35**	-0,78**	-0,99**	-0,40*	-0,96**	-0,80*	-0,37*	-0,84**	0,72**	0,79**	0,83**	0,98**	-0,27**	-0,52**	0,26*	0,35**	0,64**
	Sporidiobolales	-0,97**	0,84**	-0,98**	-0,79**	0,89**	0,48**	-0,71**	-0,97**	-0,52**	-0,92**	-0,75**	-0,42*	-0,80**	0,62**	0,72**	0,76**	0,95**	-0,36**	-0,56**	0,37*	0,32**	0,55**
Zycomycota	Mucorales	0,73**	-0,10*	0,44**	-0,02*	-0,17**	-0,84**	-0,16*	0,37**	0,67**	0,32*	-0,10*	0,44**	NS	0,15*	0,14*	0,07*	-0,30*	0,96**	0,85**	-0,93**	0,36**	-0,20*

NS: not significant, EC: electrical conductivity, OM: total organic matter, Lig: lignin, Cellu: cellulose, Hemi: hemicellulose, T_{OC}: total organic carbon. T_N: total nitrogen, WSC: water-soluble carbon, WSCH: water-soluble carbohydrates, HR: humification ratio, HD: humification degree, P_{AH}: percentage of humic acids.

* Significant at $p < 0.05$.

** Significant at $p < 0.01$.