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1 **Aerobiological behavior of Paleolithic rock art sites in Dordogne (France): a**
2 **comparative study in protected sites ranging from rock shelters to caves, with**
3 **and without public access**

4
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17

18 **Abstract**

19

20 Microbial organisms can cause huge crises in decorated caves, as seen in emblematic sites such
21 as Lascaux cave. The preservation of such sites involves understanding the healthy microbial
22 behavior of caves before the damage occurs. Indeed, knowledge of normal cave behavior is a
23 prerequisite to identifying potential imbalance. This study seeks to determine whether models
24 of aerobiological behavior could be identified in several caves of different sizes, ranging from
25 rock shelters to large caves that are open or closed to the public. Aerial rates of fungi and
26 bacteria were monitored over three years in nine sites in Dordogne (France). This study revealed
27 that in a context of caves where public visits were carefully managed, fungal and bacterial rates
28 were more affected by the size of the caves than by the opening of sites to the visitors. The
29 study confirmed that large caves can generally be described as “self-purifying caves” as they
30 were strongly affected by the exterior environment at their entry but much less so at locations
31 further inside the cave, while small caves can be described as “non-self-purifying caves” since
32 they were strongly affected by the exterior environment throughout their whole length. The
33 results also highlighted the difficulty to determine a limit value of microbial rates valid for all
34 caves because of the specificities of each one.

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40 **Keywords**

41

42 Cultural heritage preservation, decorated cave, air quality, microbiological airborne particles,
43 fungal diversity, environmental conditions

44

45 **1. Introduction**

46

47 The Dordogne region of France is well known for the number and the quality of human
48 occupation sites dating from the Upper Paleolithic (mainly Magdalenian, ~ 17 000 – 12 000
49 years ago; Leroi-Gourhan 1984). In particular, the Vézère valley boasts 147 prehistoric sites
50 and 25 ornamented caves, 15 of which have been UNESCO World Heritage Sites since 1979
51 (UNESCO 1979). The most famous of these is Lascaux cave. This cave is interesting for several
52 reasons. The cave was discovered in 1940 and is today considered one of the finest examples
53 of rock art paintings. This attracted many visitors as soon as the cave was opened to the public.
54 The presence of these visitors led to microbial damage and notably the strong growth of green
55 algae on the walls, and the cave was closed indefinitely in 1963 (Dupont et al. 2007). Despite
56 this closure, the Lascaux cave suffered from other microbial outbreaks, the most recent being a
57 fungal development in 2006 (Bastian et al. 2010). Lascaux is not an isolated case; other major
58 rock art sites such as Altamira cave in Spain have been affected by microbial outbreaks (Saiz-
59 Jimenez et al. 2011). This has made the preservation of such heritage a major concern.
60 Several studies have sought to characterize microbial life on the rock surface (Schabereiter-
61 Gurtner et al. 2004; Angelova and Groudeva 2014), in cave soil (Adetutu et al. 2011; Out et al.

62 2016), and in cave atmosphere (Docampo et al. 2011; Martin-Sanchez et al. 2014). The studies
63 in cave atmosphere are particularly interesting, with some authors recently trying to use the
64 quantity of airborne fungi as an indicator of disturbance in caves (Porca et al. 2011; Pusz et al.
65 2017). This approach required the initial identification of the non-disturbed signature of caves;
66 the term “ornamented cave” covers a wide range of sites that vary in terms of size, volume and
67 accessibility to the public. Identifying the non-disturbed aerobiological behavior of a cave
68 requires long-term monitoring to understand normal variations that happen over time. However,
69 very few studies have monitored airborne microbial rates at several sampling points and several
70 sampling dates in a given cave (Docampo et al. 2011; Taylor et al. 2013; Martin-Sanchez et al.
71 2014).

72 A previous study focusing on five caves in the Gard region (France) demonstrated that each
73 cave has its own specific aerobiological behavior signature (Leplat et al. 2018). It also showed
74 that caves could be divided into two groups according to that behavior, namely “self-purifying
75 caves” and “non-self-purifying caves”. The present study seeks to explore these results in depth
76 by widening the diversity of study cases at very low volumetric sites and by introducing the
77 human factor as a variable that could impact cave health, as some of the studied sites are open
78 to visitors whereas the others are not.

83 **2. Materials and Methods**

84

85 *2.1 Samplings*

86 This study monitored airborne microbiological particles in nine ornamented sites of the
87 Dordogne region (France), namely seven caves and two rock shelters (Fig. 1). Six of these nine
88 sites are listed as World Heritage Sites by UNESCO. The two rock shelters can be considered
89 as small caves as they are separated from the exterior by a wall. Some of the caves are open to
90 visitors; the others are not (Table 1). In this study, a “closed cave” is defined as a cave that is
91 inaccessible to the public, where the entrance is closed with walls and doors and equipped with
92 openings for ventilation. An “open cave” is defined as a cave that is accessible to the public,
93 but where the entrance is also closed with walls and doors and equipped with openings for
94 ventilation. No major microbiological outbreak has occurred on the walls of these caves, which
95 can therefore be considered representative of undisturbed caves. Five surveys were conducted
96 over a period of three years to evaluate the rates of airborne microorganisms in the studied
97 caves. Two surveys were carried out at the beginning of summer (June 2014 and June 2015),
98 and three others were carried out during autumn (October 2013, October 2014 and October
99 2016). All the caves were tested on each of the five dates, with the exception of Reverdit shelter,
100 which was not sampled in October 2016. At each sampling date, one to six points were sampled
101 in each cave according to the size of the caves (Fig. 2a and b). An additional sampling point
102 was added outside each cave in October 2016 to obtain an external air measurement.

103 A Duo SAS Super 360 air sampler (VWR-pbi, Milan, Italy) was used to detect cultivable
104 microorganisms in cave air. 50 L of air was collected at each sampling point using a 219-hole
105 impactor containing appropriate culture media in 55 mm Petri dishes. Bacteria were isolated on
106 nutrient agar (Merck KGaA, Darmstadt, Germany), and fungi were isolated on malt extract agar

107 (Merck KGaA, Darmstadt, Germany). The Petri dishes containing impacted media were then
108 taken to the laboratory for analysis.

109

110 *2.2 Counting of cultivable microorganisms*

111 The plates were incubated in a BD 115 incubator (Binder GmbH, Tuttlingen, Germany) for
112 three days at 30 °C for bacteria, and for seven days at 24 °C for fungi. These incubation
113 temperatures are higher than those found in the caves, and were chosen to allow rapid microbial
114 growth. The number of microbial colonies grown in each Petri dish was counted after
115 incubation. Each count was corrected using the table of the most probable count, as
116 recommended in the manufacturer instructions. The results were expressed as colony-forming
117 units per cubic meter (CFU m⁻³).

118

119 *2.3 Identification of fungal strains*

120 Bacterial strains were counted but not identified. Fungal strains were separated through sub-
121 culturing on malt extract agar. The resulting isolated fungal strains were identified to the genus
122 level by crosschecking their macroscopic and microscopic characteristics with those listed in
123 reference books (Domsch et al. 1980; Seifert et al. 2011).

124 Genera that could not be distinguished by their morphology were identified through molecular
125 identification. DNA was extracted as described by (Edel et al. 2001). ITS1F/ITS4 primers were
126 used to amplify the nuclear ribosomal internal transcribed spacer (ITS) region (Gardes and
127 Bruns 1993; White et al. 1990). PCR was performed in 25 µl reactions, with 1 µl of template
128 DNA, 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, USA), 2.5 µl of 10X Taq DNA
129 polymerase buffer, 1 µl of 2 mmol l⁻¹ dNTPs (Thermo Fisher Scientific, Waltham, USA) and

130 1.5 μl of each 10 $\mu\text{mol l}^{-1}$ primer (Eurogentec, Seraing, Belgium). Amplifications were
131 performed on a PrimeG thermocycler (Bibby Scientific, Stone, UK) using the following
132 parameters: a 4-minute step at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and
133 40 s at 72 °C, and a final 10-minute extension at 72 °C. PCR products were sequenced by
134 Genoscreen (Lille, France) using the same primer set. Isolates were identified by BLAST
135 comparison to the NCBI GenBank database (Altschul et al. 1990).

136

137 *2.4 Statistical analyses*

138 2.4.1 Analyses of airborne microbiological particles counts

139 Statistical analyses were carried out using R 3.4.2 with $\alpha = 5\%$ (R Core Team 2014). The
140 results of microbiological counts were analyzed with a generalized linear mixed model
141 (GLMM). GLMMs are now commonly used to analyze data from ecological surveys (Bolker
142 et al. 2009). They are suitable for the analysis of non-normal data collected from complicated
143 designs that involve random and fixed effects.

144 Five qualitative variables were used to design two different GLMMs: *Sampled cave* (nine
145 modalities corresponding to the nine studied caves); *Sampling season* (two modalities: *Early*
146 *summer* for June 2014 and June 2015, and *Autumn* for October 2013, October 2014 and October
147 2016); *Sampling depth* (three modalities: *Entry* for the first sampled point in each cave, *Deep*
148 *cave* for all other points in each cave, and *Outside cave* for the external air measurement); *Cave*
149 *size* (two modalities, *Small cave* for caves that are around ten meters deep, and *Large cave* for
150 caves that are around one hundred meters deep or more; and *public access* (two modalities;
151 *Open cave* or *Closed cave*). Two GLMMs were necessary because the *cave size* and *public*

152 *access* variables are redundant when combined with the *sampled cave* variables, and cannot
153 therefore be studied in the same model.

154

155 The first GLMM for this study was:

$$156 \text{Link}(p_{ijkl}) = \eta_{ijkl} = \theta + \alpha_i + \beta_j + \gamma_k + \mu_{ik} + \varepsilon_{ijkl} \quad (1)$$

157 where θ is the general effect, α_i is the *sampled cave* effect, β_j is the *sampling season* effect, γ_k
158 is the *sampling depth* effect, μ_{ik} is the experimental error associated to each sampled point and
159 ε_{ijkl} is the residual error. α, β, γ were considered as fixed effects when μ was considered a random
160 effect. The term η_{ijkl} is the linear predictor. This term is obtained by submitting the variable p_{ijkl}
161 to the link function of the model. The choice of the link function depends on the p_{ijkl} distribution
162 law. The shapes of the distributions for bacterial and fungal counts were checked before
163 applying the GLMM. The negative binomial link was then chosen to study these counts. This
164 distribution is well adapted to counts with over-dispersed data (Booth et al. 2003). The
165 parameters of GLMM were estimated through the Penalized Quasi-Likelihood technique
166 (package MASS; function `glmPQL`, Wolfinger and O'Connell 1993). A Wald chi-square test
167 was performed to determine the significance degree of each explanatory variable in the GLMM
168 (package `aod`; function `wald.test`; Fox 2015). GLMM was completed with Tukey's post-hoc
169 test in order to check the significance of differences between means (package `lsmeans`; function
170 `lsmeans`; Bretz et al. 2016).

171

172

173 The second GLMM for this study was:

174 $Link(p_{ijklm}) = \eta_{ijklm} = \theta + \beta_i + \gamma_j + \pi_k + \sigma_l + \mu_{jkl} + \varepsilon_{ijklm}$ (2)

175 where θ is the general effect, β_i is the *sampling season* effect, γ_j is the *sampling depth* effect, π_k
176 is the *cave size* effect, σ_l is the *public access* effect, μ_{jkl} is the experimental error associated to
177 each sampled point and ε_{ijklm} is the residual error. β , γ , π and σ were considered as fixed effects
178 when μ was considered as random effect.

179

180 2.4.2 Spatial distribution of airborne microbiological particles in the caves: distance index

181 A distance index was calculated to check whether the distance between the sampling spot and
182 the cave entrance, as well as the distance between this spot and the end of the caves, had an
183 effect on the counts of airborne microbiological particles. The end of the caves was defined as
184 the point from which no further progress could be made by humans. It is therefore possible that
185 although this point was not the real end of the cave, it was nevertheless a major physical obstacle
186 to going any further. The index was normalized in order to enable comparison between caves
187 of different sizes:

188
$$Id = \frac{\text{Distance from the entrance of the cave} - \text{Distance to the end of the cave}}{\text{Distance from the entrance of the cave} + \text{Distance to the end of the cave}}$$

189 Id thus ranged from -1 to 1, where (-1) was the value obtained at the cave entrance and (1) was
190 the value obtained at the end of the caves.

191 The strength of the relationship between the microorganism counts and the Id value was
192 assessed through linear and quadratic regression (package stats; function lm; Chambers and
193 Hastie 1992). Pearson's' correlation coefficient and Root Mean Squared Error (RMSE, package
194 nsRFA; function RMSE; Armstrong and Collopy 1992) were chosen to illustrate the strength
195 of the regressions. RMSE shows the average distance between the regression curve and the

196 observations. The significance of the difference between the linear and the quadratic regressions
197 was assessed through the comparison of the analysis of variance tables associated with each
198 regression model (package stats; function anova; Chambers and Hastie 1992). The significance
199 of the difference between obtained RMSE values was estimated by bootstrapping the RMSE
200 from original data (1000 replicates; package boot; function boot; Davison and Hinkley 1997).
201 The intervals containing 95 % of the obtained values were then compared and were considered
202 to be significantly different when they did not overlap.

203

204 2.4.3 Structure of fungal communities

205 The structure of fungal communities was analysed by Principal Component Analysis (PCA;
206 package FactoMinR; function PCA; Husson et al. 2017). The isolated fungal genera were used
207 as quantitative dependent variables. Genera that represented less than 0.3 % of the total fungal
208 population were grouped together under the name “Other genera” to facilitate the interpretation
209 of the model. *Sampled cave, sampling season, sampling depth, cave size and public access* were
210 used as supplementary quantitative variables.

211

212 **3. Results**

213

214 *3.1 Airborne microbiological particle counts*

215 3.1.1 Fungal particles

216 The rates of airborne fungal particles ranged from 0 to 8150 CFU m⁻³ over the five sampling
217 campaigns (Fig. 3). La Mouthe, Combarelles I and Font-de-Gaume caves showed a fixed
218 pattern of airborne fungal particles despite a small number of differences. The fungal rates were

219 clearly high at the entrances of the La Mouthe and Combarelles I caves, before decreasing and
220 possibly increasing again at the end of the caves. A similar pattern was observed in Font-de-
221 Gaume cave, but the fungal rates were the highest at the second sampling point before
222 decreasing and possibly increasing again at the end of the cave.

223 A second group of caves, consisting of Mammoth cave and possibly Combarelles II cave and
224 Reverdit shelter, showed two different patterns according to the sampling season. The pattern
225 in Mammoth cave was characterized by a decrease in fungal rates from the entrance to the
226 back of the cave in October, while in June the rates assessed at the second sampling point were
227 at the same level or higher than the rates assessed at the first sampling point then decreased at
228 the third sampling point. In case of Combarelles II cave and Reverdit shelter, the fungal rates
229 assessed in June were systematically higher than the rates assessed in October.

230 Finally, a third group of caves, consisting of Pigeonnier cave, Sorcier cave and Cap Blanc
231 shelter, showed no clearly fixed pattern of fungal particles.

232 The fungal rates assessed outside the caves in October 2016 were systematically higher than
233 the rates assessed inside the corresponding caves.

234 The Generalized Linear Mixed Models showed that the *sampled cave*, *sampling depth*, *cave*
235 *size* and *public access* variables all had an effect on fungal particles rates (Table 2). The fungal
236 rates assessed in the Sorcier and Mammoth caves were significantly higher than the rates
237 assessed in the Combarelles I, La Mouthe and Font-de-Gaume caves (Table 3). The rates
238 assessed in the other caves had intermediate values. The fungal rates were significantly higher
239 outside the caves than in the caves and were significantly higher at the entrance of the caves
240 than at depth. The fungal rates were significantly higher in small caves than in large caves.

241 Finally, the fungal rates were significantly higher in closed caves than in caves that were open
242 to the public.

243

244 3.1.2 Bacterial particles

245 The rates of airborne bacterial particles ranged from 20 to 9780 CFU m⁻³ over the five sampling
246 campaigns. As for the fungal particles, three groups of caves can be distinguished regarding the
247 bacterial rates. The Combarelles I and La Mouthe caves showed a clearly fixed pattern despite
248 a small number of differences (Fig. 4). These patterns were cave dependent while the same
249 general pattern characterized by high rates at the entrance and a decrease with depth was
250 observed in the case of fungal particles. For example, the second and the fifth sampling points
251 definitely provided high bacterial rates in Combarelle I cave, despite the abnormally low values
252 recorded in October 2014 (second sampling point) and June 2015 (fifth sampling point).

253 A second group of caves consisting of Font-de-Gaume cave, Cap Blanc shelter and possibly
254 Combarelles II cave showed two different patterns according to the sampling season despite
255 some abnormal points.

256 Finally, a third group of caves, consisting of Mammouth, cave, Sorcier cave, Pigeonnier cave
257 and Reverdit shelter, showed no clearly fixed pattern of bacterial particles.

258 Unlike the fungal rates, the bacterial rates assessed outside the caves in October 2016 were not
259 systematically higher than the rates assessed inside the corresponding caves.

260 The GLMMs showed that the *sampling depth* and the *cave size* had an effect on bacterial counts
261 (Table 2). The rates counted outside the caves were significantly higher than the rates counted
262 at depth inside the caves (Table 3). Intermediate rates were recorded at the entrance of the caves.

263 In addition, the rates in small caves were significantly higher than the rates in large caves.

264

265 *3.2 Spatial distribution of the airborne microbiological particles in the caves: distance index*

266 3.2.1 Fungal particles

267 The linear correlation coefficient between the distance index and the fungal rates was
268 significant but low, showing a very poor linear relationship (Fig. 5a, Table 4). The quadratic
269 correlation coefficient was significantly better than the linear coefficient, but also remained
270 low. The RMSE associated to quadratic regression was also better than that associated to linear
271 regression, but the difference was not significant.

272 The breakdown of results according to the size of the caves showed that the trend observed on
273 all data was supported by the results of large caves, while no linear or quadratic relationship
274 can be established between distance index and fungal rates in small caves. The correlations
275 coefficient of the linear and quadratic regressions in large caves are better than the coefficients
276 obtained from the entire data set. The quadratic model was significantly better adapted to the
277 results than the linear model, even if the correlation remained weak. The RMSE associated to
278 the quadratic regression was lower than the RMSE associated to the linear regression, but the
279 difference was not significant.

280

281 3.2.2 Bacterial particles

282 No linear or quadratic relationship was established between the distance index and the bacterial
283 rates for small or large caves (Fig. 5b, Tables 4).

284

285 *3.3 Structure of fungal communities*

286 Over forty fungal genera were isolated during the five sampling campaigns, of which
287 *Cladosporium* (44.81 % of the isolated strains) and *Penicillium* (38.43 %) were the most
288 abundant (Table 5).

289 The first dimension of the Principal Component Analysis performed on all data explained 18.3
290 % of the observation variance, whilst the second dimension explained 9.5 %. The fungal genera
291 *Botrytis*, *Cladosporium*, *Epicoccum*, *Penicillium* and *Aspergillus* provided the highest positive
292 contribution to the construction of the first dimension (Table 6); the fungal genera
293 *Paecilomyces*, *Verticillium*, the sterile strains and the strains belonging to the Basidiomycetes
294 division provided the highest positive contribution to the construction of the second dimension,
295 whereas “Other genera” provided the highest contribution to the construction of the negative
296 part of the second dimension. No difference was observed in the fungal communities according
297 to the sampling season or to public access (Fig. 6). On the other hand, the fungal communities
298 of the large caves were very different from the communities of small caves. The small caves
299 shifted to positive values for both dimensions of the PCA. We can therefore consider new PCA
300 that were produced by separating small caves and large caves.

301 The first dimension of the PCA explained 23.5 % of the observation variance and the second
302 dimension explained 11.1 % in large caves, while the first dimension explained 18.1 % of the
303 variance and the second dimension explained 12.4 % in small caves. The fungal communities
304 of all large caves were clearly different from the fungal communities identified outside the
305 caves, while the Cap Blanc shelter was the only small cave for which fungal communities were
306 different from outside caves communities (Fig. 7). The fungal communities of large caves were
307 not different from each other, but the communities of Combarelles II and Mammoth caves
308 strongly shifted to positive values on the PCA first dimension. The fungal communities at the

309 entrance of and at depth in the large caves were different from the outside communities and
310 differed amongst themselves. Conversely, the fungal communities at the entrance of the small
311 caves were not different from outside communities. Moreover, the communities at depth of
312 small caves were different from outside communities but could not have been distinguished
313 from the communities at the entrance.

314

315 **4. Discussion**

316

317 *4.1 Size of the caves as the main factor affecting their aerobiological behavior*

318 The most valuable and important result in a framework of ornamented cave preservation was
319 that the aerobiological behavior of caves was mainly driven by cave size and environmental
320 factors rather than by public access. The effects of cave visitation on airborne biological
321 particles are already known. The presence of visitors generally causes an increase in airborne
322 fungal and bacterial particles (Wang et al. 2010a; Taylor et al. 2013; Fernandez-Cortes et al.
323 2011; Wang et al. 2010b; Bercea et al. 2018; Mulec et al. 2017). The composition and the
324 richness of microbial communities are also affected, notably through the introduction of non-
325 indigenous species (Pusz et al. 2018; Griffin et al. 2014). In our study, opening caves to visitors
326 had no effect on bacterial counts and the fungal counts in caves open to the public were
327 significantly lower than these in closed caves; indeed, the size of caves had an effect on both
328 bacterial and fungal counts, which were higher in small caves than in large caves. Moreover,
329 the study of the composition of fungal populations through PCA showed that the populations
330 of closed caves and caves open to the public were not significantly different.

331 *4.2 Aerobiological behavior in large caves*

332 4.2.1 General behavior: La Mouthe, Combarelles I and Font-de-Gaume caves

333 La Mouthe cave can be compared with Combarelles I and Font-de-Gaume caves. These three
334 caves are of comparable sizes. Combarelles I and Font-de-Gaume are open to the public while
335 La Mouthe is not, yet no significant differences were found in fungal and bacterial counts. Pusz
336 et al. (2015) observed the same result when comparing data for the recently discovered cave of
337 Jarkovicka, which was not open to visitors, to data published for other caves in the region. They
338 also conclude that tourist traffic was perhaps not the main driver of particle increase in cave air.
339 In our study, sampling was carried out outside visiting hours, either in the morning before the
340 cave opened to visitors or after the last visit of the day. It is probable that microbial counts
341 increased during visits, but the recovery capacity of the Combarelles I and Font-de-Gaume
342 caves was enough to mask any difference with non-visited caves, namely due to the way these
343 caves are managed. The management of these caves is meticulous, with a limited number of
344 visitors, controlled group-size and with the lights turned on only when necessary to decrease
345 the risks of microbial outbreaks that have occurred in other caves in the past (Dupont et al.
346 2007).

347 These three caves can therefore be classified as “self-purifying caves” (Leplat et al. 2018). Their
348 fungal aerobiological behavior showed the pattern defined for that type of cave: the highest
349 rates were counted at the cave entrance, but were lower than rates counted outdoors. The rates
350 then decreased with depth, before a final possible increase at the far end of the cave. This pattern
351 has already been observed in many caves (Garcia-Anton et al. 2013; Docampo et al. 2011;
352 Fernandez-Cortes et al. 2011; Griffin et al. 2014). All these studies underlined the importance
353 of external pressure on the dispersal of fungal spores inside caves, which can consequently

354 follow the air flow in the cave and be gradually deposited, mainly through gravitation (Kuzmina
355 et al. 2012; Taylor et al. 2014).

356 The drivers of bacterial behavior in the air of the cave were different, despite the clearly fixed
357 pattern of bacterial distribution for each cave. No link can be established between outdoor rates
358 of bacteria and the rates assessed inside the cave. The rates counted inside the caves were in
359 turn higher or lower than the outdoor rates, and no link could be made between bacterial rates
360 and the distance from the cave entrances. This result has already been observed in several
361 studies (Fernandez-Cortes et al. 2011; Bercea et al. 2018). Dredge et al. (2013) explained this
362 difference by the size of the particles and therefore by the difference in their aerial transport,
363 the transport of bacterial particles being consistent with that observed for aerosol between 0.1
364 and 10 μm . The pattern of bacterial distribution in each cave could also be the reflection of
365 environmental variations of temperature and humidity inside the cave as well as local variations
366 in available input matter or in aerosolization phenomenon (Wang et al. 2010b; Mulec et al.
367 2017)

368 The analysis of the fungal community compositions confirmed the results obtained through
369 fungal counts. *Cladosporium* and *Penicillium* were the two most abundant genera identified
370 inside and outside the caves, which was consistent with previous results (Ogórek 2018; Porca
371 et al. 2011; Man et al. 2018; Pusz et al. 2018). Along with *Epicoccum*, *Botrytis* and *Aspergillus*,
372 these two genera were strongly and positively involved in the building of the first dimension of
373 the PCA associated with large caves. These five genera are all known to be markers of outside
374 air (Ogórek et al. 2014). Therefore, the representation of outdoor fungal population strongly
375 shifted to positive values on the first dimension of PCA representation. The representation of
376 the fungal population at the cave entrance was significantly different from the outdoor

377 population, despite being strongly affected by the external environment. Finally, the
378 representation of the population at depth shifted to negative values on the PCA first dimension,
379 as these populations were less affected by external environment and were certainly more
380 representative of the fungal endemic population of caves (Leplat et al. 2019).

381

382 4.2.2 Unusual behaviors: Combarelles II and Mammoth caves

383 Combarelles II and Mammoth can be considered large caves in view of the definition given at
384 the outset of this study, but they seemed to have a different behavior to those observed in La
385 Mouthe, Combarelles I and Font-de-Gaume caves. The fungal counts were moderately higher
386 in Combarelles II cave and significantly higher in Mammoth cave than in the three other large
387 caves. Moreover, the representation of the fungal populations of these two caves on the PCA
388 showed that they were more affected by the first dimension of the PCA than other large caves
389 were.

390 In case of Combarelles II cave, this observation was the result of an experimental artefact. The
391 only point sampled in Combarelles II was located at the entrance of the cave, which was not
392 tested at depth. The results about this cave were therefore only comparable with the results
393 obtained at the entrance of other large caves. We can reasonably assume that the results for this
394 cave would have been closer to the results obtained for other large caves if the sampling had been
395 more complete.

396 The results are much more interesting in case of Mammoth cave, which was sampled along
397 its full length. Several factors could explain the high rates of fungal particles counted in this
398 cave, which cannot be affected by visitors since the cave is closed to the public. First, the cave
399 is divided into two parts separated by a wall. The first part of the cave could be therefore

400 considered as a small cave. Second, the ceiling of the second part of the cave is covered by the
401 roots of the vegetation growing on ground level above the cave. This factor has already been
402 associated with high fungal rates (Leplat et al. 2018), probably by providing a favourable
403 oligotrophic habitat and a stock of available organic matter (Saiz-Jimenez 2012; Jurado et al.
404 2009; Vanderwolf et al. 2013). Along with cave size, this result underlined the importance of
405 environmental factors in the aerobiological behavior of caves and the difficulty to establish a
406 general pattern for this behavior even for caves with comparable sizes.

407

408 *4.3 Aerobiological behavior in small caves*

409 While all the large caves except the Mammoth cave could be classified as “self-purifying
410 caves”, the small caves can be classified as “non-self-purifying caves”. In other words, they
411 were directly affected by outside air along their full length. These caves exhibited no clear
412 distribution of fungal particles: for caves where several points were sampled, the highest fungal
413 rates were counted either at the entrance or the far end of the cave, so no link could be
414 established between the fungal rates and the distance from the entrance. The small size of these
415 caves certainly did not allow the gradual discharge of fungal propagules that occurred in large
416 caves where the fungal rates are high at the entry before collapsing (Griffin et al. 2014; Garcia-
417 Anton et al. 2013). Therefore, the fungal rates in small caves were mostly representative of the
418 variation of propagules amount in outside environment (Docampo et al. 2011), as well as of the
419 air entrance when opening the door (Fernandez-Cortes et al. 2011), than the reflection of a
420 behavior specific to the caves.

421 The study of the fungal population composition in small caves confirmed the strong influence
422 of outside air along their full length: there was no significant difference between the population

423 at the cave entrances and the population at depth. Nor was any significant difference observed
424 between the populations inside the small caves and the population outside the caves, with the
425 exception of the population of Cap Blanc shelter, which strongly shifted to positive values on
426 the first dimension of the PCA unlike other populations.

427 *Aspergillus*, *Verticillium*, *Paecilomyces* and *Engyodontium* were the fungal genera involved the
428 most in the building of this first PCA dimension for small caves, and were isolated in the Cap
429 Blanc Shelter. The genera *Verticillium*, *Paecilomyces* and *Engyodontium* could effectively be
430 representative of the cave environment, since several species in these genera are known to be
431 enthomogenous (Shah and Pell 2003; Jurado et al. 2008). This kind of fungi has already been
432 found to play a major role in cave environment (Pusz et al. 2018; Bastian et al. 2009; Bastian
433 et al. 2010). The rate of fungi counted in Cap Blanc shelter was relatively low in comparison
434 with the rates observed in other small caves, even if the difference was not significant. Cap
435 Blanc shelter therefore appeared to be less affected by the outside environment than other small
436 caves. This certainly due to the very particular layout of the shelter, which is open to visitors.
437 A building has been built directly around the shelter. Visitors first enter the building and then
438 pass a second door to enter the shelter. In this way, the shelter is never in direct contact with
439 the outside environment, as the building plays the role of a large airlock. Airlocks have already
440 been proved to be a suitable barrier to limit the impact of external environment in caves, both
441 in terms of climatological stability and of inputs of fungal and bacterial particles (Cigna 1993;
442 Houillon et al. 2017; Porca et al. 2011).

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446 *4.4 Sorting of the caves according to risk index*

447 (Porca et al. 2011) proposed an index based on the concentration of fungal spores in cave air,
448 sorting them into five risk categories ranging from caves with no fungal problem to irreversibly
449 disturbed caves. Based on averages values, four out of the nine caves in our study would be
450 classified in category 3, two in category 4 and three in category 5. Porca et al. (2011) explained
451 that categories 4 and 5 seem to occur when the cave is suffering a fungal outbreak or massive
452 visits. In our study, three of the five caves sorted in categories 4 and 5 are not open to the public,
453 while three out of the four caves sorted in category 3 are open to the public. Indeed, the caves
454 were almost sorted according to their total volume with the exceptions noted before: counts
455 were unusually high in Mammoth cave, which is a large cave, and counts were unusually low
456 in Cap Blanc shelter, which is a small cave. In general, it may even be difficult to attain the
457 safest category of the index, defined as fungal rates under 50 CFU m^{-3} , in caves that are closed
458 to visitors (Pusz et al. 2015; Leplat et al. 2018). As interesting as this index is, it probably does
459 not sufficiently consider the total volumes of the caves and the high specificity that can occur
460 in each cave, as already noted in the literature (Pusz et al. 2018). It nevertheless remains crucial
461 to carry out meticulous monitoring, particularly in small caves that are open to the public.

462

463 **5. Conclusions**

464

465 This study shows that in a context of decorated caves that are carefully managed for public
466 access, the size of the cave has more effect on the fungal and bacterial rates in the air of the
467 caves than opening the sites to visitors. The results confirm that aerobiological behavior in large
468 caves follows a cave-dependent fixed pattern. The large caves can generally be classified as

469 “self-purifying caves” since they are strongly affected by the exterior environment at their
470 entrance but much less so at depth. On the contrary, it was practically impossible to determine
471 a fixed pattern of aerobiological behavior in small caves that are strongly affected by the
472 outdoor environment. The small caves can therefore be classified as “non-self-purifying caves”.
473 The presence of an airlock could buffer the influence of outdoor environment, as observed in
474 Cap Blanc shelter. Finally, this study also confirms that the fungal airborne rates proposed in
475 past studies for healthy caves should be reviewed in greater detail to take the individual
476 characteristics of caves into account.

477

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479

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652 **Table 1** Description of caves managed during the study.

Cave	Discovery	Historical monuments list	Public access	Number of sampled points	Date of sampling				
					October 2013	June 2014	October 2014	June 2015	October 2016
Cap Blanc shelter	1909	1910 ¹	Open	2 (+1)					
Combarelles I cave	1901	1902 ¹	Open	6 (+1)	*				
Combarelles II cave	1934	1943 ¹	Closed	1 (+1)					
Font-de-Gaume cave	1901	1902 ¹	Open	5 (+1)					
La Mouthe cave	1895	1953 ¹	Closed	5 (+1)					
Mammoth cave	1978	1983	Closed	3 (+1)					
Pigeonnier cave	1978	1983	Closed	1 (+1)					
Reverdit shelter	1923	1924	Open	1					
Sorcier cave	1952	1958 ¹	Open	2 (+1)					

653 The sampling dates are indicated by grey boxes.

654 ¹ Site designated as World Heritage Site by UNESCO in 1979

655 () Control points were added outside the caves in October 2016.

656 *The fifth sampling point of Combarelles I cave was not assessed in October 2013.

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669 **Table 2** Effect of qualitative variables on fungal and bacterial counts assessed with Wald χ^2
 670 test associated to GLMMs.

<i>Source of variation</i>	df	<i>Fungi</i>		<i>Bacteria</i>	
		χ^2 -value	p-value	χ^2 -value	p-value
<i>Sampled Cave (C)</i>	8	62.0	***	13.4	0.1
<i>Sampling depth (D)</i>	2	61.3	***	6.7	0.003
<i>Sampling season (S)</i>	1	3.0	0.09	0.03	0.9
<i>Cave size (Si)</i>	1	5.8	*	8.7	**
<i>Public access (P)</i>	1	4.1	*	2.6	0.11

671 * p < 0.05, ** p < 0.01, *** p < 0.001

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689 **Table 3** Effect of significant qualitative variables on fungal and bacterial counts (CFU m⁻³) –
 690 Comparisons of the means of the different modalities assessed with Tukey’s post-hoc test
 691 associated to GLMMs.

Fungi							
<i>Sampled cave (***)</i>		<i>Sampling depth (***)</i>		<i>Cave size (*)</i>		<i>Opening (*)</i>	
Sorcier cave	1854b	Outside cave	3318c	Small caves	1103b	Closed cave	739b
Mammoth cave	1309b	Cave entry	1130b	Large caves	491a	Open cave	560a
Reverdit shelter	1005ab	Deep cave	367a				
Pigeonnier cave	919ab						
Combarelles II cave	846ab						
Cap Blanc shelter	482ab						
Combarelles I cave	379a						
La Mouthe cave	339a						
Font-de-Gaume cave	210a						

Bacteria			
<i>Sampling depth (*)</i>		<i>Cave size (**)</i>	
Outside cave	780b	Small caves	543b
Cave entry	436ab	Large caves	324a
Deep cave	345a		

692 * p < 0.05, ** p < 0.01, *** p < 0.001

693 Different letters in the columns indicate significant differences.

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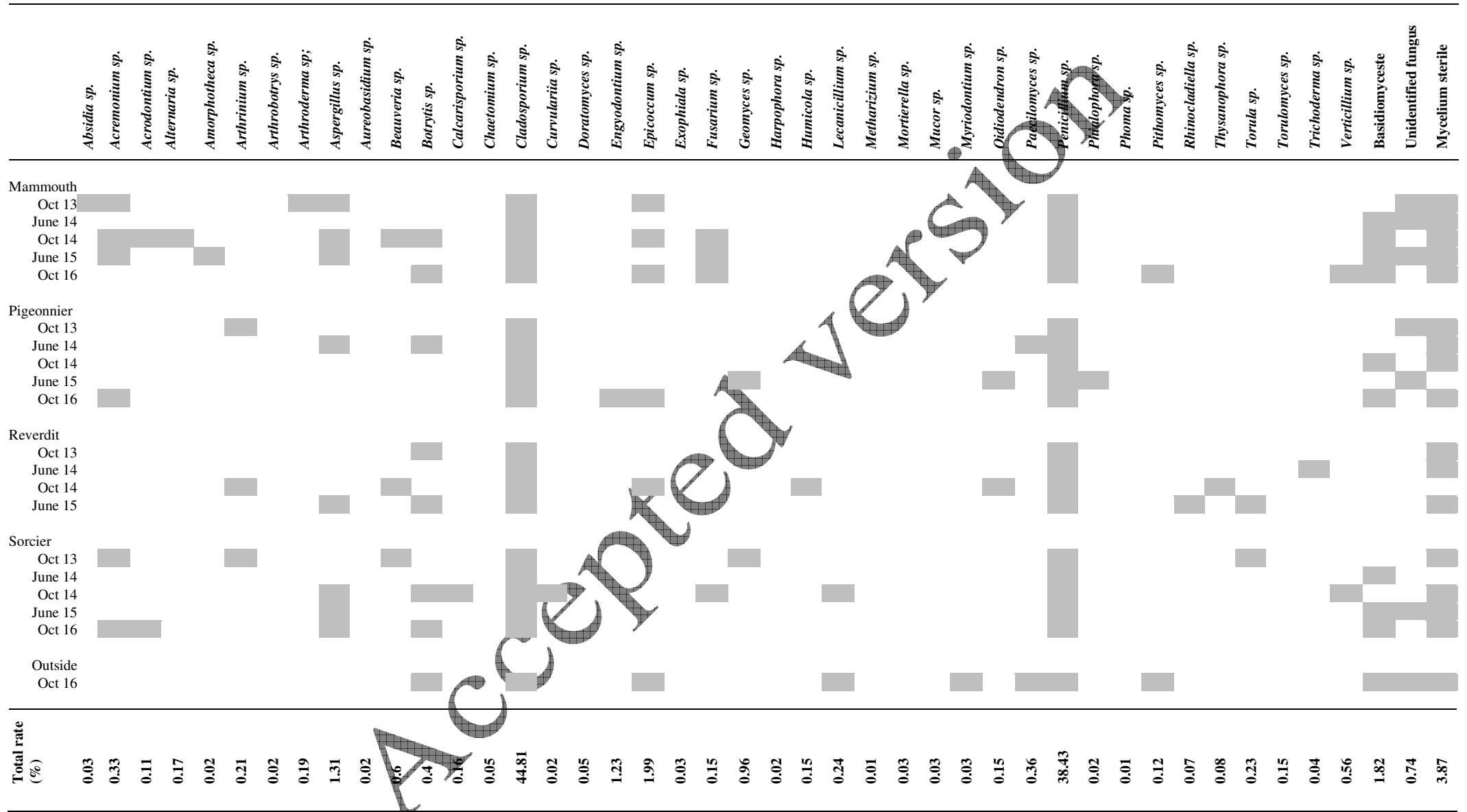
703

704 **Table 4** Comparison of the regression models between distance index and counts of airborne
 705 microorganisms.

Fungi						
Type of regression	<i>All data</i>		<i>Large caves</i>		<i>Small caves</i>	
	r ²	RMSE (CFU m ⁻³)	r ²	RMSE (CFU m ⁻³)	r ²	RMSE (CFU m ⁻³)
Linear regression	0.09***a	636a	0.15***a	613a	0.004a	589a
Quadratic regression	0.15***b	613a	0.21***b	590a	0.02a	582a
Bacteria						
Type of regression	<i>All data</i>		<i>Large caves</i>		<i>Small caves</i>	
	r ²	RMSE (spores.m ⁻³)	r ²	RMSE (spores.m ⁻³)	r ²	RMSE (spores.m ⁻³)
Linear regression	0.005a	346a	0.005a	269a	7.10 ⁻⁴ a	482a
Quadratic regression	0.02a	342a	0.04a	265a	8.10 ⁻⁴ a	482a

706 * p < 0.05, ** p < 0.01, *** p < 0.001

707 Different letters in the columns indicate significant differences.



GenBank accession number
MF788181
J86
MF788183
MF788184
J22
J114
MF788185
MF788186
MF788188
MF788189
MF788191
MF788192
MF788196
MF788197
MF788198
MF788199
MF788200
MF788201
MF788203
MF788204
MF788210
J27
MF788211
MF788213

Grey boxes indicate the identification of fungal genera.

Fungal genera without accession number were identified according to their morphological features only.

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711 **Table 6** Contribution of the most valuable variables to the construction of the two PCA
 712 dimensions – Supplementary qualitative variables significantly affected by the two PCA
 713 dimensions

All data											
Dimension 1						Dimension 2					
Quantitative variable		Cor	p-value			Quantitative variable		Cor	p-value		
<i>Botrytis sp.</i>		0.82	***			<i>Paecilomyces sp.</i>		0.55	***		
<i>Cladosporium sp.</i>		0.82	***			<i>Mycelium sterile</i>		0.54	***		
<i>Epicoccum sp.</i>		0.79	***			<i>Verticillium sp.</i>		0.47	***		
<i>Penicillium sp.</i>		0.51	***			Basidiomycete		0.44	***		
<i>Aspergillus sp.</i>		0.39	***			"Other genera"		-0.28	***		

Large caves						Small caves					
Dimension 1			Dimension 2			Dimension 1			Dimension 2		
Quantitative variable	Cor	p-value	Quantitative variable	Cor	p-value	Quantitative variable	Cor	p-value	Quantitative variable	Cor	p-value
<i>Botrytis sp.</i>	0.9	***	"Other genera"	0.66	***	<i>Aspergillus sp.</i>	0.85	***	<i>Botrytis sp.</i>	0.62	***
<i>Epicoccum sp.</i>	0.89	***	<i>Engyodontium sp.</i>	0.46	***	<i>Verticillium sp.</i>	0.76	***	<i>Beauveria sp.</i>	-0.59	***
<i>Cladosporium sp.</i>	0.85	***	<i>Paecilomyces sp.</i>	-0.45	***	<i>Cladosporium sp.</i>	-0.43	*	<i>Epicoccum sp.</i>	0.52	**
<i>Penicillium sp.</i>	0.77	***	<i>Acremonium sp.</i>	0.43	***	<i>Paecilomyces sp.</i>	0.43	*	<i>Cladosporium sp.</i>	0.48	**
<i>Aspergillus sp.</i>	0.51	***	Basidiomycete	-0.41	***	<i>Engyodontium sp.</i>	0.40	*	"Other genera"	-0.47	*

714 Cor: Correlation coefficient

715 * p < 0.05, ** p < 0.01, *** p < 0.001

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726 **Fig. 1** Cave locations (graphics programs: R, package ggmap + Inkscape).

727 The nine sites are located in the Dordogne area, France. Seven of the caves, i.e. Sorcier cave,
728 La Mouthe cave, Font-de-Gaumes caves, Combarelles I and II caves Cap Blanc shelter and
729 Reverdit shelter are located in the valley of the Vézère. The two others, i.e. Mammouth cave
730 and Pigeonnier cave, are located in the Dordogne valley.

731 The maps were generated from Google Earth (Google, Mountain View, USA, (Kahle and
732 Wickham 2013) on the basis of geographical coordinates supplied by the French Ministry of
733 Culture.

734

735 **Fig. 2a and b** Sampling maps of the nine studied caves from Leroi-Gourhan (1984; graphics
736 program: Inkscape).

737 Each number represents one sampling point where fungal and bacterial rates were assessed.
738 The sampling points were chosen in such a way as to cover the widest possible surface of the
739 caves.

740

741 **Fig. 3** Counts of airborne fungal particles in each cave for the five sampling dates (graphics
742 program: R).

743 The term *NA* is used for *Non Available* data: it indicates that the count has not been assessed.

744

745 **Fig. 4** Counts of airborne bacterial particles in each cave for the five sampling dates (graphics
746 program: R).

747 The term *NA* is used for *Non Available* data: it indicates that the count has not been assessed.

748 **Fig. 5** Study of the relationship between airborne microbiological particles and the distance
749 index: fungi (a), bacteria (b) (graphics program: R).

750 The study first considered all data, then divided the data according to cave size. In each case,
751 linear and quadratic regressions were applied to the data collected.

752

753 **Fig. 6** Representation of the fungal communities according to selected supplementary
754 qualitative variables of the PCA performed on all data (graphics program: R).

755 All data are represented according to three supplementary qualitative variables used in PCA:
756 Sampling season (a), cave size (b) and public access (c). The colored circles represent the
757 probable position of the observation barycenter associated to each modality.

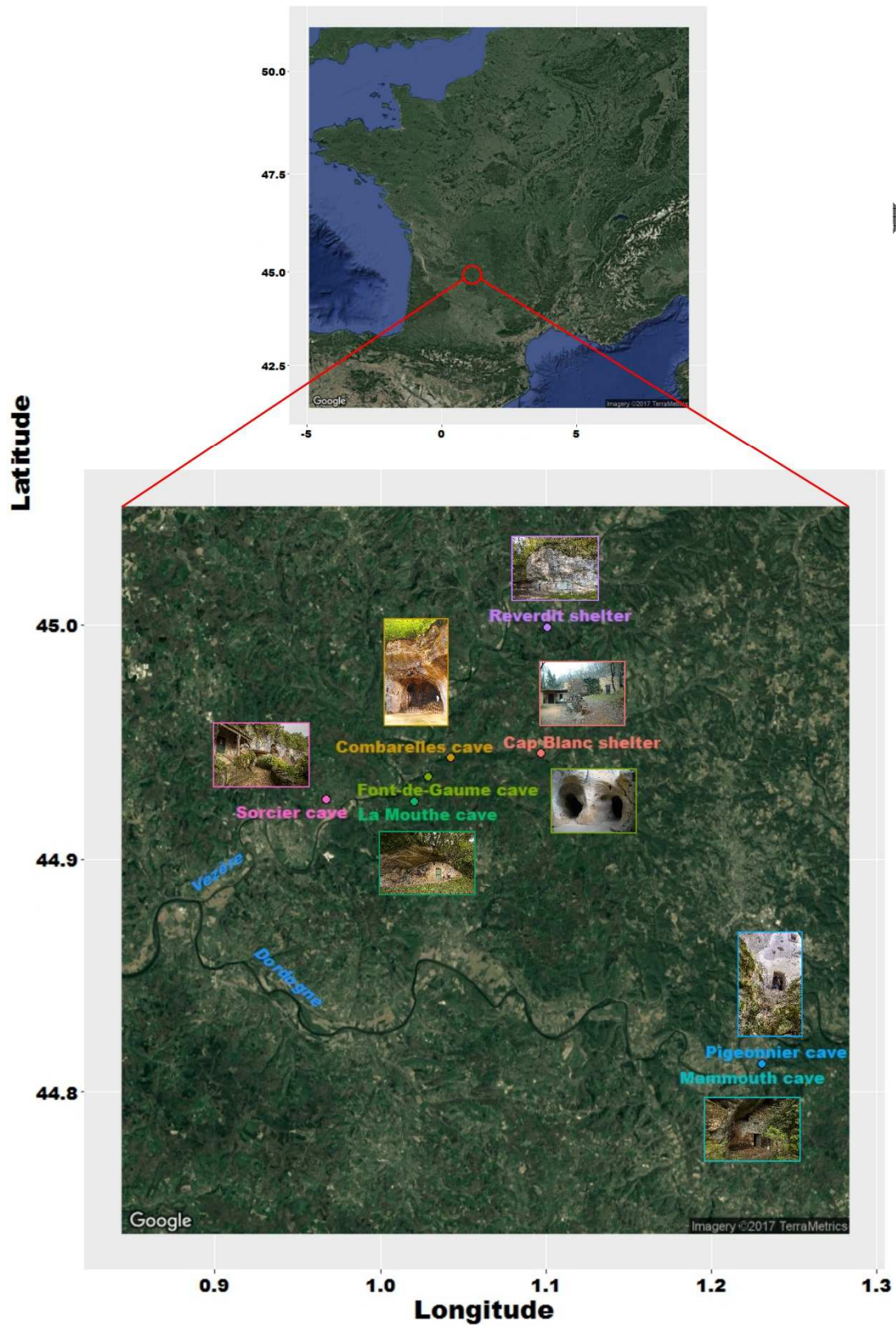
758

759 **Fig. 7** Representation of the fungal communities according to selected supplementary
760 qualitative variables of the PCA performed according to cave size (graphics program: R).

761 The data are shown for large caves (a) and small caves (b) according to two supplementary
762 qualitative variables used in PCA: *sampled cave* and *sampling depth*. The colored circles
763 represent the probable position of the observation barycenter associated to each modality.

764 Fig. 1

765



766 **Fig. 2**

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Cap Blanc shelter

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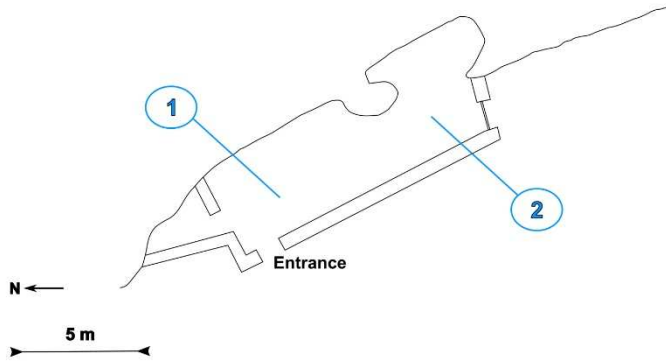
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Combarelles caves

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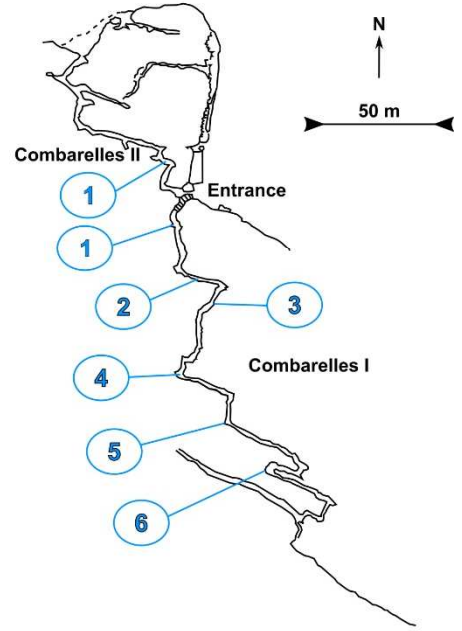
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Font-de-Gaume cave

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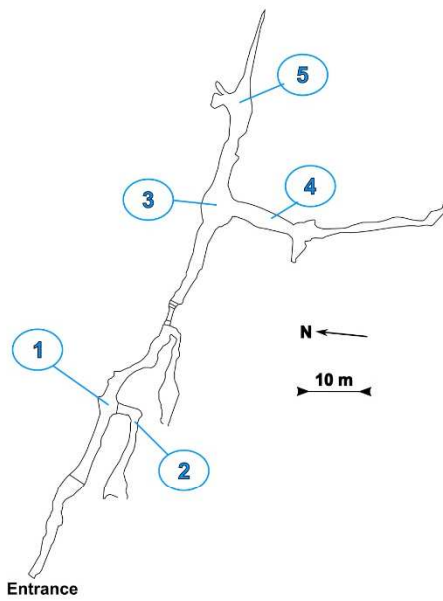
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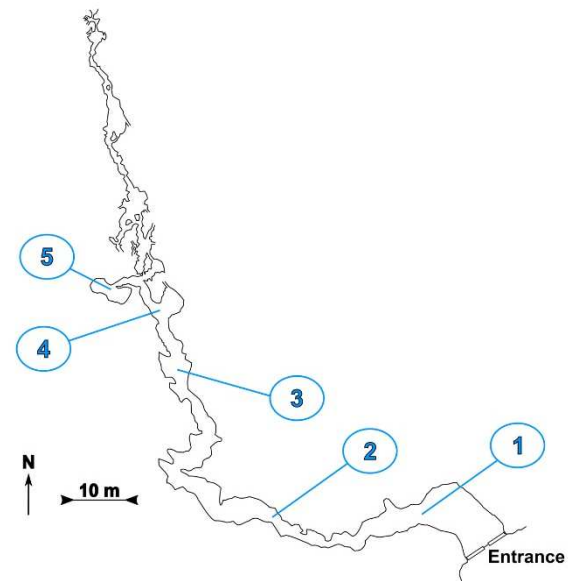
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La Mouthe cave

(a)

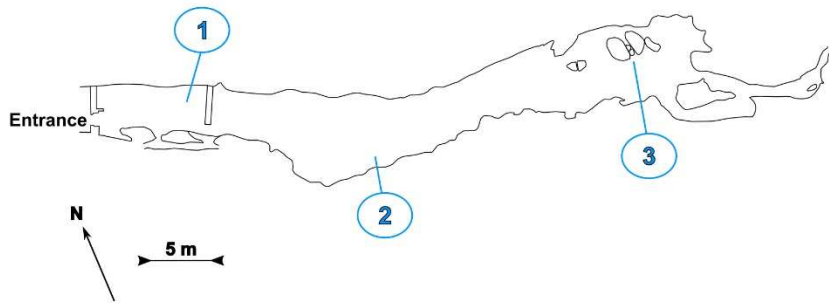


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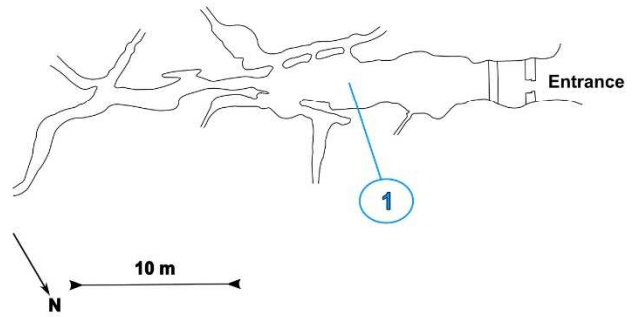
Mammouth cave

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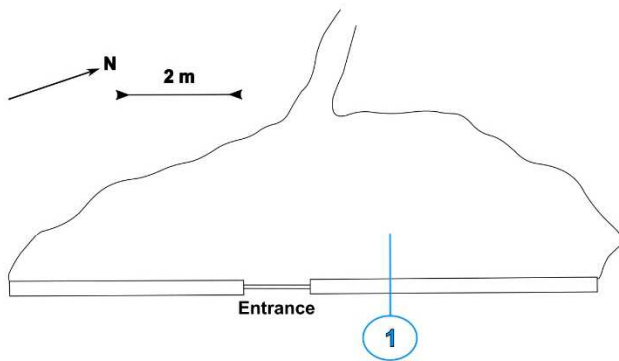
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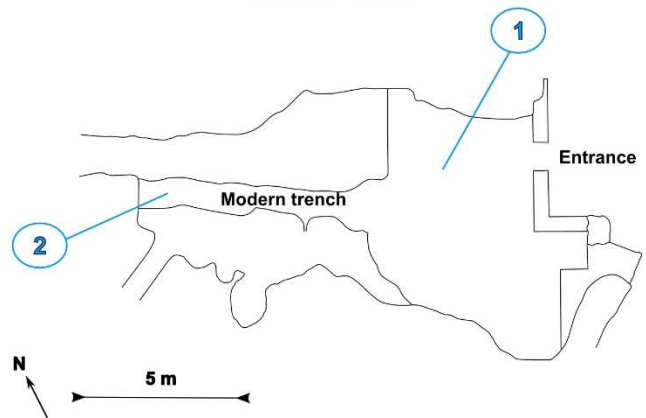
Pigeonnier cave



Reverdit shelter

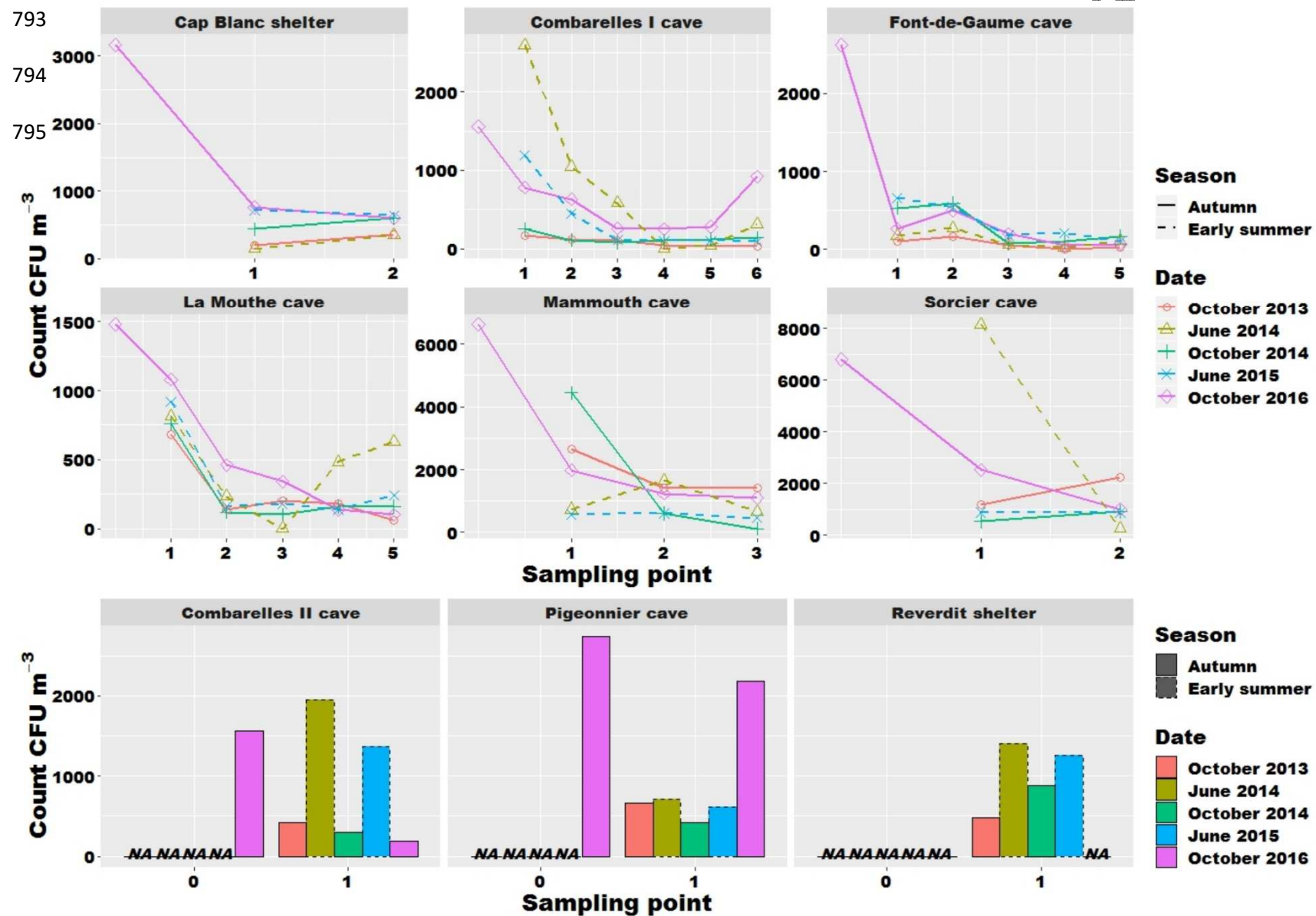


Sorcier cave

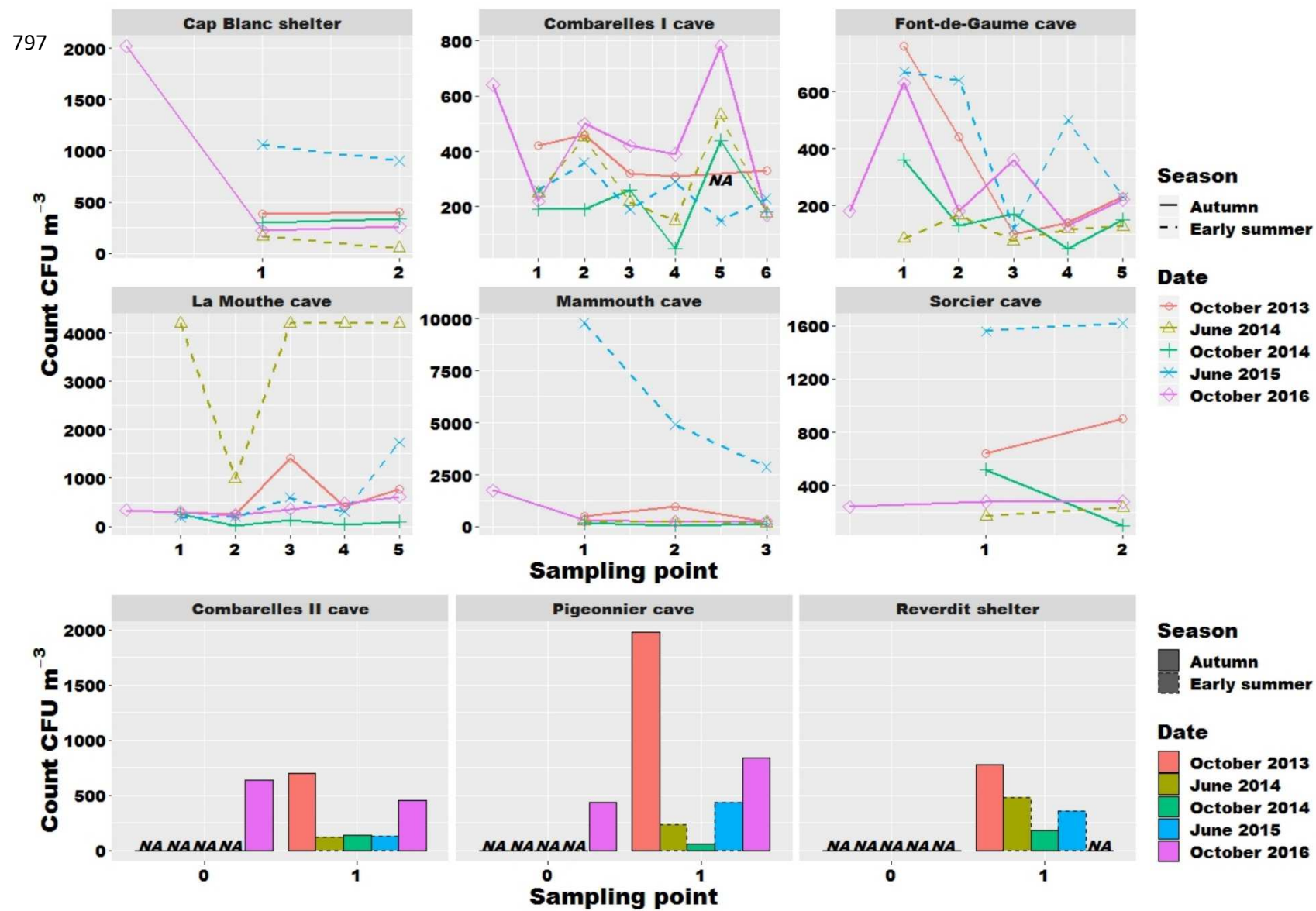


(b)

792 Fig. 3

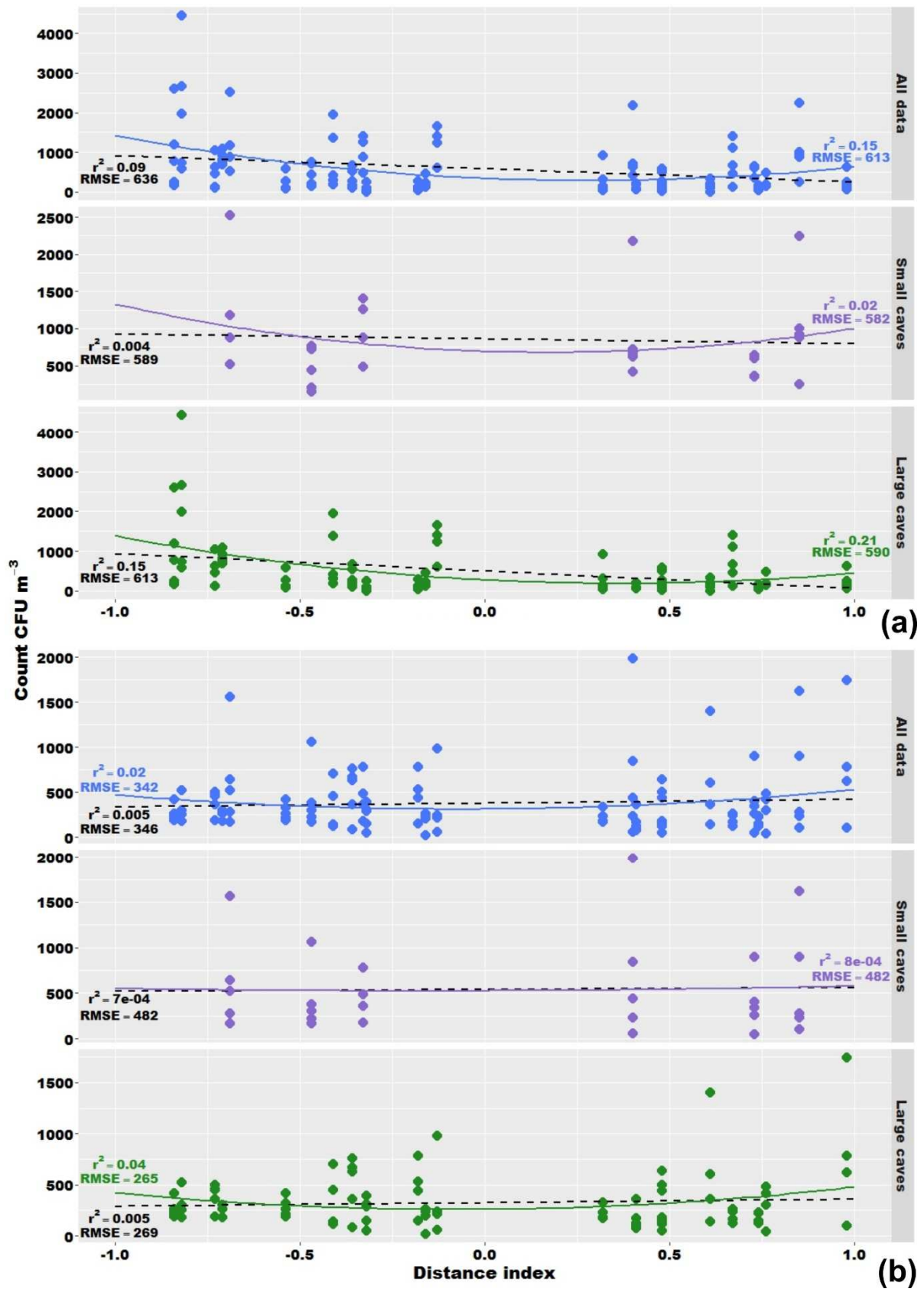


796 Fig. 4



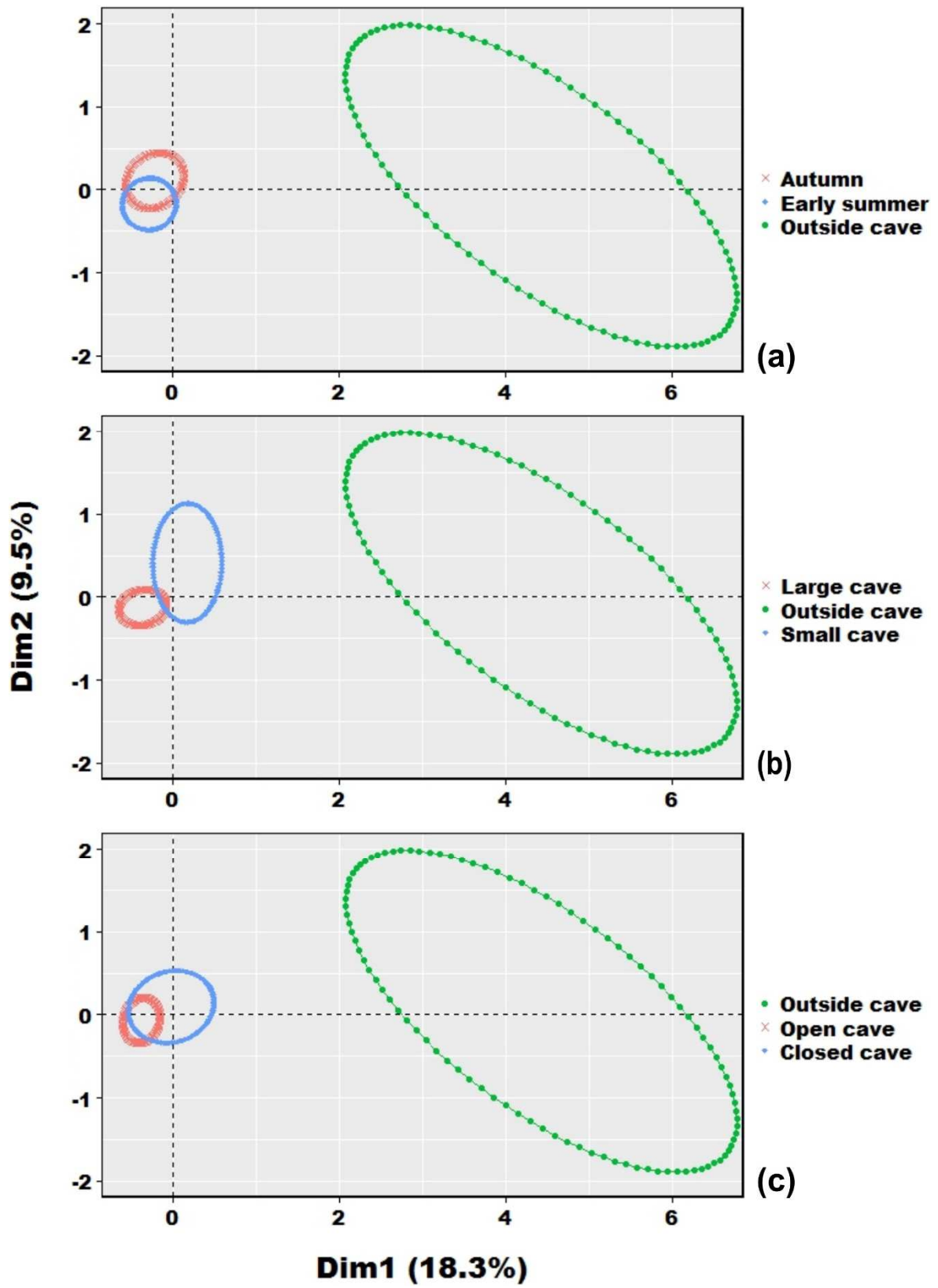
798 Fig. 5

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800 Fig. 6

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802 Fig. 7

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