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1 **Aerobiological behavior of Paleolithic decorated caves: a comparative study**
2 **of five caves in the Gard department (France)**

3

4 Johann Leplat^{ab1}, Alexandre François^{ab}, Stéphanie Touron^{ab}, Philippe Galant^c, Faïçal Boust^{ab}

5

6 ^a Laboratoire de Recherche des Monuments Historiques (LRMH), Ministère la Culture et de la
7 Communication, 29 rue de Paris, 77420 Champs-sur-Marne, France

8 ^b Sorbonne Universités, Centre de Recherche sur la Conservation (CRC, USR 3224), Muséum
9 national d'Histoire naturelle, Ministère de la Culture et de la Communication, CNRS; CP21, 36
10 rue Geoffroy-Saint-Hilaire, 75005 Paris, France

11 ^c Direction Régionale des Affaires Culturelles de la région Occitanie, Service Régional de
12 l'Archéologie, 5 rue Salle l'Evêque, 34000 Montpellier

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14

15

16 **Keywords**

17

18 Preservation of cultural heritage, decorated cave, air quality, microbiological airborne particles,
19 fungal diversity, environmental conditions

20

¹ Corresponding author: johann.leplat@culture.gouv.fr

Laboratoire de Recherche des Monuments Historiques (LRMH), Ministère la Culture et de la
Communication, 29 rue de Paris, 77420 Champs-sur-Marne, France

Tel: +33 1 60 37 77 97

21 **Abstract**

22 The preservation of decorated caves is a great challenge that has been reinforced by the
23 microbial crisis occurring at emblematic sites such as the Lascaux and Altamira caves. To date,
24 little is known about the microbial behavior of caves. One difficulty is that the term ‘cave’
25 covers a wide variety of sites and structures. Past studies concentrated on caves with microbial
26 disorders and were generally carried out in a time frame that was too short to gain an adequate
27 picture of healthy microbial behaviors, the *sine qua non* of detecting potential imbalance. The
28 aim of this study was to determine whether models of aerobiological behavior could be
29 identified in several decorated caves. We monitored aerial rates of fungi and bacteria in five
30 decorated caves of the Gard department (France) for periods of two years or more. A model
31 behavior was identified in each cave. These models were cave dependent, and were also season-
32 dependent in some caves. Following our fungal results, we suggest separating the caves into
33 two groups. The caves which were strongly affected by the external environment throughout
34 their entire length are the “non-self-purifying caves”. The caves which were only affected by
35 external environment at the entrance are the “self-purifying caves”.

37 **1. Introduction**

38
39 *Homo sapiens* (modern human) is currently believed to have appeared 200,000 years ago in
40 Africa (Stringer and Galway-Witham 2017). Several migrations from this original cradle are
41 thought to have led to the colonization of the whole Earth. Thus, the arrival of *Homo sapiens*
42 in Europe is estimated to have occurred around 45,000 years ago at the end of the Middle
43 Paleolithic (deMenocal and Stringer 2016). Numerous examples of the Paleolithic activity of
44 *Homo sapiens*, remain in France, of which caves rock art is the most striking example. The
45 Occitanie region is particularly noteworthy due to the quantity and the quality of its sites,

46 particularly in Ardèche and Gardon canyons (Leroi-Gourhan 1984). Some of the first
47 inventoried decorated sites are located in this region, including the Chabot cave, which was
48 discovered in 1878 (Abadía and Morales 2004). The region is remarkable for the age of its cave
49 art, which dates back to the first part of the Upper Paleolithic (Aurignacian, ~ 43 000 – 26 000
50 before present).

51 The preservation of this heritage is a major concern, particularly since the occurrence of
52 microbial outbreaks on the walls of the emblematic sites of Altamira and Lascaux caused by
53 algal, bacterial and/or fungal biofilms (Saiz-Jimenez et al. 2011; Bastian and Alabouvette
54 2009). These outbreaks were attributed to elements such as lighting for visiting tourists, which
55 favored algal growth, and excessive human presence in the caves and ventilation problems,
56 which favored bacterial and fungal growth (Dupont et al. 2007). All these factors represent
57 disturbances for an habitat which was often confined and had experienced very few exchanges
58 with the outdoor environment over several thousand years (Bourges et al. 2014). Although
59 caves are a distinctive habitat characterized by darkness, relatively stable low temperatures,
60 high moisture levels and limited amounts of easily degradable organic matter (Northup and
61 Lavoie 2001), microbial life can nevertheless be abundant (Pedersen 2000). Several studies
62 have sought to characterize this microbial life on the rock surface (Canaveras et al. 2001;
63 Schabereiter-Gurtner et al. 2004) as well as in cave air (Martin-Sanchez et al. 2014; Docampo
64 et al. 2011).

65 Despite this interest in cave microbiology, the relationship between microbiological rates and
66 the risks of rock art deterioration remains to be studied. Indeed, most of the studies focused on
67 caves after a microbial outbreak or investigated microbial presence in caves that were open to
68 visitors. Porca et al. (2011) made a first step towards explaining this relation by proposing to
69 rank the caves according to their airborne fungal rates into five categories of risk, ranging from
70 caves with no fungal problem to caves that have been irreversibly disturbed. The study mainly

71 focused on caves that had previously been visited by tourists or were still open to visitors.
72 However, the difficulty in the preservation of rock art heritage does not only depend on human
73 presence or absence, but also lies in the diversity of situations that are met in the field. Decorated
74 sites vary in length, ranging from rock shelters to caves that are several hundred meters long.
75 The volumes of caves are also very diverse, with some sites that have low ceiling tunnels and
76 others with “cathedral” halls. The geometry of caves strongly affects their climatic
77 characteristics (De Freitas and Littlejohn 1987; De Freitas et al. 1982). Cave art is also diverse,
78 including paintings composed of different pigments, but also engravings or sculptures. It is
79 therefore difficult to produce general rules of preservation from study cases. There is a real need
80 to improve current knowledge of microbiological behavior in caves, including healthy caves
81 which are not open to the public, to understand their natural behavior.

82 The aim of this study was to increase the knowledge of the aerobiological behavior in rock art
83 caves by monitoring the five decorated caves of the Gard department (Occitanie region, France)
84 for two and a half years. The study sought to determine if a model behavior was identifiable for
85 each cave for fungi and bacteria. The study should also determine if a generalization could be
86 obtained from these models.

87

88 **2. Materials and Methods**

89

90 *2.1 Samplings*

91 Airborne microbiological particles of the five decorated caves of the Gard department (France),
92 i.e. Bayol cave, Chabot cave, Latrone cave, Oulen cave and Points cave, were monitored during
93 the study (Fig. 1). These five caves have never been open to the public (excepted for scientific
94 studies). No major microbiological outbreak has occurred in these caves, which can therefore
95 be considered representative of undisturbed caves. Five season-based surveys were conducted

96 to evaluate the rates of airborne microorganisms in each of the caves studied. Two sampling
97 periods were carried out during winter (January 2014 and February 2015); two others were
98 carried out during late summer (September 2014 and September 2015); and the final sampling
99 period was carried out during late spring (June 2016). According to the accessibility of the
100 caves, three out of the five caves were tested during the five sampling periods. One cave was
101 tested in four sampling periods; and the remaining cave was tested in just two sampling periods.
102 At each sampling period, three to five points were sampled in each cave according to the size
103 of the caves (Table 1; Fig. 2).

104 A Duo SAS Super 360 air sampler (VWR-pbi, Milan, Italy) was used to detect cultivable
105 microorganisms in cave air. At each sampling point, 50 L of air was drawn through a 219-hole
106 impactor containing appropriate culture media in 55 mm Petri dishes. Bacteria were isolated on
107 nutrient agar (Merck KGaA, Darmstadt, Germany), and fungi were isolated on malt extract agar
108 (Merck KGaA, Darmstadt, Germany). The Petri dishes containing impacted media were then
109 taken to the laboratory for analysis.

111 *2.2 Counting of cultivable microorganisms*

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

119

120

121 *2.3 Identification of fungal strains*

122 Fungal strains were identified up to the genus, whereas bacterial strains were only counted but
123 not identified.

124 For identifications, fungal strains were sub-cultured on malt extract agar and separated from
125 each other. The resulting isolated fungal strains were identified to the genus level by
126 crosschecking their macroscopic and microscopic characteristics with those listed in reference
127 books (Domsch et al. 1980; Seifert et al. 2011).

128 Genera that could not be distinguished by their morphology were identified through molecular
129 identification. DNA was extracted as described by (Edel et al. 2001). ITS1F/ITS4 primers
130 (Gardes and Bruns 1993; White et al. 1990) were used to amplify the nuclear ribosomal internal
131 transcribed spacer (ITS) region. PCR was performed in 25 μ l reactions, with 1 μ l of template
132 DNA, 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, USA), 2.5 μ l of 10X Taq DNA
133 polymerase buffer, 1 μ l of 2 mmol l⁻¹ dNTPs (Thermo Scientific, Waltham, USA) and 1.5 μ l of
134 each 10 μ mol l⁻¹ primer (Eurogentec, Seraing, Belgium). Amplifications were performed on a
135 PrimeG thermocycler (Bibby scientific, Stone, UK) using the following parameters: a 4-minute
136 step at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 40 s at 72 °C, and a
137 final 10- minute extension at 72 °C. PCR products were sequenced by Genoscreen (Lille,
138 France) using the same ITS1/ITS4 primer set. Isolates were identified by BLAST comparison
139 to the NCBI GenBank database (Altschul et al. 1990).

140

141 *2.4 Statistical analyses*

142 2.4.1 Analyses of airborne microbiological particle counts

143 Statistical analyses were carried out using R 3.2.5 with $\alpha = 5 \%$ (R Core Team 2014). The
144 results of microbiological counts were analyzed by a generalized linear mixed model (GLMM).
145 GLMMs are commonly used to analyze data from ecological surveys (Bolker et al. 2009). They

146 are suitable means to analyze non-normal data collected from complicated designs, which
147 involve random and fixed effects.

148 Three qualitative variables were used to design the GLMM, namely the *sampled cave* (five
149 modalities, each corresponding to one of the five studied caves); the *sampling season* (three
150 modalities: *Winter* for January 2014 and February 2015, *Late spring* for June 2016, and *Late*
151 *summer* for September 2014 and 2015); and the *sampling depth* (*Entrance* for the first sampled
152 point in each cave, and *Deep cave* for all other points).

153 The GLMM for this study was:

$$154 \text{Link}(p_{ijkl}) = \eta_{ijkl} = \theta + \alpha_i + \beta_j + \gamma_k + \alpha_i:\gamma_k + \beta_j:\gamma_k + \mu_{ik} + \varepsilon_{ijkl}$$

155 where θ is the general effect, α_i is the effect of the *sampled cave*, β_j is the effect of the *sampling*
156 *season*, γ_k is the effect of the *sampling depth*, $\alpha_i:\gamma_k$ is the effect of the interaction between the
157 *sampled cave* and the *sampling depth*, $\beta_j:\gamma_k$ is the effect of the interaction between the *sampling*
158 *season* and the *sampling depth*, μ_{ik} is the experimental error associated to each sampled point
159 and ε_{ijkl} is the residual error. The effect of the interaction between the *sampled cave* and the
160 *sampling season* has not been included, because Oulen cave was not sampled during late spring.
161 α , β , γ , $\alpha:\gamma$ and $\beta:\gamma$ were considered as fixed effects while μ was considered as a random effect.

162 The term η_{ijkl} is known as the linear predictor. This term is obtained by submitting the variable
163 p_{ijkl} to the link function of the model. The choice of the link function depends on the distribution
164 law of p_{ijkl} . The shapes of the distributions for bacterial and fungal counts were checked before
165 applying the GLMM. The negative binomial link was then chosen to study these counts. This
166 distribution is well adapted to counts with over dispersed data (Booth et al. 2003). The
167 parameters of GLMM were estimated using the penalized quasi-likelihood (PQL) technique
168 (package MASS; glmmPQL function; Wolfinger and O'Connell 1993). A Wald chi-square test
169 was performed to determine the significance degree of each explanatory variable in the GLMM
170 (package car; Anova function; Fox 2015). GLMM was completed with a Tukey's post-hoc test

171 in order to check the significance of differences between means (package lsmeans; lsmeans
172 function; Bretz et al. 2016).

173

174 2.4.2 Spatial distribution of the airborne microbiological particles in the caves: distance index

175 A distance index was calculated to check whether the distance between the sampling spot and
176 the cave entrance, as well as the distance between this spot and the end of the caves, had an
177 effect on the counts of airborne microbiological particles. The end of the caves was defined as
178 the point from which humans could no longer progress. Although it is possible that this point
179 was not the real end of the cave, it nevertheless represented a major physical obstacle for
180 microorganisms. The index was normalized to compare caves of different sizes:

$$181 \text{Id} = \frac{\text{Distance from the entrance of the cave} - \text{Distance to the end of the cave}}{\text{Distance from the entrance of the cave} + \text{Distance to the end of the cave}}$$

182 Id thus ranged from -1 to 1, where (-1) was the value obtained at the cave entrances and (1) was
183 the value obtained at the end of the caves.

184 The strength of the relation between the number of microorganisms and the Id was assessed
185 using linear and quadratic regression (package stats; lm function; Chambers and Hastie 1992).

186 The Pearson correlation coefficient and root-mean-square error (RMSE, package nsRFA;
187 RMSE function; Armstrong and Collopy 1992) were chosen to illustrate the power of the
188 regressions. RMSE shows the average distance between the regression curve and the
189 observations. The significance of the difference between the linear and the quadratic regressions
190 was assessed by comparing the analysis of the variance tables for each regression model
191 (package stats; anova function; Chambers and Hastie 1992). The significance of the difference
192 between obtained RMSE was estimated by bootstrapping the RMSE from original data (1000
193 replicates; package boot; boot function; Davison and Hinkley 1997). The intervals containing
194 95 % of the obtained values were then compared and were considered as significantly different
195 when they did not overlap.

196 2.4.3 Structure of fungal communities

197 The structure of fungal communities was analysed by Principal Component Analysis (PCA;
198 package FactoMinR; PCA function; Husson et al. 2017). The isolated fungal genera were used
199 as quantitative dependent variables. Genera that represented less than 0.5 % of the total fungal
200 population were grouped together under the name “Other genera” to facilitate the interpretation
201 of the model. The *sampled cave*, the *sampling season* and the *sampling depth* were used as
202 supplementary qualitative variables.

203

204 **3. Results**

205

206 *3.1 Airborne microbiological particle counts*

207 3.1.1 Fungal particles

208 The rates of airborne fungal particles ranged from 0 to 6340 CFU m⁻³ over the five sampling
209 periods (Fig. 3a). Bayol, Latrone and Points caves each showed a specific fixed pattern of
210 airborne fungal particles over the five sampling periods, despite a small number of differences
211 (e.g. Points cave in June 2016). The fungal rates were clearly higher at the entrance of these
212 caves, before decreasing with depth, and showing a slight increase again at the end of the caves
213 in some cases. Chabot cave showed two different patterns according to the sampling season: a
214 high rate of fungal particles was found at the second sampling point during late summer
215 showing the maximum rate of the cave, whereas lower fungal rates were recorded during winter
216 and late spring at the same location. The two different patterns recorded in Oulen cave could
217 be season-dependant, but this cannot be confirmed as the cave was tested once per season.

218 The general linear mixed model showed that *sampled cave*, *sampling season* and *sampling*
219 *depth* and the interaction between *sampled cave* and *sampling depth* affected fungal particles
220 rates (Table 2). The effect of the interaction between *sampling season* and *sampling depth* was

221 not significant. The rates of fungal particles were highest during late summer and lowest during
222 late spring, and intermediate rates were observed during winter. The difference between late
223 summer and late spring was significantly supported by the statistical tests whereas the
224 differences between winter and the two other sampling periods were not (Table 3). The
225 significant interaction takes priority over the single effects regarding *sampled cave* and
226 *sampling depth* variables. No significant difference was observed between the fungal rates at
227 the entrances of the five caves. Rates at sampling locations in the deeper parts of the caves were
228 significantly lower than at the entrance for the Bayol and Latrone caves, and were lower than
229 at the entrance for the Chabot and Points caves, not significantly though. Finally, the rates were
230 higher in deep cave locations than at the entrance for Oulen cave, not significantly though.

231

232 3.1.2 Bacterial particles

233 The rates of airborne bacterial particles ranged from 10 to 5040 CFU m⁻³ over the five sampling
234 periods (Fig. 3b). As observed for fungal counts, the patterns of bacterial distribution in each
235 cave were fixed, despite a small number of differences. The patterns in the Chabot and Latrone
236 caves were season dependent: one pattern in late summer and another in winter and late spring
237 for Chabot cave, whilst Latrone cave showed one pattern in late summer, a second in winter
238 and possibly a third in late spring. In the Bayol and Points cave, the pattern of bacterial rates
239 was similar to that observed for fungal counts (with the exception of a diverging data at the 3rd
240 sampling point in Points cave in September 2015), i.e. high bacterial rates at the entrance of the
241 caves, before decreasing with depth, and finally increasing again at the end of the caves. The
242 difference with fungal rates was that the final increase in growth was much stronger in the case
243 of bacterial rates. It is important to note that these rates were often higher at the end of the cave
244 than at the entrance. No particular pattern was observed for Oulen cave.

245 The GLMM showed that all tested effects, including interaction effects, influenced the bacterial
246 rates (Table 2). The bacterial rates in the deeper parts of the caves during winter were
247 significantly lower than the rates for all other combinations of sampling date / sampling depth,
248 which were not significantly different from each other (Table 3). No significant difference was
249 observed in the bacterial rates between the entrance and the deeper parts of the caves for each
250 site, or between the entrances of the different caves. In contrast, the rates assessed in the deeper
251 parts of the Latrone and Points caves were significantly lower than the rates assessed in the
252 deeper parts of the Bayol, Chabot and Oulen caves.

253

254 *3.2 Spatial distribution of the airborne microbiological particles in the caves: distance index*

255 3.2.1 Fungal particles

256 The linear correlation coefficient between the distance index and the fungal rates was
257 statistically significant but low, showing a very poor linear relation (Fig. 4a, Table 4). The
258 quadratic correlation coefficient was significantly higher than the linear coefficient, although it
259 was also low. The RMSE associated to quadratic regression was better than the RMSE
260 associated to linear regression, but the difference was not significant.

261 The breakdown of results per sampling season confirmed the preceding observations, with more
262 pronounced differences (Fig.5, Table 4). The quadratic correlation coefficient was much higher
263 than the linear coefficient in winter, showing a link between Id and fungal rates. The quadratic
264 correlation coefficient was higher than the linear coefficient in late summer, but the difference
265 was not significant. Finally, the quadratic correlation coefficient was higher than the linear
266 coefficient in late spring, but neither of them was statistically significant. The RMSE associated
267 to quadratic regression was lower than the RMSE associated to linear regression for each season
268 studied, but the differences were not significant. The turning points of the quadratic regressions

269 were obtained for Id values of 0.17, 0.21 and 0.35 in winter, late spring and late summer,
270 respectively.

271

272 3.2.2 Bacterial particles

273 No linear or quadratic relation was found between the distance index and the bacterial rates
274 (Fig. 4b, Tables 4).

275

276 *3.3 Structure of fungal communities*

277 Over forty fungal genera were isolated during the five sampling periods, the most abundant of
278 which were *Penicillium* (47.46 % of the isolated strains) and *Cladosporium* (24.99 %; Table 5).

279 The first dimension of the principal component analysis explained 22.7 % of the variance of
280 observations and the second dimension explained 10.3 %. The fungal genera *Arthinium*,
281 *Cladosporium*, *Penicillium*, *Engyodontium* and *Alternaria* provided the highest positive
282 contributions to the construction of the first dimension (Table 6) and the fungal genera
283 *Metarhizium*, *Alternaria* and *Paecilomyces* provided the highest positive contributions to the
284 construction of the second dimension, whereas fungal strains belonging to Basidiomycete
285 division and the genera *Aspergillus* provided the highest contribution to the construction of the
286 negative part of the second dimension.

287 The five caves were clearly separated in the PCA first dimension (Table 6, Fig. 6a). Points and
288 Latrone caves shifted to the negative values, whereas Oulen and Chabot caves shifted to the
289 positive ones. Bayol cave overlapped all other caves. Chabot cave was the only site significantly
290 correlated to the second dimension of the PCA, which meant that the construction of this
291 dimension was totally dependent on the Chabot cave fungal community. Chabot cave therefore
292 shifted to the positive values on the second dimension.

293 The sampling seasons were poorly separated by the two PCA dimensions (Fig. 6b). Late
294 summer observations slightly shifted to positive values on the first dimension in comparison
295 with winter observations, and the observations of these two seasons were almost entirely located
296 in the ellipse for late spring observations.

297 The sampling depth was clearly separated on the first dimension of the PCA (Fig. 6c). The
298 observations at the entrance of the caves shifted to positive values, whereas the observations
299 in the deeper parts of the caves shifted to negative values.

300

301 **4. Discussion**

302

303 *4.1. General aerobiological behavior of the caves*

304 The first purpose of this study was to evaluate whether a model of aerobiological behavior could
305 be identified for each monitored cave. Indeed, the aerobiology of five caves was monitored for
306 two and a half years. Previous studies focusing on the aerobiology of caves generally studied a
307 “picture” of caves by sampling several points, but took samples on one date (Garcia-Anton et
308 al. 2013; Porca et al. 2011; Ogórek et al. 2014; Ogórek et al. 2015; Pusz et al. 2015). Other
309 studies focused on seasonal variations by monitoring caves for a complete year, but took
310 samples from just one sampling point (Wang et al. 2010a; Wang et al. 2010b). These studies
311 cannot, therefore, determine if there was a model of aerobiological behavior in the monitored
312 caves. After all, there are very few studies that monitored several sampling points at several
313 sampling dates (Docampo et al. 2011; Martin-Sanchez et al. 2014; Taylor et al. 2013), and
314 established a model behavior was generally not their purpose. Our study showed that a model
315 of aerobiological behavior can actually be identified in most of the monitored caves, for both
316 fungi and bacteria. Indeed, the relative distribution of aerobiological rates in a given cave did
317 not vary over time. This result is consistent with the observations made in studies that monitored

318 several sampling points at several sampling dates and showed no variation in the distributions
319 of aerobiological rates over time, at least in the parts of the caves that were not open to the
320 public (Taylor et al. 2013; Martin-Sanchez et al. 2014). This result is important because it means
321 that variations in the shape of the distributions could be an indication of a microbiological
322 disorder. In the case of Chabot cave, the shape of the distribution was also clearly season
323 dependent.

324

325 *4.2 Fungal behavior of the caves*

326 The fungal rates counted in the cave air samples seemed closely linked to the capacity of the
327 propagules to move and drop inside the caves, and therefore were closely linked to the airflow.
328 This flow occurs to a greater or lesser extent in all caves, depending on several factors such as
329 the passage geometry and configuration (De Freitas et al. 1982). The direction of air movement
330 is strongly affected by the difference between the density of air inside the cave and the outside
331 air, and thus by differences in temperature. The particles carried by the air movement drop via
332 several phenomena that depend on the characteristics of particles as well as the characteristics
333 of the support on which they drop (Davies 1967). In our study, no significant difference was
334 observed in the fungal rates counted at the entrance of the caves, which suggests that all five
335 caves had similar conditions at this point, namely the pressure of the external environment
336 (Docampo et al. 2011; Mulec et al. 2012). However, the caves were then separated into two
337 groups when samples were taken further away from the entrance: caves in which these rates
338 counted at this second sampling spot were significantly lower than the rates counted at the
339 entrance, i.e. the Bayol, Latrone and Points caves; and the caves in which these rates were not
340 lower than those counted at the entrance, i.e. the Chabot and Oulen caves. From a
341 microbiological point of view, we propose to call these two groups “Self-purifying caves” and
342 “non-self-purifying caves”.

343 4.2.1 “Self-purifying caves”

344 The “self-purifying caves” were characterized by high fungal rates at the entrance which
345 dramatically decreased as the distance from the entrance increased. A slight increase was often
346 observed in these rates at the far end of the caves. This phenomenon was regularly observed in
347 past studies (Pusz et al. 2015; Garcia-Anton et al. 2013). While Garcia-Anton et al. (2013)
348 interpreted it as a direct connection between the far end of the cave and the exterior, Gázquez
349 et al. (2016) found no evidence of such a link. The final increase in fungal rates seemed to be a
350 general rule in our study. Indeed, the study of fungal rates in the function of Distance index
351 showed that however low the correlations were, the squared regression was always better
352 adapted than the linear regression. It is important to note that the difference between the
353 correlation coefficients of these two regressions was statistically significant when studying all
354 data and winter data. These results are consistent with the hypothesis of a progressive discharge
355 of fungal propagules from cave entrances, mainly through gravity (Kuzmina et al. 2012). At the
356 far end of the caves, the propagules could be accumulated when a large physical obstacle was
357 encountered, leading to the stagnation of air. The inflection point of the squared regression
358 would therefore indicate the location from which there was more accumulation than discharge.
359 Although the fungal rates were the highest at the entrance in late summer, which is consistent
360 with previous studies (Docampo et al. 2011; Wang et al. 2010a), the balance between discharge
361 and accumulation occurred further from the entrance than it did in winter and late spring. A
362 future study using a computational fluidic dynamics model similar to that describing Lascaux
363 cave could help to validate this hypothesis (Martin-Sanchez et al. 2014).

364 The study of the fungal community composition was in accordance with the conclusions drawn
365 from the study of the fungal rates. *Penicillium* and *Cladosporium* were the two most frequently
366 isolated genera in the cave air, which is consistent with previous studies (Fernandez-Cortes et
367 al. 2011; Pusz et al. 2015; Vanderwolf et al. 2013). These two genera, along with the genera

368 *Arthrimum*, *Engyodontium* and *Alternaria*, were the most correlated with the first dimension of
369 the principal component analysis. All of these genera except *Engyodontium* are usually
370 abundant in the outside air (Wang et al. 2010a; Seifert et al. 2011). The samplings at the cave
371 entrances were strongly affected by the first dimension of PCA while the depth samplings were
372 not, suggesting that the sampling at the cave entrances was affected by outside air while the
373 samplings at depth were not. Similarly, Latrone and Points caves were weakly correlated to the
374 first dimension of PCA, which justifies their inclusion in the “self-purifying caves” group. The
375 case of Bayol cave was slightly more complex. The representation of this caves in the PCA
376 embraced both positive and negative values of the first dimension. The fungal rates at this cave
377 entrance were the highest of all counted rates and were particularly dominated by the genera
378 *Penicillium* and *Cladosporium* which strongly bias the representation. The same behavior was
379 then observed at depths in Bayol cave as in Latrone and Points caves. The ceiling of the entrance
380 in Bayol cave is covered by roots, and this oligotrophic habitat and the consequent stock of
381 organic matter could provide a high concentration of fungal propagules (Saiz-Jimenez 2012).

382

383 4.2.2 “Non-self-purifying caves”

384 The study of fungal community composition in the Chabot and Oulen caves confirmed their
385 status of “non-self-purifying”. No difference was observed in fungal rates at the entrance and
386 at depth in these two caves, nor was any difference recorded in the composition of their fungal
387 communities. The two caves were strongly correlated to the first dimension of the PCA,
388 indicating that they were entirely affected by fungal genera that are typical of the outside
389 environment. Two factors could explain the differences observed between the “self-purifying
390 caves” and the “non-self-purifying caves”, namely the contact surface with the outside
391 environment, and the cave volume. De Freitas et al. (1982) highlighted how the timing and
392 frequency of opening and closing barriers with outside environment affects airflow intensity.

393 The three “self-purifying caves” in this study are almost completely isolated from the outside
394 environment by a wall, while the two “non-self-purifying caves” are directly linked to the
395 external environment through large openings. Similarly, De Freitas et al. (1982) and Dredge et
396 al. (2013) underlined the effect of cave geometry and configuration on airflow. The three “self-
397 purifying caves” are characterized by large volumes with relatively high ceilings and downward
398 slopes, while it is impossible to stand up in Oulen cave, which is also the case in Chabot cave
399 after a relatively large first chamber. The high fungal rates in Oulen and Chabot caves could
400 therefore be explained by greater exchange with the outside environment and by volumes that
401 are too small to permit the progressive discharge of fungal propagules. The geometry of “self-
402 purifying caves” could conversely lead to a slow air flow throughout the caves, favoring a rapid
403 discharge of propagules just inside the entrance (Tuttle and Stevenson 2011).

404 The geometry of Chabot cave could also explain why two different behaviors were observed
405 according to the season. The late summer behavior was characterized by high fungal rates at
406 the second sampling point. This second point was located at the end of the entrance chamber,
407 which is quite large. The passage then turns at a right angle and becomes low and narrow. The
408 fungal rates were the highest in late summer: under these circumstances, this particular
409 geometry could block the air flow, leading to an accumulation of propagules in the air (Tuttle
410 and Stevenson 2011).

411 Finally, the attempt by Porca et al. (2011) to establish an index to describe the health of a show
412 cave from the quantity of fungal particles in its atmosphere was very interesting but is hardly
413 applicable in the light of our results. According to this index, the two “non-self-purifying caves”
414 would be sorted in the fifth category ($>1000 \text{ CFU m}^{-3}$), i.e. caves with an irreversible ecological
415 disturbance, yet the absence of fungal outbreak indicates that both caves seemed to be
416 functioning healthily. Moreover, Latrone would be the only cave considered to have no fungal
417 problem after the entrance ($<50 \text{ CFU m}^{-3}$). Points cave would be sorted as an “alarming cave”

418 requiring periodic controls and studies to identify the problem (<150 CFU m⁻³), whilst Bayol
419 cave would be sorted as a “threatened cave” requiring a different type of management (<500
420 CFU m⁻³). This index therefore appears to be too restrictive, and should be moderated according
421 to the individual characteristics of caves. Incidentally, Porca et al. (2011) explained that their
422 index was not suitable in case of huge caves. The question of air quality has been abundantly
423 studied indoors because of health concerns (Cabral 2010). As compared with these studies, the
424 fungal rates measured in depth in “non-self-purifying caves” were slightly high, but not
425 uncommon, whereas the rates measured in depth in “self-purifying caves” matched with those
426 measured in house without visible molds (Ren et al. 2001; Haas et al. 2007). Moreover, Cabral
427 (2010) evaluated that the qualitative composition of fungal community was more indicative
428 than the absolute concentration of fungi in determining the indoor air quality. The mycoflora is
429 dominated by the genus *Cladosporium* in healthy building, whereas *Penicillium* and *Aspergillus*
430 are largely dominant in unhealthy buildings. This usually results from the existence of indoor
431 humidity in unhealthy buildings, which is enough to allow the sporulation of xerophilic fungi
432 such as *Penicillium* and *Aspergillus*. Indeed, *Penicillium* was the dominant fungal genus in the
433 present study. A relative humidity above 95 % is the norm in caves, while it indicates serious
434 problems of humidity in buildings. Thus, the dominance of the genus *Penicillium* can hardly be
435 used as an indicator of bad air quality in caves.

436

437 4.2.3 Fungal signature of the caves

438 Despite the strong influence of external conditions, Chabot cave still has its own specific
439 signature. Chabot cave was the only parameter correlated to the second dimension of the PCA,
440 which meant that this dimension solely represented the particular fungal population of this cave.
441 The fungal genera that contributed the most to the construction of this second dimension was
442 *Metarhizium*. This fungal genus was occasionally isolated in samples from other caves, but

443 Chabot cave was the only one in which *Metarhizium* was frequently and abundantly found. This
444 genus is known to be strongly associated with the presence of insects (Roberts and Leger 2004).
445 It is important to note that the fungal communities of the five studied caves were strongly
446 marked by the presence of insects, spiders and nematode-related genera such as *Engyodontium*,
447 *Beauveria*, *Isaria* and *Paecylomyces* (Humber et al. 2005), which is consistent with existing
448 knowledge of fungal communities in caves (Bastian et al. 2009; Jurado et al. 2008). Other
449 fungal genera found in the cave environment, such as *Geomyces*, *Doratomyces* and
450 *Oidiodenron*, were also frequently isolated. These genera are generally closely related to the
451 presence of bats and bat droppings/guano (Saiz-Jimenez 2012; Nováková 2009; Vanderwolf et
452 al. 2013).

454 4.3 Bacterial behavior of the caves

455 As for fungus, a bacterial aerobiological behavior was identified in each cave. No global
456 relation was identified between the distance index and the bacterial rates, while a clear relation
457 appeared between the fungal rates and the distance of the sampling point from the entrances of
458 the caves, despite low correlation coefficients. Dredge et al. (2013) found the same results for
459 both fungi and bacteria and explained them by the difference in the size of the particles, and
460 therefore by the difference in their aerial transport. Fungal transport was consistent with that
461 observed for large-sized aerosols while bacterial transport was consistent with that observed for
462 aerosols between 0.1 μm and 10 μm . Similarly, the distinction between “self-purifying caves”
463 and “non-self-purifying” cannot be easily established on the basis of the bacterial count, since
464 no statistically significant difference occurred in each cave between the rates counted at the
465 entrance and the rates counted at depth. The only significant difference observed was that
466 Latrone and Points had lower bacterial rates in depth than Bayol, Chabot and Oulen caves, and
467 that the rates counted inside the cave at a distance from the cave entrance in winter were lower

468 than the rates observed for other seasons and other locations. The seasonal effect on aerial
469 bacterial rates has already been described in numerous studies (Martin-Sanchez et al. 2014;
470 Wang et al. 2010b; Cuezva et al. 2009) which all showed that temperature, relative humidity
471 and ventilation had an effect on bacterial rates. Therefore, the differences observed in our
472 studies could also be attributed to microclimatic variation according to the season and to
473 changes in the ventilation system occurring during the year. Interestingly, the bacterial rates
474 were lower at sample points at a distance from the entrance of “self-purifying caves” except
475 for Bayol cave, where the same level was observed for these rates as in “non-self-purifying”
476 caves.

477
478 In conclusion, the aim of this study was to determine if a model of aerobiological behavior was
479 identifiable for each cave for fungi and bacteria. The study should also have determined if a
480 generalization could be obtained from these models in a framework of cultural heritage
481 preservation. This study revealed the existence of a model behavior for airborne fungi and
482 bacteria in each decorated cave. This result is interesting in a framework of decorated cave
483 preservation because each future deviation from this model could be interpreted as a microbial
484 imbalance. The microbial airborne behavior is cave dependent and also season dependent in
485 some caves. On the basis of the fungal study, the caves can be divided into two groups: “self-
486 purifying caves” and “non-self-purifying caves”. Both groups were strongly affected by the
487 external environment at the entrance of the caves. The “self-purifying caves” were much less
488 affected by external environment at depth, whereas “non-self-purifying caves” were directly
489 affected by the outside air, even at depth. The differences between these two groups could be
490 due to the size of the caves and the intensity of the exchanges with the external environment.
491 Finally, the microbial airborne rates proposed in past studies for healthy caves should be
492 redesigned to take the individual characteristics of caves into account. Indeed, the rates

493 measured in our study exceeded the alarm thresholds in almost all five caves, despite the caves
494 being healthy and closed to the public.

495

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497

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671

672

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

Cave	Discovery	Historical monuments list	Number of sampled points	Date of sampling				
				January 2014	September 2014	February 2015	September 2015	June 2016
Bayol cave	1927	1931	3					
Chabot cave	1878	1903	5					
Latrone cave	1940	1941	5					
Oulen cave	1907	1910	4					
Points cave	1993	1995	4					*

674 The sampling dates are represented by grey boxes.

675 *The fourth sampling point of Points cave was not assessed in June 2016 due to a high CO₂ rate at the end of the cave.

676

677 **Table 2** Effect of qualitative variables on fungal and bacterial counts, assessed with a Wald χ^2

678 test associated to GLMMs.

Source of variation	df	Fungi		Bacteria	
		χ^2 -value	p-value	χ^2 -value	p-value
Sampled Cave (C)	4	112	<0.001***	91.0	<0.001***
Sampling depth (D)	1	32.5	<0.001***	5.78	0.02*
Sampling season (S)	2	11.6	0.004**	30.3	<0.001***
C: D	4	14.1	0.002*	17.0	0.02**
S: D	2	5.26	0.07	18.8	<0.001***

679 * p < 0.05, ** p < 0.01, *** p < 0.001

680

681 **Table 3** Effect of significant qualitative variables on fungal and bacterial counts – comparisons

682 of the means of the different modalities.

Sampling season	Fungi			Bacteria		
	Sampled Cave: Sampling depth	Sampling season: Sampling depth	Sampled cave: Sampling depth	Sampled Cave: Sampling depth	Sampling season: Sampling depth	Sampled cave: Sampling depth
Late summer 1024a	Bayol cave: Entrance	3196a	Late summer: Deep cave	1517a	Oulen cave: Deep cave	3361a
Winter 620ab	Chabot cave: Entrance	1820ab	Late spring: Deep cave	1223a	Chabot cave: Entrance	2260a
Late spring 491b	Chabot cave: Deep cave	1452ab	Winter: Entrance	1386a	Bayol cave: Entrance	2008a
	Oulen cave: Deep cave	1256ab	Late spring: Entrance	1695a	Bayol cave: Deep cave	1333a
	Oulen cave: Entrance	1125ab	Late summer: Entrance	1144ab	Chabot cave: Deep cave	1573a
	Points cave: Entrance	1036ab	Winter: Deep cave	539b	Latrone cave: Entrance	858abc
	Latrone cave: Entrance	302ab			Points cave: Entrance	718abc
	Bayol cave: Deep cave	166bc			Oulen cave: Entrance	605abc
	Points cave: Deep cave	146bc			Points cave: Deep cave	430bc
	Latrone cave: Deep cave	38c			Latrone cave: Deep cave	372c

683 Different letters in the columns indicate significant differences.

684

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

Type of regression	Fungi						Bacteria			
	<i>All data</i>		<i>Winter</i>		<i>Late spring</i>		<i>Late summer</i>		<i>All data</i>	
	r^2	RMSE (spores.m ⁻³)	r^2	RMSE (spores.m ⁻³)	r^2	RMSE (spores.m ⁻³)	r^2	RMSE (spores.m ⁻³)	r^2	RMSE (spores.m ⁻³)
Linear regression	0.14***b	1088a	0.19*b	682a	0.22a	700a	0.15*a	1390a	0.001a	1255a
Quadratic regression	0.24***a	1021a	0.53***a	520a	0.29a	667a	0.21*a	1342a	0.04a	1229a

687 * p < 0.05, ** p < 0.01, *** p < 0.001

688 Different letters in the columns indicate significant differences.

689

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Table 5 Identification of fungal strains isolated during the five sampling campaigns.

Fungal species	Points cave					Oulen cave		Latrone cave					Bayol cave					Chabot cave				Total rate (%)	BLAST similarity (%)	GenBank Accession number
	Jan 14	Sept 14	Feb 15	Sept 15	June 16	Jan 14	Sept 14	Jan 14	Sept 14	Feb 15	Sept 15	June 16	Jan 14	Sept 14	Feb 15	Sept 15	June 16	Sept 14	Feb 15	Sept 15	June 16			
<i>Absidia</i> sp.																						0.06		
<i>Acremonium</i> sp.																						0.17	99	MF788181
<i>Albifimbria</i> sp.																						0.04	99	MF788182
<i>Alternaria</i> sp.																						1.68	99	MF788183
<i>Arthrinium</i> sp.																						0.94	99	MF788184
<i>Aspergillus</i> sp.																						1.22		
<i>Aureobasidium</i> sp.																						0.08		
<i>Beauveria</i> sp.																						1.47		
<i>Botrytis</i> sp.																						0.1		
<i>Chaetomium</i> sp.																						0.07	99	MF788185
<i>Cladosporium</i> sp.																						24.99		
<i>Colletotrichum</i> sp.																						0.05		
<i>Doratomyces</i> sp.																						0.2	99	MF788186
<i>Drechslera</i> sp.																						0.04	98	MF788187
<i>Engyodontium</i> sp.																						5.37	100	MF788188
<i>Epicoccum</i> sp.																						0.27	100	MF788189
<i>Forliomyces</i> sp.																						0.04	98	MF788190
<i>Fusarium</i> sp.																						0.08	99	MF788191
<i>Gabarnaudia</i> sp.																						0.09		
<i>Geomyces</i> sp.																						0.38	100	MF788192
<i>Geosmithia</i> sp.																						0.04	99	MF788193
<i>Gliomastix</i> sp.																						0.15	99	MF788194
<i>Isaria</i> sp.																						0.09	99	MF788195
<i>Lecanicillium</i> sp.																						0.12	99	MF788196
<i>Metarhizium</i> sp.																						1.12	99	MF788197
<i>Mortierella</i> sp.																						0.03	100	MF788198

704 **Table 6** Contribution of the most valuable variables to the construction of the two dimensions
 705 of the PCA – supplementary qualitative variables significantly affected by the two dimensions
 706 of the PCA.

Dimension 1			Dimension 2		
Quantitative variable	Correlation	p-value	Quantitative variable	Correlation	p-value
<i>Arthrinium sp.</i>	0.8	<0.001***	<i>Metharizium sp.</i>	0.59	<0.001***
<i>Cladosporium sp.</i>	0.76	<0.001***	<i>Alternaria sp.</i>	0.48	<0.001***
<i>Penicillium sp.</i>	0.74	<0.001***	<i>Paecilomyces sp.</i>	0.47	<0.001***
<i>Engyodontium sp.</i>	0.69	<0.001***	<i>Aspergillus sp.</i>	-0.45	<0.001***
<i>Alternaria sp.</i>	0.56	<0.001***	Basidiomycetes	-0.54	<0.001***

Supplementary qualitative variable	Estimate	p-value	Supplementary qualitative variable	Estimate	p-value
Chabot cave	0.61	0.1*	Chabot cave	0.78	<0.001***
Bayol cave	0.66	0.3*			
Points cave	-0.93	0.04*			
Latrone cave	-1.15	<0.001***			

Entrance	0.73	<0.001***			
Deep cave	-0.73	<0.001***			

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

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

721 The five caves are located in the Gard department, France. Three of the caves, namely Chabot
722 cave, Oulen cave and Points cave, are located in the Ardèche valley. Bayol cave and Latrone
723 cave are located in the Gardon valley.

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

727

728 **Fig. 2** Sampling maps of the five studied caves (graphics program: Inkscape)

729 Each number represents one sampling point where fungal and bacterial rates were assessed. The
730 sampling points were spread out to cover the widest possible surface of the caves.

731 The topography of Chabot cave was supplied by Galant, P. and Bigot, J.Y.; other Cave maps
732 are extracted of Leroi-Gourhan (1984).

733

734 **Fig. 3** Count of airborne microbiological particles in each cave for the five sampling dates:
735 fungi (a), bacteria (b) (graphics program: R).

736

737 **Fig. 4** Study of the relation between airborne microbiological particles and the distance index:
738 fungi (a), bacteria (b) (graphics program: R).

739 The study was carried out for fungal data (a), and then for bacterial data (b). In each case, linear
740 and quadratic regressions were applied to data collected in all caves at all sampling dates.

741

742 **Fig. 5** Study of the relation between airborne fungal particles and the distance index according
743 to the sampling season (graphics program: R).

744 The linear and the quadratic regressions were applied to fungal data collected in all caves
745 according to the sampling season.

746

747 **Fig. 6** Representation of the data according to supplementary qualitative variables of the PCA
748 (graphics program: R).

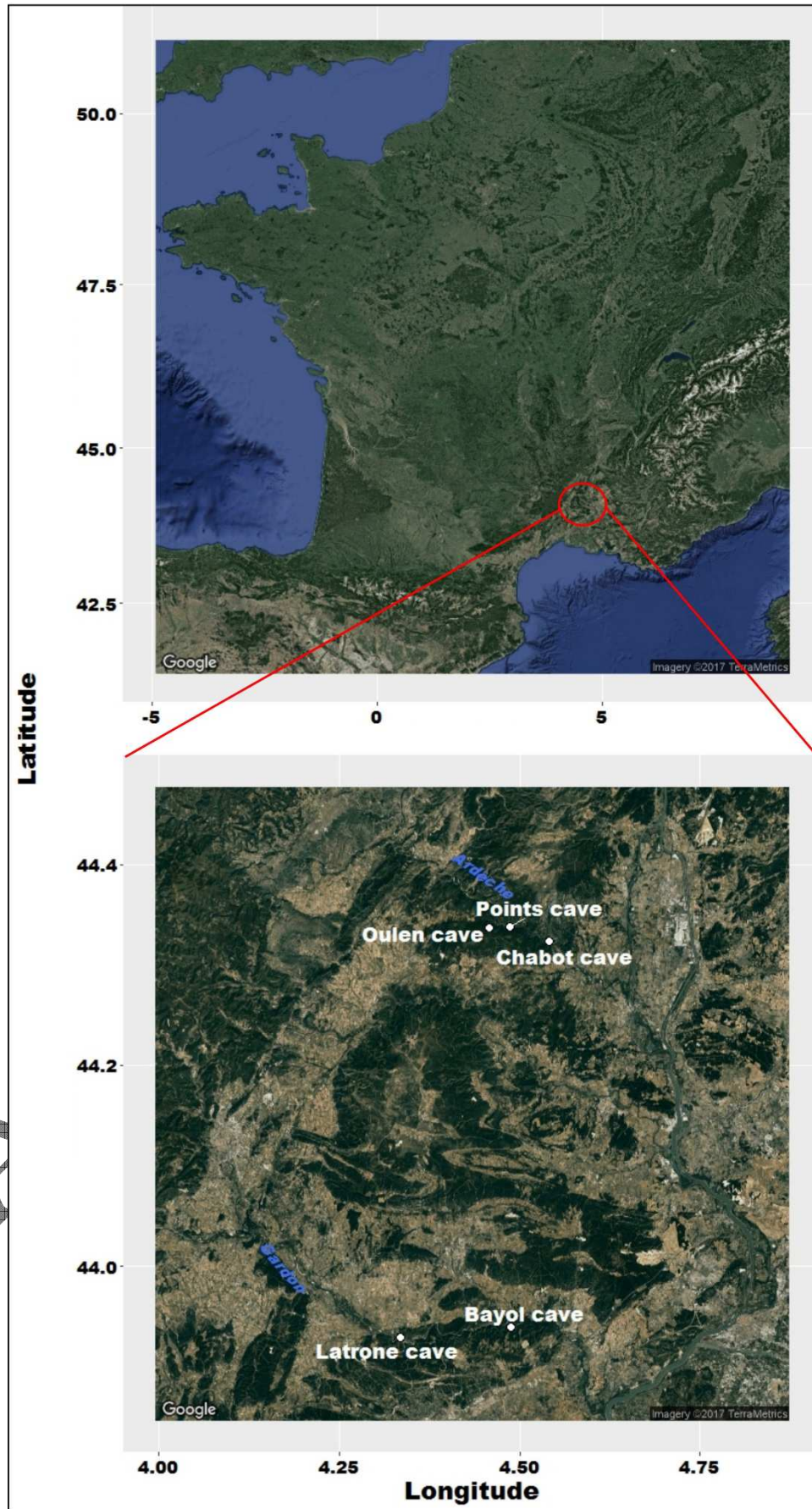
749 The data are represented according to the three supplementary qualitative variables used in
750 PCA: Sampled cave (a), sampling season (b) and sampling depth (c). The coloured circles
751 indicate the probable position of the barycenter of observations associated to each modality.

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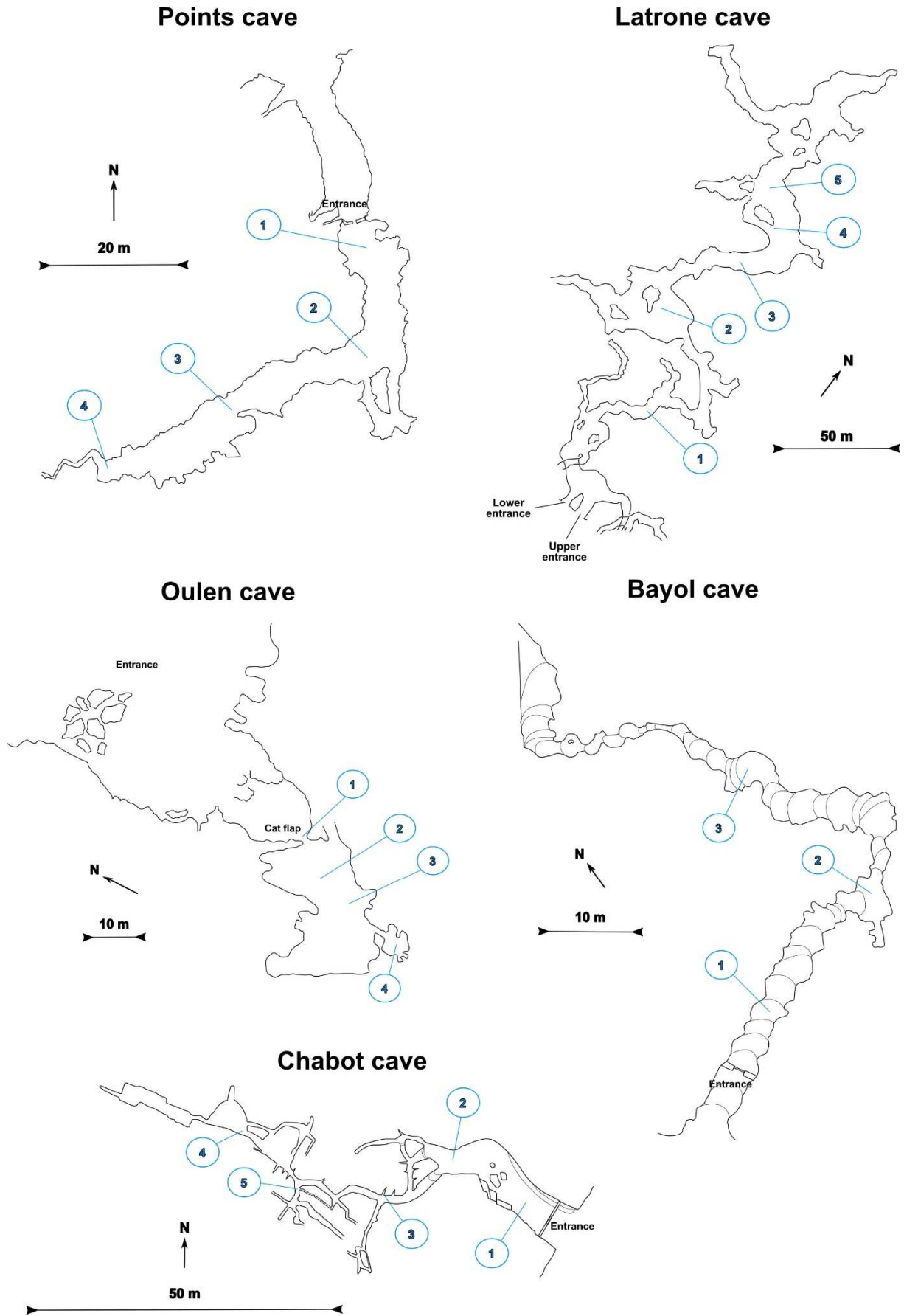
753 Fig. 1

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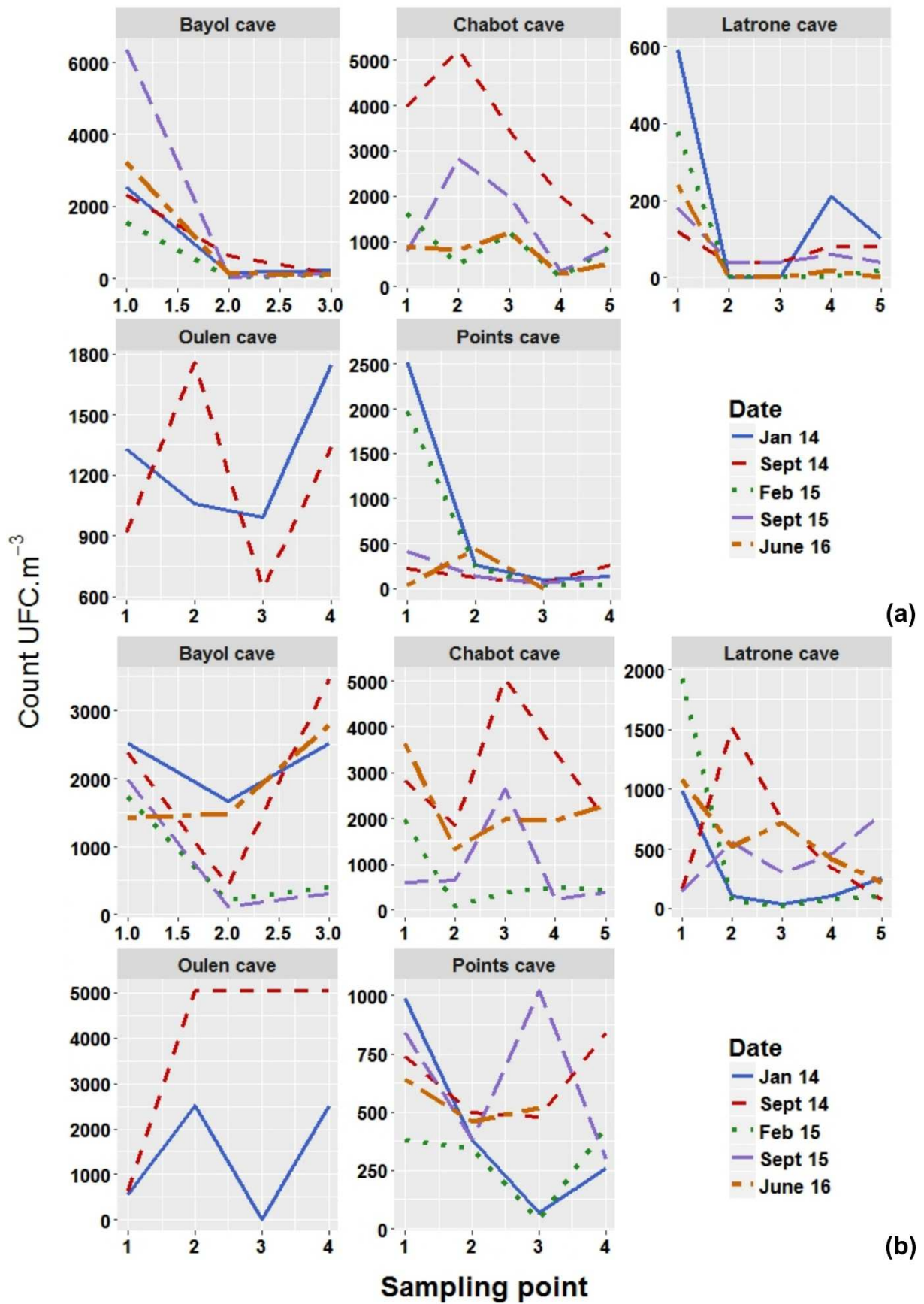
755 **Fig. 2**

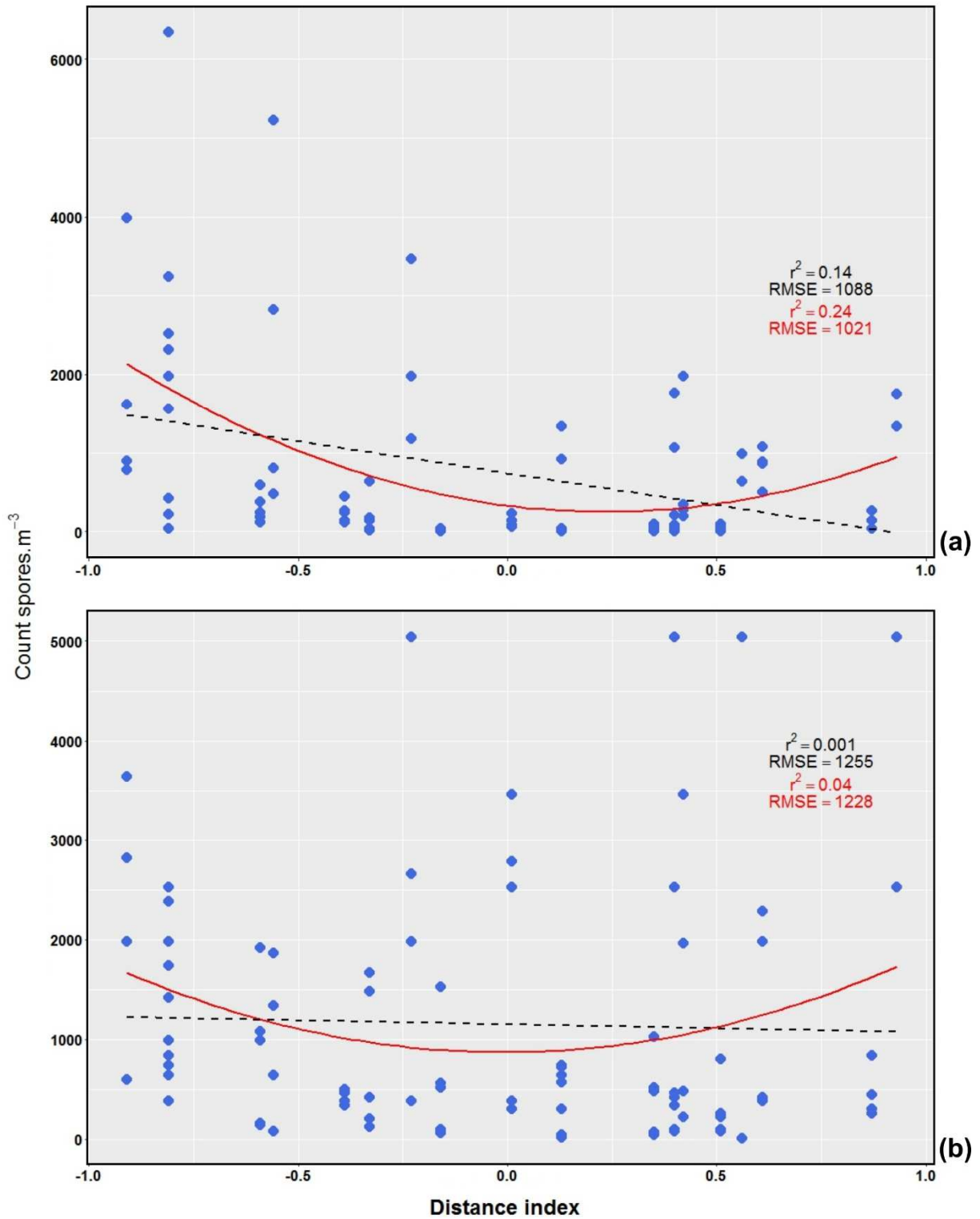
756



757 Fig. 3

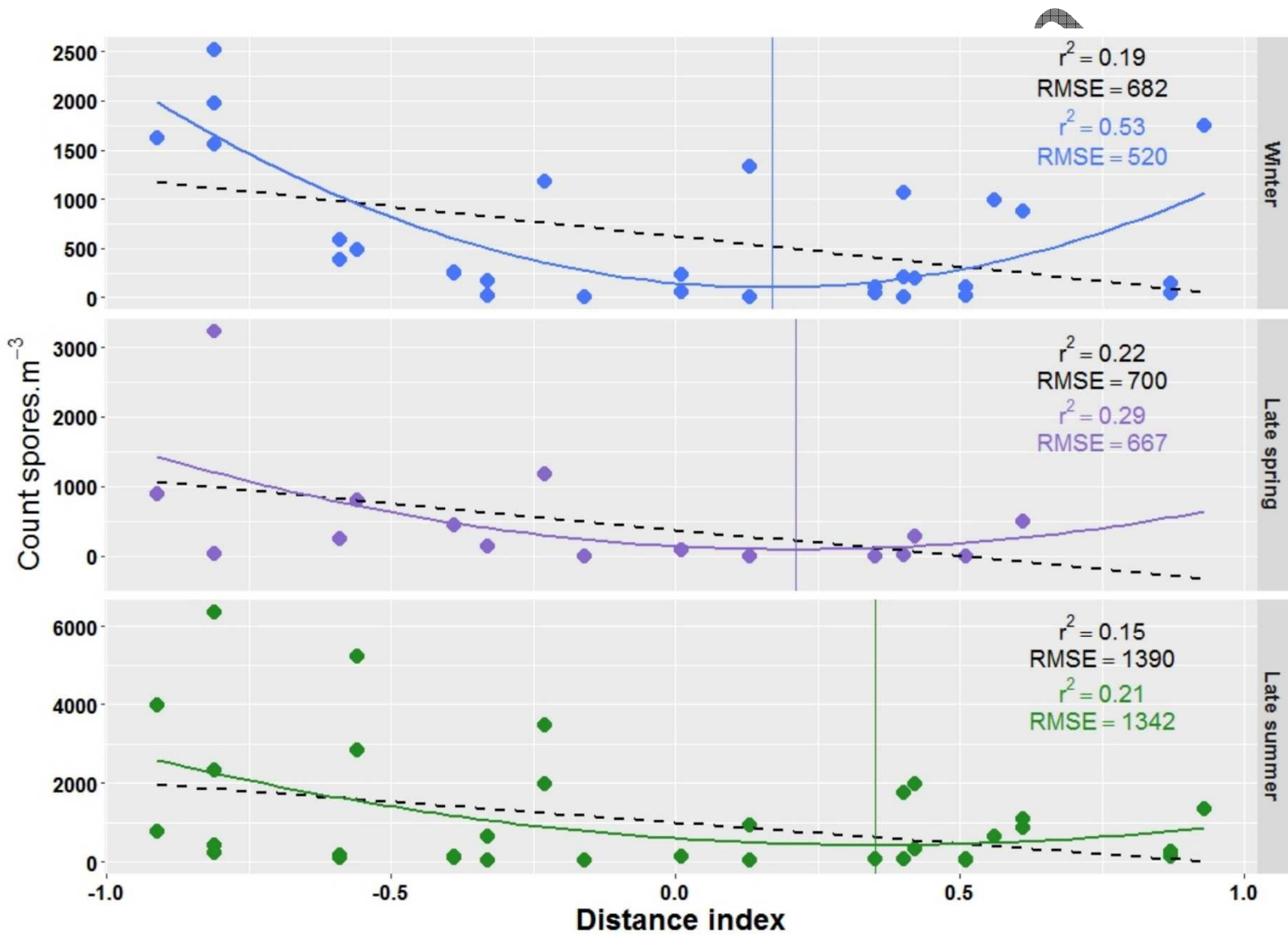
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761 Fig. 5

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763 Fig. 6

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