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1 **High diversity of microsporidian parasites and new planktonic hosts in freshwater and**
2 **marine ecosystems**

3

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16

17 ***Abstract***

18

19 Microsporidia are a large group of obligate intracellular eukaryotic parasites related to Fungi
20 primarily known as parasites of vertebrates and invertebrates. They are well described as parasites of
21 organisms of interest (e.g., edible fish and crustaceans, honeybees, bioindicators such as daphnia,
22 humans) on which they can have an important impact (e.g., reduced survival or fecundity and sex
23 ratio distortion). However, their diversity in aquatic environments, especially in marine ecosystems,
24 has been greatly understudied since they are not targeted by classical eukaryotic primers used in
25 metabarcoding studies. Moreover, little is known about their hosts among protists or micro-
26 zooplankton and therefore about their impact on the trophic food web functioning. In this work, we
27 sampled 15 different sites across marine and freshwater environments, size-fractionated the samples
28 and used microsporidian specific primers associated with metabarcoding to study the microsporidian
29 diversity (and the associated spatial variation). Co-occurrence networks as well as Tyramide Signal
30 Amplification-Fluorescent In Situ Hybridization were used to link potential hosts (planktonic
31 eukaryotes < 150 μ m) and Microsporidia diversity. Our analysis unraveled a large microsporidian
32 diversity which was widely divergent between the two environments studied. In both of them, an
33 important part of this diversity was not affiliated to a genus, suggesting an important reservoir of new
34 microsporidian species and thus new hosts among planktonic eukaryotes. Co-occurrence networks
35 and fluorescence microscopy showed for the first time associations between Microsporidia and
36 dinoflagellates in freshwater and marine environments.

37

38 ***Introduction***

39 Parasitism has been described as one of the oldest and most ubiquitous interactions in nature
40 (Cavalier-Smith 1993), however the diversity of parasites and their effects on the aquatic trophic food
41 web functioning have long been neglected (Marcogliese and Cone 1997). In the last decades, high
42 throughput sequencing allowed the description of an increasing number of parasites (e.g., Singer et
43 al. 2021) and revolutionized our understanding of aquatic food webs in term of trophic interactions
44 as well as of energy and biomass flows (Lafferty et al. 2008; Kagami et al. 2014). Among these
45 parasites, Microsporidia (obligate intracellular eukaryotic parasites related to Fungi (Karpov et al.
46 2014)) were known in aquatic ecosystems only through the search of infectious agent in organisms
47 of interest (e.g., edible fish and crustaceans, bioindicators, humans, etc.). These host-targeted studies
48 show that Microsporidia can have a considerable impact on host populations that are of great
49 importance in freshwater ecosystems, by causing a decrease in the survival and fecundity rates of
50 daphnia for example (Ebert 2005). However, they were missing from metabarcoding studies focusing
51 on microbial eukaryotes diversity (Bass et al. 2015) especially due to their divergence from other
52 eukaryotes (rapid evolution and compaction of their genome (Keeling and Fast 2002)) making the
53 primers unable to detect them (Hadziavdic et al. 2014; Hugerth et al. 2014). As a result, the overall
54 microsporidian diversity in aquatic (freshwater and marine) ecosystems is largely unknown and thus
55 their impact on ecosystem functioning is neglected.

56 In 2018, a targeted metabarcoding study (i.e. using specific primer for Microsporidia) presented the
57 first description of the microsporidian diversity in various environments including freshwater and
58 marine samples (Williams et al. 2018), but this allowed only a limited analysis due to the low number
59 of operational taxonomic units (OTUs) obtained. This study nevertheless allowed the identification
60 of a new diversity across the microsporidian tree and encouraged further work. Indeed, two others
61 metabarcoding studies focusing on lacustrine ecosystems followed and allowed the discovery of an
62 unsuspected diversity of Microsporidia (Dubuffet et al. 2021; Chauvet et al. 2022a). In these studies,

63 between 40% and 50% of the uncovered diversity couldn't be affiliated to a genus suggesting that the
64 microsporidian phylum hide a large amount of undiscovered diversity. These studies also provided
65 temporal variation data and highlighted new microsporidian planktonic hosts in a small size fraction
66 (i.e. $<150\mu\text{m}$) (Chauvet et al. 2022a,b). Moreover, few Microsporidia are currently described as
67 parasites of protists or microzooplankton (Murareanu et al. 2021), even though they are major
68 components of food webs.

69
70 Altogether, the previous studies did not allow a broad comparison of the diversity of Microsporidia
71 across aquatic ecosystems and thus lead to an incomplete picture of their diversity. Although Dubuffet
72 et al. (2021) and Chauvet et al. (2022a) showed an in-depth description of the microsporidian
73 diversity, these studies were limited to two lakes (Aydat and Pavin, Massif Central, France).
74 Moreover, the microsporidian diversity in marine ecosystems is probably still underestimated as it
75 has been neglected so far. Here, spatial variation of the microsporidian diversity (canonical (or long-
76 branch) Microsporidia (Bass et al. 2018)) was assessed through targeted metabarcoding in nine
77 different freshwater and six marine ecosystems. Co-occurrence networks and fluorescence
78 microscopy were eventually used to identify potential new microsporidian hosts among
79 microeukaryotes.

80

81 ***Materials and methods***

82 **Study sites and sampling procedure**

83 Water samples were collected in France from different freshwater lakes (n = 9) and marine
84 environments (n = 6; Mediterranean Sea and Atlantic Ocean) (Fig. 1) between 18/06/21 and 27/09/21
85 (Supporting Information Table S1). Then, 2.5 to 22 litres of water were sampled at the surface with
86 a Niskin bottle or along the euphotic zone using an integrated sampler IWS (HYDRO-BIOS)
87 (Supporting Information Table S1).

88 For most of the samples we collected the 0.8-150 μm fraction to concentrate potentially infected
89 planktonic hosts except for the Brest and Roscoff samples where we were able to collect the $>3 \mu\text{m}$
90 fraction (Supporting Information Table S1). Part of the microbial biomass of each size-fraction was
91 collected on 0.8- μm -pore-size polycarbonate filters (Millipore) at a very low vacuum to prevent cell
92 damage ($<15 \text{ kPa}$) and stored at -80°C until nucleic acids extraction. For some samples (Supporting
93 Information Table S1), the rest of the microbial biomass was fixed with paraformaldehyde (final
94 concentration, 2%), incubated at 4°C for 24 h and collected on 0.8- μm -pore-size polycarbonate filters
95 (Millipore; pressure $<15 \text{ kPa}$). Filters were preserved by dehydration in an ethanol series (50, 80, and
96 100% ethanol for three min each) and stored at -20°C until tyramide signal amplification-fluorescent
97 in situ hybridization (TSA-FISH) staining.

98

99 **Nucleic acids extraction, amplification and sequencing**

100 Filters prepared for DNA extraction were processed as described in Dubuffet et al. (2021).
101 Amplification of the V1-V3 region of the microsporidian gene coding for the SSU rRNA was
102 performed using the universal microsporidian V1F and 530R primers set (Zhu et al. 1993; Baker et
103 al. 1995). Even though other recently developed primers have shown their efficiency (i.e. CM-V5F
104 CM-V5R, $\sim 200 \text{ bp}$, Trzebny et al. 2020), this set of primers amplify a fragment of $\sim 400 \text{ bp}$ and has
105 shown its efficiency in the detection of aquatic Microsporidia (Dubuffet et al. 2021; Chauvet et al.

2022). Each PCR was performed in a total volume of 30 μ L containing 6 μ L of 5x Green GoTaq Flexi buffer, 2.4 μ L of 25 mM $MgCl_2$, 0.18 μ L of GoTaqG2 Flexi DNA Polymerase (Promega, 5 U/ μ L), 0.6 μ L of 10 mM each dNTP, 0.3 μ L of 50 mg/mL BSA and 0.6 μ L of each 10 μ M primer. The amplification conditions consisted of initial denaturation at 95 °C for 5 min followed by 35 cycles for 1 min at 95 °C, 1 min 30 s at 62 °C and 1 min 30 s at 72 °C, and a final elongation of 7 min at 72 °C. Amplification of the V4 region of total eukaryotes (excluding Microsporidia) was performed using the universal primer 515F (Caporaso et al. 2011) and the eukaryotic primer 951R (Mangot et al. 2013). Each PCR was performed in the mix described above. The amplification conditions consisted of initial denaturation at 95 °C for 5 min followed by 33 cycles for 1 min at 95 °C, 1 min at 55 °C and 1 min 30 s at 72 °C, and a final elongation of 10 min at 72 °C. The amplicons were purified and concentrated using the QIAquick Gel Extraction kit (Qiagen). Samples were then sequenced on a MiSeq Illumina instrument (2x250 bp) (Microsynth AG).

118

119 **Amplicon analysis and taxonomic affiliation**

120 Sequencing data were processed using the PANAM2 (Phylogenetic Analysis of Next-generation
121 AMplicons v2 - <https://github.com/meb-team/PANAM2>) pipeline (Taib et al. 2013). Sequences with
122 ambiguous 'N' bases, those below 200 bp, chimeras or sequencing errors in the primers were
123 eliminated. The sequences were then assembled (overlap of 50 bp minimum between forward and
124 reverse reads, maximum 2 mismatches in the overlap) and clustered into OTUs with a similarity
125 threshold of 99% for Microsporidia (Dubuffet et al. 2021) and 95% for other eukaryotes (Mangot et
126 al. 2013; Debroas et al. 2017). The representative sequences of each OTU were affiliated using a
127 custom curated reference database (Freshwater-database: [https://github.com/panammeb/Freshwater-](https://github.com/panammeb/Freshwater-database)
128 database). Affiliations to microsporidian groups and genera were considered as valid when the
129 identity scores were above 85% and 94.5%, respectively (Dubuffet et al. 2021). As clustering
130 threshold used here (especially for Microsporidia) may generate OTUs resulting from sequencing

131 errors, we only kept for the final analysis OTUs representing more than 0.005% (Bokulich et al. 2013)
132 of the total reads. Data were normalized using the SRS (Scaling with Ranked Subsampling) method
133 (Beule and Karlovsky 2020).
134 Phylogenetic analyses were performed to show the placement of the sequences obtained in this study
135 among the different microsporidian clades (Supporting Information Fig. S1, S2). We focused our
136 analyses on abundant OTUs (100 most abundant of each clade, when more than 100 OTUs were
137 identified) and on the five major clades of this study (I, III, IV1, IV2 and V). After an alignment using
138 MAFFT algorithm (Katoh et al. 2005), ML trees were obtained using the best predicted model of
139 evolution according to the BIC score (general time-reversible (GTR) model with gamma-distributed
140 rate heterogeneity (G)) with 200 bootstrap replications and partial deletion (85%), using MEGA 7
141 (Kumar et al. 2016).

142

143 **Diversity and correlation analyses**

144 Diversity analyses were performed using R (version 3.6.3, R Development Core Team 2012).
145 Community richness and diversity indices (Richness observed, Chao1, Shannon and Simpson) were
146 used to infer the taxa richness of Microsporidia. These estimators were computed with the phyloseq
147 package (version 1.30.0, McMurdie and Holmes 2013). Principal coordinates analysis (PCoA)
148 ordination based on a Bray-Curtis dissimilarity matrix (Bray and Curtis 1957; Paliy and Shankar
149 2016) was computed using ape (version 5.6-2, Paradis and Schliep 2019) and vegan (version 2.5-7,
150 Oksanen et al. 2020) packages. An Upset plot was done to show the sets and intersections of OTUs
151 between samples with the UpSetR package (version 1.4.0, Conway et al. 2017).

152 As microsporidian and eukaryotic communities' composition were largely different between marine
153 and freshwater samples, the correlation analysis was done on each dataset. To reduce the number of
154 tested hypotheses and focus the analysis on 'abundant OTUs', only those representing more than
155 0.05% of the reads were retained, representing 302 eukaryotic OTUs and 253 microsporidian OTUs

156 in freshwater and 237 eukaryotic OTUs and 268 microsporidian OTUs in marine ecosystems.
157 Spearman's ρ correlations were calculated, and to decrease false discovery rate due to multiple testing
158 we used Benjamini-Hochberg corrections (Benjamini and Hochberg 1995). A correlation was
159 considered as valid when $\rho > 0.8$ and the associated p-value < 0.01 . Correlations were then visualized
160 with chord diagrams using the circlize package (version 0.4.14) on R (R Development Core Team
161 2012; Gu et al. 2014).

162

163 **Tyramide Signal Amplification-Fluorescent In Situ Hybridization**

164 TSA-FISH was performed using a Microsporidia specific probe (USP01) as described in Chauvet et
165 al. (2022a). This probe is suitable for the detection and observation of aquatic Microsporidia, being
166 able to hybridize (in silico) with 100% of the microsporidian sequences obtained during recent
167 metabarcoding studies (Dubuffet et al. 2021; Chauvet et al. 2022a) as well as all the sequences
168 obtained in the current study. In silico, this probe does not hybridize with any sequence other than
169 microsporidian. Hybridization conditions were determined on a target species (*Encephalitozoon*
170 *cuniculi*) and a non-target species (*Anncaliia algerae*) having one mismatch with the probe sequence.
171 The probe was also tested on two other Microsporidia in culture: *Nosema ceranae* and *Nematocida*
172 *parisii*. Observations were performed using a Zeiss Axio Imager 2 microscope (Carl Zeiss).

173 ***Results and discussion***

174 In this work, we sampled 15 different sites across freshwater (n = 9) and marine (n = 6) environments
175 (Fig. 1, Supporting Information Table S1), size-fractionated the samples and used microsporidian
176 specific primers to unravel microsporidian diversity which once associated with total eukaryotic
177 diversity allow the identification of potential host-parasite associations.

178 After quality filtering, clustering at 99% and cleaning steps, a total of 1 853 microsporidian OTUs
179 were obtained from 2 944 084 SSU rRNA gene sequencing reads (1 045 560 reads after
180 normalization). OTUs obtained in this study belonged to the canonical (or long-branch (Bass et al.
181 2018)) Microsporidia and were all affiliated at least to a clade. Among the 10 clades defined for
182 canonical Microsporidia (Dubuffet et al. 2021), our OTUs were distributed in seven clades: I, II, III,
183 IV1, IV2, V, VIII (Fig. 1, Supporting Information Fig. S1, S2). 63.9% of the OTUs were affiliated to
184 a group and 9 groups were identified. Less than half (45.5%) of the OTUs were affiliated to a genus
185 among the 20 genera identified, demonstrating that a large part of the microsporidian diversity
186 remains to be described.

187

188 **Microsporidian diversity across freshwater environments**

189 So far, few studies have investigated the global diversity of Microsporidia in freshwater ecosystems
190 using high-throughput sequencing (i.e. metabarcoding). However, these studies (Williams et al. 2018;
191 Dubuffet et al. 2021; Chauvet et al. 2022a) allowed the identification of a huge and new
192 microsporidian diversity but did not allow a broad comparison of this diversity across different
193 freshwater ecosystems.

194 Here, we studied this diversity in nine different freshwater sites: eight lakes and one temporary marsh
195 (Fig. 1, Supporting Information Table S1). The freshwater microsporidian diversity was largely
196 represented by clade IV2, accounting for over 87% of the total reads (Fig. 2a). This clade is highly
197 diverse and is known to parasitize a wide range of hosts such as insects, nematodes, crustaceans and

198 fish, as well as humans, and its members have been identified in terrestrial, freshwater, and marine
199 environments (Dubuffet et al. 2021). *Euplotespora binucleata* and *Globosporidium paramecii* (Fokin
200 et al. 2008; Yakovleva et al. 2020), which are among the few microsporidian species known (SSU
201 sequences available) to parasitize protists (i.e. ciliates), are also found in this clade. Clades IV1 and I
202 were the next most abundant representing 6% and 5% of the OTUs, respectively. The first one mainly
203 includes species described as insects' parasites in terrestrial environments (Park and Poulin 2021),
204 including also human-infecting species. Clade I, on the other hand, is commonly described as a
205 freshwater clade, since it is mainly found in freshwater crustaceans and insects in various
206 environments such as lakes, rivers, ponds, etc. (Andreadis et al. 2012; Vossbrinck et al. 2014). The
207 other clades were less than 1% abundant (Fig. 2a). These findings partly overlap those from Dubuffet
208 et al. (2021) and Chauvet et al. (2022a) in which clade IV2 and I were the most abundant. However,
209 in these two studies clade IV1 was not well represented (~2%), whereas it was the second most
210 abundant clade in the present dataset, largely due to its overrepresentation in the Estivadoux sample
211 (Fig. 1, 3). Among the sampled freshwater ecosystems which are mostly lakes, the Estivadoux is quite
212 different, it is indeed a temporary marsh that can be completely dried during summer. The typical
213 hosts of clade IV1 are mainly insects of terrestrial environments, it therefore seems consistent to
214 retrieve this clade with a higher relative abundance in this sample.

215 The Arlequin group (clade IV2) was the most abundant (49% of the reads), this group being largely
216 represented by the *Crispospora* genus (40% of the reads), as already found in previous studies
217 (Dubuffet et al. 2021; Chauvet et al. 2022a) (Fig. 1). It's noteworthy that the only one OTU shared
218 by the nine freshwater samples was affiliated to the genus *Crispospora* and was actually the most
219 abundant OTU in the whole dataset. In this genus only one species is described (*Crispospora*
220 *chironomi*) and known to parasite chironomid larvae (*Chironomus plumosus*) in freshwater
221 ecosystems (Tokarev et al. 2010). Moreover, a recent study suggests that a new *Crispospora* species
222 might be able to parasitize *Kellicottia* rotifer in lake environments (Chauvet et al. 2022b).

223 Also in agreement with previous studies, *Pseudoberwaldia* and *Conglomerata* (Aquatic outgroup;
224 clade I) were the next most abundant genera (Fig. 2a). These two genera are, each, described by only
225 one species, both of which are known to be parasites of cladocerans in freshwater (Vávra et al. 2018,
226 2019).

227 Some groups and/or genera were recorded with a significant relative abundance only in two or three
228 samples as the *Orthosomella*-like group (clade IV2), mainly found in samples from lakes Chambon,
229 Saint-Gervais and Aydat. This group is so far only described as parasite of insects and nematodes in
230 terrestrial environments (Nishikori et al. 2018). *Conglomerata* (Aquatic outgroup; clade I) was
231 mainly found in lakes Chambon, Pavin and Chauvet, and *Pseudoberwaldia* (Aquatic outgroup; clade
232 I) in lakes Bourdouze and Chambon (Fig. 3). These sites may be particularly rich in daphnia
233 (cladoceran), which is the only described host of the latter two genera. Surprisingly, the *Vittaforma*
234 genus (Arlequin group; clade IV2) has been mainly found in lake Bourdouze and not in the recreative
235 lakes (Aydat, Chambon, Leman, Bourget), whereas it is currently described through a single species
236 that is a human parasite (Silveira and Canning 1995).

237 It therefore appeared that the microsporidian diversity varied across freshwater sampling sites even
238 at a high taxonomic rank (i.e. clade level (Fig. 1, 3, 4)). In addition, a surprising result compared to
239 previous studies was the significant proportion of clade IV2 OTUs not affiliated to any group (33%),
240 especially in samples from lakes Bourget and Leman where these OTUs accounted for 83.2% and
241 75%, respectively (Fig. 3). These two lakes could represent an important reservoir of new
242 microsporidian genera and species and thus potential new hosts. Altogether, these results illustrated
243 the importance to increase the sampling effort in freshwater to unravel the full extent of the
244 microsporidian diversity in these environments.

245

246

247

248 **Extent of the marine microsporidian diversity**

249 To date, the overall diversity of Microsporidia in marine ecosystems has received little attention.
250 Indeed, to our best knowledge, only Williams et al. (2018) have initiated the study of the global
251 microsporidian diversity in the marine environment, through coastal, estuary and rock pool samples,
252 however with a low number of OTUs. Here, we studied this diversity in six different marine sites
253 close to the coast: three bays, one estuary and two coastal ecosystems (Fig. 1, Supporting Information
254 Table S1). The marine microsporidian diversity was largely dominated by clade IV2 and III,
255 accounting for 49% and 40% of the total reads (Fig. 2b), the latter being a clade commonly described
256 as ‘marine’, but which includes species parasite of crustaceans and fish, in both marine and freshwater
257 environments (Vossbrinck et al. 2014; Park and Poulin 2021 (clade V in these studies = clade III
258 here)). This clade also includes a protist parasite, the species *Hyperspora aquatica* hyperparasite of
259 paramyxids (Stentiford et al. 2017). Clades IV1, V and I followed with 7%, 3% and 2% of the reads,
260 respectively. Clade II was the least abundant with 0.1% of the reads and was identified only in Corsica
261 (Fig. 2b). Due to the low number of OTUs (50) obtained by Williams et al. (2018) the comparison
262 with our data is limited. However, we can mention that higher proportion of clade III was recorded
263 in our samples but lower proportions of clades I, V and especially II and no OTUs belonging to clade
264 IX was found. The latter clades (I, II, V and IX) are known to be parasites of terrestrial and/or
265 freshwater hosts including crustaceans, insects, nematodes, annelids, bryozoans, and humans
266 (Vossbrinck et al. 2014; Dubuffet et al. 2021), it is thus not surprising to found them with a low
267 relative abundance here. Clades IV2 and III on the other hand are the ones with the most members
268 found in marine ecosystems (Park and Poulin 2021).

269 The *Ameson*-like group (clade III) and Arlequin group (clade IV2) were the most abundant with 38%
270 and 35% of the total reads, respectively (Fig. 2b). Three samples were mostly represented by the
271 *Ameson*-like group (Roscoff, Corsica and Arcachon), while two other samples were dominated by
272 the Arlequin group (Brest and La Rochelle) (Fig. 3).

273 Three genera were particularly abundant: *Crispospora* (Arlequin group), *Ameson* and *Nadelspora*
274 (*Ameson*-like group), accounting for 31%, 23% and 15% of the reads, respectively (Fig. 2b).
275 *Crispospora* has never been identified in hosts living in marine environments, and neither
276 chironomids nor rotifers are abundant in these systems, thus raising the hypothesis of one or many
277 other hosts for the *Crispospora* genus. Moreover, the proportion of *Crispospora* identified here in the
278 two ecosystems studied, both in terms of OTUs and abundance, suggests that a significant diversity
279 remains to be discovered within this genus where only one species is currently described. This relative
280 abundance may also reveal significant host diversity, as ~80% of microsporidian species are
281 specialists (Murareanu et al. 2021).

282 The *Ameson* genus includes different species all described as parasitizing arthropods in the marine
283 environment. The hosts described are mainly crustaceans (crab, lobster, shrimp) and one species is
284 described as an insect parasite (Murareanu et al. 2021). *Nadelspora* is known to parasite the crab
285 *Metacarcinus magister* through the only described species: *Nadelspora canceri* (Olson et al. 1994).

286 Other genera identified in these samples were much less abundant such as *Sporanauta* (clade IV1),
287 *Octosporea* and *Pseudoberwaldia* (Aquatic outgroup; clade I), each representing about 0.5% of the
288 reads (Fig. 2b). *Sporanauta* and *Pseudoberwaldia* are described by one species each as parasites of
289 nematodes and cladocerans, respectively (Ardila-Garcia and Fast 2012; Vávra et al. 2019).

290 *Octosporea* genus, on the other hand, includes many species that are parasitic on different hosts, all
291 of which belong to the arthropods. *Octosporea* species are mainly described as insect parasites
292 (Diptera and Hemiptera) and are also found in some cladocerans (*Daphnia*) and amphipods
293 (*Gammarus*) (Murareanu et al. 2021).

294 In these marine samples, a higher proportion of the diversity was common between the samples
295 compared to freshwater ecosystems as 402 OTUs were shared by the six marine samples; 84% of
296 these OTUs were affiliated to *Crispospora*. Despite these shared OTUs, some samples could be quite
297 different from each other considering some clades/groups/genera (Fig. 3). The sample from Brest, for

example, had only one OTU belonging to clade III. This was surprising since this was the second most abundant clade in our study and the clade in which the highest number of marine Microsporidia are found.

Microsporidian community composition in aquatic ecosystems in relation to their hosts

Although marine samples contained less reads than freshwater samples, all diversity index showed a more diverse community in marine sites (Supporting Information Fig. S3, S4). The marine sample from La Rochelle for example was the one with the highest number of OTUs (1 157) but the fewest reads (71 122). 63.4% of the OTUs were common to both ecosystem types (the majority belonging to the *Crispospora* genus, or being unaffiliated beyond clade IV2), 16.1% and 20.5% were specific to marine and freshwater ecosystems, respectively. Considering only OTUs richness, ecosystem types were indistinguishable, whereas differences were identified when considering the relative abundance of OTUs (Fig. 4). Most of the OTUs (82%) found only in freshwater samples belong to the clade IV2, whereas OTUs specific to marine samples were mostly found in clade III (72%) (Supporting Information Fig. S2b, S2D). Only one OTU (the most abundant of the whole dataset), affiliated to *Crispospora* (clade IV2; Arlequin group), was shared by all the samples. Some OTUs were shared by different samples (Supporting Information Fig. S5), but all the 15 samples showed specific OTUs (on average 1% of the OTUs). The affiliation was better in marine samples, as 58.4% of OTUs were affiliated to a group and 38.9% to a genus in freshwater versus 62.0% and 53.6% in marine samples suggesting that a large part of the diversity is unknown. It therefore seems essential to continue the sampling effort across aquatic ecosystems to unravel the global microsporidian diversity (without the bias of targeting hosts).

At the clade level, the major differences were the absence of clade VIII in the marine samples and the very low relative abundance (0.003%) of clade III in freshwater whereas it represents 40% of the microsporidian abundance in marine samples (Fig. 2a, 2b). Species belonging to clade VIII are

described as parasites of insects (mainly Diptera), affecting larvae that are present in freshwater (Murareanu et al. 2021). Clade III is mostly described as parasitizing fishes and crustaceans in marine and freshwater environments and is sometimes described as the ‘marinosporidia’ clade (Vossbrinck et al. 2014) even if Williams et al. (2018) showed the inconsistency in the classification based on the ecological niche that Microsporidia and/or their hosts inhabit. However, the primers used in this study target a wide microsporidian diversity, except in clade III (data not shown). The environmental diversity of Microsporidia affiliated to clade III is therefore likely underestimated in the two types of ecosystems.

Other differences at the genus level (apart from the genera belonging to clade III) were notable between the two types of environments such as the significantly higher relative abundance of *Conglomerata* (Aquatic outgroup; clade I) in freshwater (Supporting Information Fig. S6) and the absence of *Octosporea* (Aquatic outgroup; clade I) as well as *Mrazekia* and *Helmichia* (both in Arlequin group; clade IV2) in freshwater and marine samples, respectively (Fig 2a, 2b, 3). Some of these results are consistent with the type of host described for these genera, such as *Conglomerata* which is currently described as parasitizing only the freshwater *Daphnia pulex* (Vávra et al. 2018). These differences nevertheless involve genera found at low relative abundance, not allowing us to clearly establish their real absence in the second ecosystem and to draw conclusions on these differences.

The environmental variables studied here (salinity and temperature), trophic states, ecosystem type, size fraction, and geographical location, do not seem to explain the distribution of the microsporidian communities (Fig. 4, Supporting Information Table S1). Although the number of parameters considered here are low, it has also never been shown that abiotic parameter can directly influence Microsporidia in their environment. The diversity observed was rather in agreement with the hosts described so far, at least in terms of habitat, even though some genera have been surprisingly

348 identified in an environment that does not harbor their (often single) described hosts. Ultimately, it
349 appears that microsporidian communities are probably driven by the dynamic of their hosts and not
350 directly influenced by environmental variables. Most of the Microsporidia identified in this study are
351 so far known to parasite large hosts such as fish, crustaceans, and insects. It therefore appears that the
352 Microsporidia found in our study can infect smaller and so far, unknown hosts and/or that their free-
353 living forms, the spores, actively circulate in the water column *via* ingestion/filtration by planktonic
354 organisms of different sizes. Approximately 80% of the microsporidian species described (with host
355 data) are specialists (i.e. described as infecting only a single host), the remaining species are
356 generalists with some capable of infecting more than ten hosts (Murareanu et al. 2021), some having
357 the ability to infect both vertebrate and invertebrate (Stentiford et al. 2016). The hypothesis of a
358 second host is already suggested for *Pseudoberwaldia* (Vávra et al. 2019). Our results thus also
359 suggest that a significant proportion of Microsporidia could infect more than one host, especially in
360 the overlooked microeukaryotic compartment.

361
362 We studied the diversity of Microsporidia and planktonic eukaryotes ($< 150\ \mu\text{m}$) in parallel (Fig. 5).
363 The eukaryotic communities identified were quite different between the two types of ecosystems
364 studied (Fig. 5, Supporting Information Fig. S7). The freshwater diversity was mainly represented by
365 dinoflagellates (16%), Chlorophyta (11%), ciliates (9%) and rotifers (5%), whereas marine diversity
366 was mainly composed of arthropods (29%), dinoflagellates (14%), Chlorophyta (10%) and Bryozoa
367 (6%) (Fig. 5).

368 As for Microsporidia, some sites showed a specificity in their eukaryotic diversity that could explain
369 the distribution of the microsporidian communities. Lakes Lemane and Bourget, for example, were
370 particularly rich in rotifers compared to other lakes (Fig. 5), these rotifers belonging mainly to the
371 Brachionidae and Notommatidae. Similarly, dinoflagellates were most abundant in lake Saint Gervais
372 and Bryozoa in the Brest sampling point. To identify new host-parasite associations, OTUs

373 representing more than 0.05% of reads in both datasets were used to reveal co-occurrences. Only
374 positive associations were considered as it has already been showed that parasitism is retrieved as a
375 positive link between the partners in co-occurrence network despite the detrimental impact of the
376 parasite on the host (Weiss et al. 2016). Moreover, potential associations were searched by ecosystem
377 types since it is better to have homogeneous environments to identify biotic interactions (Faust 2021).
378 In freshwater, 205 potential associations were identified between 32 microsporidian OTUs and 14
379 eukaryotic OTUs, mostly involving metazoans (platyhelminths, gastrotrichs and arthropods) and
380 alveolates (dinoflagellates, apicomplexans, and ciliates) (Fig. 6a). These correlations largely involved
381 OTUs unaffiliated beyond the clade (IV1, IV2 and I) or the group (Arlequin and Aquatic outgroup),
382 as well as two genera: *Cystosporogenes* and *Crispospora* (both in the Arlequin group; clade IV2). In
383 marine environments, 128 potential associations were identified between 69 microsporidian OTUs
384 and 35 eukaryotic OTUs (Fig. 6b). As well as for freshwater, a lot of potential associations involved
385 Microsporidia OTUs unaffiliated beyond the clade (V, IV2, IV1, II and III) or the group (Arlequin,
386 *Orthosomella*-like, Enterocytozoonidae, *Cucumispora*-like and Aquatic outgroup), and some genera:
387 *Crispospora*, *Ameson*, *Obruspora*, *Nadelspora*, *Vittaforma*, *Sporanauta* and *Octosporea*. Eukaryotic
388 OTUs involved belong mostly to the alveolates (dinoflagellates, apicomplexans, and ciliates).
389 Metazoans (cnidarians, arthropods, annelids, mollusks, and Bryozoa) and Fungi (Ascomycota,
390 Basidiomycota, chytrids) were also involved in these co-occurrences (Fig. 6b). Metazoans represent
391 most of the described microsporidian host. Among them, arthropods, platyhelminths, gastrotrichs,
392 cnidarians, annelids, mollusks, and Bryozoa (identified here in the co-occurrences) have already been
393 described as hosts (Murareanu et al. 2021). Similarly, the potential associations identified with
394 alveolates were previously described for some, as Microsporidia have been shown to parasitize
395 apicomplexans and ciliates although these descriptions are anecdotal and generally only
396 morphological (e.g., only two microsporidian SSU sequences parasites of ciliates (Fokin et al. 2008;
397 Yakovleva et al. 2020)). In contrast, there are currently no descriptions of Microsporidia parasitizing

398 dinoflagellates. Fungi, also retrieved in these co-occurrences in potential association with
399 Microsporidia, have also never been described as their hosts. In both ecosystems, some correlations
400 were also found with phytoplanktonic organisms (Fig 6a, 6b), whereas to date, infection by
401 Microsporidia in phytoplankton has never been recorded. Interestingly, some of the potential
402 associations identified here were also retrieved in co-occurrence networks involving short-branch
403 Microsporidia (Doliwa et al. 2021).

404 Although co-occurrences network analyses should be interpreted with caution (Carr et al. 2019;
405 Blanchet et al. 2020) they are increasingly used to identify potential associations within
406 microorganisms. The method used here has its limitations, as other tools are more efficient and
407 adapted to such datasets (e.g., Kurtz et al. 2015) but often require more samples. However, this
408 correlation-based method can show comparable efficiency to other more sophisticated methods
409 (Hirano and Takemoto 2019 ; Faust 2021). However, associations between Microsporidia and
410 eukaryotes suggested by co-occurrences network should be considered as working hypothesis and
411 need to be confirmed by other approaches allowing the observation and the identification of both
412 partners (e.g., fluorescence microscopy or host/parasite genomic analysis).

413 Using TSA-FISH with a universal microsporidian probe (USP01, Chauvet et al. 2022a) we confirmed
414 the Microsporidia-*Kellicottia* (rotifer) association already observed in another lake (Chauvet et al.
415 2022a,b), here in lake Chauvet. We also identified microsporidian infections in dinoflagellates
416 (probably belonging the genus *Ceratium*) both in freshwater and marine ecosystems (Fig. 7,
417 Supporting Information Fig. S8). Indeed, these infections were observed in lakes Lemans (prevalence,
418 49% of infected individuals), Bourget (53%) and Saint Gervais (20%), and in the La Rochelle (38%)
419 marine sample. These observed Microsporidia-dinoflagellates associations in both ecosystems
420 significantly expand the host range of Microsporidia and their impact on food web functioning.
421 Indeed, dinoflagellates are relatively abundant in freshwater and marine environments, and show a
422 diversity of trophic modes (autotrophy, heterotrophy or mixotrophy). Thanks to this diversity,

423 dinoflagellates are among the major primary producers (with diatoms), they can as well be grazers,
424 and have symbiotic or parasitic relationships, while others are known to be important toxin producers
425 (Carty and Parrow 2015).

426

427 **Conclusions**

428

429 In conclusion, this study highlighted for the first time a huge microsporidian diversity in marine
430 samples, with an important part (46%) of the OTUs not affiliated to a genus suggesting an important
431 reservoir of new microsporidian species and thus potential new hosts, especially among clade IV2.
432 This diversity was also widely divergent from the diversity identified in freshwater. Interestingly,
433 most of the identified genera are described as parasites of organisms much larger than the studied size
434 fraction ($<150\ \mu\text{m}$). It therefore appears that these Microsporidia infect smaller and, so far, unknown
435 hosts such as dinoflagellates both in marine and freshwater ecosystems. These findings also greatly
436 expand the host range of Microsporidia which were never described in association with
437 phytoplanktonic hosts. Also, the proportion of the *Crispospora* genus raises questions about their
438 hosts and impact especially in marine environments and should be the subject of further research.
439 Altogether, these results indicate that the microsporidian diversity and host spectrum could be
440 therefore more important than previously described, and their role in the functioning of aquatic
441 ecosystems and especially marine ecosystems is undoubtedly largely unknown.

442

443 *References*

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632

Competing Interests

The authors declare they have no competing interests.

Author contributions

MC and CL designed the study. MC, AM, and CL performed sampling and experiments. MC and CL wrote the manuscript. All authors have read and approved the manuscript.

Data availability statement

All data and material are available in this publication and additional information online (Supporting Information). The data have been deposited with links to BioProject accession number PRJNA922474 in the NCBI BioProject database.

634 **Figure legends**

635

636 Fig. 1. Sample collection sites. Samples were collected in France from freshwater lakes (green stars):
637 1 Saint-Gervais (hypereutrophic), 2 Chambon (eutrophic), 3 Aydat (eutrophic), 4 Bourget
638 (oligomesotrophic), 5 Lemans (mesotrophic), 6 Chauvet (oligotrophic), 7 Pavin (oligomesotrophic), 8
639 Estivadoux (temporary marsh), 9 Bourdouze (oligotrophic); and marine environments (blue stars): 10
640 Corsica (bay), 11 Royan (estuary), 12 Roscoff (coastal), 13 Brest (bay), 14 Arcachon (bay), 15 La
641 Rochelle (coastal). Pie charts represent the proportions of microsporidian clades identified in each
642 sample.

643

644 Fig. 2. Krona charts representing the taxonomic affiliation of the microsporidian OTUs retrieved in
645 freshwater (a) and marine (b) samples. Inner circle represents clades. Deeper taxonomic ranks (groups
646 and genera) are presented in outer circles. Genera names are given in *italic*. Hatched patterns represent
647 OTUs unaffiliated beyond the group/clade. Genera listed are ranked from most to least abundant.

648

649 Fig. 3. Relative abundances of the most abundant ($>0.1\%$ of the total reads) microsporidian genera
650 across freshwater (left) and marine (right) samples. The black zone at the bottom of each bar
651 represents the cumulative proportion of other genera with low abundances.

652

653 Fig. 4. PCoA based on Bray-Curtis distances of the microsporidian OTUs richness (left) and relative
654 abundances (right) across samples (first three PCoA axes shown). Colors of the data points indicate
655 the ecosystem (freshwater or marine). Ellipses in the plots denote 95% confidence intervals, points
656 that are not overlapped by the two ellipses are considered significantly different.

657

658 Fig. 5. Relative abundances of the most abundant (>1% of the total reads) eukaryotic phyla across
659 freshwater (left) and marine (right) samples. The black zone at the bottom of each bar represents the
660 cumulative proportion of other phyla with low abundances.

661

662 Fig. 6. Diagrams showing the positive correlations between microsporidian OTUs (bottom) and
663 eukaryotic OTUs (top) in freshwater (a) and marine (b) ecosystems. Microsporidian OTUs are
664 grouped by genus, eukaryotic OTUs are grouped by phylum or class. One line represents one positive
665 correlation between two OTUs. un. = unclassified

666

667 Fig. 7. Microsporidia (targeted by TSA-FISH with the USP01 probe) infecting dinoflagellates in
668 freshwater samples. Under UV showing nucleus after DAPI staining (a), under blue light showing
669 Microsporidia in green fluorescence (b), overlay (c, d). Magnification on the microsporidian cells
670 inside the dinoflagellate with 3D reconstruction (d). Scale bars, 20 μ m.

671

672

673

Fig. 1

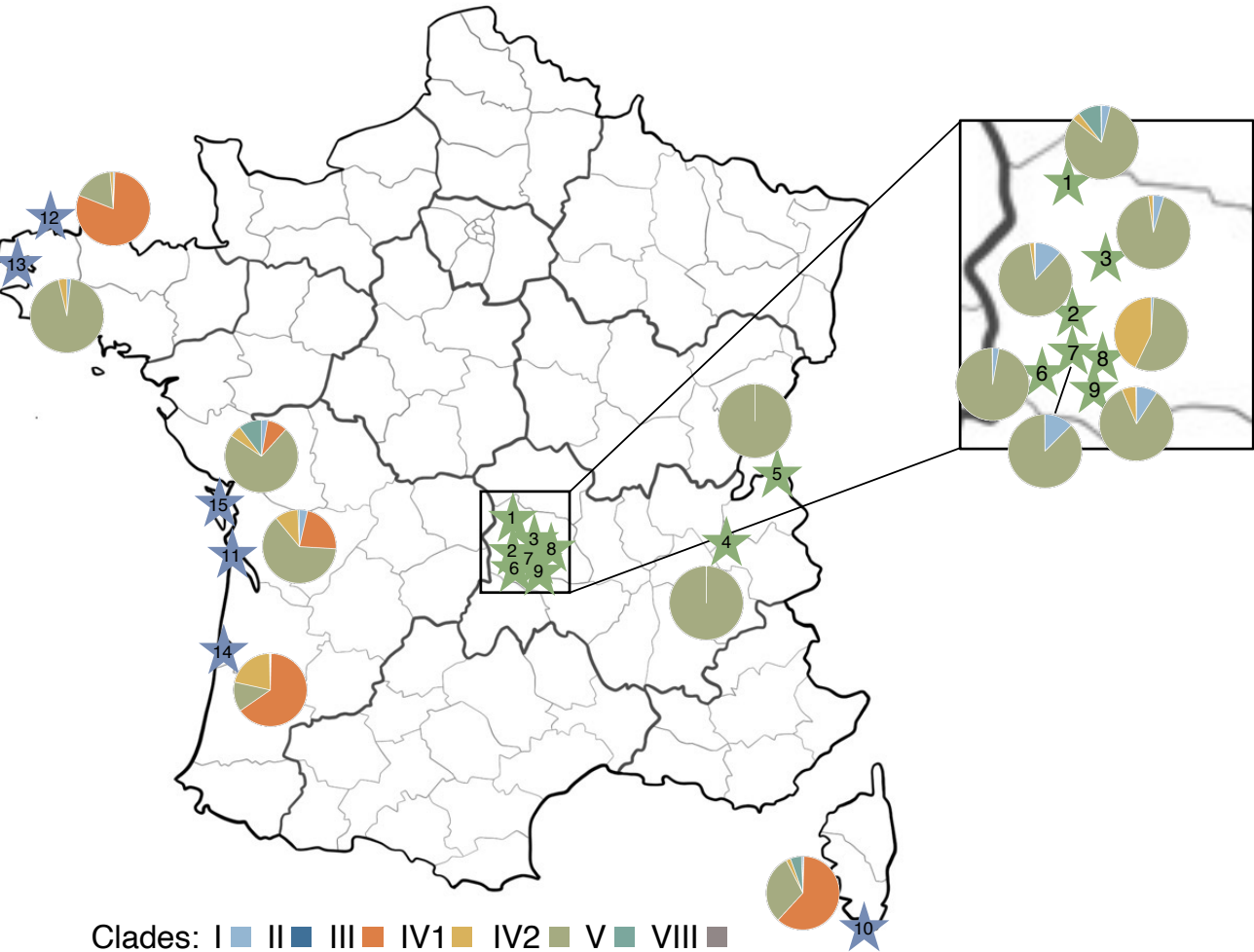


Fig. 2

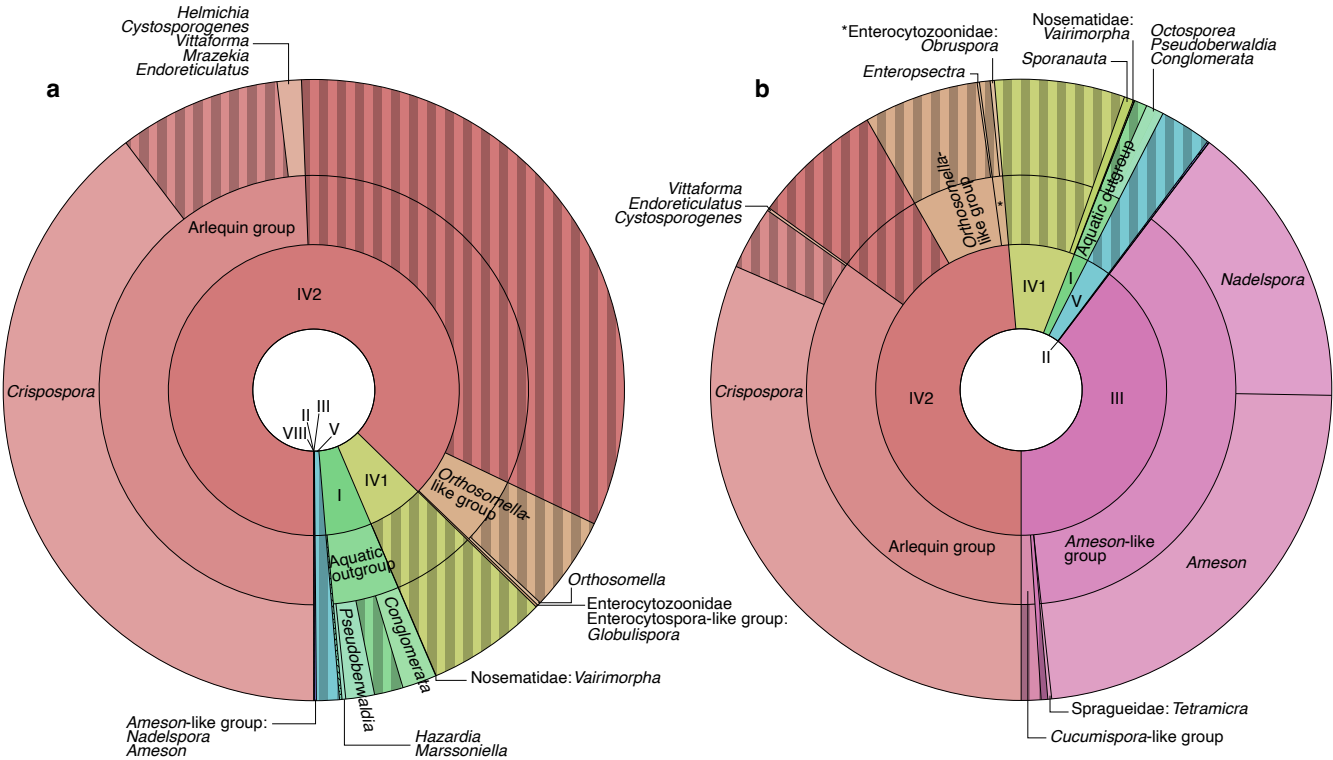


Fig. 3

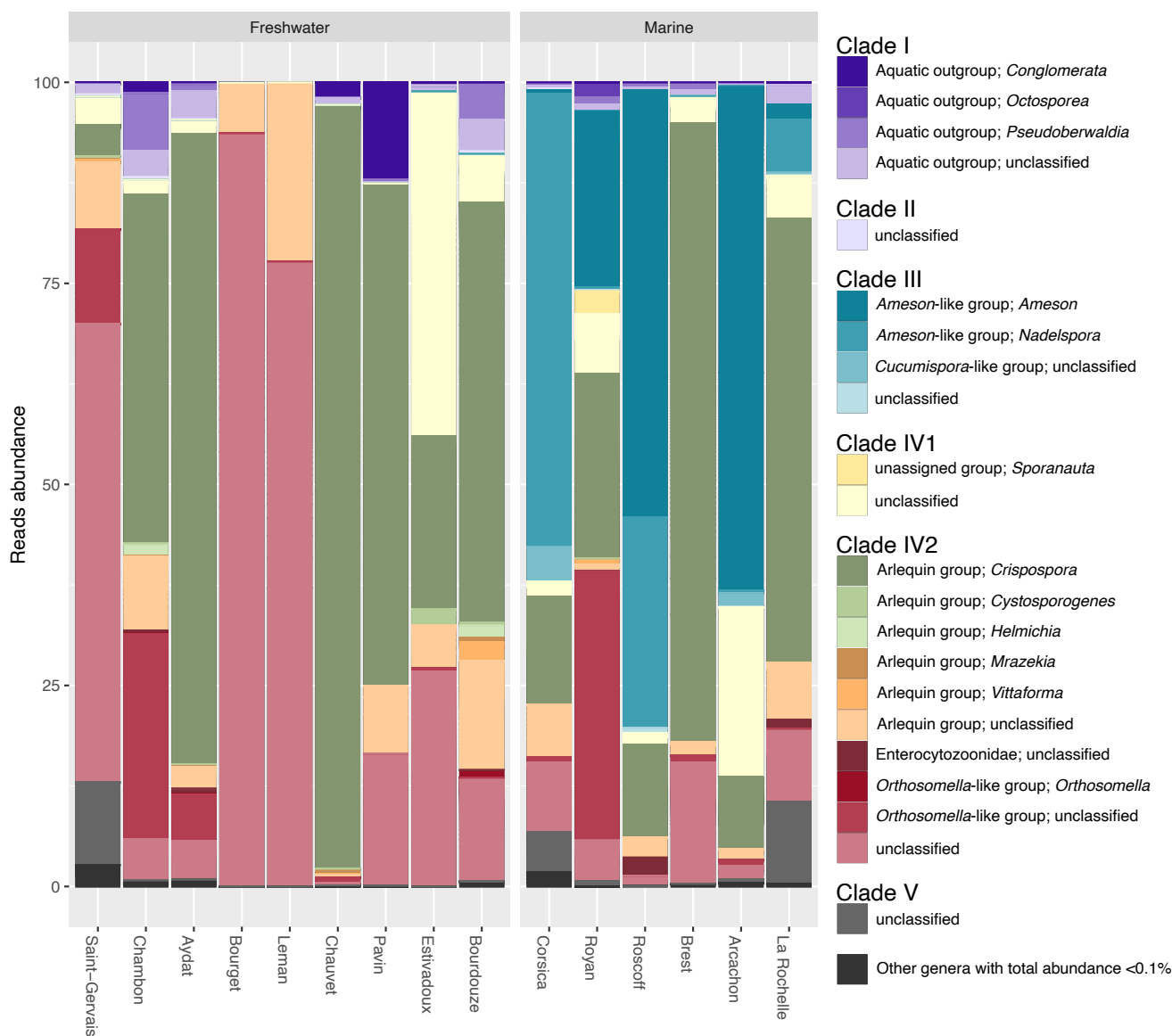


Fig. 4

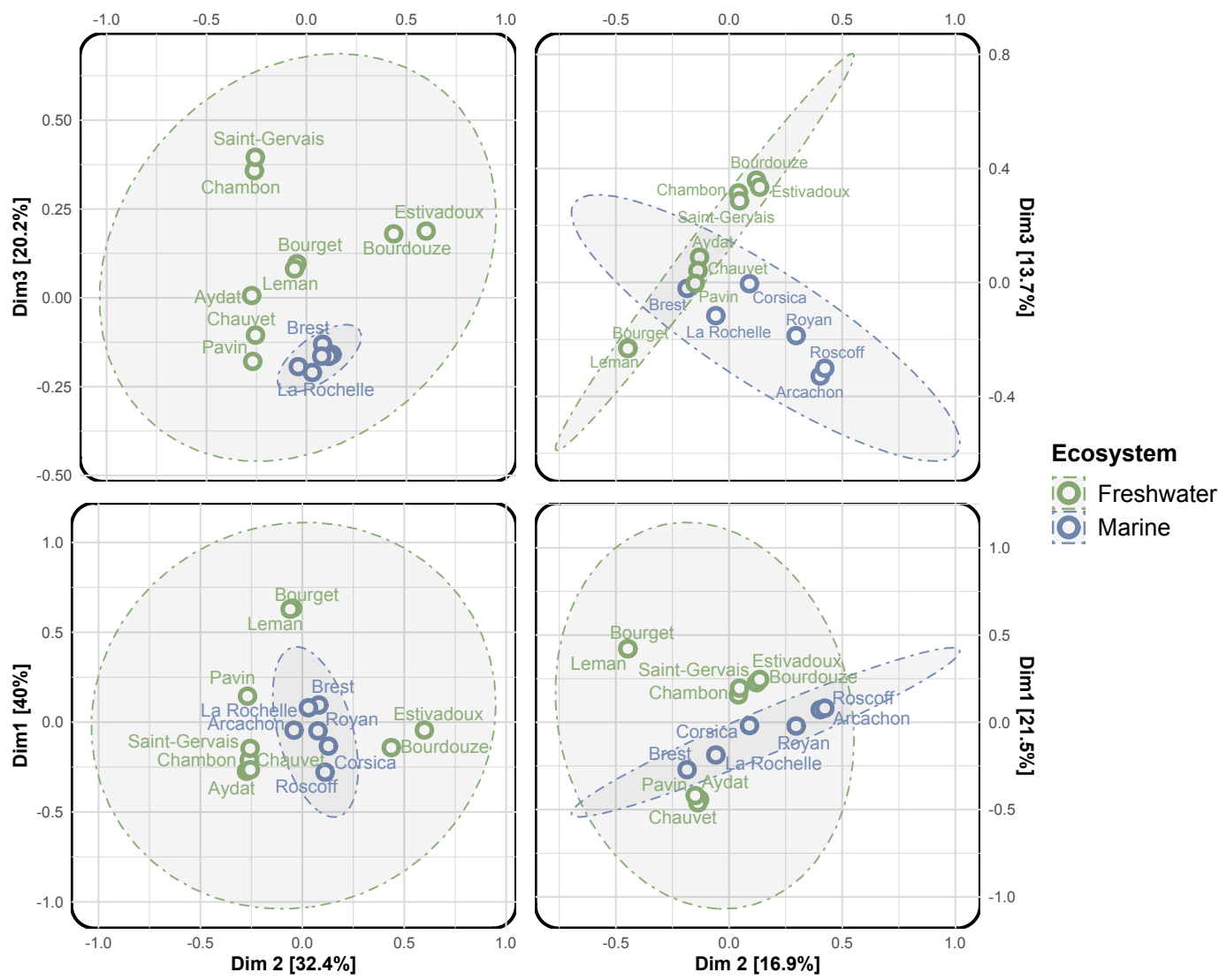


Fig. 5

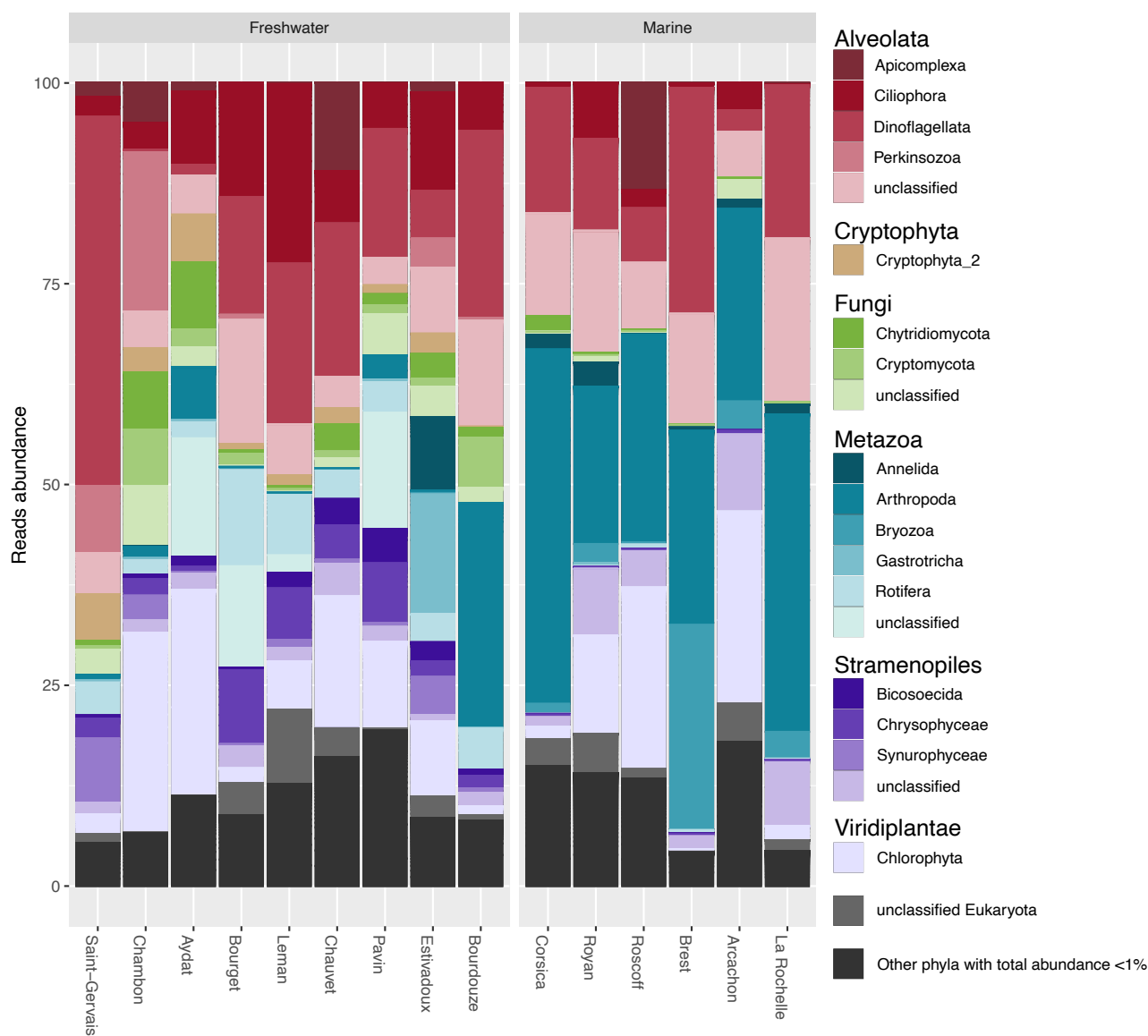


Fig. 6

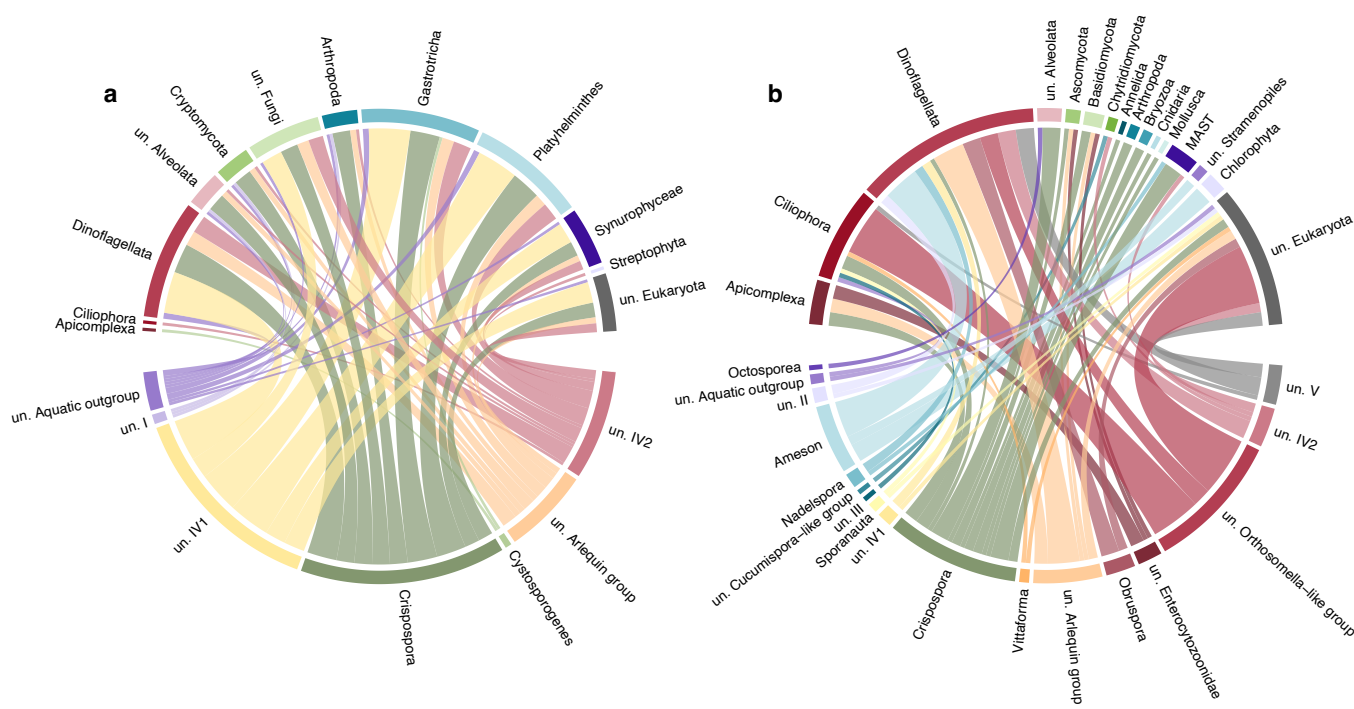


Fig. 7

