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1 **Some mixotrophic flagellate species selectively graze on Archaea**

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19 **ABSTRACT**

20 Many phototrophic flagellates ingest prokaryotes. This mixotrophic trait
21 becomes a critical aspect of the microbial loop in planktonic food webs because
22 of the typical high abundance of these flagellates. Our knowledge of their
23 selective feeding upon different groups of prokaryotes, particularly in field
24 conditions, is still quite limited. In this study, we investigate the feeding behavior
25 of three species (*Rhodomonas* sp., *Cryptomonas ovata* and *Dinobryon*
26 *cylindricum*) using their food vacuole content in field populations of a high
27 mountain lake. We used the Catalyzed Reporter Deposition - Fluorescent *in situ*
28 Hybridization protocol (CARD-FISH) with specific probes for the domain
29 Archaea and three groups of Eubacteria: β-Proteobacteria, Actinobacteria, and
30 Cytophaga-Flavobacteria of Bacteroidetes. Our results provide field evidence
31 that contrasting selective feeding exists between coexisting mixotrophic
32 flagellates under the same environmental conditions, and that some prokaryotic
33 groups may be preferentially impacted by phagotrophic pressure in aquatic
34 microbial food webs. In our study, Archaea was the preferred prey, chiefly in the
35 case of *Rhodomonas* sp., which rarely fed on any other prokaryotic group. In
36 general, prey selection did not relate to prey size among the grazed groups.
37 However, Actinobacteria, which were clearly avoided, mostly showed a size
38 <0.5 μm, markedly smaller than cells from the other groups.

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42 **IMPORTANCE:** That mixotrophic flagellates are not randomly feeding in the main
43 prokaryotic groups in field conditions is a pioneer finding in species-specific
44 behavior that paves the way for future studies according to this new paradigm.
45 The particular case that Archaea were preferentially affected in the situation
46 studied shows that phagotrophic pressure cannot be disregarded when
47 considering the distribution of this group in freshwater oligotrophic systems.

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49 **INTRODUCTION**

50 Mixotrophic behavior, the combination of phototrophic and phagotrophic
51 nutritional modes within a single cell, has been increasingly documented in
52 aquatic systems (1, 2). Phagotrophy occurs in a variety of phytoplankton
53 flagellates groups including Chrysophyceae, dinoflagellates, prymnesiophytes
54 and cryptophytes, and comprising picoeukaryotes (3-5). Currently, there is no
55 doubt on the ubiquity of mixotrophy and its significance in the functioning of
56 planktonic systems. In oligotrophic conditions, phototrophic flagellates can
57 account for up to 80 % of total bacterial grazing (4, 6, 7).
58 Predation by protists is among the primary mortality factors of prokaryotes in
59 planktonic communities, and thus an important selective pressure. It becomes a
60 structuring factor of the abundance, morphology, composition and activity of
61 bacterial assemblages (8, 9). The impact of protist predation appears to be
62 modulated by the characteristics of the system (e.g. productivity) and predator
63 and prey traits (10). Over the last few decades, efforts have been made to
64 understand the selective feeding behavior of protists. General selection
65 mechanisms have been identified (11). However, the current view is still mainly
66 based on laboratory data, using readily growing species (12). On the other
67 hand, the limited number of field experiments use general grazer groups rather
68 than evaluating predation at the species level (12-14). There is a need to
69 evaluate prey selection in natural conditions comparing flagellate species to
70 determine more specific interactions between microbial predators and preys
71 and assessing the relevance of selective microbial predation in the microbial
72 loop dynamics. The scarcity of studies are in great part due to the difficulties in
73 i) prokaryote prey identification and ii) establishing the prey and predator links at

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74 the highest possible taxonomic resolution. Prey identification can be addressed
75 by techniques based on DNA fingerprinting (15, 16), which allow following
76 taxonomic changes in prey assemblages (10, 17). Linking prey and predator
77 can be addressed using techniques based on fluorescence *in situ* hybridization
78 (FISH), which allow detecting targeted preys inside of protist food vacuoles (5,
79 14, 18, 19). These techniques have been mostly performed under experimental
80 conditions (18, 20), and results suggest a high selectivity in the feeding of
81 heterotrophic flagellates and some ciliate species investigated. In contrast, the
82 few *in situ* measurements showed not clear ingestion patterns for lake
83 flagellates, even random feeding was proposed for some bacterial groups (14).
84 All in all, there is still limited information on the selective feeding of phagotrophic
85 protists in natural conditions and, more remarkably, there is a huge gap of
86 knowledge about mixotrophic flagellates species.
87 The catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-
88 FISH) protocol (21) is particularly suited to assess the phagotrophy of
89 mixotrophic protist on prokaryotes since it maintains cell and plastid integrity
90 and allows the visualization of labeled preys against plastid autofluorescence.
91 CARD-FISH can be easily applied to natural assemblages to evaluate *in situ*
92 prey preference of mixotrophic species, and other prokaryotic grazers (14, 19),
93 using the appropriate bacterial and archaeal probes. In the present study, we
94 examine phagotrophic selectivity of three mixotrophic species in natural
95 conditions. We sampled a deep high-mountain lake in which we expected
96 mixotrophic activity to be enhanced by the ultraoligotrophic conditions. Samples
97 were obtained at different times of the day to take into account potential feeding
98 variation and to assess the mean behavior better. The phytoplankton species

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99 were identified by the size and shape of the autofluorescent plastid based on a
100 prior taxonomic knowledge of the assemblages, which is an advantage of
101 investigating mixotrophic flagellates at the species level respect to the
102 heterotrophic ones. The heterotrophic flagellates are usually grouped into
103 operational functional groups (14, 20, 22). In our study, we used fluorescent
104 probes for the domain Archaea and three groups of Eubacteria: β-
105 Proteobacteria, Actinobacteria, and the Cytophaga-Flavobacteria group of
106 Bacteroidetes. These groups dominate aquatic prokaryotes in the Pyrenean
107 lakes and account for more of the 85% of clades present in the lake studied
108 (23).
109
110 **MATERIAL AND METHODS**
111 *Sampling site:*
112 Lake Redon is an ultraoligotrophic high mountain lake located at 2,240 m above
113 sea level in the Central Pyrenees. It has a surface area of 24 ha and maximum
114 and mean depth of 73 and 32 m, respectively. The lake is dimictic, well
115 oxygenated throughout the water column and is usually covered by ice and
116 snow during six months a year (24). The productivity patterns and seasonal
117 changes in the water column are typical for high mountain lakes (25, 26). This
118 lake has been widely studied in the last 30 years, a general description of its
119 physical, chemical and temporal changes can be found in Catalan (27, 28) and
120 a detailed description of the microbial plankton composition in Felip et al. (29).
121 *Sampling:*
122 On 9 August 2004, during the summer stratification period, integrated samples
123 (0 to 60 m) of the water column were taken at the deepest point of the lake at

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124 midnight (0 a.m.), dawn (7 a.m.), morning (11 a.m.) and afternoon (3 p.m.). The
125 four sampling times aimed to an estimation of the consistency of potential
126 selective behaviors and roughly approximate the time scale of digestion, in case
127 the number of ingested preys markedly fluctuated. The goal was not to
128 investigate daily patterns with only one sampling day. The water samples were
129 screened through a 40 µm mesh net to remove large zooplankton and
130 subsequently divided into two subsamples. One of the subsamples was fixed
131 with 0.5% (vol/vol) alkaline Lugol's solution followed by 2% buffered (pH 7) 0.2-
132 µm-pore-size-filtered formaldehyde, and several drops of 3% sodium thiosulfate
133 to decolor Lugol´s fixation, following Medina-Sánchez et al. (21). After 1 h of
134 fixation at room temperature, 24 aliquots of each sample (12 of 90 ml for
135 protists and 12 of 10 ml for prokaryotes) were gently filtered (<100 mm Hg) onto
136 respective 25 mm diameter polycarbonate Millipore membrane filters (type
137 RTTP, 1 µm pore size for protists; type GTTP, 0.2 µm pore size for
138 prokaryotes). Filters were then rinsed twice with Milli-Q water, allowed to air dry,
139 and stored at -20°C until further processing. The second subsample was
140 preserved with 0.5% (vol/vol) alkaline Lugol's solution to identify algal species
141 (30).

142 *Phagotrophic activity*:

143 Three different HRP-labeled probes for the main Bacteria groups found in the
144 plankton of the Pyrenean lakes (23, 31, 32); and one for the domain Archaea
145 were used to hybridize the filters (Table 1). The probe EUB338 that targets
146 most Bacteria was used only to evaluate the hybridization yield of the other
147 probes. Two replicate filters were processed for each probe and sample.
148 Alexa488-labeled tyramide was used for signal amplification and filters were

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149 counterstained with DAPI (4´,6´-diamidino-2-phenylindole; 1µg ml^{-1} final
150 concentration), and mounted on glass slides by using Citifluor (Citifluor Ltd.,
151 UK). Slides were stored at -20 °C in the dark until subsequent counting.
152 Slides were examined at x1000 magnifications for bacterial groups, Archaea
153 and smaller protists (<10 µm) and at x400 magnifications for larger protists (>10
154 µm) under a Zeiss Axio Imager epifluorescence microscope. The microscope
155 was equipped with an X-Cite 120 light, appropriate filter sets for DAPI (Zeiss
156 filter set 01, BP365/12 FT396 LP397) and Alexa-Fluor488 (Zeiss filter sets 09,
157 BP450-490 FT510 LP515, o 24 DBP485/20 DFT500/600 BP515-540 + LP610),
158 a coupled camera Axio Cm Mrm and a PC-based image analysis software Axio
159 Vision 4.8. For prokaryotes, a minimum of 500 cells was counted to establish
160 the total abundance (DAPI counts) and the number of hybridized cells for each
161 specific probe to estimate their abundance and percentage of hybridization.
162 Also, cell area and perimeter were measured by image analysis software in at
163 least 100 cells of each filter. A characteristic length was calculated using the
164 square root of the cell area.
165 Protists were identified based on their plastid size and shape observed by the
166 chlorophyll auto-fluorescence under blue excitation. The identification was
167 facilitated by parallel observation of the Lugol's fixed subsample under an
168 inverted light microscope (x600 and x1000). A minimum of 100 individuals of
169 the most abundant protist species was assessed, for each, the number of
170 hybridized prokaryotic cells inside was counted.
171 *Data analysis:*
172 Protist species prey selectivity was analyzed according to Chesson's α-index
173 (33):

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$$\alpha_i = \frac{\frac{r_i}{p_i}}{\sum_{i=1}^{n} \frac{r_i}{p_i}}$$

174 where r_i and p_i are the mean percentages of each prokaryotic group (i) inside
175 the protist assessed and in the lake water assemblage, respectively; n is the
176 number of prokaryotic groups distinguished (four in our case). When $\alpha_i = 1/n$
177 unselective feeding occurs; when $\alpha_i < 1/n$ negative selection occurs, less of the
178 prokaryotic group *i* occurs inside the protist than expected from random feeding;
179 when $\alpha_i > 1/n$, positive selection occurs, more individuals of the prokaryotic
180 group *i* were ingested by the protist than expected from random feeding. One-
181 way and two-way ANOVAs, were performed to compare prokaryotic
182 assemblages among samplings. To test changes on protist ingestion, Kruskal-
183 Wallis non-parametric tests were used (STATISTICA 7.1; StatSoft, Inc.).
184

185 **RESULTS**

186 Total DAPI counts ranged from 2.8 x 10^5 to 6.3 x 10^5 cell ml^{-1}, and between the
187 27-37 % of such total counts were hybridized with the four probes used in this
188 study (Fig 1a). No significant differences were found in the total amount of
189 hybridized cells between samplings (Fig 1a, ANOVA $p>0.05$). The three groups
190 of Bacteria considered, all together, accounted for 99-104% of cells hybridized
191 by the probe EUB338, which is generic for the domain Bacteria, indicating that
192 not any major Bacteria group were missing in our study.
193 From the four tested prokaryotic groups, β-Proteobacteria was often the most
194 abundant, ranging from 12.4 ± 3.3 to 20.2 ± 8.8 % of total DAPI counts. Only at
195 the last sampling time the group Actinobacteria showed higher abundance

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196 reaching 15.6 ± 2.7 % of total DAPI counts (Fig 1b). Cytophaga-Flavobacteria of
197 Bacteroidetes proportions were slightly variable representing between 4.0 ± 2
198 and 7.2 ± 4.5 % of total DAPI counts, whereas Archaea ranged between 2.9 ±
199 0.8 and 6 ± 0.4 %.
200 Cell length frequency distributions, evidenced that Actinobacteria were always
201 smaller and with more uniform sizes than the others prokaryotic groups (Fig 2).
202 A temporal tendency can be observed in Archaea and Cytophaga-Flavobacteria
203 distributions: cells were larger at night and progressively declined during the
204 day.
205 Three mixotrophic species, two Cryptophyta: *Rhodomonas* sp. and
206 *Cryptomonas ovata*; and the Chrysophyceae *Dinobryon cylindricum*, were
207 selected to assess the phagotrophic activity and describe their feeding
208 behavior. These species were easy to recognize by fluorescence microscopy
209 and large enough (Table 2) to accurately quantify the hybridized prokaryote
210 cells inside them (Fig 3). Their abundance and the percentage of feeding cells
211 observed for each probe were included in Table 2. In some cases, cells actively
212 feeding accounted for more than half of the population (e.g., *Rhodomonas* sp.
213 feeding on Archaea or *C.ovata* on β-Proteobacteria), whereas in others most
214 individuals showed no prey inside (e.g. *Rhodomonas* sp. feeding on
215 Actinobacteria or Cytophaga-Flavobacteria). The average food vacuole content
216 of the three flagellate species changed among the different surveys performed
217 (Fig 4). *C. ovata* and *D. cylindricum* presented a similar feeding variation.
218 *Rhodomonas* vacuole content had an elevated variation with significant
219 differences among surveys (Kruskal-Wallis $P<0.05$, Fig 4).

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220 The main ingested prokaryotic group differed between the three protists (Fig 4)
221 and the Chessons' selectivity index (α_i) clearly indicates that the three species
222 were not randomly feeding (Fig. 5). Archaea were usually positively selected by
223 the three protists. The preference was extreme in the case of *Rhodomonas sp.*,
224 which hardly grazed on any other prokaryotic group. In contrast, Actinobacteria
225 were always apparently avoided, only once *D. cylindricum* selected this group
226 positively. Finally, β-Proteobacteria and Cytophaga-Flavobacteria were only
227 strongly avoided by *Rhodomonas* and randomly grazed or slightly avoided by *C.*
228 *ovata* and *D. cylindricum*.
229

230 **DISCUSSION**

231 *CARD-FISH performance*

232 Technical capacity for distinguishing the food vacuole content of protists is
233 crucial to study their selective feeding. The CARD-FISH protocol (21) offers this
234 possibility. Critical steps in the procedure are the probe selection and the
235 percentage of hybridized cells achieved. The probes used in the present study
236 included the most abundant prokaryotic groups described in the plankton of the
237 Pyrenean high-mountain lakes (31). Previous studies in Lake Redon and on 17
238 lakes more in the same area of the Pyrenees have shown that β-Proteobacteria,
239 Bacteroidetes, and Actinobacteria always account for more than 75% of the
240 total Bacteria clades, more than 85% in the Lake Redon (see Fig S2 from (23)).
241 The remaining clades proportion divided into 7 secondary groups and other
242 minority ones. Our results show that no primary Bacteria group was missing in
243 our assessment. The three groups of Bacteria considered accounted for 99-
244 104% of cells hybridized by the probe EUB338. There is no reason to assume

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245 that we were missing any large part of the prokaryotic community with our
246 probes, as long as we considered the three most important groups of Bacteria
247 in addition to Archaea. The same probes were used to characterize Bacteria
248 composition in alpine lakes plankton of other ranges (34, 35).
249 The relative low hybridization compared with DAPI counts (27-37%) is due to
250 the efficiency of the procedure. Low efficiency may be due to detection technical
251 issues and to the cell physiological states (36). Cells with low rRNA content are
252 common in samples from oligotrophic environments due to cell low metabolic
253 activity or dormant state. Different assessments provide a small percentage of
254 active cells in the plankton of the Pyrenean lakes, respiring prokaryotes ranged
255 between 2–7 %, whereas viable cells were seldom higher than 50 % in deep
256 Pyrenean lakes (37). Even though CARD-FISH appears suitable for the
257 detection of prokaryotes with small quantities of rRNA molecules (38), if cells
258 are scarcely active or small, the percentage of hybridized cells compared to
259 DAPI counts decline (36, 39). Thereby, the ratio between CARD-FISH, and
260 DAPI counts is often low and highly variable in lake plankton samples (19, 35,
261 40) and are not unexpected to be particularly low in an ultraoligotrophic lake
262 such as Redon. In a recently phosphorous enrichment experiment performed in
263 this lake, the % hybridized cells (using the same four probes we applied here)
264 raised from 36% of DAPI counts in low P and lake conditions to near 100% in
265 the most productive mesocosm (Supplemental material). This is clear evidence
266 that the apparent low hybridizing efficiency we obtained is not a probe problem
267 but a constraint resulting from the little activity of many cells. In our study, the
268 percentage of hybridization did not significantly change over time (Fig 1) and

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269 thus did not affect our objective to compare the food vacuole content against
270 the plankton assemblage composition.
271 The ARCH915 probe has been widely used to detect archaeal cells.
272 Occasionally, some biases of the probe hybridizing members of the phylum
273 Bacteroidetes have been reported (41-43). This doubling hybridization can
274 inflate the Archaea counts. It does not seem to occur in our case, for several
275 reasons. No correlation was found between ARCH915 and CF319a counts
276 neither in water samples nor in vacuoles. Indeed, contrasting extreme values
277 between the two groups were found in some protist food vacuoles (Fig 4). Also,
278 the Archaea proportions in the water samples of our study (3-6%) were similar
279 to those in previous studies in Lake Redon and other Pyrenean lakes using 16S
280 rRNA gene tag sequencing: 0-8 %,(44); 0-6 %, (45).
281
282 *Selective predation*
283 The significance of predation on bacterial activity and community structure in
284 natural aquatic systems has been demonstrated (10, 46-49). Consequently, the
285 effort is currently placed on understanding the details and dynamics of the
286 grazing by protists (11). From lab experiments, evidence exists that certain taxa
287 feed selectively (50, 51). In natural conditions little is known about flagellate
288 grazing preferences, and whether they tend to be specialists or generalists
289 predators. In a recent microcosm study, Glücksman et al. (17) showed that
290 closely related and morphologically similar flagellated species can have
291 different impacts on natural bacterial communities. Beyond phylogeny, some
292 general protist traits, namely, cell size and morphological plasticity, explained
293 variation in prey composition. Accordingly, our results show that two highly

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294 related species (i.e., the two cryptophytes), inhabiting the same system and
295 feeding in the same prokaryotic assemblage, markedly differ in their selectivity
296 (Fig 4 and 5).
297 The species studied are closer to specialist predators than generalist (Fig 5).
298 *Rhodomonas* sp., the smallest species analyzed, showed a more differentiated
299 feeding pattern (Fig 4). Unfortunately, the comparison of only three species
300 does not have the statistical power to evaluate the relationship between traits
301 and selectivity. In any case, we show that selectivity may be extreme and thus
302 with substantial implications for the dynamics of the prokaryotic assemblages.
303 The three protist species analyzed were the most abundant mixotrophs in the
304 phytoplankton community at the time of the study (Table 2). The selectivity, at
305 least, on high taxonomic levels shown by our results pave the way to studies
306 focusing on finer prokaryotic taxonomy, using more specific probes, and
307 evaluating whether grazing by protist constitutes a differential selective pressure
308 within each of the large prokaryotic groups with ecological and evolutionary
309 implications.
310
311 *Cell size and grazing pressure*
312 Protist prey selection may occur at various feeding steps, namely capture, prey
313 processing, ingestion and digestion (11, 52). Prey size is a trait easy to
314 measure. There is evidence indicating that flagellates tend to graze on a limited
315 size range of prokaryotic cells, thus removing medium-sized cells and shifting
316 the size distribution of the preys towards larger and smaller cells (53-55). In our
317 case, the positively selected Archaea showed similar average cell size than β-
318 Proteobacteria and Cytophaga-Flavobacteria, although large cells (i.e., quartile

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319 75% in Fig 2) were slightly smaller in Archaea than in the other two groups.
320 Very likely, other factors than cell size must explain the strong preference for
321 Archaea. During the sampling day, the cell length declined with time in both
322 Archaea and Cytophaga-Flavobacteria, which may reflect the dynamic effect of
323 grazing throughout the day (Fig 2), yet other factors may influence such as cell
324 division. In all the samples, Actinobacteria were markedly smaller than the other
325 prokaryotic groups (Fig 2) and were less affected by protist grazing (Fig 5). This
326 observation agrees with previous studies that indicate lower grazing pressure
327 on Actinobacteria by heterotrophic nanoflagellates (18, 20) and by the
328 mixotrophic Chrysophyceae *Ochromonas* sp. (56). Indeed, if small size (i.e.,
329 <0.5 μm) constitutes a refuge against grazing, Actinobacteria may be negatively
330 selected only in appearance. Either because the limiting size might be an
331 evolved defense mechanism and thus cells tend to be always smaller than in
332 other groups or, on the contrary, because preference by grazers is so high that
333 the group is permanently confined to the small size refuge. It has been
334 suggested that cell miniaturization alone might not be sufficient to explain
335 grazing avoidance (57) and that other resistance mechanisms, such as wall
336 structures present in Gram-positive Actinobacteria, could be involved in
337 determining a limited edibility (58). Research on prokaryotic grazing is still in its
338 infancy, but our results indicate that random grazing cannot be the paradigm.
339
340 *Preference for Archaea*
341 In the last decade, several studies have shown the broad distribution and
342 abundance of Archaea in aquatic ecosystems (59-61), and their potential
343 relevance in the sulfur, nitrogen and carbon cycles (62-65). However, their

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344 specific ecological interactions within the microbial communities remain largely
345 unknown. There was no evidence that Archaea behaved fundamentally different
346 than Bacteria on predatory interactions (66). Our results indicate that this may
347 not always be the case. The three mixotrophic flagellate species studied here
348 selected Archaea positively. The amount of such prokaryotic group inside the
349 protist food vacuoles was always higher than the expected by random feeding
350 (Fig 5). In *Rhodomonas* sp., the preference for Archaea was extreme. It could
351 be thought that the Archaea dominance in the vacuoles could respond to a
352 stable symbiosis. However, this appears unlikely. The average Archaea
353 densities within the vacuoles and the number of *Rhodomonas* cells without any
354 Archaea inside markedly changed between the four sampling times (Fig 4, table
355 2). This pattern favors the view of selective predation and relatively quick
356 digestion rather than a stable symbiosis.
357 The differences among the three protist species suggest that the strength of
358 grazing on Archaea can highly depend on the protist present. Nevertheless, the
359 three species show a preference for Archaea, which opens the question about
360 how important is grazing in determining the low proportion of this group in many
361 planktonic microbial assemblages.
362 The reason for high positive selectivity on Archaea remains highly speculative.
363 It could be related to a poor development of resistance mechanisms in this
364 domain. Or, it could be related to unknown stoichiometric features of the
365 Archaea, it has been shown that bacteria with low C:P ratio may be ingested at
366 higher rates by flagellates (67, 68). Or, it could be the chemical composition of
367 some particular group of the Archaea within them, as they differ in the cell wall
368 structure. Or even, it could be some chemical cues released to water than could

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369 enhance encounter rates between prey and predator (69). Archaea studies in
370 Lake Redon indicated that most of the 16S rRNA gene sequences matched
371 Thaumarchaeota (close to 90%) and only a few Euryarchaeota were detected.
372 MG 1.1 dominated most of the water column in summer. This clone has a >97%
373 identity with *Nitrosoarchaeum limnia*, an ammonium oxidizing Archaea (44).
374 However, as new primers are applied new groups of Euryarchaeota are found in
375 the lake (70). In any case, the high positive selectivity on Archaea observed in
376 the mixotrophs of Lake Redon pave the way towards new ecological and
377 physiological studies on Archaea. Protist grazing is a factor that cannot be
378 ignored for understanding the Archaea's distribution and abundance in
379 planktonic microbial assemblages.

380

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593 **FIGURE LEGENDS**

594 **Figure 1.** (a) Prokaryotic cell abundance (average ± SD of total
595 DAPI counts) and the total amount of hybridized cells by the four
596 probes used in this study (average ± SD, n=8), at the four sampling
597 times. (b) Prokaryotic assemblage composition as hybridized cell
598 abundance (average ± SD, n=2) of the four targeted groups at the
599 four sampling times.

600 **Figure 2.** Cell length cumulative distributions for the four targeted
601 prokaryotic groups at the four sampling times.

602 **Figure 3.** Epifluorescence microscope photographs of the three
603 mixotrophic flagellates species studied (a) *Rhodomonas* sp., (b)
604 *Cryptomonas ovata* and (c) *Dinobryon cylindricum*. CARD-FISH
605 staining with ARCH915 probe (a and c) and BET42a probe (b),
606 showing chloroplast autofluorescence (red), DAPI stained nucleus
607 (blue) and targeted prokaryotes (green). Dashed white lines draw
608 cell outlines and green line surround food vacuoles. Scale bar = 12
609 µm (a) and scale bar = 20 µm (b and c).

610 **Figure 4.** Food vacuole content, average ± SD (n=2) of hybridized
611 cells by the four probes, for the three mixotrophic flagellates at the
612 four sampling times.

613 **Figure 5.** Chesson's selectivity index for the three mixotrophic
614 species (plots distributed vertically) and for each of the targeted
615 prokaryotic group (plots distributed horizontally) at each sampling
616 times. The horizontal dashed lines indicate the value above which

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617 positive selection on a particular prokaryotic group is assumed (0.25

618 = 1/n, being n the number of prokaryotic groups).

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619 **Table 1.** Oligonucleotide probes used in this study

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Probe name	Specificity	Sequence (5´- 3´) of probe	% FA*	References
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	55	**(71)**
ARCH915	*Archaea*	GTGCTCCCCCGCCAATTCCT	40	**(21)**
CF319a	*Cytophaga-Flavobacteria of Bacteroidetes*	TGGTCCGTGTCTCAGTAC	55	**(72)**
HGC69a	*Actinobacteria*	TATAGTTACCACCGCCGT	30	**(73)**
BET42a	*β-Proteobacteria*	GCCTTCCCACTTCGTTT	55	**(73)**

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628 * Formamide (FA) concentration in the hybridization buffer.

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633 **Table 2.** Protist average data: mean size, mean abundance and percentage of active feeding cells for each probe analyzed

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SPECIES	SIZE (µm)	ABUNDANCE (cells ml $^{-1}$)	PERCENTAGE OF ACTIVE FEEDING CELLS			
			β-Proteobacteria	Actinobacteria	Cytophaga-Flavobacteria	Archaea
***Rhodomonas* sp.**	11 x 6	28 ± 8	9.4 ± 12.3	3.3 ± 2.6	2.9 ± 3.2	52.5 ± 47.7
Cryptomonas ovata	30 x 14	16 ± 6	50 ± 19.4	19.2 ± 14.7	44.1 ± 9.9	36.6 ± 16
Dinobryon cylindricum	14 x 6	139 ± 47	28.5 ± 9	7.8 ± 7.3	11.5 ± 5.4	13.3 ± 10.2

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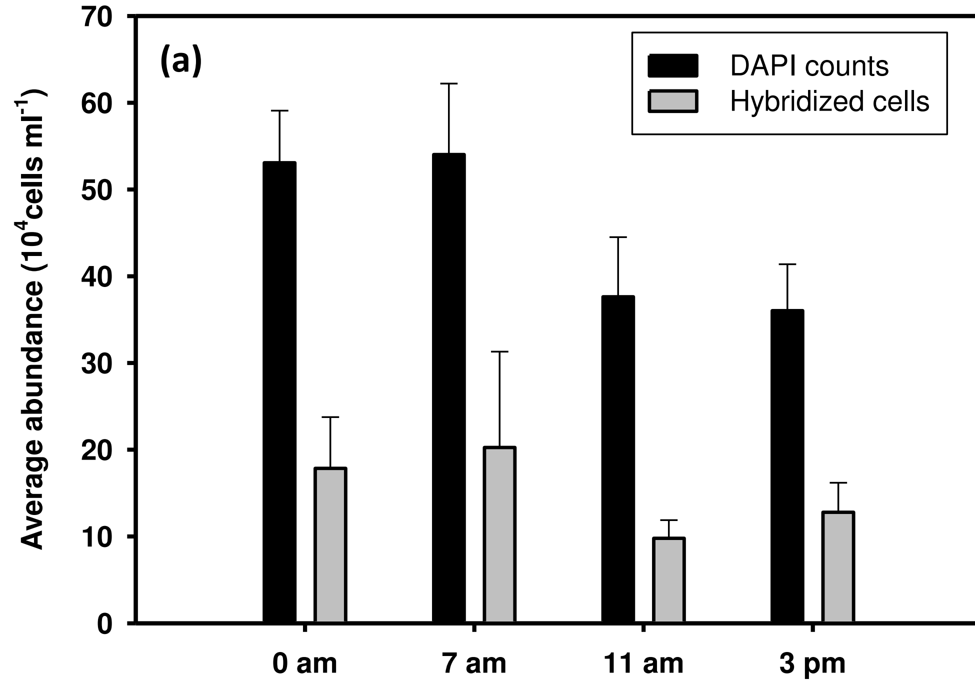

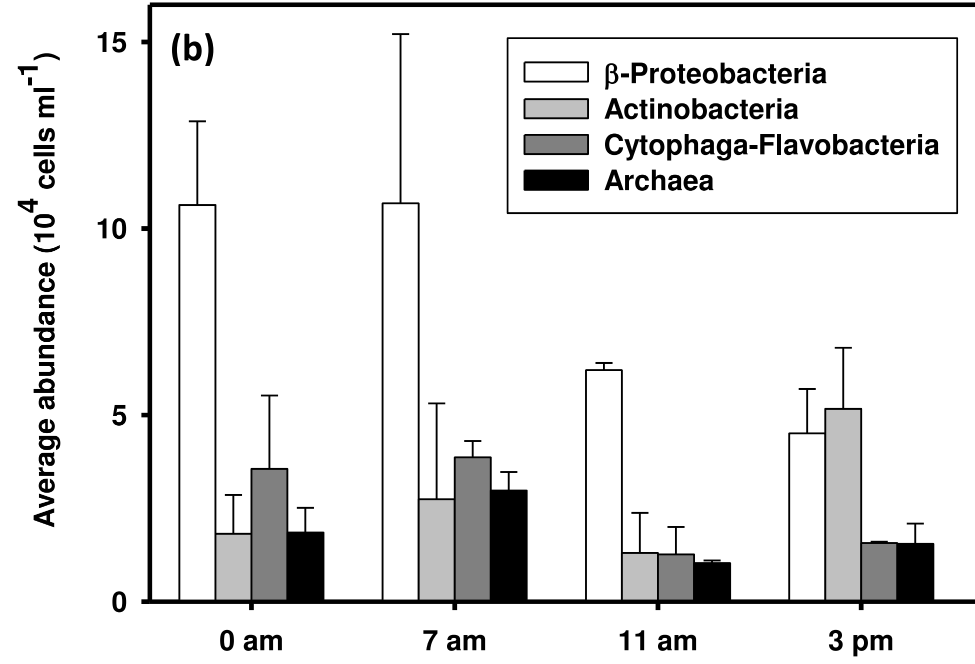

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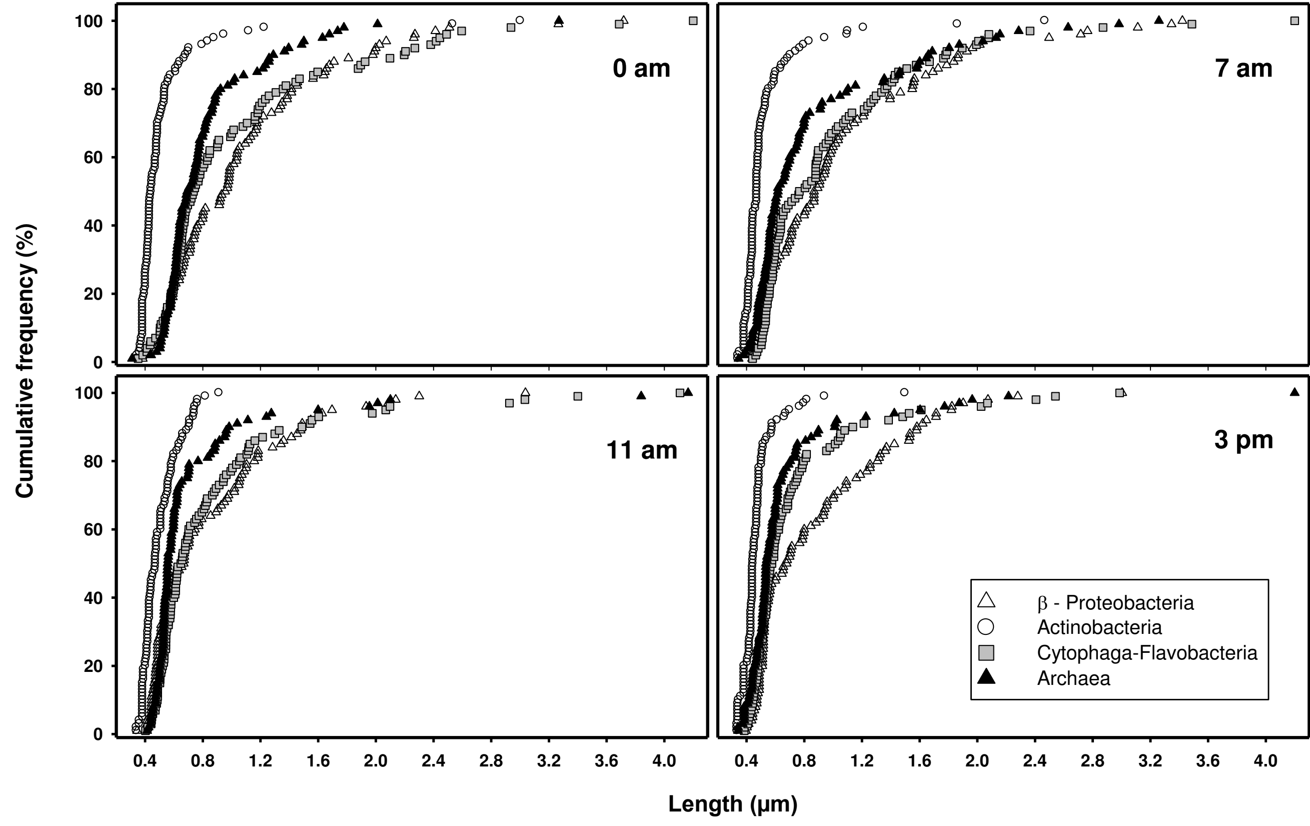

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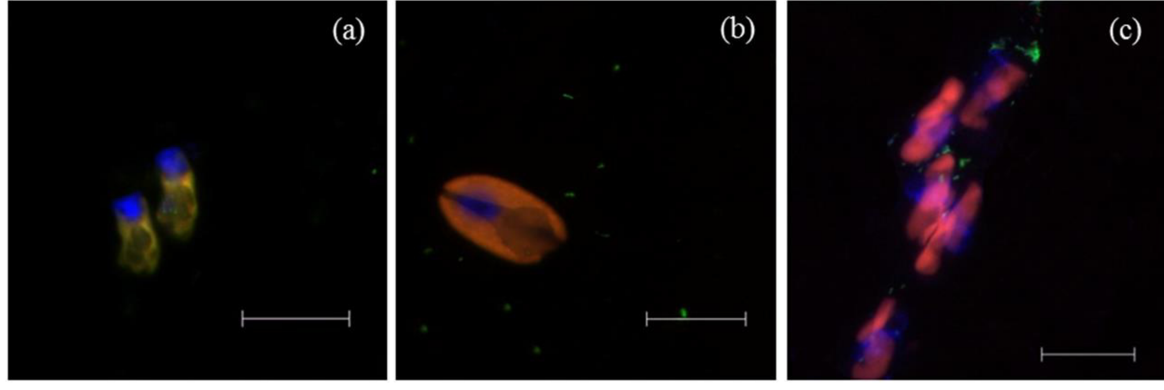

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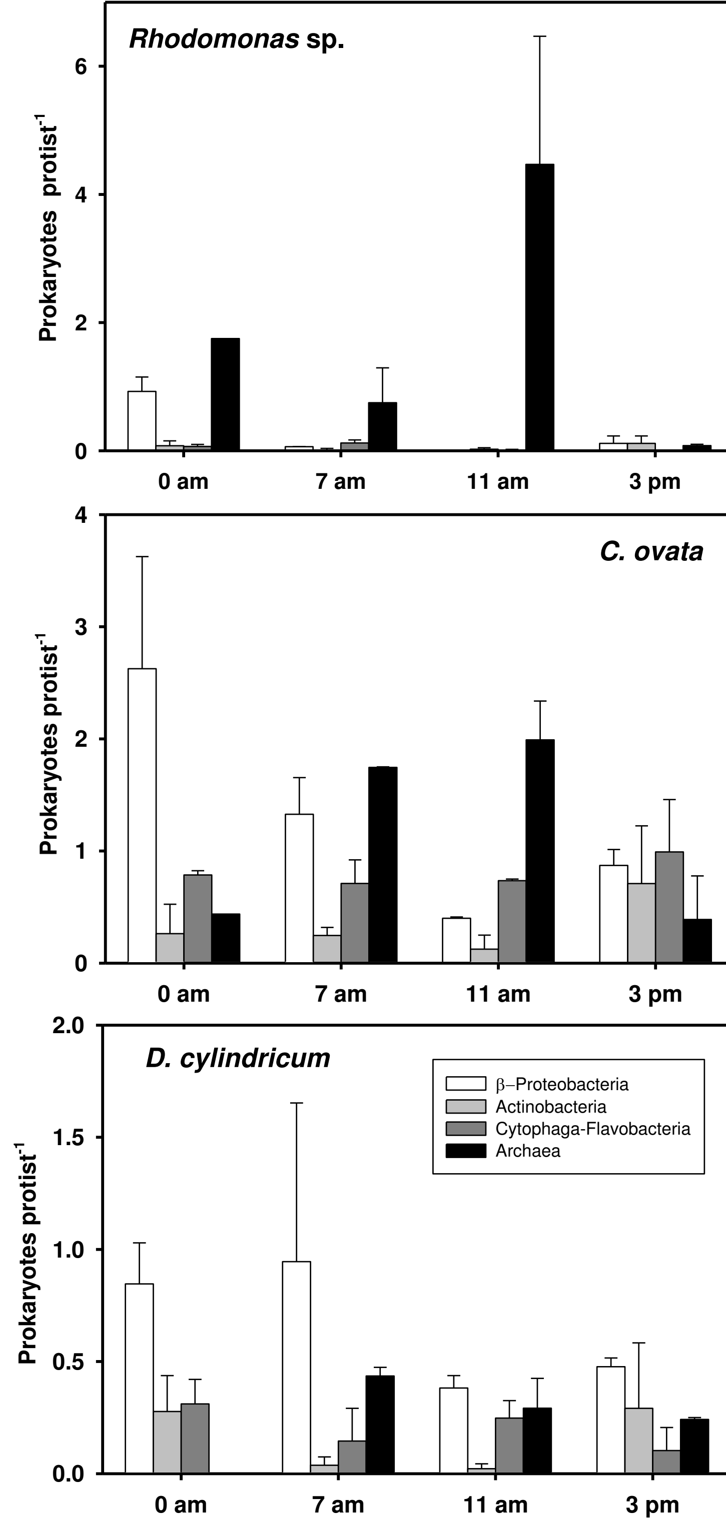

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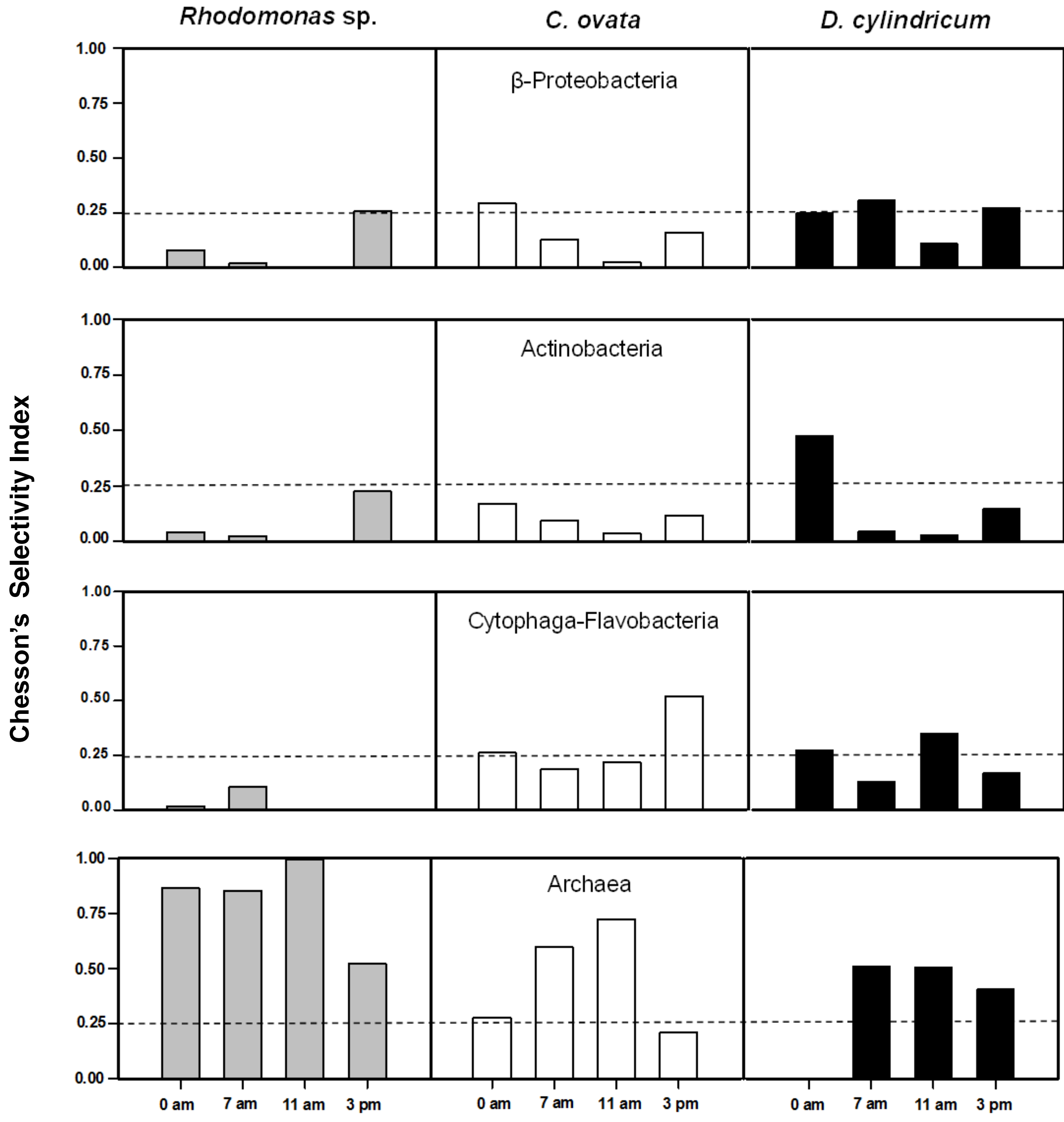

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