



# Some Mixotrophic Flagellate Species Selectively Graze on Archaea

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**ABSTRACT** Many phototrophic flagellates ingest prokaryotes. This mixotrophic trait becomes a critical aspect of the microbial loop in planktonic food webs because of the typical high abundance of these flagellates. Our knowledge of their selective feeding upon different groups of prokaryotes, particularly under field conditions, is still quite limited. In this study, we investigated the feeding behavior of three species (*Rhodomonas* sp., *Cryptomonas ovata*, and *Dinobryon cylindricum*) via their food vacuole content in field populations of a high mountain lake. We used the catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) protocol with probes specific for the domain *Archaea* and three groups of *Eubacteria*: *Betaproteobacteria*, *Actinobacteria*, and *Cytophaga-Flavobacteria* of *Bacteroidetes*. Our results provide field evidence that contrasting selective feeding exists between coexisting mixotrophic flagellates under the same environmental conditions and that some prokaryotic groups may be preferentially impacted by phagotrophic pressure in aquatic microbial food webs. In our study, *Archaea* were the preferred prey, chiefly in the case of *Rhodomonas* sp., which rarely fed on any other prokaryotic group. In general, prey selection did not relate to prey size among the grazed groups. However, *Actinobacteria*, which were clearly avoided, mostly showed a size of  $<0.5 \mu\text{m}$ , markedly smaller than cells from the other groups.

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

**KEYWORDS** *Archaea*, mixotrophic protist, selective feeding

Mixotrophic behavior, the combination of phototrophic and phagotrophic nutritional modes within a single cell, has been increasingly documented in aquatic systems (1, 2). Phagotrophy occurs in a variety of phytoplankton flagellate groups, including *Chrysophyceae*, dinoflagellates, prymnesiophytes, and cryptophytes, which comprise some picoeukaryotes (3–5). Currently, there is no doubt of the ubiquity of mixotrophy and its significance in the functioning of planktonic systems. Under oligotrophic conditions, phototrophic flagellates can account for up to 80% of total bacterial grazing (4, 6, 7).

Predation by protists is among the primary mortality factors of prokaryotes in planktonic communities and thus is an important selective pressure. It becomes a structuring factor of the abundance, morphology, composition, and activity of bacterial assemblages (8, 9). The impact of protist predation appears to be modulated by the characteristics of the system (e.g., productivity) and predator and prey traits (10). Over

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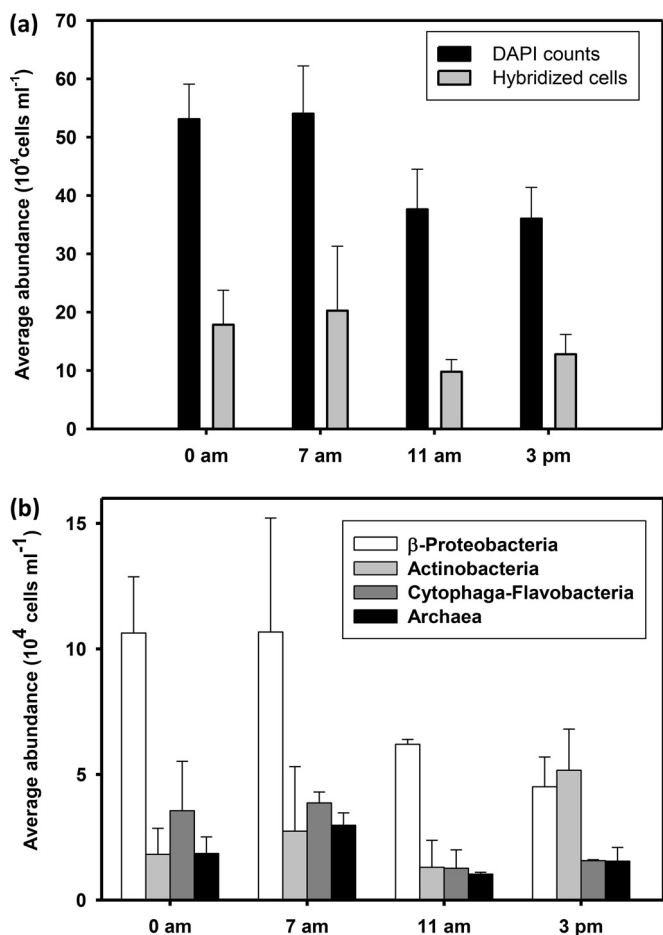
the last few decades, efforts have been made to understand the selective feeding behavior of protists. General selection mechanisms have been identified (11). However, the current view is still mainly based on laboratory data, using readily growing species (12). On the other hand, a limited number of field experiments use general grazer groups rather than evaluating predation at the species level (12–14). There is a need to evaluate prey selection under natural conditions comparing flagellate species to determine more specific interactions between microbial predators and prey and assessing the relevance of selective microbial predation in the microbial loop dynamics. The scarcity of studies is due in great part to the difficulties in (i) prokaryote prey identification and (ii) establishing the prey and predator links at the highest possible taxonomic resolution. Prey identification can be addressed by techniques based on DNA fingerprinting (15, 16), which allow ensuing taxonomic changes in prey assemblages (10, 17). Linking prey and predator can be addressed using techniques based on fluorescence *in situ* hybridization (FISH), which allow detecting targeted prey inside protist food vacuoles (5, 14, 18, 19). These techniques have been performed mostly under experimental conditions (18, 20), and results suggest a high selectivity in the feeding of heterotrophic flagellates and some ciliate species investigated. In contrast, the few *in situ* measurements showed unclear ingestion patterns for lake flagellates, and even random feeding was proposed for some bacterial groups (14). All in all, there is still limited information on the selective feeding of phagotrophic protists under natural conditions, and more remarkably, there is a huge gap of knowledge about mixotrophic flagellate species.

The catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) protocol (21) is particularly suited to assess the phagotrophy of mixotrophic protists on prokaryotes, since it maintains cell and plastid integrity and allows the visualization of labeled prey against plastid autofluorescence. CARD-FISH can be easily applied to natural assemblages to evaluate *in situ* prey preference of mixotrophic species and other prokaryotic grazers (14, 19) using the appropriate bacterial and archaeal probes. In the present study, we examined the phagotrophic selectivity of three mixotrophic species under natural conditions. We sampled a deep high-mountain lake in which we expected mixotrophic activity to be enhanced by the ultraoligotrophic conditions. Samples were obtained at different times of day to take into account potential feeding variation and to assess the mean behavior better. The phytoplankton species were identified by the size and shape of the autofluorescent plastid based on a prior taxonomic knowledge of the assemblages, which is an advantage of investigating mixotrophic flagellates at the species level with respect to the heterotrophic ones. The heterotrophic flagellates are usually grouped into operational functional groups (14, 20, 22). In our study, we used fluorescent probes for the domain *Archaea* and three groups of *Eubacteria*: *Betaproteobacteria*, *Actinobacteria*, and the *Cytophaga-Flavobacteria* group of *Bacteroidetes*. These groups dominate aquatic prokaryotes in the Pyrenean lakes and account for more of the 85% of clades present in the lake studied (23).

## RESULTS

Total 4',6-diamidino-2-phenylindole (DAPI) counts ranged from  $2.8 \times 10^5$  to  $6.3 \times 10^5$  cells  $\text{ml}^{-1}$ , and 27 to 37% of such total counts were hybridized with the four probes used in this study (Fig. 1a). No significant differences were found in the total amount of hybridized cells between samplings (Fig. 1a) ( $P$  value of  $>0.05$  by analysis of variance [ANOVA]). The three groups of *Bacteria* considered together accounted for 99 to 104% of cells hybridized by probe EUB338, which is generic for the domain *Bacteria*, indicating that no major *Bacteria* group was missing from our study.

From the four tested prokaryotic groups, *Betaproteobacteria* was often the most abundant, ranging from  $12.4\% \pm 3.3\%$  to  $20.2\% \pm 8.8\%$  of total DAPI counts. Only at the last sampling time did the group *Actinobacteria* show higher abundance, reaching  $15.6\% \pm 2.7\%$  of total DAPI counts (Fig. 1b). Proportions of *Cytophaga-Flavobacteria* of *Bacteroidetes* were slightly variable, representing between  $4.0\% \pm 2\%$  and  $7.2\% \pm 4.5\%$  of total DAPI counts, whereas *Archaea* ranged between  $2.9\% \pm 0.8\%$  and  $6\% \pm 0.4\%$ .



**FIG 1** (a) Prokaryotic cell abundance (average  $\pm$  standard deviation [SD] total DAPI counts) and the total amount of cells hybridized by the four probes used in this study (averages  $\pm$  SD;  $n = 8$ ) at the four sampling times. (b) Prokaryotic assemblage composition as hybridized cell abundance (averages  $\pm$  SD;  $n = 2$ ) of the four targeted groups at the four sampling times.

Cell length frequency distributions evidenced that *Actinobacteria* were always smaller and had more uniform sizes than the others prokaryotic groups (Fig. 2). A temporal tendency can be observed in *Archaea* and *Cytophaga-Flavobacteria* distributions: cells were larger at night and progressively declined in size during the day.

Three mixotrophic species (two species of *Cryptophyta* [*Rhodomonas* sp. and *Cryptomonas ovata*] and one species of *Chrysophyceae* [*Dinobryon cylindricum*]) were selected to assess the phagotrophic activity and describe their feeding behavior. These species were easy to recognize by fluorescence microscopy and large enough (Table 1) to accurately quantify the hybridized prokaryote cells inside them (Fig. 3). Their abundance and the percentage of feeding cells observed for each probe are included in Table 1. In some cases, cells actively feeding accounted for more than half of the population (e.g., *Rhodomonas* sp. feeding on *Archaea* or *C. ovata* on *Betaproteobacteria*), whereas in others most individuals showed no prey inside (e.g., *Rhodomonas* sp. feeding on *Actinobacteria* or *Cytophaga-Flavobacteria*). The average food vacuole content of the three flagellate species changed among the different surveys performed (Fig. 4). *C. ovata* and *D. cylindricum* presented a similar feeding variation. *Rhodomonas* vacuole content had an elevated variation with significant differences among surveys ( $P$  value of  $<0.05$  by Kruskal-Wallis test) (Fig. 4).

The main ingested prokaryotic group differed among the three protists (Fig. 4), and Chesson's selectivity index ( $\alpha_i$ ) clearly indicates that the three species were not randomly feeding (Fig. 5). *Archaea* were usually positively selected by the three protists.

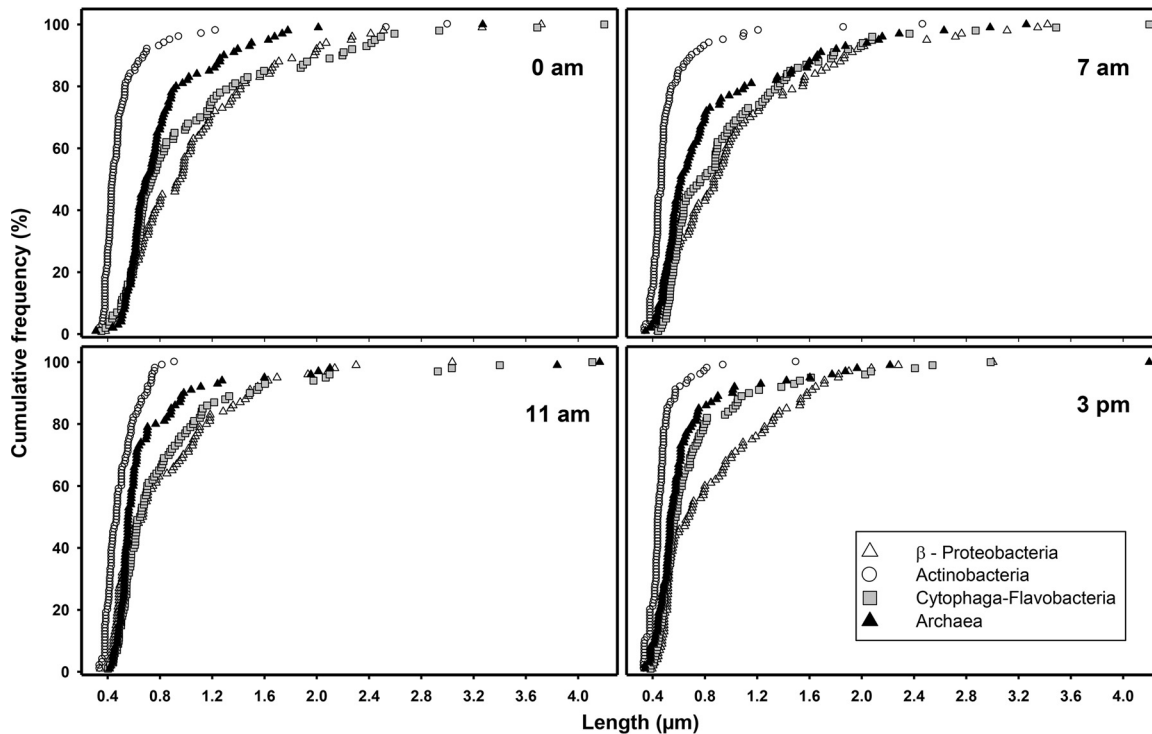


FIG 2 Cell length cumulative distributions for the four targeted prokaryotic groups at the four sampling times.

The preference was extreme in the case of *Rhodomonas* sp., which hardly grazed on any other prokaryotic group. In contrast, *Actinobacteria* were always apparently avoided, and only once did *D. cylindricum* positively select this group. Finally, *Betaproteobacteria* and *Cytophaga-Flavobacteria* were only strongly avoided by *Rhodomonas* and randomly grazed or slightly avoided by *C. ovata* and *D. cylindricum*.

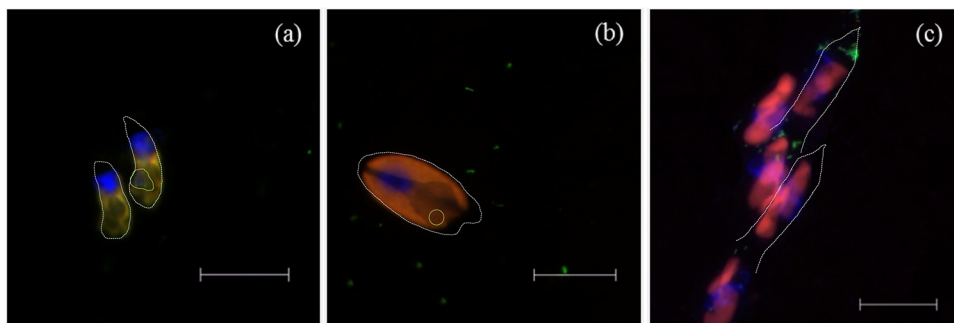
## DISCUSSION

**CARD-FISH performance.** Technical capacity for distinguishing the food vacuole content of protists is crucial to study their selective feeding. The CARD-FISH protocol (21) offers this possibility. Critical steps in the procedure are probe selection and the percentage of hybridized cells achieved. The probes used in the present study included the most abundant prokaryotic groups described in the plankton of the Pyrenean high-mountain lakes (24). Previous studies in Lake Redon and in 17 lakes in the same area of the Pyrenees have shown that *Betaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* always account for more than 75% of the total *Bacteria* clades and more than 85% in Lake Redon (see Fig. S2 from reference 23). The remaining clades were divided into 7 secondary groups and other minority ones. Our results show that no primary *Bacteria* group was missing from our assessment. The three groups of *Bacteria* considered accounted for 99 to 104% of cells hybridized by probe EUB338. There is no reason to assume that we were missing any large part of the prokaryotic community with our probes, as long as we considered the three most important groups of *Bacteria* in

TABLE 1 Protist average data

Species	Size ( $\mu\text{m}$ )	Abundance (cells $\text{ml}^{-1}$ ) <sup>a</sup>	Percentage of active feeding cells <sup>a</sup>			
			<i>Betaproteobacteria</i>	<i>Actinobacteria</i>	<i>Cytophaga-Flavobacteria</i>	<i>Archaea</i>
<i>Rhodomonas</i> sp.	11 by 6	28 $\pm$ 8	9.4 $\pm$ 12.3	3.3 $\pm$ 2.6	2.9 $\pm$ 3.2	52.5 $\pm$ 47.7
<i>Cryptomonas ovata</i>	30 by 14	16 $\pm$ 6	50 $\pm$ 19.4	19.2 $\pm$ 14.7	44.1 $\pm$ 9.9	36.6 $\pm$ 16
<i>Dinobryon cylindricum</i>	14 by 6	139 $\pm$ 47	28.5 $\pm$ 9	7.8 $\pm$ 7.3	11.5 $\pm$ 5.4	13.3 $\pm$ 10.2

<sup>a</sup>Data are averages  $\pm$  standard deviations.



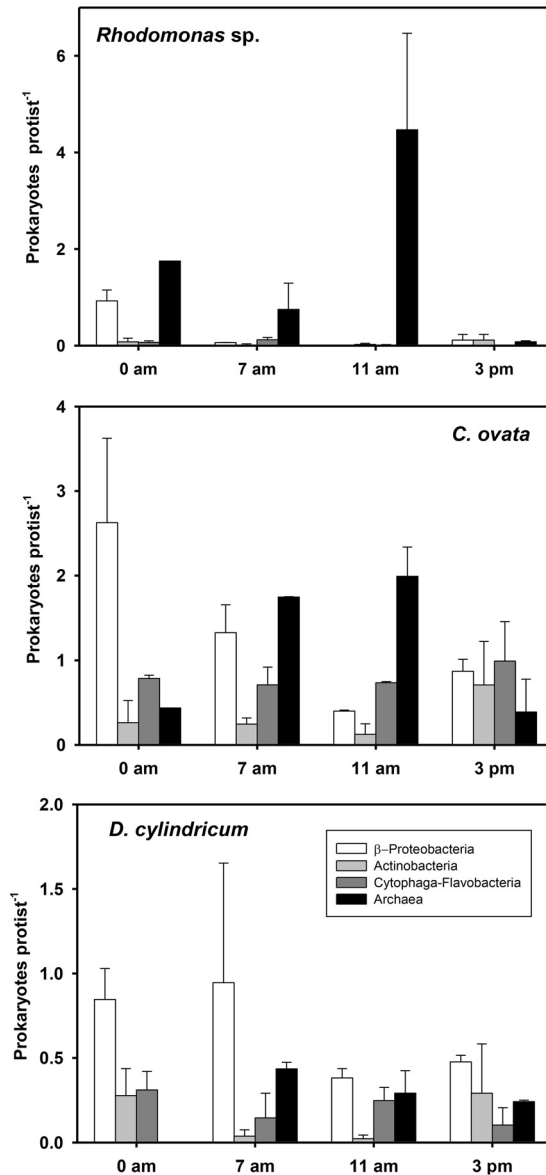
**FIG 3** Epifluorescence microscope photographs of the three mixotrophic flagellate species studied. (a) *Rhodomonas* sp. (b) *Cryptomonas ovata*. (c) *Dinobryon cylindricum*. Presented is CARD-FISH staining with ARCH915 probe (a and c) and BET42a probe (b), showing chloroplast autofluorescence (red), DAPI-stained nucleus (blue), and targeted prokaryotes (green). Dashed white lines represent cell outlines, and green lines surround food vacuoles. Scale bars, 12  $\mu\text{m}$  (a) and 20  $\mu\text{m}$  (b and c).

addition to *Archaea*. The same probes were used to characterize *Bacteria* composition in plankton from alpine lakes of other ranges (25, 26).

The relatively low hybridization compared with DAPI counts (27 to 37%) is due to the efficiency of the procedure. Low efficiency may be due to detection technical issues and to the cell physiological states (27). Cells with low rRNA content are common in samples from oligotrophic environments due to low cell metabolic activity or dormant state. Different assessments provide a small percentage of active cells in the plankton of the Pyrenean lakes, with respiring prokaryotes ranging between 2 and 7%, whereas levels of viable cells were seldom higher than 50% in deep Pyrenean lakes (28). Even though CARD-FISH appears suitable for the detection of prokaryotes with small quantities of rRNA molecules (29), if cells are scarcely active or small, the percentage of hybridized cells compared to DAPI counts declines (27, 30). Therefore, the ratio of CARD-FISH to DAPI counts is often low and highly variable in lake plankton samples (19, 26, 31) and is expected to be particularly low in an ultraoligotrophic lake such as Redon. In a recent phosphorus enrichment experiment performed in this lake, the percentage of hybridized cells (using the same four probes we applied here) increased from 36% of DAPI counts under low-P and lake conditions to nearly 100% in the most productive mesocosm (see the supplemental material). This is clear evidence that the apparent low hybridizing efficiency we obtained is not a probe problem but a constraint resulting from the low level of activity of many cells. In our study, the percentage of hybridization did not significantly change over time (Fig. 1) and thus did not affect our objective to compare the food vacuole content to the plankton assemblage composition.

The ARCH915 probe has been widely used to detect archaeal cells. Occasionally, some biases of the probe-hybridizing members of the phylum *Bacteroidetes* have been reported (32–34). This doubling hybridization can inflate the *Archaea* counts. It does not seem to occur in our case for several reasons. No correlation was found between ARCH915 and CF319a counts in water samples or in vacuoles. Indeed, contrasting extreme values between the two groups were found in some protist food vacuoles (Fig. 4). Also, the *Archaea* proportions in the water samples of our study (3 to 6%) were similar to those in previous studies in Lake Redon and other Pyrenean lakes using 16S rRNA gene tag sequencing, namely, 0 to 8% (35) and 0 to 6% (36).

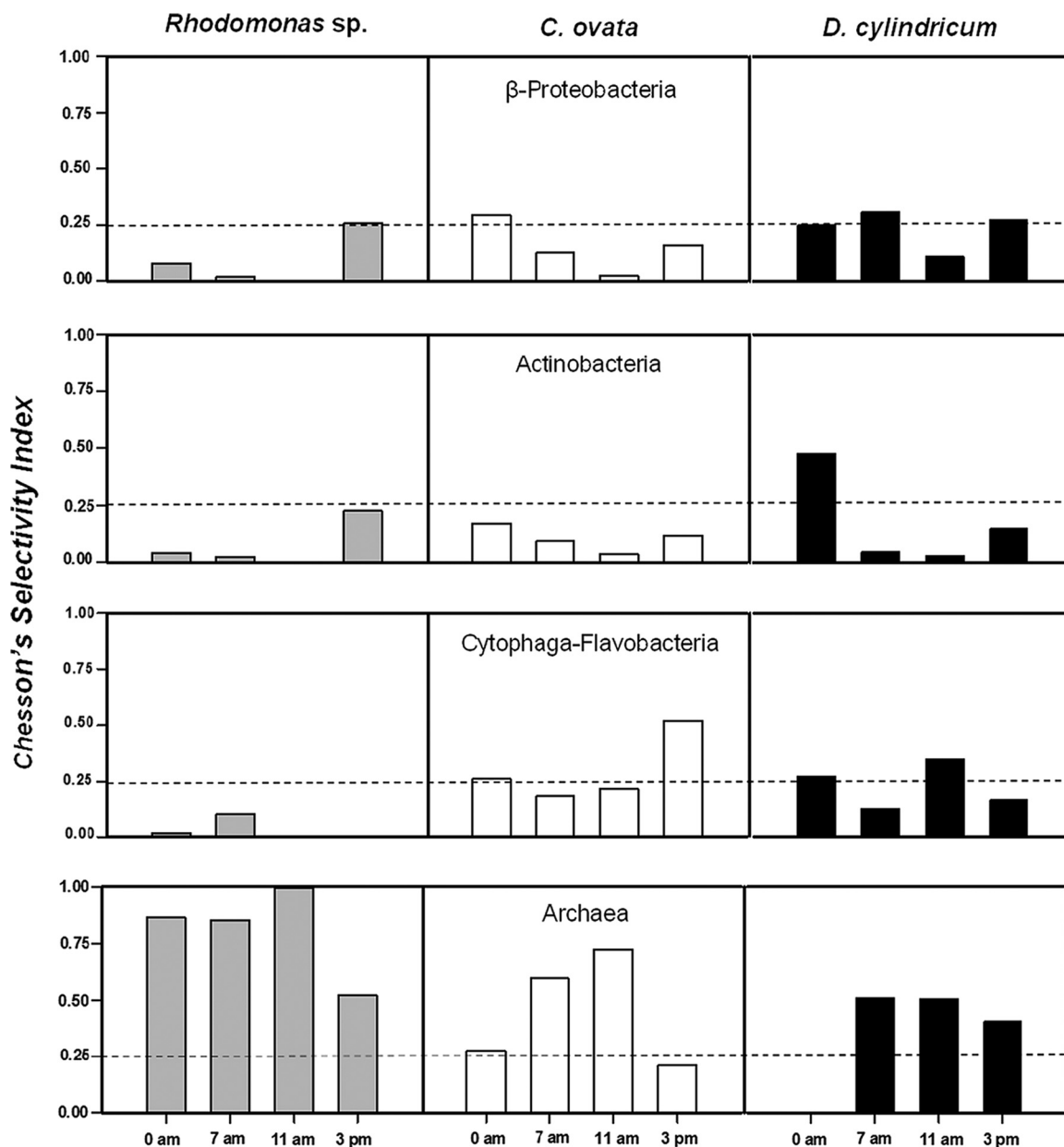
**Selective predation.** The significance of predation on bacterial activity and community structure in natural aquatic systems has been demonstrated (10, 37–40). Consequently, effort is currently placed on understanding the details and dynamics of grazing by protists (11). From laboratory experiments, evidence exists that certain taxa feed selectively (41, 42). Under natural conditions, little is known about flagellate grazing preferences and whether they tend to be specialist or generalist predators. In a recent microcosm study, Glücksman et al. (17) showed that closely related and morphologically similar flagellated species can have different impacts on natural bacterial communities. Beyond phylogeny, some general protist traits, namely, cell size



**FIG 4** Food vacuole content (average  $\pm$  SD [ $n = 2$ ] number of cells hybridized by the four probes) for the three mixotrophic flagellates at the four sampling times.

and morphological plasticity, explained variation in prey composition. Accordingly, our results show that two highly related species (i.e., the two cryptophytes), inhabiting the same system and feeding in the same prokaryotic assemblage, markedly differ in their selectivity (Fig. 4 and 5).

The species studied are closer to specialist predators than generalists (Fig. 5). *Rhodomonas sp.*, the smallest species analyzed, showed a more differentiated feeding pattern (Fig. 4). Unfortunately, the comparison of only three species does not have the statistical power to evaluate the relationship between traits and selectivity. In any case, we show that selectivity may be extreme and thus has substantial implications for the dynamics of the prokaryotic assemblages. The three protist species analyzed were the most abundant mixotrophs in the phytoplankton community at the time of the study (Table 1). The selectivity, at least at the high taxonomic levels shown by our results, paves the way for studies focusing on finer prokaryotic taxonomy, using more specific probes and evaluating whether grazing by protists constitutes a differential selective pressure within each of the large prokaryotic groups, with ecological and evolutionary implications.



**FIG 5** Chesson's selectivity index for the three mixotrophic species (plots distributed vertically) and for each of the targeted prokaryotic groups (plots distributed horizontally) at each sampling time. The horizontal dashed lines indicate the value above which positive selection of a particular prokaryotic group is assumed ( $0.25 = 1/n$ , where  $n$  is the number of prokaryotic groups).

**Cell size and grazing pressure.** Protist prey selection may occur at various feeding steps, namely, capture, prey processing, ingestion, and digestion (11, 43). Prey size is a trait that is easy to measure. There is evidence indicating that flagellates tend to graze on a limited size range of prokaryotic cells, thus removing medium-sized cells and shifting the size distribution of the prey toward larger and smaller cells (44–46). In our case, the positively selected *Archaea* showed average cell sizes similar to those of *Betaproteobacteria* and *Cytophaga-Flavobacteria*, although large cells (i.e., quartile 75% in Fig. 2) were slightly smaller in *Archaea* than in the other two groups. Very likely, factors other than cell size must explain the strong preference for *Archaea*. During the sampling day, the cell length declined with time in both *Archaea* and *Cytophaga-Flavobacteria*, which may reflect the dynamic effect of grazing throughout the day (Fig. 2), yet other factors may have an influence, such as cell division. In all samples, *Actinobacteria* were markedly smaller than the members of the other prokaryotic

groups (Fig. 2) and were less affected by protist grazing (Fig. 5). This observation agrees with previous studies that indicate lower grazing pressure on *Actinobacteria* by heterotrophic nanoflagellates (18, 20) and by the mixotrophic *Chrysophyceae* *Ochromonas* sp. (47). Indeed, if small size (i.e.,  $<0.5 \mu\text{m}$ ) constitutes a refuge against grazing, *Actinobacteria* may be negatively selected only in appearance, either because the limiting size might be an evolved defense mechanism (and thus cells tend to be smaller than those in other groups) or, on the contrary, because preference by grazers is so high that the group is permanently confined to the small-size refuge. It has been suggested that cell miniaturization alone is not sufficient to explain grazing avoidance (48) and that other resistance mechanisms, such as wall structures present in Gram-positive *Actinobacteria*, are involved in determining a limited edibility (49). Research on prokaryotic grazing is still in its infancy, but our results indicate that random grazing cannot be the paradigm.

**Preference for Archaea.** In the last decade, several studies have shown the broad distribution and abundance of *Archaea* in aquatic ecosystems (50–52) and their potential relevance in the sulfur, nitrogen, and carbon cycles (33, 53–55). However, their specific ecological interactions within the microbial communities remain largely unknown. There was no evidence that *Archaea* behaved fundamentally differently from *Bacteria* on predatory interactions (56). Our results indicate that this is not always the case. The three mixotrophic flagellate species studied here selected *Archaea* positively. The amount of such a prokaryotic group inside the protist food vacuoles was always larger than that expected by random feeding (Fig. 5). In *Rhodomonas* sp., the preference for *Archaea* was extreme. It might be thought that the *Archaea* dominance in the vacuoles responds to a stable symbiosis. However, this appears unlikely. The average *Archaea* densities within the vacuoles and the number of *Rhodomonas* cells without any *Archaea* inside markedly changed between the four sampling times (Fig. 4 and Table 1). This pattern favors the view of selective predation and relatively quick digestion rather than a stable symbiosis.

The differences among the three protist species suggest that the strength of grazing on *Archaea* can highly depend on the protist present. Nevertheless, the three species show a preference for *Archaea*, which raises questions on the importance of grazing in determining the low proportion of this group in many planktonic microbial assemblages.

The reason for high positive selectivity on *Archaea* remains highly speculative. It could be related to a poor development of resistance mechanisms in this domain, or it could be related to unknown stoichiometric features of the *Archaea*; it has been shown that bacteria with a low C-to-P ratio may be ingested at higher rates by flagellates (57, 58). The reason also could be the chemical composition of some particular group of the *Archaea* within them, as they differ in cell wall structure. It could even be some chemical cues released to water that could enhance encounter rates between prey and predator (59). *Archaea* studies in Lake Redon indicated that most of the 16S rRNA gene sequences matched *Thaumarchaeota* (close to 90%), and only a few *Euryarchaeota* were detected. MG 1.1 dominated most of the water column in summer. This clone has  $>97\%$  identity with *Nitrosoarchaeum limnia*, an ammonium-oxidizing archaeon (35). However, as new primers are applied, new groups of *Euryarchaeota* are found in the lake (60). In any case, the high positive selectivity on *Archaea* observed in the mixotrophs of Lake Redon paves the way toward new ecological and physiological studies on *Archaea*. Protist grazing is a factor that cannot be ignored for understanding the distribution and abundance of *Archaea* in planktonic microbial assemblages.

## MATERIALS AND METHODS

**Sampling site.** Lake Redon is an ultraoligotrophic high mountain lake located at 2,240 m above sea level in the central Pyrenees. It has a surface area of 24 ha and maximum and mean depths of 73 and 32 m, respectively. The lake is dimictic, well oxygenated throughout the water column, and usually covered by ice and snow 6 months a year (61). The productivity patterns and seasonal changes in the water column are typical for high mountain lakes (62, 63). This lake has been widely studied in the last 30 years. A general description of its physical, chemical, and temporal changes can be found in references 64 and 65, and a detailed description of the microbial plankton composition is in reference 66.

**Sampling.** On 9 August 2004, during the summer stratification period, integrated samples (0 to 60 m) of the water column were taken at the deepest point of the lake at midnight (12 a.m.), dawn (7 a.m.),



**TABLE 2** Oligonucleotide probes used in this study

Probe name	Specificity	Sequence (5'–3')	% FA <sup>a</sup>	Reference
EUB338	<i>Bacteria</i>	GCTGCCTCCCGTAGGAGT	55	69
ARCH915	<i>Archaea</i>	GTGCTCCCCGCCAATTCCT	40	21
CF319a	<i>Cytophaga-Flavobacteria</i> of <i>Bacteroidetes</i>	TGGTCCGTGTCTCAGTAC	55	70
HGC69a	<i>Actinobacteria</i>	TATAGTTACCACCGCCGT	30	71
BET42a	<i>Betaproteobacteria</i>	GCCTCCCACTTCGTTT	55	71

<sup>a</sup>Formamide (FA) concentration in the hybridization buffer.

morning (11 a.m.), and afternoon (3 p.m.). The four sampling times aimed to estimate the consistency of potential selective behaviors and roughly approximate the time scale of digestion in case the number of ingested prey markedly fluctuated. The goal was not to investigate daily patterns with only one sampling day. The water samples were screened through a 40- $\mu\text{m}$  mesh net to remove large zooplankton and subsequently divided into two subsamples. One of the subsamples was fixed with 0.5% (vol/vol) alkaline Lugol's solution followed by 2% buffered (pH 7) 0.2- $\mu\text{m}$ -pore-size filtered formaldehyde, and several drops of 3% sodium thiosulfate were used to decolor Lugol's fixation by following the method of Medina-Sánchez et al. (21). After 1 h of fixation at room temperature, 24 aliquots of each sample (12 of 90 ml each for protists and 12 of 10 ml each for prokaryotes) were gently filtered (<100 mm Hg) onto respective 25-mm-diameter polycarbonate Millipore membrane filters (type RTTP, 1- $\mu\text{m}$  pore size for protists; type GTPP, 0.2- $\mu\text{m}$  pore size for prokaryotes). Filters were then rinsed twice with Milli-Q water, allowed to air dry, and stored at  $-20^{\circ}\text{C}$  until further processing. The second subsample was preserved with 0.5% (vol/vol) alkaline Lugol's solution to identify algal species (67).

**Phagotrophic activity.** Three different horseradish peroxidase (HRP)-labeled probes for the main *Bacteria* groups found in the plankton of the Pyrenean lakes (23, 24, 68) and one for the domain *Archaea* were used to hybridize the filters (Table 2). The probe EUB338, which targets most *Bacteria*, was used only to evaluate the hybridization yield of the other probes. Two replicate filters were processed for each probe and sample. Alexa 488-labeled tyramide was used for signal amplification, and filters were counterstained with DAPI (1  $\mu\text{g ml}^{-1}$  final concentration) and mounted on glass slides by using Citifluor (Citifluor Ltd., United Kingdom). Slides were stored at  $-20^{\circ}\text{C}$  in the dark until subsequent counting.

Slides were examined with a Zeiss Axio Imager epifluorescence microscope at  $\times 1,000$  magnification for bacterial groups, *Archaea*, and smaller protists (<10  $\mu\text{m}$ ) and at  $\times 400$  magnification for larger protists (>10  $\mu\text{m}$ ). The microscope was equipped with an X-Cite 120 light, appropriate filter sets for DAPI (Zeiss filter set 01; BP365/12 FT396 LP397) and Alexa-Fluor 488 (Zeiss filter set 09; BP450-490 FT510 LP515, o24 DBP485/20 DFT500/600 BP515-540 + LP610), a coupled camera (AxioCam MRm), and PC-based image analysis software (AxioVision 4.8). For prokaryotes, a minimum of 500 cells was counted to establish the total abundance (DAPI counts) and the number of hybridized cells for each specific probe to estimate their abundance and percentage of hybridization. Also, cell area and perimeter were measured by image analysis software in at least 100 cells of each filter. A characteristic length was calculated using the square root of the cell area.

Protists were identified based on their plastid size and shape, as observed by chlorophyll autofluorescence under blue excitation. The identification was facilitated by parallel observation of Lugol's fixed subsample under an inverted light microscope ( $\times 600$  and  $\times 1,000$ ). A minimum of 100 individuals of the most abundant protist species was assessed for each, and the number of hybridized prokaryotic cells inside was counted.

**Data analysis.** Protist species prey selectivity was analyzed according to Chesson's  $\alpha$  index (72), as follows:

$$\alpha_i = \frac{(r_i/p_i)}{\left[ \sum_{i=1}^n r_i/p_i \right]}$$

where  $r_i$  and  $p_i$  are the mean percentages of each prokaryotic group ( $i$ ) inside the protist assessed and in the lake water assemblage, respectively;  $n$  is the number of prokaryotic groups distinguished (four in our case). When  $\alpha_i$  equals  $1/n$ , unselective feeding occurs; when  $\alpha_i$  is less than  $1/n$ , negative selection occurs, i.e., less of the prokaryotic group  $i$  occurs inside the protist than expected from random feeding; when  $\alpha_i$  is greater than  $1/n$ , positive selection occurs, i.e., more individuals of the prokaryotic group  $i$  were ingested by the protist than expected from random feeding. One-way and two-way ANOVAs were performed to compare prokaryotic assemblages among samplings. To test changes on protist ingestion, Kruskal-Wallis nonparametric tests were used (STATISTICA 7.1; StatSoft, Inc.).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02317-16>.

**TEXT S1**, PDF file, 0.1 MB.

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