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In situ prey selection of mixotrophic and heterotrophic flagellates in Antarctic oligotrophic lakes: an analysis of the digestive vacuole content

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We investigated the selective predation of mixotrophic and heterotrophic flagellates (MF and HF) on different heterotrophic prokaryote phylotypes (HPP; *Bacteria* + *Archaea*) living in natural assemblages from oligotrophic Antarctic lakes. *In situ* prey preference was analyzed for the first time on different mixotrophic taxa (*Pseudopedinella* sp., *Ochromonas*-like cells, Chrysophyceae >5 μm). The relative abundances of seven different HPP hybridized by CARD-FISH (catalyzed reporter deposition-fluorescent *in situ* hybridization) in natural community were compared with the proportions of hybridized cells inside digestive vacuoles. Our results showed some general trends to selectivity over some HPP. *Alphaproteobacteria* and *Betaproteobacteria* were the most abundant groups, and strikingly, a negative selection trend was detected in most samples by all bacterivorous protists. In contrast, for *Actinobacteria* a positive selection trend was observed in most samples, whereas *Bacteroidetes* seemed to be randomly preyed upon. Interestingly, similar prey preferences were observed in all bacterivorous flagellates. Our results suggest that

phylogenetic affiliation determines part of the process of prey selection by protists in these lakes. Nevertheless, other features, such as cell size, morphology and the presence of the S-layer, might also significantly contribute to prey selectivity on the HPP.

KEYWORDS: mixotrophic flagellates; heterotrophic flagellates; prey selection; CARD-FISH; oligotrophic lakes

INTRODUCTION

Planktonic protists [mixotrophic and heterotrophic flagellates (MF and HF) and ciliates] are considered the main bacterivorous organisms in aquatic ecosystems (Azam *et al.*, 1983; Jürgens and Matz, 2002; Pernthaler, 2005). Besides controlling bacterial abundances in a wide range of ecosystem conditions, they also channel organic carbon to higher trophic levels and release inorganic nutrients that often limit primary production (Pernthaler, 2005; Jürgens and Massana, 2008). Selective feeding of bacterivorous protists is recognized as an important mechanism for the structuring of planktonic food webs (Strom and Loukos, 1998). Size-structured predator–prey interactions are of particular importance (Lampert, 1987), and bacterial cell size must be considered a major feature that influences susceptibility towards different grazers (Corte, 1962; Güde, 1989). Different protists might have different prey preferences, being adapted to consume a specific part of the bacterial assemblage. Thus, each predator may exert specific and a highly complex top-down pressure shaping the bacterial community composition and diversity (Jardillier *et al.*, 2004; Šimek *et al.*, 2005; Vázquez-Domínguez *et al.*, 2005). Food selection by HF mostly depends on prey size and morphology (Pernthaler *et al.*, 1997; Šimek *et al.*, 1997). However, prey features other than size and shape, such as motility, digestibility, cell surface, physiological state, and food quality (as determined by the C:N:P ratio) can also mediate selective grazing (González *et al.*, 1990a; Boenigk *et al.*, 2001a, b; Matz *et al.*, 2002; Jezbera *et al.*, 2005, 2006; Matz and Jürgens, 2005; Shannon *et al.*, 2007; Massana *et al.*, 2009; Jousset, 2012).

Despite the fact that HF were formerly considered the main bacterivores in aquatic systems, several studies support the idea that MF, which combine phototrophy and phagotrophy, can be as important grazers as HF, especially in oligotrophic systems (Havskum and Riemann, 1996; Safi and Hall, 1999; Unrein *et al.*, 2007; Zubkov and Tarran, 2008). Mixotrophy is a nutritional strategy widely distributed among phytoplankton; it presents an advantage over strictly autotrophic and phagotrophic organisms when resources (e.g. nutrients) are scarce (Nygaard and Tobiesen, 1993; Marshall and Laybourn-Parry, 2002). Prey selection by mixotrophs has been poorly studied, only culture experiments have been performed on prey-size preference for some Chrysophyceae (Posch *et al.*, 1999; Boenigk *et al.*, 2001a, b; Šimek *et al.*, 2005).

In the last years, a modification of a molecular technique was developed to identify different bacterial phylotypes with a better resolution. The catalyzed reporter deposition-fluorescent *in situ* hybridization (CARD-FISH) protocol (Pernthaler *et al.*, 2002) was recently used to identify bacteria inside digestive vacuoles of certain bacterivorous protists (Jezbera *et al.*, 2005, 2006; Medina-Sánchez *et al.*, 2005; Modenutti *et al.*, 2008; Massana *et al.*, 2009). In particular, recent studies have analyzed the *in situ* prey selection of HF considered as a unique entity (Jezbera *et al.*, 2005, 2006), by comparing the relative abundances of specific prey phylotypes *in situ* and within food vacuoles. The power of this approach is that no incubations are needed. In relation to MF, it is worth noting that Medina-Sánchez *et al.* (Medina-Sánchez *et al.*, 2005) showed that the CARD-FISH technique enabled the identification of bacteria inside the digestive vacuoles without losing the autofluorescence of algal chloroplasts, although they did not perform a detailed study on prey preference of MF. Remarkably, the prey selectivity of MF has not been determined, until now.

In this investigation, we evaluated for the first time the prey preference of different taxa within the natural mixotrophic assemblage. The *in situ* prey selectivity on different prokaryote phylotypes (*Bacteria* and *Archaea*) was analyzed for MF and also HF in oligotrophic Antarctic lakes dominated by mixotrophic organisms (Allende and Izaguirre, 2003; Izaguirre *et al.*, 2003; Unrein *et al.*, 2005). We applied the CARD-FISH technique in order to estimate the relative proportions of seven different heterotrophic prokaryote phylotypes (HPP) in the natural community compared with their proportions inside protistan digestive vacuoles.

METHOD

Study site

Four lakes were sampled in Hope Bay: Esperanza, Flora, Encantado and Chico. Hope Bay is situated at the northern end of the Antarctic Peninsula (63°24′S, 57°00′ W), where most of the lakes are of glacial origin. During summer, lakes are ice-free or covered by a thin ice layer ranging between 1 and 35 cm. Esperanza, Flora and Encantado are situated in the Five Lakes Valley, and are connected by a small stream; Chico is located on the Mount Flora shelf. Morphometric and physicochemical features of these Antarctic lakes have been previously described (Izaguirre *et al.*, 1998; Unrein *et al.*, 2005; Schiaffino *et al.*, 2009). All these lakes are oligotrophic (<1 μg chlorophyll *a* L^{-1}) and shallow (maximum depth between 1 and 7 m), and share a roughly similar phytoplankton composition dominated by mixotrophic chrysophytes (Izaguirre *et al.*, 1998, 2003; Allende and Izaguirre, 2003; Unrein *et al.*, 2005).

Sample collection

Samples of Antarctic lakes were collected during the austral summer period, between January and March 2004 on two different dates. Samples were collected in acid-washed plastic bottles pre-rinsed with lake water from a single site near the shore of the lake, beneath the surface during the ice-free periods, or immediately below the ice layer when the lakes were frozen. Physical–chemical parameters measured on these dates have already been published by Schiaffino *et al.* (Schiaffino *et al.*, 2009).

Heterotrophic prokaryote and flagellate abundance

Samples for heterotrophic prokaryotes (*Bacteria* + *Archaea*) and flagellates were fixed with ice-cold filtered 10% glutaraldehyde (final concentration 1%). For heterotrophic prokaryote enumeration, between 2 and 5 mL of fixed sample were stained with 10 μg mL^{-1} (final concentration) of 4′,6-diamidino-2-phenylindole (DAPI) according to Porter and Feig (Porter and Feig, 1980), and then filtered through a 0.22-μm black polycarbonate membrane filters (Millipore). Samples for flagellate enumeration were processed in a similar way, except that the filtered volume was 10 or 20 mL and the filters had a 0.8-μm pore size. All filters were mounted on a microscope slide with a drop of immersion oil for fluorescence microscopy (Immersol 518 F) and stored at −20°C. Samples were inspected at ×1000 magnification using an epifluorescence microscope (Olympus BX50, Japan) equipped with an HBO 50W lamp, and a filter set for blue light excitation (BP 420–480 nm, BA 515 nm), green light excitation (BP 480–550 nm, BA 590 nm) and UV excitation (BP 330–385 nm, BA 420 nm). Flagellates smaller than 10 μm were first detected by UV excitation, and then inspected by blue light excitation in order to identify the presence or absence of chloroplast autofluorescence (phototrophic and heterotrophic flagellates, respectively). Coccoid picoeukaryotic chlorophytes (<2 μm and without flagellae) were frequently observed in our samples, though they were not considered in the present study. The phytoplankton cells >10 μm and ciliates were counted following the Utermöhl technique, for which additional water samples were fixed with acid Lugol's solution and were counted under an inverted microscope (Olympus CKX41, Japan) using 50-mL Utermöhl chambers. Phytoplankton and ciliates were enumerated by scanning the entire chamber surface at ×400.

Identification of heterotrophic prokaryote phylotypes in the plankton

The term HPP includes *Bacteria* and *Archaea*, making reference to all the microorganisms which are neither autotrophic nor eukaryotic cells. HPP were identified by applying the CARD-FISH technique. Water samples were fixed with formaldehyde (2% final concentration), and aliquots between 2 and 10 mL were filtered through 0.22-μm pore size (47 mm diameter) white polycarbonate filters (for prokaryotes in the plankton), dried and kept frozen until processed. Samples for identification of the HPP inside the flagellate's digestive vacuoles were processed in a similar way, except for the sample volume (10 and 20 mL) and the pore size filter (0.8 μm). Sections of filters were hybridized according to Pernthaler *et al.* (Pernthaler *et al.*, 2002) and Sekar *et al.* (Sekar *et al.*, 2003) using the following oligonucleotide probes: EUB338-II-III, to target most *Bacteria* including *Verrucomicrobia* and *Planctomycetes* (Amann *et al.*, 1990; Daims *et al.*, 1999); ALF968, specific for *Alphaproteobacteria* (Neef, 1997); BET42a, specific for *Betaproteobacteria* (Medina-Sánchez *et al.*, 2005); GAM42a, to target *Gammaproteobacteria* (Manz *et al.*, 1992); CF319a, to target the *Bacteroidetes* (Manz *et al.*, 1996); HGC69a, specific for *Actinobacteria* (Amann *et al.*, 1990); CREN554, to target *Crenarchaeota* (Massana *et al.*, 1997a) and EURY806, specific for *Euryarchaeota* (Teira *et al.*, 2004). Probes were supplied by Thermo Electron Corporation (Waltham, MA, USA) with an aminolink (C6) at the 5′ end, bound with a horseradish peroxidase enzyme (Urdea *et al.*, 1988). After hybridization, the signal was amplified with Alexa 488-labeled tyramide and

counter-stained with DAPI. Filter pieces were mounted on a slide with Vectashield with DAPI and observed by epifluorescence microscopy (Olympus BX50, Japan) under blue and UV light excitation.

Average cell size of the prokaryotes targeted by specific oligonucleotide probes was measured on each sample using Image Pro Plus, following the image analysis protocol proposed by Massana *et al.* (Massana *et al.*, 1997b). Dimensions of cells were overestimated because they were measured from the specific probe signal. Previously, we performed comparisons between bacterial size determinations obtained from DAPI and CARD-FISH signals in the study lakes, and no significant differences were found in the enlargement of cells among all probes (data not shown). Despite this, in the present investigation, bacterial dimensions were determined from CARD-FISH, and size values are comparable with other studies that analyzed bacterial dimension in a similar way (e.g. Jezbera *et al.*, 2005, 2006). Cells longer than 3 μm were assumed to be protected against grazing by most bacterivorous protists (Hahn and Höfle, 2001; Pernthaler, 2005; Šimek *et al.*, 2005).

Enumeration of HPP inside flagellate digestive vacuoles

We identified three different MF: *Pseudopedinella* sp., Chrysophyceae <5 μm (*Ochromonas*-like cells), and Chrysophyceae >5 μm. The identification of these flagellates was achieved based on previous studies (Izaguirre *et al.*, 1998, 2003; Allende and Izaguirre, 2003; Unrein *et al.*, 2005). The HF were considered as a single group. Seventy-five cells of each flagellate type were inspected for evaluating the presence of each HPP directly in the digestive vacuoles on each sample.

To determine the preference of each bacterivorous flagellate for a given HPP, we compared the percentage of each of the seven HPP inside digestive vacuoles in relation to their percentage in the plankton. In previous studies, these percentages were calculated with respect to the abundance of EUB338 targeted cells (Šimek *et al.*, 1997; Jezbera *et al.*, 2005, 2006). However, in the present investigation we found that the EUB338-II-III probe also hybridized the chloroplasts of MF, making it impossible to identify ingested *Bacteria* in food vacuoles of these organisms. To solve this drawback, we referred the estimations to the sum of all the identified HPP. In this sense, we defined total identified prokaryotes (TIP) as the sum of the following seven HPP, five of *Bacteria* (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*) and two of *Archaea* (*Crenarchaeota* and *Euryarchaeota*). TIP in plankton samples represented on average 80.2% (between 65.2 and 95.2%) of the total heterotrophic prokaryotes detected by DAPI. To validate this assumption, in the case of HF we calculated all the parameters using TIP and EUB338-II-III targeted cells values, and compared the obtained results.

Selectivity index

For assessing prey selection, we applied the Chesson selectivity index (α) (Chesson, 1978, 1983):

$$\alpha i = \frac{(ri/ni)}{\sum_{i=1}^{m}(ri/ni)},$$

where i is the number of items of food type i in the consumer's diet, which can vary between 1 and m. The ri and ni are the percentage of food type "i" in the diet and the environment, respectively (expressed as percentage of TIP). This index varies between 0 and 1; values of 1/m represent random feeding, $<1/m$ indicate negative selection, $>1/m$ indicate positive selection. This index was calculated in all the cases when HPP was above 4% of the TIP. As the number of prey types varies as a function of the abundance percentage, and the limit for the selection should be different in each case, we calculated the parameter ε (Chesson, 1983), which becomes independent of m. The α values were transformed in ε values as follows:

$$\varepsilon i = \frac{(m\alpha i - 1)}{(m-2)\alpha i + 1}.$$

This parameter varies between -1 and 1; $\varepsilon = 0$ implies random selection, negative values represent negative selection, whereas positive values represent positive selection.

Statistical analysis

Pearson correlation indexes were calculated relating the α value of Chesson selectivity index among all bacterivorous flagellates, and between the Selectivity indexes calculated for HF in relation to TIP and EUB338-II-III-targeted cells. A linear regression was used to compare the relation between the EUB338-II-III-targeted cells and the TIP abundances in all samples.

RESULTS

Characterization of the heterotrophic prokaryote assemblage

HPP abundance ranged from 0.9×10^5 to 6.8×10^5 cells mL^{-1} (Fig. 1a). *Alphaproteobacteria* (ALF968

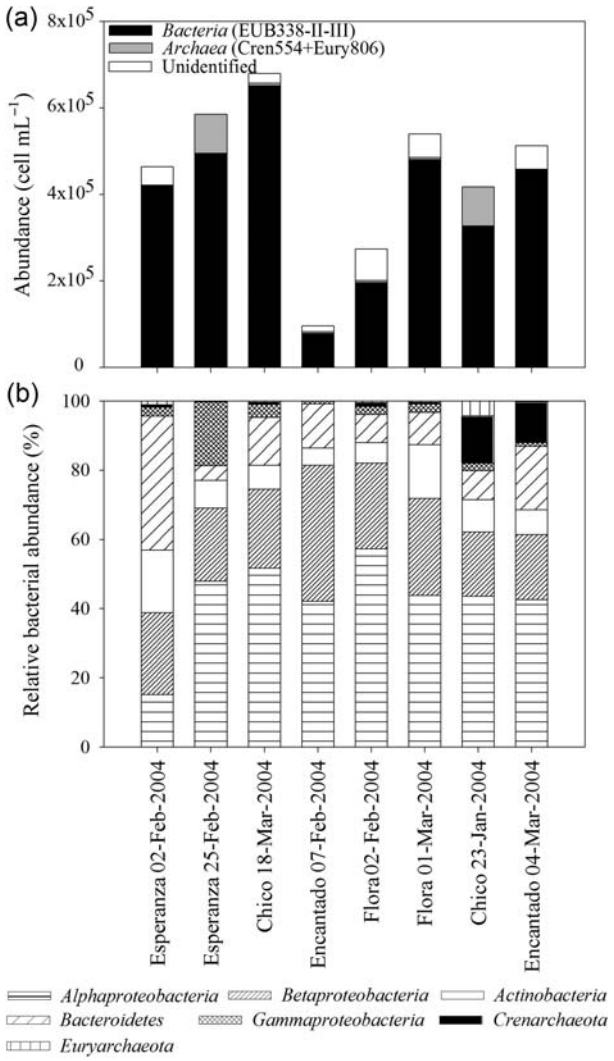

Fig. 1. (**a**) Abundance of heterotrophic prokaryotes. Unidentified heterotrophic prokaryote cells were estimated from the difference between DAPI counts and the sum of *Bacteria* plus *Archaea*. (**b**) Relative abundance of the seven heterotrophic prokaryote phylotypes (HPP) related to total identified prokaryotes (TIP) (see text for further explanations); samples are ordered by increasing Chl *a* concentration.

probe) and *Betaproteobacteria* (BET42a probe) were the most abundant groups, and together accounted for >60% of TIP (Fig. 1b). In particular, *Alphaproteobacteria* presented values >40% in most systems. The abundance of bacteria targeted by *Bacteroidetes*, *Actinobacteria* and *Gammaproteobacteria* probes averaged 15, 10, 4% of TIP, respectively. The *Archaea* abundance was low in all samples (<1%) except in two lakes where their relative abundance reached 11% (Fig. 1b).

HPP cell size was measured for the four most abundant groups (*Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes* and *Actinobacteria*) in the samples where they represented >4% of the TIP. The largest cell biovolumes and lengths were registered for *Betaproteobacteria*, followed by *Alphaproteobacteria*, whereas *Actinobacteria* were always the smallest (Table I). In addition, cells >3 μm of both phylotypes were recurrently observed in all samples (Fig. 2). The relative contribution of cells >3 μm to the total biovolume ranged from 11 to 66% (mean, 32 ± 18%) and 2–57% (mean, 25 ± 18%) in *Alphaproteobacteria* and *Betaproteobacteria*, respectively. In the other two groups, the percentages averaged 15% in the case of *Bacteroidetes* and 10% in the case of *Actinobacteria*. *Bacteroidetes* had slightly lower cell size, and it was represented by typically rod-shaped cells.

Protistan community assemblage

In all cases the protist community was numerically dominated by bacterivorous flagellates. A few truly autotrophic species of Chlorophyceae (mostly *Chlamydomonas*-like species) were registered always in very low abundances (<5%). All lakes were dominated by MF, except one particular date for Flora (02 February 2004) when HF prevailed; Ciliate abundance was very low (<10 ind L^{-1}) in all cases, and for this reason we considered that their contribution was negligible (Fig. 3). In all lakes, MF were dominated by Chrysophyceae (Fig. 3), which were categorized in two size classes: <5 μm (*Ochromonas*-like cells) and >5 μm. Although most *Ochromonas*-like cells were morphologically similar (round shaped and ~3–4 μm in diameter), larger Chrysophyceae (>5 μm) were represented by a heterogeneous group of cells with different morphologies. *Pseudopedinella* sp. (Dictyochophyceae) was present in five out of eight samples, although almost always in low abundances. Cells were radially symmetrical, with three to six peripheral chloroplasts, and ~7 μm in diameter in all lakes.

Selectivity index

The Chesson index was calculated for all bacterivorous flagellates, both the MF taxa (*Ochromonas*-like cells; Chrysophyceae >5 μm; *Pseudopedinella* sp.) and the whole HF assemblage. The number of ingested prey found inside digestive vacuoles in each bacterivorous flagellate usually varied between one and three cells, although most flagellates had only one cell inside the digestive vacuoles. The real number of each HPP found inside digestive vacuoles of each flagellate are detailed in the Supplementary data, Table SA.

The results of the Chesson index calculated for HF with the percentage of HPP in relation to the EUB338-II-III-targeted cells were similar to those

Table I: Cell volume (μm^3) of different heterotrophic prokaryotes phylotypes (HPP) targeted by oligonucleotide probes in all studied lakes

	Esperanza 2 February 2004	Esperanza 25 February 2004	Chico 18 March 2004	Encantado 7 February 2004	Flora 2 February 2004	Flora 1 March 2004	Chico 23 January 2004	Encantado 4 March 2004
Alpha								
n	305	709	478		481	649	662	1242
Mean	1.450	0.686	1.291	ND	0.479	0.680	1.104	0.989
SD	4.706	1.763	2.575		0.676	0.907	2.458	1.243
Median	0.298	0.237	0.456		0.244	0.344	0.372	0.638
Beta								
n	297	243	240	455	167	209	189	433
Mean	1.393	0.833	1.348	2.433	0.409	0.562	0.616	0.855
SD	2.154	1.145	1.944	2.644	0.367	0.558	0.769	0.837
Median	0.585	0.528	0.749	1.665	0.316	0.391	0.358	0.584
Actino								
n	119	168	145		44	288	522	128
Mean	0.174	0.167	0.515	ND	0.121	0.401	0.140	0.239
SD	0.518	0.371	0.838		0.133	0.569	0.863	0.275
Median	0.060	0.055	0.136		0.086	0.196	0.019	0.137
Bacteroi								
n	104	107		190	86	125	295	380
Mean	0.347	0.941	ND	0.838	0.272	0.847	0.743	0.847
SD	0.532	1.467		0.588	0.275	1.283	0.955	0.950
Median	0.235	0.510		0.676	0.186	0.400	0.317	0.373

Alpha, *Alphaproteobacteria*; Beta, *Betaproteobacteria*; Actino, *Actinobacteria*; Bacteroi, *Bacteroidetes*; ND, not determined. The samples are ordered by increasing Chl *a* concentration.

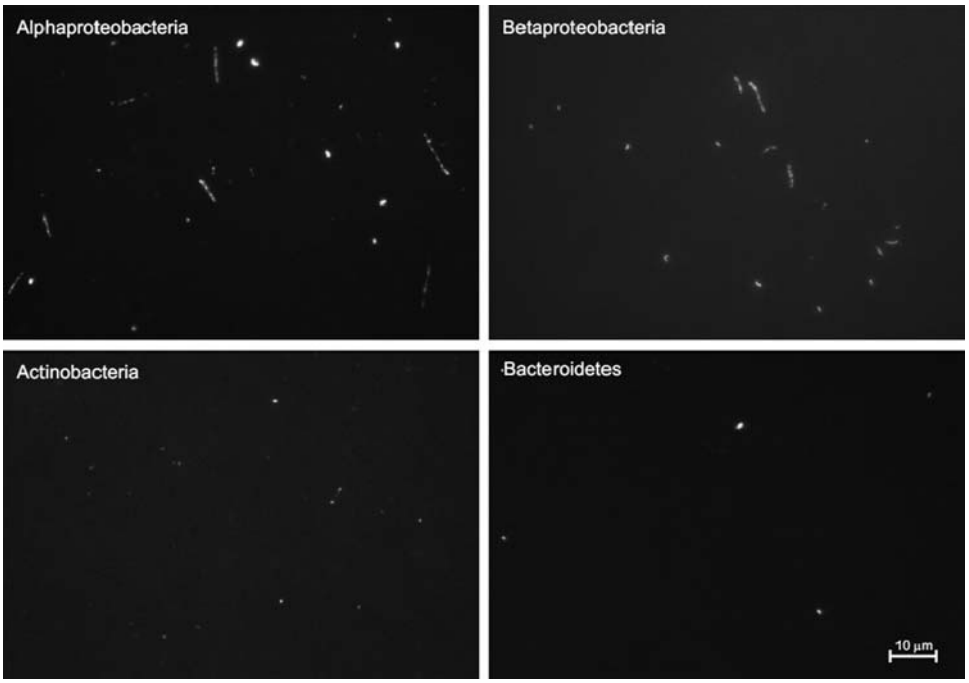

Fig. 2. Epifluorescence micrographs of CARD-FISH hybridized cells of the most abundant heterotrophic prokaryote phylotypes (HPP) observed under blue-light excitation. Scale bar is 10 µm and applies to all pictures.

calculated based on TIP abundance, and both estimations were significantly correlated (Supplementary data, Fig. SA). The significant linear regression analysis between the TIP and the EUB338-II-III-targeted cells abundances in all studied lakes ($R^2 = 0.937$; $P < 0.001$; $F = 89.773$), supported our assumption that TIP are a good proxy of the EUB338-II-III-targeted cells in these samples.

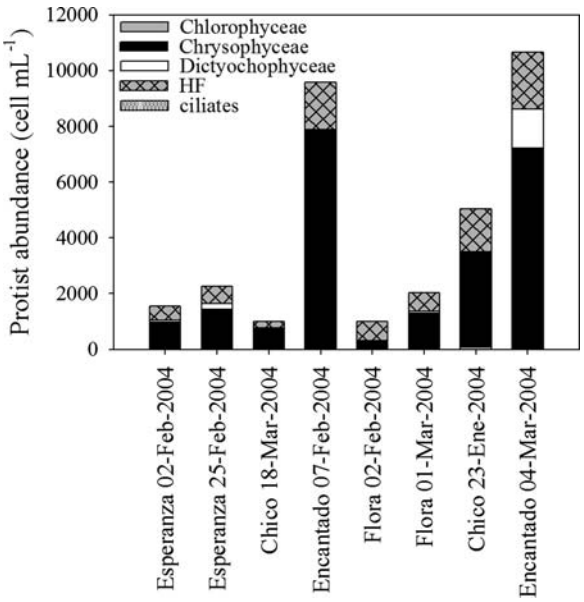

Fig. 3. Abundance of autotrophic (Chlorophyceae), mixotrophic (Chrysophyceae, Dictyochophyceae), heterotrophic flagellates (HF) and ciliates at each sampling site. The samples are ordered by increasing Chl *a* concentration.

A negative trend of selection was observed by all MF and HF in relation to the *Betaproteobacteria* and *Alphaproteobacteria* group (Fig. 4). In contrast, *Actinobacteria* were, in general, positively selected by HF and *Pseudopedinella* sp., whereas *Ochromonas*-like cells and Chrysophyceae >5 μm did not show a clear preference. For *Bacteroidetes* no clear trend was observed in any of the bacterivorous flagellates analyzed (Fig. 4). On the other hand, the two index values calculated for the *Archaea* group suggested a negative selection by all predators (data not shown). Overall, we found that all flagellates generally showed a very similar feeding preference with respect to the majority of HPP (Fig. 4). This finding was further supported by the significant correlations obtained when comparing the Chesson selectivity index values between each pair of flagellate taxa (Table II).

DISCUSSION

The HPP assemblage was generally dominated by *Alphaproteobacteria*. Although this dominance has already been reported in other lakes around the world (Kirchman *et al.*, 2004; Nishimura and Nagata, 2007; Salcher *et al.*, 2011), the general assumption is that *Alphaproteobacteria* dominates marine waters, whereas freshwater environments are dominated by *Betaproteobacteria* (Glöckner *et al.*, 1999) and, to a lesser extent, by *Actinobacteria*, *Cytophaga* and *Verrucomicrobia* (Glöckner *et al.*, 1999; Urbach *et al.*, 2001; Warnecke *et al.*, 2005). It has been observed that a high water residence time (Lindström *et al.*, 2005) and a high grazing pressure (Pernthaler *et al.*, 1997; Salcher *et al.*, 2005) seems to increase the abundance of *Alphaproteobacteria* in freshwater environments. In this sense, a recent study comparing the bacterial community composition in 45 lakes located in Antarctica and the southernmost region of South America revealed that 78% of the surveyed lakes are dominated by the *Alphaproteobacteria* group (Schiaffino, 2011).

The CARD-FISH technique was successfully applied in this study allowing us to evaluate the predation of MF and HF on different HPP in natural environments. As a methodological comment, we note that these good results were obtained when analyzing the cells fixed with 2% formaldehyde. However, in previous investigations we have used paraformaldehyde (1%) as fixative, as suggested by Medina-Sánchez *et al.* (Medina-Sánchez *et al.*, 2005), and we observed that this caused a systematic deformation of the mixotrophic algal cells present in our samples. So, we discourage the application of paraformaldehyde in studies that assess prey selectivity of mixotrophic algae.

In relation to the selectivity behavior, our results showed general trends of selectivity over different HPP, although we did not find a general pattern applicable in all systems. In the case of *Alphaproteobacteria* and *Betaproteobacteria*, the two more abundant bacterial groups, a negative selection trend (73 and 67% of the cases, respectively) was generally observed in all grazers. These groups had longer cells than other HPP, including filaments (>3 μm), which have been previously described as grazing-resistant morphologies (Jürgens *et al.*, 1999; Jürgens and Matz, 2002; Pernthaler, 2005; Salcher *et al.*, 2005). However, the presence of these resistant morphotypes could only partially explain the negative selection trend observed, since they were present in relatively low abundance. It is well known that other factors such as food quality could influence the prey selectivity over the HPP (Boenigk *et al.*, 2001a; Shannon *et al.*, 2007), although further studies are necessary to analyze the food quality of HPP in these environments. On the contrary, in the case of *Bacteroidetes* there was a similar number of cases with positive and negative selection in all the flagellates (11 samples with positive selection and 13 samples with negatives values). This lack of pattern lead us to propose a randomly selectivity behavior.

In relation to *Actinobacteria*, our results show a positive selection trend by *Pseudopedinella* sp. (four samples positive and one negative) and HF (six samples positive and

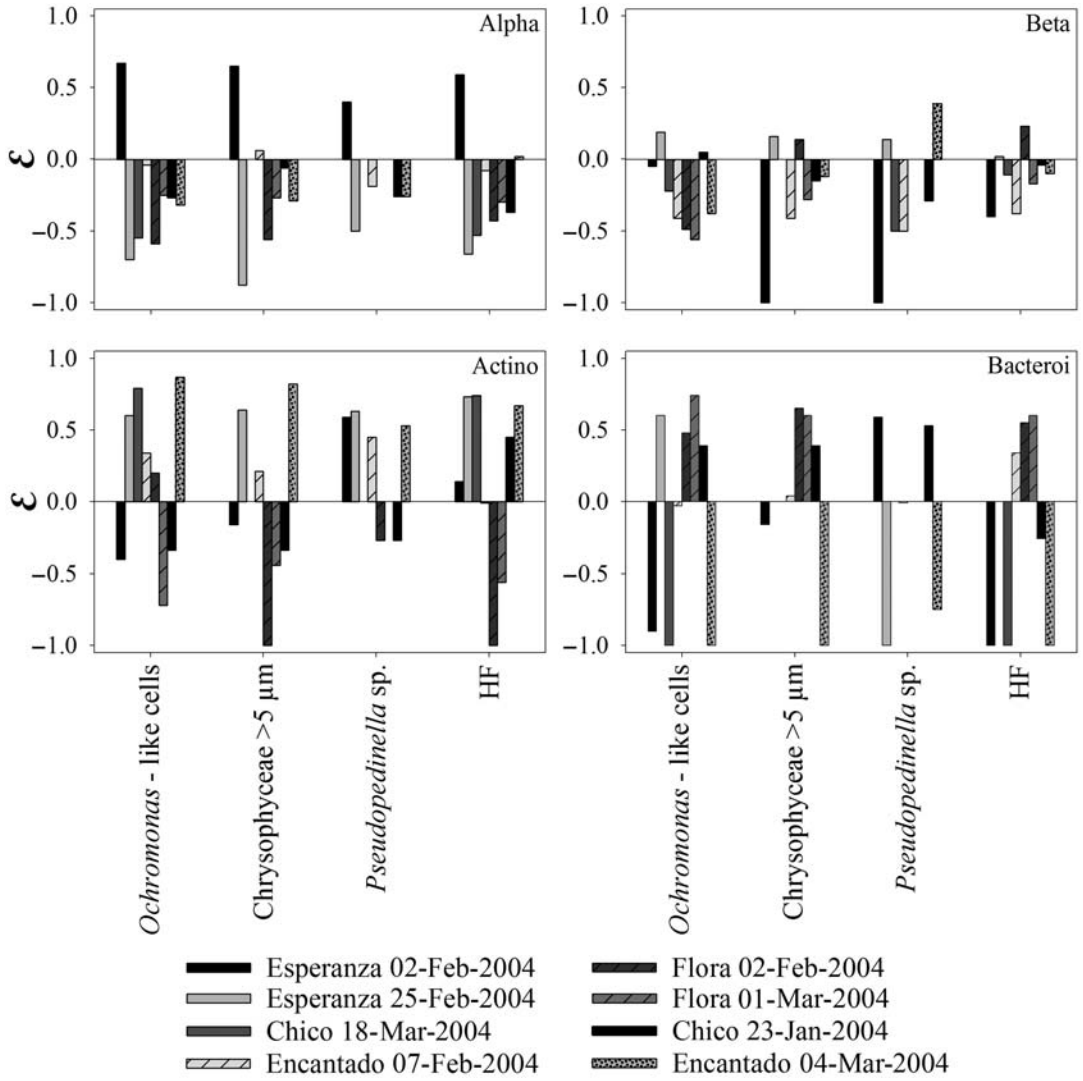

Fig. 4. Chesson selectivity index (ε) of each protist for the most abundant heterotrophic prokaryote phylotypes (HPP). Alpha, *Alphaproteobacteria* group (ALF968); Beta, *Betaproteobacteria* group (BET42a); Actino, *Actinobacteria* group (HGC69a); Bacteroi, *Bacteroidetes* group (CF319a); HF, heterotrophic flagellates. The samples are ordered by increasing Chl *a* concentration.

Table II: Pearson correlation values among selectivity indexes of all bacterivorous flagellates

	Chrysophyceae >5 µm	*Pseudopedinella* sp.	HF
Ochromonas-like cells	0.878* ($n = 29$)	0.718* ($n = 21$)	0.817* ($n = 33$)
Chrysophyceae >5 µm		0.782* ($n = 21$)	0.845* ($n = 29$)
Pseudopedinella sp.			0.703* ($n = 21$)

*$P < 0.001$.

two negative). These results do not agree with previous investigations which have shown that *Actinobacteria* are avoided by predators (Hahn *et al.*, 2003; Jezbera *et al.*, 2005; Šimek *et al.*, 2005). It was suggested that the small cell size and the thick cell wall of these bacteria conferred resistance against protistan grazing (Warnecke *et al.*, 2004). Nevertheless, this is not the first study that has shown grazing over *Actinobacteria*. Pernthaler *et al.* (Pernthaler *et al.*, 2001) observed the size-selective grazer *Ochromonas* (*Poterioochromonas*) sp. grazing upon the *Actinobacteria* (acI) lineage and other freshwater lake lineages. Other authors have also noted a decrease in the abundance of the *Actinobacteria* during periods of enhanced grazing pressure (Newton *et al.*, 2006). Experimentally, an increase of the number of *Poterioochromonas* cells has been observed in the presence of *Actinobacteria* cells, probably associated with the availability of essential lipids in these prey (Tarao *et al.*, 2009).

Table III: Estimated half-life ($t_{1/2}$, in minutes) of bacteria contained in food vacuoles of flagellates

				Average prey $t_{1/2}$ in minutes (*n*)								
				Abs S-layer		Presence of S-layer					Unkn S-layer	
Source	Predator	Prey[a]	T(°C)	Beta	Gama	Beta	Gama	Actin	Firm	Cyan	Gama	MBA
Sherr *et al.* (Sherr *et al.*, 1988)	Mixed HF assemblage	MBA	19.5–20									24 (5)
González *et al.* (González *et al.*, 1990b)	Mixed HF assemblage	*Escherichia coli*[b]	17–19		27 (4)							
	Mixed HF assemblage	*Enterococcus faecalis*[c]	17–19						80 (4)			
González *et al.* (González *et al.*, 1993)	*Cafeteria* sp.	MBA	15									56 (8)
Dolan and Šimek (Dolan and Šimek, 1998)	*Bodo saltans*	*Synechococcus*-like[d]	22							64 (8)		
Boenigk *et al.* (Boenigk *et al.*, 2001b)	*Spumella* sp.	*Pseudomonas putida*	17								12 (2)	
	Cafeteria roenbergensis	*Pseudomonas putida*	17								25 (2)	
	Ochromonas sp. (mixotrophic chrysophyte)	*Pseudomonas putida*	17								8 (2)	
Jezbera *et al.* (Jezbera *et al.*, 2005)	*Bodo saltans*	*Aeromonas hydrophila*[e]	22			52 (1)						
	Bodo saltans	*P. fluorescens*[f]	22				20 (1)					
	Goniomonas sp.	*Aeromonas hydrophila*	22			38 (1)						
	Goniomonas sp.	*P. fluorescens*	22				34 (1)					
Shannon *et al.* (Shannon *et al.*, 2007)	*Ochromonas danica* (mixotrophic chrysophyte)	*P. fluorescens*	ND				77 (30)					
Tarao *et al.* (Tarao *et al.*, 2009)	*Poterioochromonas* sp.	*Polynucleobacter cosmopolitanus*[g]	20–22	18 (2)								
	Poterioochromonas sp.	*Actinobacteria* strain MWH-Po1 (Luna-2 cluster)[g]	20–22					95 (2)				
Weighted average (range)				24 (16–56)				72 (20–144)			15 (6–39)	44 (17–148)

Abs S-layer, absence of S-layer; Unkn S-layer, unknown presence or absence of S-layer; T (°C), temperature in Celsius degrees; the number between brackets indicates the number of samples considered for the calculations; Beta, *Betaproteobacteria*; Gama, *Gammaproteobacteria*; Actin, *Actinobacteria*; Firm, *Firmicutes*; Cyan, *Cyanobacteria*; MBA, mixed bacterial assemblage; HF, heterotrophic flagellates.

[a]The following references show the relation between each prey with the S-layer:

[b]Lederer *et al.* (Lederer *et al.*, 2011);

[c]Brinster *et al.* (Brinster *et al.*, 2007);

[d]Šmarda *et al.* (Šmarda *et al.*, 2002);

[e]Murray *et al.* (Murray *et al.*, 1988), Thomas and Trust (Thomas and Trust, 1995);

[f]*Pseudomonas* Genome Database: http://www.pseudomonas.com/genomeMenu.do?strain_id=113&submit=Submit;

[g]Tarao *et al.* (Tarao *et al.*, 2009).

Besides, the existence of *Actinobacteria* cells with a thin cell wall has recently been demonstrated (Hahn *et al.*, 2003), contrary to what was known. Further, it is important to note that our results relate to *in situ* samples, and that the *Actinobacteria* strains of the study lakes are included in the size range of edible prey by flagellates. Moreover, the relatively high number of *Actinobacteria* cells observed inside digestive vacuoles could also be related to a high digestion time. Recently, the existence of a surface protein layer (S-layer) was described associated to the cell wall, present in several species including some strains of *Actinobacteria* (Tarao *et al.*, 2009). It has been suggested that the S-layer of *Actinobateria* impairs digestion by flagellates (Jousset *et al.*, 2012). Considering such evidence, we analyzed all the available information about the digestion times (half-life of prey inside flagellate) of different predators, in relation to the presence or absence of the S-layer in each prey (Table III, and references therein). The estimated half-time of prey inside digestive vacuoles was three times greater when the S-layer was present in the prey. This supports the idea that the S-layer effectively increases digestion times. Summarizing, our results show that *Actinobacteria* were grazed by flagellates in these Antarctic lakes, and the positive selection by the flagellates could be explained by a higher digestion time, probably due to the presence of the S-layer in these prey.

Despite these general trends, HPP were not always positively or negatively selected by a given protist. This suggests that phylogenetic affiliation determines part of the process of prey selection by protists. Even though cell size and morphology, that are sometimes linked to phylogenetic affiliation (e.g. *Betaproteobacteria* usually form inedible filaments under high grazing pressure or *Actinobacteria* reduce cell size to escape from grazing; Jürgens and Matz, 2002), partially explained the observed patterns. Other features (chemical, behavioral, physiological, etc.) might also significantly contribute to the prey selectivity over the HPP (Boenigk *et al.*, 2001a; Shannon *et al.*, 2007; Jousset, 2012).

Interestingly, the four groups of protists had a very similar trend in the selectivity for each HPP (Fig. 4 and Table II). This result was unexpected since, due to the heterogeneity of sizes, nutritional strategies (i.e. mixotrophs and heterotrophs) and taxonomic affiliation among predators, we expected to find different selectivity behaviors among the flagellates assessed. Accordingly, experimental studies have illustrated different prey preferences by different protistan grazers, which were mostly related to predator–prey size relationship (e.g. Andersson *et al.*, 1986; Jürgens and Matz, 2002; Massana *et al.*, 2009). The similarity of *in situ* prey preference observed in our study for the four group of flagellates, suggests the existence of some features of HPP cells (e.g. cell size, morphology, presence of the S-layer, etc.) that interacts with the different bacterivores in the same way.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://*plankt.oxfordjournals.org*.

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