The ISME Journal (2009) 3, 588–596

www.nature.com/ismej

ORIGINAL ARTICLE

Grazing rates and functional diversity of uncultured heterotrophic flagellates

Ramon Massana[1], Fernando Unrein[1,3], Raquel Rodríguez-Martínez[1], Irene Forn[1], Thomas Lefort[1], Jarone Pinhassi[2] and Fabrice Not[1,4]

[1]*Department of Marine Biology and Oceanography, Institut de Ciències del Mar (CSIC), Barcelona, Catalonia, Spain and* [2]*Marine Microbiology, Department of Pure and Applied Natural Sciences, University of Kalmar, Kalmar, Sweden*

Aquatic assemblages of heterotrophic protists are very diverse and formed primarily by organisms that remain uncultured. Thus, a critical issue is assigning a functional role to this unknown biota. Here we measured grazing rates of uncultured protists in natural assemblages (detected by fluorescent *in situ* hybridization (FISH)), and investigated their prey preference over several bacterial tracers in short-term ingestion experiments. These included fluorescently labeled bacteria (FLB) and two strains of the *Roseobacter* lineage and the family *Flavobacteriaceae*, of various cell sizes, which were offered alive and detected by catalyzed reporter deposition-FISH after the ingestion. We obtained grazing rates of the globally distributed and uncultured marine stramenopiles groups 4 and 1 (MAST-4 and MAST-1C) flagellates. Using FLB, the grazing rate of MAST-4 was somewhat lower than whole community rates, consistent with its small size. MAST-4 preferred live bacteria, and clearance rates with these tracers were up to 2 nl per predator per h. On the other hand, grazing rates of MAST-1C differed strongly depending on the tracer prey used, and these differences could not be explained by cell viability. Highest rates were obtained using FLB whereas the flavobacteria strain was hardly ingested. Possible explanations would be that the small flavobacteria cells were outside the effective size range of edible prey, or that MAST-1C selects against this particular strain. Our original dual FISH protocol applied to grazing experiments reveals important functional differences between distinct uncultured protists and offers the possibility to disentangle the complexity of microbial food webs.

The ISME Journal (2009) **3,** 588–596; doi:10.1038/ismej.2008.130; published online 8 January 2009
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: functional diversity; grazing rates; MAST-4; MAST-1C; prey preference; uncultured flagellates

Introduction

Bacterial grazing is of fundamental importance in aquatic ecosystems and is carried out mostly by small flagellated protists up to 5 μm in diameter (Sherr and Sherr, 2002). It controls bacterial abundances in a wide range of ecosystem conditions, channels organic carbon to higher trophic levels, and releases inorganic nutrients that often are limiting primary production (Pernthaler, 2005; Jürgens and Massana, 2008). There are two main approaches to estimate community bacterivory rates: tracer techniques that follow the fate of an added bacterial surrogate and manipulation techniques that uncouple predator and prey populations (Strom, 2000). At the community level, sound information is available on how bacterial grazing relates to system productivity, temperature or other environmental variables in a wide range of oceanographic conditions (Sanders *et al.*, 1992; Vaqué *et al.*, 1994). Recently, a large phylogenetic diversity of small marine protists, mostly uncultured, has been unveiled (Moon-van der Staay *et al.*, 2001; Epstein and López-García, 2008), which likely implies a large functional diversity that needs to be considered for a better understanding of microbial food webs. Some studies have tried to tell apart the black box of bacterial grazers. For instance, the most popular tracer technique to estimate bacterivory, which inspects fluorescently labeled bacteria (FLB) inside protistan food vacuoles after short-term incubations (Sherr *et al.*, 1987), allows categorizing the grazers according to cell size, pigmentation or conspicuous morphologies (Simek *et al.*, 2004;

Correspondence: R Massana, Department of Marine Biology and Oceanography, Institut de Ciències del Mar (CSIC), Passeig Marítim de la Barceloneta 37-49, Barcelona, Catalonia 08003, Spain.
E-mail: ramonm@icm.csic.es
[3]Current address: Instituto de Investigaciones Biotecnológicas—Instituto Tecnológico de Chascomús (CONICET), Argentina.
[4]Current address: Groupe Plancton Océanique, Station Biologique de Roscoff, France.
Received 20 October 2008; revised 1 December 2008; accepted 1 December 2008; published online 8 January 2009

Unrein *et al.*, 2007). Nowadays molecular techniques offer new tools to address simultaneously the phylogenetic and functional diversity of bacterial grazers.

There are several ways to explain how different grazers apparently using the same resource actually coexist and occupy separate ecological niches. First, each species might have different environmental optimum, being better adapted to a given range of physicochemical or biotic parameters. When this applies to strains from the same species the term ecotype is used (Rodríguez *et al.*, 2005; Boenigk *et al.*, 2007). Second, each species might have different prey preferences, being adapted to consume a specific part of the bacterial assemblage. The most critical parameter to define the grazing vulnerability of a given bacteria is its cell size (González *et al.*, 1990), but other factors such as cell viability (Landry *et al.*, 1991), surface properties (Matz and Jürgens, 2001), motility (Matz and Jürgens, 2005), phylogenetic affiliation (Jezbera *et al.*, 2005) or food quality (Shannon *et al.*, 2007) have been also demonstrated. Finally, intrinsic physiological parameters like the functional response (relationship of grazing rates with prey concentration) or the growth efficiency (conversion of ingested food to biomass) might explain adaptations to specific environmental settings. These functional features have been studied on model organisms grown in cultures (Fenchel, 1982; Eccleston-Parry and Leadbeater, 1994; Mohapatra and Fukami, 2004), but it has been suggested that these do not represent the dominant grazers in the sea (Massana *et al.*, 2006a).

Here we present an approach to study the grazing rates and prey preferences of uncultured heterotrophic flagellates (HFs) living in natural assemblages. It is based on the estimation of the feeding activity of specific grazers detected by fluorescent *in situ* hybridization (FISH) after short-term ingestion experiments with tracer preys. As grazers, we targeted two marine stramenopiles (MAST) lineages, each one including a significant and similar phylogenetic diversity (up to 3–4% in the 18S rDNA gene). These protists represent a noticeable fraction of *in situ* HFs, are globally distributed, and are bacterivorous (Massana *et al.*, 2006a), but little is known with respect to their feeding behavior. Besides the commonly used FLB as tracer, we also used live bacteria that were stained after the ingestion by a secondary FISH step. Our combined use of short-term ingestion experiments and double FISH procedure targeting both prey and predators shows that phylogenetic diversity is indeed contributing to functional diversity within marine bacterivorous assemblages.

Materials and methods

Natural microbial assemblages

Surface water from the Blanes Bay Microbial Observatory was taken on 13 June 2006 and carried to the laboratory in less than 2 h. This sample was prefiltered through a 100 μm mesh by an inverse filtration and used to perform a first grazing experiment with the *in situ* assemblage of protistan predators, mostly HFs and mixotrophic algae smaller than 5 μm. Simultaneously, 12 l of seawater was gently filtered by gravity through 3 μm polycarbonate filters and incubated in the dark at *in situ* temperature (20 °C) as explained before (Massana *et al.*, 2006b). After 2 days of unamended incubation, this sample was used to carry out a second grazing experiment with the incubated protistan assemblage.

Counts of bacteria (including heterotrophic bacteria and archaea), *Synechococcus*, HFs and phototrophic flagellates were carried out by epifluorescence microscopy (Porter and Feig, 1980). Glutaraldehyde-fixed aliquots (1% final concentration) were stained with 4′,6-diamidino-2-phenylindole (DAPI; 5 μg μl^{-1}) and filtered on 0.2 (for bacteria) or 0.6 μm (for flagellates) pore size polycarbonate filters. The filters were kept frozen until observed by ultraviolet irradiance and blue light in an Olympus BX61 microscope. Pictures of DAPI-stained bacteria were taken with a digital camera (Spot RT Slider; Diagnostic Instruments Inc., Sterling Heights, MI, USA) and processed with the Image Pro Plus software analyzer (Media Cybernetics Inc., Bethesda, MD, USA) to calculate the biovolume of 100–500 cells after the measured area and perimeter (Massana *et al.*, 1997). Bacterial viability was assessed with the nucleic acid double-staining (NADS) protocol (Grégori *et al.*, 2001) that uses SYBR Green to stain all cells and propidium iodide to stain cells with compromised membranes. Both populations were counted by flow cytometry and cells with intact membranes were considered 'alive' (Falcioni *et al.*, 2008).

Specific protist taxa were detected by FISH. Aliquots were fixed with formaldehyde (3.7% final concentration), filtered on 0.6 μm pore size polycarbonate filters and kept frozen until processed. Oligonucleotide probes for five MAST groups were used, NS4 for MAST-4 (Massana *et al.*, 2002), NS1A for MAST-1A, NS1B for MAST-1B, NS1C for MAST-1C and NS2 for MAST-2 (Massana *et al.*, 2006a), together with the general eukaryotic probe Euk502 (Lim *et al.*, 1999). Probes labeled with the fluorescent dye CY3 at the 5′ end were supplied by Thermo Electron Corporation (Waltham, MA, USA). For FISH we followed the protocol and conditions detailed previously (Pernthaler *et al.*, 2001; Massana *et al.*, 2006a). Briefly, filter portions with protist cells were hybridized for 3 h at 46 °C in the appropriate buffer (with 30% formamide), washed at 48 °C in a second buffer, counter-stained with DAPI and mounted in a slide. Cells were then observed by epifluorescence microscopy under green light excitation.

Bacterial strains as prey

Brevundimonas diminuta (syn. *Pseudomonas diminuta*; *Caulobacteraceae*, α-*Proteobacteria*) was

obtained from Colección Española de Cultivos Tipo (Valencia, Spain), grown in Luria-Bertrani agar plates and used to prepare FLB (Sherr *et al.*, 1987). Two-week-old colonies were scraped, diluted in carbonate–bicarbonate buffer, stained with 5-[4,6-dichlorotriazinyl]aminofluorescein for 2 h at 60 °C, kept at −20 °C, and thawed and sonicated before use as explained before (Unrein *et al.*, 2007). Strains MED479 (*Nereida* sp., *Roseobacter* lineage, *Rhodobacteraceae*, α-*Proteobacteria*) and MED134 (*Dokdonia* sp., *Flavobacteriaceae*, *Bacteroidetes*) were isolated in 2003 from the Blanes Bay Microbial Observatory in Zobell agar plates and kept since then in glycerol frozen stocks (Lekunberri *et al.*, submitted). Before the experiments, cells were grown on agar plates with Marine Broth 2216 (Difco, Lawrence, KS, USA) and then regrown in diluted (1:10) liquid Marine Broth (MED479) or in filtered autoclaved seawater (MED134).

Complete 16S rDNA sequences of MED479 (FJ482233) and MED134 (DQ481462) were imported to ARB (http://www.arb-home.de) to design specific oligonucleotide probes: NER380 (5′-GCATCGCTA GATCAGGGTTT-3′; *Escherichia coli* positions 381–400), and DOK196 (5′-TCTTATACCGCCGAAACT-3′; *E. coli* positions 197–228). Besides MED479, probe NER380 targets 22 GenBank entries of uncultured marine bacteria (including one clone from Blanes Bay) and five cultured strains (*Nereida ignava*, an isolate from the surface microlayer, and three other Blanes Bay strains). Besides MED134, probe DOK196 targets 27 uncultured marine clones and 28 cultured strains (5 *Dokdonia* sp., 5 *Krokinobacter* sp., 1 *Flexibacter* sp., 2 isolates from marine sponges and 15 from coastal bacterioplankton). Probes were supplied by Thermo Electron Corporation with an aminolink (C6) at the 5′ end, ligated with a horseradish peroxidase enzyme (Urdea *et al.*, 1988), and then optimized for catalyzed reporter deposition (CARD)-FISH (Pernthaler *et al.*, 2002) with bacterial cells (fixed and filtered as before) from the target culture. Filter pieces were permeabilized with lysozyme and achromopeptidase before hybridizing overnight at 35 °C in a buffer containing 30% formamide (which gave the best results after trying a range from 10% to 60%). After hybridization the signal was amplified with Alexa 488-labeled tyramide and counter-stained with DAPI. Filter pieces were mounted on a slide and observed by epifluorescence microscopy under blue light excitation.

Grazing experiments

Samples with the natural assemblages of bacteria and protists were acclimated in a large container (>10 l) for 2–4 h at *in situ* temperature (20 °C) and mean light intensity in the water column (200 μmol m^{-2} s^{-1}). Several 2-l bottles were filled-up, inoculated with a different bacterial suspension added at tracer concentrations (ca 15% of total bacteria), and dispensed into three 0.5-l bottles (triplicates). *In situ* abundance of strains MED479 and MED134 was checked. At time 0 and after 40 min of incubation, aliquots for DAPI-stained microbial counts and FISH analysis were taken as before, with the exception that fixation was carried out with an equal volume of diluted fixative to reduce cell egestion (Sieracki *et al.*, 1987) and that the final glutaraldehyde concentration was 2%. The incubation time (40 min) was chosen based on a previous time series that showed a plateau in the number of ingested bacteria at 45 min (Unrein *et al.*, 2007). In one case, grazing was estimated by counting FLB inside HFs in the DAPI-stained samples. In all other cases, counting ingestion involved a FISH step targeting specific predators. A single FISH step sufficed when using FLB as tracer, but an additional CARD-FISH hybridization was required to assess the ingestion of MED479 and MED134 cells that were offered alive and unstained. Optimal signals were obtained when CARD-FISH for bacteria was carried out first, followed by FISH for protists, according to the protocols explained above.

After the single or dual FISH procedures, the filter was inspected by epifluorescence at 1000 × under green light excitation to detect positive predator cells. When one was detected, the excitation was changed to blue light to count the tracer items ingested. On average, 125 predator cells were observed for each data point. The average number of tracer bacteria per predator was estimated for the initial sample (I_0) and the sample at 40 min (I_{40}), and used to calculate clearance rates (CRs: nl per predator per h) according to

$$CR = (I_{40} - I_0) \times (60/40) \times 1/(T)$$

where (T) represents the tracer prey concentration (in cells per nl). CRs were converted to ingestion rates (bacteria per predator per h) by multiplying by the bacterial concentration (native plus tracer cells, in cells per nl), assuming that both bacterial types were ingested at similar rates. Each grazing rate estimate (three replicate bottles × two times × two separate hybridizations) represents approximately 12 h of microscopy.

Results

To infer grazing rates of uncultured protist taxa, ingestion experiments were carried out with two starting microbial assemblages, the *in situ* assemblage from Blanes Bay and the assemblage resulting after 2 days of unamended dark incubation (Table 1). During this incubation, bacteria initially increased (Figure 1a) and then decreased concomitantly with the growth of HFs (Figure 1b). The protistan assemblage changed from one dominated by phototrophic cells (78%) to one dominated by heterotrophic cells (84%). Specific HF taxa belonging to different MAST lineages were quantified by FISH along the incubation. Two MAST groups (-4 and -1C) had abundances high enough (Table 1) to allow

Table 1 Microbial counts and bacterial parameters of the *in situ* (Blanes Bay, 13 June 2006) and incubated (unamended dark incubation for 2 days) samples used in the short-term ingestion experiments

	In situ *sample*	*Incubated sample*
Counts by DAPI staining (cells per ml)		
Bacteria	1203 582	1714 615
Synechococcus	25 598	14 195
Heterotrophic flagellates $<5\,\mu m$	973	11 868
Phototrophic flagellates $<5\,\mu m$	3421	2327
Counts by FISH (cells per ml)		
MAST-4	70	890
MAST-1C	11	217
MED479 (*Roseobacter* strain)	367	5350
MED134 (flavobacteria strain)	1749	0
Bacterial parameters		
Biovolume in μm^3 (s.e.)	0.099 (0.007)	0.069 (0.001)
Cells alive (%)	86	ND

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescent *in situ* hybridization; MAST, marine stramenopiles; ND, not determined.

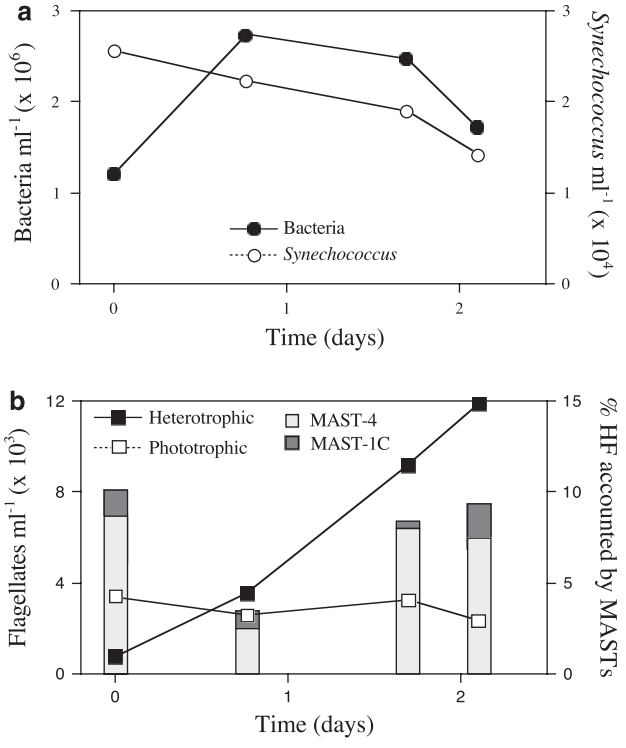

Figure 1 Abundance of bacteria and *Synechococcus* (**a**) and heterotrophic and phototrophic flagellates (**b**) during an unamended dark incubation started with Blanes Bay surface seawater. The percentage of heterotrophic flagellates accounted for marine stramenopile group 4 (MAST-4) and MAST-1C during the incubation is shown in (**b**).

determining their grazing rates, whereas the other three groups examined (-1A, -1B and -2) were too rare for this purpose (less than 20 cells per ml). Note that even though the number of HF increased during incubation, the relative contribution of MAST-4 and MAST-1C to the HF assemblage was maintained (Figure 1b).

The *Roseobacter* (*Nereida* sp. MED479) and the flavobacteria (*Dokdonia* sp. MED134) strains to be used as tracers in ingestion experiments were found at low *in situ* abundance (less than 0.3% of total bacteria; Table 1). The mean cell volume of MED479 and MED134 was 0.21 and 0.26 μm^3 when grown in standard rich media, and after starving for 1 or 2 weeks they reached a volume similar to that of natural bacteria (ca 0.1 μm^3; Tables 1 and 2). Besides getting smaller, the starvation compromised the viability of MED479 cells (only 5–25% of cells kept intact membranes), but not of MED134 cells. The double FISH hybridization we performed to estimate specific protistan grazing gave optimal signal, allowing easy detection of specific ingested bacteria (MED134 or MED479 cells) within specific predators (MAST-4 or MAST-1 cells; Figure 2).

Short-term ingestion experiments using FLB and live MED479 and MED134 (of different cell size) were carried out with the *in situ* sample (experiments 1–5; Table 2), and the incubated sample (experiments 6–8; Table 2). All the experiments yielded grazing rates for the uncultured MAST-4 protists (Figure 3). To compare rates obtained at different prey abundance (especially between *in situ* and incubated samples) we calculated both CRs (volume cleared) and ingestion rates (bacteria ingested), and both estimates gave a similar picture. CRs of MAST-4 were virtually identical in experiments 1 and 6 (Figure 3a; see FLB estimates), resulting in somewhat higher ingestion rates in the incubated sample because of the higher prey abundance (Figure 3c). This suggests that the specific activity of MAST-4 was not artificially stimulated by the incubation. Moreover, important differences in grazing rates were seen when using different tracers (analysis of variance (ANOVA), $F(7, 14) = 4.69$, $P = 0.0068$), with FLB giving lowest clearance and ingestion rates (0.7 nl per predator per h and 1.0 bacteria per predator per h) and MED134 highest rates (1.9 nl per predator per h and 2.9 bacteria per predator per h). These differences could not be explained by the cell size of tracers because rates can vary highly using tracers of similar biovolume (Figures 3a and c). A clear and statistically significant pattern ($R > 0.70$; $P < 0.05$) emerged when relating grazing rates with the percentage of live cells (as determined by NADS) in the tracer (Figures 3b and d). MAST-4 appears to prefer bacteria that are in good physiological condition. Clearance and ingestion rates of the *in situ* HF assemblage (cells $<5\,\mu m$) measured with FLB in experiment 1 (2.1 nl per predator per h and 3.1 bacteria per predator per h) were higher than that of MAST-4, which therefore seemed to be less active than the average HF cell.

In experiments 6–8, another uncultured flagellate (MAST-1C) was relatively abundant and targeting

Table 2 Summary of grazing experiments performed, showing the sample used, the bacterial strains employed as tracer prey (biovolume in μm^3 and s.e. in brackets) and the predators assessed in each experiment

Experiment	*Sample*	*Prey used as tracer*			*Predators*
		Name	*Biovolume*	*% alive*	
1	*In situ*	FLB	0.179 (0.006)	0.0	MAST-4, heterotrophic flagellates
2	*In situ*	MED479	0.080 (0.001)	5.0	MAST-4
3	*In situ*	MED479	0.121 (0.004)	25.2	MAST-4
4	*In situ*	MED134	0.090 (0.005)	79.4	MAST-4
5	*In situ*	MED134	0.119 (0.003)	99.3	MAST-4
6	Incubated	FLB	0.179 (0.006)	0.0	MAST-4, MAST-1C, eukaryotes
7	Incubated	MED479	0.109 (0.003)	6.8	MAST-4, MAST-1C, eukaryotes
8	Incubated	MED134	0.096 (0.004)	94.2	MAST-4, MAST-1C, eukaryotes

Abbreviation: MAST, marine stramenopiles.
FLB refers to fluorescently labeled bacteria, MED479 to the *Roseobacter* strain and MED134 to the flavobacteria strain.

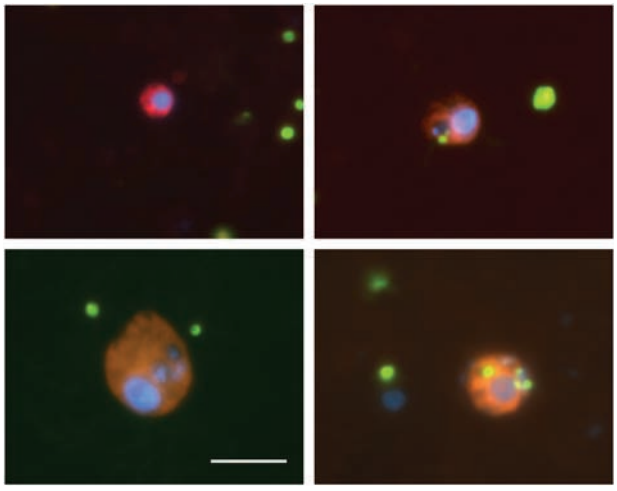

Figure 2 Epifluorescence micrographs of marine stramenopile group 4 (MAST-4; upper panels) and MAST-1C (lower panels) cells at the beginning (left panels) and at the end (right panels) of the ingestion experiment. Each image is an overlay of three pictures of the same cell observed under ultraviolet (UV) radiation (showing the blue nucleus after 4′,6-diamidino-2-phenylindole (DAPI) staining), green light (red cytoplasm after fluorescent *in situ* hybridization (FISH)) and blue light excitation (green MED134 or MED479 cells after catalyzed reporter deposition (CARD)-FISH). Scale bar is 5 μm and applies to all figures.

the assemblage with an eukaryotic probe provided rates mostly attributable to HF cells (Table 1). Grazing rates of these three predators (MAST-4, MAST-1C and eukaryotes) were estimated using different tracers (Figure 4). As these experiments were carried out at similar prey abundance, ingestion rates paralleled CRs and are not shown here. MAST-4 CRs are a subset of those presented in Figure 3, but under another display format, and showed again that live bacteria were preferred over FLB. The pattern observed for the whole eukaryotic community was comparable to that of MAST-4, with MED134 giving highest rates, although community rates were generally higher than MAST-4 rates, as observed in the *in situ* sample. On the other hand, MAST-1C deviated clearly from this picture, with FLB yielding the highest CR (with a value 2.5 times higher than that of MAST-4), whereas live bacteria were ingested at much lower rates, especially MED134 that was almost not ingested. All predator groups showed a significant difference between rates obtained with FLB and MED134 (ANOVA, *post hoc* Fisher's least significant difference test, $P<0.05$). Clearly, the food preferences of MAST-4 and MAST-1C were distinct and the latter did not behave as the average HF cell in the assemblage.

Finally, we did additional experiments with the incubated sample using other cell suspensions as tracers (data not shown). We employed MED134 cells that were damaged by heating the culture at 60 °C for 1 h. Cell viability was compromised (70% of cells lost membrane integrity), but cells preserved their shape and size and were readily detected by CARD-FISH. To our surprise, we did not detect any ingestion by any of the predators investigated (that is, MAST-4, MAST-1C or eukaryotes) when using these dead MED134 cells as tracers. We also used cultures of *Micromonas pusilla* and *Ostreococcus* sp. in other experiments, applying CARD-FISH with probes MICRO01 and OSTREO01 (Not *et al.*, 2004) to detect ingestion of these picoprasinophytes. The results are not formally presented because these cells were added at unrealistically high abundance (10^4–10^5 cells per ml, whereas *in situ* abundance was 10^2–10^3 cells per ml) but they were still too scarce to obtain robust ingestion data (not enough ingestion cases were seen). Nevertheless, the experiments suggest that the two picoalgae were readily ingested by eukaryotes, MAST-4 and MAST-1C at CRs similar to the highest rates measured with bacterial tracers.

Discussion

Experimental settings

The aim of this study was to estimate *in situ* grazing rates of specific taxa of uncultured HFs and to study the putative prey preference (that is, functional diversity) among these taxa. To address our first objective we performed short-term ingestion

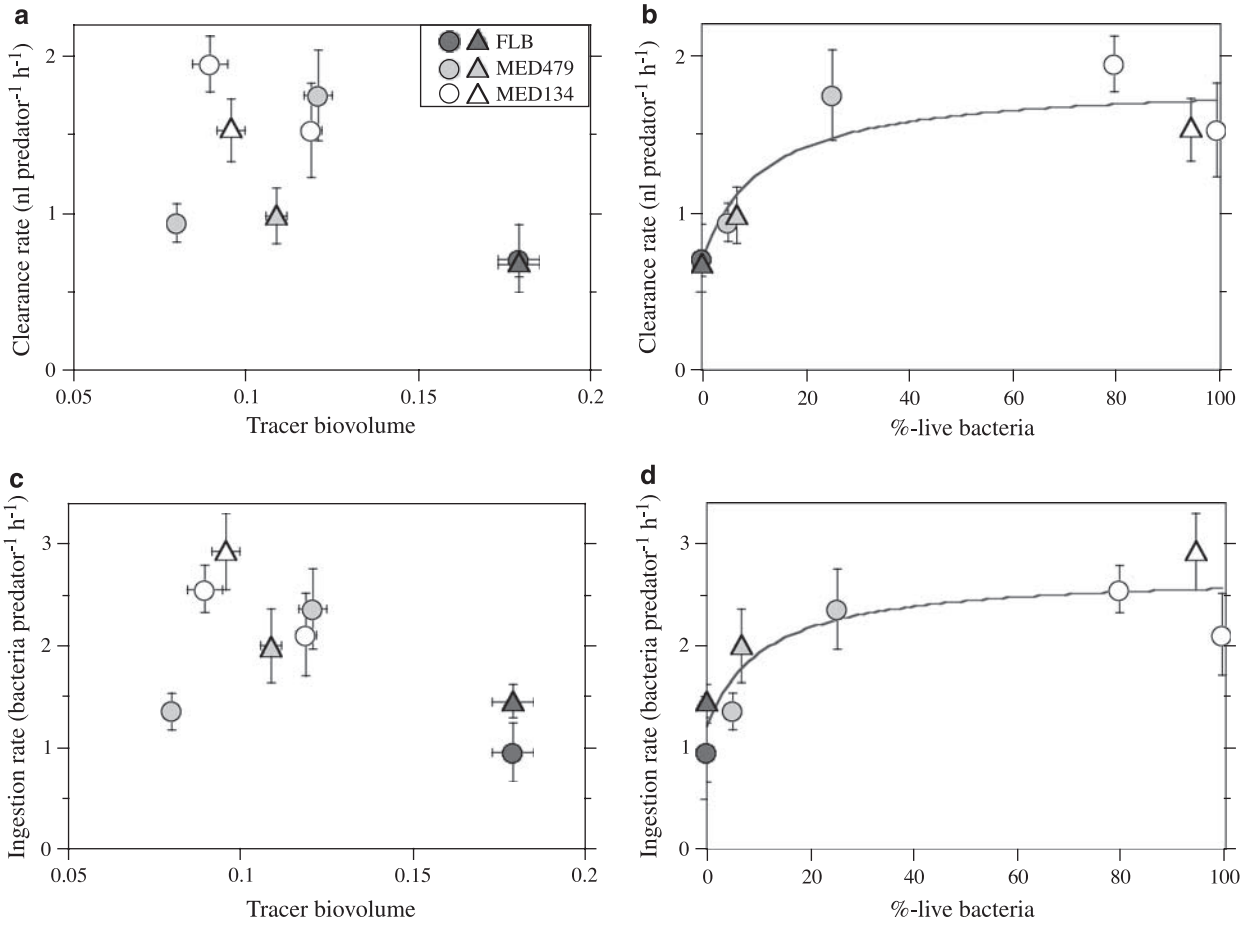

Figure 3 Clearance rates (**a**, **b**) and ingestion rates (**c**, **d**) of marine stramenopile group 4 (MAST-4) cells plotted against the biovolume (**a**, **c**) and the percentage of nucleic acid double-staining (NADS)-positive 'live' cells (**b**, **d**) of the bacterial tracer used in each of the eight independent experiments (circles when carried out with the *in situ* sample; triangles with the incubated sample). FLB refers to fluorescently labeled bacteria, MED479 to the *Roseobacter* strain and MED134 to the flavobacteria strain. A hyperbolic fit (Michaelis–Menten equation with an initial constant) was applied in (**b**) and (**d**). Bars represent standard errors.

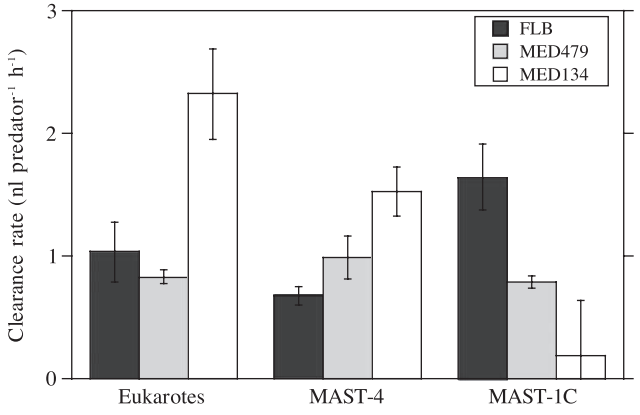

Figure 4 Clearance rates of the eukaryotic assemblage (mostly heterotrophic flagellate (HF) cells) and the specific marine stramenopile group 4 (MAST-4) and MAST-1C taxa in the incubated sample estimated with three different bacterial tracer preys: fluorescently labeled bacteria (FLB), MED479 (the *Roseobacter* strain) and MED134 (the flavobacteria strain). Bars represent standard errors.

experiments with natural microbial assemblages and applied FISH to measure the feeding rates of specific taxa. In addition to the *in situ* sample we analyzed a sample from an unamended incubation (Massana *et al.*, 2006b), which promote the growth of uncultured HF and therefore increase the chances of finding specific predators. For the second objective, besides the standard FLB, we used live bacteria that affiliate to the well-represented marine groups *Roseobacter* and *Flavobacteriaceae* (Kirchman, 2002; Buchan *et al.*, 2005; Alonso-Sáez *et al.*, 2007). The strains we used as tracers were isolated from the sampling point (Blanes Bay) and were too scarce in the original sample to interfere with the grazing experiments. We starved the bacterial cultures to reach a cell size comparable to that of natural bacteria, and assessed if this was accompanied by changes in other cell properties, such as membrane integrity. Also, we developed a dual FISH method to target simultaneously the predators and the ingested prey. This methodological setup led to a reliable protocol to estimate grazing rates and prey preferences of uncultured HF taxa on specific live bacteria. Until now, grazing rates and prey preferences were only known for cultured HF taxa under controlled laboratory conditions (Eccleston-Parry and Leadbeater, 1994; Mohapatra

and Fukami, 2004; Shannon *et al.*, 2007). Prey preference has been recently studied using FISH against specific bacterial prey within food vacuoles (Jezbera *et al.*, 2005), but this approach could not provide concurrently grazing rates nor specific activity for particular grazers.

Grazing rates of uncultured flagellates

We obtained *in situ* grazing rates of the uncultured HF taxa MAST-4 and MAST-1C. These have been detected only in molecular surveys (18S rDNA sequences and FISH-targeted cells) and are important members of marine assemblages, accounting for 9.2% and 2.7% of HF cells globally (Massana *et al.*, 2006a). Using the classical FLB procedure, clearance and ingestion rates for MAST-4 were 0.7 nl per predator per h and 1.0–1.5 bacteria per predator per h, and rates for MAST-1C were 1.6 nl per predator per h and 3.6 bacteria per predator per h. As their functional responses are still unknown, these values likely underestimate maximal CRs (if half-saturation constant (km) is low relative to actual bacterial abundances), or maximal ingestion rates (if km is high). Comparing these rates with those from the whole HF assemblage, it appears that MAST-4 is less active and MAST-1C more active than the average HF cell. This is consistent with a larger cell size of MAST-1C than MAST-4 (5.6 and 3.3 μm diameter on average in these samples). The specific grazing rates of these MAST taxa are comparable to *in situ* rates measured in Blanes Bay (Unrein *et al.*, 2007) and worldwide (Vaqué *et al.*, 1994), but much lower than most estimates derived from cultured strains. Indeed, maximal CRs from cultured HF strains range from 1 to 58 nl per predator per h, and maximal ingestion rates range from 5 to 259 bacteria per predator per h (Eccleston-Parry and Leadbeater, 1994). So, these cultured HF could be poor models of natural and dominant HF taxa.

Comparing FLB and live bacteria in ingestion experiments with MAST-4

Several studies have analyzed the effect of using dead bacteria as tracers in ingestion experiments. These generally show that using live bacteria result in grazing rates significantly higher than FLB (Landry *et al.*, 1991; Boenigk *et al.*, 2001). Differences can also be seen when comparing growing versus starving bacteria, the first being preferentially consumed (González *et al.*, 1993). Our results fit well with this general picture, and higher grazing rates of MAST-4 were obtained when using live bacteria over FLB (2–3 times higher). Moreover, MAST-4 grazing rates could be plotted to respect cell viability of the tracer suspensions, with maximal values being reached from ca 20% of live cells. An extreme case of negative selection is shown by the experiments using heat-killed MED134 cells. Those were not ingested at all, and the underlying mechanism for this prey avoidance is unknown. Finally, besides differences related to cell viability, no other differences in measured grazing rates were seen when using the two bacterial strains, even though they belong to distant phylogenetic groups with different life strategies. Members of the *Roseobacter* lineage tend to be free-living bacteria and typical of somewhat rich conditions (Buchan *et al.*, 2005), whereas flavobacteria tend to be particle-associated bacteria with high exoenzymatic activity (Kirchman, 2002). In our study, *Roseobacter* and flavobacteria cells were ingested equally by MAST-4, so these important differences in phylogeny and life strategy did not determine prey preference.

Functional differences between MAST-4 and MAST-1C

There were clear functional differences between these two taxa. MAST-4 cells preferred live prey and somehow represented the average *in situ* HF. MAST-1C cells, on the other hand, behaved very differently and the dead FLB yielded the highest rates. Most strikingly, the flavobacteria MED134 were almost not ingested by MAST-1C, which was unexpected because these bacterial cells showed the best physiological state and, in the same bottle (experiment 8), gave highest rates for MAST-4 and eukaryotes. A possible explanation would be that MAST-1C does not like this particular strain as food, opening an interesting and complex scenario of specific trophic interactions for some flagellates (but not for others). However, a more plausible explanation would be that the boundary of optimal prey size for MAST-1C falls within the size range of the tested bacteria. MED134, being the smallest of the bacteria tested, could be outside the size range of edible bacteria and escape predation. This could also explain the moderate rates measured with MED479 and the highest rates with the largest FLB. The fact that MAST-1C could be adapted to feed on larger bacteria than MAST-4 is consistent with its larger size, following the established relationship between predator and prey size (Fenchel, 1987). Our results clearly show functional differences between MAST-4 and MAST-1C, but the underlying mechanisms for such differences remain to be elucidated. The functional diversity we observed gives an ecological meaning to the high phylogenetic diversity of marine heterotrophic protists (Vaulot *et al.*, 2002).

Our study opens up the black box of bacterivory in marine ecosystems by showing different specific activity and prey preferences in distinct uncultured taxa. If our interpretation is correct, cell size was the main factor in prey vulnerability, as commonly accepted (González *et al.*, 1990), and there were sharp size boundaries outside which preys could not be ingested (Fenchel, 1987; Jürgens and Matz, 2002). Secondarily, when all preys fell within the edible size range then other factors interplayed with a less dramatic impact. For instance, MAST-4

preferred live bacteria in good physiological state, but still fed on dead FLB at one third of the maximal rate. Our data did not reveal differential feeding behavior related to the phylogenetic affiliation of the tested bacteria. This study assigned differential functional roles to distinct uncultured HF taxa, effectively linking phylogenetic and functional diversity within a natural assemblage. Our combination of FISH for specific predators with the use of live bacteria as prey surrogates allows addressing the huge complexity of microbial food webs.

Acknowledgements

This study was supported by the project ESTRAMAR (CTM2004-12631/MAR, MEC) to RM. FN was supported by the Marie-Curie fellowship ESUMAST (MEIF-CT-2005-025000) and TL by the project METAOCEANS (MEST-CT-2005-019678). We thank Josep M Gasol for help in flow cytometry, Matthias Engel for microscopic counts and Marta Ribes for statistical advice.

References

Alonso-Sáez L, Balagué V, Sà EL, Sánchez O, González JM, Pinhassi J *et al.* (2007). Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. *FEMS Microbiol Ecol* **60**: 98–112.

Boenigk J, Arndt H, Cleven EJ. (2001). The problematic nature of fluorescently labeled bacteria (FLB) in *Spumella* feeding experiments—an explanation by using video microscopy. *Arch Hydrobiol* **152**: 329–338.

Boenigk J, Jost S, Stoeck T, Garstecki T. (2007). Differential thermal adaptation of clonal strains of a protist morphospecies originating from different climatic zones. *Environ Microbiol* **9**: 593–602.

Buchan A, González JM, Moran MA. (2005). Overview of the marine *Roseobacter* lineage. *Appl Environ Microbiol* **71**: 5665–5677.

Eccleston-Parry JD, Leadbeater BSC. (1994). A comparison of the growth kinetics of six marine heterotrophic nanoflagellates fed with one bacterial species. *Mar Ecol Prog Ser* **105**: 167–177.

Epstein S, López-García P. (2008). 'Missing' protists: a molecular prospective. *Biodivers Conserv* **17**: 261–276.

Falcioni T, Papa S, Gasol JM. (2008). Evaluating the flow-cytometric nucleic acid double-staining protocol in realistic situations of planktonic bacterial death. *Appl Environ Microbiol* **74**: 1767–1779.

Fenchel T. (1982). Ecology of heterotrophic microflagellates. II. Bioenergetics and growth. *Mar Ecol Prog Ser* **8**: 225–231.

Fenchel T. (1987). *Ecology of Protozoa: The Biology of Free-Living Phagotrophic Protists*. Brock/Springer Series in Contemporary Bioscience, Springer: Berlin.

González JM, Sherr EB, Sherr BF. (1990). Size-selective grazing on bacteria by natural assemblages of estuarine flagellates and ciliates. *Appl Environ Microbiol* **56**: 583–589.

González JM, Sherr EB, Sherr BF. (1993). Differential feeding by marine flagellates on growing versus starving, and on motile versus nonmotile, bacterial prey. *Mar Ecol Prog Ser* **102**: 257–267.

Grégori G, Citterio S, Ghiani A, Labra M, Sgorbati S, Brown S *et al.* (2001). Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining. *Appl Environ Microbiol* **67**: 4662–4670.

Jezbera J, Hornak K, Simek K. (2005). Food selection by bacterivorous protists: insight from the analysis of the food vacuole content by means of fluorescence *in situ* hybridization. *FEMS Microbiol Ecol* **52**: 351–363.

Jürgens K, Matz C. (2002). Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. *Antonie van Leeuwenhoek* **81**: 413–434.

Jürgens K, Massana R. (2008). Protistan grazing on marine bacterioplankton. In: Kirchman DL (ed). *Microbial Ecology of the Oceans*, 2nd edn. Wiley: New York, pp 383–441.

Kirchman DL. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**: 91–100.

Landry MR, Lehner-Fournier JM, Sundstrom JA, Fagerness VL, Selph KE. (1991). Discrimination between living and heat-killed prey by a marine zooflagellate *Paraphysomonas vestita* (Stokes). *J Exp Mar Biol Ecol* **146**: 139–152.

Lim EL, Dennet MR, Caron DA. (1999). The ecology of *Paraphysomonas imperforata* based on studies employing oligonucleotide probe identification in coastal water samples and enrichment cultures. *Limnol Oceanogr* **44**: 37–51.

Massana R, Gasol JM, Bjørnsen PK, Blackburn N, Hagström Å, Hietanen S *et al.* (1997). Measurement of bacterial size via image analysis of epifluorescence preparations: description of an inexpensive system and solutions to some of the most common problems. *Sci Mar* **61**: 397–407.

Massana R, Guillou L, Díez B, Pedrós-Alió C. (2002). Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. *Appl Environ Microbiol* **68**: 4554–4558.

Massana R, Terrado R, Forn I, Lovejoy C, Pedrós-Alió C. (2006a). Distribution and abundance of uncultured heterotrophic flagellates in the world oceans. *Environ Microbiol* **8**: 1515–1522.

Massana R, Guillou L, Terrado R, Forn I, Pedrós-Alió C. (2006b). Growth of uncultured heterotrophic flagellates in unamended seawater incubations. *Aquat Microb Ecol* **45**: 171–180.

Matz C, Jürgens K. (2001). Effects of hydrophobic and electrostatic cell surface properties of bacteria on feeding rates of heterotrophic nanoflagellates. *Appl Environ Microbiol* **67**: 814–820.

Matz C, Jürgens K. (2005). High motility reduces grazing mortality of planktonic bacteria. *Appl Environ Microbiol* **71**: 921–929.

Mohapatra BR, Fukami K. (2004). Comparison of the numerical grazing response of two marine heterotrophic nanoflagellates fed with different bacteria. *J Sea Res* **52**: 99–107.

Moon-van der Staay SY, De Wachter R, Vaulot D. (2001). Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* **409**: 607–610.

Not F, Latasa M, Marie D, Cariou T, Vaulot D, Simon N. (2004). A single species, *Micromonas pusilla* (Prasinophyceae), dominates the eukaryotic picoplankton in

the Western English Channel. *Appl Environ Microbiol* **70**: 4064–4072.

Pernthaler A, Pernthaler J, Amann R. (2002). Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Env Microbiol* **68**: 3094–3101.

Pernthaler J, Glöckner FO, Schönhuber W, Amann R. (2001). Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. In: Paul JH (ed). *Marine Microbiology*. Academic Press: London, pp 207–226.

Pernthaler J. (2005). Predation on prokaryotes in the water column and its ecological implications. *Nat Rev Microbiol* **3**: 537–546.

Porter KG, Feig YS. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* **25**: 943–948.

Rodríguez F, Derelle E, Guillou L, Le Gall F, Vaulot D, Moreau H. (2005). Ecotype diversity in the marine picoeukaryote *Ostreococcus* (Chlorophyta, Prasinophyceae). *Environ Microbiol* **7**: 853–859.

Sanders RW, Caron DA, Berninger UG. (1992). Relationships between bacteria and heterotrophic nanoplankton in marine and fresh waters: an inter-ecosystem comparison. *Mar Ecol Prog Ser* **86**: 1–14.

Shannon SP, Chrzanowski TH, Grover JP. (2007). Prey food quality affects flagellate ingestion rates. *Microb Ecol* **53**: 66–73.

Sherr BF, Sherr EB, Fallon RD. (1987). Use of monodispersed, fluorescently labeled bacteria to estimate *in situ* protozoan bacterivory. *Appl Environ Microbiol* **53**: 958–965.

Sherr EB, Sherr BF. (2002). Significance of predation by protists in aquatic microbial food webs. *Antonie van Leeuwenhoek* **81**: 293–308.

Sieracki ME, Haas LW, Caron DA, Lessard EJ. (1987). Effect of fixation on particle retention by microflagellates: underestimation of grazing rates. *Mar Ecol Prog Ser* **38**: 251–258.

Simek K, Jezbera J, Hornák K, Vrba J, Sed′a J. (2004). Role of diatom-attached choanoflagellate of the genus *Salpingoeca* as pelagic bacterivores. *Aquat Microb Ecol* **36**: 257–269.

Strom SL. (2000). Bacterivory: interactions between bacteria and their grazers. In: Kirchman DL (ed). *Microbial Ecology of the Oceans*. Wiley-Liss Inc: New York, pp 351–386.

Unrein F, Massana R, Alonso-Sáez L, Gasol JM. (2007). Significant year-round effect of small mixotrophic flagellates on bacterioplankton in an oligotrophic coastal system. *Limnol Oceanogr* **52**: 456–469.

Urdea MS, Warner BD, Running JA, Stempien M, Clyne J, Horn T. (1988). A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. *Nucleic Acids Res* **16**: 4937–4956.

Vaqué D, Gasol JM, Marrasé C. (1994). Grazing rates on bacteria: the significance of methodology and ecological factors. *Mar Ecol Prog Ser* **109**: 263–274.

Vaulot D, Romari K, Not F. (2002). Are autotrophs less diverse than heterotrophs in marine picoplankton? *Trends Microbiol* **10**: 266–267.