Dominant marine heterotrophic flagellates are adapted to natural planktonic bacterial abundances

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Summary
Grazing controls bacterial abundances and composition in many ecosystems. In marine systems, heterotrophic flagellates (HFs) are important predators. Assemblages of HFs are primarily formed by species still uncultured; therefore, many aspects of their trophic behaviour are poorly known. Here, we assessed the functional response of the whole assemblage and of four taxa grown in an unamended seawater incubation. We used fluorescently labelled bacteria to create a prey gradient of two orders of magnitude in abundance and estimated ingestion rates. Natural HFs had a half-saturation constant of $6.7 \times 10^5$ prey ml$^{-1}$, a value lower than that of cultured flagellates and within the range of marine planktonic bacterial abundances. *Minorisa minuta* was well adapted to low prey abundances and very efficient in ingesting bacteria. MAST-4 and MAST-7 were also well adapted to the typical marine abundances but less voracious. In contrast, *Paraphysomonas imperforata*, a typical cultured species, did not achieve ingestion rate saturation even at the highest prey concentration assayed. Our study, beside to set the basis for the fundamental differences between cultured and uncultured bacterial grazers, indicate that the examined predator taxa have different functional responses, suggesting that they occupy distinct ecological niches according to their grazing strategies and prey preferences.

Introduction
Heterotrophic flagellates (HFs) are colourless small protists that are active predators of aquatic bacteria (Sherr and Sherr, 2002). Grazing by HFs controls bacterial abundance and diversity in a wide range of ecosystem conditions, channels organic carbon to higher trophic levels, and releases inorganic nutrients that controls regenerated primary production (Fuhrman and Noble, 1995; Šimek et al., 2001; Pernthaler, 2005). In fact, HFs are central in the microbial loop concept (Azam et al., 1983). Grazing rates of natural HF assemblages are estimated using tracer techniques that follow the fate of an added bacterial surrogate or by manipulation techniques that uncouple predators and preys (Vaqué et al., 1994; Strom, 2000; Jürgens and Massana, 2008). Community grazing rates may be then used to calculate growth rates of HFs (Fenchel, 1987) and to evaluate their contribution to bacterial mortality. However, these rates average the activities of the different taxa in the community, each one perhaps having different grazing rates and prey preferences. Indeed, molecular surveys have unveiled a large diversity of marine protists assemblages (de Vargas et al., 2015; Massana et al., 2015), including HFs (Jürgens and Massana, 2008; Logares et al., 2012). Recent studies conducted mostly in freshwater systems have been dealing with characterizing the growth rates of major bacterivorous taxa (Grujicic et al., 2018; Šimek et al., 2018, 2020). So, for a better understanding of the bacterial grazing and its impact on microbial food web structure, it is still necessary to investigate the physiological parameters of the dominant marine HF taxa (Piwosz et al., 2021).

Grazing rates directly depend on prey abundance. This dependence, named functional response, has been determined in a variety of marine and freshwater predators (Weisse et al., 2016), including copepods (Henriksen et al., 2007; Isari and Saiz, 2011), dinoflagellates (Kim and Jeong, 2004; Jeong et al., 2005; Roberts et al., 2011), ciliates (Jonsson, 1986; Jürgens and Šimek, 2000; Gismervik, 2005; Lu et al., 2021) and heterotrophic
flagellates (Jeong et al., 2008). Functional responses can be fitted to different mathematical models (Holling, 1959), being the most popular among ecologists the equivalent to the enzyme kinetic model developed in 1913 by Leonor Michaelis and Maude Menten. This model includes two parameters, the maximum ingestion rate (\( IR_{\text{max}} \)), determined by the capacity of the predator to capture, handle and digest preys, and the half-saturation constant (\( K_s \): prey concentration that allows half \( IR_{\text{max}} \)), which is a proxy of the prey abundance at which the predator is adapted to live (Fenchel, 1980). Only few studies have measured the functional response of small flagellates, due to the difficulty in obtaining grazing rates, while it is more common to measure the numerical response, the relationship between growth rates and prey abundance (Jürgens and Matz, 2002). As the growth efficiency in these predators is considered constant regardless prey abundance, growth rates are proportional to ingestion rates, and the numerical and functional responses have the same form (Fenchel, 1987). Numerical response reports exist for cultured heterotrophic flagellates (Fenchel, 1982a; Eccleston-Parry and Leadbeater, 1994; Mohapatra and Fukami, 2004; Anderson et al., 2011), but it has been suggested that these cultured species may not represent the dominant grazers in the sea, many of which are still uncultured (Massana et al., 2014). A few trophic experiments (grazing rates and prey preferences) have been done with uncultured species (Massana et al., 2009; Piwosz and Pernthaler, 2010; Meira et al., 2018), but it is still unknown if they have a fundamentally different functional response than cultured ones. There is an overall need to increase the effort on functional studies on ecologically relevant organisms, including HF, placed in natural multispecies assemblages under in situ conditions (Caron et al., 2012; Worden et al., 2015; Weis et al., 2016).

The aim of this study is to determine the functional responses of uncultured flagellates living in natural assemblages. We combined short-term ingestion experiments based on counting fluorescently labelled bacteria (FLB) inside protist food vacuoles (Sherr et al., 1987), with specific FISH counts of HF taxa within mixed assemblages. This time-consuming approach is so far the only way to provide specific ingestion rates for individual flagellate taxa. To overcome the typically low in situ abundances of HFs, we carried out this experiment in an unamended seawater incubation known to promote the growth of uncultured HFs (Massana et al., 2006a). By preparing a gradient of FLB abundance, we obtained the functional responses of the whole HF assemblage and of four distinct taxa (MAST-4, MAST-7, Minorisa minuta and Paraphysomonas imperforata). This is the first report of the functional response of uncultured HF taxa and highlights intrinsic features that might explain why they have not been cultured by classical approaches.

**Experimental procedures**

**Enrichment of heterotrophic flagellates by an unamended incubation**

Surface seawater from the Blanes Bay Microbial Observatory (BBMO) was taken on 16 October 2007 and transported to the laboratory in less than 2 h. Six litres of seawater were filtered by gravity through a 200-μm nylon mesh and then through 3-μm pore size polycarbonate filters. The resulting community (bacteria plus eukaryotes ≤ 3 μm) was incubated into a Nalgene polycarbonate bottle at in situ temperature (19°C) in the dark, to prevent the growth of phototrophic cells (Massana et al., 2006a), and sampled daily during 4 days (Fig. 1). Glutaraldehyde fixed aliquots (1% final concentration) were stained with 4,6-diamidino-2-phenylindole (DAPI; 5 μg ml⁻¹ according to Sieracki et al., 1985) and filtered on 0.2 μm (for bacteria) or 0.6 μm (for flagellates) pore size black polycarbonate filters (DHI Lab Products). Counts of heterotrophic bacteria (including archaea), Synnechococcus, and phototrophic (PF) and heterotrophic flagellates (HF) were carried out in an Olympus BX61 microscope at 1000× magnification using UV irradiance (DAPI-stained DNA signal) and blue light (chlorophyll signal) (Porter and Feig, 1980). Samples for FISH were taken daily by filtering formaldehyde fixed aliquots (3.7% final concentration) on 0.8 or 1-μm pore size polycarbonate filters, which were then kept at −80°C. A sample for DNA extraction was taken at day 3 of the incubation by filtering 100 ml onto a 0.2-μm pore size Durapore.

**Detection of possible predators by clone library and FISH**

DNA extraction was done by using lysozyme, proteinase K and SDS for cell lysis, phenol:chloroform:isoamyl alcohol for DNA extraction, and a Centricon-100 (Millipore) for DNA purification (Massana et al., 2000). Two nanogram of the DNA extract was added to a PCR mixture (50 μl) containing 0.5 μM of each primer, 200 μM of dNTP, 1.5 mM MgCl₂, and 1.25 units of a Taq DNA polymerase (Promega). We used eukaryotic 18S rDNA primers 528F (Elwood et al., 1985) and EUKR (Medlin et al., 1988) and the following PCR cycle: initial denaturation at 94°C for 3 min; 30 cycles with denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension at 72°C for 3 min; and a final extension at 72°C for 10 min. PCR products were purified with the QiAquick PCR Purification kit (Qiagen) and cloned using the TOPO-TA cloning kit (Invitrogen). The presence of correct insert in the bacterial clones was checked by PCR reamplification with the same primers and PCR amplicons were sequenced at the Macrogen sequencing service (Korea). Chimera detection and phylogenetic
affiliation of sequences were obtained by a basic local alignment search tool (BLAST).

Oligonucleotide probes for FISH or CARD-FISH (Table 1) were labelled at the 5′ end with the fluorescent dye CY3 or the enzyme HRP, respectively, and supplied by Thermo Electron Corporation (Waltham, MA, USA). We generally used CARD-FISH except for three probes (NS4, CRN 02 and CET1). For FISH, filter portions were hybridized for 3 h at 46 °C in the appropriate buffer (with 30% formamide) and washed at 48 °C in a second buffer, following the protocol and conditions detailed elsewhere (Pernthaler et al., 2001; Massana et al., 2006b). For CARD-FISH, we followed the protocol and conditions detailed in the study by Pernice et al. (2015). Briefly, filters with protist cells were first embedded in 1% (w/v) low-gelling-point agarose to minimize cell loss. Then, filter portions were hybridized overnight at 35 °C, washed at 37 °C, and tyramide signal amplification was done for 60 min at 46 °C using Alexa 594-labelled tyramide. A final washing step of 1 h with 1:1 ethanol : PBS at room temperature was done to remove background fluorescence. After hybridization, filters were counter-stained with DAPI, mounted in a slide, and cells were observed by epifluorescence microscopy at 1000× under green light excitation. Cell biovolumes were calculated by measuring two dimensions (length and width) in about 100 cells of the target group stained by FISH or CARD-FISH, and applying the prolate spheroid formula (Hillebrand et al. 1999) considering the third dimension (height) as two-thirds of the width.

Fluorescently labelled bacteria used as prey

_Brevundimonas diminuta_ (syn. _Pseudomonas diminuta_; Caulobacteraceae, α-Proteobacteria) was obtained from the Colección Española de Cultivos Tipo (Valencia, Spain), grown in LB agar plates and used to prepare FLBs (Sherr et al., 1987). _B. diminuta_ has already been used to prepare FLB (Vazquez-Dominguez et al., 1999) because of their small size close to that of natural marine bacteria. Two-week-old colonies were scraped, diluted in carbonate–bicarbonate buffer (pH 9.5), and stained with 5-(4,6-dichlorotriazinyl)-aminofluorescein (DTAF; 100 pg ml⁻¹) for 2 h in a water bath at 60°C. Stained cells were centrifuged five times (10 min, 10 000 rpm) and resuspended in 0.2 μm-filtered carbonate–bicarbonate buffer to prevent the transfer of leftover dye to experimental samples. Cell suspensions (average cell biovolume
Table 1. Probes used to visualize specific taxa within the unamended seawater incubation by FISH or CARD-FISH.

| Species | Group | References | Group | Species | Group | References |
|---------|-------|------------|-------|---------|-------|------------|
| Minorisa minuta | Chlorarachniophyta | del Campo et al. (2002) | | Paraphysomonas imperforata | Chrysophyta | Massana et al. (2009) |
| Caecitellus parvulus, C. paraparvulus | Bicosoecida | Massana et al. (2009) | | Bathymonas sp. | Raphidophyta | Giner et al. (2006) |
| Chlorarachniophyta | Chlorarachniophyta | Massana et al. (2006) | | Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) |
| Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) | | Stramenopiles | Stramenopiles | Massana et al. (2006) |
| Stramenopiles | Stramenopiles | Massana et al. (2006) | | Rhodomonas sp. | Rhodomonas | Massana et al. (2006) |
| Rhodomonas sp. | Rhodomonas | Massana et al. (2006) | | Haptophyta | Haptophyta | Massana et al. (2006) |
| Haptophyta | Haptophyta | Massana et al. (2006) | | Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) |
| Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) | | Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) |
| Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) | | Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) |
| Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) | | Phaeocystis antarctica, P. antarctica | Phaeocystis | Massana et al. (2006) |

IR (ingestion rate) = (I_{40} - I_0) \times (100/40)

Data for IR and prey abundance were fitted by iteration to the hyperbolic Michaelis–Menten equation:

IR = N \times IR_{max}/(KS + N)
where $I_{R_{\text{max}}}$ is the maximum ingestion rate, $N$ is the prey concentration (prey ml$^{-1}$) and $K_s$ is the half-saturation constant (prey ml$^{-1}$). To fit this model, we used the R package ‘drc’ (analysis of dose–response curves) (Ritz et al., 2015) with functions ‘ drm ’ and ‘nls’. Results were visualized using the ggplot2 package for R (Gómez-Rubio, 2017). Growth efficiency was calculated as the percentage of protist biovolume produced ($\mu \times$ predator biovolume) to the bacteria biovolume ingested ($I_{R_{\text{max}}} \times$ prey biovolume).

Results
The grazing experiment was done with a natural community incubated for 3 days in the dark, in which predators from higher trophic levels had been filtered out and the growth of bacterivorous HFs was promoted. During this unamended incubation in situ bacterial abundance ($5.9 \times 10^5$ cells ml$^{-1}$) increased to $1.1 \times 10^6$ cells ml$^{-1}$ and in situ HF abundance (804 cells ml$^{-1}$) increased to 6200 cells ml$^{-1}$, being in exponential growth at the moment of the experiment (Fig. 2A). The cell abundance of photosynthetic flagellates and Synechococcus decreased continuously during the incubation (Fig. 2A), as a result of the inhibition of the photosynthesis during the dark incubation and perhaps of grazing mortality. Short-term ingestion experiments with the HF-enriched assemblage were prepared along a gradient of prey abundance (native bacteria plus FLB) covering almost two orders of magnitude ($10^5$–$10^7$ preys ml$^{-1}$) in 13 bottles (Fig. 1). To cover properly this gradient, some of the conditions were prepared with diluted samples. The gradient covers the natural marine bacterial abundance, typically around $10^6$.

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cells ml⁻¹. This experimental set-up allowed to measure grazing rates at different prey abundances and therefore estimate the functional responses of the whole natural assemblage and of specific heterotrophic flagellate taxa.

In order to characterize the protist species composition of the assemblage used for the grazing experiment (mostly formed by HFs cells, as shown by microscopic inspections), we did first a simple clone library with a sample taken at the same time of the grazing experiments (day 3). This clone library yielded 44 sequences distributed in 15 phylogenetic groups, being most of them highly similar to environmental sequences from previous marine surveys (Massana et al., 2004). More than half of the clones affiliated to uncultured MAST (Marine Stramenopiles) with similarities with the closest match in GenBank generally at the range 99%–100%: 10 clones to MAST-2, 5 to MAST-8, 4 to MAST-7 and MAST-12, and a single one to MAST-4. Another three clones affiliated to uncultured MALV (Marine Alveolates) I and II (similarities above 99%). Six clones were almost identical to Minorisa minuta, an heterotrophic flagellate basal to Chlorarachniophyceae and cultured in oligotrophic conditions (del Campo et al., 2013). Five clones affiliated to chrysophytes, two highly similar to Paraphysomonas imperforata and P. foraminifera, one belonging to the cultured clade C and two other to the uncultured clades H and I. The single bicoseocidae clone was moderately related to Caecitellus sp. (96.5%). The remaining groups (Haptophyte, Fungi, Cercozoa, and Bolidomonas) presented only one clone.

We then applied a battery of FISH and CARD-FISH probes to directly quantify the presence of these taxa (and others) in our experimental sample. We used 10 FISH probes from uncultured and cultured heterotrophic flagellates available at the moment (Table 1). Four groups appeared at a reasonable abundance at the day 3 of the experiment, which made them suitable for grazing inspections. M. minuta was the most abundant flagellate of the four (782 cells ml⁻¹), followed by MAST-4 (372 cells ml⁻¹), MAST-7 (362 cells ml⁻¹), and P. imperforata (165 cells ml⁻¹). Probe sequences matched perfectly the sequences retrieved in the clone libraries (probes NS4, CRN 02) or with a single mismatch (NS7 and PIMP 663), indicating that these probes targeted properly these populations. Some probes gave none or very little signal, consistent with the absence of the corresponding taxa in clone libraries: MAST-1A, -1B and -1C and Cafeteria burkhardae. Finally, two groups gave inconsistent results, despite the probes matched perfectly the sequences from the sample: MAST-2, the group dominant at the clone library was detected in low cell abundance (22.5 cells ml⁻¹), whereas Caecitellus spp. was not detected at all. After the selection of the four candidates for grazing analysis, they were counted during the unamended incubation to estimate their growth dynamics. The four taxa were in exponential growth at the moment of the experiment (Fig. 2B). The derived growth rates for these taxa ranged from 0.7 to 1.9 day⁻¹ (Table 2).

In the grazing experiments, we calculated the ingestion rate in the different bottles having a gradient of prey abundance, allowing the delineation of the functional response of the grazer (Table 2). Images of FISH-stained cells with ingested FLBs are exemplified in Fig. 3 for the four taxa analysed. We did first this analysis for the community of heterotrophic flagellates (Fig. 4A), just by inspecting DAPI-filters without FISH hybridization. The fit of the Michaelis–Menten curve to the ingestion data yielded a maximum ingestion rate IRₘₐₓ of 2.3 prey HF⁻¹ h⁻¹ and a half-saturation constant Kₛ of 6.7 x 10⁵ prey ml⁻¹. Then, we estimated the functional response of four different heterotrophic flagellate taxa. M. minuta presented an IRₘₐₓ more than double the community rates (Fig. 4B), and a Kₛ only slightly lower. Both parameters were estimated with a high significance. For the MAST-4, the IRₘₐₓ was rather low, only 1.0 prey HF⁻¹ h⁻¹ and the Kₛ was slightly higher than that of the HF community, although in this fit the Kₛ parameter was not significant in the model (Fig. 4C). The situation of the MAST-7 was similar, with an IRₘₐₓ similar to the HF community and a Kₛ also slightly higher than the HF community (Fig. 4D). In this case, the significance of the Kₛ value is low, close to the 0.01 level. Finally, the curve of P. imperfecta was different from the other four, as there was not clear sign of saturation of the ingestion rates along the prey concentration used in this experiment.

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**Table 2.** A summary of specific functional parameters for each HF taxa: growth rates (μ, d⁻¹), biovolume (Size, μm³, mean values), maximum ingestion rates (IRₘₐₓ, prey cell⁻¹ h⁻¹), and half-saturation constant (Kₛ, prey ml⁻¹, 10⁶).

| Organisms      | μ     | Size (μm³) | IRₘₐₓ  | Kₛ     | IRₑₓᵖ   | IR₉₄₄₀ |
|----------------|-------|------------|--------|--------|---------|--------|
| HF             | 0.71  | 8.6        | 2.3    | 0.67   | 1.5     | 6.4    |
| Minorisa minuta| 1.54  | 6.3        | 5.3    | 0.62   | 3.4     | 10.2   |
| MAST-4         | 0.90  | 3.3        | 1.0    | 0.87   | 0.6     | 3.1    |
| MAST-7         | 0.86  | 9.0        | 2.0    | 0.97   | 1.1     | 8.14   |
| P. imperfecta  | 1.95  | 21.2       | 2.1    | 6.10   | 0.3     | 43.5   |

From the functional response, the ingestion rate of the day of the experiment was estimated (IRₑₓᵖ). The last column (IR₉₄₄₀) shows ingestion rates needed to explain the observed growth rates with a growth efficiency of 40%.

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Even though the parameters estimated were not significant, the $K_s$ shown for this species was one order of magnitude higher than that of all the other taxa.

We then evaluated the distribution of ingested FLBs (estimated at time 40 subtracting time 0) within individual cells for the whole community and for the four individual taxa, considering only the six bottles with highest prey abundance (Fig. 5). When considering the maximal number of FLBs ingested, the number was highest for HF (one cell had 15 FLBs), and relatively similar in the other cases: seven in Minorisa, five in MAST-4, 8 in MAST-7, and seven in Paraphysomonas. Except in one case, the majority of cells appeared with any FLB ingested. The percentage of cells without ingestion was highest in Paraphysomonas (82%), followed by MAST-4 (about 70%) and MAST-7 and the HF assemblage (about 60%). As a striking contrast, Minorisa presented only about 20% of cells without ingestion. When observing the ingested cells, in the case of MAST-4 most cells presented only one FLB ingested, while several had two FLBs. In the case of MAST-7 a similar number of cells exhibited one or two ingested FLBs, while for Minorisa the majority of cells presented between two and four ingested FLBs. Paraphysomonas presented few cells with ingestion, but with a constant decrease until seven ingested FLBs per cell. The HF community presented a less marked profile, with many cells having between one and four ingested FLBs.

Finally, we evaluated the fit between the observed growth rates and the measured ingestion rates (Table 2). From the functional response equations and the measured bacterial abundance of the day of the experiment ($1.1 \times 10^6$ prey ml$^{-1}$) we calculated the ingestion rate of that day and used this value to calculate the growth efficiency, which resulted in values unrealistically high. We then calculated which ingestion rate was needed to get a typical growth efficiency of 40% (Table 2).

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A main challenge in microbial ecology is to shed light on the black box approach. Indeed, for decades the abundance and activity of microbial components (such as bacteria, bacterial grazers, picoalgae, etc.), have been studied as bulk properties, ignoring the different capacities and performances of the composing species in the assemblage. A very relevant topic when investigating predation in nature is the shape of the functional or numerical responses, i.e., the changes of grazing rates or growth rates with food abundance (Weisse et al., 2016). These physiological features have been studied in cultured heterotrophic flagellate strains grazing.

Fig. 4. Functional responses (relationship of ingestion rates and prey abundance) of the natural community of heterotrophic flagellates (A), Minorisa minuta (B), MAST-4 (C), MAST-7 (D) and Paraphysomonas imperfecta (E). Ks = half-saturation constant (prey ml⁻¹) and IRmax = maximum ingestion rate (prey cell⁻¹ h⁻¹). Significance of the estimate in the fit: ‘+++’(P ≤ 0.001), ‘++’(P ≤ 0.01), ‘+’(P ≤ 0.05), ‘.’(P ≤ 0.1), ‘’(P ≤ 1).
on bacteria (summarized in Table 3). However, to our knowledge, there are no studies analysing functional responses of natural assemblages, including uncultured species. This was the motivation of our study.

Our grazing experiments were based in short-term incubations adding FLBs, which were then counted inside protist food vacuoles by epifluorescence. This technique has been highly used (Jezbera et al., 2005; Ng and Liu, 2016; Šimek and Sirova, 2019) and is the best to calculate specific ingestion rates for taxonomic classes of protists. Major limitations of this approach are the negative selection against fluorochrome labelled and heat-killed bacteria (Landry et al., 1991; Fu et al., 2003; Massana et al., 2009), and statistical problems in obtaining reliable counts of ingested FLBs at low predator densities (McManus and Okubo, 1991). The first limitation can be solved by using alive monospecific bacteria as food and CARD-FISH detection in food vacuoles (Jezbera et al., 2005; Massana et al., 2009). In the later paper, we found that MAST-4 ingested alive bacteria two to three times faster than FLBs. However, using alive bacteria would have added an extra layer of complexity to our experiment. At any rate, the underestimation because using FLB would be similar along the prey gradient, therefore not affecting the shape of the functional response. To minimize the problem of low predator densities, we did our grazing experiment with a sample enriched in HF. As expected (Massana et al., 2006a), our unamended seawater incubation selected for heterotrophic flagellates abundant in situ, many of them being uncultured MAST taxa, but also including cultured species like M. minuta, which had been cultured mimicking natural conditions (del Campo et al., 2013). Luckily enough, some typical cultured HF also developed in our incubation, highlighting a very interesting and contrasting behaviour.

The growth rate (Table 2) during the incubation of the whole HF assemblage (0.71 day⁻¹) was similar to previous incubations studies (between 0.66 and 1.25 day⁻¹) (Massana et al., 2006a), and faster than typical growth rates of natural assemblages, 0.05–0.50 day⁻¹ (Jürgens and Massana, 2008; Piwosz and Pernthaler, 2010), in accordance with the promotion of growth in the incubation. Moreover, each flagellate taxa presented a slightly higher growth rate than the community rate, particularly for M. minuta (1.54 day⁻¹). Specific growth rates measured here were similar to those from previous studies: M. minuta (1.56 day⁻¹; del Campo et al., 2013) and MAST-4 (0.62 day⁻¹ on average; Massana et al., 2006a). Moreover, growth rates of cultured species in the laboratory were remarkably larger (up to 10 times) than the rates reported in our unamended incubations.

The maximal ingestion rate of M. minuta was several times higher than that of MAST-4 and MAST-7 (Fig. 4). Using FLB as prey surrogates and the concentration range assayed, M. minuta results to be very efficient and well adapted to low prey abundances. The low feeding rates for MAST-4 measured here are consistent with previous estimates, also obtained with FLB, of ingestion rates of 1.0–1.5 bacteria predator⁻¹ h⁻¹ (Massana

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Fig. 5. Percentage of cells within five HF groups having different number of ingested FLBs. These numbers are calculated with all cells observed per group at saturating food abundances (the last six bottles of the gradient).
These rates are about half the ingestion rates of the whole HF assemblage, even though a high expression of genes involved in phagocytosis has been reported for MAST-4 (Labarre et al., 2020). It has been inferred before that MAST-4 biases against heat-killed FLB, preferring alive bacteria in good physiological state (Massana et al., 2009), but this likely holds true for most HF taxa in the assemblage (Landry et al., 1991; Fu et al., 2003). Another explanation for lower ingestion rates in MAST-4 could be that it is adapted to graze on a specific prey, as small *Pelagibacter ubique* detected within MAST-4 cells by single-cell sequencing (Martinez-Garcia et al., 2012), therefore potentially occupying a different ecological niche than *M. minuta*. Finally, *P. imperforata* represents a very contrasting case, consistent with the fact that it is easily cultured feeding on large bacteria at very high densities (Lim et al., 1999). Studies with *Paraphysomonas* species have shown different results, including a strain with relatively low Ks, 1.1 × 10⁶ bacteria ml⁻¹ (Eccleston-Parry and Leadbeater, 1994) and another that ceased to multiply at prey abundances below ~2 × 10⁶ cells ml⁻¹ (Ishigaki and Seligh, 2001). Our data seemed to agree with the later case, since *P. imperforata* is the taxa that grows faster but did not achieve ingestion rate saturation even at the highest prey concentrations assayed (Fig. 4).
these data, we should expect a high Ks, similar to other cultured flagellates (Table 3).

The growth efficiencies calculated from the measured ingestion rates and the observed growth rates were unrealistically high, above 100% in all cases and extremely high in *P. imperforata*, something vitally impossible and out of the range of 30%–60% in previous estimates (Snyder and Hoch, 1996; Zubkov and Sleigh, 2000). This inconsistency is caused by underestimation of the ingestion rates, which can be explained by several reasons: a negative selection against FLBs as previously commented (Massana et al., 2009), an incubation time in ingestion experiments being too close to the plateau (Unrein et al., 2007), or the predation on alternative preys, such as *Synechococcus* and phototrophic flagellates that in fact decreased during the incubation, which could contribute to biomass ingestion and therefore to the growth rates. Our calculations of the ingestion rates needed to explain the growth observed with a GE of 40% provided very realistic estimates, within the range of 2–20 bacteria prey⁻¹ h⁻¹ measured for *in situ* HF assemblages (Jürgens and Massana, 2008), while *P. imperforata* displayed values similar to other cultured flagellates (Table 3).

The most remarkable finding of this work was that the Ks of the functional responses for the whole community and for three flagellate taxa were in the narrow range of 6.2–9.7 × 10⁵ prey ml⁻¹ (Fig. 4). MAST-4 (Rodriguez-Martínez et al., 2012), MAST-7 (Giner et al., 2016) and *M. minuta* (del Campo et al., 2013) represent heterotrophic flagellates that are widely distributed and abundant in natural marine assemblages (Mangot et al., 2018). Interestingly, our data indicate that they are very well adapted to the bacterial abundances of marine planktonic environments, typically around 10⁶ bacteria ml⁻¹ (Fuhrman and Hagström, 2008). For instance, in the oligotrophic coastal system sampled here (Blanes Bay, NW Mediterranean), the averaged bacterial concentration during the last 20 years (monthly sampling) was 0.90 × 10⁶ bacteria ml⁻¹, with a typical bacterial cell size of 0.06 μm³ (Gasol et al., 1995). In contrast, the Ks of cultured heterotrophic flagellates is typically at least one order of magnitude higher, ranging from 0.1 to 4.5 × 10⁷ bacteria ml⁻¹ with only one exception, *Pfiesteria piscicida* (Table 3). These higher Ks of cultured flagellates are the expected values for organisms that grow efficiently in rich media, and at the same time establish an obvious limitation for their development at the prevailing low in situ bacterial abundances. The results shown here for *P. imperforata* agree with this scenario, since this species exhibited a low grazing capacity and no food saturation in the range of prey abundance tested.

In conclusion, we have shown here that the assemblage of heterotrophic flagellates derived from a marine coastal station (BBMO) presents a functional response with a Ks of 6.7 × 10⁶ bacteria ml⁻¹, which is much lower than the Ks of typical cultured flagellates. This indicates that cells of the community are generally well adapted to in situ marine bacterial abundance. Inside this mixed community, there are taxa with different functional responses, therefore delineating ecological niches, perhaps with different predation strategies and prey preferences. We have shown that *Minorisa minuta* is well adapted to low prey abundances (Ks of 6.2 × 10⁵ bacteria ml⁻¹) and is very efficient in ingesting bacteria (IRmax 5.3 prey h⁻¹). MAST-4 is less voracious but is also well adapted to typical planktonic bacterial abundances (Ks of 8.7 × 10⁵ bacteria ml⁻¹). MAST-7 has a IRmax similar to the natural HF community and a Ks slightly higher. In contrast, *Paraphysomonas imperforata* is food limited all along the prey gradient tested, suggesting a general poor performance in natural marine planktonic environments. Our study sets the basis for the fundamental differences between cultured and uncultured bacterial grazers.

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