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Culturing Bias in Marine Heterotrophic Flagellates Analyzed Through Seawater Enrichment Incubations

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Abstract The diversity of heterotrophic flagellates is generally based on cultivated strains, on which ultrastructural, physiological, and molecular studies have been performed. However, the relevance of these cultured strains as models of the dominant heterotrophic flagellates in the marine planktonic environment is unclear. In fact, molecular surveys typically recover novel eukaryotic lineages that have refused cultivation so far. This study was designed to directly address the culturing bias in planktonic marine heterotrophic flagellates. Several microcosms were established adding increasing amounts and sources of organic matter to a confined natural microbial community pre-filtered by 3 μm. Growth dynamics were followed by epifluorescence microscopy and showed the expected higher yield of bacteria and heterotrophic flagellates at increased organic matter additions. Moreover, protist diversity analyzed by molecular tools showed a clear substitution in the community, which differed more and more from the initial sample as the organic matter increased. Within this gradient, there was also an increase of sequences related to cultured organisms as well as a decrease in diversity. Culturing bias is partly explained by the use of organic matter in the isolation process, which drives a shift in the community to conditions closer to laboratory cultures. An intensive culturing effort using alternative isolation methods is necessary to allow the access to the missing heterotrophic flagellates that constitute the abundant and active taxa in marine systems.

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Introduction

Marine heterotrophic flagellates (HF) perform key roles in microbial food webs and global biogeochemical cycles as trophic linkers and nutrient remineralizers [1]. HF are distributed all along the world oceans at concentrations between 10^2 and 10^4 cells ml^{-1}, representing 10–30 % of protist cells in upper marine waters [2]. The majority of HF cells are bacterivorous [3], but may also include dispersal stages of parasites of other marine organisms [4]. The central role of HF in marine ecosystems has fueled a great interest in studying these organisms under controlled conditions in the laboratory [5, 6]. Cultures have been essential for physiological and phylogenetic studies but the ecological relevance of cultured strains is not clear.

Bacterivorous HF have been cultivated using seawater supplemented typically with cereal grains, cerophyll or yeast extract that promote the growth of bacteria as food [7]. When trying to isolate free-living marine HF, it is expectable that this rich media would promote bacteria that are much larger than regular ones, and also at a much higher abundance [8]. This strategy applied to enrichment studies or culturing retrieves mainly the same pool of species such as *Cafeteria* spp., *Paraphysomonas* spp., or *Neobodo* spp. [9–13], which are considered to be generally rare in marine plankton based on cultured independent approaches [2, 14]. In contrast, some of the most abundant and representative HF in the marine

environment refuse cultivation [15, 16] reflecting what has been named as culturing bias: cultured strains from a given system do not necessarily represent their dominant members.

The existence of culturing bias in bacteriology and virology is well known and accepted by the scientific community [17, 18], whereas it has attracted less attention in protistology. There are no published studies where the effect of the quantity and quality of the added substrates on the same microbial community is comprehensively compared. Previous studies have analyzed the dynamics of protists in microcosms [10, 16, 19] revealing a community shift and giving us the first solid clues about the existence of certain bias and the development of HF enrichments. In these studies, the abundance of enriched taxa was very low (sometimes even below detection) in the original samples, based on molecular data. The results of these works encouraged us to carry on a deeper and exhaustive analysis.

The aim of our work was to report the effects of organic matter enrichments on microbial dynamics and community structure of HF (>3 μm). We expected that by increasing organic matter, the enrichment would result in bacterial assemblages more similar to the ones obtained by classical culturing conditions, yielding more HF that are closely related to commonly cultured types. For the unamended incubations (no additions), we would expect HF species similar to the ones dominating in the environment [16]. Several microcosms were prepared with the same microbial inocula, and the dynamics of bacteria and HF were traced by epifluorescence microscopy. The diversity and identity of the HF proliferating in each condition was analyzed by DGGE fingerprinting and 18S rDNA clone libraries. The enrichment effect on the abundance and composition of HF assemblages was clear and consistent with the culturing bias scenario.

Material and Methods

Sampling and Incubations

Water samples from the Blanes Bay Microbial Observatory (NW Mediterranean Sea) were collected 800 m offshore in March 6th, 2006. Surface seawater was filtered by gravity first through a nylon mesh of 200 μm and later through polycarbonate filters (Poretics) of 3 μm pore-size. Five treatments were prepared with two replicas each: Unamended Incubation (O condition), 0.01 and 0.1 % (*w*/*v*) of yeast extract (L and M respectively), 0.1 % (*w*/*v*) of rice extract (R) and 0.1 % (*w*/*v*) of nutrients in known proportion (P). Rice extract was obtained after boiling rice grains, discarding rice grains and lyophilizing the remaining water. The organic matter in treatment P was a mix of glucose, KH_2PO_4 and NH_4Cl in a C/N/P ratio of 106:16:1. For each treatment, 5 L of the 3 μm-filtered seawater were dispensed into 8 L containers and incubated in the dark at 13 °C in a laboratory chamber. Bottles were sampled every day during 8 days. Subsamples for epifluorescence microscopy were fixed with cold glutaraldehyde (1 % final concentration), stained with DAPI and filtered onto 0.2 or 0.6 μm pore-size black polycarbonate filters (Poretics) for enumeration of bacteria and flagellates. Bacterial counts included both free-living and aggregated cells. Subsamples of 100 ml were filtered onto 0.2 μm pore-size Durapore filters, submerged in lysis buffer (40 mM EDTA, 50 mM Tris–HCl and 0.75 M sucrose) and kept frozen at −80 °C until DNA extraction.

Image Analysis of Bacterial Cell Biovolume

Filters for microscopy 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 (Olympus DP72; Olympus Europa GmbH, Hamburg) and processed with the Image Pro Plus software analyzer (Media Cybernetics Inc., Bethesda, MD, USA) to calculate the biovolume of 100–500 free-living cells after the measured area and perimeter [20]. The percentage of bacterial cells forming aggregates was estimated by direct manual counts from the same pictures.

Fingerprint Analysis Using DGGE

Cell lysis was performed by digestion with lysozyme followed by proteinase K and SDS treatments. DNA was purified by phenol/chloroform extraction and concentrated with a Centricon-100 (Millipore, Billerica, MA, USA) as described previously [21]. About 10 ng of extracted DNA was used as template in a PCR in which eukaryotic 18S ribosomal DNA (rDNA)-specific primers Euk1A and Euk516r-GC were used to amplify a fragment approximately 560 bp long. Details of the primers and PCR conditions are explained elsewhere [21]. Denaturing Gradient Gel Electrophoresis (DGGE) was performed with a DGGE-2000 system (CBS Scientific Company, Del Mar, CA, USA) as described previously [22] using described settings [23]. The resulting gel was visualized with UV radiation by using a Fluor-S MultiImager and the MultiAnalyst imaging software (Bio-Rad, Hercules, CA, USA). Digitized DGGE images were analyzed with the Chemidoc software (Bio-Rad, Hercules, CA, USA) to obtain the relative contribution (in percentage) of each DGGE band to the total band signal in each lane of the gel. Bands occupying the same position in the different samples were identified. A matrix was then constructed taking into account the presence and relative intensity of individual bands in each sample. Based on this matrix (arcsin transformed), we produced a Bray–Curtis dissimilarity matrix and samples were grouped based on a dendogram (CLUSTER analysis, PRIMER, Plymouth, UK).

18S rDNA Cloning and Sequencing

PCR was performed with primers EukA and EukB as detailed in 21. The PCR product was used to construct a clone library with a TA cloning kit (Invitrogen, Carlsbad, CA, USA). The presence of an 18S rDNA insert was confirmed by PCR reamplification with the same primers, and positive clones were partially sequenced with the primer 528f [24] by the MACROGEN Genomics Sequencing Services (Seoul, South Korea). Sequences were identified and inspected for chimeras by BLAST [25] and KeyDNATools [26], yielding 202 sequences (accession numbers KC147206–KC147388). Clone sequences where blasted again in order to determine their similarity against their closest cultured match, CCM [14]. Based on previous observations [14], sequences with a similarity value against its CCM over 94 % were considered as deriving from cultured protists, whereas those below 94 % were considered from uncultured protists.

Phylogenetic Analyses

A dataset of published 18S rDNA sequences and those from this study were aligned using MAFFT [27]. Alignments were checked with Seaview 3.2 [28] and highly variable regions of the alignment were removed using Gblocks [29]. Maximum likelihood (ML) phylogenetic trees were constructed with the fast ML method RAxML [30] using the evolutionary model GTRMIXI. Phylogenetic analyses were done in the freely available University of Oslo Bioportal (www.bioportal.uio.no). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one having the best Likelihood of 1,000 alternative trees). Bootstrap ML analysis was done with 1,000 pseudoreplicates and the consensus tree was computed with MrBayes [31]. Trees were edited with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

A phylogenetic tree has been constructed by the method described above using the 202 sequences from the study. The obtained tree has been analyzed using Fast Unifrac [32] in order to determine wich samples in the tree have a similar eukaryotic composition. A clustering analysis has been performed using the weighted UniFrac after a normalization step.

Results

We aimed to report the differences on the community composition of HF among incubations differing in the amount and origin of organic matter and started with the same initial community of bacteria and small protists (<3 μm). We prepared five different microcosms (two replicas for each of them). All microcosms were incubated in the dark at in situ temperature and sampled daily for 8 days to follow the growth and diversity dynamics by several analytical tools, including epifluorescence microscopy, image analysis, molecular techniques (DGGE and clone libraries) and molecular phylogeny. The objective of the first three incubations (O–L–M) was to analyze the effect of the increase of the same source of organic matter in the incubated community, whereas the last two incubations (R–P) were done to determine the effect of alternative organic matter sources added at the same concentration.

Heterotrophic Flagellates and Bacterial Growth Dynamics

In all of the incubations we detected a bacterial peak of abundance occurring the first 1–2 days, which was followed by a peak of HF that typically consumed bacterial cells (Fig. 1). All treatments showed a second bacterial peak occurring after 4–6 days of incubation. When we increased the amount of organic matter added we observed that the peaks of bacteria and HF were larger, together with a delay in the occurrence of both peaks. Replicated treatments always exhibited very coincident dynamics. The abundance of phototrophic flagellates decreased during the eight days of the experiment, typically becoming a very low percentage (<1 %) of the eukaryotic cells at the moment of the HF peak (data not shown).

Bacterial and HF peaks appeared earlier in treatment O (day 1) than in the other treatments, and exhibited the lower abundance, 2.5×10^6 bacteria mL^{-1} and 12×10^3 HF mL^{-1}. The second bacterial peak occurred at day 5.5 and was rather minor. Microbial peaks appeared later in treatment L, on day 1.5 for bacteria and day 4 for HF. In addition of being more separated, these two peaks had higher cell abundance: 10×10^6 bacteria mL^{-1} and $50–100\times10^3$ HF mL^{-1}. In this case, the second bacterial peak appeared at the same time as in treatment O but it was much higher. In treatment M there were also two bacterial peaks, on days 2.5 and 5.5, however here both were of comparable abundance. Interestingly, the HF peak did not appear until day 6 or 7 (depending on the replica) and seemed to feed on bacteria from the second peak. Treatment M exhibited the highest cell abundance at the peaks: 40×10^6 bacteria mL^{-1} and 400×10^3 HF mL^{-1}. For treatment R, the timing of the peaks and its cell abundance were remarkably similar to treatment L, which had ten times less organic matter in the form of yeast extract. Finally, for treatment P, the timing of the peaks was very similar to treatment M, so HF were also feeding on the second bacterial peak, whereas the attained cell abundances were similar to treatments L and R. An overview of measured growth rates of HF assemblages indicated faster growth when increasing organic matter (Table 1). We also observed that the ratio between new flagellates and bacteria consumed (yield) was lower in the unamended incubation than in the other three treatments.

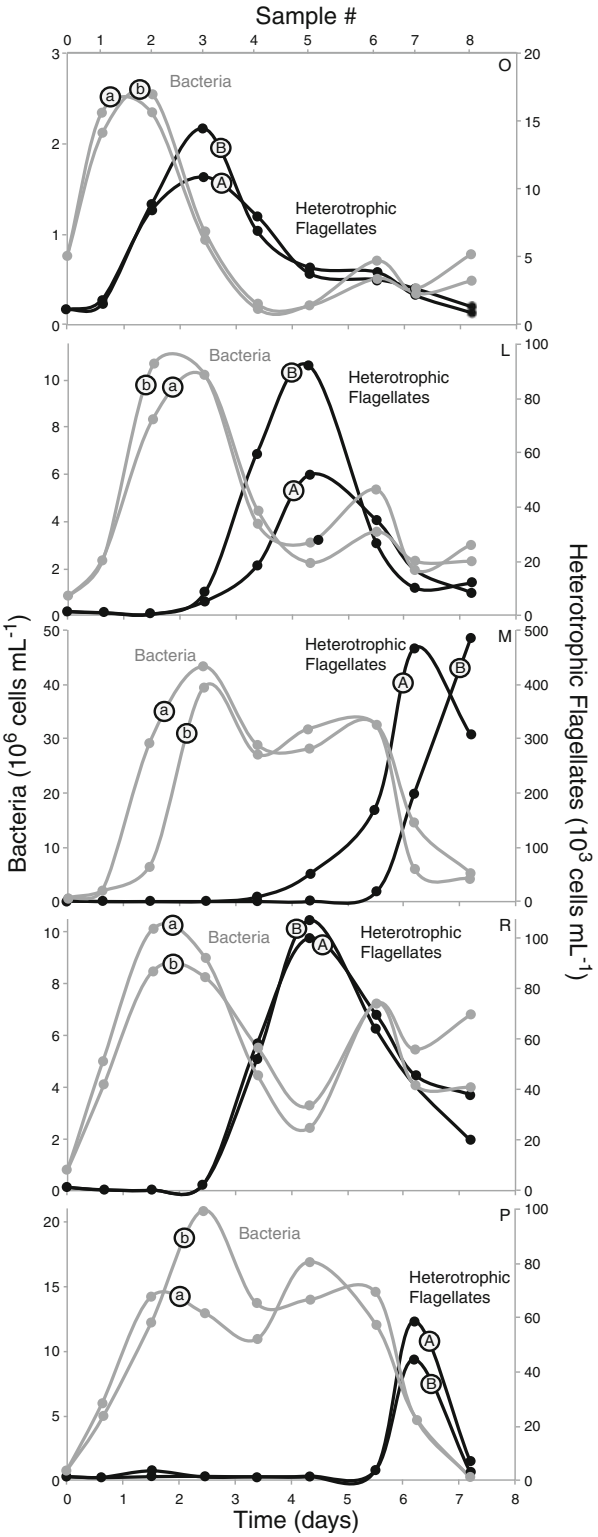

Fig. 1 Growth dynamics of HF (*black dots*) and bacteria (*gray dots*) in the five organic matter enrichments followed by epifluorescence microscopy. Treatment O corresponds to an unamended incubation, L to 0.01 % yeast extract addition, M to 0.1 % yeast extract addition, R to 0.1 % rice extract addition and P to 0.1 % glucose based enrichment. There are two replicas for each treatment (HF: *A* and *B*, bacteria: *a* and *b*). Sample numbers are shown at the *top of the graphs*. Note the different scale in vertical axis in each panel

Table 1 Growth rate (μ), doubling time (T_d), and flagellate yield of each incubation (calculated as the ratio of flagellates appeared and the decrease of 10^3 bacteria) based on cell abundance dynamics. Treatment O corresponds to an unamended incubation, L to 0.01 % yeast extract addition, M to 0.1 % yeast extract addition, R to 0.1 % rice extract addition and P to 0.1 % glucose based enrichment. There are two replicas for each treatment, A and B

Sample	μ (day-1)	T_d (hours)	Yield (f/10^3 b)
OA	1.38	12.10	4.5
OB	1.43	11.67	6.3
LA	2.22	7.49	8.2
LB	2.75	6.05	13.3
MA	3.40	4.89	18.5
MB	4.12	4.04	21.5
RA	3.02	5.52	16.4
RB	3.06	5.43	27.1
PA	4.13	4.02	11.3
PB	3.72	4.47	8.5

In order to describe the morphology of food prey for HF, we analyzed bacterial cell sizes and the percentage of bacterial cells in aggregates at two given time points (Fig. 2). In samples before the HF development, both bacterial descriptors generally were higher in incubations with high concentrations of organic matter, indicating that the morphology of bacteria was being modified along the enrichment gradient, becoming larger and more aggregated (Fig. 3). The effect of HF grazing on bacterial assemblages became apparent following the analysis of samples after HF peaks, which in general showed smaller bacterial sizes and lower percentages of bacteria in aggregates. Nevertheless, there were some noticeable exceptions: treatment L present slightly smaller cells than treatment O, bacterial size was not reduced by HF grazing in treatments P and R, and bacterial aggregation was not reduced by HF grazing in treatment R.

Fingerprinting Analysis of Eukaryotic Community Structure

The DGGE image displayed a fingerprint of the protist composition in the initial assemblage and in samples at the HF peaks (Fig. 4a). It was obvious from the image that the eukaryotic diversity was very similar in replicated incubations and very distinct among each one of the treatments. The cluster analysis revealed that O samples grouped with the initial sample (T0) whereas the remaining samples with organic matter additions clustered together (Fig. 4b). Among these, samples that received yeast extract were related, as well as samples that received alternative organic matter additions.

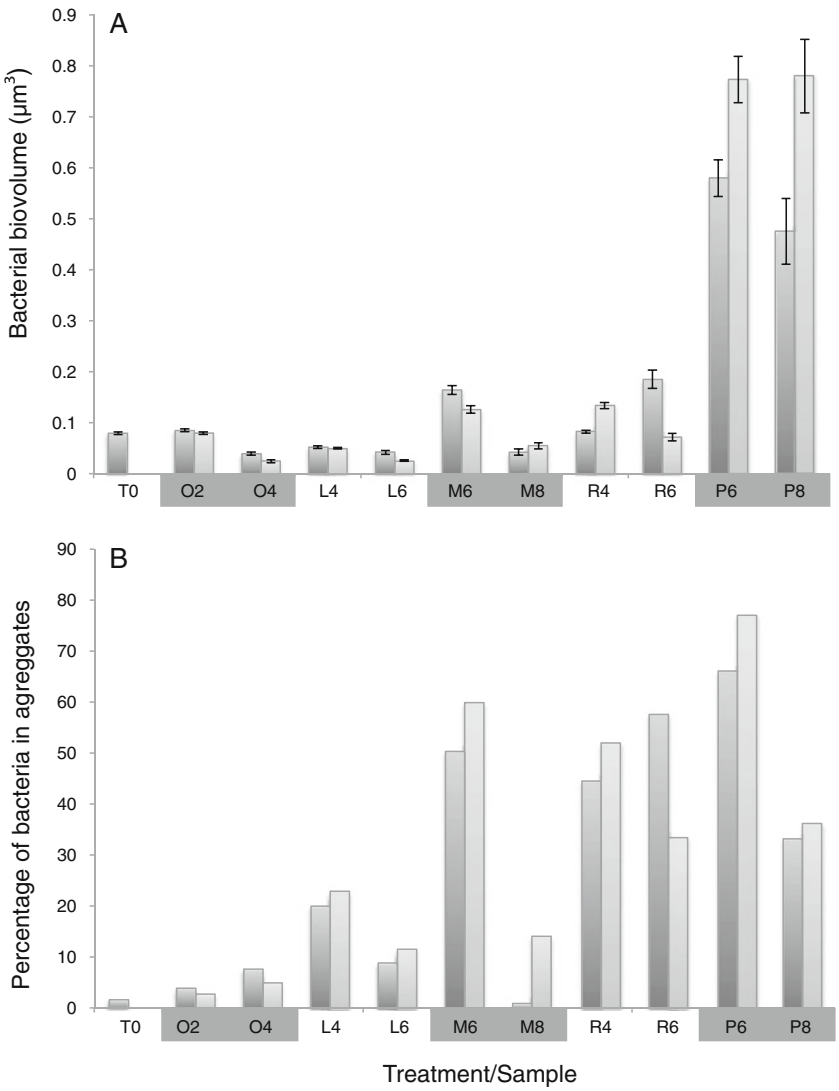

Fig. 2 **a** Averaged biovolume of bacterial cells in the original sample (T0) and in the two replicas (indicated in different tones of *gray*) of the five treatments before and after HF peaks. *Error bars* show SE of individual measurements. **b** Percentage of bacterial cells forming aggregates in the same samples

Effect of Organic matter on the Development of HF Diversity

We prepared five 18S rDNA clone libraries at the HF peak in order to determine the phylogenetic affiliation of the dominant organisms developing in each incubation. For this analysis, we chose only one microcosm per treatment (replica A) given that DGGE fingerprints revealed a high reproducibility among replicas. The obtained sequences were clustered in operational taxonomic units (OTUs) using a 99 % similarity criterion in order to determine the diversity in each sample. Chrysophytes and MAST (Marine Stramenopiles [33]) were the dominant groups as most clones affiliated within these two stramenopile groups (Table 2). For the unamended incubation sample (OA3), we sequenced 32 clones and identified 15 different OTUs, 9 corresponding to chrysophytes, 4 to MAST, 1 to prasinophytes and 1 to dinoflagellates. In treatment L (LA5) we sequenced 73 clones and found 15 different OTUs: 11 corresponded to chrysophytes, 3 to MAST and 1 to radiolaria. In treatment M (MA7; 50 clones sequenced), there was a striking decrease in the number of OTUs, only 5 chrysophytes and 1 MAST. Treatments R and P (RA5 and PA7) also yielded a low OTU number, as the 14 and 26 clones sequenced resulted in 4 OTUs each, most of the clones affiliating with chrysophytes. Although the sequencing effort in R5A and PA7 was lower than in the other samples, the pattern of low diversity was clear. The Shannon diversity indices [34] calculated from the number of clones belonging to each OTU indeed showed the lower diversity in the treatments with higher organic matter. Shannon indices decreased from 2.5 at the unamended incubation to less than 1 in treatments with 0.1 % organic matter (Fig. 5).

Trying to define the role of organic matter in altering the protist community and driving culturing bias, we classified the sequences (and OTUs) in two categories according to their similarity values to its CCM in GenBank (Table 2). This classification was done under the hypothesis that organic matter was increasing the number of sequences similar to

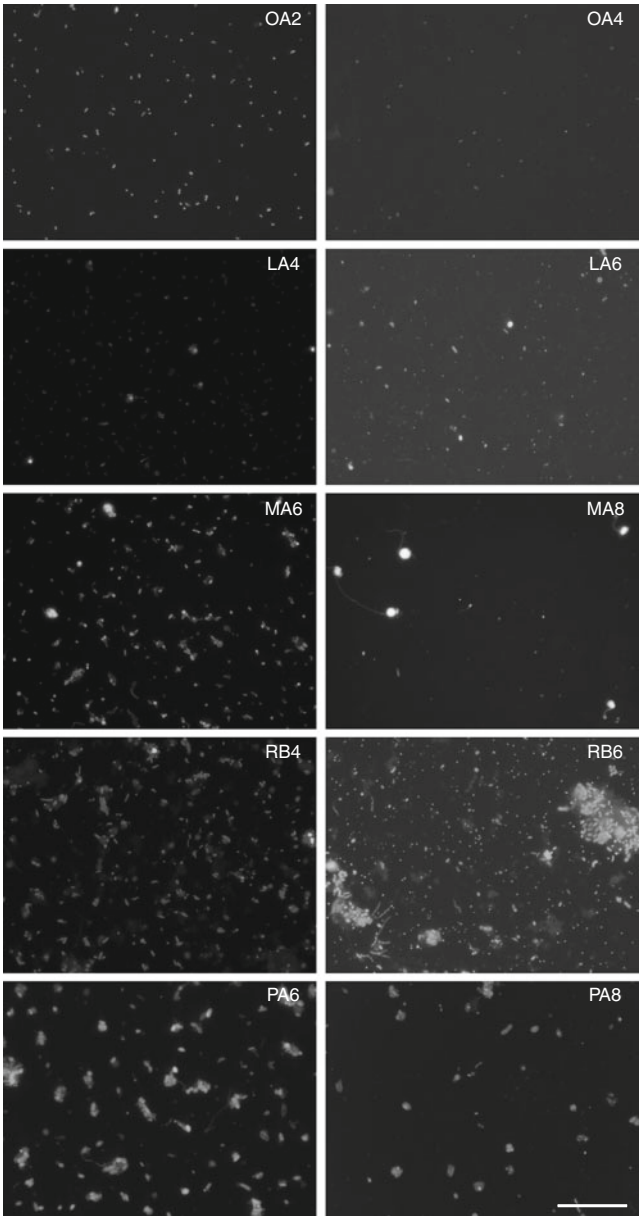

Fig. 3 Micrographs of the bacterial assemblages taken from the different treatments before and after the HF peak. *Scale bar* represents 20 μm

cultured protists (<94 % similar to its CCM). In treatment O, most of the defined OTUs (10 out of 15) have similarity values below 94 % to its CCM. An intermediate situation occurred in the moderately amended treatment (L), in which 6 of 15 OTUs had similarity values below 94 % to its CCM. For the highly amended treatments (M, R, and P) almost all sequences belong to OTUs that had similarity values above 94 % to its CCM (Fig. 5). So, the shift from "uncultured" to "cultured" HF by increasing organic matter was clear.

The cloning and sequencing analysis revealed that most sequences belonged to chrysophytes. Therefore, we constructed a chrysophyte ML phylogenetic tree in order to go deeper in the phylogenetic affiliation of these sequences, compare them in different treatments, and investigate if they belong to cultured or uncultured clades (Fig. 6). Most chrysophyte sequences from the unamended incubation belonged to environmental clades such as Clade H and Clade I, whereas sequences obtained from enriched treatments belonged mainly to Clades F1 and J, which contain the well-known *Paraphysomonas* and *Oikomonas* species [14].

In order to determine the relationship between the different treatments in terms of community composition we analyzed the five clone libraries altogether using UniFrac (Suppl. Fig. 1). We observed a clear separation between unamended and enriched samples. Among the latter, the relationship between samples agreed with the microscopic observations, with treatments R and P being more related to L than to M.

Discussion

To determine the effect of organic matter additions on the heterotrophic components of the microbial loop (organic matter–heterotrophic bacteria–heterotrophic flagellates), we compared the original community with its development under different conditions. We used several methods to give an integrated view of the effect of organic matter in enrichments and its direct link with the culturing bias in heterotrophic flagellates.

In incubations where we added the same organic matter source at different concentrations (O, L, and M), the peak abundance of bacteria and HF increased from O to M and this was accompanied by a delay in the timing of bacterial and HF peaks (and a longer gap between both peaks). These delays could be explained by the time needed by the community to adapt to the enrichment conditions. In unamended incubations (treatment O) the original bacterial populations, typically adapted to low nutrient scenarios, found the right conditions to start growing. On the contrary, these original bacteria could be inhibited to grow in enriched incubation (L and M) and seems plausible that some minority population, adapted to high nutrient concentrations, could develop quickly and dominate in the enriched community [35, 36]. So, this bacterial community will derive from a smaller inoculum size, delaying the apparition of the peak. A similar reasoning can be invoked for the delay in the appearance of HF peaks. In particular, HF cells in high organic matter incubations needed to adapt to high bacterial abundance, large bacteria, and often the formation of bacterial aggregates. Most likely, the original dominant marine HF species were not prepared for these enriched conditions. The differences in growth rate were consistent with different species growing in different incubations (Table 1), with faster growing populations in enriched conditions. Again, these fast-growing opportunistic HF species

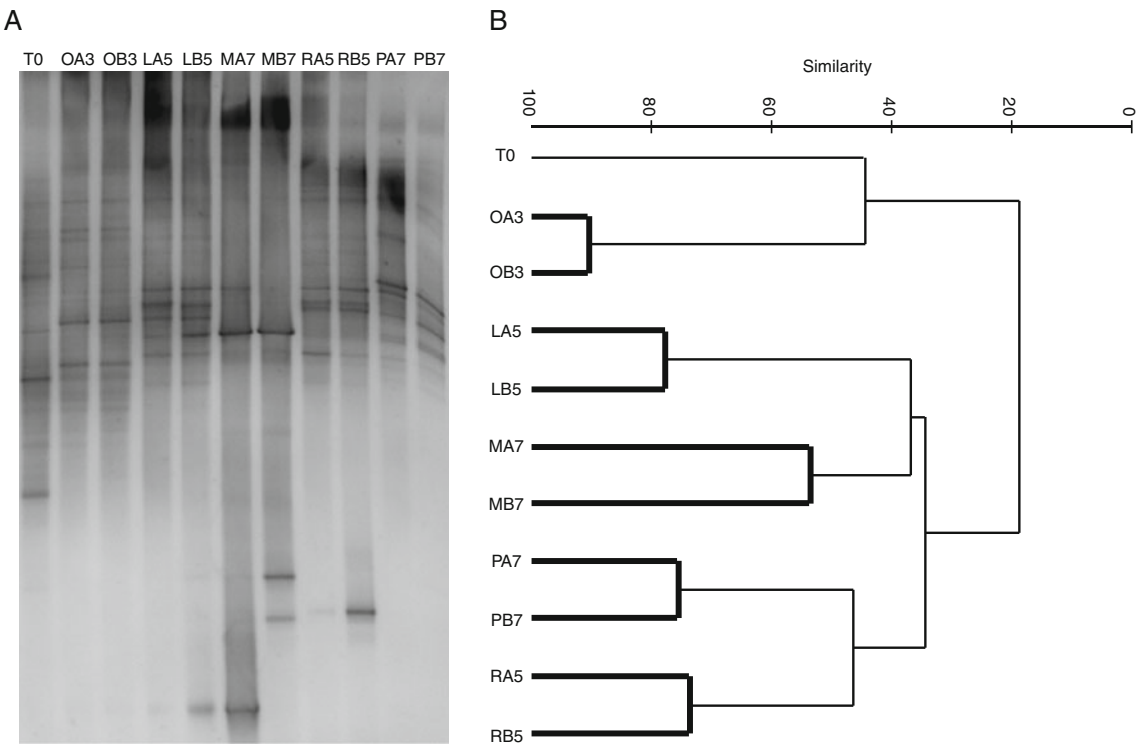

Fig. 4 **a** DGGE fingerprint of eukaryotic assemblages (based on 18S rDNA genes) obtained from the original sample (T0) and the enrichments at HF peaks. **b** Dendrogram relating the samples calculated on the basis of Bray–Curtis dissimilarity. *Bold lines* indicate no significant differences among clustered samples ($p<0.05$)

were likely in low abundance in the original oligotrophic sample [37], and needed extra time to form the HF peak.

All treatments had two bacterial peaks, with the second being more important as the organic matter increased. This second bacterial peak could be the result of a substitution in the bacterial community, as has been previously noted [38]. These authors found that in similar microcosm experiments, Alteromonadaceae formed the first peak and the second peak was formed by Rhodobacteraceae. Whereas flagellates in treatments O and L were feeding from bacteria in the first peak, those from treatment M were feeding bacteria from the second peak. Only some specialized species can consume large and aggregated bacteria and proliferate in these conditions [39], as the dominating *Paraphysomonas* spp. and *Oikomonas* spp. taxa in treatment M, which are known to be easily isolated from marine snow [40]. These flagellates were clearly able to consume this bacterial field, composed by large free-living cells (mean volumes of 0.2 μm^3, three times larger than in situ sizes) and up to 60 % of bacteria in aggregates. The consumption of large bacteria was also reflected on in the yield data, where the same amount of bacterial cells were yielding more flagellates when increasing of organic matter.

In treatments R and P, which had an alternative source of organic matter (at the same concentration as M), the timing of the peaks coincided with treatments L and M, respectively. Hence, HF in treatment P were also feeding on the second bacterial peak. In addition, cell abundance at the peaks was similar between R and P and much lower than the

Table 2 18S rDNA sequences obtained from clone libraries for the different samples at the HF peak. Sequences have been classified based on their phylogenetic position and depending on the blastn similarity value to its Closest Cultured Match (CCM) (94 % as the boundary). Number within brackets display the number of OTUs (grouped at a 99 % similarity criteria) of each group of sequences. Abbreviations are the same as informed in Table 1, plus a number indicating the day when samples were taken

Sample	CCM	Total	Chrysophytes	MAST	Other
OA3	>94 %	8 (5)	5 (3)	1 (1)	2 (1)
	<94 %	24 (10)	13 (6)	10 (3)	1 (1)
LA5	>94 %	62 (9)	62 (9)	–	–
	<94 %	11 (6)	4 (2)	6 (3)	–
MA7	>94 %	47 (5)	47 (5)	–	–
	<94 %	3 (1)	–	3 (1)	–
RA5	>94 %	13 (3)	12 (2)	–	1 (1)
	<94 %	1 (1)	–	1 (1)	–
PA7	>94 %	24 (3)	24 (3)	–	–
	<94 %	2 (1)	–	2 (1)	–

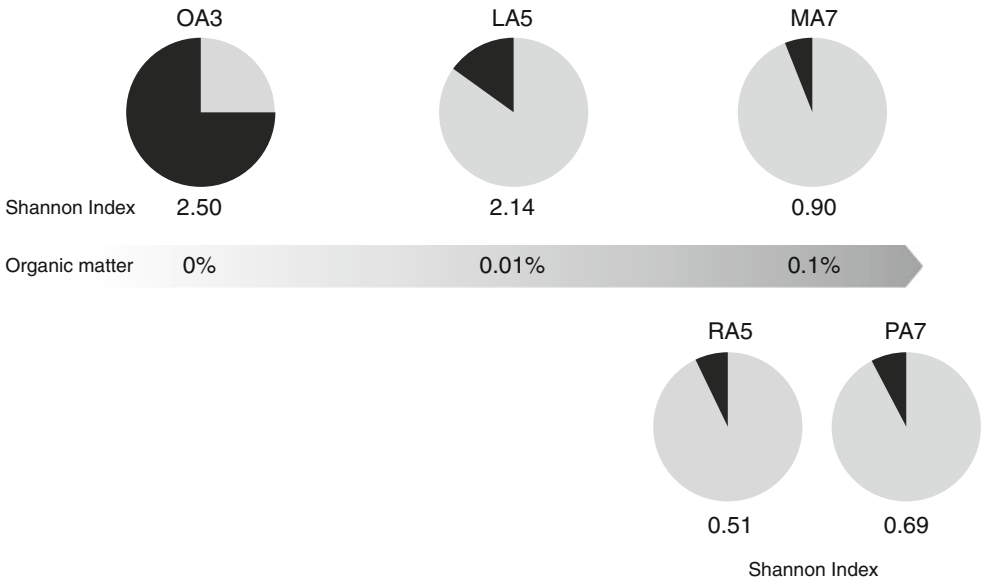

Fig. 5 Pie charts representing the proportion of clones in five 18S rDNA libraries affiliating to uncultured (similarity against its CCM <94 %; *dark gray*) or cultured (similarity against its CCM >94 %; *light gray*) protists. The Shannon diversity index is also shown for each library under its corresponding pie chart

abundances attained in M. This clearly highlighted that the type of organic matter strongly influenced HF dynamics.

The organic matter source in treatment R, rice extract, is rich in starch. The use of these large molecules requires production of starch hydrolyzing exoenzymes, and not all bacteria have this enzymatic machinery. So for most bacteria, starch is less accessible and desirable than yeast extract. This could explain why this treatment had bacterial abundances closer to treatment L, which had one order of magnitude less organic matter but used a more attractive source. In fact, microbial dynamics in treatments R and L were strikingly similar. In the case of treatment P, although glucose is an accessible carbon source and was properly supplemented with N and P, this treatment might lack some oligoelements (such as amino acids or vitamins) that are present in yeast extract. This could again explain the lower bacterial numbers in comparison with treatment M. Moreover, the very large bacteria (0.6–0.8 μm^3) in the second peak, which is unique in this treatment (Fig. 2), seemed to become a grazing refuge that avoided efficient HF exploitation [39, 41, 42]. Flagellate yield values for the P treatment did not reflect the consumption of very large cells and deviated from the consistent values from the other treatments (Table 1). This suggested that other mechanisms might be involved in the crash of the bacterial peak in treatment P.

A general analysis of HF diversity by DGGE fingerprinting revealed that the unamended treatments grouped with the original sample and the enriched treatments grouped together in another cluster. This agrees with previous studies that showed that unamended incubations promote the growth of HF already present in the natural assemblage and prevent a large modification of the community structure [16], whereas the organic matter enrichments selected populations which were not very abundant in the original sample [10, 13, 43]. A detailed analysis of the sequences highlighted clearly the bias effect caused by the organic matter. While in OA3 clone library there was a dominance of uncultured protists, the increase of organic matter reversed this trend and cultured protists became dominant in treatments M, R, and P (with L being at an intermediate condition). This fact is even more obvious when we look at the chrysophyte phylogenetic tree: sequences from enriched samples belong to clades with a high representation of cultured organisms and sequences from the unamended incubation belong to environmental clades [14].

The selective and homogenizer role of the organic matter was confirmed by a clear decrease in the diversity when increasing organic matter (Fig. 5). Whereas the Shannon index in OA3 library was 2.5, this index decreased to less than one in MA7, RA5, and PA7 (with LA5 again being an intermediate condition). Organic matter enrichments resulted in communities not only dominated by cultured organisms but also less diverse, here dominated by *Paraphysomonas* spp. and *Oikomonas* spp. Those species are adapted to eat bacteria in high abundances [44–46] and potentially were able to outcompete the organisms that were originally more abundant in the oligotrophic initial sample.

Culturing bias is an important obstacle for both evolutionary biologists that intend to obtain a complete picture of the eukaryotic tree of life and for microbial ecologists looking to improve the understanding of marine ecosystem functioning.

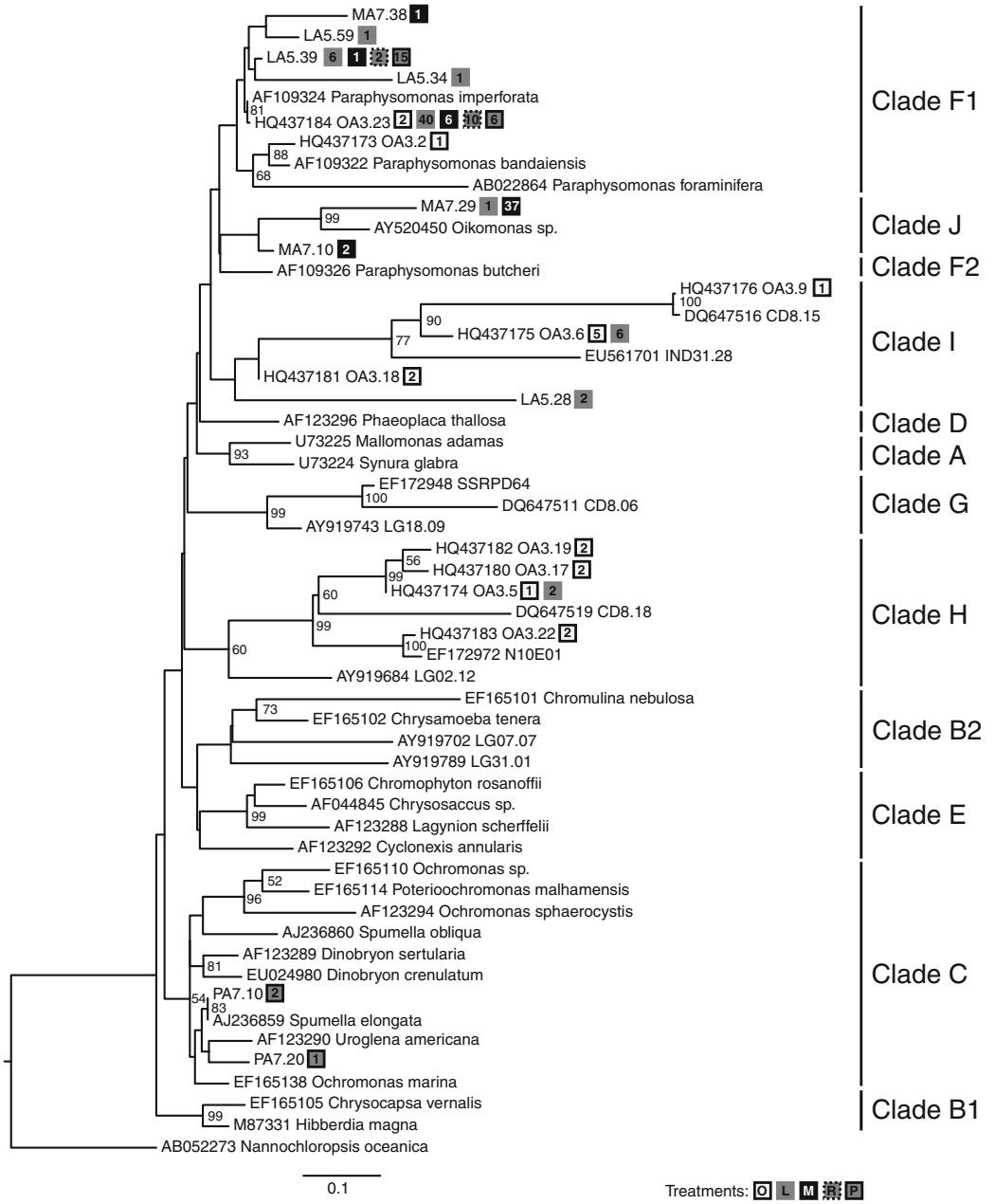

Fig. 6 Maximum Likelihood phylogenetic tree of chrysophytes constructed with 38 complete and 16 partial 18S rDNA sequences (1,444 informative positions). ML bootstrap values over 50 % are shown. Only one sequence representative of each OTU (delineated at 99 % similarity) is shown in the tree. The number of sequences from different libraries assigned to each OTU appears after the corresponding sequence name. The *scale bar* indicates 0.1 substitutions per position

There have been many indirect signs of culturing bias in HF. For example, different species are retrieved by using culturing independent approaches [21, 47, 48] than by using culturing dependent ones [49–54]. More direct evidence derives from the fact that some cultured species are easily enriched but found at very low abundance in the original sample [10, 13]. Our data highlighted that classical culturing techniques based on supplementing with organic matter need to be complemented with novel culturing strategies in order to increase our knowledge on the protists responsible for most processes in the sea [55]. Culturing efforts attempted up to now have been extremely important, but a new culturing impulse is needed to advance our understanding of protist evolution and ecology.

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