

Unveiling the Organisms behind Novel Eukaryotic Ribosomal DNA Sequences from the Ocean

Ramon Massana,* Laure Guillou, Beatriz Díez, and Carlos Pedrós-Alió

Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CMIMA, CSIC, 08003 Barcelona, Catalonia, Spain

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Despite the fact that the smallest eukaryotes (cells less than 5 μm in diameter) play key roles in marine food webs, particularly in open oligotrophic areas, the study of their in situ diversity started just one year ago. Perhaps the most remarkable finding of the most recent studies has been the discovery of completely new phylogenetic lineages, such as novel clades belonging to the stramenopile and alveolate phyla. The two new groups account for a significant fraction of clones in genetic libraries from North Atlantic, equatorial Pacific, Antarctic, and Mediterranean Sea waters. However, the identities and ecological relevance of these organisms remain unknown. Here we investigate the phylogenetic relationships, morphology, in situ abundance, and ecological role of novel stramenopiles. They form at least eight independent clades within the stramenopile basal branches, indicating a large phylogenetic diversity within the group. Two lineages were visualized and enumerated in field samples and enrichments by fluorescent in situ hybridization using specific rRNA-targeted oligonucleotide probes. The targeted organisms were 2- to 3- μm -diameter, round-shaped, nonpigmented flagellates. Further, they were found to be bacterivorous. One lineage accounted for up to 46% (average during an annual cycle, 19%) of heterotrophic flagellates in a coastal environment, providing evidence that novel stramenopiles are important and unrecognized components of the total stock of bacterial grazers.

Taxonomic identification of aquatic microorganisms has been historically a difficult task due to their lack of conspicuous morphological features and the selectivity of culturing approaches. During the last decade, the introduction of molecular techniques in microbial ecology has greatly increased our knowledge on this topic (2). Thus, marine prokaryotic assemblages have been found to be largely made of novel bacterial and archaeal lineages without any known cultured representative (6, 10, 12). Small marine eukaryotes are also recognized as fundamental components of marine food webs (5, 15). This assemblage is formed mostly by chlorophyll-containing phototrophic cells, which participate in primary production, and unpigmented heterotrophic cells, which are generally considered to be grazers of prokaryotic cells. Despite the fact that small marine eukaryotes contribute significantly to community biomass and activity (5, 15), the study of their phylogenetic composition lagged one decade with respect to that of marine prokaryotic assemblages. Three recent publications analyzed 18S ribosomal DNA (rDNA) genetic libraries from eukaryotic microbes (cells smaller than 3 to 5 μm in diameter) collected in different marine systems and found a remarkably high phylogenetic diversity (7, 18, 20). In each system, slightly over half of the clones belonged to groups of organisms known to be mainly photosynthetic and a much smaller fraction belonged to typically heterotrophic groups, whereas a large proportion of clones belonged to completely novel phylogenetic lineages within the stramenopile (*Heterokonta*) and alveolate phyla. Whereas these studies point to the ubiquity and clonal abundance of these novel phylogenetic lineages, they do not give a

clue about their cellular identity, in situ abundance, and ecological role.

In this paper we focus on unveiling the organisms behind one of these novel phylogenetic groups, the novel stramenopiles. Clones affiliating with this group were present in all systems investigated and accounted for 10 to 27% of all clones from North Atlantic, Mediterranean, equatorial Pacific, and Antarctic genetic libraries (7, 18, 20). Novel stramenopile sequences, therefore, appeared to be widespread in marine waters all over the world. We first performed a phylogenetic reconstruction to find out the exact placement of these novel sequences in the eukaryotic tree and the genetic diversity within the group. We then developed oligonucleotide probes against several of the genetic clusters identified and applied the probes to natural and enrichment samples through fluorescent in situ hybridization (FISH). This allowed us to identify their morphology under epifluorescence microscopy and their abundance in the marine environment. Finally, we conducted several experiments to determine their trophic role in the marine environment, specifically addressing the question of whether they are phototrophic or heterotrophic organisms.

MATERIALS AND METHODS

Sequencing and phylogenetic analyses. Genetic libraries that we had described previously (7) were screened for more novel stramenopile clones. These libraries had been obtained from samples in three distant marine regions, the Mediterranean Sea (library ME1), the Antarctic Ocean (libraries ANT37 and ANT12), and the North Atlantic Ocean (libraries NA11 and NA37). Relevant parameters of the sites sampled are presented in the above-mentioned study (7). We obtained 8 complete and 17 partial new 18S rDNA sequences. They belonged to the stramenopile phylum, according to the results of a BLAST search (1), and were not chimeric, according to the results of a CHECK_CHIMERA search (19). Complete sequences, together with others already published (7, 18, 20), were manually aligned to a general stramenopile alignment (kindly provided by Susan Loiseaux-de Goër), previously optimized by using primary and secondary rRNA structures. Three dinoflagellate sequences were used as an outgroup. Highly variable gene regions were removed, leaving 1,490 unambiguously aligned posi-

* Corresponding author. Mailing address: Institut de Ciències del Mar, CMIMA, CSIC, Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalonia, Spain. Phone: 34-93-2309500. Fax: 34-93-2309555. E-mail: ramonm@icm.csic.es.

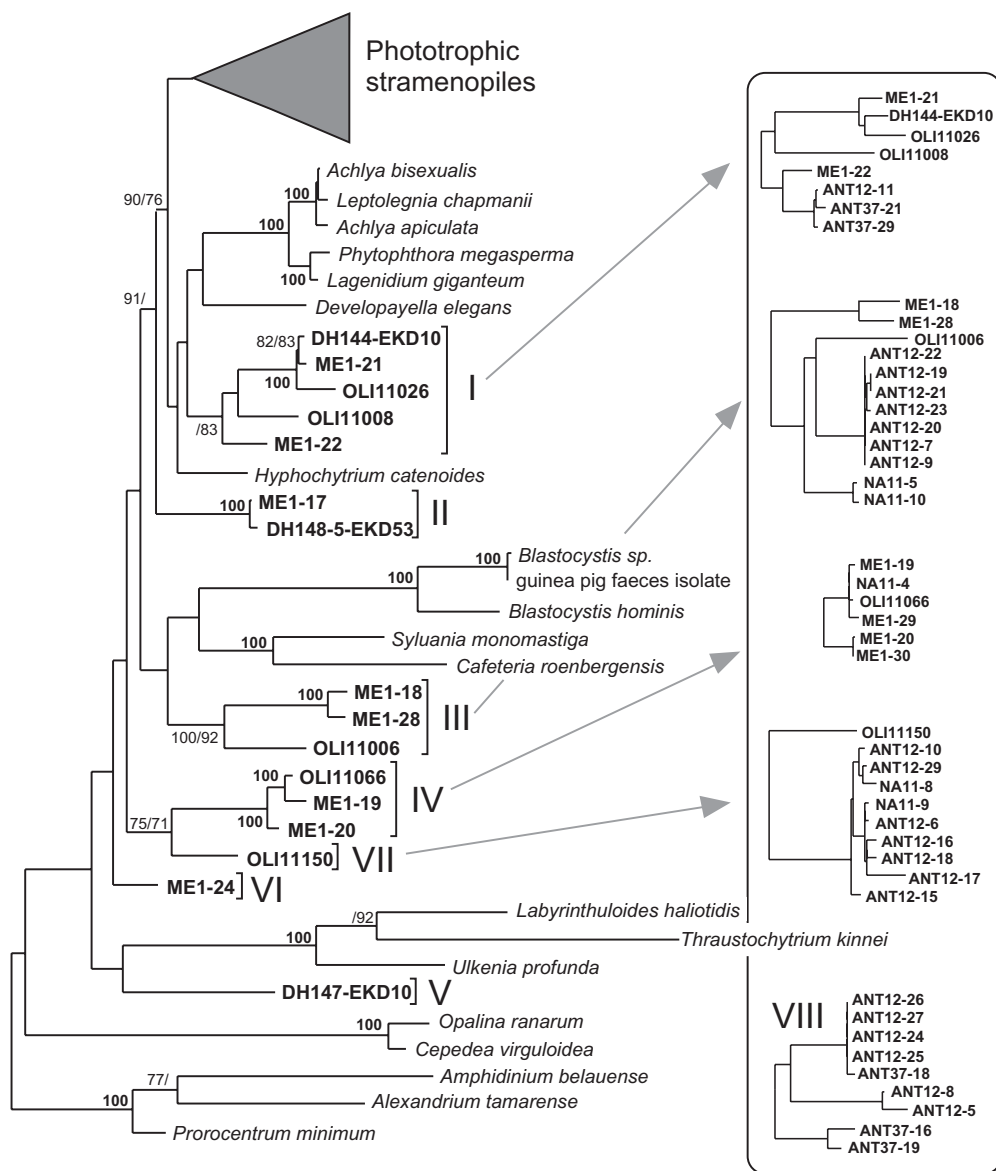


FIG. 1. Maximum-likelihood phylogenetic tree with complete 18S rDNA sequences of novel stramenopiles. The scale bar indicates 0.1% sequence divergence. Neighbor-joining and maximum-parsimony bootstrap values (left to right, respectively) are shown at the internal branches (100 replicates; values >70% shown). Bold numbers indicate identical bootstrap values in both analyses. Novel stramenopile lineages are marked with roman numerals (from I to VIII). The right box shows subtrees of several lineages with additional partial sequences. The position of lineage VIII is intermediate between lineages IV and VII. The code of the clones indicates their origin as follows: Mediterranean Sea (ME), Equatorial Pacific (OLI), North Atlantic (NA), and surface (ANT) and deep (DH) Antarctic waters.

tions for subsequent phylogenetic analyses. Maximum-likelihood analysis (for tree, see Fig. 1) and neighbor-joining bootstrap values (with the Kimura two-parameter option) were carried out using the PHYLO_WIN software (11). Maximum-parsimony bootstrap values were computed with PAUP 3.1.1 software (29), using a heuristic search method with a tree-bisection-reconnection branch-swapping option with random taxon addition. The 49 phototrophic stramenopile sequences used were monophyletic and were boxed together under the heading "Phototrophic stramenopiles" to simplify the tree. Partial sequences (around 500 bp) were aligned to the same data set, and a neighbor-joining phylogenetic tree was obtained as described above. Particular lineages were extracted from this tree and are shown in the box to the right in Fig. 1.

FISH. We used the same alignment to design 18S rDNA probes against novel stramenopile lineages III (probe NS3, 5'-ATTACCTTGGCCTCCAAC-3'; *Ochromonas danica*, positions 846 to 829) and IV (probe NS4, 5'-TACTTCGG TCTGCAAAACC-3'; positions 844 to 828). A BLAST search (1) revealed that these probes had at least three mismatches with every known sequence in Gen-

Bank. Probes, labeled with a CY3 fluorophore at the 5' end, were ordered from Interactiva (<http://www.interactiva.de/>). For FISH, we followed the protocol of Pernthaler et al. (22). Briefly, cells were fixed with formaldehyde, collected on 0.6- μ m-pore-diameter polycarbonate filters, hybridized with the CY3 probe, and stained with DAPI (4'6'-diamidino-2-phenylindole). Filters were then observed by epifluorescence with UV radiation (DAPI staining) and green light-specific excitation (CY3 signal). Since none of the novel stramenopile lineages are yet available in culture, the probes were tested against environmental samples as positive controls (i.e., we used a sample from the enrichment culture). During probe testing, temperature was kept constant at 46°C and the stringency was varied with different formamide concentrations (from 0 to 50%) in the hybridization buffer. Optimal formamide concentration was found to be 30% for both probes. Probes were also tested against the following battery of nontargeted cultures, obtained from the Roscoff Culture Collection (<http://www.sb-roscoff.fr/Phyto/collect.html>): the prasinophyte *Ostreococcus tauri* (RCC116), an unidentified prymnesiophyte (RCC437), an unidentified cryptophyte (RCC440), the

eustigmatophyte *Nannochloropsis* sp. (RCC438), the chryomonad *Picophagus flagellatus* (RCC22), and the bicosoecid *Symbiomonas scintillans* (RCC24). All these cultures gave a negative signal with both probes, even at the lowest stringency level tested (0% formamide).

Coastal sampling. Surface seawater was collected in Blanes Bay (Catalan coast, northwestern Mediterranean Sea, at 41°40'N, 2°48'E), at 800 m offshore. Seawater was kept in 25-liter plastic carboys for less than 2 h during transport before processing in the laboratory. During the monthly sampling, a filter for FISH was prepared and stored at -70°C until used. The abundance of heterotrophic flagellates (HF) and phototrophic eukaryotes (PE) was estimated on the day of sampling in DAPI-stained preparations (23). An aliquot of glutaraldehyde-fixed sample was filtered on a 0.6- μm -pore-size polycarbonate filter, stained with DAPI, and observed by epifluorescence microscopy with UV radiation (DAPI staining) and blue light excitation (chlorophyll fluorescence of PE).

Enrichment cultures. Seawater was gravity filtered through a 2- μm -pore-size polycarbonate filter and incubated in replicate 2-liter bottles at near-in situ temperature in the dark. For 1 week, we estimated the abundance of HF and PE (using DAPI-stained preparations) and of NS3- and NS4-positive cells (using FISH) daily. A fluorescently labeled bacteria (FLB) uptake experiment (25) was performed using heat-killed, DTAF-stained *Pseudomonas diminuta* cells. They were added to the enrichment at tracer concentrations (10^6 FLB ml^{-1}), and the sample was then incubated for 2 h and processed for FISH as before. The presence of FLB inside flagellates was observed under blue light epifluorescence (previous tests showed that FLB fluorescence is not washed out during the FISH process).

Nucleotide sequence accession numbers. Sequences newly determined for this study have been deposited in the GenBank database under the accession numbers AY116219 to AY116241.

RESULTS AND DISCUSSION

Phylogenetic analyses of novel stramenopiles were carried out using published complete 18S rDNA sequences obtained from equatorial Pacific Ocean (OLI clones [20]) and deep Antarctic (DH clones [18]) genetic libraries and sequences newly presented here that were obtained from an open Mediterranean Sea genetic library (ME1 clones). Maximum-likelihood, neighbor-joining, and maximum-parsimony analyses consistently placed these clones among the basal branches of the stramenopile radiation, forming at least eight independent lineages based on high bootstrap values and specific nucleotide signatures (Fig. 1). Stramenopiles form a phylogenetic group that is extremely diverse in metabolisms and cell types, including unicellular and multicellular algae, fungi-like cells, and HF (21). It is assumed that photosynthetic stramenopiles arose from a secondary endosymbiosis between a heterotrophic eukaryote and a primitive red alga (4). Thus, all photosynthetic stramenopiles are monophyletic, whereas distinct heterotrophic lineages, such as oomycetes, bicosoecids, labyrinthulids, thraustochytrids, and opalinids, appear at the basal branches of the stramenopile radiation (14). The fact that novel stramenopiles appear among these heterotrophic groups (Fig. 1) suggests (although it does not demonstrate) that they are heterotrophic organisms as well.

Additional partial sequences obtained from North Atlantic, Mediterranean, and Antarctic genetic libraries (NA, ME, and ANT clones, respectively) fit within the previously described lineages (see box at right in Fig. 1). The genetic diversity of novel stramenopiles within each environment was rather high, with different clones, often belonging to different lineages, appearing in the same library. Some lineages were represented by only one or two clones. Other lineages (I, III, IV, and VII) contained clones from distant marine areas, indicating that very similar phylotypes may have a widespread geographic distribution. In particular, lineage IV contained nearly identical (99% similarity) clones from the North Atlantic (NA11-4),

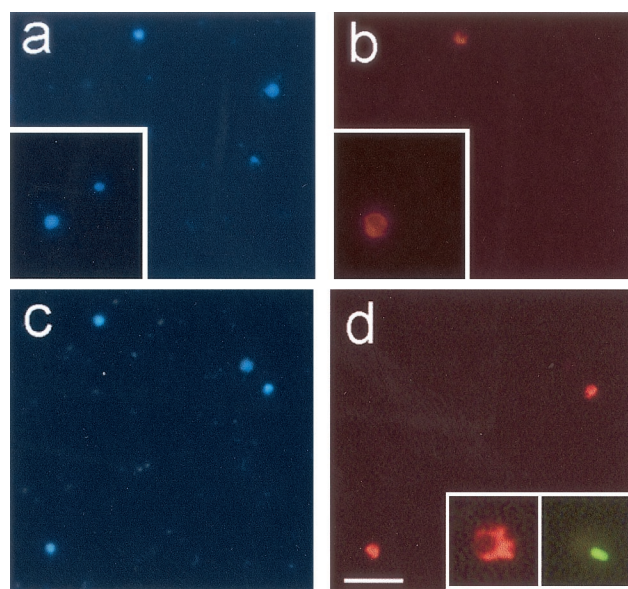


FIG. 2. Epifluorescence micrographs of novel stramenopiles. (a and c) DAPI-stained cells and the corresponding microscopic field showing NS3 (b) and NS4 (d) cells after FISH. Panels a and b also include insets showing a different microscopic field. The scale bar is 10 μm . Two of the five eukaryotes were NS3 cells (compare a and b), and two of the four eukaryotes were NS4 cells (compare c and d). The nuclear region, brightest by DAPI staining, is dimmer by FISH fluorescence, which is consistent with the cytoplasmic localization of ribosomes. The inset in panel d shows a NS4 cell (magnified three times) with one ingested FLB.

Mediterranean (ME1-29), and equatorial Pacific (OLI11066) genetic libraries. Finally, lineage VIII contained clones that were restricted to Antarctica (this lineage was not placed in the general tree, since the corresponding libraries [9] were obtained with partial rDNA inserts). Overall, novel stramenopiles are highly diverse and reveal a mosaic of cosmopolitan and habitat-restricted phylotypes.

We tried to obtain pure cultures of novel stramenopiles to properly characterize these organisms. We started cultures of small eukaryotes from different stations and seasons in the Mediterranean Sea. Most phototrophic cultures were prasinophytes and prymnesiophytes, whereas most heterotrophic cultures were bicosoecids (L. Guillou, unpublished results). Thus, we were unable to retrieve novel stramenopiles in stable culture. As an alternative method for visualizing and enumerating these cells, we resorted to FISH. This method has already been applied to marine eukaryotes but not very extensively (16, 17, 28). Due to the genetic heterogeneity of novel stramenopiles, it was not possible to find a single probe targeting this complex group. Thus, we designed probes against lineages III and IV, because these were widely represented in genetic libraries, especially those from the Mediterranean coast (unpublished results) whose samples were tested by FISH. Organisms from field samples and enrichment cultures that returned positive results with either of the two probes (NS3 and NS4 cells) were, indeed, very small eukaryotes (Fig. 2). These eukaryotes were visible as round-shaped cells with a bright nucleus, due to DAPI staining, and with bright and unevenly distributed orange fluorescence, due to the CY3-labeled probe. Probe NS4

revealed a homogeneous assemblage of cells 2 to 3 μm in diameter (Fig. 2c and d), whereas probe NS3 hybridized with a more heterogeneous assemblage, with most cells measuring 2 to 3 μm but also some cells measuring up to 5 μm in diameter (Fig. 2a and b). This is consistent with a larger phylogenetic diversity in cluster III (see inset in Fig. 1), which may accommodate different morphotypes.

FISH was extremely useful for visualizing novel stramenopiles. However, during the hybridization, a variable amount of chlorophyll was washed out and it was not possible to assess with confidence whether they were heterotrophic or phototrophic organisms. This point was addressed by following the development of several microbial groups in an enrichment culture in the dark, which was started, using surface Blanes seawater, on 27 September 2001. During the course of the experiment, the number of PE decreased continuously whereas that of HF increased 2 orders of magnitude (Fig. 3). The number of NS4 cells also increased, reaching maximal concentrations of 10^4 cells ml^{-1} and up to 30% of HF cell levels. NS3 cells responded in a similar way, although they were always less abundant (up to 400 cells ml^{-1}). Cells from both lineages grew very fast, with doubling times of 8 h. The fact that NS3 and NS4 cells became more abundant than PE unequivocally demonstrates that they form part of the HF assemblage. Electron microscopy was not attempted at this point, since NS4 cells never made up the largest fraction of the assemblage.

The first enrichment experiment demonstrated that the NS4 cells, and most likely the NS3 cells also, were heterotrophic (unpigmented) organisms and formed part of the HF assemblage. In a second enrichment culture carried out on 6 November 2001, we did a test of the ability of these organisms to ingest bacteria. In this experiment, we obtained a similar development of the HF assemblage and NS4 cells (unpublished results). An FLB uptake experiment was performed at a time of high NS4 cell abundance. Of a total of 43 NS4 cells inspected, 21% contained presumably ingested FLBs. An example of an NS4 cell with one FLB in the cytoplasm is shown in the inset in Fig. 2d. These observations strongly argue for these organisms being phagotrophic and bacterial grazers.

The fact that NS4 cells develop so well in enrichments without any addition prompted us to use these enrichments to retrieve the cells in pure culture. Thus, a third enrichment was started with Blanes seawater on 14 January 2002. In this case, the yield of HF was not as high (up to 3×10^3 cells ml^{-1}) but NS4 cells also developed significantly and reached 36% of the HF count. Then we used this NS4-cell-enriched sample to start a serial dilution culture battery using two different media, yeast extract (0.5 g liter^{-1}) and rice ($40 \text{ grains liter}^{-1}$). After two weeks, the tubes with positive growth were checked by FISH and we did not detect any NS4 or NS3 cells. Apparently, these organisms are not willing to grow in rich media or are outcompeted by other flagellates that grow faster under such conditions. In fact, in the first enrichment (Fig. 3), NS4 cells grew faster than the other flagellates (they started at 9% of the HF count and increased to 27% during the exponential growth phase) but also died off faster (they ended up at 8% of the HF count). It is clear that all these behavioral aspects of NS4 cells must be considered in future attempts to obtain them in stable culture.

Finally, we investigated the relevance in the environment of

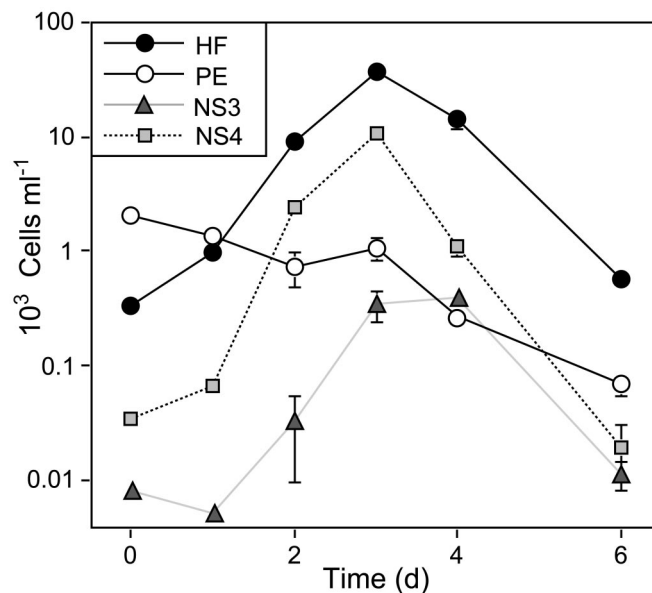


FIG. 3. Counts of HF and PE and NS3 and NS4 cells in an enrichment culture, which was started with 2- μm -diameter-pore-filtered Blanes seawater in September 2001. Average values and standard errors from two replicates are shown. Note that both NS3 and NS4 numbers became higher than the total number of PE.

the two novel stramenopile lineages for which we had probes. Thus far we had data only from genetic libraries regarding their clonal abundance, and this could be affected by well-known PCR biases (31). We monitored the abundance of NS3 and NS4 cells, together with that of HF and PE, in Blanes Bay surface waters during an annual cycle (Fig. 4). HF were always less abundant than PE, averaging 730 and 4,500 cells ml^{-1} , respectively, during the studied period. NS4 cells ranged from 19 to 327 cells ml^{-1} (average, 116 cells ml^{-1}), and NS3 cells ranged from 3 to 36 cells ml^{-1} (average, 12 cells ml^{-1}). In Fig. 4, the abundance of NS cells is shown with respect to the HF count. NS4 cells accounted on average for 19% of total HF,

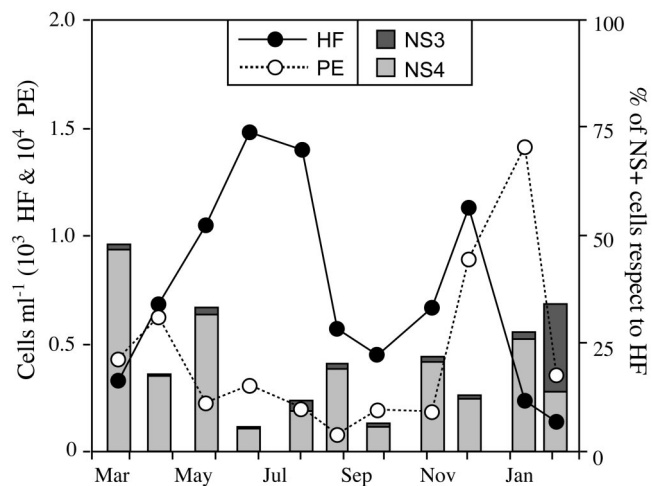


FIG. 4. In situ abundance of HF and PE and of NS3 and NS4 cells (expressed as percentages of HF numbers) in samples collected in Blanes Bay during an annual cycle from March 2001 to February 2002.

and on some days they composed up to 46% of the HF stock. NS3 cells accounted on average for only 3% of HF stock, but on the last sampling date they composed up to 20% of HF stock. Therefore, NS3 and NS4 cells appear to be quantitatively important components of marine HF assemblages.

HF are ubiquitous and play key roles in planktonic marine food webs. They are the main consumers of prokaryotes and participate directly in nutrient remineralization (9, 27). Both direct measurements (5, 26) and size-fractionation grazing experiments (5, 24) have revealed that marine HF assemblages are numerically dominated by very small cells of 2 to 3 μm in diameter. Up to now, there has been a significant lack of knowledge about the populations forming these assemblages (3). Direct light and electron microscopy observations usually failed to identify these very small cells (8, 30). A few HF cultures of this size are presently available (13). However, it is well known that culturing or enrichment can strongly bias the in situ diversity, as was exemplified by *Paraphysomonas imperforata*, which dominated in enrichment cultures from a coastal environment but was never abundant in the original sample (17). In fact, of the analyzed clones from the genetic libraries, very few (5.5% of total) affiliated to known HF such as cercomonads, choanoflagellates, or chryomonads (7, 18, 20). Our approach provides insight into the dominant components of this functional group.

Genetic analyses of the smallest marine eukaryotes are quickly reshaping our understanding and perception of microbial diversity. As was found for bacteria and archaea a decade ago (6, 10, 12), novel lineages appear to be very important in marine eukaryotic picoplankton (7, 18, 20). We have demonstrated here that novel stramenopiles, whose existence was unrecognized just one year ago, are bacterivorous HF and are essential components of this functional group in marine samples. The question remains open whether other lineages, such as the novel alveolates, may also have an important share in the HF assemblage. These results have implications for the estimates of carbon flow in the ocean, since parameters inferred from cultured HF (such as growth efficiencies or functional responses) may not coincide with those of flagellates important in nature. This underscores the importance of renovated attempts to culture them.

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