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Growth of uncultured heterotrophic flagellates in unamended seawater incubations

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ABSTRACT: Unamended dark incubations of 3 µm-filtered seawater were prepared with surface samples from the Norwegian Sea, the NW Mediterranean and the Indian Ocean. Except for the very oligotrophic Indian Ocean samples, this simple treatment promoted the growth of heterotrophic flagellates, with rates (0.3 to 1.2 d^{-1}) typical of natural assemblages. Marine stramenopile (MAST)-1 and MAST-4 cell counts, obtained by fluorescence *in situ* hybridization (FISH), increased in most incubations. These yet uncultured protists were first detected in molecular surveys of marine picoplankton and have been recently shown to be globally distributed heterotrophic flagellates. Three incubations from the Norwegian Sea were studied in more detail by denaturing gradient gel electrophoresis (DGGE), 18S rRNA gene clone libraries and FISH counts for 5 MAST groups. Protist diversity changed gradually during the incubation, but the DGGE bands selected were already present at the beginning of the incubation. Clone libraries from the peaks in abundance of heterotrophic flagellates were dominated by MAST sequences. FISH counts revealed MAST-1B to be a very successful organism in the 3 incubations, accounting for 15 to 30% of heterotrophic flagellates after 6 to 8 d; MAST-1A and -1C cells were also abundant. MAST-4 cells peaked before the other groups and MAST-2 was the least represented. We concluded that unamended seawater incubations can select for heterotrophic flagellates abundant *in situ* but not yet isolated in pure culture. Therefore, they allow investigation of the growth requirements and dynamics of these uncultured protists, and provide promising preliminary stages for their isolation.

KEY WORDS: Heterotrophic flagellates · Unamended incubations · MAST · Uncultured protists · FISH · DGGE

INTRODUCTION

Conceptual models of carbon flow in marine planktonic food webs changed drastically with the realization of the importance of heterotrophic bacteria growing on dissolved organic matter (Pomeroy 1974). In this new paradigm, bacteria were consumed by small predators, loosely referred to as heterotrophic flagellates (Fenchel 1986), which linked bacteria with higher trophic levels in the so-called microbial loop (Azam et al. 1983). This view was complicated by the discovery of multiple trophic levels within heterotrophic flagellates (Wikner & Hagström 1988), of their ability to graze on picophytoplankton (Caron et al. 1991), and of viruses as additional players in bacterial mortality (Proctor & Fuhrman 1990). Although the significance of heterotrophic flagellates in carbon transfer is still debated, it is widely accepted that they exert strong grazing pressure on picoplankton populations and are central in nutrient recycling (Sherr & Sherr 2002, Pernthaler 2005). Given this central importance and considering that different species surely imply functional differences in terms of food spectra, prey–predator interactions, and biogeochemical cycling (Boenigk & Arndt 2002), it is surprising how little we know about the diversity of *in situ* marine heterotrophic flagellates (Arndt et al. 2000).

Heterotrophic flagellates are routinely quantified by epifluorescence microscopy after DAPI staining (Porter & Feig 1980). In marine systems, cells smaller than

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5 µm in diameter dominate in numbers (Sherr et al. 1997, Caron et al. 1999, Jürgens et al. 2000), but these remain mostly unidentified in terms of phylogenetic affiliation owing to the few observable morphological features. The long list of formally described marine species (Lee & Patterson 1998) derives mainly from cultures or enrichments started by adding a substrate for bacterial growth that, in turn, are the food for the flagellates. While it is obvious that these easily enriched strains live in the sea, it is doubtful that they are dominant members of natural assemblages. A now classic study demonstrated that the heterotrophic flagellate dominating several enrichments was rare in the original samples (Lim et al. 1999). On the other hand, recent molecular surveys of surface marine picoplankton (Díez et al. 2001a, Moon-van der Staay et al. 2001) retrieved very few sequences related to known heterotrophic flagellates. Instead, these surveys revealed a large number of uncultured lineages, such as the marine stramenopiles (MAST) that formed up to 12 independent lineages (Massana et al. 2004). It has recently been shown that some of these MAST lineages are heterotrophic flagellates with a global distribution and significant abundance in the oceans (Massana et al. 2006). It is thus clear that culture isolations and molecular surveys are providing different views on the species composition of marine heterotrophic flagellates.

Unamended seawater incubations with size-fractionated microbial assemblages can establish a bridge between both approaches. By incubating 3 µm-filtered seawater in the dark, bacteria growing on autochthonous substrates allow for a moderate pulse of heterotrophic flagellates, which can then be identified by molecular tools. Although short-lived, these events can provide interesting information on marine protists, especially if growth of uncultured organisms is promoted. In an incubation performed on a sample from Blanes Bay (NW Mediterranean), we observed growth of MAST-3 and MAST-4 cells and demonstrated that they were heterotrophic flagellates (Massana et al. 2002). This study provided useful phenotypic and functional information on uncultured protists and showed the potential of this approximation, but it remained to be known whether it represented a peculiarity of the sample analyzed or an example of a more general phenomenon.

We conducted unamended seawater incubations in 3 oceanographic areas: the Blanes Bay Microbial Observatory (5 times throughout the year), the Norwegian Sea (at 1 coastal and 2 offshore stations) and the Indian Ocean (at 5 stations along a transect from South Africa to Australia). The growth of MAST cells was checked by fluorescence *in situ* hybridization (FISH) at the beginning and at the time of maximal abundance of heterotrophic flagellates. In addition, the 3 incubations from the Norwegian Sea were studied in more detail. This is a complex oceanographic region where Atlantic and Arctic waters meet, and data on abundance and community structure of picoeukaryotes from the same cruise were presented elsewhere (Not et al. 2005, Lovejoy et al. 2006). Population dynamics of protists was followed by denaturing gradient gel electrophoresis (DGGE), and the major populations of heterotrophic flagellates growing were identified in 18S rRNA gene clone libraries. Since MAST lineages were well represented in clone libraries, we followed the dynamics of these cells by FISH.

MATERIALS AND METHODS

Sampling. Water samples from the Blanes Bay Microbial Observatory (NW Mediterranean Sea) were retrieved 800 m offshore at 5 different times of the year, including fall, winter and summer. Water samples from the Norwegian Sea and the Indian Ocean were collected with Niskin bottles attached to a CTD rosette on cruises with RV 'Johan Hjort' (Norwegian Institute of Marine Research) and RV 'Melville' (Scripps Institution of Oceanography), respectively. Three stations were sampled in the Norwegian Sea: near the coast (Coastal), southeast of Spitzbergen with Barents Sea influence (Polar), and southwest of the island (Atlantic). Five stations were sampled in the Indian Ocean between the African coast and Keeling Island. Station locations, date of sampling and temperature and chlorophyll *a* (chl *a*) concentration of surface samples used for incubations are shown in Table 1.

Unamended seawater incubations. 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 (except for the first Blanes sample, which was filtered through 2 µm). Subsequently, 2 l of the filtered seawater were dispensed into Nalgene polycarbonate bottles and incubated in the dark at near *in situ* temperature (in a laboratory chamber for Blanes or inside a tank with running surface seawater for the 2 cruises). Mean temperature for the incubations in the Norwegian Sea and the Indian Ocean was 9 and 24°C, respectively. Bottles were sampled every 1 to 2 d. Subsamples for epifluorescence microscopy were fixed with ice-cold glutaraldehyde (1% final concentration), stained with DAPI and filtered onto 0.2 or 0.6 µm pore-size black polycarbonate filters (Poretics) for numeration of bacteria (but potentially also including Archaea and *Prochlorococcus*), *Synechococcus*, and flagellates (heterotrophic and phototrophic). 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 (–20°C during the cruises and –70°C afterwards). 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) as described previously (Díez et al. 2001a). Subsamples for FISH counts (100 ml) were fixed with 0.2 µm-filtered formaldehyde (2% final concentration) at 4°C for 1 to 24 h, subsequently filtered onto 0.6 µm pore size polycarbonate filters (Poretics), and stored frozen.

Denaturing gradient gel electrophoresis (DGGE). We used 1 µl of DNA extract as a template for PCR amplification of a 560 bp-long fragment of 18S rRNA genes using primers Euk1A and Euk516r-GC (Díez et al. 2001b). DGGE was performed with a DGGE-2000 system (CBS Scientific Company) as described previously (Díez et al. 2001b). Gels of 6% polyacrylamide were prepared with a gradient of denaturant agent from 40 to 65% (100% denaturant agent being 7 M urea and 40% deionized formamide). PCR products (800 ng per sample) were loaded and the gel was run at 100 V for 16 h at 60°C in 1× TAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) and DNA fragments were visualized in a Fluor-S Multi-Imager (Bio-Rad). High-resolution images were analyzed with the software Quantity One (Bio-Rad) in order to detect DGGE bands, quantify their intensity, and identify the same band position across the different lanes of the gel. A matrix was constructed with the presence and relative intensity of individual bands in each lane. This matrix was used to calculate a distance matrix with Euclidean distances and a dendrogram with UPGMA (Unweighted Pair-Group Method with Arithmetic averages) using Statistica 6.0 (StatSoft).

Gene clone libraries. 18S rRNA genes were amplified by PCR using 2 eukaryotic primer sets (Díez et al. 2001a,b): EukA and EukB that amplify the complete gene (ca. 1780 bp), and the DGGE set (without the GC clamp in the reverse primer) that amplifies only one-third of the gene (ca. 560 bp). PCR products from several reactions were cleaned with the QIAGEN PCR purification kit and cloned with the TOPO-TA cloning kit (Invitrogen). The presence of the 18S rRNA gene insert in the positive colonies was checked by PCR amplification with the same primers. In libraries with the first primer set (complete 18S rRNA genes), amplified inserts from single clones were digested with the restriction enzyme *Hae*III (Invitrogen), electrophorized in 2.5% low-melting-point agarose (Invitrogen), and clones with the same restriction pattern were considered to belong to the same operational taxonomic unit (OTU). One clone per OTU was sequenced with the Bigdye Terminator Cycle Sequencing Kit version 3.0 (PE Biosystems) and an ABI PRISM model 377 (version 3.3) automated sequencer using the internal primer Euk528f (Elwood et al. 1985). Thirteen clones were completely sequenced with 3 to 6 universal primers (the PCR primers plus 336f and 1209f). Sequence similarity of clones from the same OTU was above 99%. In libraries with the second primer set (partial 18S rRNA genes), clones were directly sequenced with the vector primer M13f. In this case, clones were affiliated to the same OTU when their sequence similarity was above 99%. This threshold was also applied to compare sequences from different libraries. The phylogenetic affiliation of clones was obtained by a basic local alignment search tool (BLAST) search (Altschul et al. 1997). Sequences were also submitted to BLAST searches with different regions for detecting chimeric artifacts (6 out of 207 sequences). Sequences were deposited in GenBank under accession numbers DQ121419 to DQ121431 and DQ647509 to DQ647544.

Fluorescent *in situ* hybridization (FISH). Five oligonucleotide probes specific to different MAST lineages were used: NS4 (Massana et al. 2002) and NS1A, NS1B, NS1C, and NS2 (Massana et al. 2006). Probes were supplied by Thermo Electron Corporation with a CY3 fluorophore at the 5′ end. For FISH we followed the protocol detailed previously (Pernthaler et al. 2001). Filter portions (2 to 4 per sample) with protist cells were hybridized for 3 h at 46°C with each probe at 5 ng μl^{-1} in a buffer of 900 nM NaCl, 20 mM Tris-HCl, 0.01% SDS and 30% formamide. Filters were washed for 15 min at 48°C in a buffer of 110 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA and 0.01% SDS. Filters were then dried, counter-stained with DAPI, mounted on a slide with a mix of Citifluor and Vecta Shield (at a ratio of 4:1), and observed by epifluorescence microscopy under UV excitation (DAPI signal) and green light excitation (CY3 signal). Several transects (8 per sample on average) were inspected and mean MAST counts and SE were calculated; SE were typically 20% of the mean.

RESULTS

Unamended dark incubations with 3 µm-filtered surface seawater were conducted in 3 oceanographic areas: the Norwegian Sea, the NW Mediterranean and the Indian Ocean (Table 1). Most incubations resulted in a substantial growth of heterotrophic flagellates (HF), which increased from *in situ* concentrations of 0.4 to 1.2×10^3 cells ml^{-1} to 4 to 16×10^3 cells ml^{-1} after 2 to 8 d. The growth rate of HF assemblages was related to temperature, being around 0.5 d^{-1} below 10°C and around 1 d^{-1} above 20°C. Only 3 incubations from the

Table 1. Unamended dark incubations conducted in 3 marine areas. Station locations, sampling dates, and temperature (°C) and chl *a* concentration (µg l^{-1}) of surface seawater are given. Dynamics of HF (heterotrophic flagellates) assemblages are indicated by abundance (cells ml^{-1}) at the beginning and at the peak in HF, day of the peak, and growth rate (d^{-1}). Abundance (cells ml^{-1}) of MAST-1 and -4 cells estimated by FISH on the same 2 dates are also shown. The latter 3 incubations in the Indian Ocean did not show a significant increase in HF

Incubation	Location	Date (d/mo/yr)	Temp.	Chl *a*	HF				MAST-1		MAST-4	
					Day 0	Peak	Peak day	µ	Day 0	Peak	Day 0	Peak
Norwegian Sea												
Coastal	70°30′N, 20°04′E	24/8/02	12.6	0.936	652	5020	8	0.50	14	1136	73	78
Polar	76°19′N, 23°45′E	27/8/02	4.5	2.815	1003	4287	6	0.28	69	1401	90	190
Atlantic	76°20′N, 03°59′E	29/8/02	6.0	0.606	584	4196	6	0.46	8	1131	43	134
Blanes Bay (NW Mediterranean)												
Nov 2001	41°40′N, 02°48′E	06/11/01	18.0	0.410	710	8330	3	0.84	17	128	104	1812
Jan 2005	41°40′N, 02°48′E	20/1/05	13.0	0.510	412	4021	7	0.66	1	1423	26	65
Feb 2005	41°40′N, 02°48′E	15/2/05	12.1	1.340	495	3055	3	0.90	9	43	54	858
Jul 2005	41°40′N, 02°48′E	05/7/05	24.0	0.110	1425	11228	3	0.75	25	169	80	1811
Sep 2005	41°40′N, 02°48′E	13/9/05	26.1	0.270	1222	16546	2	1.25	31	86	18	2526
Indian Ocean												
Coastal	35°03′S, 23°44′E	16/5/03	20.9	1.300	687	4597	2	0.98	25	267	101	621
Madagascar	33°18′S, 45°22′E	21/5/03	20.9	0.171	499	1212	4	0.87	6	415	19	9
Gyre	31°50′S, 52°37′E	23/5/03	21.6	0.147	626	–						
Osborn	17°11′S, 83°41′E	01/6/03	25.2	0.073	450	–						
Keeling	12°13′S, 96°47′E	06/6/03	28.5	0.122	719	–						

Central Indian Ocean did not show significant growth of HF. The detailed microbial dynamics (direct counts of DAPI stained cells) of the 3 incubations from the Norwegian Sea are shown in Fig. 1. The other incubations followed similar dynamics (not shown). Initially, bacterial numbers increased 2 to 3 times, followed by increasing numbers of HF a few days later coincident with the decrease in bacterial numbers. Photosynthetic flagellates and *Synechococcus* decreased continuously in numbers during the incubations, owing to dark conditions and perhaps to predation by HF.

The potential for growth of uncultured HF was analyzed by counting MAST-1 (with a mix of 3 probes against 3 related groups) and MAST-4 cells by FISH at the beginning and at the peak of HF (Table 1). In most incubations, including the 3 oceanographic areas, there was substantial growth of at least 1 of the 2 groups, and both groups often grew together. Initially, average MAST-1 and MAST-4 concentrations were 21 and 61 cells ml^{-1}, and during the peak they reached values above 10^3 cells ml^{-1} (in 4 and 3 incubations, respectively) and between 10^2 and 10^3 cells ml^{-1} (in 4 incubations each). At the peak of HF, these 2 MAST groups accounted on average for 27% of heterotrophic flagellates, indicating that they form a significant fraction of the grown HF.

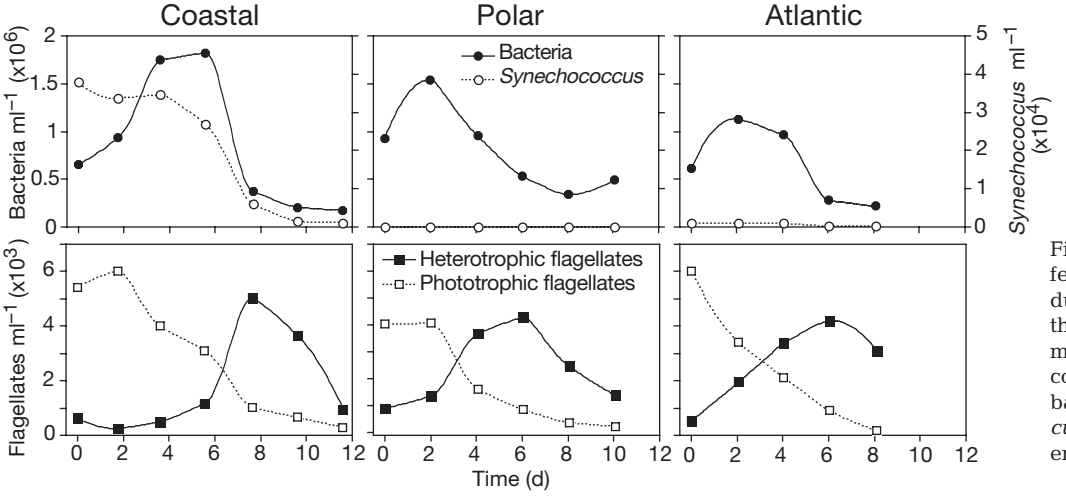

Fig. 1. Abundance of different microbial groups during 3 incubations from the Norwegian Sea estimated by DAPI cell counts. Upper panels: bacteria and *Synechococcus*. Lower panels: heterotrophic and phototrophic flagellates

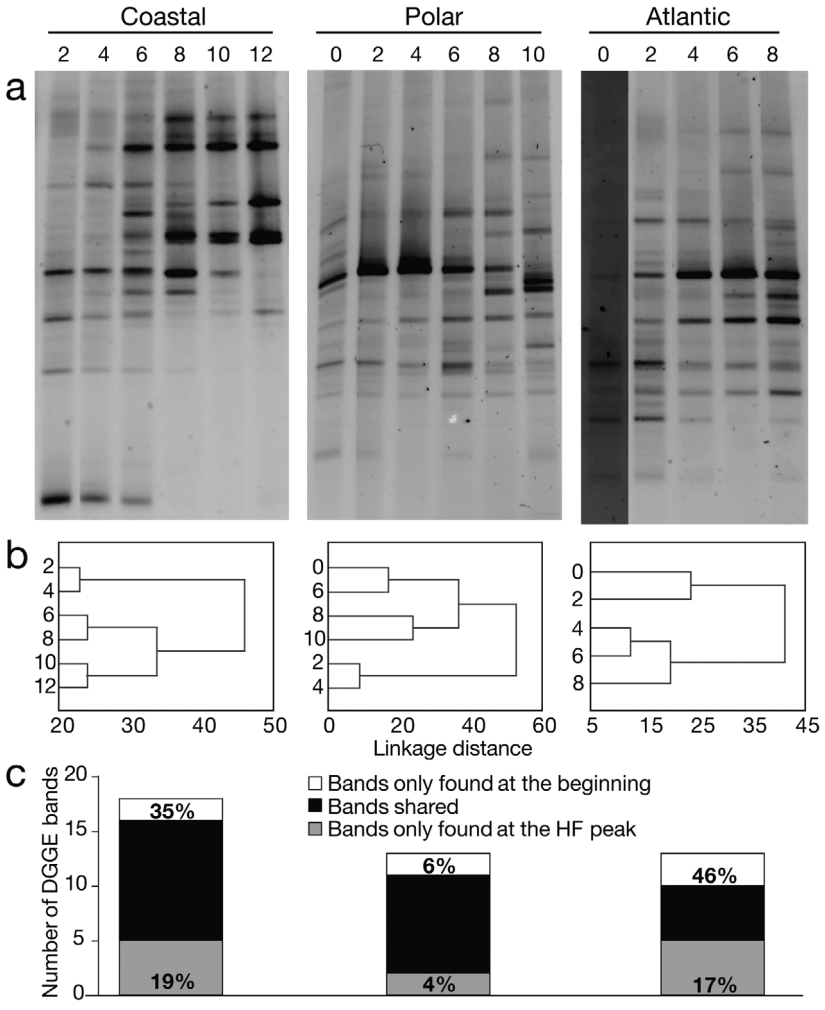

Fig. 2. (a) Inverted images of DGGE gels showing the fingerprint each day during incubations from the Norwegian Sea. (b) Cluster analysis relating DGGE fingerprints. (c) Comparison of DGGE fingerprints (number and band intensity) between the initial sample and sample with maximal HF abundance (Day 8 in Coastal and Day 6 in Polar and Atlantic). Number of bands is separated among those found only at the beginning, those found only at the HF peak, and those shared. Values above histograms are % of band intensity accounted for by unique bands

The changes in protist diversity during the incubations from the Norwegian Sea were followed by DGGE (Fig. 2a). Protist diversity varied over time, and the changes were gradual. DGGE bands were normally observed on consecutive dates, and each sample was generally more similar to the previous and the next one than to others, as shown in cluster analyses of DGGE fingerprints (Fig. 2b). A finer analysis of the protists growing in each incubation was conducted by comparing the number and intensity of DGGE bands between the initial sample and the sample at the peak of HF (Fig. 2c). Minor changes in protist composition were observed in the Polar incubation: most band positions were shared between these 2 samples, and the few DGGE bands disappearing or appearing during the incubation were weak (6 and 4% of signal intensity). Coastal and Atlantic incubations behaved differently, with a few intense bands (35 and 46% band intensity) disappearing from the original assemblage and the bands that appeared being relatively weak. This implied that most of the protists selected during these incubations were already well represented in the original sample. For instance, in the Atlantic incubation, shared bands accounted for 54% of intensity at the beginning and 83% of intensity at the date of maximal HF abundance.

To obtain phylogenetic information on the growing protists, we prepared 18S rRNA gene clone libraries from the date of maximal HF abundance (Day 8 in Coastal and Day 6 in Polar and Atlantic incubations). Three libraries contained the complete 18S rRNA genes (CD8, PD6 and AD6; 36, 52 and 54 clones, respectively) and 2 libraries contained just one-third of the gene (CD8S and AD6S; 30 and 32 clones, respectively). In the 5 libraries, 10 to 20% of clones affiliated with putative photosynthetic groups (Fig. 3), which agreed with the dominance of HF observed by microscopy in

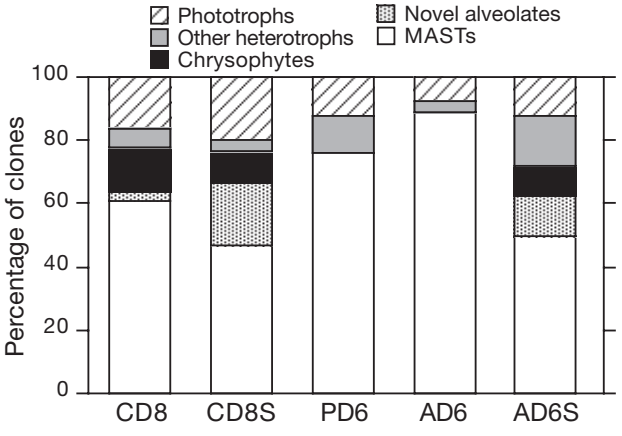

Fig. 3. Percentage of clones within phototrophic groups and putative heterotrophic groups (MASTs, novel alveolates, chrysophytes and others) in 18S rRNA gene libraries from Norwegian samples with maximal HF abundance. Libraries CD8, PD6 and AD6 contain the whole 18S rRNA gene and libraries CD8S and AD6S only one-third

these samples (more than 80% of the cells). The putative heterotrophic groups were dominated by uncultured protists found in marine molecular surveys, particularly MASTs (which accounted for 50 to 90% of clones) and novel alveolates. Chrysophytes also formed a significant fraction of clones. Libraries from the same sample with the 2 primer sets gave somewhat different pictures of protist diversity. For example, in the Atlantic sample, novel alveolates and chrysophytes were not found in the AD6 library.

The phylogenetic affiliation of our sequences is given in Table 2, with each row indicating a single OTU and its clonal representation in the 5 libraries. Several OTUs were found in the 2 libraries from the same sample, showing that different primer sets were retrieving the same sequences (but at different clonal abundance). Important exceptions were the OTUs represented by the sequences CD8.09 (7 clones in AD6S and none in AD6) and CD8.03 (10 clones in CD8 and none in CD8S). In addition, the OTUs represented by a single clone were seldom found in the other libraries, an indication of library under-sampling. When the 3 samples are compared, similar MASTs were selected in Polar and Atlantic incubations, whereas a somewhat

Table 2. Phylogenetic affiliation of clones in incubations from the Norwegian Sea. Each row represents a different operational taxonomic unit (OTU), with its representative clone and best match in GenBank and the number of clones in each genetic library. Libraries CD8, PD6 and AD6 contain the whole 18S rRNA gene (ca. 1780 bp) and libraries CD8S and AD6S only 560 bp

Group	Clone name	Best match (%)	Coastal, Day 8		Polar, Day 6	Atlantic, Day 6	
			CD8	CD8S	PD6	AD6	AD6S
MAST-1A	AD6.02	DH144-EKD10 (99.4)	–	–	32	35	5
	AD6.05	ME1-21 (98.0)	–	–	–	5	–
MAST-1B	CD8.09	RA000412.91 (99.4)	5	12	3	–	7
MAST-1C	CD8.02	ME1-22 (99.2)	6	2	–	–	–
	AD6.03	FV18_2F12 (99.8)	–	–	3	8	3
MAST-2	CD8.03	DH148-5-EKD53 (99.7)	10	–	2	–	1
MAST-7	CD8.12	UEPACLp5 (99.7)	1	–	–	–	–
Marine alveolates-I	CD8.17	UEPAC36p4 (98.9)	1	3	–	–	–
	CD8S.30	OLI11511 (98.0)	–	1	–	–	–
Marine alveolates-II	CD8S.12	UEPACFp5 (99.8)	–	1	–	–	2
	CD8S.16	TWS096 (95.8)	–	1	–	–	-
	AD6S.01	UEPACEp3 (94.3)	–	–	–	–	1
	AD6S.30	UEPACCp3 (99.5)	–	–	–	–	1
Chrysophyceae	CD8.06	*Spumella* JBAF33 (91.2)	1	–	–	–	–
	CD8.15	NOR50.37 (98.4)	2	–	–	–	1
	CD8.18	ENI42482.00342 (99.2)	2	1	–	–	–
	CD8S.27	NS51A157 (95.0)	–	2	–	–	–
	AD6S.11	LG44-07 (95.9)	–	–	–	–	1
	AD6S.18	LG35-09 (95.7)	–	–	–	–	1
Telonema	PD6.20	*Telonema antarcticum* (97.3)	–	–	1	–	–
	AD6S.04	*Telonema antarcticum* (94.0)	–	–	–	–	2
	AD6S.06	*Telonema antarcticum* (97.3)	–	–	–	–	1
Choanoflagellates	PD6.09	M1_18E10 (97.1)	–	–	1	–	–
Cercozoa	CD8S.31	*Allas* sp. (93.1)	–	1	–	–	–
	AD6.07	NOR26.21 (98.7)	–	–	–	1	1
Ciliates	CD8.05	MD65.05 (98.7)	1	–	–	1	–
	CD8.16	*Strombidium* SNB99-2 (97.2)	1	–	–	–	–
	AD6S.28	NS371C63 (96.9)	–	–	–	–	1
Fungi	PD6.12	RA000609.30 (99.3)	–	–	4	–	–
Prasinophyceae	AD6.08	A1_E023 (99.3)	–	–	–	2	–
Prymnesiophyceae	CD8S.01	*Phaeocystis jahnii* (95.9)	–	1	–	–	–
	PD6.17	*Chrysochromulina scutellum* (97.4)	–	–	4	1	–
Cryptophyceae	AD6.09	*Plagioselmis prolonga* (100)	–	–	–	1	–
	CD8S.08	LG07-11 (94.9)	–	2	–	–	–
Novel algae	AD6S.10	He000803-72 (98.4)	–	–	–	–	3
Diatoms	CD8S.05	*Chaetoceros rostratus* (97.8)	–	1	–	–	1
	PD6.14	*Skeletonema costatum* (99.5)	–	–	2	–	–
Bolidophyceae	CD8S.09	*Bolidomonas pacifica* (99.3)	–	1	–	–	–
Dictyochophyceae	CD8.07	*Pteridomonas danica* (96.3)	1	1	–	–	–
	CD8.08	*Florenciella parvula* (99.7)	1	–	–	–	–
Dinoflagellates	CD8.04	*Prorocentrum mexicanum* (99.3)	3	–	–	–	–
	CD8.10	FV23_1E1 (97.4)	1	–	–	–	–

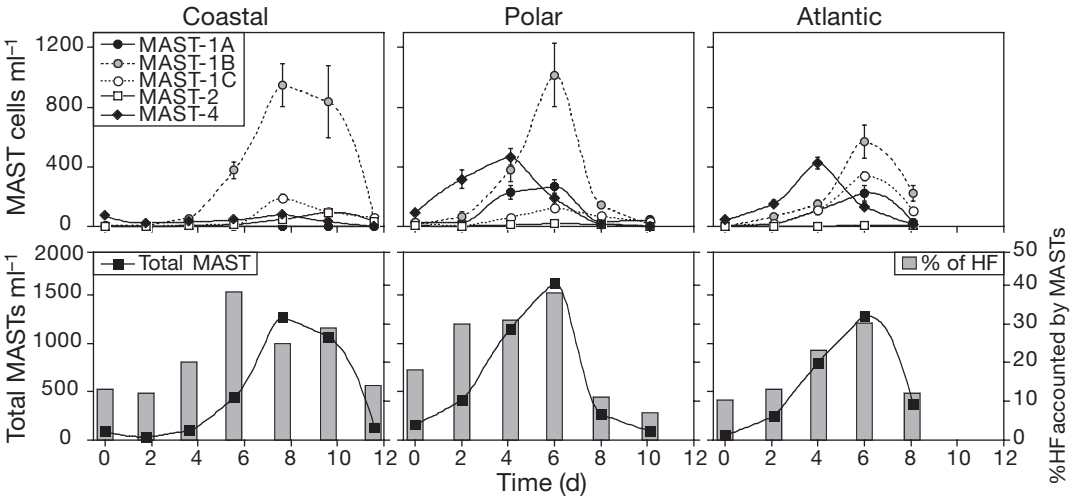

Fig. 4. Dynamics of 5 MAST groups during incubations from the Norwegian Sea. Upper panels: cell abundance determined by FISH counts (mean and SE). Lower panels: total MAST abundance and contribution of MAST cells to the HF count

different assemblage developed in the Coastal incubation. Thus, the dominant OTU in the Polar-Atlantic libraries (clone AD6.02 within MAST-1A) was not found in the Coastal library, and a different MAST-1C was found in Coastal (CD8.02) and Polar-Atlantic incubations (AD6.03). Nevertheless, the same MAST-1B sequence was found in the 3 cases. Other uncultured protists found in the libraries were the novel alveolates, both Group I and II, and the chrysophytes. Sequences from the later group were unrelated to known chrysophytes and formed 3 independent lineages represented by CD8.06 (including AD6S.11), CD8.15 (including CD8S.27), and CD8.18 (including AD6S.18). Other heterotrophic groups found at low clonal abundance were *Telonema*, choanoflagellates, cercozoans, fungi, and ciliates.

The dynamics of MAST cells during the Norwegian Sea incubations were followed by FISH counts with probes specific to 5 lineages. The targeted cells were spherical and measured around 2 to 3 µm (MAST-4), 4 to 5 µm (MAST-1B and -2), and 6 to 7 µm (MAST-1A and -1C). These groups grew substantially in at least 2 of the 3 incubations (Fig. 4), with growth rates of 0.4 to 1 d^{-1}, and attained 10^2 to 10^3 cells ml^{-1} (Table 3). At the peak of abundance of HF, the 5 MAST groups accounted for around 1500 cells ml^{-1} or 30 to 40% of HF cells (Fig. 4). The proportion of HF comprised of MASTs doubled during the incubations, as these cells already accounted for 10 to 20% of HF in the initial sample. However, the dynamics of the 5 groups considered together obscured particular trends. MAST-4 cells were the most abundant *in situ*, but they did not thrive well in the incubations and always decreased their contribution to HF (Table 3). These cells did not grow in the Coastal incubation and peaked before the other groups in the other 2 cases. In contrast, the other MAST groups showed a marked increase in their contribution to HF during the incubations. MAST-1B cells were the most successful in all 3 cases and reached the highest abundance (up to 10^3 cells ml^{-1}). MAST-1A and MAST-1C cells grew at similar rates in the 3 incubations (maximal abundances of 100 to 300 cells ml^{-1}), with the remarkable absence of MAST-1A cells from the Coastal incubation. MAST-2 cells grew moderately in the 3 incubations and never reached abundances above 100 cells ml^{-1}.

Finally, our data set allowed comparison of the representation of specific MAST groups in clone libraries with their actual cell percentage estimated by FISH direct counts (Fig. 5). The primer set amplifying the complete 18S rRNA gene gave a very biased view of MAST presence in the analyzed samples, with MAST-1A and -2 severely overestimated and MAST-1B

Table 3. Abundance (cells ml^{-1}) and growth rates (d^{-1}) of MAST cells in incubations from the Norwegian Sea. Growth rates were calculated using 3 to 4 time points of exponential increase. The last column shows the quotient between the % contribution of the specific group to total HF at the peak and at the beginning of the experiment. nd: not detected

		Abundance		Growth	Increase
		Day 0	Maximal	rate	% HF
MAST-1A	Coastal	1	nd	nd	nd
	Polar	28	266	0.59	2.5
	Atlantic	2	225	0.81	9.9
MAST-1B	Coastal	3	947	0.79	9.9
	Polar	12	1016	0.75	7.7
	Atlantic	1	567	0.54	7.7
MAST-1C	Coastal	9	189	0.76	5.4
	Polar	29	124	0.40	1.7
	Atlantic	5	338	0.71	8.3
MAST-2	Coastal	2	95	0.52	8.1
	Polar	5	19	0.57	1.3
	Atlantic	2	9	nd	nd
MAST-4	Coastal	73	78	nd	nd
	Polar	90	467	0.40	0.3
	Atlantic	43	423	0.57	0.4

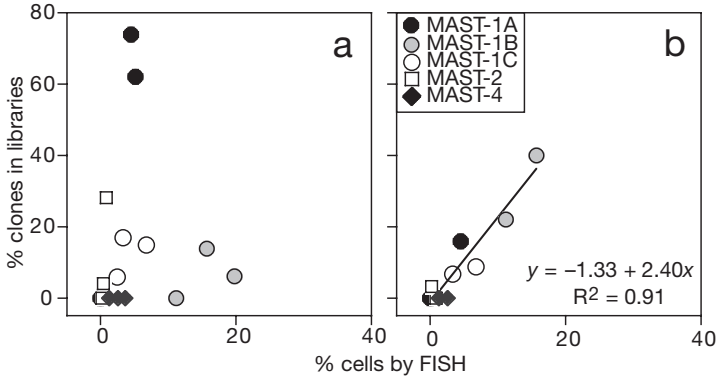

Fig. 5. Comparison of MAST signals found by FISH (% of cells with respect to total protist counts) and clone libraries (% of clonal abundance) using (a) primers for the complete gene (libraries CD8, PD6 and AD6) and (b) primers for partial genes (libraries CDS8 and ADS6)

underestimated (Fig. 5a). In contrast, the primer set amplifying one-third of the gene (in fact, the set that is regularly used in DGGE studies) provided very good correlation between clonal representation and cell abundance of the 5 MAST groups, where all groups are similarly over-represented 2.4 times (Fig. 5b).

DISCUSSION

Seawater incubations enriched with organic matter additions are typically conducted as first stages in the isolation of HF and to facilitate live and electronic microscopy observations (Fenchel 1986, Vørs et al. 1995). This addition usually selects for fast-growing bacteria, typically larger than *in situ* bacteria, and only some flagellates able to graze on these develop. This was nicely exemplified in a series of enrichment cultures done in North Atlantic coastal waters (Lim et al. 1999). In the latter study, addition of yeast extract increased bacterial abundance by 2 orders of magnitude (up to 10^8 cells ml^{-1}), and *Paraphysomonas imperforata* dominated most enrichments (up to 98% of cells). However, this flagellate was barely found in the environment. Thus, culture bias in rich media would explain why cultured HF are scarce in molecular surveys (they are readily enriched but have low *in situ* abundance) and why these surveys retrieve uncultured lineages (they are outcompeted during isolation). This is a well-known scenario for marine prokaryotes: the numerically dominant cells do not belong to easily cultured strains but to lineages first discovered in molecular surveys (Giovannoni et al. 1990). These prokaryotes were later found to be very abundant in FISH counts (Morris et al. 2002), and some were eventually cultured by serial dilution in low-nutrient media (Rappé et al. 2002). This illustrates the fruitful convergence of molecular surveys and culture attempts to study the microbial world, and the potential advantage of simulating the oligotrophic nature of the marine environment.

Our seawater incubations were unamended: bacteria grew on autochthonous substrates, and maintained their abundance at realistic levels; thus, we expected the growth of typical *in situ* flagellates. The preparation was easy, including only a prefiltration, which simplified the assemblage by leaving small protists without their predators, and dark incubation, which prevented the growth of phototrophic cells. In our samples, this setup resulted in a change from an *in situ* protist assemblage dominated by phototrophic cells to another dominated by heterotrophic cells, which increased 3 to 30 times. Since the incubations were unamended, there was the possibility that bacteria might not be stimulated and that flagellates consequently might not grow, as occurred in samples from very poor areas of the Central Indian (Table 1) or Atlantic Oceans (Jürgens et al. 2000). In most instances, however, growth was obtained. The main drawback of our approach is that these events are short-lived and unstable: flagellate numbers decrease rapidly after their maximal abundance, likely because of prey exhaustion. If cells of uncultured protists develop, these incubations can hardly lead directly to successful isolations. Instead, they can be used to infer metabolic and kinetic features of uncultured protists, and provide information and become the first stages for more focused isolating attempts.

The significant increase in HF in our unamended incubations (Table 1) was paralleled by an increase in MAST-1 and/or MAST-4 cells. These organisms are yet uncultured, but recent studies indicate that they are heterotrophic flagellates with a global distribution and significant abundance in the world's oceans (Massana et al. 2006). It seemed that MAST-1 cells were favored in colder systems, and MAST-4 in warmer systems, but more data are needed to support this observation. These cells also appear to grow in North Atlantic water incubated without prefiltration (Countway et al. 2005): even though FISH counts were not provided, the authors reported a marked change in diversity and MAST-1 and -4 sequences were still found after 3 days of incubation. A closer look at the incubations from the Norwegian Sea by DGGE, clone libraries and FISH revealed that a diverse assemblage of MAST cells was growing in each case. However, the same MAST-1B cell appeared to be the most important in the 3 cases, and 2 of the incubations (Polar and Atlantic) revealed very similar dynamics. This is surprising given the particularity of each initial sample (with coastal, polar and Atlantic influences) and

the geographic distance between them (hundreds of nautical miles).

A detailed analysis of clone libraries revealed which protists besides MASTs could grow in the incubations. Other uncultured groups common in picoplankton surveys were retrieved, such as the marine alveolates Groups I and II. The trophic role of these cells remains unknown; however, it has been suggested that most of them are parasites (Groisillier et al. 2006). This deserves further research, because the growth of parasites in our exclusively picoplanktonic incubations was unexpected. A significant set of sequences belonged to chrysophytes, a group known to contain phototrophic, mixotrophic and heterotrophic flagellates, but they did not affiliate with any of the phylogenetic clusters described so far (Andersen et al. 1999). Interestingly, these uncultured chrysophytes are also seldom found in environmental surveys, highlighting the large extent of protist diversity and the different picture obtained depending on the window of observation. Finally, a few sequences were affiliated with known heterotrophic flagellates, such as choanoflagellates, cercozoans and *Telonema*, whereas ciliate sequences likely derived from cell breakage during the initial prefiltration.

Clone libraries are essential to obtain a phylogenetic description of the microbial taxa in a given sample, but provide poor estimates of actual abundance owing to PCR biases (von Wintzingerode et al. 1997, Stoeck et al. 2006) and the varying copy number of the rRNA operon in different organisms (Zhu et al. 2005). In our study, we prepared clone libraries using 2 primer sets from the same samples. Some sequences were found with both sets, but others were not. The primer bias detected here was not as extreme as described recently (Stoeck et al. 2006), because the most frequent OTUs were generally found in the 2 libraries and most non-shared OTUs also had low clonal abundance (so their uniqueness was a consequence of undersampling). When comparing the clonal representation (even in these libraries with only 30 to 50 clones) of MAST groups with their true cell abundance estimated by FISH, it is clear that the 2 primer sets did not perform equally. The first set (Fig. 5a) gave a very biased view of MAST presence, whereas the second set (Fig. 5b) gave a good correlation where all groups were similarly overrepresented. In summary, we see the clone libraries as the only way to obtain a list of taxa in a given assemblage, with the use of different primer sets improving the length and accuracy of the list; however, the quantification of each taxa requires other methods such as FISH.

We used 5 FISH probes to follow the dynamics of the corresponding MAST cells in incubations from the Norwegian Sea. These 5 groups have been shown to be heterotrophic flagellates with mean global abundances of 5 to 10 (MAST-1A and -2), 30 to 40 (MAST-1B and -1C) and 130 cells ml^{-1} (MAST-4) (Massana et al. 2006). MAST-1B cells were the most successful in the 3 incubations (sequence analyses suggest that they were always the same genotype, both within and among incubations), followed by MAST-1A cells (only 1 genotype) and MAST-1C cells (2 different genotypes). MAST-2 cells grew moderately and MAST-4 cells peaked before the other groups, which could explain why they were not detected in clone libraries. Thus, the FISH data identified the particular dynamics of each MAST group, with different groups peaking at different times or developing only in given incubations. The growth rates of MAST cells ranged from 0.4 to 1 d^{-1}, values that are several times lower than the maximal growth rates of cultured strains (3 to 6 d^{-1}, Fenchel 1986), even if corrected for differences in experimental temperatures (Montagnes et al. 2003). In fact, the measured growth rates of MAST cells were remarkably similar to growth rates of marine HF assemblages, both in this study (Table 1) and others (summarized by Laybourn-Parry & Parry 2000). This indicates that MAST cells have growth dynamics typical of *in situ* heterotrophic flagellates.

We observed the growth of uncultured protists in unamended incubations prepared to promote the development of HF with coastal and offshore surface waters from the Mediterranean Sea, the Norwegian Sea and the Indian Ocean. These protists, including different MAST cells, have been found in molecular surveys from distant oceanic areas, indicating a global distribution, but have not yet been retrieved in culture. Incubations such as those performed in this study help to assess some of their biological properties and growth requirements, and will be used as first steps in more focused culturing attempts. This experimental protocol could in principle be used also for other uncultured protists, both heterotrophic and phototrophic.

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