

Distribution of the uncultured protist MAST-4 in the Indian Ocean, Drake Passage and Mediterranean Sea assessed by real-time quantitative PCR

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Summary

Molecular surveys of marine picoeukaryotes have revealed a large number of sequences unrelated to cultured organisms, such as those forming the marine stramenopile (MAST)-4 clade. Recent FISH (fluorescent *in situ* hybridization) data have shown that MAST-4 cells are uncultured heterotrophic flagellates of 2–3 µm in size that have a global distribution in non-polar marine waters. However, FISH is time-consuming and hard to apply to the many samples generated during oceanographic cruises, so we developed a real-time quantitative polymerase chain reaction (Q-PCR) protocol to determine rapidly the abundance of this group using environmental DNA. We designed a primer set targeting the 18S rRNA genes (rDNA) of MAST-4 and optimized and calibrated the Q-PCR protocol using a plasmid with the target sequence as insert. The Q-PCR was then applied to quantify MAST-4 rDNA molecules along three marine transects, longitudinal in the Indian Ocean, latitudinal in the Drake Passage and coastal–offshore in the Mediterranean Sea, and to a temporal study in a Mediterranean Sea coastal station. MAST-4 was detected in all samples processed (averaged abundances between 500 and 1000 rDNA molecules ml⁻¹) except in mesopelagic and Antarctic samples, where it was virtually absent. In general, it was more abundant in the coast than offshore and in the deep chlorophyll maximum than at surface. A comparison of Q-PCR and FISH signals in well-controlled microbial incuba-

tions indicated that MAST-4 cells have around 30 copies of the rDNA operon. This Q-PCR assay quickly yielded quantitative data of uncultured MAST-4 cells and confirmed their wide distribution and putative ecological importance.

Introduction

Marine heterotrophic flagellates are small unpigmented protists that are the main picoplankton grazers and nutrient remineralizers in aquatic ecosystems (Fenchel, 1986). Together with viruses (Suttle, 2005), they maintain picoplankton populations at relatively stable abundances in seawater (Pernthaler, 2005). Heterotrophic flagellates are routinely quantified by epifluorescence microscopy after DAPI (4,6-diamidino-2-phenylindole) staining (Porter and Feig, 1980), but this reveals few morphological features, so they remain generally unidentified. Recent molecular surveys are providing new insights into the phylogenetic affiliation of these minute protists. Clone libraries of 18S rDNA from marine eukaryotic picoplankton have revealed a large *in situ* diversity and the existence of novel groups unrelated to cultured protists (Díez *et al.*, 2001; López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001). Among them, marine stramenopiles (MAST) appear recurrently and include more than 10 different phylogenetic lineages affiliating among the basal stramenopile groups (Massana *et al.*, 2004). Oligonucleotide probes against several MAST lineages have been designed (Massana *et al.*, 2002; 2006a) and used by FISH (fluorescence *in situ* hybridization) to identify their morphology and infer their trophic mode. The MAST cells resulted to be heterotrophic flagellates of various sizes (from 2 to 8 µm), able to grow in the dark and graze bacteria. Further studies of their distribution and abundance revealed that they were globally distributed, and a single group, MAST-4, contributed to 9% of heterotrophic flagellates in all surface marine systems except polar waters (Massana *et al.*, 2006a).

Thus, MAST-4 is an abundant protistan group in marine ecosystems and likely contributes to picoplankton grazing and nutrient remineralization. Concerted efforts without success until now are being made to obtain a represen-

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tative in culture. So far, MAST-4 has been quantified by FISH, which is very reliable but too time-consuming to process the large number of samples generated during oceanographic cruises. For this reason, we developed a quantitative polymerase chain reaction (Q-PCR) assay, based on the 18S rDNA, to quantify this protistan group in marine samples. This technique allows an accurate estimation of the copy number of a target gene in a given DNA extract by measuring the increase of its PCR product in real time. Three different detection systems are generally used: the SYBR Green I stain that binds unspecifically to double-stranded DNA (Schneeberger *et al.*, 1995) and the more specific 'Taqman' and 'Molecular Beacon' technologies (Walker, 2002). The Q-PCR was initially developed for clinical applications (Heid *et al.*, 1996), and recently has been applied to marine research. Several studies have dealt with planktonic prokaryotes, targeting broad bacterial groups (Suzuki *et al.*, 2000), an uncultured bacteria (Labrenz *et al.*, 2004), and several ecotypes of marine picocyanobacteria (Becker *et al.*, 2002; Ahlgren *et al.*, 2006; Johnson *et al.*, 2006). Other Q-PCR studies quantified marine protists, always targeting cultured organisms: the prasinophytes *Bathycoccus*, *Micromonas* and *Ostreococcus* (Zhu *et al.*, 2005; Marie *et al.*, 2006; Countway and Caron, 2006), the dinoflagellates *Pfiesteria piscicida* (Bowers *et al.*, 2000) and *Alexandrium minutum* (Galluzzi *et al.*, 2004) and the alveolate *Perkinsus marinus* (Audemard *et al.*, 2004).

The aim of this study was to develop a fast and sensitive technique to assess the abundance of the uncultured heterotrophic flagellate MAST-4 in large sets of environmental samples, based on the Q-PCR detection of its 18S rRNA gene. This technique takes advantage of the DNA extracts stored in our lab and collected in oceanographic cruises done during the last 10 years. One problem when targeting uncultured protists is the variable copy number of the rDNA operon in different species (Prokopowich *et al.*, 2003; Zhu *et al.*, 2005). To estimate this value, we prepared unamended seawater incubations (Massana *et al.*, 2006b), where we expected to find varying amounts of MAST-4 cells, and sampled them carefully to compare Q-PCR and FISH results in the best conditions. To our knowledge, this is the first application of Q-PCR to assess the distribution and abundance of an uncultured protist and provides a general view of its broad distribution.

Results

Optimization of Q-PCR for MAST-4

DNA cleaning for optimal amplification. The different DNA extracts (from oceanic cruises, incubations or plasmids) were used as templates for Q-PCR with the specific 18S rDNA MAST-4 primer set. Amplifications were posi-

tive with the plasmid but initially negative with environmental samples. In order to eliminate potential PCR inhibitors, DNA extracts were further purified with an ethanol precipitation step. Virtually all environmental samples yielded positive amplifications after this cleaning step. To check for potential DNA loss during precipitation, the plasmid was processed before and after precipitation (two replicates from seven dilutions), and no significant differences in the quantification were seen (data not shown).

Q-PCR efficiency and melting curve. After processing the DNA extracts with the cleaning step, the efficiencies of each Q-PCR run were calculated by comparing the signal in the different dilutions of the standard (plasmid) and the relative standard (one environmental sample). Both efficiencies were always close to 100% (98% on average; range of 93–101% in the standard and of 94–102% in the relative standard). Moreover, in the same run the efficiencies of both standards were highly similar (0.8% difference on average, 3.2% being the maximal difference). Thus, the plasmid standard could be used to quantify MAST-4 sequences in environmental samples. To confirm that the measured PCR product derives from the target sequence, a melting curve was done after the last cycle in all tubes. A single peak at 84°C appears in all samples, implying that the fluorescence readings derive integrally from the specific PCR product. Primer-dimer signals only appeared in some tubes with milliQ water or with environmental samples that did not amplify. Non-specific PCR products [melting temperature (T_m) = 89°C] were only observed in the coldest samples from the Drake passage and were considered negative. In fact, the size of this PCR product was around 700 bp (as measured in an agarose gel), confirming that they were clearly non-target amplicons.

DNA storage and thawing tests. Some of the DNA extracts processed by Q-PCR were kept at -80°C during almost 10 years and defrost several times for particular analysis, with the risk of an unknown level of DNA degradation. The possible effect of freeze-thaw cycles on DNA degradation and Q-PCR results was studied on three samples, the plasmid and two environmental samples. These were thawed, an aliquot taken for Q-PCR, frozen again at -80°C and the process repeated several times. All samples were then processed in the same Q-PCR run (Fig. 1). Data were arctan-transformed, tested for normality (Kolmogorov-Smirnov test) and heteroscedasticity (Bartlett test), and then processed using a repeated measures ANOVA with the STATISTICA software (version 5.1; StatSoft). The two environmental samples did not show any sign of DNA degradation, as the number of MAST-4 molecules was always similar even when defrosted

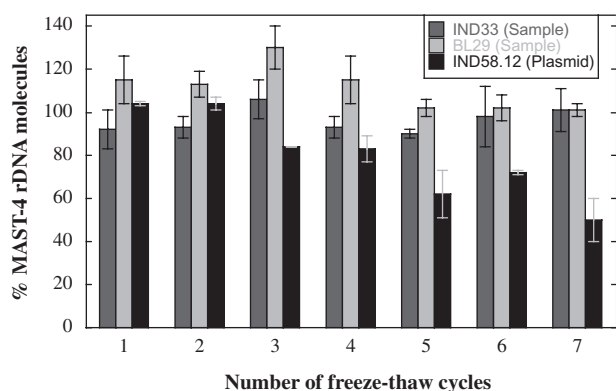


Fig. 1. Effect of freeze/thaw cycles on DNA recovery of the standard (plasmid IND58.12) and environmental samples from the Indian Ocean (IND33) and Blanes Bay (BL29). Shown are the mean (and SE; $n = 3$) of the percentage of MAST-4 rDNA molecules assessed by Q-PCR after several cycles relative to the initial quantification.

seven times (IND33, $F = 0.45$, $p = 0.89$; BL29, $F = 1.77$, $p = 0.16$). On the other hand, DNA degradation occurred in the plasmid in the last cycle (IND58.12, $F = 7.36$, $p = 0.006$; the *post hoc* test Scheffe detected significant differences between the initial cycle and the last cycle). To prevent a biased calibration the plasmid was never used as standard after being defrosted twice.

Specificity of the primer set. The specificity of the MAST-4 primer set was assessed with 18S rDNA clones from the Indian Ocean libraries, including two MAST-4 (with one mismatch with primer M41f) and seven non-target clones representative of the groups with highest clonal abundance (dinoflagellates: 14%; marine alveolate-I: 25%; marine alveolate-II: 11%; MAST: 11%; radiolaria: 15%) (Not *et al.*, 2008). The pelagophyte clone was included because it presented the lowest number of mismatches with a given primer (two with the reverse primer). These clones were compared with the standard (perfect match with both primers), by adding the same number of rDNA molecules (10^8 copies) and quantifying the MAST-4 molecules after the Q-PCR run. The positive clones with one mismatch amplified close to 100% whereas the negative clones amplified always less than 0.01% of the molecules added and often did not amplify at all (Fig. 2), showing that the primer set was highly specific for MAST-4 sequences.

Spiking experiments. These were done to address two questions: the putative presence of PCR inhibitors in samples with negative amplification, and the putative inhibition of the PCR when increasing non-target DNA. For the first question, a known amount of target molecules (standard plasmid) was added to three environmental samples that did not amplify. In these mixtures,

the number of target molecules quantified by Q-PCR was similar to the molecules added (data not shown), indicating that the negative amplification in the original samples was not due to the presence of inhibitors but to the absence of target sequences. For the second question, four different mixtures with known and variable amounts of target and non-target molecules were prepared. Again, the target molecules quantified by Q-PCR in these mixtures were similar to the molecules added (data not shown). So, the TaqDNA polymerase did not modify its efficacy depending on the proportion of target DNA.

Overall success of the method. A total of 214 samples were processed in 22 PCR runs. Most of them gave a good quantification the first time (144 samples), whereas others had to be repeated to obtain a reliable estimate (26 samples). Some gave a clear negative signal, indicating the absence of target sequences (34 samples, 22 with no amplification and 12 with an unspecific PCR product). Finally, a few samples (10 samples) appeared positive but the signal was too low to be properly quantified. This quantification success was related to the amount of target molecules in the sample. We assumed a detection limit of our protocol of 100 molecules per PCR tube (negative milliQ samples yielded between 0 and 90 molecules, 10 on average). This translates that the natural sample needs to have at least 20 molecules ml^{-1} for a proper quantification, assuming the volumes typically processed (10 l of seawater and 200 μl of DNA extract).

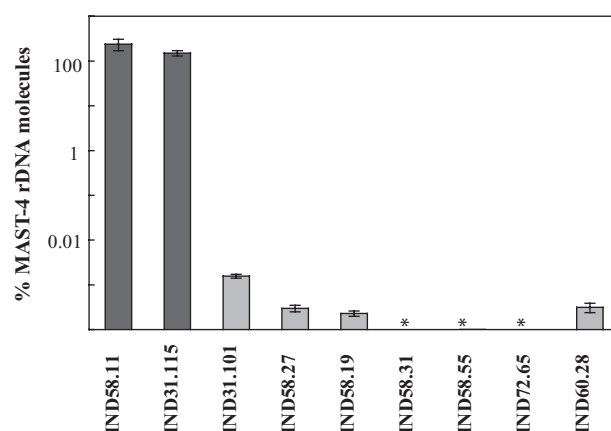


Fig. 2. Percentage (mean and SE; $n = 4$) of MAST-4 rDNA molecules (with respect to rDNA molecules added) recovered from target and non-target clones: IND58.11 (MAST-4; 1 mismatch with M41f and 0 mismatch with NS4), IND31.115 (MAST-4; 1 and 0), IND31.101 (dinoflagellate; 8 and 5), IND58.27 (marine alveolate-I; 8 and 6), IND58.19 (MAST-3; 6 and 5), IND58.31 (MAST-1C; 12 and 3), IND58.55 (marine alveolate-II; 14 and 9), IND72.65 (radiolaria; 15 and 5), IND60.28 (pelagophyte; 10 and 2). Samples with an asterisk present negative amplification.

Quantification of MAST-4 molecules in environmental samples

The MAST-4 rDNA molecules were quantified by Q-PCR using DNA extracts from three oceanographic cruises (Fig. 3A). The first cruise was a transect from South Africa to Australia crossing the Indian Ocean Gyre, where 15 stations were sampled at five to six depths (from 5 to 1000 m). The second cruise was a coastal-offshore transect crossing the northern part of the Western Alboran Gyre, where three stations (shelf, slope and deep) were sampled at five depths (from 5 to 500 m). The third cruise was a transect across the Polar Front in the Drake Passage, from South Atlantic to Antarctic waters. This transect was formed by nine stations sampled at three depths (from 5 to 60 m).

The abundance of MAST-4 rDNA molecules in the Indian Ocean was higher in the coastal samples and lower in the centre of the transect, in the most oligotrophic central gyre (Fig. 3B). Regarding the vertical gradient, MAST-4 was present all along the photic zone, being more abundant around the DCM (deep chlorophyll maximum) and with low values in the upper aphotic zone, represented here by the 200 m depth. The figure only shows data from surface to 200 m, as deeper samples (between 650 and 1000 m) generally gave negative amplification. The abundance of MAST-4 in the Alboran Sea shows a similar tendency, being more abundant at the coastal station than the offshore station (Fig. 3C). Similarly, MAST-4 also occupies the whole photic zone, being more abundant at 50–100 m depth than at surface, and being very low (250 m) or virtually absent (500 m) in the aphotic zone. Considering both cruises, 11 of the 15 deeper samples (500 to 1000 m) gave clear negative signal, and the remaining

four gave unclear signals. The pattern found in the Drake Passage was remarkably different from the other two cruises (Fig. 3D). The MAST-4 molecules were only detected in the two northernmost stations, those with warmer temperatures (5.5°C and 5.8°C at the surface of stations 30 and 32 respectively). In the other stations, with temperatures below 5°C, target molecules were undetected. Here, only the upper water column was quantified, so no clear vertical pattern could be described.

Besides the three oceanographic cruises, samples from a coastal Microbial Observatory were also analysed. These were collected monthly at the surface of Blanes Bay (North-western Mediterranean), with a total of 62 samples during 6 years (Fig. 4). A clear seasonal pattern was not observed, and abrupt changes occurred between consecutive dates. What was apparent, though, was an important interannual variation, with some years (2002 and 2005) with low mean abundance (350 molecules ml⁻¹) and other years (2003 and 2004) with high mean abundance (1450 molecules ml⁻¹). The other 2 years (2001 and 2006) had an intermediate mean abundance (800 molecules ml⁻¹).

An overview of the abundances of MAST-4 in the four marine systems investigated is shown in Table 1. The average abundances of MAST-4 in the Indian Ocean, Alboran Sea and Blanes Bay, systems with comparable mean temperatures (16–24°C), were similar (between 500 and 1000 molecules ml⁻¹). MAST-4 seems to be more abundant near the coast than at the open sea (see Fig. 3B and C). Also, Table 1 shows that the abundance of MAST-4 is higher (almost twice) in the DCM as compared with the surface (5 m). The upper photic region (200–250 m) generally gives clear signals, although very low (40–50 molecules ml⁻¹), whereas the lower mesope

Table 1. Molecule and cell numbers quantified by Q-PCR and FISH respectively from the different places analysed in this study and their relationship with the temperature.

	18S rDNA molecules ml ⁻¹			Cells ml ⁻¹			Temperature (°C)	
	Mean	Range	<i>n</i>	Mean	Range	<i>n</i>	Mean	Range
Indian Ocean								
Above DCM	504	52–2484	28	98	82–118	5	24	21–24
DCM	864	91–2845	14	155	79–262	3	22	19–25
Upper aphotic (200 m)	48	0–285	13	0	0	3	16	8–20
Alboran Sea								
Surface (5 m)	706	469–826	3				16.5	16.5
Subsurface (50–100 m)	954	320–2205	6				15	14–16.5
Upper aphotic (250 m)	38	21–48	3					
Drake Passage								
Above SAF (5–100 m)	150	31–351	6				5.7	5.5–5.8
Below SAF (5–60 m)	0	0	20				1.3	(–1)–4.4
Blanes Bay								
Surface (5 m)	926	34–4500	62	91	18–244	26	17.6	11–26

SAF, Sub Antarctic Front.

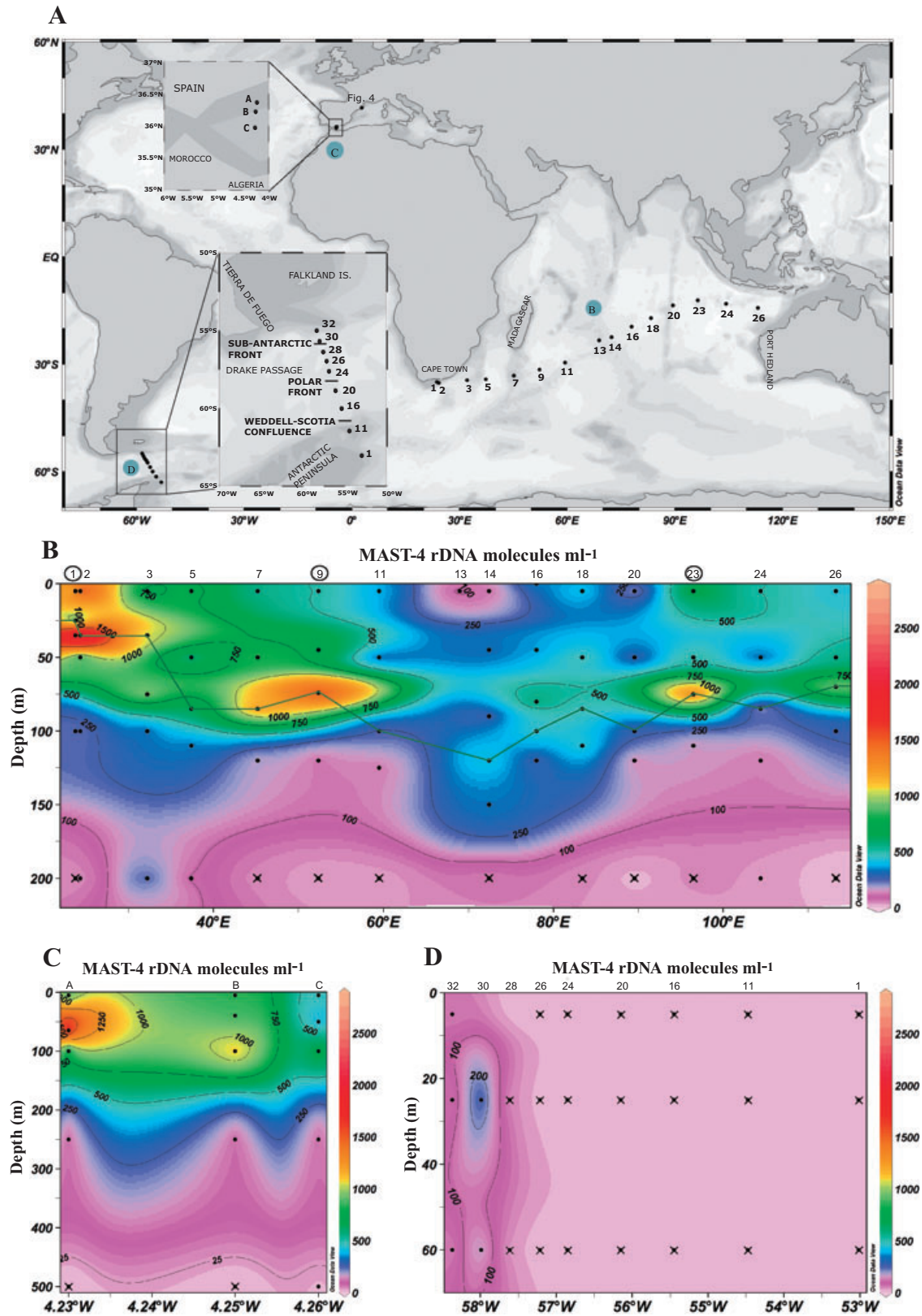


Fig. 3. Overview of the marine systems investigated (A) and abundance of MAST-4 (18S rDNA molecules ml⁻¹) at several depths in three of them: (B) Indian Ocean transect. The green line marks the DCM and stations analysed by FISH are encircled. (C) Alboran Sea transect. (D) Drake Passage transect. Samples with a cross in (B), (C) and (D) indicate negative amplification.

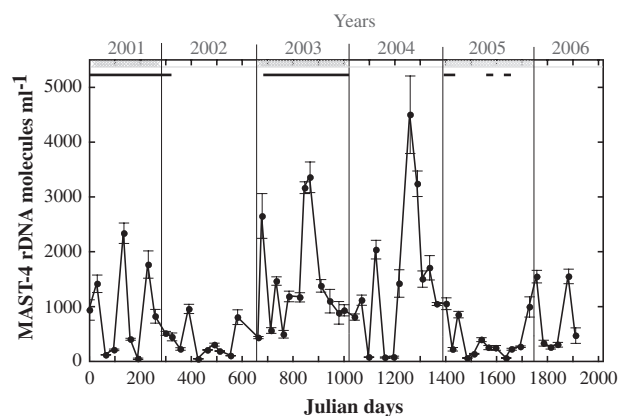


Fig. 4. Abundance (mean and SE; $n = 4$) of MAST-4 molecules in Blanes Bay during a 6-year study sampled monthly. Black lines at the top of the figure mark the periods where FISH data were also obtained.

logic region generally gives no or very low amplification. Finally, MAST-4 was absent from the cold Antarctic waters below 5°C and present at low abundances (150 molecules ml^{-1}) north of the South Atlantic Front.

Comparison of Q-PCR and FISH quantifications

The FISH counts were done in a subset of samples from Blanes Bay (monthly during years 2001 and 2003 and some more during 2005, Fig. 4) and from the Indian

Ocean (vertical profiles at stations 1, 9 and 23, Fig. 3). Except the deepest Indian samples investigated (200 m), all samples yielded a significant MAST-4 count by FISH, with concentrations ranging from 18 to 244 cells ml^{-1} (Table 1). Correlating Q-PCR and FISH estimates for these two data sets was moderate, with a R^2 of 0.47 for Blanes samples (Fig. 5A) and of 0.65 for Indian samples (Fig. 5B). The slope of these correlations, representing the rDNA copy number per MAST-4 cell, was estimated to be 11 and 6 respectively.

In the unamended seawater incubations, samples to be processed by Q-PCR were collected to maximize DNA recovery, in contrast with environmental samples that were generally collected to optimize DNA quality (see *Discussion*). In the Indian Ocean incubations, MAST-4 peaked after 2 days (2×10^4 molecules ml^{-1}), whereas in the Blanes Bay incubations the peak occurred the fourth day (3×10^4 molecules ml^{-1}) (data not shown). As expected in these incubations, the abundance of MAST-4 molecules decreased after the peak to very low levels in both cases. The comparison of Q-PCR signal and FISH counts was much better with these samples, with a R^2 of 0.95 for Blanes samples (Fig. 5C) and of 0.99 for Indian samples (Fig. 5D). The number of the rDNA copies per cell was estimated to be 29 in the Blanes and 37 in the Indian incubation data sets. Interestingly, in an additional incubation performed in Blanes Bay on July 2005, the comparison of Q-PCR and FISH data yielded an rDNA copy number of 22 ($R^2 = 0.99$; $n = 4$) (data not shown).

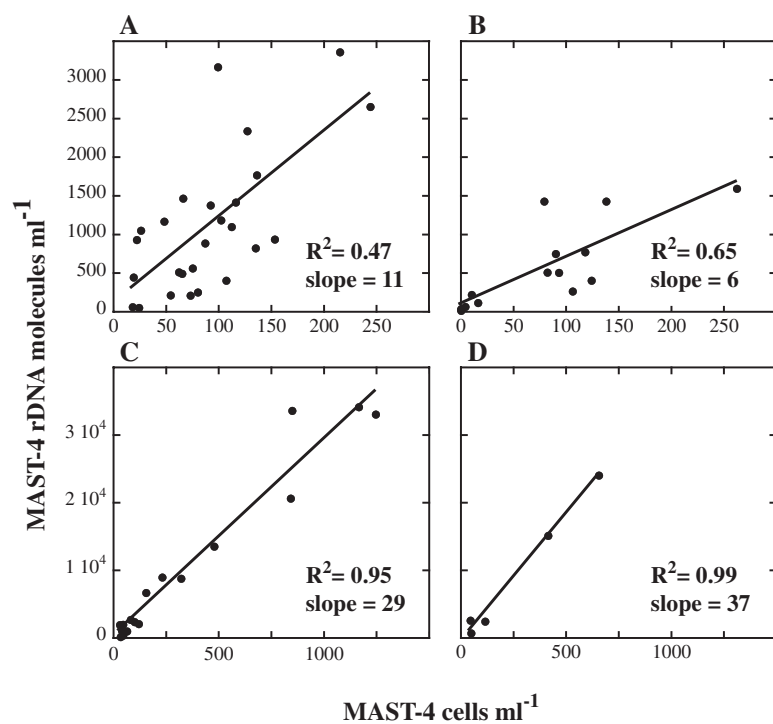


Fig. 5. Relationship between the MAST-4 signal estimated by Q-PCR (rDNA molecules ml^{-1}) and by FISH (cells ml^{-1}) in environmental samples from the Mediterranean Sea (A) and the Indian Ocean (B), and samples from unamended seawater incubations from the Mediterranean Sea (C) and the Indian Ocean (D).

Discussion

Quantification of an uncultured protist taxa by Q-PCR

Here we have optimized, calibrated and validated a Q-PCR protocol to assess the abundance in marine waters of the uncultured protist MAST-4. Whereas some previous studies have applied Q-PCR for uncultured marine prokaryotes, such as *Sulphurimonas denitrificans*-like (Labrenz *et al.*, 2004), our study represents the first application of Q-PCR to uncultured protists, whose presence has been only inferred by molecular tools (environmental sequencing and FISH probing). The main difference when dealing with uncultured organisms is that the standard to calibrate the Q-PCR signal cannot be cultured cells. Here we have used a plasmid carrying the target sequence and, therefore, target molecules are quantified instead of target cells. To convert the abundance of rDNA molecules to cells, the rDNA operon copy number in the genome must be known, and this can be derived by comparing the Q-PCR and the FISH signals from the same samples (see later).

The reliability of the Q-PCR data depends on several critical aspects, such as the quality of the DNA extract, the specificity of the amplified PCR product and obtaining optimal amplification efficiencies (Cankar *et al.*, 2006). In our study, it has been essential to further purify the DNA extracts from environmental samples, which did not amplify initially, by a DNA precipitation step with ethanol. The plasmid DNA samples, extracted with a different system, did not need the precipitation step to amplify properly. This confirms that different extraction methods can influence the purity of the DNA and have a great impact on the results obtained by Q-PCR (Peano *et al.*, 2004). After this cleaning step, the regular Q-PCR checks gave very satisfactory results. Thus, the primer set used was highly specific for MAST-4, the efficiencies of the standard and the environmental samples were similar and close to 100%, and the melting curve analysis indicate that a single PCR product ($T_m = 84^\circ\text{C}$) was always generated in the samples with positive signal.

Moreover, once the Q-PCR protocol was stabilized, we performed additional tests to assess its applicability on environmental samples. First, we studied the influence of freeze/thaw cycles on the percentage of recovered target molecules, as it is known that this process can compromise the integrity of DNA (Bellete *et al.*, 2003). Here we show that up to seven cycles do not affect the MAST-4 quantified in environmental samples. This result gives confidence that the numbers obtained are realistic despite years of storage of the DNA extracts, including several thawing events. This might be due to the use of a very short amplicon (only 188 bp), which would still be amplified properly even if some DNA breakage occurs. Other studies have shown the stability of DNA extracts during

long-term storage (Jerome *et al.*, 2002). Second, with the spiking experiments we demonstrated the absence of PCR inhibitors in the negative samples (so they really lacked target molecules) and that the same number of target molecules amplify roughly equally even when diluted with a lot of non-target DNA (Bellete *et al.*, 2003). Thus, the Q-PCR protocol presented here appears robust and adequate to quantify the rDNA molecules of MAST-4 in marine samples.

Q-PCR and FISH comparison and estimated rDNA copy number

There is a very good correlation between Q-PCR and FISH signals in the incubation samples, particularly robust in the Blanes incubation (Fig. 5C). With these results we can estimate that MAST-4 cells have around 30 copies of the rDNA operon. This value is fundamental to interpret the Q-PCR results targeting the rDNA genes, specially in the light that the rDNA copy number can vary orders of magnitude in protists, from 1–4 (some green algae) to more than 10 000 (some dinoflagellates). Comparing different eukaryotic species, a strong correlation has been found between the rDNA copy number and genome size (Prokopowich *et al.*, 2003) and cell length (Zhu *et al.*, 2005). Having around 30 copies of the rDNA operon (a comparatively low number) in MAST-4 cells is consistent with its small size, 2–3 μm in diameter (Massana *et al.*, 2006a), and fits well within the relationship described for 18 phytoplankton strains (Zhu *et al.*, 2005).

The correlation between Q-PCR and FISH signals in environmental samples is considerably less robust (Fig. 5A and B) and the copy number is lower (around 10). A possible explanation for this noisier signal is that the distinct MAST-4 lineages in the environmental samples (the probes target a phylogenetic group with up to 3% divergence in the complete 18S rDNA) also vary in their rDNA copy number, whereas the incubations are likely selecting a single genotype. Nevertheless, we consider that the main cause of the larger variability and the lower rDNA copy number in the environmental samples was that they were not collected to be quantitatively processed (as, for instance, detailed in Boström *et al.*, 2004). First, in some samples the volume of seawater filtered and the volume of DNA extract obtained were only approximate. Second, samples were collected on encapsulated Sterivex filters that are known to be less efficient in DNA recovery. We have seen that Sterivex units might recover only half of the DNA quantity as compared with regular filters (R. Massana, unpubl. data). Third, during the DNA extraction, emphasis was done on the quality of DNA and this surely caused significant DNA losses, particularly during the phenolization step. These factors could cause a less efficient and inexact DNA recovery, therefore

yielding a lower and noisier Q-PCR signal. In fact, when these issues were properly addressed in the incubation samples, both techniques correlated very well and the rDNA copy number was higher. So, the Q-PCR is very suitable to quantify MAST-4 as long as it is combined with a careful sample collection and DNA extraction. Taking into account the constraints of applying Q-PCR with our environmental samples, it is clear that the data obtained provides interesting insights into the distribution and abundance of MAST-4 (Fig. 3). Samples were processed similarly, so they can be compared to provide useful global views of MAST-4 distributional patterns (presence and predominance). In addition, the data generated can be regarded as minimal estimates of the abundance of MAST-4 molecules.

Distribution of MAST-4 in the oceans

In a previous study, 24 surface samples from different world oceans were processed by FISH to estimate the abundance of MAST-4 cells (Massana *et al.*, 2006a). This protist appeared in all samples investigated, except the polar ones, with averaged abundances of 131 cells ml⁻¹. The Q-PCR protocol allows a faster sample processing, so we have increased the number of samples analysed by one order of magnitude (214 samples), addressing new aspects such as the depth distribution or the interannual variation. Our data confirm and expand the FISH data indicating that MAST-4 is a widespread protist and, interestingly, also finds some environmental constraints that limit this broad distribution.

The vertical profiles in the Alboran Sea and the Indian Ocean show that MAST-4 is found in the upper ocean, including the photic zone and the upper aphotic zone. MAST-4 seems to be more abundant at subsurface (near the DCM) than at surface (5 m). Perhaps there is a negative effect of UV light at surface (Moran and Zepp, 2000) or simply the DCM is a more active and convenient habitat for MAST-4. Conversely, MAST-4 was hardly found in the deeper aphotic region, which is in accordance to the fact that heterotrophic flagellates and their bacterial food are becoming more scarce in mesopelagic waters (Tanaka and Rassoulzadegan, 2002; Fukuda *et al.*, 2007). So, if present, MAST-4 cells were likely below the detection limit of Q-PCR. In fact, the three samples quantified by FISH at 200 m in the Indian Ocean did not reveal any target cell (abundance < 2 cells ml⁻¹). Moreover, cloning and sequencing of mesopelagic and bathypelagic waters has never retrieved a MAST-4 sequence (López-García *et al.*, 2001; Countway *et al.*, 2007; Not *et al.*, 2007).

Seawater temperature seems to be a second constraint in the distribution of MAST-4. In a previous study, this protist could not be detected by FISH in polar samples (Massana *et al.*, 2006a) and here we had a unique oppor-

tunity to identify its real boundary by analysing a transect in the Drake Passage from South Atlantic to Antarctic waters. Indeed, the coldest samples from this transect, with temperatures up to 4°C, gave negative signal for MAST-4. Only the northernmost stations of the transect, with warmer temperatures (5–6°C), show MAST-4 signal, although rather low as compared with that from the other systems. Thus, MAST-4 seems to be excluded in polar waters below 5°C, an intriguing feature shared by other microorganisms such as marine picocyanobacteria (Partensky *et al.*, 1999).

So, combining the FISH data with the Q-PCR data it appears that MAST-4 is a structural component of protist assemblages in marine temperate photic waters. Virtually all samples from epipelagic waters (surface to 120 m) and with temperatures above 5°C have MAST-4 molecules. This broad and systematic presence is shared with several marine bacteria like SAR11 (Morris *et al.*, 2002), and *Roseobacter* (Selje *et al.*, 2004) and could be a common trait of smallest marine protists, as clone libraries of picoeukaryotes retrieve similar groups in distant oceans (Epstein and López-García, 2008). There are few reports on the abundance and distribution of picoeukaryotes at large oceanographic scales. Recently, small prasinophytes have been studied (Not *et al.*, 2005; Not *et al.*, 2008), and they change orders of magnitude from the coast, where they are more abundant, to the open sea. For larger protists, those that can be identified by microscopy, it is well known that they are not always present in marine samples, and temporality seems to be extremely important. Nevertheless, we have to keep in mind the significant phylogenetic diversity of MAST-4. So, although they look the same by FISH (Massana *et al.*, 2006a), they can include distinct lineages with different and complementary ecological adaptations that might explain this broad distribution, as has been proposed for other picoeukaryotes (Rodríguez *et al.*, 2005).

Sampling a coastal station allows a detailed temporal assessment of microbial dynamics. This cannot be easily done in the open sea, where each cruise represents a single temporal snapshot. We could not identify a seasonal pattern for the abundance of MAST-4 in the Mediterranean coastal station. There was a large variation (up to 3000 molecules ml⁻¹) between consecutive dates, and averaging different periods did not yield systematic trends. Probably MAST-4 varies on a shorter time scale and sampling only once a month does not properly describe its temporal variation. Data from the incubation experiments indicate that MAST-4 might be a typical r-strategist that responds with high growth rates to increase in prey and declines rapidly when prey diminishes. This could explain the irregular peaks during the season. Surprisingly, we detected important interannual variations, and we could not explain these by the environmental parameters currently taken,

such as temperature, salinity, inorganic nutrients, chlorophyll or microbial counts.

In summary, we present a very robust Q-PCR protocol for a fast quantification of rDNA molecules of the uncultured protist MAST-4. The extent that this protocol gives absolute abundance on environmental samples depends on the care with which sample collection and DNA extraction were done. The application of this protocol to a large sample collection from different oceanographic cruises, including some where only DNA extracts were available, yields a global vision of the distribution of this taxon. MAST-4 appears as a constitutive member (always found) of most marine systems and also identifies some habitats where it is excluded, such as mesopelagic and polar waters.

Experimental procedures

Environmental DNA from marine assemblages of small protists

Samples from three oceanographic cruises were taken at different depths with Niskin bottles attached to a CTD rosette. Alboran Sea samples were collected on 2–4 May 1998 during cruise MTP-II-MATER/HESP/04-98 on board the Spanish RV *Hespérides*. Drake Passage samples were collected on 6–14 December 1998 during cruise DHARMA on board RV *Hespérides*. Indian Ocean samples were collected on 16 May to 11 June 2003 on board RV *Melville* (Scripps Institution of Oceanography, US). Some physico-chemical (temperature, salinity, inorganic nutrients, chlorophyll) and biological data have already been published for the Alboran Sea (Arin *et al.*, 2002), Drake Passage (Díez *et al.*, 2004) and Indian Ocean (Not *et al.*, 2008) cruises. We also collected surface samples from the Blanes Bay Microbial Observatory in the Mediterranean Sea (41°40'N, 2°48'E), monthly from March 2001 to June 2006. Samples (5–20 l) were first prefiltered through a 200 µm nylon mesh and then collected in Sterivex filter units of 0.2 µm pore size (Durapore; Millipore) after being prefiltered through 3 µm (Indian Ocean and Blanes Bay) or 5 µm (Alboran Sea and Drake Passage). Sterivex units were filled up with lysis buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose) and kept frozen (–20°C during the cruises and –80°C afterwards) until DNA extraction.

Unamended incubations were prepared by filtering surface seawater by gravity first through a nylon mesh of 200 µm and second through 3-µm-pore-size polycarbonate filters (Massana *et al.*, 2006b). Subsequently, the filtered seawater was dispensed into Nalgene polycarbonate bottles and incubated at near *in situ* temperature. Two bottles were prepared in the Indian Ocean (this is the Coastal incubation in Massana *et al.*, 2006b), one incubated at ambient light and the other in the dark. Another two bottles were prepared in the Blanes Bay (sampled on 7 March 2006) and incubated in the dark. Mean temperatures for the Indian Ocean and Blanes Bay incubations were 21°C and 13°C respectively. Bottles were sampled every 1–2 days: 100–200 ml of seawater was filtered onto 25 mm Durapore filters of 0.2 µm pore size, submerged in lysis buffer and kept as before.

DNA extraction was done as described before (Massana *et al.*, 2000). Cell lysis was performed by digestion with lysozyme followed by proteinase K and SDS treatments. DNA was purified twice with phenol : chloroform : isoamyl alcohol (25:24:1, pH 8) and once with chloroform : isoamyl alcohol (24:1), desalted and concentrated with a Centricon-100 (Millipore). Special effort was done with the samples from the unamended incubations to maximize the quantity of DNA recovered during the phenolization step. The integrity of the DNA was checked by agarose gel electrophoresis. Nucleic acid extracts were stored at –80°C until they were analysed. To obtain a positive amplification, all the environmental samples needed a DNA precipitation step. The DNA extract was mixed with 2.8 vols of precipitation mix (absolute ethanol, 2 M NaAc and 1 M MgCl₂) and kept more than half an hour at –80°C. Samples were then centrifuged at 14.000 r.p.m. for 15 min, washed twice with ethanol 70%, and re-suspended in milliQ water.

Development and optimization of the Q-PCR protocol

Design of specific primers for 18S rDNA of MAST-4. The forward primer M41f (5'-GTC TGC ACT GGA GTC GG-3') was designed in base of all MAST-4 sequences available so far (34 clones from nine different marine sites). It matches perfectly all these clones, except two from the Indian Ocean (IND 31.115 and IND 58.11) that have one mismatch. It has more than five mismatches to all non-target sequences in GenBank. The reverse primer has the sequence of the probe NS4 designed for FISH (5'-TAC TTC GGT CTG CAA ACC-3'), which matches all target sequences and has at least two to three internal mismatches with all non-target sequences in GenBank (Massana *et al.*, 2002). Primers were optimized using the PerlPrimer software (Marshall, 2004) in order to check the no formation of primer-dimers, the GC content and the theoretical melting temperature. The amplicon was 188 bp of length. The specificity of the primer set was checked by standard PCR. It gave negative amplification for 15 non-target clones and positive amplification for six target clones (including the two clones with one mismatch).

Preparation of the standard plasmid. 18S rRNA genes from an Indian Ocean sample (Not *et al.*, 2008) were amplified with the universal eukaryotic primers EukA and EukB (Medlin *et al.*, 1988) and cloned with the TOPO TA cloning kit (Invitrogen). A clone (IND58.12) having a MAST-4 insert was used as standard for the Q-PCR. Its plasmid was extracted with the Plasmid DNA Purification kit (QIAGEN) and linearized by digesting the supercoiled plasmid with the restriction endonuclease NotI (Sigma). Similar to other studies (Suzuki *et al.*, 2000), we have seen that the Q-PCR signal of the linearized plasmid is 10 times higher than the supercoiled plasmid (data not shown). The plasmid extract was purified with the precipitation step (described before) to process it equally to the environmental samples. The DNA concentration and purity of the plasmid extract were assessed with a NanoDrop (ND-1000 Spectrophotometer). The number of rDNA molecules in the plasmid extract was calculated using the following formula:

$$\text{molecules } \mu\text{l}^{-1} = [a/(5736 \times 660)] \times 6.022 \times 10^{23}$$

where *a* is the plasmid DNA concentration (g µl⁻¹), 5736 is the plasmid length (3931 bp of the vector plus 1805 bp of the 18S

rDNA insert), 660 is the average molecular weight of one base pair and 6.022×10^{23} is the molar constant (Avogadro constant).

Optimization of the Q-PCR conditions. The Q-PCR reactions (final volume of 15 μ l) were done with 0.45 μ l of forward and reverse primers (both 10 μ M), 7.5 μ l of iQ SYBR Green Supermix (Bio-Rad), 5.6 μ l of sterile milliQ water and 1 μ l of DNA template. The reaction mixtures were prepared in thin-wall tubes and cap strips (Bio-Rad) and filter Safeseal-Tips (Biozym), DNase, RNase, Pyrogen-free, inside a UV-sterilized chamber. Reactions were performed in a iCycler iQ Multi-Color Detection System (Bio-Rad) programmed with an enzyme activation step (95°C, 3 min) and 40 cycles of 10 s of denaturation at 94°C, 30 s of annealing extension at 59°C and 30 s of data collection at 72°C. Data were analysed using the Multicolor Real-Time PCR Detection System v 3.1 software (Bio-Rad). These conditions were decided after different tests done with the positive plasmid. The optimal primer concentration (0.3 μ M each) was the one that gave the earliest target amplification and the lowest amount of primer-dimer (nine combinations tested). The optimal annealing-extension temperature (58.9°C) was found by testing a gradient from 55°C to 65°C.

For each Q-PCR run (96 tubes) we prepared three replicates of seven serial dilutions (from 10^8 to 10^2 rDNA molecules) from the standard (IND58.12 plasmid), three replicates of four serial dilutions (10^{-1} – 10^{-5} μ l of DNA extract) from the environmental sample used as relative standard and PCR efficiency control, three negative samples with milliQ water and two replicates of two dilutions (10^{-1} and 10^{-2} μ l of DNA extract; undiluted samples generally did not amplify) from each environmental sample to assay (15 in total). After the Q-PCR run, the number of rDNA molecules in the tubes with environmental samples was obtained. These values were converted to true concentration (molecules ml⁻¹ of seawater) in three steps: (i) considering the dilution factor to find out the number of molecules μ l⁻¹ of DNA extract, (ii) multiplying the later number by the volume of DNA extract (100–300 μ l) to obtain the total number of molecules in the extract and (iii) dividing the later number by the volume of seawater collected (5–20 l).

Melting curve analysis. The SYBR Green I binds all double-stranded DNA, including specific and unspecific PCR products and primer-dimers. These can be distinguished by their different melting temperatures, which depend on their base composition and length. In the melting curve analysis the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At T_m , the two DNA strands separate and the fluorescence rapidly decreases. The software plots the rate of change of the relative fluorescence units (RFU) with temperature (T) ($-d(\text{RFU})/dT$) on the *y*-axis versus the temperature on the *x*-axis, and this will peak at the T_m . A dissociation curve from 55°C to 94°C was measured after the last Q-PCR cycle in all samples.

Q-PCR efficiency with plasmid and environmental samples. The Q-PCR efficiency was assessed by comparing the number of molecules estimated in the dilutions from the

same sample. With an optimal efficiency of 100%, a 1–10 dilution should yield 10% of the molecules (or a C_t difference between dilutions of 3.3). The efficiency of the standard IND58.12 plasmid (three replicates in seven serial dilutions) was compared each time with the efficiency of one environmental sample used as relative standard (three replicates in four serial dilutions). Samples with different PCR efficiencies (or too far from 100%) were excluded from further analyses.

FISH

Samples for FISH were collected during the Indian Ocean cruise and the seasonal sampling in Blanes Bay. Seawater (100–200 ml) was fixed with filtered formaldehyde (3.7% final concentration) and filtered through 0.6 μ m pore diameter polycarbonate filters. The FISH samples (80–150 ml) during the unamended seawater incubations were similarly collected. Filters were kept at -80°C until processed. For FISH we used the probe NS4, specific for MAST-4 (Massana *et al.*, 2002), supplied with a CY3 fluorophore at the 5' end. Thin pieces of filters were hybridized with the CY3-NS4 probe following the protocol described before (Perthaler *et al.*, 2001; Massana *et al.*, 2002) and counter-stained with DAPI. Positive cells were then observed by epifluorescence with green light excitation (CY3-specific signal) and checked with UV radiation (DAPI staining).

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