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Ecological niche partitioning in the picoplanktonic green alga *Micromonas pusilla*: evidence from environmental surveys using phylogenetic probes

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Summary

Very few studies have analysed the niches of pelagic protist in details. This is because for most protists, both an accurate species definition and methods for routine detection and quantification of cells are lacking. The morphospecies *Micromonas pusilla*, a marine unicellular green alga, is the most ubiquitous and cosmopolitan picoeukaryote described to date. This species comprises several independent genetic lineages or clades, which are not currently distinguishable based on comparison of their morphology or biogeographical distribution. Molecular probes were used to detect and quantify the genetic clades of *M. pusilla* in samples from temperate, polar and tropical environments in order to assess potential ecological niche partitioning. The three clades were detected in all biogeographical regions studied and were commonly found in sympatry. Cell abundances recorded for clades A and B were high, especially at coastal stations. Clade C, when detected, was always at low abundances and is suggested to be a low-light clade. Shifts in the contribution of clades to total *M. pusilla* abundance were observed along environmental gradients, both at local and basin-wide scales. This suggests that the phylogenetic clades occupy specific niches and confirms the existence of cryptic species within the morphospecies *M. pusilla*. Parameters which can precisely explain the distribution of these cryptic species remain to be elucidated.

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Introduction

Picoeukaryotic algae represent a compartment of major importance, in terms of contribution to both biomass and primary productivity in various marine systems (Maranon *et al*., 2001; Worden *et al*., 2004). The genetic diversity of picoeukaryotes, recently revealed by culture-independent molecular surveys, appears to be high at all taxonomic levels (Diez *et al*., 2001; López-García *et al*., 2001; Moon-van der Staay *et al*., 2001; Guillou *et al*., 2004; Not *et al*., 2007). Almost all major eukaryotic divisions with known photosynthetic taxa are generally represented in natural picoplanktonic assemblages (Worden and Not, 2008). At lower taxonomic scale, some taxa (species or genera) exhibit high molecular microdiversity in environmental sequence libraries (Guillou *et al*., 2004; Worden, 2006). However, even though ribosomal gene sequences databases (and more recently genome sequences databases) are expanding, the ecology and function of the newly identified diversity, and in particular of the microdiversified taxa, have seldom been studied.

The Prasinophyceae are regarded as modern representatives of the earliest green algae (Sym and Pienaar, 1993) and this class includes some of the smallest eukaryotic marine species. The prasinophyte *Micromonas pusilla* (Butcher; Manton et Parke 1960) was the first picoplanktonic species described, initially as *Chromulina pusilla* (Butcher, 1952), and belongs to the order Mamiellales. *Micromonas pusilla* is a minute (1–2 μm) green alga with a pear-shaped naked cell body, a single flagellum and a characteristic swimming behaviour (Manton and Parke, 1960). According to the literature, *M. pusilla* appears to be the most ubiquitous and cosmopolitan species of all picoeukaryotes described to date (Thomsen and Buck, 1998). It dominates the picoeukaryotic community all year long in coastal systems such as the English Channel (Not *et al*., 2004). Recent studies based on the phylogenetic analysis of several genes from culture isolates of this species collected worldwide revealed the existence of three (Guillou *et al*., 2004) to five (Slapeta *et al*., 2006) phylogenetically discrete

clades, indicating that this taxon is a complex of cryptic species which started to diverge as long ago as during the late Cretaceous (Slapeta *et al*., 2006; Worden, 2006). However, to date no clear morphological, ecophysiological or biogeographical differentiation between strains or clades of this species have been reported, except for one lineage described as purely Arctic (Lovejoy *et al*., 2007). We designed three oligonucleotide probes in order to identify potential ecological niche partitioning among genetic clades of *M. pusilla*, each specific for one of the three main genetic lineages designated as clades A.ABC.12, B.E.3 and C.D.5 (Worden, 2006) abbreviated as clades A, B and C respectively in this study. These probes, coupled to fluorescent *in situ* hybridization associated with tyramide signal amplification (TSA-FISH) (Not *et al*., 2002), were used to study the spatial distribution (both vertical and horizontal) and temporal dynamics of the three genetic clades along environmental gradients in temperate (English Channel and Mediterranean Sea), polar (Arctic seas) and subtropical to tropical (Indian Ocean) waters.

Results

Design and specificity tests of 18S rDNA probes targeting Micromonas *clades*

To distinguish and quantify the different *M. pusilla* clades in natural samples, we designed three oligonucleotide probes, MICROA01 (5′-CCGTCAAGAGGCCGCGGT-3′), MICROB01 (5′-CACGACCAACAGACGGTT-3′) and MICROC01 (5′-ACGGCGGCGAACCGCAAT-3′), specific for clades A, B and C respectively. The probes hybridize to the helix 49 at the end of the 18S rRNA molecule and each probe has at least three mismatches with the homologous region of the non-target clades. The three clade-specific probes targeted all full-length sequences identified as belonging to *M. pusilla* in databases, except for the three environmental sequences forming the recently identified minor lineage B._.4 (Worden, 2006), and not available when the probes were designed. The fluorescent signal of each probe was bright and specificity was high when tested by TSA-FISH on a range of *M. pusilla* strains in culture (Table 1). While MICROA01 did not exhibit any non-specific labelling with the strains tested, MICROB01 and MICROC01 showed slight non-specific hybridization with cells belonging, respectively, to the clades C and B. This issue was solved by the use of competitor oligonucleotides (Simon *et al*., 1995) consisting of unlabelled MICROC01 and MICROB01 probes which were systematically mixed with labelled MICROB01 and MICROC01 respectively in order to prevent unspecific labelling of the labelled probes to non-targeted cells.

To identify potential shifts in the abundance of *M. pusilla* genetic clades over seasonal cycles, the dynamics of the three clades were monitored along time series at three coastal stations: the mesotrophic station SOMLIT-Astan and the eutrophic estuarine station Dourduff, both located in the English Channel, as well as the oligotrophic station Blanes in the western Mediterranean Sea (Fig. 1). Potential correlations between distribution of genetic types and conditions encountered at different depths were also studied at a temperate Mediterranean coastal station (SOMLIT-Point B) during a stratification event, and along oceanographic transects in polar (Artic seas) and tropical (Indian Ocean) waters that exhibited clear environmental gradients.

Seasonal cycle in temperate coastal surface waters

During the sampling periods at the SOMLIT-Astan station (2001, 2002, 2005), the temperature varied between 9.2°C (March 2005) and 16.6°C (August 2001), and chlorophyll *a* (Chl *a*) concentrations varied according to the classical pattern observed in this region with minima in winter (0.07 μg l^{-1} in December 2005) and maxima corresponding to diatom blooms in late spring or early summer (5.79 μg l^{-1} in May 2002) (Fig. 2A). At the estuarine station Dourduff, temperature and Chl *a* exhibited larger

Table 1. Specificity tests of the clade-specific oligonucleotide probes on *M. pusilla* cultures.

RCC number[a]	Strain	Origin	Phylogenetic clade[b]	Probes[c]		
				MICROA01	MICROB01 + competitor	MICROC01 + competitor
804	NECCP 29	Pacific ocean	A	+	–	–
836	Mnorbal	Mediterranean sea	A	+	–	–
746	A1_Arousa 90	Atlantic ocean	B	–	+	–
828	MP1	Mediterranean sea	B	–	+	–
829	MP Part	Mediterranean sea	B	–	+	–
497	BL_105-7	Mediterranean sea	C	–	–	+
498	BL_74-8	Mediterranean sea	C	–	–	+

a. Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/RCC/).
b. Phylogenetic position of strains according to E. Foulan and A. Houdan (unpublished data).
c. + and – indicate the results of hybridization tests (+ bright fluorescent signal and – no fluorescent signal).

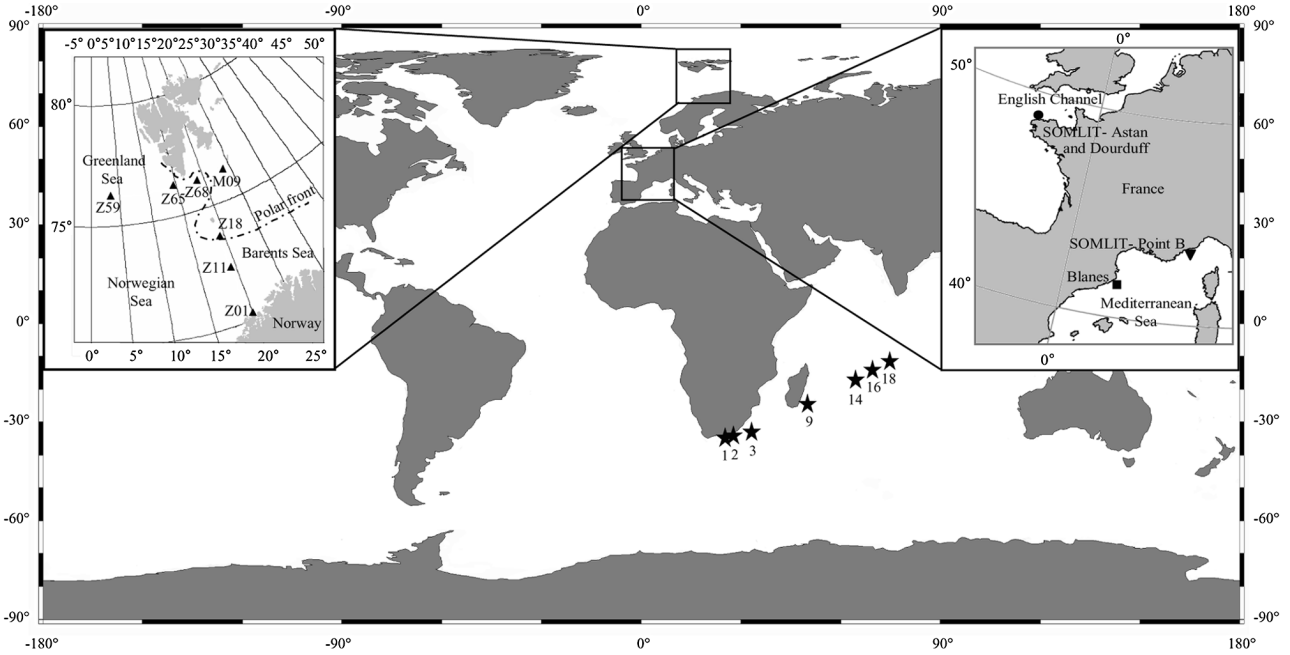

Fig. 1. Location of the different stations studied.

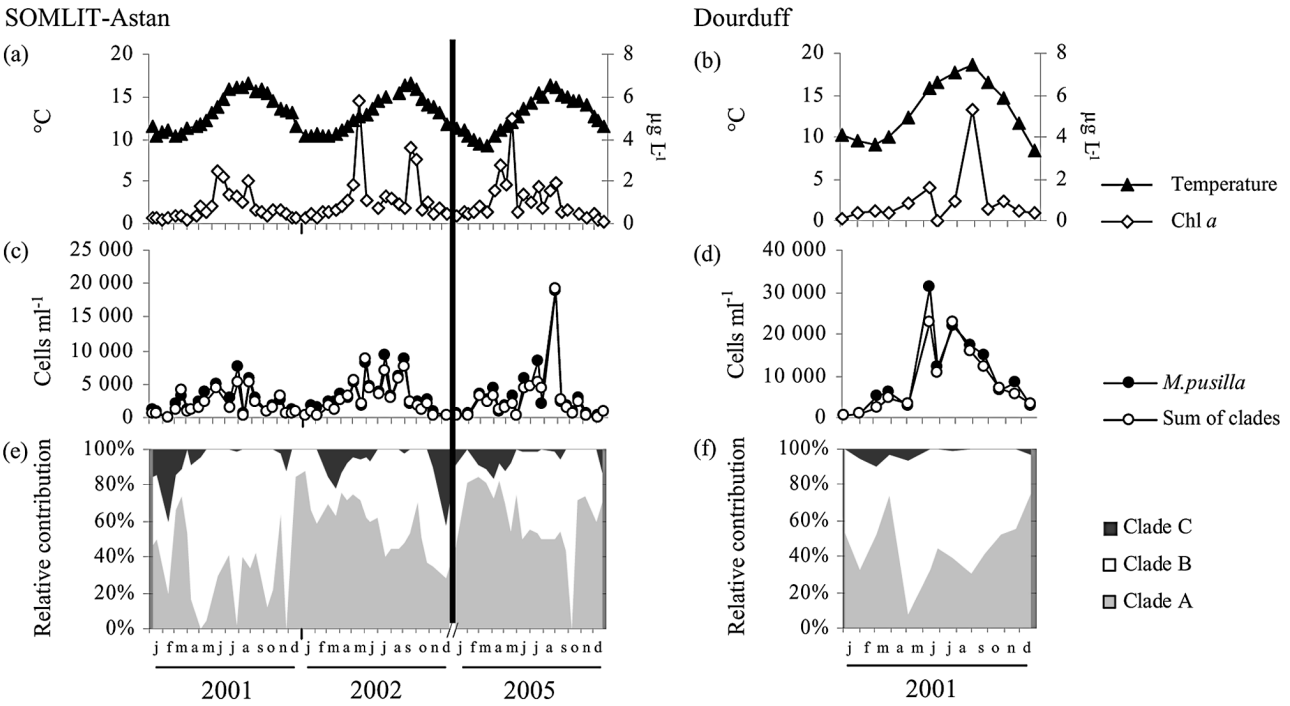

Fig. 2. A and B. Variations of temperature and Chl *a* biomass at the English Channel coastal station SOMLIT-Astan over the years 2001, 2002 and 2005, and at the estuarine station Dourduff during the year 2001.
C and D. *Micromonas pusilla* (MICRO01 probe counts) cell abundances over the time series. Both the MICRO01 probe counts and the sum of the MICROA01, MICROB01 and MICROC01 probe counts (clade sum) are shown.
E and F. Contribution of the three *M. pusilla* genetic clades, A (MICROA01 probe), B (MICROB01 probe) and C (MICROC01 probe), to total clade counts.

amplitudes (8.4°C to 18.5°C and 0.14–5.30 μg l^{-1} Chl *a*) within the same year than at the SOMLIT-Astan station (Fig. 2B). At both stations, the abundance of *M. pusilla*, targeted by the general probe MICRO01, exhibited a marked seasonal cycle every year. For instance, at the SOMLIT-Astan station, minimal abundances occurred in February 2001 (< 100 cells ml^{-1}) and maxima in August 2005 (1.8×10^4 cells ml^{-1}) (Fig. 2C). *Micromonas pusilla* typically exhibited higher cell abundance at the estuarine station Dourduff with an annual average of 9.9×10^3 cells ml^{-1} (Fig. 2D). Over shorter time scales, several peaks were observed each year between the beginning of June and the end of October. The counts obtained with MICRO01 and the cumulated counts obtained with the clade-specific probes for the Dourduff station were not statistically different (Wilcoxon test, $\alpha = 0.05$, $n = 13$ and $P = 0.075$). Although the counts were statistically different for the SOMLIT-Astan station with the counts obtained with the specific probes inferior to those using MICRO01 (Wilcoxon test, $\alpha = 0.05$, $n = 64$ and $P = 0$), temporal patterns over the time series were very similar (Fig. 2C). All three clades were detected at both stations all year round (Fig. 2E and F). Clades A and B were the dominant genetic types representing, respectively, 52% and 43% of total *Micromonas* cells at the SOMLIT-Astan station, and 45.5% and 52.5% of total *Micromonas* cells at the Dourduff station. Clade C accounted for only 5% and 2% on average at the SOMLIT-Astan and Dourduff stations respectively. Interannual variations were observed at the SOMLIT-Astan station, with clade B dominating the *Micromonas* assemblage in 2001 (58% of the total cells detected by the three probes), whereas clade A was dominant in the two other years analysed (58% in 2002 and 61% in 2005) (Fig. 2E). At both marine and estuarine stations, for each of the years studied, a seasonal signal was observed with clade A dominating the spring and early summer assemblages and clade B proliferating later in summer, to become dominant or codominant with clade A (year 2005 at SOMLIT-Astan) during the *M. pusilla* 'bloom' (Fig. 2E and F). The contribution of clade C cells was lowest (< 3.5%) from July to October, when total *M. pusilla* cell abundances were highest, and was highest in winter or early spring (40% in February 2001 and 43% in December 2002 at SOMLIT-Astan), when total cell abundances of *M. pusilla* were lowest.

During the 2 years studied (2003 and 2006) at the Blanes station, the temperature varied from 11.0°C (March 2003) to 25.3°C (August 2006). Chl *a* concentration peaked in winter and spring with maximal concentration detected in December 2003 (3.93 μg l^{-1}) with lower and less variable values for the rest of the year (Fig. 3A). The abundance of *M. pusilla* showed a marked seasonal cycle with maxima in winter (7377 cells ml^{-1} in December 2003) and minima in summer. *Micromonas pusilla* was not detected in September and October 2006 (Fig. 3B). As for the SOMLIT-Astan station, the counts obtained with MICRO01 and the cumulated counts obtained with the clade-specific probes were statistically different, with the counts obtained with the specific probes inferior to those using MICRO01 (Wilcoxon test, $\alpha = 0.05$, $n = 25$ and $P = 0.002$), but trends were very similar (Fig. 3B). Through most of the 2 years studied, clade B was the only clade detected. Clade A was only detected in October 2003 and November 2006 with a contribution reaching, respectively, 38% and 17% of the cumulated counts

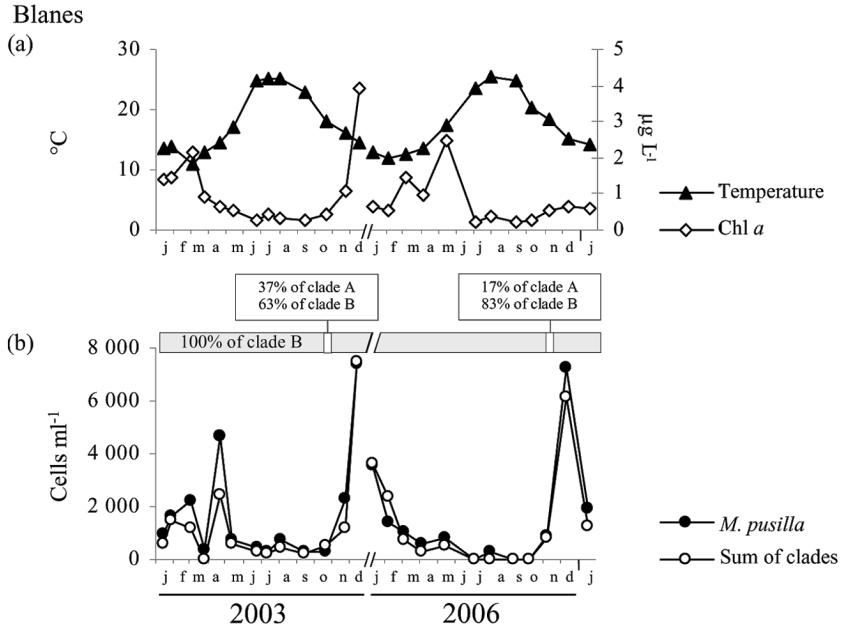

Fig. 3. A. Variations of temperature and Chl *a* biomass at the Mediterranean Blanes station during the years 2003 and 2006.
B. Variations in the cell abundances of *M. pusilla* over the time series. Both the MICRO01 probe counts and the sum of the MICROA01, MICROB01 and MICROC01 probe counts (clade sum) are shown. Contribution (%) of each clade to the sum of clades is indicated.

obtained with the three probes (Fig. 3B). Clade C was never detected.

Stratification event in Mediterranean Sea coastal waters

The dynamics of the three clades at the Mediterranean coastal station SOMLIT-Point B were studied during a stratification event (from 30 March to 20 June 2006). At the end of March, temperature was homogenous throughout the water column (12.6°C on average). In June, the water column was clearly stratified, temperature being > 20°C at the surface and decreasing rapidly with depth (Fig. 4A). Chl *a* peaked at the beginning of the stratification event with a maximum of 1.73 μg l^{-1} reached on 4 April at 20 m depth and decreased as the stratification developed (Fig. 4B). Maximum cell abundances of *M. pusilla* reached 3.7×10^3 cell ml^{-1} (4 April) at the beginning of the stratification event (Fig. 4C). Cell abundances gradually decreased during the establishment of the thermocline and *M. pusilla* was absent at depth on 2 May. The counts obtained with MICRO01 and the cumulated counts obtained with the clade-specific probes for this station were not statistically different (Wilcoxon test, $\alpha = 0.05$, $n = 48$ and $P = 0.643$). Over the period studied, the three clades A, B and C were detected (Fig. 4D–F). Clades B and A were codominant at the beginning of the stratification event but, with the onset of stratification, the contribution of clade A decreased and clade B was the only clade detected for some dates (2, 16 May and 20 June) (Fig. 4D and E). Clade C was detected only once (30 May) below the thermocline at very low abundances (< 50 cells ml^{-1}), when the stratification began to be clearly established (Fig. 4F).

Arctic waters

Seven stations distributed along two transects in Arctic waters were selected (Fig. 1). Hydrological parameters enabled distinction of waters of Arctic origin at stations Z18 and M09, with temperature ranging from 1.7°C to 4.7°C and maximum Chl *a* concentration of 2.04 μg l^{-1}. All other stations (Z01, Z11, Z59, Z65, Z68) were in zones of North Atlantic-influenced waters with temperatures ranging from 2.2°C to 12.5°C and a maximum Chl *a* concentration of 2.54 μg l^{-1} (Fig. 5A and B). Among the latter stations, station Z01 presented hydrological features typical of coastal waters (see details in Not *et al.*, 2005). *Micromonas pusilla* was detected at all stations with maximal abundances recorded at stations Z65 and Z68 (6×10^3 and 8×10^3 cells ml^{-1} respectively) near the surface (Fig. 5C). *Micromonas pusilla* abundances were generally highest near the surface and decreased with depth. The counts obtained with MICRO01 and the cumulated counts obtained with the clade-specific probes were not statistically different for these stations (Wilcoxon test, $\alpha = 0.05$, $n = 35$ and $P = 0.106$). The three clades were found at the coastal station Z01, with clade A dominating at all depths and clades B and C representing 11% and

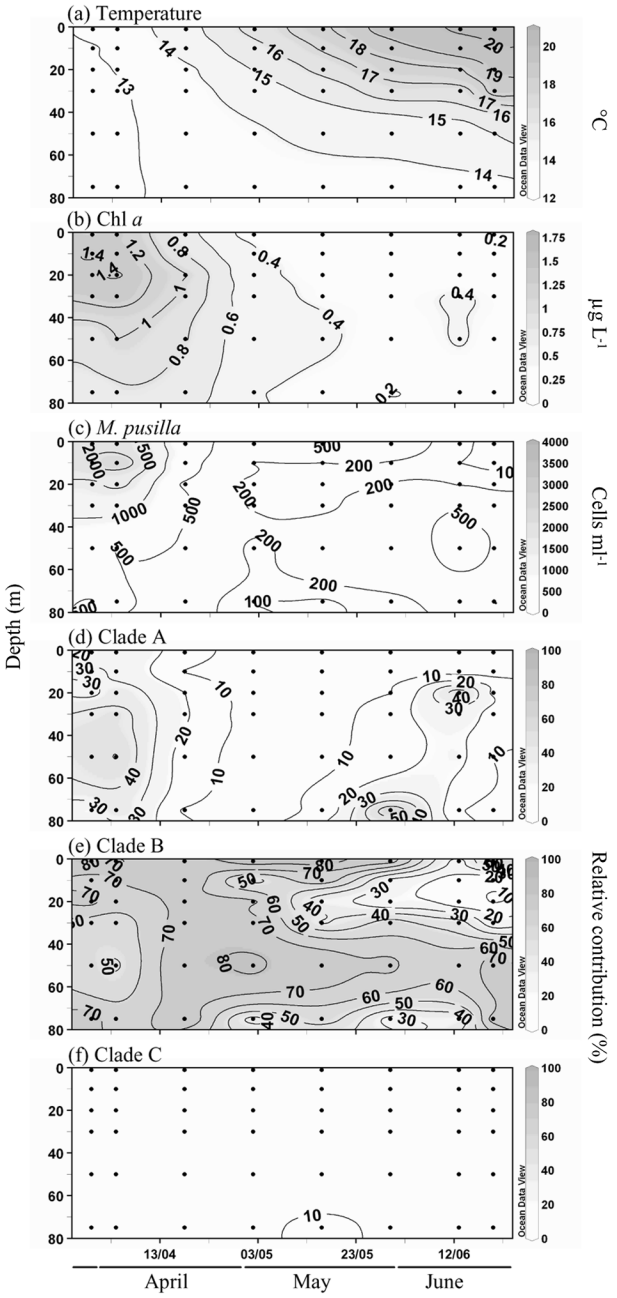

Fig. 4. A and B. Variations of temperature and Chl *a* biomass at the Mediterranean SOMLIT-Point B station during a stratification event in 2006.
C. Variations in the cell abundances of *M. pusilla* over the period studied.
D–F. Contribution of the three *M. pusilla* genetic clades, A (MICROA01 probe), B (MICROB01 probe) and C (MICROC01 probe), to total clade counts. Black dots correspond to actual samples.

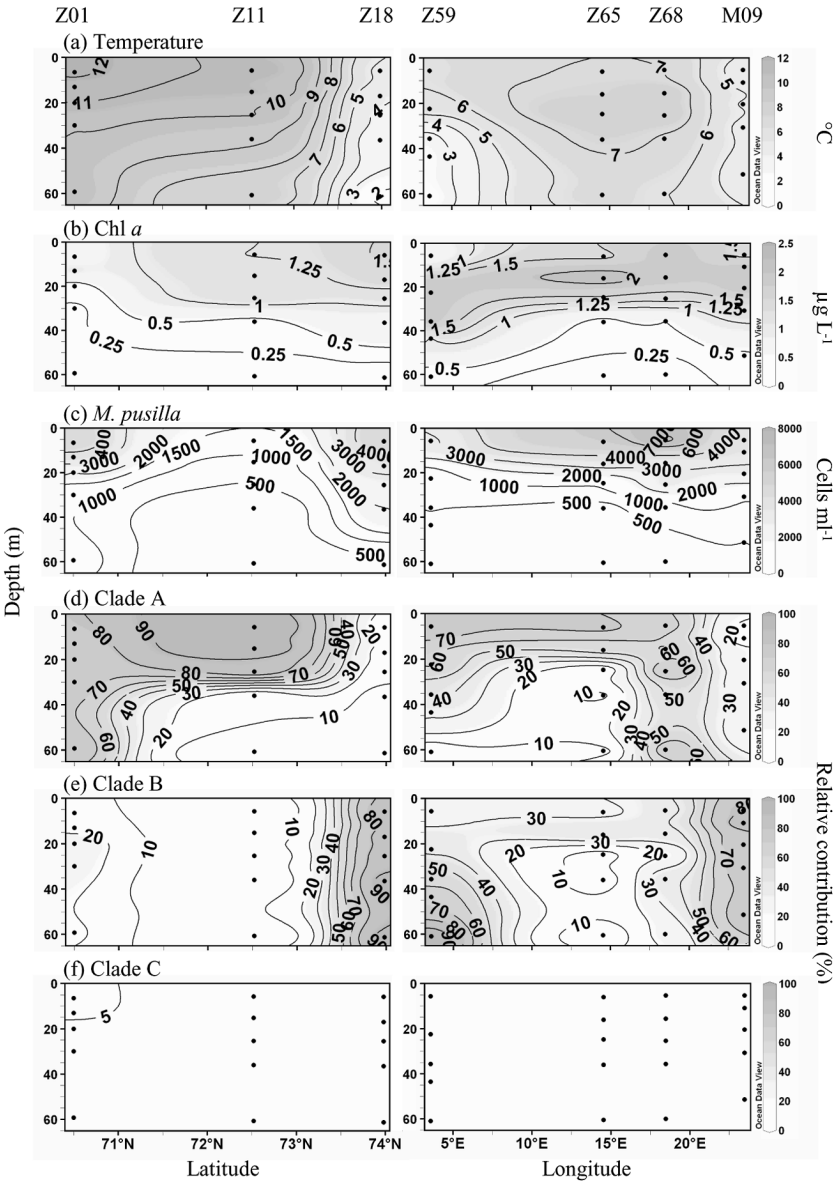

Fig. 5. Depth sections along the south/north (S/N) and east/west (E/W) transects in the Arctic seas sampled from 20 August to 8 September, 2002.
A. Temperature.
B. Chl *a* biomass.
C. Cell abundances of *M. pusilla* (MICRO01 probe counts).
D–F. Relative contribution of clades A (MICROA01 probe), B (MICROB01 probe) and C (MICROC01 probe) to total clade counts. Black dots correspond to actual samples.

5% of the cells respectively (Fig. 5D–F). At the other Atlantic-influenced stations, only clades A and B were recorded. Clade A was the only clade detected at station Z11, whereas maximum contributions of clade B were recorded at stations Z65 and Z68 (41% and 32% of total cells respectively) which are at the front between Atlantic and Arctic waters. At stations M09 and Z18 located beyond the polar front, contributions of clades were totally different, with clade B dominating (86% of total cells).

Subtropical Indian Ocean waters

Seven stations distributed along a transect across the Indian Ocean were selected (Fig. 1). Coastal stations off South Africa (01 and 02) presented temperatures ranging from 8.0°C at 200 m depth to 24.0°C at the surface with maximum Chl *a* concentrations of 1.30 µg l^{-1} at the surface of station 01 (Fig. 6A and B). Temperatures were higher (15.0°C to 26.0°C from 200 m to the surface) at the oceanic stations (03, 09, 14, 16 and 18) and maximum Chl *a* concentrations (0.92 µg l^{-1} at station 18) were found around 100 m depth. *Micromonas pusilla* was detected at each station, but abundance decreased rapidly from the coastal to the oceanic stations. Maximal cell abundance reached more than 4×10^3 cells ml^{-1} near the surface at station 01 to less than 300 cells ml^{-1} at the deep chlorophyll maximum, around 100 m depth, at station 16 (Fig. 6C). The counts obtained with MICRO01 and the cumulated counts obtained with the clade-specific probes for this transect were not statistically different (Wilcoxon

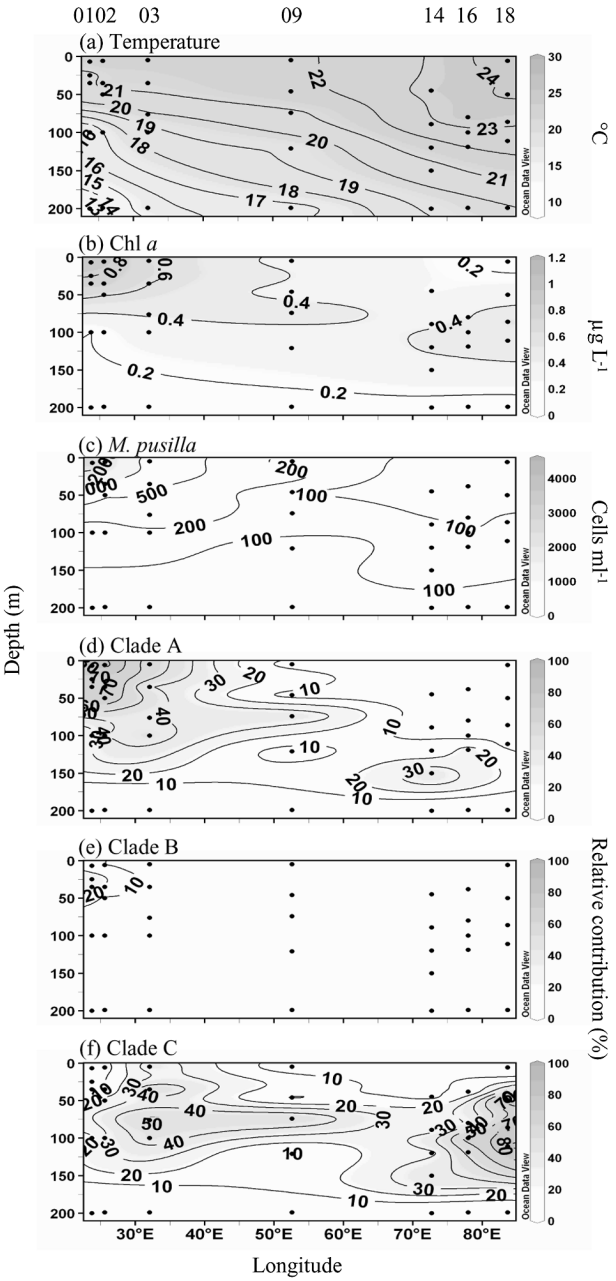

Fig. 6. Depth sections along the transect in the subtropical Indian Ocean sampled from 15 May to 15 June, 2003.
A. Temperature.
B. Chl *a* biomass.
C. Cell abundances of *M. pusilla* (MICRO01 probe counts).
D–F. Relative contribution of clade A (MICROA01 probe), B (MICROB01 probe) and C (MICROC01 probe) to total clades counts. Black dots correspond to actual samples.

test, $\alpha = 0.05$, $n = 34$ and $P = 0.411$). Clades A, B and C were all detected in the Indian Ocean. The clade composition varied drastically along the transect. While clade A was present from the coast to station 16 (Fig. 6D), clade B was present only at the most coastal station (Fig. 6E), and clade C was present only far from the coast (from station 03 to station 18) with contributions to total *M. pusilla* assemblages always above 46% (Fig. 6F). Clade C was the only clade detected at the most oceanic station 18.

Preliminary uni- and multivariate analyses (scatterplots, principal component analysis) were applied to study the distribution patterns of the clades (data not shown). Separate analyses were performed using temperature and Chl *a* data from the different sites, but no significant correlations were clearly established between the contribution of clades to total *M. pusilla* cell abundance and temperature or biomass.

Discussion

Phylogenetic analyses have demonstrated that the morphospecies *M. pusilla* includes several separate lineages with levels of genetic divergence exceeding those usually observed between different genera in the order Mamiellales (Worden, 2006; Lovejoy *et al*., 2007). Clear signatures were identified in the 18S rRNA sequences for the three main clades A, B and C. Probes designed to hybridize to the signature regions permitted clear distinction of cells belonging to each of the clades by TSA-FISH. *Micromonas pusilla* counts from cumulated abundances of the clade-specific probes and from MICRO01 showed highly similar trends for all series of samples analysed (both time series and transects), but were statistically different for two time series (SOMLIT-Astan and Blanes stations). The recently described subclade B._.4 (Worden, 2006) is not labelled by the three clade-specific probes but is targeted by MICRO01. As it contains two sequences from Blanes station, presence of this subclade at this site may explain the statistical difference observed. Errors associated with the counting process, possible unspecific binding of some of the probes or the presence of genetic diversity not yet encountered in sequence databases could also contribute to these differences. However, for most of the samples analysed, total counts of *M. pusilla* and the cumulative abundance of clade counts were not significantly different, which demonstrates that most of the genetic variability of *M. pusilla* was covered by the clade-specific probes.

The specific probes coupled to the TSA-FISH technique allowed analysis of shifts in abundance of *M. pusilla* and the variations in the contribution of clades to total counts along environmental gradients and time series at both local and basin-wide scales. Our study confirms that the morphospecies *M. pusilla* is a cosmopolitan taxon that prevails in coastal systems. It was detected in all stations sampled, with maximal cell abundances recorded from samples collected in temperate estuarine and coastal waters in summer, while cell abundances recorded in

oligotrophic stratified water masses were relatively low. These observations are consistent with previous studies in which the morphospecies *M. pusilla* has been suggested to occur worldwide (Thomsen and Buck, 1998), and has been suspected as early as 1951 to contribute significantly to phytoplankton assemblages, especially in coastal areas (Knight-Jones and Walne, 1951).

Previous studies based on the analysis of the available culture isolates and of environmental DNA sequences suggested that *M. pusilla* phylogenetic clades A, B and C are ubiquitous and have a pan-oceanic distribution (Slapeta *et al*., 2006; Worden, 2006). Our study confirms the presence of clades in all biogeographical regions analysed (temperate Atlantic, Mediterranean, Indian Ocean and Arctic). Clade A was detected in all stations sampled while clades B and C were absent from some stations of the transects studied. The possibility that clades B and C are present at these stations during other periods of the year cannot be excluded. Clade C was absent at the Blanes station throughout the 2 years of analyses. Phylogenetic analyses of environmental DNA sequences from Blanes surface waters and from a transect in the Mediterranean Sea reported only sequences belonging to clades A and B (Massana *et al*., 2004; Viprey *et al*. 2008). However, two culture isolates belonging to clade C were isolated from the Blanes station (RCC 497 and RCC 498, Roscoff Culture Collection, Roscoff, France, http://www.sb-roscoff.fr/Phyto/RCC/) and clade C was detected once in our study at the SOMLIT-Point B station. This suggests that clade C is present either very sporadically or at very low cell abundances in the Mediterranean Sea.

Our study shows that the three clades A, B and C can occur in sympatry. They even coexist all year round in permanently mixed coastal and estuarine systems, such as the western English Channel. In addition, shifts in clade abundance occurred along time series and vertical gradients. At some periods of the year in some marine systems, only a single clade was detected. For example, at the coastal Mediterranean station Blanes, clade B was the only clade detected for most of the year. Shifts in relative abundance recorded over time series in the English Channel suggest either that competition exists between clades or that biotic factors, such as differential grazing pressure or host-specific virus infection, have different impacts on the three genetic clades. This indicates that niche partitioning occurs between clades and that cryptic species within *M. pusilla* (Slapeta *et al*., 2006) may correspond to ecological species.

By analysing shifts in clade dominance over the time series and along the environmental gradients at different scales, we attempted to identify the ecological preferences of clades A, B and C. Although clade B was present at most of the stations sampled, it was not detected in offshore stations of the Indian Ocean. Inversely, it appeared to develop during summer in the English Channel, during the stratification event at the SOMLIT-Point B station, and throughout the year at the Blanes station in the Mediterranean Sea. Phylogenetic analyses of environmental DNA sequences from a North American coastal site in the Pacific Ocean also revealed the presence of clade B notably in summer (Worden, 2006). This clade seems, therefore, to be well adapted to warm (presumably well illuminated) coastal waters. Paradoxically, our results also show that clade B dominated the *M. pusilla* assemblage in Arctic waters. This could be explained by the fact that clade B can be split into two subclades, one of which, B.Ea.3, may be specific to the Arctic Ocean and could correspond to a pan-Arctic ecotype (Lovejoy *et al*., 2007). Our study, however, shows that clade A was found at all stations in the Norwegian and Barents Seas as well as in the stations identified as strictly Arctic-influenced stations. The possibility that Atlantic waters did indeed influence all stations sampled in this region can not be excluded.

Clade C exhibited the lowest cell abundances. Its contribution to the *M. pusilla* assemblage was highest in the most oligotrophic stations sampled and generally increased when total abundance of *M. pusilla* was low. The contribution of clade C to the total *M. pusilla* assemblage increased from the coast to the most offshore station in the Indian Ocean and also peaked in winter in the permanently mixed English Channel waters. In addition, it appears that this clade develops better at depth in stratified waters. The highest contributions of this clade to the total *M. pusilla* assemblages in the Indian Ocean were recorded at depth at open ocean stations, while at the SOMLIT-Point B Mediterranean station, this clade was only detected at depth on one sampling date. Overall, clade C appears to correspond to a low light-adapted clade whose contribution to total *M. pusilla* counts is highest in open ocean stratified stations. Comparative photophysiological studies of the different clades are required to test this hypothesis.

Although potential factors that may favour the development of clades B and C were identified, we were not able to develop hypotheses for the ecological preferences of clade A. Clade A was detected in all environments studied, with high contributions at coastal stations in the Indian Ocean, in the Mediterranean samples before the stratification event, and all year round in the English Channel stations. This clade appears to be the most ubiquitous and abundant. However, clade A has been shown to be composed of three sublineages (Slapeta *et al*., 2006). The design of probes for these subclades would allow testing of the potential ecological significance of the relatively recently diverged genetic diversity within clade A.

Based on our preliminary statistical analysis, no significant correlations were established between contribution of clades to total *M. pusilla* cell abundance and temperature or Chl *a* biomass. This is either because the scale of genetic differentiation analysed here was not fine enough to identity a clear correlation within these parameters, or because other parameters were involved. Studies combining physiological experiments on cultured strains (e.g. monitoring the impact of light) and genomic analyses would be valuable to determine more precisely which factors affect the growth of clades B and C, and at a finer scale for clade A. Application of such ecophysiological approaches on both the cyanobacterium *Prochlorococcus* (Moore and Chisholm, 1999) and the picoeukaryote *Ostreococcus* (Rodriguez *et al*. 2005) has demonstrated that these taxa included several ecotypes and have contributed to the description of the potential ecological niches of these ecotypes. Several studies have suggested that viruses may contribute significantly to the regulation of *M. pusilla* populations (Cottrell and Suttle, 1995; Evans *et al*., 2003). A wide variability in susceptibility to viral infection has also been reported among *M. pusilla* strains (Zingone *et al*., 2006). Hence, the combined influence of abiotic and biotic may control the dynamics of *M. pusilla* cryptic species. Deciphering precisely how these factors influence the growth of each clade would help identifying the mechanisms of speciation in the pelagic environment.

Experimental procedures

Probe design

Three 18S rDNA-specific oligonucleotide probes, MICROA01, MICROB01 and MICROC01, were developed to target, respectively, the *Micromonas* clades A, B and C distinguished originally by Guillou and colleagues (2004) and re-analysed in particular by Worden (2006). The probes were designed using the 'probe design' function of the ARB software package (Ludwig *et al*., 2004); (http://www.arb-home.de) based on a prasinophyte SSU rDNA sequence database maintained by the Oceanic Plankton group at the Station Biologique de Roscoff. The specificity of the probes was further tested on an SSU rDNA sequence database containing more than 30 000 complete and partial sequences. This database contained unpublished partial eukaryote sequences in addition to published sequences. Oligonucleotide probes were purchased with a 5′-aminolink (C6) from Eurogentec (Angers, France). The probes were then labelled with horseradish peroxidase (HRP) (Urdea *et al*., 1988; Amann *et al*., 1992).

Sampling sites

Samples were taken between 2001 and 2006 in the context of long-term time series (English Channel and Mediterranean Sea) and during oceanographic cruises (Arctic seas and Indian Ocean) (Fig. 1).

i. Samples from the English Channel were collected over time series at two distinct sampling stations. The SOMLIT-Astan station (48°46′N, 3°57′W), located off Roscoff (France), was sampled twice a month between January 2001 and December 2002 and during the year 2005. The estuarine station Dourduff (48°38′N, 3°51′W), located in the Morlaix bay (France), was sampled once a month during the year 2001. At both stations, water was collected at 1 m depth with 5 l Niskin bottles and was pre-filtered through a 200 μm mesh before further processing in the laboratory. Temperature and Chl *a* concentrations were measured by standard oceanographic methods and data were provided by the 'Service d'Observation en Milieu Littoral, INSU-CNRS, Station Biologique de Roscoff' ([http://www.domino.u-bordeaux.fr/somlit_national/]).
ii. Samples from the Mediterranean Sea were also collected over time series at two sampling sites. At the SOMLIT-Point B station (43°41′N, 7°19′E), located off Villefranche-sur-Mer (France), water was collected twice a month from 30 March to 20 June 2006 at six depths (surface, 10 m, 20 m, 30 m, 50 m and 75 m) with 5 l Niskin bottles. Temperature and Chl *a* concentrations were measured by standard oceanographic methods and data were provided by the 'Service d'Observation en Milieu Littoral, INSU-CNRS, Observatoire Océanographique de Villefranche-sur-Mer' (http://www.domino.u-bordeaux.fr/somlit_national/). At the 'Blanes' station (41°40′N, 2°48′E), located in Blanes Bay (Spain), surface water was collected monthly during the years 2003 and 2006. Seawater was first pre-filtered through a 200 μm nylon-mesh and kept in 25 l plastic carboys for less than 2 h during transport prior to processing in the laboratory. Temperature and Chl *a* concentrations were measured by standard oceanographic methods and data were provided by the 'Blanes Bay Microbial Observatory'. To determine Chl *a* concentration, 50–150 ml of seawater was filtered on GF/F filters (Whatman) and fluorescence was measured in acetone extracts with a Turner Designs fluorometer (Yentsch and Menzel, 1963).
iii. Samples from the Arctic seas were collected during a cruise on board the F/F Johan Hjort (Norwegian Institute of Marine Research, Bergen, Norway), between the Norwegian and Barents seas. Ten stations were sampled from 20 August to 8 September 2002 with 5 l Niskin bottles (for details on the sampling site see Not *et al*., 2005). Seven stations (Z01, Z11, Z18, Z59, Z65, Z68 and M09) were chosen to represent the range of environments encountered based on the results from previous analysis (Fig. 1).
iv. Samples from the Indian Ocean were collected during the VANC10MV cruise on board the R/V Melville (Scripps Institute of Oceanography, San Diego, USA) which took place between Cape Town (South Africa) and Port Hedland (Australia). Fourteen stations were sampled from 15 May to 15 June 2003 with 15 l Niskin bottles. For each station, five depths were chosen based on chlorophyll fluorescence and hydrological conditions provided by a 'Seabird' CTD/fluorometer. Water was pre-filtered through a 200 μm mesh. Among the 14 stations sampled, two coastal stations [station 01 (35°03′S, 23°44′E) and station 02 (35°03′S, 24°34′E)] and five oceanic stations [station 03 (35°49′S, 32°02′E), station 09 (31°49′S, 52°36′E),

station 14 (22°05′S, 72°44′E), station 16 (19°44′S, 78°00′) and station 18 (17°10′S, 83°40′E)] were selected for analysis in the present work (Fig. 1).

FISH-TSA

For natural samples, 90 ml of seawater was pre-filtered through a 3 μm pore size Nuclepore filter and fixed with 10 ml of 10% PFA for 1 h (1% final concentration), except for three samples from the Mediterranean Sea (30 March, 4 April and 18 April) for which 45 ml of seawater was used. Samples were then filtered onto 0.2 μm Anodisc filters (Whatman international, Maidstone, England) and dehydrated in an ethanol series (50%, 80% and 100% absolute ethanol, 3 min each). Filters were dried at room temperature and stored at −80°C. Filters from the Arctic seas and Indian Ocean were first stored at room temperature on board the research vessels and subsequently stored at −80°C in the laboratory. For cultured cells, 4.5 ml of exponentially growing culture was fixed and filtered onto a 0.2 μm Anodisc filter before dehydration using ethanol series and storage at −80°C.

In situ hybridization with HRP-labelled probes, signal amplification and target cell detection were performed as described by Not and colleagues (2002). Taxon-specific probes used were developed in the frame of this study and the MICRO01 probe that targets all clades of *M. pusilla* (Not *et al*., 2004). When needed, competitor probes were used in the hybridization buffer at the same concentration as the HRP-labelled probe (Simon *et al*., 1995). The cells were counterstained with propidium iodide (final concentration at 10 μg ml^{-1}) in order to visualize the nuclei. This allowed verification of labelling of target cell when specificity tests were conducted on selected strains. For each filter, at least 15 randomly chosen fields were manually counted. For each filter, the total number of cells counted ranged from 0 (absence of the taxon) to more than 900 cells for the MICRO01 probe and from 0 (absence of the taxon) to more than 450 cells for the specific probes. A total of approximately 19 000, 7800, 8200 and 350 cells were counted with the probes MICRO01, MICROA01, MICROB01 and MICROC01, respectively, for the samples from mesotrophic stations (English Channel, polar waters). A total of approximately 2700, 900, 1730 and 110 cells were counted with the probes MICRO01, MICROA01, MICROB01 and MICROC01, respectively, for the samples from oligotrophic stations (Mediterranean Sea and Indian Ocean). Percentages of error associated with TSA-FISH cell number estimations were variable depending on samples (15% for English Channel samples, and 27–65% for the oligotrophic waters of the Indian Ocean, the highest values being recorded for samples with the lowest concentrations of target cells, Not *et al*., 2004; 2008).

Cultures

In order to test the probes designed in this study, seven strains of *M. pusilla* belonging to the three different phylogenetic clades A, B and C were selected from the Roscoff Culture Collection (Table 1). They were grown in 50 ml flasks at 19–20°C under a 12:12 h Light : Dark regime in K medium (Keller *et al*., 1987). Light was provided by Sylvania Daylight fluorescent bulbs at an intensity of 100 μmol photon m^{-2} s^{-1}.

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