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The Roscoff Culture Collection (RCC): a collection dedicated to marine picoplankton

by

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Abstract: The Roscoff Culture Collection (RCC, http://www.sb-roscoff.fr/Phyto/RCC/index.php) holds over 500 strains of marine cyanobacteria and microalgae with a strong emphasis on picoplankton, i.e. cells with size below 2-3 microns. Most of these strains have been obtained from the Equatorial Pacific, the Tropical Atlantic, the Mediterranean Sea, the Red Sea, the North Sea and the English Channel. A large fraction of strains are characterized based on ultrastructure features, pigment analyses and SSU rDNA sequencing. With respect to prokaryotes, the RCC holds more than 100 strains of *Prochlorococcus* and *Synechococcus*. Both genera exhibit considerable ecotypic variability since they colonize virtually all oceanic waters from tropical oligotrophic regions to temperate coastal areas. These two cyanobacteria should receive increasing attention with the recent availability of complete genome sequences of four representative isolates. In recent years, we have also isolated novel picoeukaryotic strains from several algal classes (Prasinophyceae, Prymnesiophyceae, Bolidophyceae, Dictyochophyceae, Pelagophyceae...). As an example, we have now 20 strains of *Ostreococcus* (Prasinophyceae, Mamiellales), the smallest photosynthetic eukaryote initially isolated from Thau lagoon in France. These *Ostreococcus* strains originate from environments as diverse as the Red Sea or the coast of Brittany. We are currently extending our collection to heterotrophic picoplankton, since this compartment is probably very diversified, as revealed by recent molecular studies.

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Introduction

Photosynthetic picoplankton (i.e. the smallest size fraction of the phytoplankton) was discovered at the end of the 1970's, although records of very small species had been made earlier e.g. *Chromulina pusilla* Butcher, renamed later *Micromonas pusilla* (Butcher 1952, Manton & Parke 1960). Since then, a growing body of literature has emphasized the importance of picoplankton in terms of biomass and production, not only in oligotrophic waters (e.g. Campbell et al. 1994, Li 1994), but also in coastal waters (e.g. Vaquer et al. 1996). Probably the technique that contributed most to establish the ubiquity and importance of picoplankton has been flow cytometry (Olson et al. 1985) that allowed to map its abundance in vast oceanic regions (e.g. Partensky et al. 1999, Li 2002).

When fresh marine samples from subtropical waters are processed with a flow cytometer, three populations may be observed based on their size and pigment fluorescence characteristics (Fig. 1). The lowest population only emits red chlorophyll fluorescence and corresponds to *Prochlorococcus*, a tiny atypical cyanobacterium (0.6 µm cell diameter), discovered in 1988 (Chisholm et al. 1988). The middle population is characterized by a bright orange phycoerythrin fluorescence in addition to the red one and corresponds to *Synechococcus*, another coccoid cyanobacterium of size around 1 µm. Finally, the larger population that only emits red fluorescence corresponds to tiny eukaryotes, often called picoeukaryotes. In the rest of the paper, we define "picoplankton" as species or strains representative of the three populations. Flow cytometric analyses of field samples (DV, FP and DM, unpublished data) demonstrate that picoplankton populations can pass through a 3 µm filter, but are, in general, partially retained by a 2 µm filter, the original threshold defined by Sieburth (Sieburth et al. 1978). Setting a higher threshold, e.g. 5 µm would encompass genera such as *Phaeocystis* or *Emiliania*, not usually considered as part of picoplankton. We prefer the term picoplankton to that of ultraplankton (e.g. Li & Wood 1988), because it is of much wider use. Therefore, in what follows we consider as picoplankton, species or strains for which at least one of the dimensions may fall below 3 µm (Stockner & Antia 1986).

Despite the recognition of its ecological role, the diversity of picoplankton remains little studied. Although discovered the latest, it is probably *Prochlorococcus* that has attracted most interest, both for its major contribution in open ocean waters and for its unusual characters such as, the presence of modified chlorophyll *a* and *b* (Goericke & Repeta 1992). A large number of strains have been isolated from equatorial, subtropical and Mediterranean waters and characterized physiologically (e.g. Moore et al. 1995) and genetically (e.g. West et al. 2001) forming a number of different clades, two adapted to high light and several to low light. The genome sequences of three strains are currently available (Hess et al. 2001), yielding a wealth of information on the relationship between genes and niches and re-enforcing the current interest in *Prochlorococcus*. Despite an initial surge of interest in the 80's (Waterbury et al. 1986), the diversity of *Synechococcus*, probably the most ubiquitous phytoplankton genus on Earth since it is found from equatorial to polar waters (e.g. Neuer 1992), has remained little studied until very recently, but is probably very high (Honda et

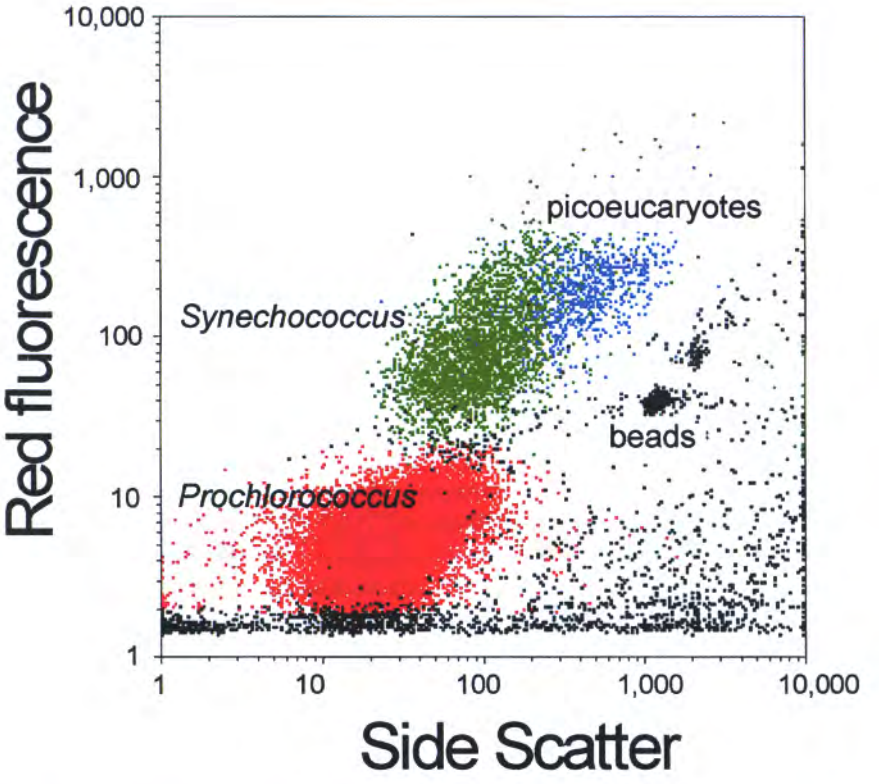

Fig. 1: Flow cytometry diagram of a natural sample from the Equatorial Pacific (OLIPAC cruise, CTD 67) taken at 75 m. Bead diameter is 0.95 µm.

al. 1999, Toledo et al. 1999, Fuller et al. 2003). In contrast to prokaryotes, no systematic effort was made until recently to describe the diversity of picoeukaryotes. Less than 50 algal species may qualify as picoplankton (Table 1). This is very few compared to the estimated 100,000 extant species of diatoms for example (van den Hoek et al. 1995). In some cases, the description of novel picoplankton species has required the creation of novel classes such as the Pelagophyceae (Andersen et al. 1993) and the Bolidophyceae (Guillou et al. 1999b). Recent work using molecular tools, in particular cloning and sequencing of 18S rDNA, revealed that many picoeukaryote sequences from the marine environment do not correspond to known sequences from described or cultured organisms (Moon-van der Staay et al. 2000, Moon-van der Staay et al. 2001).

Therefore there is a clear need to isolate cultures from all groups of marine picoplankton, first, to describe novel species not yet isolated from the marine environment (some of them may correspond to novel taxonomic groups), second, to establish ecotype diversity within ubiquitous genera such as *Prochlorococcus* or *Synechococcus*, and third, to conduct ecophysiological studies on species of ecological

Table 1: List of selected protist species for which the minimum length is smaller than or equal to 3 µm based on the available literature. In bold, we highlighted species for which the maximum size is also lower than 3 µm, i.e. those that are always part of the picoplankton fraction.

Class	Genus	Species	Authority	Min. Length (µm)	Max. Length (µm)	Hetero-trophic
Ancyromonadida	*Ancyramonas*	*sigmoides*	Kent	3	5	+
Bacillariophyceae	*Chaetoceros*	*tenuissimus*	Meunier	3	5	
Bacillariophyceae	*Thalassiosira*	*pseudonana*	Hasle & Heimdal	2.3	5.5	
Bicosoecida	*Cuvcitellus*	*parvulus*	(Griessmann) Patterson et al.	3	10	+
Bicosoecida	***Symbiomonas***	***scintillans***	Guillou & Chrétiennot-Dinet	**1.2**	**1.5**	+
Bolidophyceae	***Bolidomonas***	***mediterranea***	Guillou & Chrétiennot-Dinet	**1**	**1.7**	
Bolidophyceae	***Bolidomonas***	***pacifica***	Guillou & Chrétiennot-Dinet	**1**	**1.7**	
Cercomonadida	*Massisteria*	*marina*	Larsen & Patterson	3	7	+
Chlorophyceae	*Stichococcus*	*cylindricus*	Butcher	3	4.5	
Chrysophyceae	*Paraphysomonas*	*antarctica*	Takahashi	2	4.3	+
Chrysophyceae	*Paraphysomonas*	*imperforata*	Lucas	1.7	5.1	+
Chrysophyceae	*Paraphysomonas*	*sideriophora*	Thomsen	3	5	+
Chrysophyceae	***Picophagus***	***flagellatus***	Guillou & Chrétiennot-Dinet	**1.4**	**2.5**	+
Chrysophyceae	*Tetraparma*	*insecta*	Bravo-Sierra & Hernández-Becerril	2.8	3.8	
Chrysophyceae	***Tetraparma***	***pelagica***	Booth & Marchant	**2.2**	**2.8**	
Chrysophyceae	*Triparma*	*columacea*	Booth	2.3	4.7	
Chrysophyceae	*Triparma*	*laevis*	Booth	2.2	3.1	
Chrysophyceae	*Triparma*	*retinervis*	Booth	2.7	4.5	
Cryptophyceae	***Hillea***	***marina***	Butcher	**2.5**	**2.5**	
Eustigmatophyceae	*Nannochloropsis*	*gaditana*	Lubián	2	4	
Eustigmatophyceae	*Nannochloropsis*	*granulata*	Karlson & Potter	2	4	
Eustigmatophyceae	*Nannochloropsis*	*limnetica*	Krienitz et al.	1.5	6	
Eustigmatophyceae	*Nannochloropsis*	*oculata*	(Droop) Hibberd	2	4	
Eustigmatophyceae	*Nannochloropsis*	*salina*	Hibberd	3	4	
Pelagophyceae	***Aureococcus***	***anophagefferens***	Hargraves & Sieburth	**1.5**	**2**	
Pelagophyceae	*Aureoumbra*	*lagunensis*	Stockwell et al.	2.5	5	
Pelagophyceae	***Pelagococcus***	***subviridis***	Norris	**2.5**	**3**	
Pelagophyceae	***Pelagomonas***	***calceolata***	Andersen & Saunders	**2**	**3**	
Pinguiophyceae	***Pinguiochrysis***	***pyriformis***	Kawachi	**1**	**3**	
Pinguiophyceae	*Pinguiococcus*	*pyrenoidosus*	Andersen et al.	3	8	
Prasinophycae	***Bathycoccus***	***prasinos***	Eikrem & Throndsen	**1.5**	**2.5**	
Prasinophycae	*Crustomastix*	*stigmatica*	Zingone	3	5	
Prasinophycae	*Dolichomastix*	*eurylepidea*	Manton	3	3	
Prasinophycae	***Dolichomastix***	***lepidota***	Manton	**2.5**	**2.5**	
Prasinophycae	*Dolichomastix*	*tenuilepis*	Throndsen & Zingone	3	4.5	
Prasinophycae	*Mantoniella*	*squamata*	(Manton & Parke) Desikachary	3	5	
Prasinophycae	***Marsupiomonas***	***pelliculata***	Jones et al.	**3**	**3**	
Prasinophycae	***Micromonas***	***pusilla***	(Butcher) Manton & Parke	**1**	**3**	
Prasinophycae	***Ostreococcus***	***tauri***	Courties & Chrétiennot-Dinet	**0.8**	**1.1**	
Prasinophycae	***Picocystis***	***salinarum***	Lewin	**2**	**3**	
Prasinophycae	*Prasinococcus*	*capsulatus*	Miyashita & Chihara	3	5.5	
Prasinophycae	*Prasinoderma*	*coloniale*	Hasegawa & Chihara	2.5	5.5	
Prasinophycae	*Pseudoscourfieldia*	*marina*	(Throndsen) Manton	3	3.5	
Prasinophycae	*Pycnococcus*	*provasolii*	Guillard	1.5	4	
Prasinophycae	***Resultor***	***micron***	(Throndsen) Moestrup	**1.5**	**2.5**	
Prymnesiophyceae	*Chrysochromulina*	*elegans*	Estep et al.	3	4	
Prymnesiophyceae	*Chrysochromulina*	*leadbeateri*	Estep et al.	1.5	4	

Class	Genus	Species	Authority	Min. Length (µm)	Max. Length (µm)	Hetero-trophic
Prymnesiophyceae	*Chrysochromulina*	*simplex*	Estep et al.	2	6	
Prymnesiophyceae	*Chrysochromulina*	*tenuisquama*	Estep et al.	2	5	
Prymnesiophyceae	*Dicrateria*	*inornata*	Parke	3	5.5	
Prymnesiophyceae	*Imantonia*	*rotunda*	Reynolds	3	4	
Prymnesiophyceae	*Phaeocystis*	*cordata*	Zingone	3	3.5	
Prymnesiophyceae	*Trigonaspis*	*minutissima*	Thomsen	2	3.6	
Trebouxiophyceae	***Chlorella***	***nana***	Andreoli et al.	**1.5**	**3**	
Trebouxiophyceae	*Choricystis*	*minor*	(Skuja) Fott	1.5	3.5	
Trebouxiophyceae	***Nannochloris***	***atomus***	Butcher	**2**	**3**	
Trebouxiophyceae	***Nannochloris***	***eucaryotum***	(Wilhelm et al.) Menzel & Wild	**3**	**3**	
Trebouxiophyceae	***Nannochloris***	***maculata***	Butcher	**3**	**3**	

or biological interest especially for key parameters of the marine environment such as light or nutrient assimilation. Only few algal collections detain significant holdings in this area. A notable exception is the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) in the USA (http://ccmp.bigelow.org/index.html) that has a large set of picoplankton strains (Andersen et al. 1997), some of them not yet described formerly (Potter et al. 1997). Some picoplankton strains are also available from the Marine Biotechnology Institute Culture collection (MBIC) in Japan (http://www.mbio.jp/mbic). However no such facility was existing in Europe and our goal in setting up the Roscoff Culture Collection (RCC) was to fill up this gap.

In this paper, we first present the RCC facility and discuss our isolation, purification and characterization procedures. We summarize then our current holdings and taking two examples of large isolation efforts, discuss what are the organisms most commonly isolated from marine picoplankton. Finally, we discuss future directions that could be followed in order to increase the representativeness of our collection.

Collection history

The Roscoff Culture Collection started informally in the late 80's and early 90's, when at the favor of several cruises, in particular CHLOMAX conducted in 1987 in the Sargasso Sea (Neveux et al. 1989), EROS in 1989 in the Mediterranean Sea (Vaulot & Partensky 1992) and EUMELI in 1991 in the Tropical East Atlantic (Partensky et al. 1996), members of the Roscoff Phytoplankton team (DV, FP, C. Courties) began to isolate picoplankton cultures. Following the isolation of a first *Prochlorococcus* strain from the chlorophyll maximum in the Sargasso Sea by B. Palenik, then at the Massachusetts Institute of Technology, we were lucky enough (with the help of S. W. Chisholm who supplied us with the medium recipe) to isolate a second *Prochlorococcus* strain (MED), representative of one of the high light clades. One clonal culture obtained from the latter (MED4) is the first *Prochlorococcus* strain for which the full genome sequence became available (http://www.jgi.doe.gov/). Unbeknown to us, at the same time, we isolated strains that would only be

described several years later, such as strain MaxEuk71 (RCC 101) isolated in 1987 that can be assigned to *Pelagomonas calceolata* described in 1993 (Andersen et al. 1993) or strain EUM13B (RCC 143) isolated in 1991 that belongs to the genus *Ostreococcus*, only described in 1995 (Chrétiennot-Dinet et al. 1995). As our number of strains increased following new cruises, in particular OLIPAC in 1994 in the Equatorial Pacific, MINOS in 1996 and PROSOPE in 1999 in the Mediterranean Sea and systematic isolation of strains off Roscoff more recently in 2000 and 2001, we progressively decided to formalize the collection starting in 1998. The RCC is now registered with the World Data Center for Microorganisms under accession number 829 (http://wdcm.nig.ac.jp/CCINFO/CCINFO.xml?829). It is also part of the "Souchothèque de Bretagne", a regional assemblage of four microbial collections from Brittany including algae, bacteria and fungi. The RCC current director is Daniel Vaulot and curator, Florence Le Gall.

Facilities and culture maintenance

The RCC is hosted by the Station Biologique de Roscoff, one of the oldest marine laboratories in the world, founded in 1872. It is held in two medium-sized constant temperature rooms, one for tropical and subtropical cultures set at 20°C and one for temperate cultures set at 15°C. These rooms are equipped with several racks fitted with fluorescent tubes, that can provide light levels between 4 and 100 µmol quanta m^{-2} s^{-1}. For the lower intensities used to isolate and maintain tropical and subtropical strains, fluorescent tubes are wrapped in blue plastic paper (Lee Filters, Moonlight Blue n. 183) to better reproduce the light spectrum encountered at depth in oligotrophic "blue" waters. In addition, we have also two small light cabinets; one for high temperature adapted strains (30°C) and one for polar strains (4°C).

All our cultures are maintained in plasticware. We use routinely the following recipients:

- 12 mL disposable polystyrene tubes (CML, Nemours, France) for maintaining strains.
- 50 mL and 250 mL disposable polystyrene tissue culture flasks (Sarstedt, Orsay, France). These flasks are used to grow cultures for analyses such as DNA or pigment extraction, ecophysiological experiments as well to establish pre-cultures from field samples prior to isolation (see below).
- 250 mL to 10 L Nalgene polycarbonate bottles (VWR International, Strasbourg, France). These bottles are used for analyses and experiments that require larger volumes such as pigment or protein analyses.

All chemicals for medium preparation are purchased, except when noted, from Sigma (Saint-Quentin Fallavier, France). The following media are used for transfer and experiments:

- K medium (Keller et al. 1987) for most eukaryote strains, except for diatoms for which we add Si.
- f/2 (Guillard 1975) for a few eukaryotic strains. These strains often come from other collections that used f/2 for transfer. We try to acclimate them progressively to K if possible.

- PCR-S11 (Rippka et al. 2000) for all *Synechococcus* and *Prochlorococcus* strains.

As our attention has recently focused towards the isolation of heterotrophic picoeukaryotes, we are also using media based on rice (40 rice grains added per 1 L sea water followed by autoclaving 2 to 3 times) and yeast extract (0.5 g of yeast extract added to 1 L seawater followed by autoclaving 2 to 3 times).

All our media are based on natural sea water processed as follows. Water is collected in 20-30 L polypropylene carboys, mostly offshore Roscoff in surface and sometimes from oligotrophic locations (such as the Mediterranean Sea) depending on cruises of opportunity. The water is left to age at room temperature for at least one month and then filtered through a 90 mm diameter 0.2 µm filter and an AP15 prefilter (both from Millipore) using a peristaltic pump. Medium components are prepared as independent stock solutions of N, P, metals, vitamins, and in some cases buffer (e.g. Hepes), except f/2 for which we use a pre-weighted mix from Sigma. These stock solutions (except vitamins) are autoclaved for 20 min at 120°C whereas the seawater (generally 10L is prepared at a time in polypropylene Nalgene carboys) is autoclaved for 1 h 30 at 120°C. Stock solutions are then added sterilely to the cooled seawater together with vitamins which are simply filter sterilized through 0.2 µm with a syringe. Then the mixture is again filter sterilized under a flow hood directly into a sterile Nalgene polycarbonate carboy through a 90 mm diameter system autoclaved in advance with a 0.2 µm Millipore filter. This last step proves useful for preventing formation of particles and for the long term conservation of media.

All cultures are maintained by transfer at frequencies varying between 2 and 10 weeks. Series of up to 4 consecutive transfers are kept at any given time to prevent as much as possible culture loss. During transfer, cultures are verified either visually (for color, presence of a pellet), or by inverted microscopy, or by flow cytometry for the smallest sized strains such as *Prochlorococcus*.

All strains are given a unique RCC number when entered in the collection. They also have a strain name that encapsulates their origin (e.g., MINOS01 for a strain isolated during the MINOS cruise). All available data for each individual strain are stored in an Access database, including in particular: taxonomy, isolation and purification history, images, phenotypic and genotypic characters. In the same database, miscellaneous information is also stored such as, the origin of sea water, the date of preparation for each medium batch, or the distribution of strains to outside institutions.

A subset of the culture collection (about 270 strains, excluding those obtained from other collections such as the CCMP) is available to interested researchers. A periodically updated catalog as well as ordering information is available from the web (http://www.sb-roscoff.fr/Phyto/RCC/index.php). At present, we do not charge for strains, but pickup of the strains from our laboratory must be arranged by the receiving party.

Strain isolation procedures

Since our major aim is to isolate picoplankton sized cultures, we have developed a number of procedures and achieve this goal taking into account several considerations.

Many picoplankton species grow in oligotrophic waters where fairly constant environmental conditions prevail. Since they may not resist very well to stresses often associated with isolation, we try to reduce these at all cost. During oceanographic cruises, we bring on board lighting equipment and we try to match both ambient light levels and light spectra (e.g. using blue filters for deep samples). We also try to minimize time between sample collection and set up of pre-cultures (see below) down to typically one or two hours. We also minimize transfer time of pre-cultures from the ship to the laboratory. This is indeed one of the most critical parameters to ensure that fragile cultures will not be lost. As an example, for the OLIPAC cruise from which we isolated at least three novel picoplanktonic species, *Bolidomonas pacifica* (Guillou et al. 1999b), *Picophagus flagellatus*, and *Symbiomonas scintillans* (Guillou et al. 1999a), transfer from the ship in Tahiti to the laboratory in Roscoff took less than 24 hours, the pre-cultures being kept at room temperature in an ice box during that period. When on board culture isolation is not feasible, we try to keep the sampled water in a large container (20 to 50 L) and to bring it rapidly to the laboratory. This strategy was successful to isolate *Prochlorococcus* strain MED: a large carboy of water was sampled from surface waters of the Mediterranean Sea through the ship clean water supply line on the last day of the EROS cruise in January 1989 and brought back to Roscoff, where we started the pre-cultures.

Pre-cultures are usually established by gravity filtering the initial sample through one or two superimposed filters(s) (0.6, 2 or 3 µm) and then adding 1/10 or 1/100 of full medium (typically K and PCR-S11). The filtration step is critical to remove larger species such as diatoms that would take over very quickly following nutrient addition. We have tried to vary the form of the nutrients supplied (e.g. ammonia vs. nitrates), but we did not see any systematic influence, although the effect may be very subtle. Pre-cultures are usually set-up in 50 mL tissue flasks with which we have had much better success than in smaller volumes such as, 15 mL tubes or 96 well plates. Pre-cultures are then periodically screened by flow cytometry to monitor any cell growth. Again, the use of flow cytometry is critical because we are able to detect growth at a very early stage before any color is developing in the tubes. In some lucky cases, a single species may ultimately survive. However in most cases, mixed communities occur and then the culture needs to be purified further. If we are interested in smaller cells (e.g. *Prochlorococcus* or *Synechococcus*), then several round of filtration through 0.6 µm is often very efficient to get rid of the larger cells. We have also attempted to sort the cells of interest by flow cytometry. However, the instrument we currently own (Becton Dickinson FACSort) proved to be very poorly suited for this task, yielding a very high number of contaminated cultures (typically over 50%), despite taking as much care as possible to clean up and sterilize the instrument immediately prior to sorting. High speed sorters with a jet in air are clearly a pre-requisite for this task (Sensen et al. 1993, Moore et al. 1998). Finally in most cases, the classical dilution approach (Throndsen 1978) using 1 to 5 cells per tube is best to obtain pure cultures, although for some groups like *Prochlorococcus*, growth is rarely observed in the most diluted tubes of the dilution series. One major problem that we encounter is contamination of algal cultures by heterotrophic eukaryotes. Although the latter may be very commonly isolated species such as, *Paraphysomonas* spp. (Lim et al. 1999), these contaminants may also turn out to be

very interesting organisms, more valuable than their autotrophic companion. As an example, we have repeatedly isolated *Imantonia rotunda* from local English Channel waters, which was contaminated by a biflagellated cell that turned out to be *Telonema subtilis* Griessmann (Griessmann 1913). These mixed cultures allowed to start an interesting study of the latter, which until now had not been isolated and could not be assigned to a defined protist group (Shalchian-Tabrizi et al., submitted).

Characterization and monitoring of cultures

Since it is difficult to identify picoplankton species because of their small cell size and of the absence, in most cases, of clear distinctive morphological features, we rely on several different approaches first to characterize, and then to monitor our cultures (e.g. to detect contamination).

Optical microscopy. We document all cultures with digital pictures (Fig. 2) taken by a Spot RT-Slider cooled CCD camera (Diagnostic Instruments Inc., Sterling Heights, Michigan) on an Olympus BX51 microscope. We try to note important features such as cell size, presence of flagella, swimming behavior, or cell refractive index. Careful and frequent microscopical observations are of particular importance to rapidly detect drift in cultures not yet fully purified (i.e. a cryptic species replacing a dominant species) as well as to check for contaminants.

Electron microscopy. Access to a transmission electron microscope is necessary when dealing with very small cells. Simple whole mount preparations can reveal critical features such as the number, size and insertion of flagella, organic scale covering, etc.

Flow cytometry. Flow cytometry is critical to detect and monitor small-sized strains such as *Prochlorococcus* that are almost impossible to distinguish from heterotrophic bacteria by light microscopy. Moreover *Prochlorococcus* strains grow often quite slowly and do not reach high cell densities (at least as long as they are not yet fully acclimated to the laboratory conditions) and one cannot rely on the color of the culture to assess the status of the cultures. We also use flow cytometry to rapidly assess the axenic status of algal cultures by staining heterotrophic bacteria with SYBR Green after fixation with glutaraldehyde 0.25% (a modification from Marie et al. 1999).

Spectrofluorometry. This technique is applied mostly to *Synechococcus* and *Prochlorococcus* cyanobacteria to distinguish different pigment phenotypes. For example, marine *Synechococcus*, contain two chromophores, phycourobilin (PUB) and phycoerythrobilin (PEB), and their ratio is a fairly stable phenotypic feature (Wood et al. 1985), although it has been shown, for some strains, to change with light quality (Palenik 2001).

Pigment analysis by High Precision Liquid Chromatography (HPLC). Eukaryotes contain a wide diversity of photosynthetic pigments, which are best characterized by HPLC analysis. Although pigment signature is useful for class level

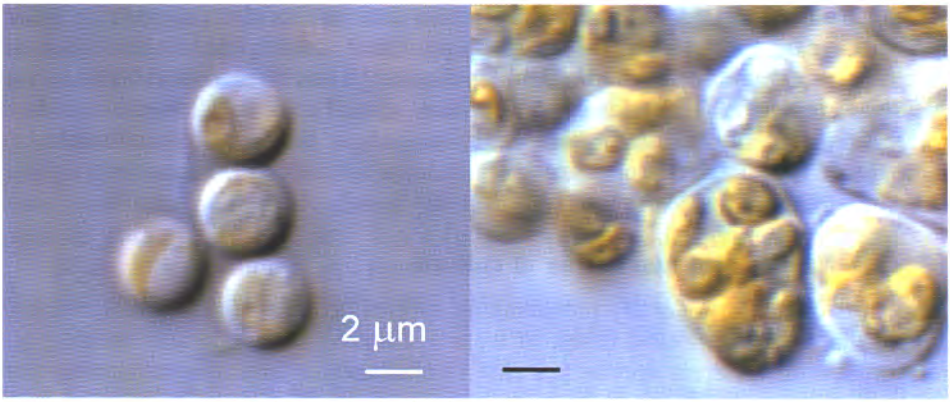

Fig. 2: Microscopy image (differential interference contrast) of two picoplanktonic species, *Pycnococcus provasolii* (left, RCC 444) and *Prasinoderma coloniale* (right, RCC 137). Scale bars are 2 µm.

assignment when certain pigments are present (e.g. prasinoxanthin for Prasinophyceae or alloxanthin for Cryptophyceae), it finds quickly its limits. For example, some Prasinophyceae do not contain prasinoxanthin or the carotenoid signature of several classes (Pelagophyceae, Chrysophyceae, Dictyochophyceae, Prymnesiophyceae) is similar (presence of fucoxanthin and of its two derivatives 19'hexanoyl- and 19'butanoyloxyfucoxanthin).

Genetic markers. We have recently switched to the systematic use of genetic markers since they usually provide a clean and definite answer to identify organisms at the genus/species level. We found the following markers most useful from a practical point of view.

- *SSU rDNA (small sub-unit ribosomal RNA gene)*. Because of the large amount of data available, this gene is by far the most useful. For eukaryotes, a short 500 bp sequence in the most variable region (roughly from position 500 to 1000) of the 18S rDNA gene allows to assign a strain at the genus/species level. Further differentiation, of ecotypes for example, requires the use of intergenic spacers (ITS).
- *pcb*. This photosynthetic antenna gene that may be present in several copies in *Prochlorococcus* (Garczarek et al. 2000) is very useful for distinguishing ecotypes within this important genus by RFLP (see below).
- *ntcA*. This gene implicated in the response to nitrogen starvation (Lindell et al. 1998) can be used to distinguish *Synechococcus* ecotypes by RFLP (Fuller et al. 2003).

For each genetic marker, several alternate techniques can be used.

- *Restriction Fragment Length Polymorphism (RFLP)*. This very simple approach proves fairly useful, once algae are characterized, to verify that the culture is not

contaminated or has not drifted. After PCR amplification, the gene is restricted with one enzyme (e.g. *Hae III* for 18S rDNA and *pcb*) and loaded onto a 2% agarose gel. The obtained banding pattern is compared to a reference database of both observed and theoretical RFLP patterns running under Access.

- *Denaturing Gradient Gel Electrophoresis (DGGE).* Although more complex to set up than RFLP, DGGE (Muyzer 1999) can be a useful tool to screen enrichment cultures in order to estimate the number of occurring species and to determine whether interesting organisms are present. One should be cautious however that a single species may give several bands, e.g. if it contains several slightly different copies of the target gene. We use DGGE with the 18S rDNA gene. A 500 bp fragment is amplified by PCR, one of the primers being fitted at the 5' end by a long GC clamp. The products are then loaded on a denaturing gradient polyacrylamide gel that separates products of the same size, but that have different base pair composition, following the protocol of Díez et al. (2001). Interesting bands can then be sequenced.
- *Gene cloning and sequencing.* This is by far the most informative approach. With the dramatic decrease in the cost of sequencing, we are now systematically sequencing a short variable region of 18 rDNA (see above) for all eukaryotes. After amplification of the full length 18S rDNA gene with universal eukaryotic primers (Moon-van der Staay et al. 2000), the products are cloned into a TOPO-TA vector (InVitrogen) and individual clones are screened by RFLP and sequenced using an internal primer (Elwood et al. 1985), Euk528f (5'-CCG CGG TAA TTC CAG CTC-3'), on an ABI 3700 sequencer, yielding about 500-600 bp starting from position 528 (*E. coli*). The sequence data are compared to public and RCC eukaryotic sequences stored in a large database running under the ARB software (http://www.arb-home.de/).

Overview of the available strains

At the time of writing (November 2002), the RCC contains 533 non-redundant cultures. A limited number of RCC strains are duplicated, e.g. because the same strain has been maintained historically at two different temperatures and given two different RCC numbers. Table 2 provides a summary of our current holdings arranged by Class. Among our entries, 104 have been obtained from other collections such as the CCMP. The remaining 429 strains have been isolated either by us or by partners of the European PICODIV program and therefore are unique to our collection (although in some cases, e.g. new species, we have deposited them to the CCMP for safety reasons). Picoplanktonic strains (i.e. with an average size below 3 µm) constitute about half of our holdings (Table 2). The major groups represented are cyanobacteria (*Prochlorococcus* and *Synechococcus*), and Prasinophyceae (especially *Bathycoccus*, *Micromonas*, *Ostreococcus* and *Pycnococcus*). In fact, the number of picoplanktonic genera in our collection is fairly limited (18, Table 3) although we have at present a dozen of picoplanktonic strains that will require formal description probably as novel species. Moreover, species diversity within a given genus can be high. For example, we have at present 20 strains of *Ostreococcus*, for which 18S rDNA gene phylogeny suggests at least 4 clades (Guillou et al. 2004) and chromosome separation and sizing

Table 2: Number of strains organized according to algal classes held by RCC (November 2002).

Division	Class	Total	Specific to RCC	Pico sized (< 3 µm)
Bacteria	Cyanophyceae	143	124	134
Chlorophyta	Chlorophyceae	10	5	
	Trebouxiophyceae	26	22	21
	Prasinophyceae	103	69	70
Rhodophyta	Rhodophyceae	1	1	
Chlorarachniophyta	Chlorarachniophyceae	4	4	
Cryptophyta	Cryptophyceae	5	3	
Haptophyta	Prymnesiophyceae	34	23	10
	Pavlovophyceae	2	1	
Stramenopiles	Bolidophyceae	8	8	8
	Diatomophyceae	19	7	
	Fucophyceae	1	1	
	Chrysophyceae	7	6	2
	Dictyochophyceae	13	12	5
	Eustigmatophyceae	4	3	4
	Pelagophyceae	13	8	10
	Pinguiophyceae	6	6	1
	Bicosoecida	5	5	2
	Stramenopiles (heterotrophic)	5	5	
Alveolates	Dinophyceae	8	1	
	Ciliophora	1	1	
Euglenozoa	Euglenophyceae	1	1	
	Bodonids	1	1	
Incertae sedis	*Telonema*	3	3	
Unknown		110	110	13
	Total	**533**	**430**	**280**

by pulse field gel electrophoresis (Derelle et al. 2002) reveal at least 9 genotypes (Derelle & Moreau, unpublished). For *Micromonas*, 18S rDNA sequences cluster into three well-defined clades (Guillou et al. 2004). For marine *Synechococcus*, the situation is even more complex with at least 10 different clades based on 16S rDNA and within each clade, major phenotypic variability (e.g. presence or absence of motility) is observed (Fuller et al. 2003).

The geographic localization of our picoplankton strains is quite diverse (Fig. 3), but still mostly limited to regions where we had the opportunity to conduct cruises. Vast oceanic areas are not represented yet in our collection such as, the Indian Ocean, the South Atlantic, or polar waters. Moreover, specific habitats such as coastal lagoons, coastal upwellings, or ice edges will need to be investigated since they may harbor interesting, although probably marginal, picoplankton species. Many genera are very ubiquitous. One good example is *Ostreococcus* that has been isolated from the tropical Atlantic, the Red Sea, the Mediterranean Sea and English Channel coastal waters.

Table 3: Number of picoplanktonic strains organized according to genus held by RCC (November 2002).

Class	Genus	Count
Cyanophyceae	*Synechococcus*	106
Cyanophyceae	*Prochlorococcus*	22
Trebouxiophyceae	*Nannochloris*	19
Prasinophyceae	*Ostreococcus*	20
Prasinophyceae	*Micromonas*	17
Prasinophyceae	*Bathycoccus*	11
Prasinophyceae	*Pycnococcus*	13
Prasinophyceae	*Prasinoderma*	1
Prasinophyceae	*Mantoniella*	1
Prymnesiophyceae	*Imantonia*	8
Bolidophyceae	*Bolidomonas*	8
Chrysophyceae	*Picophagus*	2
Eustigmatophyceae	*Nannochloropsis*	4
Pelagophyceae	*Aureococcus*	1
Pelagophyceae	*Aureoumbra*	1
Pelagophyceae	*Pelagococcus*	1
Pelagophyceae	*Pelagomonas*	7
Bicosoecida	*Symbiomonas*	2
	Total	**244**

Large scale isolation efforts of picoplankton strains: two examples

During some of the cruises and projects, to which we participated, we have made systematic efforts to isolate picoeukaryotes. In the following paragraphs, we try to determine, based on two examples (one cruise in the Mediterranean Sea and seasonal isolation off Roscoff), whether any pattern emerges relating isolated strains to environmental conditions and isolation strategy.

1. The PROSOPE cruise (Mediterranean Sea)

During the PROSOPE cruise conducted through the Mediterranean Sea in the fall of 1999, we established pre-cultures at 9 different stations and at 4-6 depths over the euphotic zone. Each sample was pre-filtered through either 3 or 0.6 µm. The former filtrate was enriched with K/10, K/10+urea or K/100 medium, while the latter was enriched with PRO2/10 (Moore & Chisholm 1999) with or without nitrates as N-source. Pre-cultures from the surface layer and the deep chlorophyll maximum were illuminated at 100-150 and 5-20 µmol quanta m^{-2} s^{-1}, respectively. Overall 160 pre-cultures were obtained that were monitored and purified as explained above (see Strain isolation procedures). The 55 unialgal strains obtained belong mostly to cyanobacteria (*Synechococcus*) and Prasinophyceae (Table 4), reflecting the dominance of these groups among picoplankton. However, we also had some interesting novel strains not corresponding to any described species from the Dictyochophyceae (e.g. RCC 446) and the Chlorarachniophyceae (RCC 365). The genus/species diversity of strains isolated in surface was higher than at depth (roughly

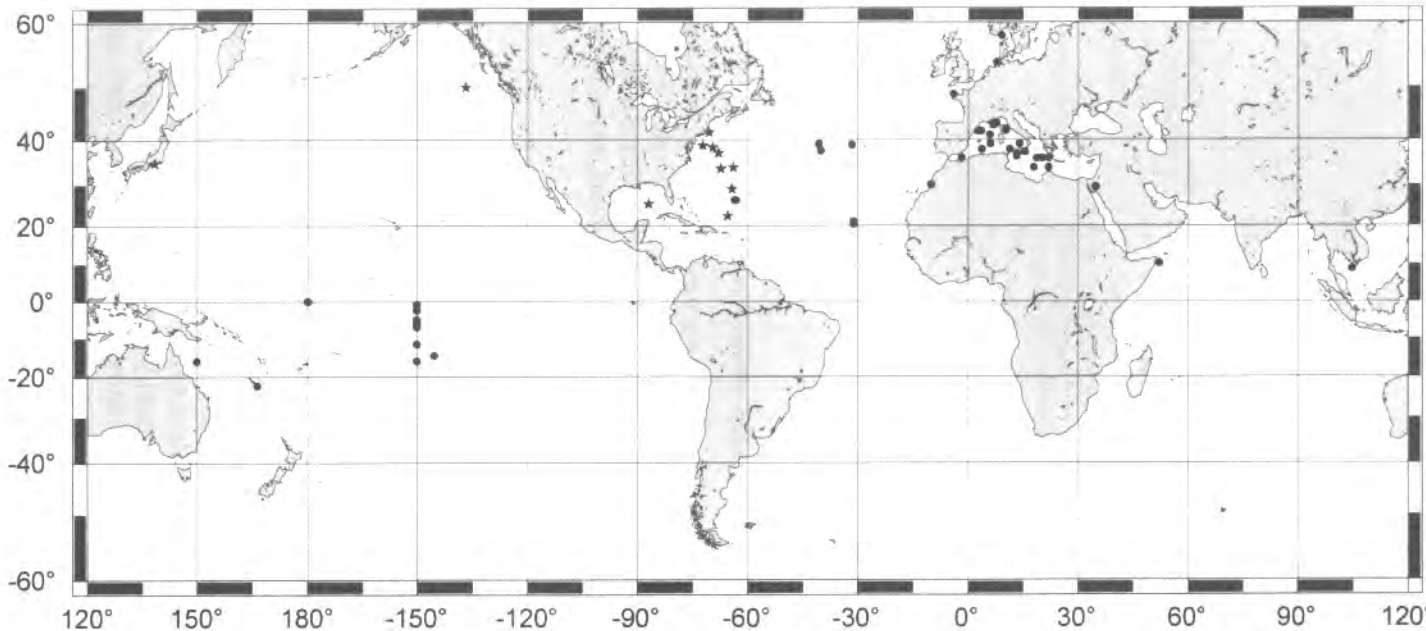

Fig. 3: Geographic localization of the RCC picoplankton strains. Squares correspond to strains that are unique to our collections while stars correspond to strains obtained from various collections (in particular the CCMP).

Table 4: Summary of identified cultures isolated during the PROSOPE cruise conducted in the Mediterranean Sea in fall 1999 according to station, depth, and filtration. No identified cultures were recovered from Station 1 (West Basin).

Location	Station	Latitude	Longitude	Surface < 0.6 µm	Surface < 3 µm	Deep < 0.6 µm	Deep < 3 µm
Morocco upwelling	UPW	30°N	10°W	*Ostreococcus*	*Synechococcus* *Mamiella* sp. *Mesopedinella arctica* *Chrysochromulina acantha*	*Ostreococcus*	
West Basin	3	38°N	3°E	*Synechococcus* Dictyochophyceae	*Synechococcus* Dictyochophyceae	*Pycnococcus*	*Synechococcus*
	8	39°N	14°E		*Synechococcus* *Bathycoccus*	*Synechococcus*	*Synechococcus*
	9	41°N	10°E	*Synechococcus*			*Synechococcus* *Ostreococcus*
	DYF	43°N	7°E		*Synechococcus* Chlorarachniophyceae		
East Basin	5	36°N	13°E		*Synechococcus*	*Ostreococcus*	*Synechococcus*
	7	37°N	15°E	Chlorarachniophyceae Dictyochophyceae *Chlamydomonas* sp.	*Synechococcus* *Chlorarachnion* sp.		*Synechococcus*
	MIO	33°N	22°E		*Synechococcus*	*Pelagomonas*	*Synechococcus*

below the thermocline) where only representatives of *Synechococcus*, *Ostreococcus*, *Pycnococcus* and *Pelagomonas* were recovered. The chlorophyll-rich Moroccan upwelling waters (Station UPW) clearly yielded a wider variety of strains. Filtration through two superimposed 0.6 mm filters (aimed initially at isolating *Prochlorococcus*, although none were recovered in fine) did not favor specifically smaller-sized strains. In fact, *Synechococcus* strains were more readily recovered from < 3 µm pre-cultures and conversely, nanoplankton strains (*Chlamydomonas* sp.) could be recovered from < 0.6 µm pre-cultures.

2. **The English Channel off Roscoff**

As part of an extensive study to assess and monitor picoplankton diversity in coastal waters (PICODIV), we made systematic efforts to isolate cultures from two stations off Roscoff, one located a few miles offshore in permanently mixed English Channel waters (Astan), and one in the bay of Morlaix (Dourduff). On 7 different occasions (once per season in 2000 and 3 times in Spring 2001), we started between 6 and 9 pre-cultures using 3 µm filtered samples. We tried to vary as much as possible pre-cultures conditions (K/10 with or without Si; K1/00; f/20; direct isolation on agar; immediate serial dilution at 1 or 5 cells per tube in K/10) exposed at 150-200 µmol quanta m^{-2} s^{-1} at 15°C. A total of 33 strains were recovered (Table 5). By far the most successful approach to obtain pure cultures, especially for the small Prasinophyceae,

Table 5: Summary of the genera isolated during the PICODIV project off Roscoff sorted according to season and date.

Class	Genus	Count	Date Isolation							
			Spring	Summer		Fall		Winter		
Cyanophyceae	*Synechococcus*	2		9-Jun-00	11-Jul-00					
Prasinophyceae	*Ostreococcus*	7	12-Apr-00	13-Jun-01		7-Sep-00	25-Sep-01			
	Bathycoccus	3		14-Jun-01		7-Sep-00				
	Micromonas	2		14-Jun-01						
	Mantoniella	1			11-Jul-00					
	Pycnococcus	1						19-Dec-00		
Prymnesiophyceae	*Chrysochromulina*	2		9-Jun-00	11-Jul-00					
	Emiliania	1	16-May-01							
	Imantonia	5	16-May-01	9-Jun-00				19-Dec-00		
Bacilliarophyceae	*Navicula*	4	17-Apr-01	9-Jun-00		7-Sep-00				
Dictyochophyceae	undescribed species	1	12-Apr-00							
Eustigmatophyceae	*Nannochloropsis*	1			11-Jul-00					
Incertae sedis	*Telonema*	3	12-Apr-00		11-Jul-00	7-Sep-00				

was immediate serial dilution. However, some interesting strains such as *Telonema* could not be obtained this way. Although Prasinophyceae, especially *Ostreococcus*, were dominating as in the case of the Mediterranean Sea, Prymnesiophyceae, in particular *Imantonia rotunda*, were also important. The absence of *Synechococcus* cyanobacteria, despite their constant presence off Roscoff, is explained by the fact that we did not retain them at the purification step. In general, more diverse cultures were isolated at a given date in summer compared to winter. Several genera such as *Ostreococcus* were recovered throughout the year. It is interesting to compare these data with 18S rDNA sequences directly obtained from natural picoplankton samples (filtered through 3 µm) for the same dates (Romari & Vaulot, 2004). In fact the majority of sequences falling into photosynthetic groups corresponded to Prasinophyceae (mostly *Ostreococcus*, *Bathycoccus* and *Micromonas*), Prymnesiophyceae, Cryptophyceae and Dinophyceae. While we recovered representatives from the first two groups, the latter were absent from our cultures. The use of taxon specific 18S rDNA oligonucleotide probes detected by fluorescent *in situ* hybridization (Not et al. 2002) permitted a more precise quantification of the abundance of three Prasinophyceae genera (*Micromonas*, *Bathycoccus*, and *Ostreococcus*), during an annual cycle (Not et al., unpublished data). While *Micromonas* was dominant throughout the year, *Bathycoccus* appeared only on sporadic occasions, but at noticeable concentrations, while *Ostreococcus* was always very marginal. Paradoxically, *Ostreococcus* was most often isolated, while *Micromonas* was only recovered from a single sample (Table 5).

3. **Representativeness of cultures**

The data from these two intensive isolation efforts clearly demonstrate that we largely fail to isolate representative strains from the environment. Often one strategy (or one operator) will favor isolation of the same species from very different environments.

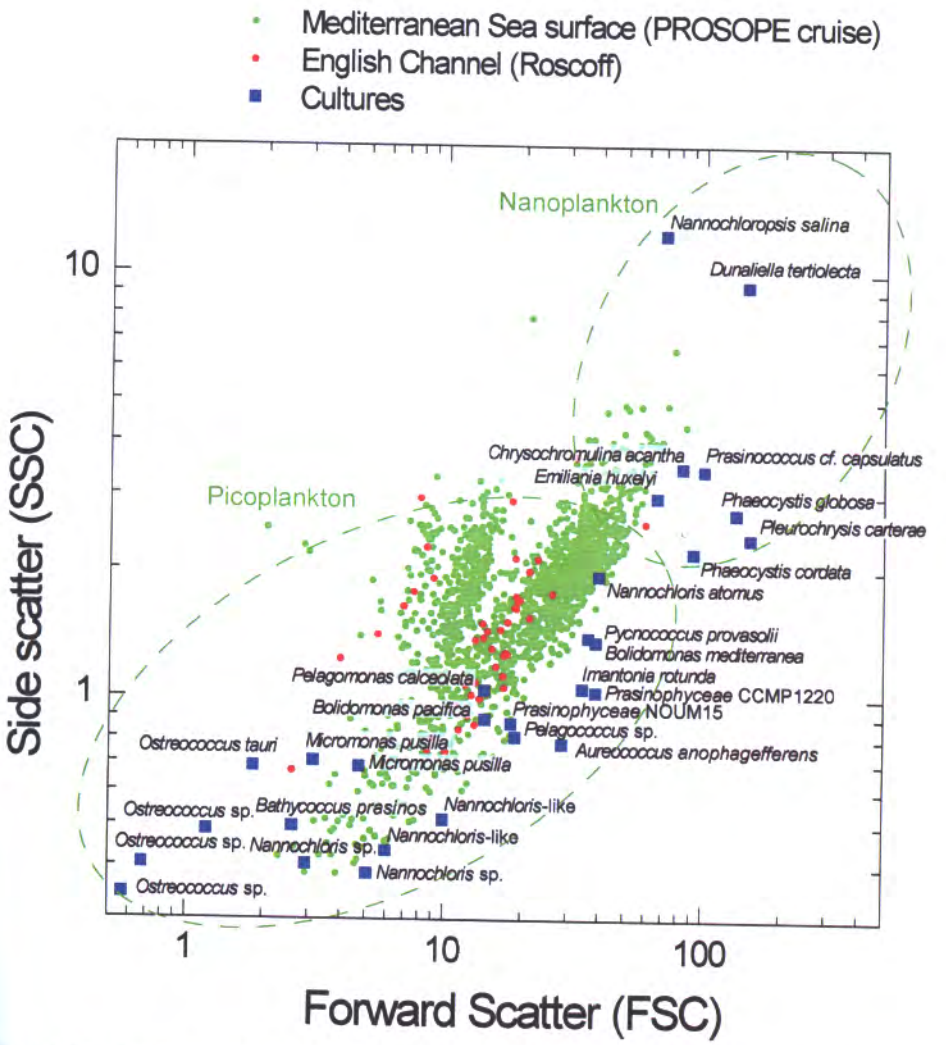

Fig. 4: Mean flow cytometry signature (forward vs. side scatter) of some pico- and nanoplankton strains from the RCC (blue squares) compared with the signature of environmental picoeukaryote communities sampled from surface water in the Mediterranean Sea (PROSOPE cruise, green circles) and the English Channel (Astan station off Roscoff, red circles). All data are normalized to 0.95 µm beads.

One striking example is *Ostreococcus* that went unnoticed until 1994 when it was found in the Thau lagoon (Courties et al. 1994) and that we now isolate routinely from many different locations.

We compared the flow cytometry signature of typical picoplankton strains from the RCC to that of surface picoeukaryote field populations (Fig. 4). We used forward

and side scatter, two parameters that are mostly influenced by cell size and refractive index and should depend less on culture conditions, in contrast to chlorophyll fluorescence that varies drastically with light intensity. Genera such as *Micromonas*, *Pelagomonas*, *Pycnococcus*, *Imantonia* or undescribed coccoid Prasinophyceae belonging to the same phylogenetic clade as strain CCMP 1205 have signatures roughly matching that of natural picoeukaryote populations. In contrast, genera such as *Ostreococcus* and *Bathycoccus* fall at the lower limit of natural population average signatures and are probably not typical of open ocean waters. On the high end of the size spectrum, the boundary between pico- and nanoplankton is somewhat loose and it is difficult to interpret field flow cytometric data by direct comparison to that of cultivated strains. More sophisticated approaches such as the use of FISH probes (Simon et al. 1995) or of cell sorting followed by molecular analyses are needed (Zubkov et al. 2002).

Perspectives

Clearly the effort to bring picoplanktonic strains into culture must continue. First, molecular approaches indicate that the marine environment harbors organisms which rDNA sequences have no match among cultivated strains. For photosynthetic groups, this is particularly true for Prasinophyceae and Prymnesiophyceae, for which several uncultivated clades have been detected for example in the Pacific Ocean (Moon-van der Staay et al. 2000, Moon-van der Staay et al. 2001), but also for Cryptophyceae and Dinophyceae, as well as for some classes for which no picoplanktonic species have been described yet (Dictyochophyceae, Chlorarachniophyceae). Still some uncultivated photosynthetic classes may remain to be discovered. For example, we detected a new set of 18S rDNA sequences related to red algae in European coastal waters (Romari and Vaulot, 2004). However, it is probably in the non-photosynthetic picoplankton that the largest effort must be achieved. The majority of environmental sequences found in marine picoplankton samples corresponds to non-photosynthetic groups falling into two divisions, the alveolates and the stramenopiles (Vaulot et al. 2002). Recent work clearly demonstrates that the corresponding organisms are heterotrophic (Massana et al. 2002). Isolating these organisms is critical to better understand their role in the marine ecosystem (predation, symbiosis, parasitism) as well as to shed light on protist phylogeny through a detailed look at their ultrastructure.

Besides these yet to be cultivated organisms, flow cytometry, culture isolation and molecular approaches have clearly established that a few genera are very ubiquitous (*Prochlorococcus*, *Synechococcus*, *Micromonas*, *Ostreococcus*). For each of these genera, a large ecotype diversity is observed and we need to increase significantly the number of available strains for these genera so that we can better decipher the relationship between ecotypes and environmental conditions as it was successfully done for *Prochlorococcus* (Moore et al. 1998, Moore & Chisholm 1999, Partensky et al. 1999). This will be even more important since these species have small genomes that are currently targeted for sequencing (e.g. *Prochlorococcus*, *Ostreococcus*). These genome data will make comparative physiology very attractive.

In order to bring new organisms into culture, we need to vary our isolation procedures. A few important parameters to be played with include, among others, the initial filtration method, the medium added (concentration and composition), the light and temperature levels. Immediate (i.e. without prior enrichment) isolation of picoplankton cells by serial dilution, plating or flow cytometry sorting should probably be tested on a large scale. Finally pre-cultures could be closely monitored with molecular methods such as DGGE or phylogenetic probes to better catch interesting organisms as they appear (Massana et al. 2002) and to favor their growth.

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