REVIEW ARTICLE

The diversity of small eukaryotic phytoplankton ($\leq 3\,\mu m$) in marine ecosystems

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Abstract

Small cells dominate photosynthetic biomass and primary production in many marine ecosystems. Traditionally, picoplankton refers to cells $\leq 2\,\mu m$. Here we extend the size range of the organisms considered to 3 μm, a threshold often used operationally in field studies. While the prokaryotic component of picophytoplankton is dominated by two genera, *Prochlorococcus* and *Synechococcus*, the eukaryotic fraction is much more diverse. Since the discovery of the ubiquitous *Micromonas pusilla* in the early 1950s, just over 70 species that can be $< 3\,\mu m$ have been described. In fact, most algal classes contain such species. Less than a decade ago, culture-independent approaches (in particular, cloning and sequencing, denaturing gradient gel electrophoresis, FISH) have demonstrated that the diversity of eukaryotic picoplankton is much more extensive than could be assumed from described taxa alone. These approaches revealed the importance of certain classes such as the Prasinophyceae but also unearthed novel divisions such as the recently described picobiliphytes. In the last couple of years, the first genomes of photosynthetic picoplankton have become available, providing key information on their physiological capabilities. In this paper, we discuss the range of methods that can be used to assess small phytoplankton diversity, present the species described to date, review the existing molecular data obtained on field populations, and end up by looking at the promises offered by genomics.

Introduction

The presence of tiny cells in the ocean had been suspected long before the term picoplankton was established. More than 150 years ago, Nägeli (1849) described the tiny green alga *Stichococcus bacillaris*. At the beginning of the 20th century, Lohmann (1908, 1911) realized that organisms still smaller than what was caught with plankton nets were present in the oceans. One of the first descriptions of a 'pico' cyanobacterium, *Synechocystis salina*, appeared in 1924 (Wislough, 1924). In the early 1930s, the importance of very small cells in the food chain was realized when Gaarder (1932) found small green algae (1–3 μm) to be the main food source of oyster larvae on the West Coast of Norway. In 1938, Ruinen described the heterotrophic *Cafeteria minuta* and in 1952, Butcher described the ubiquitous *Micromonas pusilla*. Knight-Jones (1951) calculated the abundance of ultra and nanoplankton in British coastal waters using the serial dilution method and found that picoplankton species like *Micromonas pusilla* and *Hillea marina* could be present in large numbers.

However, it was only in the late 1970s that the use of epifluorescence microscopy (Hobbie *et al.*, 1977) led to the realization of the abundance of bacteria in all marine systems. This was soon followed by the discovery of very small primary producers (Johnson & Sieburth, 1979, 1982; Waterbury *et al.*, 1979), leading to the conceptualization of the microbial loop (Azam *et al.*, 1983), which changed our view of marine ecosystems and shifted the scientific emphasis from the larger to the smaller sized organisms. At that time, picoplankton was formally defined as those cells whose size lies between 0.2 and 2 μm (Sieburth *et al.*, 1978), while ultraplankton was defined as cells $< 5\,\mu m$ (Murphy & Haugen, 1985). Since then, numerous scientific papers have

demonstrated the importance of these small cells in terms of biomass and production, in particular for its photosynthetic component (e.g. Li, 1994; Worden *et al.*, 2004).

A key advance with respect to picoplankton diversity was constituted by the landmark paper of Giovannoni *et al.* (1990). The use of molecular techniques to directly analyze gene sequences in natural samples led to the realization that our image of the diversity of these organisms obtained previously from cultivation approaches was heavily biased. This led to a flurry of studies analyzing the diversity of all components of the microbial loop (prokaryotes, viruses, and eukaryotes), leading to the discovery of many new taxonomic groups for which cultures were (and often still are) unavailable. However, gene sequences provide little information on the actual nature and role of the organisms they originate from and it is critical to obtain cultures to advance further. This is illustrated by the isolation of the key marine bacterial species '*Pelagibacter ubique*' (Rappé *et al.*, 2002), 12 years after its sequence was determined as SAR11 (Giovannoni *et al.*, 1990).

A new era is opening now with the development of genomics not only to sequence the genome of important marine small organisms that can be cultivated such as *Prochlorococcus* (Dufresne *et al.*, 2003; Rocap *et al.*, 2003) but also to determine directly fragments of the genome of natural populations (Béjà *et al.*, 2000; Venter *et al.*, 2004). Clearly, this creates new avenues to analyze the diversity of the microbial loop components.

However, in almost 30 years of development of the field of marine microbiology, small eukaryotes have received much less attention than prokaryotes. In fact, no taxonomic overview of marine eukaryotic picoplankton has been published since the work of Thomsen (1986), more than 20 years ago. With respect to molecular tools, 11 years elapsed between the paper of Giovannoni *et al.* (1990) and the widespread application of similar approaches to picoeukaryotes (Díez *et al.*, 2001b; López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001). One reason for this lack of attention is probably due to the fact that eukaryotes are perceived as possessing morphological features that allow identification. However, molecular approaches have revealed that the extent of the diversity of eukaryotes is probably as wide as that of bacteria and that many groups do not correspond to established taxa and are not available in culture.

In this review, we focus on the diversity of small eukaryotic marine phytoplankton without considering broader questions concerning its ecology or physiology. We extend the size range of the organisms considered to 3 μm, i.e. beyond that formerly assigned to picoplankton (≤ 2 μm), because recent field studies have often used 3-μm filters to separate small plankton from larger cells (e.g. Moon-van der Staay *et al.*, 2001).

Approaches to assess the diversity of small eukaryotic phytoplankton

Cultures vs. natural samples

A first strategy that can be used to examine the diversity of small eukaryotic plankton involves establishing cultures favoring smaller organisms. One of the most classical techniques is the dilution culture, which consists in transferring in a repeated fashion a subvolume of a culture (1/10) to fresh medium (9/10) to obtain statistically one cell per tube at the end of the series (Knight-Jones, 1951; Throndsen, 1978). This approach, which allowed the first cultivation of a true eukaryotic picoplankton species, *Micromonas pusilla* (Butcher, 1952), is still one of the most easily applicable to establish novel cultures or to follow the presence of a specific organism in the environment (Throndsen & Kristiansen, 1991; Zingone *et al.*, 1999b). However, the dilution approach will always favor the most abundant organisms, because the rarer ones will be diluted away before being separated from the more abundant ones. In order to facilitate the growth of small cells, samples can be prefiltered through two overlapping 3-μm filters or even 0.6-μm filters (Vaulot *et al.*, 2004). Other techniques for isolation include micropipette isolation (quite challenging for very small cells), flow cytometry sorting, and growth on solid medium (Andersen & Kawachi, 2005; Sieracki *et al.*, 2005). Culturing can be performed on enrichment cultures, field samples brought back to the laboratory, or even on board ships (Le Gall *et al.*, 2008). Isolated strains can be found in many algal collections but some facilities have a higher proportion of picoplanktonic species, in particular the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, http://ccmp.bigelow.org/) in the USA, the Marine Biotechnology Institute Culture collection (MBIC, now transferred at National Institute of Technology and Evaluation, http://www.nbrc.nite.go.jp/) in Japan, and the Roscoff Culture Collection (RCC, http://www.sb-roscoff.fr/Phyto/RCC/) in France.

A second strategy consists in physically separating small cells from the rest of the community in natural samples. This is most easily done with filters. Many of the filters used in the early studies (e.g. glass–fiber filters) had a very variable mesh size. It is only with the development of nuclear bombardment that more uniformly pore-sized filters became widely available (Nuclepore filters). Quite various filtration thresholds have been used: 0.6, 0.8, 1, 2, 3, or 5 μm. The use of these filters led in the early 1980s to the recognition of picoplankton as a major component of the photosynthetic community in open ocean waters (Herbland & Voituriez, 1979; Li *et al.*, 1983). Filter thresholds are, however, somewhat theoretical and, depending on their shape and plasticity, cells may squeeze through pores that

are smaller than their actual size (Li, 1990). Another way to separate cells is flow cytometry sorting (see Flow cytometry), but this has rarely been used for picoeukaryotes (Li, 1994).

Culturing and physical separation strategies have distinct advantages but both produce biases that should always be taken into account when interpreting the resulting data. It is now well known that culture methods favor fast-growing 'weed' species. Yet many of the important picoplankton groups such as the Prasinophyceae, the Pelagophyceae or the Bolidophyceae have been discovered through this approach. Conversely, size-separated field samples can suffer from cell loss due to breakage during filtration (Fahnenstiel *et al.*, 1994) or may not really reflect the picoplankton community as larger cells may be forced through the filters because of too strong filtration pressure or because they are extremely thin and elongated (e.g. long diatoms such as *Cylindrotheca closterium*).

Microscopy

For many years, light microscopy has been the only way to observe and describe phytoplankton cells. However, it has very limited use to assess picoplankton diversity in the field. In contrast, for cultures, it can provide valuable information on the size and shape of the cells, the number of chloroplasts, or the swimming behavior, allowing identification of species. Specific fixatives, such as lugol or osmium vapors, can make certain cellular features, in particular flagella (Fig. 1), more easy to observe.

It is the development of electron microscopy in the 1960s that really allowed visualization of important diagnostic features in small phytoplankton cells. Whole-cell mounts with proper shadow cast or uranyl acetate contrasting can reveal important features (e.g. Andersen *et al.*, 1993; Eikrem & Edvardsen, 1999), such as the presence and shape of flagellar hairs or body scales (Fig. 2f, h, i and k). In fact, the ornamentation of body scales allows unambiguous determination of picoplanktonic species such as *Bathycoccus prasinos* (Eikrem & Throndsen, 1990) or *Imantonia rotunda* (Fig. 2h and i). Thin sections are critical to establish chloroplast organization (Fig. 2a and e), membrane configuration, and the presence of storage products such as starch (Ral *et al.*, 2004). Flagellar insertion features (Fig. 2a) and flagellar root three-dimensional architecture, a key for phylogenetic analysis, require serial sections (e.g. Moestrup & Sengco, 2001). Scanning electron microscopy (SEM), which probes only the cell surface (Fig. 2c and d), has, in contrast, been much less useful because many small species lack body ornamentation such as organic, calcified, or silicified scales and for those that have such surface features, SEM resolution was until recently not sufficient to resolve scale details. However, the newly developed field emission SEM (Fig. 2d) offers a much better resolution (Probert *et al.*, 2007).

Widespread use of electron microscopy on field samples is restricted by some key limitations. Cells need to be fixed and concentrated before embedding or mounting, which often results in the loss of the smallest and most delicate forms. Still, some early landmark papers have relied on electron microscopy of thin sections to establish the widespread occurrence of picophytoplankton (Johnson & Sieburth, 1982). A good technique for making whole mounts of live samples is concentration by gravity filtering combined with gentle centrifugation, followed by fixation on a grid using osmic vapor (e.g. Thomsen, 1980). Later electron microscopy studies have established the importance of certain groups, such as Pelagophyceae, in oligotrophic waters (Andersen *et al.*, 1996). Moreover, some picophytoplankton species in particular in the order Parmales (see Described species) are only known from the analysis of field samples by SEM. Fixing agents (like glutaraldehyde and Lugol's solution) and air drying shrink cells and caution should be exercised when measuring cell size from such preparations. Many of the scale-bearing species are described from whole mounts alone and their live size may be underestimated (e.g. Estep *et al.*, 1984).

Epifluorescence microscopy relies on the emission of light by cellular compounds (e.g. pigments such as chlorophyll) or by stains specific for certain components such as DNA. This technique was key to establish a widespread importance of picophytoplankton (Murphy & Haugen, 1985) and was initially widely used to enumerate different types of cells discriminated on the basis of their pigment content (e.g. phycoerythrin is present in cyanobacteria and cryptophytes and makes them to fluoresce orange) or the number and shape of their chloroplasts (Thomsen *et al.*, 1994). In addition, staining with chemicals such as 4′,6-diamidino-2-phenylindol (DAPI), specific of DNA, or primulin (Caron, 1983) that stains mainly phospholipids, may reveal features, such as flagella and scale outline, that are critical for the identification of many taxa. Despite its replacement by flow cytometry (see Flow cytometry), epifluorescence microscopy is still widely used in conjunction with cell labeling by antibodies and molecular probes (see Immunological approaches and Molecular approaches). Moreover, automation, such as solid-phase cytometry (West *et al.*, 2006) or image analysis software, can speed up analysis.

Flow cytometry

In flow cytometry, samples are directly injected into a fluid stream, forcing cells to align in front of a light source (usually laser). Light scattered by each individual cell (a function of cell size and refractive index) and fluorescence from pigments such as chlorophyll or phycoerythrin are recorded in real time (Marie *et al.*, 1999). Major advantages of flow cytometry include speed, accuracy, and absence of

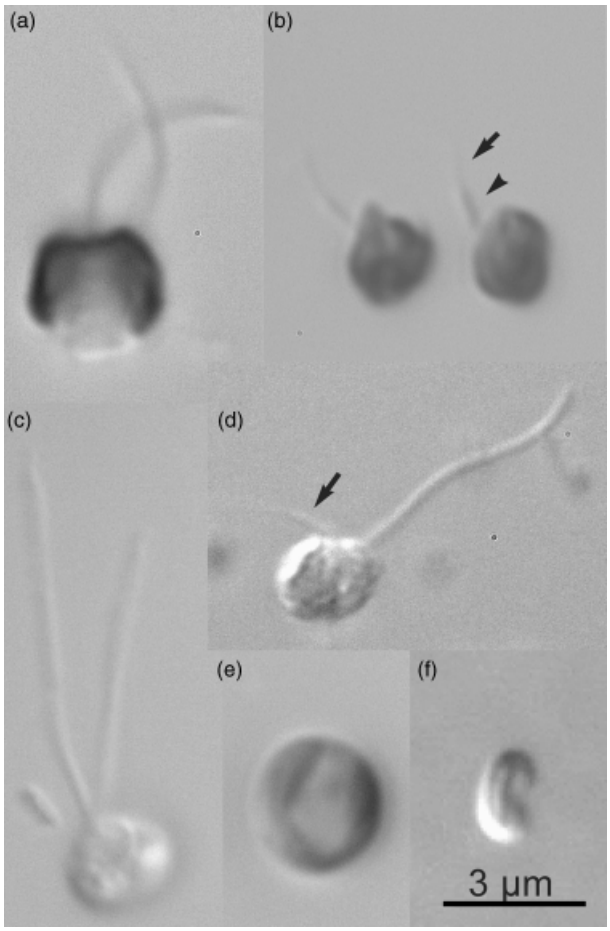

Fig. 1. (a–f) Light micrographs of some small phytoplankton species. (a) *Imantonia rotunda*. Cell with two chloroplasts and two flagella. Fixed in osmium tetroxide vapor. (b) *Micromonas pusilla*. Cells with flagellum (arrow and arrowhead), only proximal part (arrowhead) clearly visible, extension barely discernable (arrow). Fixed with Lugol's solution. (c) RCC 391 (Mamiellales). Cell with two unequal flagella. Fixed in osmium tetroxide vapor. (d) *Florenciella parvula*. Cell with heterokont flagella, short smooth (arrow), and long flagellum bearing nonvisible hairs. Fixed in osmium tetroxide vapour. (e) RCC 287 (Prasinophyceae clade VII). Spherical and nonmotile live cell. (f) *Bathycoccus prasinos*. Comma-shaped and nonmotile live cell.

sample preparation, at least to analyze photosynthetic pigments. Since its first application in oceanography (Olson *et al.*, 1985), flow cytometry has been the method of choice to estimate picoplankton abundance in oceanic waters (Collier, 2000). In fact, the use of flow cytometry led to the discovery of very important picoplanktonic organisms such as *Prochlorococcus* (Chisholm *et al.*, 1988) and *Ostreococcus* (Courties *et al.*, 1994). Still, scattering and fluorescence properties are not sufficient to discriminate taxa within picoeukaryotes, with the exception of cryptophytes that contain phycoerythrin (Li & Dickie, 2001). Therefore, potential applications of flow cytometry are considerably enhanced when samples are stained with fluorescent markers binding to specific cell compounds. For example the use of DNA stains allows an estimation of genome size and therefore permits to separate species or strains with closely related cell properties (Simon *et al.*, 1994). Some other stains can separate live from dead cells (Veldhuis *et al.*, 2001). As for epifluorescence, flow cytometry can be used in conjunction with antibodies and molecular probes (see Immunological approaches and Molecular approaches), but applications are still limited (Simon *et al.*, 1995; Biegala *et al.*, 2003). Flow cytometry also provides the capacity to sort cells of interest physically, allowing one to obtain pure cultures from natural assemblages (Moore *et al.*, 1998) or to perform further measurements on specific groups (Li, 1994).

Photosynthetic pigments

Photosynthetic pigments are key taxonomic diagnostic features for microalgae. At the simplest level, pigment composition allows distinguishing green, brown, and red algae characterized by chlorophyll *b*, chlorophyll *c*, and phycobiliproteins, respectively. HPLC pigment signature is often indicative of the class (e.g. prasinoxanthin is only present in prasinophytes), but in general cannot resolve lower taxonomic levels. Several classes may share the same suite of pigments (e.g. diatoms and bolidophytes, Guillou *et al.*, 1999b) and a given class may contain several pigment signatures (e.g. prasinophytes, Latasa *et al.*, 2004). Pigment analyses are now a standard requirement for the description of novel phytoplankton species (Guillou *et al.*, 1999a) and can be used to distinguish ecotypes within a given species (Rodríguez *et al.*, 2005). Relative pigment ratios in filter fractionated samples allow estimating dominant picophytoplankton groups in specific oceanic areas (Brunet *et al.*, 2006) based on algorithms such as CHEMTAX (Mackey *et al.*, 1996).

Immunological approaches

Soon after the discovery of picoplankton, it was realized that sensitive methods were needed to determine the abundance of specific eukaryotic taxa that could not be distinguished using fluorescence methods (see Microscopy and Flow cytometry). Fluorescent antibodies combine a strong signal and a relatively low cost. Initial applications involved polyclonal antibodies raised against whole cells (Shapiro *et al.*, 1989). However, their exact specificity can be difficult to determine and more recently, monoclonal antibodies have been used to detect economically important species such as *Aureococcus anophagefferens* (Caron *et al.*, 2003) that causes brown tides in coastal environments. Detection of labeled cells can be made more rapid and quantitative by flow cytometry (Vrieling *et al.*, 1996) or solid-phase

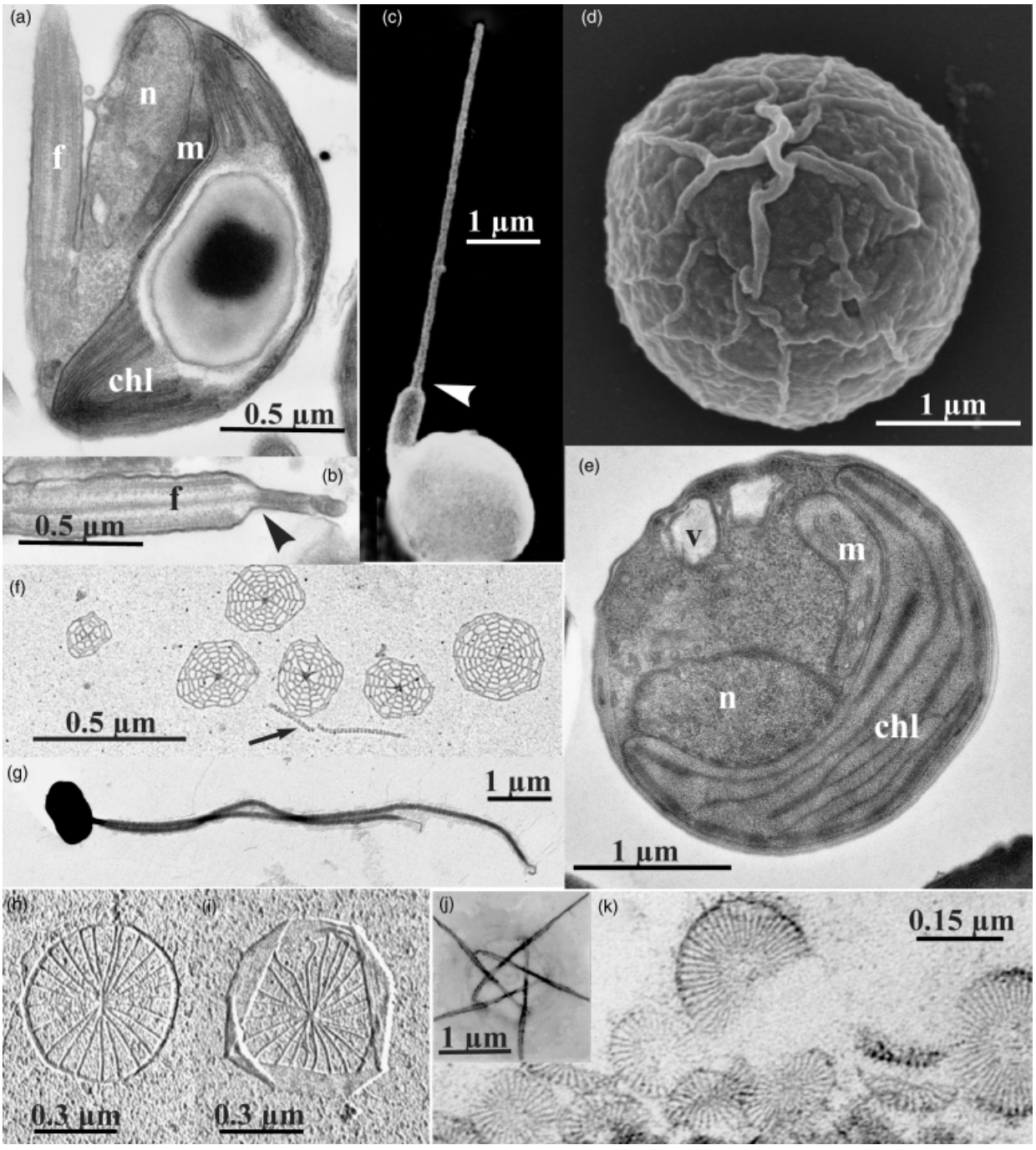

Fig. 2. Electron micrographs (SEM, TEM) of some small phytoplankton species. (a–c) *Micromonas pusilla*. (a) TEM of section through cell revealing the chloroplast (chl), nucleus (n), mitochondrion (m), and flagellum (f). (b) TEM of longitudinal section through the flagellum showing the mucronate extension (arrowhead). (c) SEM of cell with flagellum and its mucronate extension (arrowhead). (d–e) RCC 287 (Prasinophyceae clade VII). (d) Field emission SEM of whole cell. (e) TEM of section showing the single chloroplast (chl), mitochondrion (m), nucleus (n), and vesicles (v). (f–g) TEM whole mounts of RCC 391 (Mamiellales). (f) Scales and hair-scale (arrow) stained with uranyl acetate. (g) Whole cell with unequal flagella shadow-cast with gold-palladium. (h–i) *Imantonia rotunda*. TEM of whole mounts shadowed with chromium. (h) Inner layer scale. (i) Outer layer scale. (j–k) *Phaeocystis pouchetii*. (j) Uranyl acetate stained whole mount of ejected extrusome. (k) TEM of section through scaly covering.

cytometry (West *et al.*, 2006), although these techniques have not yet been applied for antibody detection in eukaryotic picophytoplankton.

Molecular approaches

In recent years, the introduction of molecular biology approaches making use of genetic information directly obtained from environmental samples provided a new way to analyze the genetic diversity of the picoplankton. Among the different genetic markers that have been used, the most popular by far is the nuclear-encoded small subunit rRNA gene (18S rRNA gene) that is present in all eukaryotes. Its rate of evolution is sufficiently slow to allow assessment of evolutionary relationships between distantly related organisms, and there is no evidence for lateral transfer that could introduce incongruent phylogenies. This gene presents both well-conserved and rapidly evolving regions allowing discrimination of organisms at many different taxonomic levels from the phylum to the species level. Another advantage of this gene is that its transcribed product (rRNA) is a building block of ribosomes, and is therefore very abundant inside individual cells (typically more than 1000 copies in prokaryotes, Amann & Kuhl, 1998), making it amenable to direct detection by probes even in small cells. In contrast, a major problem for PCR-based approaches using the 18S rRNA gene is the presence of several copies of the rRNA operon (Prokopowich *et al.*, 2003; Zhu *et al.*, 2005) that may not be all identical and therefore may introduce some spurious organism diversity. However, such artifacts are probably minimal for picoplankton because of their small genome sizes and therefore limited rRNA gene copy numbers. For example, *Ostreococcus tauri* has only four copies (Derelle *et al.*, 2006), all identical.

For discrimination at lower taxonomical levels, e.g. subspecies or ecotypes, the rRNA operon internal transcribed spacers (ITS1 and ITS2) are preferred (Rodríguez *et al.*, 2005). Plastid genes offer the advantage of targeting only photosynthetic plankton, in contrast to rRNA genes that target both autotrophs and heterotrophs. *rbcL*, coding for a large subunit of the ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO), which is involved in CO_2 fixation (Paul *et al.*, 2000), *psbA*, coding for a major protein of photosystem II reaction center (Zeidner *et al.*, 2003), and plastid 16S rRNA gene (Rappé *et al.*, 1997; Fuller *et al.*, 2006a) have all been used successfully on natural populations. However, in contrast to 18S, some of these genes such as *rbcL* are subject to horizontal transfer (Delwiche & Palmer, 1996).

When applied to field populations, techniques making use of these genetic markers fall into two categories: qualitative and quantitative. The former approaches (clone libraries, DGGE, etc.) allow assessment of the diversity and composition of the overall community. The latter [FISH, quantitative PCR (QPCR), etc.] can be used to quantify the abundance of specific groups.

In qualitative approaches, the first step consists in amplifying the gene of interest by the PCR using either universal primers (Moon-van der Staay *et al.*, 2001) or primers specific to a taxonomic group (Berglund *et al.*, 2005). Once the gene has been amplified, several approaches can be used to assess the sequence diversity within a sample. Generally, PCR products are cloned and a given number of clones (typically 100) are sequenced. Partial sequences from variable regions of the 18S rRNA gene are often sufficient to assign a clone to a taxonomic group, usually down to the genus level, while full-length sequences are necessary to narrow affiliation down to the species level and for phylogeny reconstruction. Alternative techniques, such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE), single strand conformation polymorphism (SSCP) and terminal restriction fragment length polymorphism (TRFLP) (van Hannen *et al.*, 1998; Díez *et al.*, 2001a; Marie *et al.*, 2006; Medlin *et al.*, 2006), can be used to compare rapidly community structure from different samples based on a band pattern without the need for cloning and sequencing. However, supplementary steps (extraction and sequencing) are required for the taxonomic affiliation of the bands.

The major advantage of the environmental gene sequencing approach is that it allows for the discovery of new groups and that the data obtained contribute to the growth of gene sequence databases, allowing global distribution studies. This is particularly important for novel uncultivated groups in which reality is reinforced if they are observed at different locations by different research groups (e.g. uncultivated alveolates, López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001). However, gene sequencing approaches have several drawbacks (von Wintzingerode *et al.*, 1997) such as biases and errors in PCR amplification (Acinas *et al.*, 1997) and production of chimerical sequences (Berney *et al.*, 2004). Software tools can now be used to detect the latter (Ashelford *et al.*, 2006). The diversity image obtained is often biased by the fact that certain groups are better amplified than others. In particular, photosynthetic groups are often underrepresented in clone libraries despite the fact that small eukaryotic plankton is dominated by phototrophs (Not *et al.*, 2004; Romari & Vaulot, 2004). Such biases can be counteracted by using multiple sets of primers (Stoeck *et al.*, 2006) or primers that are specific for certain phylogenetic groups (Bass & Cavalier-Smith, 2004). However, thorough methodological studies investigating possible biases are clearly lacking. In particular, it would be interesting to apply the cloning approach to complex mixtures of cultures to determine how representative the sequences obtained are.

For quantifying specific taxonomic groups, oligonucleotide probes targeting the 18S rRNA gene (DeLong *et al.*, 1989) were seen quite early as a key technique. When detected by FISH, they allow determination of the identity and abundance of the very small eukaryotes that are part of picophytoplankton (Simon *et al.*, 1995). However, it is only with the improvement of FISH with catalyzed reporter deposition (CARD), such as tyramide signal amplification (TSA, Not *et al.*, 2002) that it became possible to estimate taxa abundance easily by epifluorescence microscopy in field samples (Not *et al.*, 2004). TSA–FISH has been coupled with flow cytometry (Biegala *et al.*, 2003), but protocols are quite complex and analysis is not as amenable to routine counting as microscopy. Another approach involves the use of probes hybridizing to PCR-amplified products (Moon-van der Staay *et al.*, 2000; Fuller *et al.*, 2006b). In recent years, QPCR approaches have been developing rapidly in plankton ecology (Suzuki *et al.*, 2000). Application of QPCR to eukaryotic picophytoplankton has allowed to map key groups and species spatially and temporally (Countway & Caron, 2006; Marie *et al.*, 2006). However, calibration of probes targeting wide groups appears to be difficult because of the wide variation of the number of 18S rRNA gene copies among species of different sizes (Zhu *et al.*, 2005), while probes targeting specific species or genera displaying a narrow size range are more promising (Countway & Caron, 2006). DNA chips harboring phylogenetic probes have begun to be used but still appear to be qualitative (Medlin *et al.*, 2006).

Described species of small eukaryotic marine phytoplankton

Table 1 presents the 71 photosynthetic species described to date that have a minimum size of 3 μm or less. Among these, over 30 qualify as picoplanktonic (minimum size $\leq 2\,\mu m$). The smallest marine eukaryote is the Prasinophyceae *Ostreococcus tauri*, which, with a minimum size at 0.8 μm, can be quite undistinguishable from prokaryotes. It should be emphasized that size is a parameter that is quite difficult to measure and displays environmental and physiological variability. The maximum size of some species included in Table 1 can be considerably $> 3\,\mu m$, e.g. some of the *Skeletonema* species. Size remains uncertain for species that are not available in cultures and therefore only described from field material. First, in many cases when the species is described, the size of only a few individuals is measured. Second, it is often difficult to assess size variability, because the species is often described from a single or, at best, a small number of samples. Third, techniques such as whole-mount electron microscopy induce severe cell shrinking, leading to drastic underestimates of cell size.

Historically, some species that can be $< 3\,\mu m$, such as *Stichococcus bacillaris* or *Phaeocystis pouchetii*, were described as early as the 19th century (Fig. 3). However, the real breakthrough took place with the paper of Butcher (1952) in which he described several species in the 2–3 μm range including *Chromulina pusilla*, later renamed *Micromonas pusilla*, which proved to be one of the most widespread species in temperate latitudes. A clear impetus for the discovery of novel picoplanktonic species from coastal waters was provided by the application of electron microscopy, especially at the hand of Irene Manton and Mary Parke from England in the early 1960s (Fig. 3). A second wave of descriptions emerged when it was realized that picoplankton was dominating biomass and primary production in many oceanic areas, leading to an effort to isolate representative cultures in particular through the efforts of Bob Guillard in Woods Hole, which led to the creation of the CCMP (Andersen *et al.*, 1997), which constitutes to date the reference collection for picoplankton cultures. Finally, a new impetus emerged in the late 1990s with the widespread application of molecular phylogeny methods that allowed placing unambiguously some of the small planktonic species lacking diagnostic morphologically features. In fact, this led to recognition of the existence of novel algal classes, some of which were erected based on picoplanktonic species such as the Pelagophyceae (Andersen *et al.*, 1993) or the Bolidophyceae (Guillou *et al.*, 1999a).

Small plankton species are found in most algal classes (Fig. 4). The largest number of small photosynthetic eukaryotic species described to date (Table 2) belongs to the Heterokontophyta (also called heterokonts or stramenopiles, characterized by the presence of two flagella of differing morphology and function), followed by Chlorophyta (green algae) in particular from the class Prasinophyceae. Other groups represented include the Haptophyta (characterized by the presence of a third appendage called the haptonema) and Cryptophyta (containing phycobiliproteins besides chlorophyll and carotenoids).

Chlorophyta

Prasinophyceae

One of the first truly picoplanktonic species to have been described, *Micromonas pusilla*, belongs to the green lineage, more specifically to the Prasinophyceae, a class that regroups taxa at the base of the Chlorophyta. A recent analysis (Guillou *et al.*, 2004) of the 18S rRNA gene sequences available both for cultured strains and environmental sequences clearly established that this class is polyphyletic and led to the delineation of seven different clades (Fig. 4), some corresponding to known orders such as Mamiellales, some not yet formerly described. All these clades will probably be described as separate classes in the future.

Table 1. List of described small marine eukaryotic phytoplankton species, modified and expanded from Vaulot *et al*. (2004)

Division	Class	Genus	Species	Authority	Year Basionym	Min. size (μm)	Max. size (μm)	Hydrography
Picoplankton (Minimum size ≤ 2 μm)								
Chlorophyta	Pedinophyceae	*Resultor*	*micron*	(Throndsen) Moestrup	1969	1.5	2.5	Marine
Chlorophyta	Prasinophyceae	*Bathycoccus*	*prasinos*	Eikrem et Throndsen	1990	1.5	2.5	Marine
Chlorophyta	Prasinophyceae	*Micromonas*	*pusilla*	(Butcher) Manton et Parke	1952	1.0	3.0	Marine
Chlorophyta	Prasinophyceae	*Ostreococcus*	*tauri*	Courties et Chrétiennot-Dinet	1995	0.8	1.1	Marine
Chlorophyta	Prasinophyceae	*Picocystis*	*salinarum*	Lewin	2001	2.0	3.0	Hypersaline
Chlorophyta	Prasinophyceae	*Pycnococcus*	*provasolii*	Guillard	1991	1.5	4.0	Marine
Chlorophyta	Trebouxiophyceae	*Chlorella*	*nana*	Andreoli *et al*.	1978	1.5	3.0	Marine
Chlorophyta	Trebouxiophyceae	*Picochlorum*	*atomus*	(Butcher) Henley *et al*.	1952	2.0	3.0	Brackish
Chlorophyta	Trebouxiophyceae	*Picochlorum*	*oklahomensis*	Henley *et al*.	2004	2.0	2.0	Hypersaline
Cryptophyta	Cryptophyceae	*Hillea*	*marina*	Butcher	1952	2.0	2.5	Marine
Haptophyta	Prymnesiophyceae	*Chrysochromulina*	*tenuisquama*	Estep *et al*.	1984	2.0	5.0	Marine
Haptophyta	Prymnesiophyceae	*Imantonia*	*rotunda*	Reynolds	1974	2.0	4.0	Marine
Haptophyta	Prymnesiophyceae	*Trigonaspis*	*minutissima*	Thomsen	1980	2.0	3.6	Marine
Heterokontophyta	Bacillariophyceae	*Arcocellulus*	*cornucervis*	Hasle *et al*.	1983	1.0	17.0	Marine
Heterokontophyta	Bacillariophyceae	*Chaetoceros*	*minimus*	(Levander) Marino *et al*.	1991	2.0	7.0	Marine
Heterokontophyta	Bacillariophyceae	*Chaetoceros*	*throndsenii*	(Marino *et al*.) Marino *et al*.	1991	1.5	5.0	Marine
Heterokontophyta	Bacillariophyceae	*Minidiscus*	*comicus*	Takano	1981	2.0	7.0	Marine
Heterokontophyta	Bacillariophyceae	*Minutocellus*	*polymorphus*	(Hargraves et Guillard) Hasle *et al*.	1983	2.0	30.0	Marine
Heterokontophyta	Bacillariophyceae	*Skeletonema*	*grethae*	Zingone et Sarno	2005	2.0	10.5	Marine
Heterokontophyta	Bacillariophyceae	*Skeletonema*	*japonicum*	Zingone et Sarno	2005	2.0	10.0	Marine
Heterokontophyta	Bacillariophyceae	*Skeletonema*	*marinoi*	Sarno et Zingone	2005	2.0	12.0	Marine
Heterokontophyta	Bacillariophyceae	*Skeletonema*	*menzelii*	Guillard *et al*.	1974	2.0	7.0	Marine
Heterokontophyta	Bacillariophyceae	*Skeletonema*	*pseudocostatum*	Medlin	1991	2.0	9.0	Marine
Heterokontophyta	Bacillariophyceae	*Thalassiosira*	*bulbosa*	Syvertsen	1984	2.0	16.0	Marine
Heterokontophyta	Bacillariophyceae	*Thalassiosira*	*mala*	Takano	1965	2.0	10.0	Marine
Heterokontophyta	Bacillariophyceae	*Thalassiosira*	*proschkinae*	Makarova	1979	2.0	11.5	Marine
Heterokontophyta	Bolidophyceae	*Bolidomonas*	*mediterranea*	Guillou et Chrétiennot-Dinet	1999	1.0	1.7	Marine
Heterokontophyta	Bolidophyceae	*Bolidomonas*	*pacifica*	Guillou et Chrétiennot-Dinet	1999	1.0	1.7	Marine
Heterokontophyta	Eustigmatophyceae	*Nannochloropsis*	*granulata*	Karlson et Potter	1996	2.0	4.0	Marine
Heterokontophyta	Pelagophyceae	*Aureococcus*	*anophagefferens*	Hargraves et Sieburth	1988	1.5	2.0	Marine
Heterokontophyta	Pelagophyceae	*Pelagomonas*	*calceolata*	Andersen et Saunders	1993	2.0	3.0	Marine
Heterokontophyta	Pinguiophyceae	*Pinguiochrysis*	*pyriformis*	Kawachi	2002	1.0	3.0	Marine
Other small species (Minimum size ≤ 3 μm)								
Chlorophyta	Pedinophyceae	*Marsupiomonas*	*pelliculata*	Jones *et al*.	1994	3.0	3.0	Brackish, marine
Chlorophyta	Prasinophyceae	*Crustomastix*	*stigmatica*	Zingone	2002	3.0	5.0	Marine
Chlorophyta	Prasinophyceae	*Dolichomastix*	*eurylepidea*	Manton	1977	3.0	3.0	Marine
Chlorophyta	Prasinophyceae	*Dolichomastix*	*lepidota*	Manton	1977	2.5	2.5	Marine
Chlorophyta	Prasinophyceae	*Dolichomastix*	*tenuilepis*	Throndsen et Zingone	1997	3.0	4.5	Marine
Chlorophyta	Prasinophyceae	*Mantoniella*	*squamata*	(Manton et Parke) Desikachary	1960	3.0	5.0	Marine
Chlorophyta	Prasinophyceae	*Prasinococcus*	*capsulatus*	Miyashita et Chihara	1993	3.0	5.5	Marine
Chlorophyta	Prasinophyceae	*Prasinoderma*	*coloniale*	Hasegawa et Chihara	1996	2.5	5.5	Marine
Chlorophyta	Prasinophyceae	*Pseudoscourfieldia*	*marina*	(Throndsen) Manton	1969	3.0	3.5	Marine
Chlorophyta	Prasinophyceae	*Pyramimonas*	*virginica*	Pennick	1977	2.7	3.5	Marine
Chlorophyta	Trebouxiophyceae	*Chlorella*	*spärckii*	Ålvik	1934	2.8	7.0	Marine
Chlorophyta	Trebouxiophyceae	*Picochlorum*	*eukaryotum*	(Wilhelm *et al*.) Henley *et al*.	1982	3.0	3.0	Marine

Table 1. Continued.

Division	Class	Genus	Species	Authority	Year Basionym	Min. size (μm)	Max. size (μm)	Hydrography
Chlorophyta	Trebouxiophyceae	*Picochlorum*	*maculatus*	(Butcher) Henley *et al*.	1952	3.0	3.0	Brackish
Chlorophyta	Trebouxiophyceae	*Stichococcus*	*bacillaris*	Nägeli	1849	3.0	4.0	Marine
Haptophyta	Prymnesiophyceae	*Chrysochromulina*	*apheles*	Moestrup et Thomsen	1986	3.0	4.0	Marine
Haptophyta	Prymnesiophyceae	*Chrysochromulina*	*minor*	Parke et Manton	1955	2.5	7.5	Marine
Haptophyta	Prymnesiophyceae	*Chrysochromulina*	*planisquama*	Hu *et al*.	2005	3.0	6.0	Marine
Haptophyta	Prymnesiophyceae	*Dicrateria*	*inornata*	Parke	1949	3.0	5.5	Marine
Haptophyta	Prymnesiophyceae	*Ericiolus*	*spiculiger*	Thomsen	1995	3.0	3.8	Marine
Haptophyta	Prymnesiophyceae	*Phaeocystis*	*cordata*	Zingone	1999	3.0	4.0	Marine
Haptophyta	Prymnesiophyceae	*Phaeocystis*	*pouchetii*	(Hariot) Lagerheim	1892	3.0	8.0	Marine
Heterokontophyta	Bacillariophyceae	*Minidiscus*	*chilensis*	Rivera	1984	3.0	7.5	Marine
Heterokontophyta	Bacillariophyceae	*Minidiscus*	*spinulosus*	Gao *et al*.	1992	3.0	5.0	Marine
Heterokontophyta	Bacillariophyceae	*Minidiscus*	*trioculatus*	(F.J.R. Taylor) Hasle	1967	2.5	3.8	Marine
Heterokontophyta	Bacillariophyceae	*Minutocellus*	*scriptus*	Hasle *et al*.	1983	3.0	36.0	Marine
Heterokontophyta	Bacillariophyceae	*Thalassiosira*	*oceanica*	Hasle	1983	3.0	12.0	Marine
Heterokontophyta	Bacillariophyceae	*Thalassiosira*	*pseudonana*	Hasle et Heimdal	1970	2.3	5.5	Marine
Heterokontophyta	Chrysophyceae	*Ollicola*	*vangoorii*	(Conrad) Vørs	1938	3.0	5.0	Marine
Heterokontophyta	Chrysophyceae	*Tetraparma*	*insecta*	Bravo-Sierra et Hernández-Becerril	2003	2.8	3.8	Marine
Heterokontophyta	Chrysophyceae	*Tetraparma*	*pelagica*	Booth et Marchant	1987	2.2	2.8	Marine
Heterokontophyta	Chrysophyceae	*Triparma*	*columacea*	Booth	1981	2.3	4.7	Marine
Heterokontophyta	Chrysophyceae	*Triparma*	*laevis*	Booth	1981	2.2	3.1	Marine
Heterokontophyta	Chrysophyceae	*Triparma*	*retinervis*	Booth	1981	2.7	4.5	Marine
Heterokontophyta	Dictyochophyceae	*Florenciella*	*parvula*	Eikrem	2004	3.0	6.0	Marine
Heterokontophyta	Eustigmatophyceae	*Nannochloropsis*	*oceanica*	Suda et Miyashita	2002	3.0	5.0	Marine
Heterokontophyta	Eustigmatophyceae	*Nannochloropsis*	*salina*	(Bourrelly) Hibberd	1958	3.0	4.0	Brackish
Heterokontophyta	Pelagophyceae	*Aureoumbra*	*lagunensis*	Stockwell *et al*.	1997	2.5	5.0	Marine
Heterokontophyta	Pelagophyceae	*Pelagococcus*	*subviridis*	Norris	1977	2.5	5.5	Marine
Heterokontophyta	Pinguiophyceae	*Pinguiococcus*	*pyrenoidosus*	Andersen *et al*.	2002	3.0	8.0	Marine

Picoplankton species are defined as those for which minimum cell size is $\leq 2\,\mu m$. Other small species for which the minimum cell size is $\leq 3\,\mu m$ are also listed.

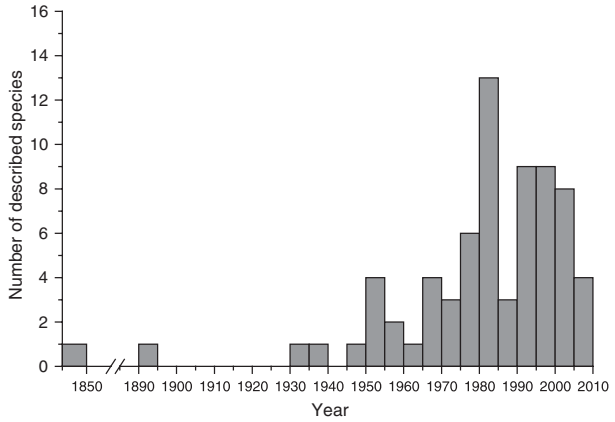

Fig. 3. Number of described small phytoplankton species (minimum size $\leq 3\,\mu m$) as a function of their year of publication.

Mamiellales

In contrast to many other phylogenetic groups that harbor both picoplanktonic and larger species, the order Mamiellales contains mostly small-sized species (Fig. 1b, c and f).

Micromonas pusilla (Fig. 1b and 2a–c) is characterized by an easily recognizable swimming pattern and a single peculiar flagellum. The proximal part of the flagellum consists of the regular 9+2 microtubules, but only the two central microtubules extend into the distal part (Fig. 2b and c). Several studies have pointed out its ubiquity in coastal temperate and near-polar waters, based on culture isolation by serial dilution (Manton & Parke, 1960; Throndsen & Kristiansen, 1991) and frequency of species-specific viruses (Cottrell & Suttle, 1991; Zingone *et al*., 1999b). More recently, the availability of fluorescent probe targeting *Micromonas* 18S rRNA gene and detected by FISH has allowed establishing that this species can account on average for 45% of the picoeukaryotes in English Channel coastal waters (Not *et al*., 2004) and for 32% in late summer arctic waters off Spitsbergen (Not *et al*., 2005) with maximum concentrations of up to $10\,000\,cell\,mL^{-1}$. In contrast, *Micromonas* appears to be absent from more oligotrophic waters such as the central Mediterranean Sea (Marie *et al*., 2006) or the Gulf of Mexico (Cottrell & Suttle, 1991), although it has been reported in the South East Pacific

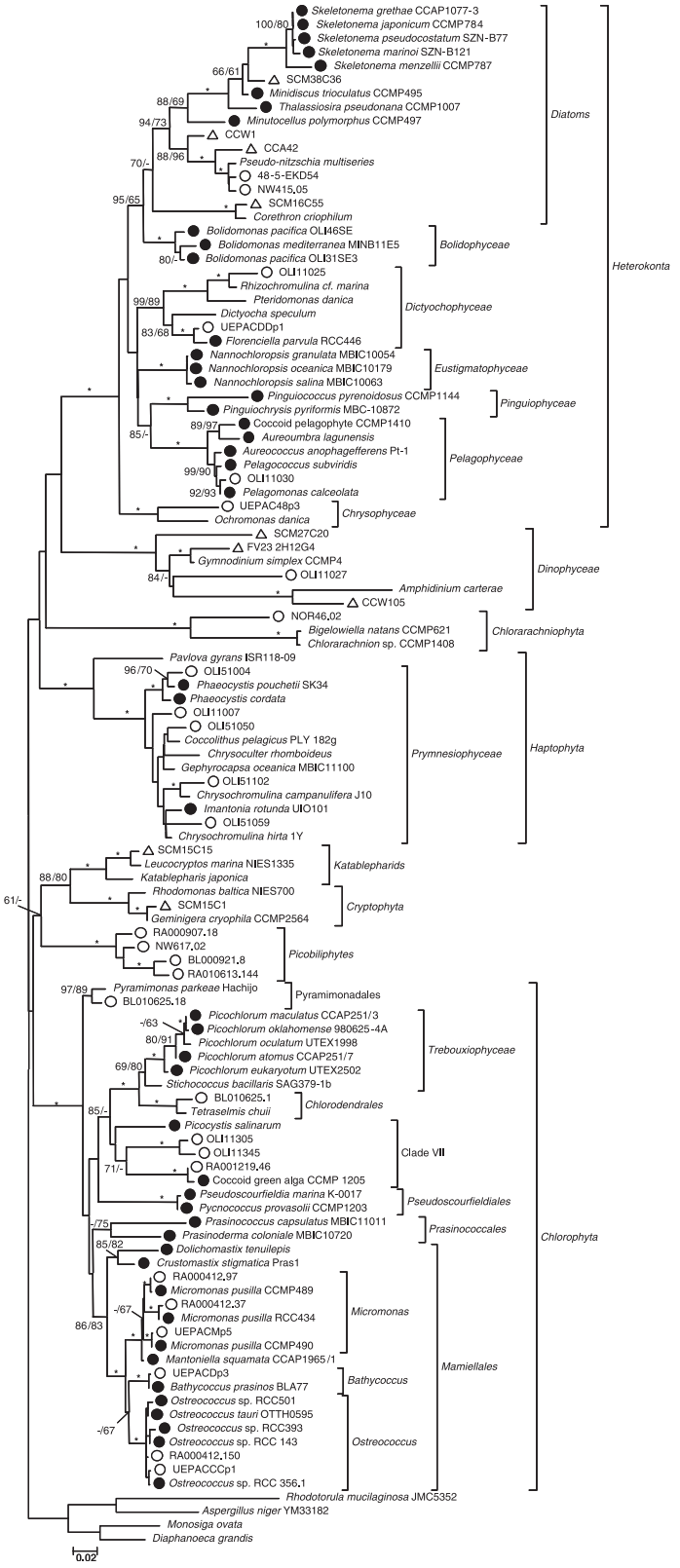

Fig. 4. Tree presenting all available sequences for described small phytoplankton species (closed circles) along with environmental sequences from $\leq$ 3 μm filtered samples (open circles) or total samples (open triangles) closely related to described species or belonging to taxonomic groups suspected to be photosynthetic and for which no species has been described yet (e.g. picobiliphytes). Phylogeny based on 113 full-length 18S rRNA gene sequences and 1353 total characters. The tree shown was inferred by the maximum likelihood (ML) method based on a TrN (Tamura & Nei, 1993) model of DNA substitutions with the following parameters: proportion of invariable sites (I) = 0.3341, gamma distribution shape parameter = 0.6293, and substitution models of R(a) [A–C] = 1.5090, R(b) [A–G] = 2.9589, R(e) [C–T] = 4.9420 and 1.0 for the remaining substitution rates. Total number of rearrangements tried = 113 703. Bootstrap values are indicated above internodes and correspond to neighbor joining (NJ) and maximum parsimony (MP), respectively. Only bootstrap values higher than 60% are reported and a 100/100 bootstrap value is represented by an asterisk (*). The scale bar indicates 0.02 substitution per site.

Table 2. Number of described small marine eukaryotic phytoplankton species by taxonomic group, tabulated from Table 1

Division	Class	Species # per class	Species # per phylum
Heterokontophyta	Bacillariophyceae	20	37
	Chrysophyceae	6	
	Pelagophyceae	4	
	Eustigmatophyceae	3	
	Bolidophyceae	2	
	Pinguiophyceae	2	
Chlorophyta	Prasinophyceae	14	23
	Pedinophyceae	2	
	Trebouxiophyceae	7	
Haptophyta	Prymnesiophyceae	10	10
Cryptophyta	Cryptophyceae	1	1

(Throndsen, 1973) and south of Japan (Throndsen, 1983). *Micromonas pusilla* appears to be quite diverse genetically and at least three clades (Fig. 4) can be identified on the basis of 18S rRNA gene sequences (Guillou *et al*., 2004) and up to five if additional genes are taken into account (Slapeta *et al*., 2006). However, it is not clear whether each clade corresponds to a specific bio-geographical or ecotypic distribution or to a well-defined phenotype (Slapeta *et al*., 2006).

Bathycoccus prasinos is a very small coccoid organism (Fig. 1f) that is covered by spider-web-like scales. Initially isolated from the bottom of the euphotic zone in the Mediterranean Sea (Eikrem & Throndsen, 1990), it has been observed with FISH to be present and sometimes quite abundant (up to 5000 cell mL^{-1}) in surface coastal waters in the Mediterranean Sea and Atlantic Ocean (Not *et al*., 2004, 2005; Zhu *et al*., 2005).

A third emblematic Mamiellales species is *Ostreococcus tauri*. It is the smallest free-living eukaryote known to date with an average size of 0.8 μm (Courties *et al*., 1994). Initially isolated from a coastal eutrophic lagoon on the French Mediterranean Sea coast, it has since been cultivated from a wide variety of oceanic environments including coastal and open ocean sites. Phylogenetic analyses of the 18S rRNA gene and intergenic spacers (ITS) led to the delineation of four clades (Guillou *et al*., 2004; Rodríguez *et al*., 2005). It is possible to make a correspondence between these clades and specific environments: clade C contains only the original strain isolated from Thau lagoon, while clade B regroups strains isolated from the bottom of the euphotic zone (90–120 m) from the Atlantic Ocean, the Mediterranean Sea, and the Red Sea. Interestingly, all B strains possess a specific chlorophyll-like pigment (Chl CS-170), not observed in the other clades. Despite the fact that *Ostreococcus* has been isolated from many oceanic locations, it seems to occur usually at very low concentrations, typically of the order of 100 cell mL^{-1}, as established by FISH in the Mediterranean Sea and Atlantic Ocean (Not *et al*., 2004, 2005; Zhu *et al*., 2005). In contrast, it can reach sporadically very high concentrations (10^5 cell mL^{-1}) within the near shore deep chlorophyll maximum (Countway & Caron, 2006) and in confined coastal environments (O'Kelly *et al*., 2003).

Three other Mamiellales genera, *Mantoniella, Crustomastix*, and *Dolichomastix*, contain small species. Among these, *Mantoniella squamata*, characterized by two unequal flagella, has been recorded in many different locations ranging from polar to subtropical waters, with an estimated abundance of up to 1000 cell mL^{-1} (Hallegraeff, 1983; Thomsen & Buck, 1998). Species belonging to the two other genera have been observed sporadically, in particular in the Mediterranean Sea (Throndsen & Zingone, 1997; Zingone *et al*., 2002), but precise abundance data are not available. A nondescribed culture belonging to this order (Figs 1c and 2f and g) has been isolated from the Mediterranean Sea (Guillou *et al*., 2004).

Pseudoscourfieldiales

The small coccoid species *Pycnococcus provasolii* was one of the first picoplanktonic eukaryotic species to be repeatedly isolated from the open ocean (Guillard *et al*., 1991), in particular from below the mixed layer (i.e. below the pycnocline, hence its name). Quite surprisingly, its 18S rRNA gene sequence is almost 100% similar to that of another small planktonic flagellate, *Pseudoscourfieldia marina* (Fawley *et al*., 1999), leading to the speculation that these two species could be alternate life-cycle stages (Guillou *et al*., 2004). In fact, flagellate stages were already reported in the original description of *P. provasolii* (Guillard *et al*., 1991). *Pseudoscourfieldia marina* has been reported off Denmark and South Africa (Manton, 1975) and appears to be quite common in the Skagerrak area based on serial dilution culture data with estimated densities up to 170 cell mL^{-1} (W. Eikrem & J. Throndsen, unpublished data). An 18S rRNA probe targeting this clade (*Pseudoscourfieldiales*) only detected a very small number of cells (< 100 cell mL^{-1}) in temperate English Channel coastal waters (Not *et al*., 2004). Antibodies directed against *P. provasolii* detected few cells in the Gulf of Maine but much higher concentrations off Hawaii, especially at the chlorophyll maximum, where it accounted for up to 23% of eukaryotes (Campbell *et al*., 1994b).

Prasinococcales

The two species *Prasinococcus capsulatus* and *Prasinoderma coloniale* that belong to clade VI ('Prasinococcales'), as defined in Guillou *et al*. (2004), are characterized by small cells embedded in a gelatinous matrix. They are quite often isolated from oceanic samples (Sieburth *et al*., 1999; Fuller *et al*., 2006a) and the use of FISH probes has shown that the

concentration of members of this order is highly variable but can reach up to 1000 cell mL^{-1} in coastal waters.

Clade VII

One group of Prasinophyceae (clade VII following Guillou *et al.* 2004) is composed of picoplankton-sized strains that have been isolated from open ocean areas (e.g. CCMP 1205 or RCC 287, Fig. 2d and e) but have not been formally described as yet. Within this clade, *Picocystis salinarum* is a species found in hypersaline environments such as Mono Lake (USA) where it can reach extremely high concentrations in excess of 4×10^5 cell mL^{-1} (Roesler *et al.*, 2002). Despite the fact that it can grow at much lower salinities (Roesler *et al.*, 2002), there is no evidence that it occurs in marine environments.

Pyramimonadales

Pyramimonas virginica is very small and has been recorded sporadically in several coastal environments ranging from New Zealand to Denmark and Greenland, through Thailand (Thomsen, 1986; Hori *et al.*, 1995). In view of the diversity of the genus *Pyramimonas* (more than 40 species described to date), it is likely that other small planktonic *Pyramimonas* species do exist.

Pedinophyceae

The two species *Resultor mikron* and *Marsupiomonas pelliculata*, characterized by a single flagellum and the absence of scales, belong to the Pedinophyceae, a class with only three genera whose relationship with the other Chlorophyta classes awaits molecular phylogenetic analysis. *Resultor mikron* has been recorded in particular in the Atlantic Ocean (Estep *et al.*, 1984), while *M. pelliculata* was isolated into culture and described from a salt marsh in Great Britain (Jones *et al.*, 1994) and may be restricted to this specific environment, because it has not been recorded since.

Trebouxiophyceae

Several species of Trebouxiophyceae may qualify as picoplanktonic. The newly established genus *Picochlorum* regroups species included previously into genus *Nannochloris* that are halotolerant, including *Picochlorum oklahomensis* isolated from hyper-saline inland waters (Henley *et al.*, 2004). The different species described to date lack obvious morphological features and probably many species do exist that would need to be defined based on molecular markers such as 18S rRNA gene or ITS. These species are easily isolated from marine environments (e.g. more than 25 strains are available from the CCMP – http://ccmp.bigelow.org/) but no data are available on their actual abundance in marine waters. Three small Trebouxiophyceae (*Chlorella nana, Stichococcus bacillaris*, and *Stichococcus cylindricus*, the latter two being probable synonyms) have been mentioned very rarely in the scientific literature since their initial description (Nägeli, 1849; Butcher, 1952; George, 1957; Throndsen, 1969; Andreoli *et al.*, 1978) and their exact taxonomical position would probably need to be re-evaluated. In fact, the genus *Stichococcus* is limited to terrestrial habitats, with the exception of *S. cylindricus/S. bacillaris*.

Heterokontophyta

Although Heterokontophyta are dominated by macroalgae species (Phaeophyceae), many classes contain small planktonic representatives (Fig. 4).

Pelagophyceae

Pelagomonas calceolata is a very small flagellate initially isolated from the North Pacific gyre. Its pigment and some ultrastructural features were typical of heterokonts. However, some of its morphological features (single basal body, bipartite flagellar hairs) as well as its 18S rRNA gene sequence did not allow easy inclusion in existing classes. As a consequence, Andersen *et al.* (1993) created a new class, the Pelagophyceae, to accommodate *Pelagomonas*. Later, three other small planktonic species were classified into the Pelagophyceae based on their 18S rRNA gene sequence. All these species possess the carotenoid 19′ butanoyloxyfucoxanthin. *Pelagococcus subviridis* is a coccoid organism also isolated from several locations in the Pacific and initially classified into the Chrysophyceae (Lewin *et al.*, 1977). It has also been recorded from the Norwegian Sea (Haltenbanken) using transmission electron microscopy (TEM) thin sections and whole mounts as well as SEM (Throndsen & Kristiansen, 1982). *Aureococcus anophagefferens* and *Aureoumbra lagunensis* are two brown tide organisms that can reach very high densities (up to 10^6 cell mL^{-1}). The former occurs in coastal embayments and lagoons of the East Coast of the USA (Caron *et al.*, 2003), while the latter has, in recent years, formed quasi-persistent blooms in Texas hypersaline environments such as the Laguna Madre (Buskey *et al.*, 2001). The extent of the distribution of the pelagic species *P. calceolata* and *P. subviridis* in the marine environment has not been investigated in detail although these species have been isolated from several oceanic locations in the Pacific and Atlantic Oceans (Andersen, 2007; Le Gall *et al.*, 2008). Antibodies directed against *P. subviridis* detected very few cells ($< 0.1\%$ of eukaryotes in general) in the Gulf of Maine and off Hawaii, although in one sample at the latter location its abundance reached 25% of eukaryotic cells (Campbell *et al.*, 1994b). However, a FISH probe targeting all

Pelagophyceae recognized up to 5000 cell mL^{-1} in English Channel coastal waters (Biegala *et al*., 2003).

Bolidophyceae

The isolation of two very small heterokont flagellates in the Pacific Ocean (*Bolidomonas pacifica*) and in the Mediterranean Sea (*Bolidomonas mediterranea*) with pigment content (fucoxanthin as the major carotenoid) and 18S rRNA gene sequences closely related to those of diatoms led to the erection of a novel class: the Bolidophyceae (Guillou *et al*., 1999a). Probe dot-blot analysis indicated that Bolidophyceae were very minor contributors to the picoplankton communities in the areas from which they were isolated (Guillou *et al*., 1999b). However, a FISH probe targeting Bolidophyceae could detect up to almost 500 cell mL^{-1} in English Channel coastal waters (F. Not, unpublished data).

Eustigmatophyceae

The genus *Nannochloropsis* is characterized by small coccoid cells that lack distinct morphological features, often requiring molecular analyses to establish phylogeny (Andersen *et al*., 1998). Some strains are a valuable source of polyunsaturated fatty acids and pigments such as astaxanthin (e.g. Lubian *et al*., 2000). Three small marine planktonic species have been described to date: *Nannochloropsis salina*, *Nannochloropsis granulata*, and *Nannochloropsis oceanica* (Suda *et al*., 2002), but their oceanic distribution remains unknown.

Chrysophyceae

The Parmales (Booth & Marchant, 1987) constitute quite a mysterious group that encompasses very small cells with a silicified cell wall. At least five described species belonging to the genera *Triparma* and *Tetraparma* can be < 3 μm in size. However, because these cells are only known from field observations, mostly by SEM (Bravo-Sierra & Hernandez-Becerril, 2003), and have never been isolated in culture, their exact taxonomic affiliation remains mysterious, although they have been provisionally classified into the Chrysophyceae. The mixotrophic species *Ollicolla vangoorii* is another marine Chrysophyceae that possesses a lorica and that has been reported from temperate and polar waters (see Novarino *et al*., 2002 and references therein).

Bacillariophyceae

Paradoxically, despite the fact that diatoms are usually considered as prominent members of the microplankton (i.e. with cell size in excess of 20 μm), almost 20 species of Bacillariophyceae can have one of their cell dimensions below 3 μm. This is the case in particular of the genera *Minidiscus* and *Minutocellus* (Ake Castillo *et al*., 2001). The species *Minidiscus chilensis* can be important in antarctic waters as it is found at a high concentration in sediment traps (Kang *et al*., 2003), while *Minutocellus polymorphus* has been shown to bloom to above 100 000 cell mL^{-1} in a Mediterranean lagoon (Sarno *et al*., 1993). Five species belonging to the genus *Skeletonema*, some of which have been created following the revision of the taxonomy of the ubiquitous species *Skeletonema costatum*, can also have a small cell size. However, because they usually occur in chains, they are unlikely to be retained by a 2- or 3-μm filter (Sarno *et al*., 2005; Zingone *et al*., 2005). Finally, *Thalassiosira pseudonana* is the first eukaryotic phytoplankton species for which the genome has been sequenced (Armbrust *et al*., 2004).

Dictyochophyceae and Pinguiophyceae

Two other heterokont classes, the Dictyochophyceae and the Pinguiophyceae, harbor small species. The flagellate *Florenciella parvula* (Fig. 1d) was initially isolated from European coastal waters (Eikrem *et al*., 2004) and a second strain has been obtained recently from the South Pacific Ocean (Le Gall *et al*., 2008). *Florenciella* is phylogenetically related to the mildly toxic genus *Pseudochattonella* (Edvardsen *et al*., 2007; Eikrem *et al*., 2008) and, more distantly, to the genus *Dictyocha*, whose characteristic spiny skeleton is often found in marine phytoplankton samples. The Pinguiophyceae constitute a novel class characterized by cells containing an unusually high amount of polyunsaturated acids (Kawachi *et al*., 2002b). *Pinguiochrysis pyriformis*, a nonmotile picoplanktonic species with a pyriform shape and a high concentration of eicosapentaenoic acid (EPA), has been isolated from the surface of the tropical Pacific Ocean (Kawachi *et al*., 2002a).

Haptophyta

Among the Haptophyta, four *Chrysochromulina* species can be < 3 μm. This highly diversified genus contains over 60 species, some of which are toxic, some of which can form blooms, and some of which are mixotrophic. The difficulty in distinguishing species by optical microscopy has prevented accurate estimates of individual species but the genus can be abundant in coastal waters (Dahl *et al*., 2005). *Phaeocystis* is a very widespread genus and several of its species can form large mucous colonies leading to accumulation of visible foam in coastal waters. Two species, the non-colony-forming *Phaeocystis cordata* described from the Mediterranean Sea (Zingone *et al*., 1999a) and the colonial *Phaeocystis pouchetii* that is restricted to cold northern hemisphere waters (Schoemann *et al*., 2005), may have cells as small as 3 μm (Fig. 2j and k). *Imantonia rotunda* (Fig. 1a and 2h and i) and *Dicrateria inornata* are two rather similar

species characterized by the absence of visible haptonema and short flagella, the latter lacking body scales, that are easily isolated from coastal waters (Backe Hansen & Throndsen, 2002). *Trigonaspis minutissima* and *Ericiolus spiculiger* have both been described from field observations in north temperate and arctic waters (Thomsen, 1980; Thomsen *et al.*, 1995) and do not seem to have been recorded since then.

Cryptophyta

A single cryptophyte species may qualify as picoplanktonic: *Hillea marina*. It was described in the early 1950s (Butcher, 1952) and has been observed to be quite abundant in some coastal waters (Chang, 1983).

Environmental diversity of small eukaryotic phytoplankton

It is quite paradoxical that although small planktonic eukaryotes are present in very diverse ecosystems and may represent a large fraction of the biomass and production in many ecosystems (Campbell *et al.*, 1994a; Worden *et al.*, 2004), very few species have been described, in particular in comparison with other microplankton groups such as diatoms. This contradiction has led in recent years to the increased use of molecular approaches based on direct gene amplification and sequencing from natural samples (see section above). This approach has now been applied to many different ecosystems including hydrothermal vents, sediments, and anoxic waters (López-García *et al.*, 2003; Stoeck & Epstein, 2003). Most studies have been focused on the 18S rRNA gene as a taxonomic marker although other genes such as those coding for the large subunit of Rubisco (*rbcL*) and plastidial 16S RNA gene have also been considered (Rappé *et al.*, 1995; Paul *et al.*, 2000).

We have compiled 3561 18S rRNA gene sequences (either full length or partial, but always longer than 350 bp) originating from 18 published data sets (Table 3). These sequences have been obtained only from water column marine plankton samples (i.e. ignoring sediments as well as fresh water environments). This set is somewhat biased towards coastal waters with fewer studies from more open ocean regions, in particular in tropical or polar regions (Table 3). A large subset of the samples have been filter fractionated to $< 3\,\mu m$ and therefore correspond to the small plankton size range considered in this review. In the analysis below we concentrate on this size fraction, while mentioning sequences recovered from total samples when they are closely related to the species listed in Table 1. These sequences have been compared by a basic local alignment search tool (BLAST) with those available in GenBank (September 2007), allowing the placement of each sequence into a taxonomic group. Further analyses have been performed with the ARB software (Ludwig *et al.*, 2004) using the Silva database (Pruesse *et al.*, 2007) containing more than 30 000 eukaryotic 18S rRNA gene sequences.

Among the 3561 18S rRNA gene sequences analyzed, about 30% are affiliated to taxonomic groups known to harbor photosynthetic organisms. The remaining sequences correspond to heterotrophic groups in particular Alveolata and stramenopiles, but also to Metazoa, likely to originate from gametes, especially when samples are unfiltered (Countway *et al.*, 2005). Among the most abundant photosynthetic divisions (Fig. 5) are green algae, more specifically Prasinophyceae, followed by dinoflagellates, cryptophytes, haptophytes, and stramenopiles. The relative frequency of these groups depends on many factors including depth, degree of oligotrophy, as well as season (Massana *et al.*, 2004; Romari & Vaulot, 2004).

Chlorophyta

Prasinophyceae constitute the dominant photosynthetic group among small plankton 18S rRNA gene sequences (Fig. 5). This fits quite well with the fact that this group is the one for which most small species have been described to date. Prasinophyceae sequences are, however, mostly restricted to the euphotic zone, suggesting that this group is not transferred to deeper layers through sedimentation. Among Prasinophyceae (Fig. 4), most sequences recovered belong to the order Mamiellales. The three picoplanktonic genera *Micromonas*, *Ostreococcus*, and *Bathycoccus* represent more than 90% of the available sequences and are especially found in temperate coastal waters (Massana *et al.*, 2004; Romari & Vaulot, 2004; Medlin *et al.*, 2006; Worden, 2006). Moreover, *Micromonas* sequences have been recovered in temperate and polar oceanic waters (Díez *et al.*, 2001b; Lovejoy *et al.*, 2006), *Bathycoccus* sequences have been found throughout an extensive range from polar (Lovejoy *et al.*, 2006) to oligotropic stations in the Mediterranean Sea (Marie *et al.*, 2006) or Sargasso Sea (Countway *et al.*, 2007), often at depth, while *Ostreococcus* sequences have only been observed sporadically in Mediterranean Sea waters (Marie *et al.*, 2006). Such trends agree with data from FISH (Not *et al.*, 2004) and QPCR (Marie *et al.*, 2006), which show that while *Micromonas* is, for example, the most abundant genus in English Channel waters, it completely disappears in the central Mediterranean Sea, where, in contrast, the two other genera occur sporadically at specific stations and depths. Other Mamiellales sequences have been recovered presenting similarities to the genera *Crustomastix*, *Mantoniella*, and *Mamiella*, the former two genera containing small plankton species (see Table 1). Mamiellales-related sequences have also been recovered using other genes such as plastid 16S rRNA gene (Fuller *et al.*, 2006a), *rbcL* (Paul *et al.*, 2000;

Table 3. Sets of marine samples analyzed in this paper for which eukaryotic environmental 18S rRNA gene sequences have been determined using either cloning, DGGE or TTGE

Sample set	Type	Filtration	Ecosystem	Region	Cruise	References	Number of sequences
Antarctic, Diez	Cruise	$< 1.6\,\mu m$	Oceanic	Antarctic	E-DOVETAIL	Díez *et al*. (2001a)	51
Drake Passage, López-García	Cruise	$< 5\,\mu m$	Oceanic	Antarctic	DHARMA 98	López-García *et al*. (2001), López-García *et al*. (2003)	49
Arctic, Lovejoy	Cruise	$< 3\,\mu m$	Oceanic	Arctic Ocean	Three cruises	Lovejoy *et al*. (2006)	267
North Atlantic, Diez	Cruise	$< 2\,\mu m$	Oceanic	North Atlantic	ACSOE NAE	Díez *et al*. (2001a)	16
Sargasso Sea, Countway	Cruise		Oceanic	Sargasso Sea; Gulf Stream	Endeavor 2000	Countway *et al*. (2007)	896
Sargasso Sea, Not	Cruise	$< 2\,\mu m$	Oceanic	Sargasso Sea	Endeavor 2001	Not *et al*. (2007a)	229
Mediterranean Sea, Diez	Cruise	$< 5\,\mu m$	Oceanic	Mediterranean Sea	MATER	Díez *et al*. (2001a)	31
Mediterranean Sea, Marie	Cruise	$< 3\,\mu m$	Oceanic	Mediterranean Sea	PROSOPE	Marie et al. (2006)	119
Nansha islands, Yuan	Cruise		Oceanic	South China Sea		Yuan *et al*. (2004)	63
Equatorial Pacific, Moon	Cruise	$< 3\,\mu m$	Oceanic	Equatorial Pacific	OLIPAC	Guillou *et al*. (1999a), Moon-van der Staay *et al*. (2000), Moon-van der Staay *et al*. (2001)	51
Cariaco Basin, Stoeck	Cruise		Oceanic	Carribean Sea	CARIACO	Stoeck & Epstein (2003), Stoeck *et al*. (2006)	233
Framvaren, Behnke	Cruise		Coastal anoxic	Norway fjord	Nordstranda	Behnke *et al*. (2006)	182
Pacific Ocean, Short	Coastal station		Coastal	Pacific Ocean		Short & Suttle (2003)	19
Helgoland, Medlin	Time series	$< 3\,\mu m$	Coastal	North Sea; North Atlantic		Medlin *et al*. (2006)	290
Roscoff, Romari	Time series	$< 3\,\mu m$	Coastal	English Channel		Romari & Vaulot (2004)	383
Blanes, Massana	Time series	$< 3\,\mu m$	Coastal	Mediterranean Sea		Massana *et al*. (2004)	148
Scripps Pier, Worden	Time series	$< 2\,\mu m$	Coastal	Pacific Ocean		Worden (2006)	72
Atlantic enrichment, Countway	Experiment		Coastal	North West Atlantic		Countway *et al*. (2005)	462
						Total	3561

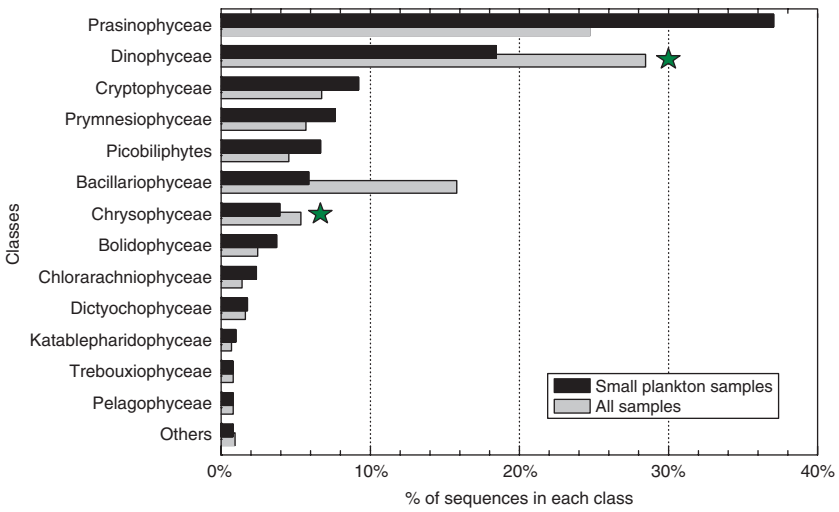

Fig. 5. Relative percentage of 18S rRNA gene sequences in each photosynthetic class from clone libraries and DGGE for sample sets listed in Table 3. Sequences from $\leq 3\,\mu m$ filtered samples ('small plankton') and from the total data set are differentiated (groups labeled with a star contain a significant fraction of heterotrophic species).

Wawrik & Paul, 2004), and *psbA* (Zeidner *et al*., 2003). Other sequences are closely related to the diversified genera *Tetraselmis* and *Pyramimonas*. The most interesting sequences correspond to clade VII (following the nomenclature of Guillou *et al*., 2004) for which no truly marine species has been described yet, although culture isolates are available. Sequences belonging to this clade are found in very different environments: oligotrophic surface waters of

the Pacific Ocean (Moon-van der Staay *et al.*, 2001) and Sargasso Sea (Countway *et al.*, 2007), coastal waters of the English Channel (Romari & Vaulot, 2004), but also deep (900 m) anoxic waters of the Cariaco Basin (Stoeck & Epstein, 2003). Two novel clades without cultured representatives have been discovered very recently using Chlorophyta-biased primers (Viprey *et al.*, 2008). Paradoxically, no 18S rRNA gene sequences related to Prasinococcales and Pseudoscourfieldiales have been recovered using general primers. This is surprising in view of the importance in culture collections of genera belonging to these orders (e.g. *Prasinococcus*, *Pycnococcus*) and of the detection of *Pycnococcus* in the field by antibody labeling (Campbell *et al.*, 1994b). This suggests that they probably occur under specific conditions not sampled in surveys available to date. In fact, *rbcL* sequences related to *Prasinococcus* have been recovered from marine waters (Wawrik & Paul, 2004).

In contrast to Prasinophyceae, the other Chlorophyta classes are very little represented in environmental samples, with only a few sequences related to the Trebouxiophyceae genus *Nannochloris*/*Picochlorum* found mostly in coastal samples (e.g. Medlin *et al.*, 2006).

Alveolates (Dinophyceae)

Despite the fact that no dinoflagellate species of minimum size below 3 μm has been described yet, this group comes second in abundance among sequences from samples filtered through 3 μm (Fig. 5). It should be noted that a significant fraction of these sequences may originate from heterotrophic species that constitute half of the described taxa (Larsen & Sournia, 1991). Still, their relative abundance in euphotic zone samples suggests that some of them are autotrophic. Among filtered sample sequences, similarity to known species ranges from 100% to $< 92\%$. A large fraction of sequences fall into the order Gymnodiniales, some into the Prorocentrales, a few into the Peridiniales, with the rest of unclear affiliation. Clearly, the Gymnodiniales probably harbors many small and hard to distinguish species that remain to be described. Moreover, dinoflagellate cells may be flexible enough to squeeze through pores smaller than their actual size. Interestingly, some sequences are closely related to *Lepidodinium viride* (Watanabe *et al.*, 1990), a dinoflagellate with prasinophyte-like scales and containing chlorophyll *b* as its major accessory pigment, which could contribute to the pigment signature of ≤ 3 μm filtered samples. Recently, dinoflagellate sequences have also been recovered from picoplankton samples using dinoflagellate-biased primers (Lin *et al.*, 2006).

Stramenopiles

Within photosynthetic stramenopiles, diatoms are paradoxically the most abundant group among the sequences from the ≤ 3 μm fraction, although of course they provide an even higher contribution when both filtered and unfiltered samples are considered (Fig. 5). Diatom sequences are relatively more important in the aphotic zone than higher in the water column, suggesting that many sequences may originate from sedimenting material (Kang *et al.*, 2003). In nonpolar waters, all diatom sequences found in ≤ 3 μm filtered samples are related to centric diatoms, with the exception of a few sequences, such as one in the Mediterranean Sea that is close to the toxic genus *Pseudo-nitzschia* (Massana *et al.*, 2004). In the Canadian Arctic Basin (Lovejoy *et al.*, 2006), in contrast, most sequences are related to pennate diatoms, especially to *Fragilariopsis*, a key genus in these waters (Kang & Fryxell, 1992). These sequences may either come from yet undescribed small diatoms, from diatoms passing through filter pores (e.g. for pennates), or alternatively from male gametes of larger diatoms (Davidovich & Bates, 1998).

Bolidophyceae, a small class closely related to diatoms, contains only two picoplanktonic species, both isolated from oligotrophic environments. Quite surprisingly, the only Bolidophyceae sequences detected in oligotrophic waters were recovered after screening clones with a specific probe, suggesting that this group may be insignificant in these environments (Guillou *et al.*, 1999b). More recently, however, sequences with high similarities to Bolidophyceae (up to 99.6%) have been obtained from the Arctic and the North Sea (Lovejoy *et al.*, 2006; Medlin *et al.*, 2006). Moreover, Bolidophyceae plastid 16S rRNA gene and *rbcL* sequences have also been recovered in the ocean (Wawrik & Paul, 2004; Fuller *et al.*, 2006a).

The second most abundant stramenopile group within small plankton sequences is constituted by dictyochophytes, which were not considered as an important group until recently. Sequences have been recovered from size-fractionated samples in quite diverse oceanic waters (North Atlantic, Equatorial Pacific, Sargasso, and Mediterranean Seas, Arctic). Interestingly, most of these sequences are closely related to the newly described species *Florenciella parvula* (Eikrem *et al.*, 2004) while one sequence from the Pacific Ocean has some similarity to the amoeboid species *Rhizochromulina marina* (Hibberd & Chrétiennot-Dinet, 1979).

Pelagophyceae, a class that contains picoplanktonic species, are paradoxically quite rare in environmental 18S rRNA gene clone libraries (Equatorial Pacific, Mediterranean, and Sargasso Seas, Arctic), with all sequences closely related to *Pelagomonas calceolata*. Pelagophyte plastid 16S rRNA gene sequences have also been obtained from various marine waters (Fuller *et al.*, 2006a).

Even more surprising is the fact that Eustigmatophyceae sequences are absent from the data set examined while they are often isolated from coastal waters.

A large fraction of environmental Chrysophyceae sequences from coastal samples filtered through 3 μm or less corresponds to the heterotrophic and highly diversified genera *Spumella* and *Paraphysomonas*. The former is typically from fresh waters while the latter has been shown to be easily isolated in culture from coastal samples despite its generally low concentration in the water (Lim *et al*., 1999). However, the remaining 18S sequences, some originating from coastal waters and some from the Sargasso Sea, form independent clades not related to any known species. Interestingly, a large set of plastid 16S rRNA gene sequences related to chrysophytes have been retrieved from Indian Ocean ≤ 3 μm filtered samples (Fuller *et al*., 2006a). Because 16S rRNA gene sequences could not be recovered from any of the several heterotrophic chrysophyte cultures tested such as *Paraphysomonas* or *Picophagus* (Fuller *et al*., 2006a), this would suggest the existence of undescribed groups of autotrophic marine Chrysophyceae.

Cryptophyta and relatives

Very interestingly, all Cryptophyta sequences from ≤ 3 μm filtered samples but one (from the Sargasso Sea, Not *et al*., 2007a) have been recovered from temperate and polar coastal samples (Mediterranean Sea, English Channel, North Sea, Beaufort Sea), suggesting that this division is probably not adapted to low nutrient conditions. Some of these environmental sequences match almost perfectly those of species available in culture, especially for the genera *Teleaulax*, *Plagioselmis*, or *Geminigera* (clade B following Deane *et al*., 2002), while others are more distantly related to *Hemiselmis* (clade D following Deane *et al*., 2002).

Two other groups related to Cryptophyta are also found in ≤ 3 μm filtered samples. Sequences from the recently described katablepharids (Okamoto & Inouye, 2005) are observed in temperate waters, some being closely related to *Leucocryptos* and *Katablepharis*.

Picobiliphytes (Not *et al*., 2007b), previously referred to as the env Rosko II group, constitute a new phylogenetic group initially only known from sequences retrieved from temperate and arctic waters (Romari & Vaulot, 2004; Lovejoy *et al*., 2006; Medlin *et al*., 2006). Recently, a couple of picobiliphyte sequences have been observed in the Sargasso Sea (Countway *et al*., 2007; Not *et al*., 2007a). Use of oligonucleotide probes has allowed visualization of the cells in natural samples. They appear to harbor phycobilin-containing plastids and possibly a nucleomorph (Not *et al*., 2007b).

Chlorarachniophyta

Sequences from Chlorarachniophyta recovered in ≤ 3 μm filtered samples display only distant relationships with known species and have been found in the coastal Mediterranean and North Seas, in the Arctic Ocean, as well as in the Sargasso Sea, suggesting that they have a wide distribution.

The next frontier: genomics and metagenomics

Genomics offers a holistic approach to microbial ecology. By sequencing all of the genes from an organism, one expects to gain an insight into the intimate relationship between this organism and its environment (Coleman *et al*., 2006). Complete genomes of five unicellular algae have been analyzed so far, two from freshwater species, the red alga *Cyanidioschyzon merolae* (Matsuzaki *et al*., 2004) and the green alga *Chlamydomonas reinhardtii* (Merchant *et al*., 2007), and three from marine environments, the diatom *T. pseudonana* (Armbrust *et al*., 2004) and the prasinophytes *Ostreococcus tauri* (Derelle *et al*., 2006) and *O. lucimarinus nomen nudum* (Palenik *et al*., 2007), all three belonging to the small plankton (see Table 1). The latter three genomes are all small, ranging from about 12 Mb for *Ostreococcus* to 30–35 Mb for diatoms (Misumi *et al*., 2008). Other characteristics, such as the G+C content, are variable and appear to depend on the phylogenetic position of the organism rather than on algal characteristics. Many other micro-algal genome projects are underway (Table 4) and the number of sequenced organisms will certainly increase rapidly.

Complete genomes yield information that can be used for taxonomic analyses. For example, the two *Ostreococcus* strains the genomes of which have been published have similar ultrastructural cell morphologies and very similar 18S sequences (99.6% of identity), but the average amino-acid identity between their orthologous genes is only 70%, which represents by far the highest difference known for species belonging to the same genus (Palenik *et al*., 2007). This indicates how the genetic diversity of picoeukaryotes, which have usually a very simple morphology, is probably largely underestimated and how genomics can be used to explore this diversity. The availability of complete genomes also provides the possibility to describe new markers of diversity or to study the evolution of a gene family in a lineage or between algae belonging to different phyla (e.g. heat shock proteins, Waters & Rioflorido, 2007).

Although the functional analysis of small phytoplankton genomes is still in its infancy, it already provides some interesting clues about the adaptation of these organisms to their environment. For example, several observations suggest that diatoms are not CO_2-limited under natural oceanic conditions. The analysis of genes potentially involved in CO_2 concentration mechanisms (CCM) such as C_4-photosynthesis or biophysical CCM leads to new hypotheses that can be tested experimentally. Genes encoding all of the enzymes required for C_4-photosynthesis have been identified both in the diatoms *T. pseudonana* and *Phaeodactylum*

tricornutum (Kroth *et al.*, 2008) and in the two *Ostreococcus* genomes (Derelle *et al.*, 2006; Palenik *et al.*, 2007). This raises exciting questions, because, despite its high energetic cost, C4-photosynthesis could confer a critical ecological advantage under CO_2-limiting conditions, such as in phytoplankton blooms. Furthermore, recent experimental work supports the idea that C_4-based CO_2-concentrating mechanisms are widely distributed in diatoms (McGinn & Morel, 2008).

The preparation of complete tiling arrays based on the genome sequence of an organism provides a useful tool for probing global gene expression under various culture conditions. This technology has been used recently for the identification of genes involved in silica metabolism of *T. pseudonana* (Mock *et al.*, 2008). Under conditions of limiting silica concentrations, a set of 75 genes showing no similarities to known proteins were induced. Another set of genes was induced when both silica and iron were limiting, showing possible interactions between these two metabolisms or, alternatively, that iron is a required cofactor for silica metabolism. Interestingly, in these experiments, the whole-genome expression profiling identified 3000 new genes that were not annotated previously and included noncoding and antisense RNAs.

Another example of possible environmental adaptations deduced from genome analyses comes from a global survey for selenoenzymes in available complete genomes, including the three small algae described above (Lobanov *et al.*, 2007). Selenoproteins are known to be more catalytically active than similar enzymes lacking selenium and thus cells having genes coding for these enzymes may require less of that protein and can thereby acquire a selective advantage in some environments. Selenoenzymes are present in both prokaryotes and eukaryotes, but the number of pathways in which they are implicated varies enormously between organisms. Interestingly, an unusually high content of selenoprotein-coding genes has been found in both *Ostreococcus* (Palenik *et al.*, 2007) and in the diatom *T. pseudonana* (Lobanov *et al.*, 2007), whereas the selenoproteomes of terrestrial organisms were reduced or completely lost. This strongly suggests that

Table 4. Marine micro-algae for which full nuclear genome sequence is or will be soon available

Class	Species	Strain	Small phytoplankton	Status or References	Web link
Prasinophyceae	*Bathycoccus prasinos*	Bban7	+	Pending	http://www.cns.fr/externe/English/corps_anglais.html
	Ostreococcus tauri	OTH95	+	Derelle *et al.* (2006)	http://bioinformatics.psb.ugent.be/genomes/view/Ostreococcus-tauri
	Ostreococcus lucimarinus nomen nudum	CC9901	+	Palenik *et al.* (2007)	http://genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html
	Ostreococcus sp.	RCC809	+	Annotation	http://www.jgi.doe.gov/sequencing/why/CSP2006/llostreococcus.html
	Micromonas pusilla	RCC827	+	Annotation	http://www.jgi.doe.gov/sequencing/DOEmicrobes2005.html
	Micromonas pusilla	CCMP1545	+	Annotation	http://www.jgi.doe.gov/sequencing/DOEmicrobes2005.html
Chlorophyceae	*Dunaliella salina*			Sequencing	http://www.jgi.doe.gov/sequencing/DOEmicrobes2006.html
Bacillariophyceae	*Thallassiosira pseudonana*	CCMP1335	+	Armbrust *et al.* (2004)	http://genome.jgi-psf.org/Thaps3/Thaps3.home.html
	Phaeodactylum tricornutum	CCAP1055/1		Finished	http://genome.jgi-psf.org/Phatr2/Phatr2.home.html
	Pseudo-nitzschia sp.			Sequencing	http://www.jgi.doe.gov/sequencing/DOEmicrobes2006.html
	Fragilariopsis cylindrus			Sequencing	http://www.jgi.doe.gov/sequencing/why/CSP2007/fragilariopsis.html
Prymnesiophyceae	*Phaeocystis globosa*			Pending	http://www.jgi.doe.gov/sequencing/why/CSP2008/pglobosa.html
	Phaeocystis antarctica			Pending	http://www.jgi.doe.gov/sequencing/why/CSP2008/pantarctica.html
	Emiliania huxleyi	CCMP1516		Annotation	http://www.jgi.doe.gov/sequencing/DOEmicrobes2003.html
Cryptophyceae	*Guillardia theta*			Assembly	http://www.jgi.doe.gov/sequencing/why/CSP2007/guillardia.html
Chlorarachniophyceae	*Bigelowiella natans*			Sequencing	http://www.jgi.doe.gov/sequencing/why/CSP2007/guillardia.html

aquatic life supports selenium utilization, whereas terrestrial habitats lead to reduced use of this trace element due to unknown environmental factors (Lobanov *et al.*, 2007).

These examples show how genomics can provide important new clues about the adaptation of small algae to their environment, although most hypotheses are still awaiting experimental support. This suggests at least two research directions: one relying on functional studies based on hypotheses emerging from genome analyses and the other supported by the acquisition of new algal genomes (Table 4). For instance, genomes of other prasinophyte strains (*Ostreococcus* RCC809, *B. prasinos*, and *M. pusilla*) isolated from different environments (Rodríguez *et al.*, 2005; Slapeta *et al.*, 2006) are currently being sequenced. Comparison of these genomes will foster new hypotheses for future functional approaches.

Nucleomorph, mitochondrial, or chloroplastic genomes of several microalgae have also been sequenced (Gilson *et al.*, 2006; de Cambiaire *et al.*, 2007; Oudot-Le Secq *et al.*, 2007). These genomes are much smaller than nuclear genomes and yield information on the organism evolution following primary or secondary endosymbiosis events. For small marine autotrophic eukaryotes, the only organelle genomes available are those of the three organisms for which the complete nuclear genome has been sequenced, i.e. *O. tauri* (Robbens *et al.*, 2007), *O. lucimarinus* (Palenik *et al.*, 2007), and *T. pseudonana* (Oudot-Le Secq *et al.*, 2007). These genomes are very compact. The mitochondrial genomes of the two *Ostreococcus* are the most gene-dense mitochondrial genomes of all Chlorophyta and are characterized by a unique segmental duplication covering 44% of the genome, not observed before in green algae. The chloroplast genomes show a classical circular quadripartite structure with both large and small single copy regions, separated by two inverted repeat sequences. Based on genome size and number of genes, the *Ostreococcus* chloroplast genomes are the smallest known among the green algae. *Thalassiosira pseudonana* chloroplast genome is also compact with small intergenic regions and no introns. Gene content is similar to that of both the centric *Odontella sinensis* and the pennate *P. tricornutum* diatoms.

Metagenomics approaches, i.e. the direct sequencing of large genome fragments from natural samples, are yielding a tremendous amount of new information on environmental genetic diversity. For example, in the Sargasso Sea study (Venter *et al.*, 2004), a total of 1045 billion base pairs of nonredundant sequences were generated, originating from an estimate of 1804 different genomic species including 148 new bacterial phylotypes. Although most samples were filtered through 0.8 μm, which strongly biased the acquired data towards prokaryotes, eukaryotic sequences nevertheless represented 4–18% of the estimated prokaryotic diversity, depending on assumptions for the average prokaryote vs. eukaryote genome size ratio. These sequences correspond to species that are broadly dispersed throughout the eukaryotic tree of life (Piganeau *et al.*, 2008). In particular, at least two new *Ostreococcus* strains have been identified. *Ostreococus* scaffolds cover 23% of the complete nuclear genome and 14% of the total number of protein-coding genes in *O. tauri* (Piganeau & Moreau, 2007). Picoeukaryote sequences have not yet been specifically analyzed in other oceanic metagenomics data sets (e.g. DeLong *et al.*, 2006), but are most probably present.

Concluding remarks

Clearly, the small number of photosynthetic picoplankton species that has been described constitutes the tip of the iceberg because many of the 18S rRNA gene sequences recovered from the environment do not match those from species in culture. In fact, the number of environmental sequences from photosynthetic picoplankton remains quite small in comparison with heterotrophic ones because clone libraries are heavily biased towards the latter. New approaches such as amplification of 18S rRNA gene with primers biased towards certain groups (e.g. Chlorophyta, Viprey *et al.*, 2008), construction of clone libraries for plastid 16S rRNA genes (Fuller *et al.*, 2006a), or from cells sorted by flow cytometry on the basis of chlorophyll fluorescence should help explore the real extent of photosynthetic picoplankton diversity. The genomes of several prasinophytes strains (*Micromonas*, *Ostreococcus*, and *Bathycoccus*) are or will soon be available and their comparison should allow fascinating insight linking genetic to functional diversity. Metagenomics approaches will also lead in the near future to the production of a tremendous amount of new data. However, because eukaryote genomes are clearly larger and less gene dense than prokaryote ones, the analysis and exploitation of environmental genomic data for eukaryotes (Piganeau *et al.*, 2008) at a functional level will require extensive effort drawing from many disciplines in particular bioinformatics, genetics, physiology, and ecology.

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