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Eukaryotic Picoplankton in Surface Oceans

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Abstract

The eukaryotic picoplankton is a heterogeneous collection of small protists 1 to 3 μm in size populating surface oceans at abundances of 10^2 to 10^4 cells ml^{-1}. Pigmented cells are important primary producers that are at the base of food webs. Colorless cells are mostly bacterivores and play key roles in channeling bacteria to higher trophic levels as well as in nutrient recycling. Mixotrophy and parasitism are relevant but less investigated trophic paths. Molecular surveys of picoeukaryotes have unveiled a large phylogenetic diversity and new lineages, and it is critical to understand the ecological and evolutionary significance of this large and novel diversity. A main goal is to assess how individuals are organized in taxonomic units and how they participate in ecological processes. Picoeukaryotes are convincingly integral members of marine ecosystems in terms of cell abundance, biomass, activity, and diversity and they play crucial roles in food webs and biogeochemical cycles.

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INTRODUCTION

Oceans cover approximately 70% of Earth's surface and play fundamental roles in processes that have global ecological and socioeconomic impacts (7). They are a vital component of the climate system and are suffering and partially attenuating anthropogenic global change. Life originated in the oceans, which have been the main sites of evolution. It is no surprise that oceans harbor organisms spanning a large range of body sizes, phylogenetic affiliations, and trophic modes (71). Photosynthesis is a critical process that allows life on Earth, and interestingly, half the global primary production occurs in the sea, mostly by planktonic microorganisms that account for only 0.2% of global primary producer biomass (24). This has many consequences for the functioning of marine ecosystems, influencing carbon and energy fluxes through organisms (food webs), affecting carbon fluxes to deep waters (biological pump), and fine-tuning of all biogeochemical cycles. The recognized importance of oceans and their microbial life has promoted an expansion of microbial ecology studies fueled by novel analytical capabilities (8).

Planktonic microorganisms are categorized into classes based on size for operational purposes (78). Initially, only prokaryotes were included in the smallest class (picoplankton: cells 0.2 to 2 μm) and microbial eukaryotes (protists) were included in the nanoplankton (2 to 20 μm) or microplankton (20 to 200 μm). Picoeukaryotes known in culture at that time were not expected to be quantitatively important in the sea. However, minute eukaryotes were soon detected by epifluorescence microscopy (23, 37) and flow cytometry (62). Picoeukaryotes are now known to be ubiquitous in surface oceans (39, 88) and form, together with prokaryotes, an ocean's veil above which larger protists and metazoans might bloom. They exemplify the ecological success of miniaturized cells prepared for independent life by keeping only the minimal cellular components, typically one mitochondrion, one Golgi apparatus, and optionally one chloroplast and flagellum (66).

For decades, picoeukaryotes were treated as a bulk assemblage owing to the inability to differentiate them (**Figure 1**). Pigmented cells account for a significant fraction of primary production, especially in oligotrophic conditions (46, 88), whereas colorless cells are mainly

bacterial grazers (39). The recent molecular revolution has shown that the eukaryotic picoplankton includes a large phylogenetic diversity and many novel lineages (22, 55, 82). Molecular methods today offer new tools for studying picoplankton biogeography, activity, biological interactions, and population control mechanisms.

This review focuses on the eukaryotic picoplankton living in the region where photosynthesis occurs (upper 200 m), because this reactive surface skin harbors the largest variety of taxa and functional modes. This review does not address the dark ocean, a biome with its own biogeochemical properties (5), nor does it address anoxic systems or lakes typically harboring a different microbial life (43, 79). Picoeukaryotes are considered cells ≤ 3 μm, a criterion widely used (82) and supported by direct observations. The ecology of marine picoeukaryotes treated as a bulk assemblage is presented first. Then, tools for opening this black box are listed, followed by navigation through the main phylogenetic groups and their putative cell abundance and ecological roles. Finally, population and community ecology issues deserving more attention are discussed.

BULK ECOLOGICAL ROLE

Distribution and Cell Abundance

The smallest eukaryotes were first quantified by epifluorescence microscopy (23, 37) and separated between chloroplast-containing phototrophs and colorless heterotrophs (**Figure 1*c–g***). These cells, often flagellated, were considered nanoflagellates. Flow cytometry, soon used to quantify phytoplankton (62), yielded phototrophic eukaryotic counts (named picoeukaryotes) roughly equivalent to epifluorescence counts of phototrophic nanoflagellates. Typical abundances by both methods are 1–3 $\times$ 10^3 cells ml^{-1} in oligotrophic systems and up to 10^5 cells ml^{-1} in coastal and nutrient-rich regions (47, 72). Within the water column, counts increase

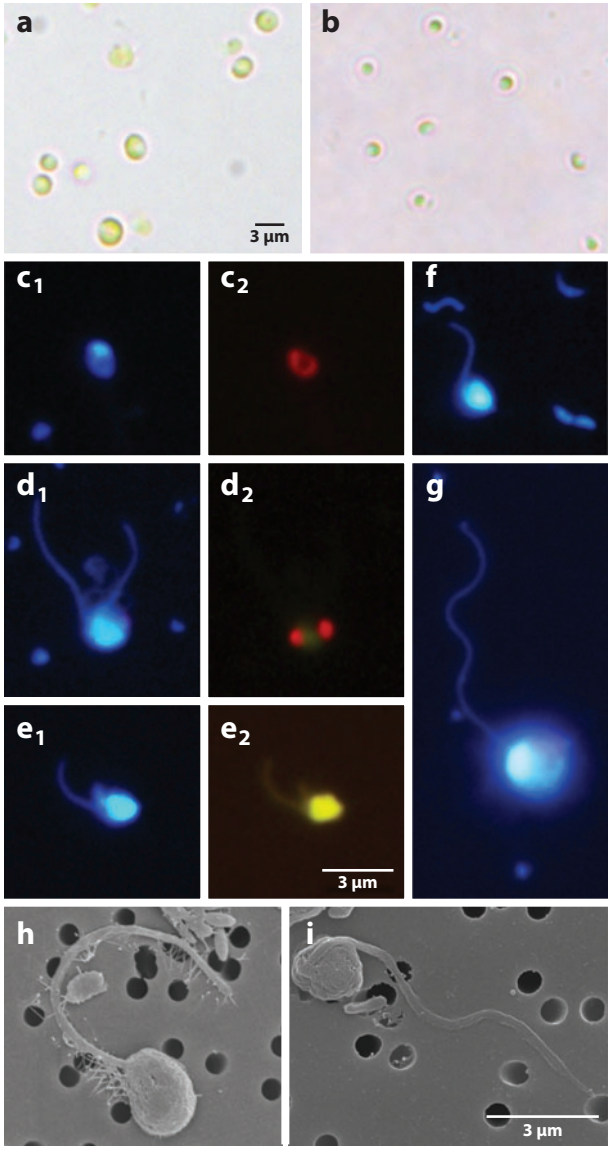

Figure 1

Marine picoeukaryotes as seen by (*a*,*b*) light microscopy, (*c–g*) epifluorescence microscopy, and (*h*,*i*) scanning electron microscopy. Epifluorescence images are taken by UV excitation for (c_1-e_1, *f*,*g*) DAPI-stained DNA blue fluorescence or by blue excitation for (c_2-e_2) Chl *a* red autofluorescence. Organisms are (*a*) *Pelagomonas calceolata*; (*b*) *Micromonas pusilla*; two unidentified phototrophic picoeukaryotes, probably (*c*) a prasinophyte and (*d*) a haptophyte; (*e*) an unidentified heterotrophic picoeukaryote; and (*f–i*) unidentified cells. Images courtesy of I. Forn, A.M. Cabello, J. del Campo, and J.M. Fortuño. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

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from surface to the deep chlorophyll maximum and then sharply decrease. Flow cytometry allows picoeukaryotes to be compared with the picocyanobacteria *Synechococcus* and *Prochlorococcus* (88). *Prochlorococcus* are more abundant in the open sea, whereas picoeukaryotes and *Synechococcus* dominate in coastal systems. Regarding small heterotrophic eukaryotes, few studies have used flow cytometry for quantification (89), so most data derive from epifluorescence (72). These small heterotrophs are less abundant than phototrophs and account for 20% to 30% of eukaryotes (39). They also exhibit a narrower range, from 3×10^2 to 3×10^3 cells ml^{-1}, and tend to be more abundant in productive areas.

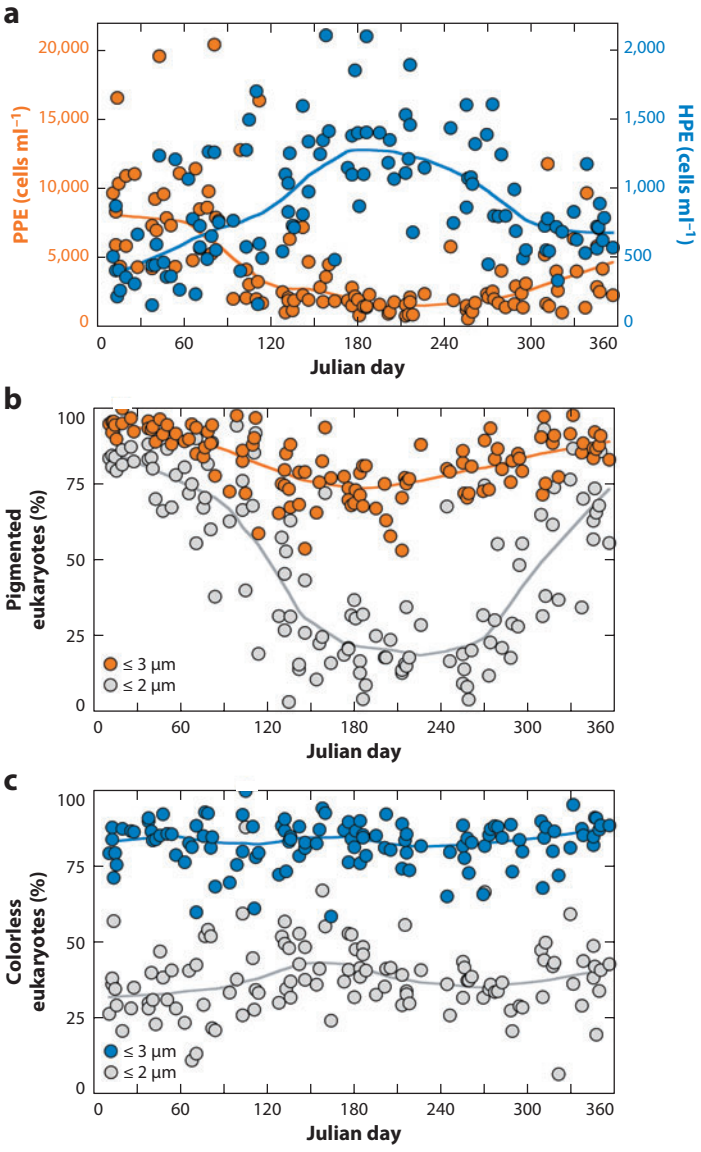

Figure 2

(*a*) Abundance of phototrophic picoeukaryotes (PPE) and heterotrophic picoeukaryotes (HPE) (protists ≤3 µm) at the Blanes Bay Microbial Observatory during nine years of monthly sampling (117 points). (*b*) Percentage of pigmented eukaryotes explained by PPE and ≤2 µm cells. (*c*) Percentage of colorless eukaryotes explained by HPE and ≤2 µm cells.

How many of these minute eukaryotes belong to picoplankton? Epifluorescence cell sizing reveals that the 2-µm limit does not mark a natural discontinuity in the eukaryotic size spectra. Instead, a coherent assemblage is delimited by the 3-µm limit, as shown by counts from the Blanes Bay Microbial Observatory, northwestern Mediterranean (**Figure 2**). In this oligotrophic coastal site (54), picoeukaryotes defined as cells ≤3 µm exhibit a clear seasonality. Phototrophic picoeukaryotes (PPE) average 4,950 cells ml^{-1} (560 to 37,500 cells ml^{-1}) and are more abundant in winter, whereas heterotrophic picoeukaryotes (HPE) average 940 cells ml^{-1} (160 to 3,850 cells ml^{-1}) and are more abundant in summer (**Figure 2*a***). They account for the largest fraction of eukaryotes year-round: PPE explain on average 82% of pigmented cells (**Figure 2*b***) and HPE explain 83% of colorless cells (**Figure 2*c***). In contrast, eukaryotic cells ≤2 µm form a variable fraction of pigmented eukaryotes (**Figure 2*b***) or constitute a minor fraction (38% on average) of colorless cells (**Figure 2*c***). These data, similar to those found in other systems (39), support using the 3-µm boundary to define picoeukaryotes.

The ubiquity and relatively stable cell abundance of marine picoeukaryotes suggest tight and efficient controlling mechanisms. On the one hand, bottom-up forces include environmental constraints (temperature, oxygen) and resource availability (food, light, nutrients) and are expected to provide an upper limit to growth rates. On the other hand, top-down forces such as predation and viral infection may control the realized abundances. Correlations to discern the interplay between both forces show

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positive trends of PPE with Chl *a* (47) and less clear positive trends between HPE and bacteria (29). In general, it seems that resource control on microbial eukaryotes is more common in oligotrophic systems and predation control is more common in productive systems. Bottom-up and top-down forces operate on individuals and populations, so intrinsic specific differences in resource acquisition and predation-viral susceptibility may affect the final observed trends.

Contribution to Primary Production and Bacterivory

Picoeukaryotes are clearly the most abundant eukaryotes in the sea, but this does not directly imply a large biogeochemical impact. PPE cells are primary producers that use CO_2, inorganic nutrients, and light for growth. Together with *Prochlorococcus* and *Synechococcus* they form the picophytoplankton, which accounts for a high fraction (80%–90%) of phytoplankton biomass in the open sea (42, 60) and ~10% in the most productive systems (2). The dominance of picophytoplankton in nutrient-poor systems reflects its advantages in resource and light acquisition with respect to larger cells (66). Within the picophytoplankton, PPE cells, slightly larger than cyanobacteria, typically dominate (60%–80%) biomass and primary production (46, 87). Therefore, PPE cells appear as important contributors of algal biomass and primary production globally. For instance, they account for 38%–50% of algal biomass in the Indian Ocean (60) and for 34% of primary production in the North Atlantic (36).

HPE cells are generally bacterial grazers (39). Their contribution to biomass requires microscopic cell sizing because they do not have a signature for cell fractionation. Such an exercise has been done in the Blanes Bay Microbial Observatory, yielding a 33% contribution of HPE to the biomass of heterotrophic eukaryotes <20 μm (H. Sarmento & R. Massana, unpublished data). Manipulations with size-fractionated natural assemblages indicate that HPE cells are the major bacterivores in marine systems (85), and this is partially confirmed by direct sizing of protists with ingested fluorescent bacteria (81). Bacterivorous HPE thus play a central role in the microbial loop (64): They keep bacterial stocks stable, transfer dissolved organic matter to higher trophic levels, and recycle nutrients that sustain regenerated primary production.

Primary production carried out by minute cells dominates in vast areas of the oceans, such as the subtropical gyres, which represent ~40% of Earth's surface (40). PPE cells are optimal prey for their grazers (87), typically flagellates, which have similar growth rates and are too small to be eaten by copepods. Thus, transfer of primary production to copepods requires at least three trophic links (PPE-flagellates-ciliates-copepods), contrary to the simple food chain in productive systems (diatoms-copepods). A large fraction of eaten biomass is respired at each link, so little carbon reaches higher trophic levels (44). Small cells do not sink passively and are regarded as poor contributors to the biological carbon pump, although this has been recently challenged (67). Thus, environments dominated by small primary producers represent almost closed systems that maintain relatively high rates of primary production, thanks to efficient internal nutrient recycling, and result in little carbon export to food webs or deep waters.

Other Trophic Attributes

The dichotomy between phototrophs and bacterivores is blurred by the functional versatility of specific organisms. Many pigmented protists are mixotrophs that combine photosynthesis with phagotrophy, presumably obtaining part of their carbon and/or nutrients from the ingested prey (38). Phagotrophy is considered a universal character in the evolution of eukaryotes and, except for green algae, all photosynthetic lineages exhibit mixotrophic species. What is perhaps more surprising is that mixotrophy explains ~50% of bacterivory in coastal (81) and open sea systems (89). Mixotrophs arrive to these figures by combining lower grazing rates than heterotrophs but

Ecological role: contribution of an assemblage of organisms to major ecosystem functions such as primary production, predation, osmotrophy, or parasitism

HPE: heterotrophic picoeukaryote(s)

PPE: phototrophic picoeukaryote(s)

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higher cell abundances. Another mechanism of algal mixotrophy is the osmotrophic incorporation of dissolved organic matter (83). Both particulate and dissolved organic matter uptake may supplement the nutritional needs of the mixotrophic cell.

Regarding HPE cells, some can ingest small phototrophs such as picocyanobacteria or PPE, thus being herbivores instead of bacterivores (75). In addition, it remains to be explored whether bacterivorous HPE can perform osmotrophy to supplement their diet, since the smallest bacterial osmotrophs are assumed to have a competitive advantage over eukaryotes in diluted systems. Nevertheless, osmotrophs such as thraustochytrids have been detected both in the coast and in the open sea (65). Finally, the importance and prevalence of parasitism in the sea, especially in pelagic habitats, probably have been underestimated, and some HPE cells could indeed be dispersal stages of parasites of larger marine organisms (12, 14, 31, 77).

BEYOND BULK ASSEMBLAGES

Need for Molecular Approaches in Diversity Studies

Fundamental knowledge has been gained through the bulk study of the abundance, biomass, and activity of marine picoeukaryotes. However, picoeukaryotes include different cells. Epifluorescence microscopy displays only a few features such as size, general shape, and the presence of plastids and flagella (**Figure 1**), so picoeukaryotes cannot be classified even into a high-ranking taxonomic group except in a few cases (**Figure 1*c*,*d***). This is far from the resolution obtained by inverted microscopy in larger protists such as dinoflagellates or diatoms. Electron microscopy has a large potential for morphological inspection, but many cells are lost or remain unidentified when processing natural assemblages (84). Phytoplankton groups can be assessed by high-performance liquid chromatography (HPLC) pigment signatures (42), but only at a broad taxonomic resolution. Culturing could also provide insights into in situ diversity. Cultured PPE belong mostly to Prasinophyceae, Pelagophyceae, Bolidophyceae and Pinguiophyceae (82), and HPE to Bicosoecida and Chrysophyceae (39). However, culture-based surveys and molecular surveys often do not agree (54). Moreover, quantification based on the most probable number (MPN) method yields low counts for heterotrophic flagellates (11) and only realistic counts for some PPE such as *Micromonas* (80). The poor MPN results, a method based on replicate cultures from serially diluted samples, further stress the culturing bias in microorganisms.

Due to inherent limitations of the above methods, picoeukaryotes were treated for decades as a black box of difficult access, as occurred for marine prokaryotes. Microscopic or pigment surveys failed to identify most cells, and only a few could be assigned to a class. Moreover, it was not clear whether the whole natural diversity was represented in culture collections or whether isolated strains were good models to explore the ecological and evolutionary significance of marine picoeukaryotes. New insights into the microbial world arrived with molecular tools, which revolutionized microbial ecology. A multifaceted approach can now be used to investigate cell abundance, diversity, and function of picoeukaryote populations (**Figure 3**).

The diversity of marine picoeukaryotes can be addressed by sequencing environmental genes. A first approach amplifies 18S rDNA from picoplankton DNA, clones PCR products, and sequences several clones. Seminal studies revealed an unexpectedly large and novel diversity (20, 50, 58), a view confirmed in posterior reports targeting other genes. Surveys based on ribosomes (instead of genomic rDNA) are adequate to detect the active community (59), whereas other studies focus on phototrophs by targeting plastid 16S rDNA (45) or photosynthetic genes (53) or focus on given lineages by using group-specific primers (49). High-throughput sequencing methods, with the power to yield millions of sequences

HPLC: high-performance liquid chromatography

Molecular survey: study of the diversity of natural assemblages by obtaining the environmental sequences of a given marker such as the rDNA gene

High-throughput sequencing: set of new technologies producing thousands or millions of DNA sequences at once by parallelizing the sequencing process

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without the cloning step, are opening new dimensions to gene-targeted studies (13). Finally, sequencing of environmental DNA obviating the PCR step can also detect phylogenetic markers that describe in situ diversity (59).

Laboratory Studies with Isolated Picoeukaryotes

Some groups detected in molecular surveys have cultured representatives (39, 82), so these strains are good candidates for ultrastructural, physiological, and genomic studies. Ecophysiology investigates the effect of environmental parameters, resources, or natural enemies on cell activity. Critical factors for PPE are the intensity and quality of light or the uptake kinetics of inorganic nutrients, whereas for HPE critical factors are the size, quality, and quantity of prey (39, 48). In general, the high surface/volume ratio of small cells derives from high absolute and specific growth rates, high affinity for diluted compounds, and better fitness with respect to larger cells in oligotrophic environments (66).

Genome projects of cultured strains are pivotal for understanding evolutionary relationships, metabolism, and development of specific traits. About 10% of the 1,508 published genomes are eukaryotic and only 38 are of protists (and 243 are in progress; **http://www.genomesonline.org/**). Free-living marine protists include four prasinophyte strains, two diatom strains, and one choanoflagellate strain (41, 63, 86). Each genome has been analyzed from a different perspective, including gene organization, speciation patterns, and the origin of multicellularity, and may lead to new hypotheses to be tested experimentally. Together, ecophysiological and genomic studies contribute to identifying the traits that make an organism successful in the environment (48).

Linking Diversity and Function for Uncultured Groups

A large fraction of in situ diversity is not represented in cultures, and it is urgent to try original strategies to isolate interesting picoeukaryotes (35). Nevertheless, some may be unculturable and, even in the best scenario, it is unlikely that we will ever have the whole in situ diversity in culture. So, methods to address the function of uncultured picoeukaryotes are essential. A first approach closing the rRNA cycle is fluorescence in situ hybridization (FISH). This technique visualizes specific cells in natural assemblages, putting a face (cell size, shape) to uncultured clades and allowing them to be quantified (56). When combined with experiments and direct observations, FISH relates uncultured clades to given ecological roles (12, 57).

Direct sequencing community DNA (metagenomics) or community RNA (metatranscriptomics) is revolutionizing microbial ecology, especially when combined with high-throughput sequencing. Metagenomics reveals community gene repertoires and metabolic potential (21), whereas metatranscriptomics provides insights into realized functions (27). Functional profiles given by -omic approaches can be used as descriptors of community properties and for comparative purposes. Indeed,

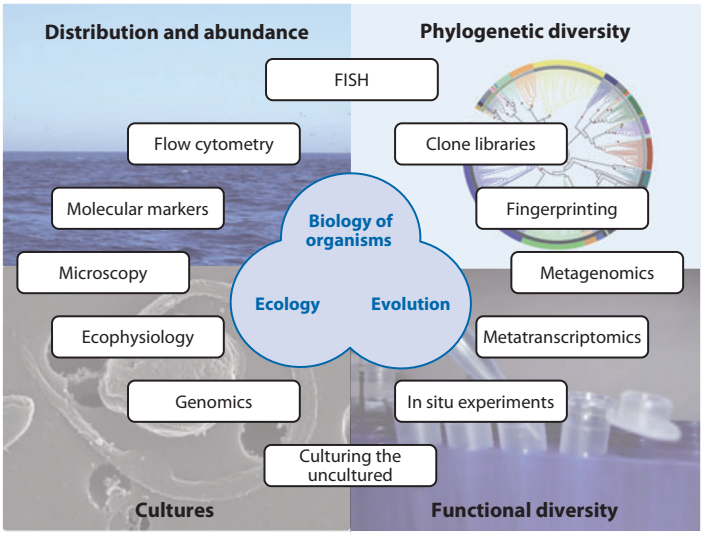

Figure 3

Overview of approaches to investigate cell biology, ecology, and evolution of marine picoeukaryotes (and microorganisms in general), treating four main study areas: abundance, phylogenetic diversity, functional diversity, and culture studies. Abbreviation: FISH, fluorescence in situ hybridization.

Ecophysiology: refers to functional properties of an organism important to understanding its environmental adaptation

Genome project: complete sequencing of the DNA of an organism to determine its gene content, metabolism, evolutionary origin, and ecological adaptation

FISH: fluorescence in situ hybridization

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Eukaryotic supergroups: major lineages of eukaryotes that join together apparently unrelated taxonomic groups on the basis of ultrastructural and molecular data

CCTH: cryptophytes, centrohelids, telonemids, plus haptophytes

MALV: marine alveolates

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the large database of marine metagenomes from the Global Ocean Sampling expedition (69) is fueling the microbial research agenda. These approaches still need to focus specifically on picoeukaryotes, which are often removed by filtration from the analysis.

Combining techniques gives further functional insights into uncultured cells. A general approach is to incubate natural assemblages with a labeled precursor and identify the taxa incorporating it. The precursor can be radioactive inorganic or organic carbon for phototrophy or osmotrophy, or labeled bacteria for grazing. Cells incorporating radioactive substrates can be seen by microautoradiography and FISH (83). Flow cytometry can be used to sort populations before assessing their radioactivity and community structure (36). Grazers with ingested fluorescent bacteria can be seen by FISH (57), whereas those ingesting isotopically labeled bacteria can be identified by sequencing labeled rRNA separated by ultrafiltration (28). The sorting capacities of modern flow cytometers are opening new possibilities for single-cell analyses. Single microbial cells can be used as inocula to start pure cultures or as template for whole genomic amplification prior to genome sequencing. This single-cell approach has been recently applied to heterotrophic flagellates (33).

NAVIGATING THROUGH THE MAIN PHYLOGENETIC GROUPS

In the past decade, advanced multigene phylogenies have delineated the eukaryotic tree of life into a few eukaryotic supergroups, each one including well-known taxonomic classes (6, 9). Although the configuration of supergroups varies, the general consensus includes unikonts (opisthokonts plus amoebozoans), archaeplastidans, SAR (stramenopiles, alveolates plus rhizarians), excavates, and CCTH (cryptophytes, centrohelids, telonemids plus haptophytes). Eukaryotic molecular surveys use this renovated tree of life as the phylogenetic frame to place environmental sequences (20, 50, 55, 58, 82). To present the phylogenetic groups detected, I use a dataset of 8,719 public 18S rDNA sequences (V4-V5 regions) mostly from surface picoeukaryotes, but also from deep sea and larger protists (**Figure 4**). The clonal representation of each group (roughly equivalent to a class) gives an initial taste of its importance but is also influenced by the variable rDNA copy number among taxa and other molecular biases. So, when available, additional data based on pigments, FISH, metagenomics, or other gene markers are added for each group. This section ends with an overview of the main phylogenetic groups comprising marine picoeukaryote assemblages.

Alveolates: MALV

This supergroup includes dinoflagellates, ciliates, apicomplexans, and MALV (marine alveolates) (31) and dominates marine eukaryote surveys with 57% of sequences (**Figure 4*b***). Dinoflagellates and ciliates are well-known free-living heterotrophs, mixotrophs, or phototrophs and are well represented (11% of sequences each). They still comprise 5% of sequences in a dataset of only picoeukaryotes (**Figure 4*c***), which is surprising because their known minimal size is 5 to 10 μm. The existence of picosized dinoflagellates or ciliates is possible, but the most plausible explanation is a combination of filtration artifacts and amplifying dissolved DNA. Thus, these two groups most likely do not contribute to marine picoeukaryotes.

Uncultured MALV lineages account for one-third of sequences, 21% MALV-II and 11% MALV-I. Soon after the description of MALV clades, *Amoebophrya* sp. was sequenced and seen to belong to MALV-II. This parasite is host specific, with different species infecting different dinoflagellates (14). Its life cycle starts when a 2- to 10-μm-dispersal dinospore infects a host and grows as a trophont that occupies the whole host volume; then the trophont leaves the host and releases dinospores. Whereas MALV-II seems to parasitize dinoflagellates only, MALV-I has a wider host spectrum, including radiolarians, ciliates, and fish eggs.

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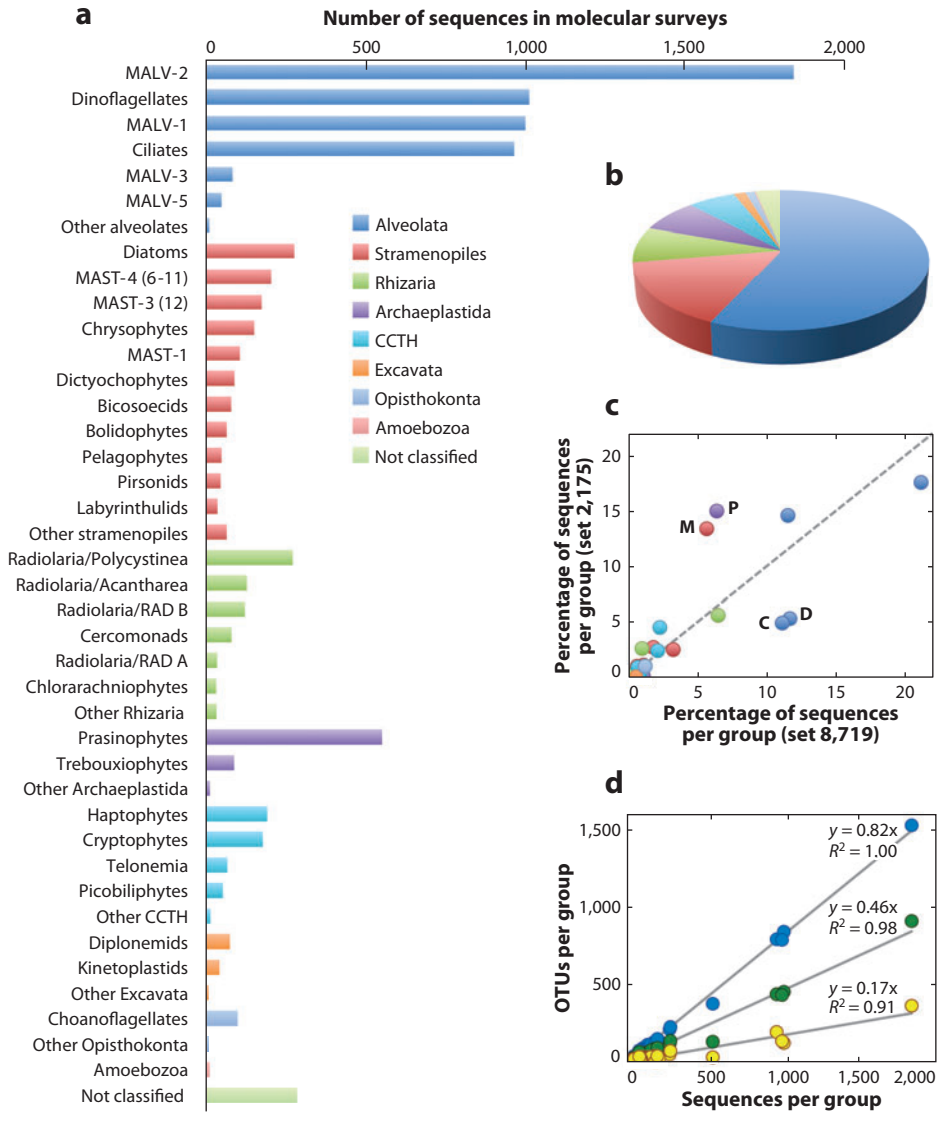

Figure 4

(*a*) Taxonomic affiliation of 18S rDNA sequences retrieved in marine protist surveys (metazoa and fungi excluded). The 8,719 sequences derive from seawater clone libraries done with eukaryotic primers (~90% from surface protists; ~67% from surface picoeukaryotes). (*b*) Sequence contribution of eukaryotic supergroups. (*c*) Comparison of a group's relative abundance with a dataset of 2,175 sequences from surface picoeukaryotes (55). Groups deviating from the 1:1 line are prasinophytes (P), MAST (M), ciliates (C), and dinoflagellates (D). (*d*) Number of operational taxonomic units (OTUs) contributed per group as a function of its number of sequences, at 100% OTU clustering level (*blue dots*), 99% (*green dots*), and 95% (*yellow dots*). Data from M. Pernice (unpublished data). Abbreviations: CCTH, cryptophytes, centrohelids, telonemids, plus haptophytes.

MAST: marine stramenopiles

Further studies demonstrate that the MALV clonal abundance severely overestimates cell abundance. MALV sequences decrease from 39% in a rDNA library to 8% in a rRNA library likely because of the high rDNA copy number in alveolates and perhaps because of the presence of detrital DNA (59). FISH counts of MALV-II dinospores in the Mediterranean Sea (77) are rather high in one coastal station (20% of eukaryotes) but low offshore (0.4%–3.1%). These moderate counts, accompanied with high prevalence (2%–10% infected cells) and a wide range of host species, suggest that MALV may be important parasites. Indeed, parasitism emerges as an interaction that can severely influence microbial food webs, community structure, and population dynamics (12).

Stramenopiles: Chrysophytes, Pelagophytes, and MAST

This supergroup accounts for 16% of sequences in our dataset (**Figure 4**). The stramenopile phylogenetic tree includes a large radiation of chloroplast-containing groups and a basal grade of heterotrophic groups. Diatoms, chrysophytes, pelagophytes, dictyochophytes, and bolidophytes are well represented in molecular surveys and could explain marine PPE, because they all have picosized cultures (82). Indeed, environmental sequences are close to cultures, although new clades are also seen (19). From these groups, chrysophytes and pelagophytes appear as especially important PPE components as deduced from plastid 16S rDNA surveys (45), the expression of the photosynthetic gene *psbA* (53), rRNA libraries (59), FISH counts on cytometrically sorted cells (21% and 26% of eukaryotes respectively; 36), and pigment analyses (42, 60). On the other hand, diatoms seem to contribute little to picoplankton. The same holds true for bolidophytes and dictyochophytes, although one study highlights these two groups as major cyanobacterial grazers (28).

Stramenopile heterotrophic groups include parasites (such as pirsonids), osmotrophs (such as labyrinthulids), and bacterivores (such as bicosoecids), groups that are detected in molecular surveys (**Figure 4**). However, most sequences in this part of the tree form new clades named MAST (marine stramenopiles) (56). They account for 6% of sequences in our survey, and 13% considering only studies analyzing picoeukaryotes (**Figure 4*c***). Moreover, MAST sequences are more represented in rRNA libraries, stressing their putative activity (59). FISH results revealed that MAST cells from clades 1, 2, and 4 have a size of 2 to 8 μm, lack chloroplasts, ingest bacteria, and are widely distributed in the sea, accounting for ~20% of heterotrophic flagellates (56). Each lineage exhibits cells of different size and with a particular distribution. In addition, combining FISH with bacterivory experiments highlights functional diversity between clades, which vary in grazing rates and prey spectra (57).

Rhizaria: Cercozoans and Radiolarians

Rhizaria include amoeboid protists within three main groups, cercozoa, radiolaria, and foraminifera, with the first two well represented in molecular surveys (**Figure 4**). Cercozoan sequences fall within flagellated cercomonads or within the algal chlorarachniophytes. Both groups are plausible picoeukaryotes. Most rhizarian sequences (6%) affiliate with radiolaria, including the novel clades RAD A and RAD B. Radiolarian sequences are even more prevalent in subsurface and deep samples (60). This clonal abundance is a current enigma, because known radiolarians are typically microplankters. Some sequences could derive from small swarmers released as reproductive stages, and others from detrital or dissolved DNA. The clonal share of radiolaria is severely reduced (from 39% to 3%) when constructing rDNA and rRNA libraries from the same sample (59), highlighting the overestimation of rDNA surveys. Further research is needed to resolve the picoradiolaria enigma.

Archaeplastida: Prasinophytes

This supergroup of obliged phototrophs includes red algae, glaucophytes, and green algae plus land plants. Only prasinophytes (green algae) are important in molecular surveys, with

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6% of sequences (**Figure 4**), a value that increases to 15% in studies that deal only with picoeukaryotes (**Figure 4*c***). The picoplankters *Micromonas*, *Ostreococcus*, and *Bathycoccus* are the best represented in cultures and clone libraries albeit other clades, including new ones, are also seen in the sea (76). *Micromonas pusilla* is easily culturable and explains a large fraction of PPE cells in coastal sites (60, 80, 88). *Ostreococcus* are widely distributed and seem more abundant at the deep chlorophyll maximum (16). Strains of *Micromonas* and *Ostreococcus* with little but measurable genetic difference appear better adapted to a given condition such as light or temperature and are regarded as ecotypes of the same species (51, 68). Pigment, plastid 16S rDNA, and FISH analyses confirm the relevance of prasinophytes in coastal sites and their lower contribution offshore (88).

Picoprasinophytes provide a unique opportunity for genome projects of ecologically relevant picoeukaryotes. The genomes of two *Ostreococcus* and two *Micromonas* strains have been sequenced and compared (86) and more are in the pipeline. *Ostreococcus*, the smallest eukaryote known (0.8 μm diameter), has a small genome with 13 Mb and 8,000 genes. This miniaturized cell maintains the complete genetic set for resource acquisition and photosynthesis and exhibits a high gene density due to low gene copy numbers and intergenic reductions. *Micromonas* strains have a larger genome (20 Mb and 10,000 genes), which provides a higher ecological flexibility with more genes for nutrient transport or chemical protection. Comparative genomics reveals a core genome shared among related species and an accessory genome unique to a given strain. Unique genes seem to provide new metabolic capabilities and are often related to distant evolutionary lineages, suggesting horizontal gene transfer in eukaryotic evolution (63).

CCTH: Haptophytes, Cryptophytes, and Picobiliphytes

This newly described supergroup (9), perhaps including picobiliphytes as well, explains 6% of sequences in our dataset (**Figure 4**). Haptophytes dominate the PPE component in offshore systems as seen by HPLC pigments (42, 60), plastid rDNA (45), *psbA* transcripts (53), and FISH counts (32% of eukaryotes; 36). Their low clonal share (2%) could be explained by suboptimal PCR amplification due to high G+C content (49). Picohaptophytes belong to several novel taxa distant from coccolithophores, a group well known by its calcium carbonate plates. Their contribution to chlorophyll *a* stock is ~25%–50% globally, among the largest for a single algal group (18, 49). A key factor for the ecological success of these tiny algae could be their phagotrophic capacity. A metagenomic survey based on flow cytometric sorted populations provides initial data about their gene content, genome structure, and ecological adaptations (18).

Cryptophytes are marine algae easily identifiable by epifluorescence microscopy by their chloroplasts with phycobilins. They are relatively abundant in coastal systems and less so offshore (45, 60). Picobiliphytes represent one of the deepest evolutionary branches without a cultured member (61). They were initially described as phycobilin-containing algae, but could also be heterotrophs (33). Microscopic observations of cryptophytes and picobiliphytes indicate that they are slightly larger than 3 μm (17), so the signal detected is probably due to small nanoplankters squeezing through the filters.

Excavates, Opisthokonts, and Amoebozoans

These supergroups of generally heterotrophic eukaryotes are poorly represented in marine surveys (**Figure 4**). The large excavate radiation of anaerobic symbionts is not detected. The second excavate radiation includes well-known heterotrophic flagellates such as kinetoplastids and diplonemids and accounts for 1.4% of sequences. These could explain a fraction of HPE cells. Opisthokonta unite metazoans and fungi with protists such as choanoflagellates. Choanoflagellates are small (3–10 μm)

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heterotrophic flagellates with a characteristic morphology that are detected at moderate abundances in marine surveys (1% of sequences) and often form new clades (19). Because they are the closest living relatives of metazoans, they have attracted the interest of evolutionary biologists to investigate the transition to multicellularity (41). Fungi, so prevalent in freshwater molecular surveys (43), are poorly represented in our database (<0.5% of sequences) and are not treated further because they are not protists. Finally, Amoebozoa, including naked lobose amoebae (10–20 μm) that can be important grazers of surface-attached bacteria, are poorly represented.

Operational taxonomic unit (OTU): a group of environmental sequences sharing a given similarity level required for quantitative and comparative sequence analyses

Overview of the Numerically Dominant Taxonomic Groups

Which phylogenetic groups dominate marine picoeukaryote assemblages? The PPE pool can be targeted specifically by analyzing pigments, photosynthetic genes, or cytometrically sorted populations. The consensus is that haptophytes, pelagophytes, and chrysophytes (in this order) dominate in offshore waters. These groups are still present in coastal sites but prasinophytes dominate (42, 45, 49, 53, 60, 76). This general trend is confirmed by FISH counts (36, 88). The HPE pool is more difficult to target, because they do not share a common marker. Using FISH, MAST contribute to ~20% of cells (56), and MALV-II contributes a lower share (77). The remaining HPE likely belong to unprobed MAST or MALV cells together with cercomonads, chrysophytes (a group with PPE and HPE), choanoflagellates, and kinetoplastids. HPE assemblages can also be addressed by single cell sequencing after flow cytometry cell sorting. Thus, 23% of heterotrophic flagellates in a coastal sample belong to MALV, 19% to cercomonads, 15% to MAST, and 15% to picobiliphytes (33). After identifying the main taxonomic groups of marine picoeukaryotes, the next challenge is determining the number and identity of lower-rank taxa (i.e., how many species and which ones). Two properties emerge: Groups are diverse and contain novel clades. With an estimated number of $\sim 10^{26}$ picoeukaryote cells in surface oceans, representing one of the largest eukaryotic pools on Earth, it is critical to understand how they organize into species and ecological functions.

SEQUENCES, POPULATIONS, AND COMMUNITIES

Crunching Sequence Datasets: Estimating Diversity and Novelty

Sequences generated during molecular surveys are best analyzed by grouping similar sequences into operational taxonomic units (OTUs), easily done with convenient software packages (74). After OTU clustering, the extent of microbial diversity can be quantified. Thus, our dataset of 8,719 sequences from clone libraries yields 6,913 OTUs clustering at 100% and 3,895 OTUs clustering at 99%. Rarefaction curves (OTUs observed with respect to sequences analyzed) seldom saturate, and the number of OTUs estimated is always higher than the number of OTUs observed (22). Thus, it seems clear that standard clone libraries are far from fully capturing picoeukaryote diversity. Clone libraries using group-specific primers (49) or high-throughput sequencing (13) typically enlarge the diversity observed. The latter study found ~30,000 OTUs (at the 100% level) and ~4,000 OTUs (at 98%) and no saturation. High-throughput tag studies are still in the phase of curating sequencing errors potentially increasing the diversity (34) and are expected to provide, for the first time, an upper bound to the number of microbial OTUs.

A second step in sequence analysis is to classify OTUs in taxonomic groups (including novel clades). The web applications GreenGenes (**http://greengenes.lbl.gov/**) and RDP (**http://rdp.cme.msu.edu/**) classify automatically prokaryote sequences but are not operational for eukaryotes, which in the meantime can be semiautomatically classified by KeyDNAtools (31). Then the diversity within each taxonomic group can be analyzed separately. For instance, in our dataset each group

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contributes to the OTU number in proportion to its relative clonal abundance (**Figure 4*d***). In addition, the group novelty can be reported by comparing the closest environmental and cultured BLAST matches of its sequences (19). This points to groups requiring more culturing and/or sequencing effort. Finally, sequences that do not belong to any taxonomic group could represent novel evolutionary lineages and thus deserve more attention.

Translating OTUs into a Taxonomic Rank

It is desirable to determine the taxonomic rank of defined OTUs and in particular the clustering level that corresponds to species, the basic unit of diversity. Defining species is often a controversial issue, and it is even more problematic among microbe-sized organisms (73). The biological species concept, grouping organisms by sexual interbreeding, applies to some diatoms or dinoflagellates and reveals that strains need 100% rDNA identity to be sexually compatible (4). This refers to the dominant rDNA sequence within a strain. Although intragenomic rDNA copies are not always identical, they are homogenized by concerted evolution, so the few variants are usually >99.5% similar to the dominant sequence (3). Little is known about sex in picoeukaryotes, but some hints suggest it may exist. On the one hand, picoeukaryote genomes reveal the complete suite of meiosis genes (41, 86), and recombination between *Ostreococcus* strains has been detected, although the estimated frequency of sexual divisions was low (30). On the other hand, if picoeukaryotes were fully asexual, they could speciate as prokaryotes. Under the ecological species concept, organisms from the same species occupying the same niche accumulate neutral mutations until one genotype outcompetes the rest due to an adaptive mutation (15). Neutral mutations with periodic purging events could explain the microdiversity found in prokaryotic surveys (1). In fact, such microdiverse clusters are also detected in picoeukaryote surveys, with ~50% of rDNA sequences closely related (>99% similarity) but not identical.

What clustering level should be used to group sequences from the same species? If picoeukaryotes had sex and considering the diatom interbreeding experiments (4), the advisable level would be 100%. So, they could engage in a sexual event periodically, perhaps triggered by environmental cues. If they were asexual, microdiverse clusters of highly related sequences could appear, and each individual variant would not correspond to a different species. However, the microdiversity detected can also be caused by other biological or methodological factors. So, clustering at 99% similarity seems to be a good compromise. This gives a conservative species number (some species will be lumped together) and avoids considering microdiversity, rDNA intragenomic variability, and PCR or sequencing errors.

Microbial biogeography: study of the distribution of microbial species with a special focus on the existence of geographic barriers and endemic species

Population Ecology and Biogeography

The species distributional range of microbes is a controversial topic. In one view, the small body size of microbial taxa implies huge population sizes and ubiquitous dispersal, leading to cosmopolitan distributions and a low number of species globally (25). The opposite view accepts cosmopolitan species but claims the existence of endemic ones, leading to a much higher microbial diversity (26). Molecular surveys provide an objective way to assess microbial biogeography and offer good arguments for global distribution of marine microbes, as exemplified by MAST-4 cells (**Figure 5**). This picoeukaryote appears in all samples and accounts for ~100 cells ml^{-1} and ~13% of heterotrophic flagellates globally (**Figure 5*a***). MAST-4 sequences form five clades that exhibit a high internal similarity (**Figure 5*b***) and a pan-oceanic distribution (**Figure 5*c***). Thus, at the 18S rDNA level, there do not seem to be geographical barriers to the distribution of marine picoeukaryotes. More variable markers indeed highlight locally restricted populations within a cosmopolitan rDNA type (70). Another relevant finding of

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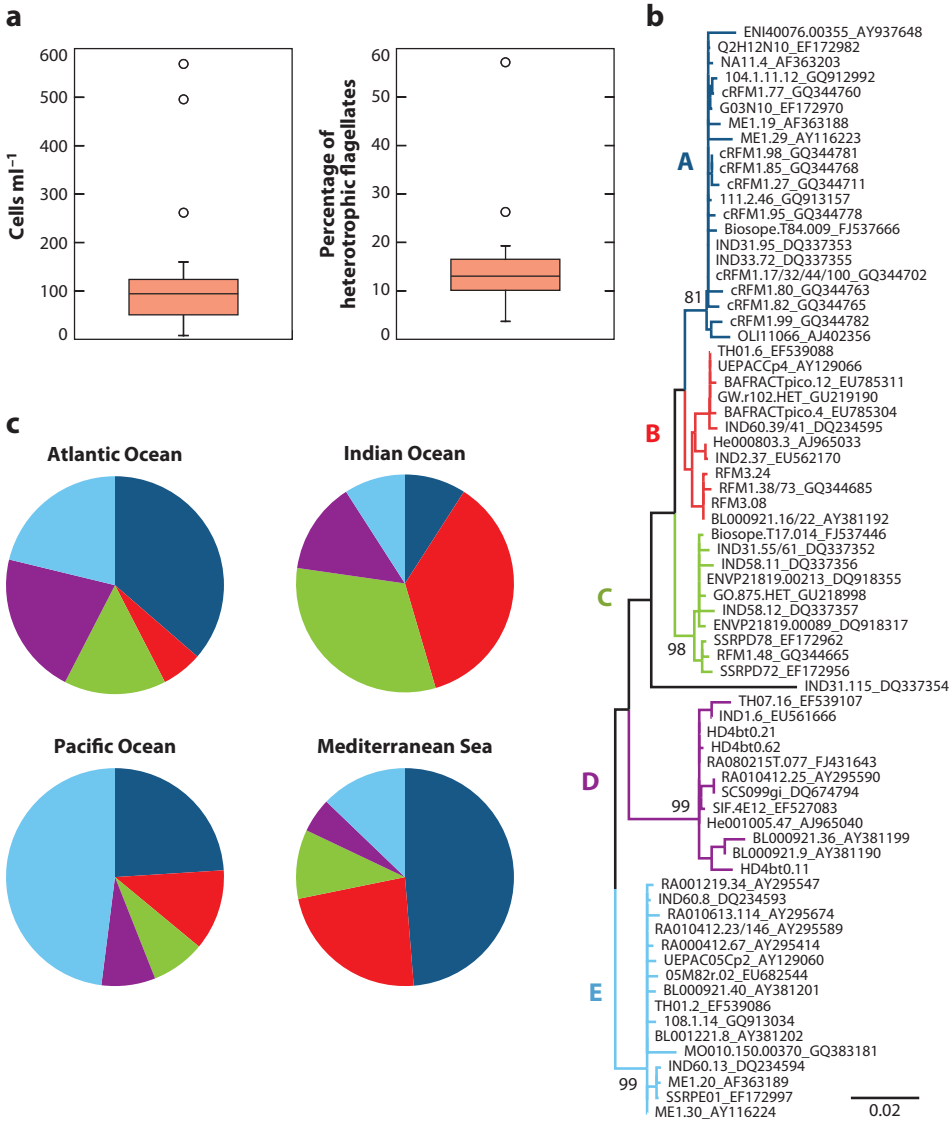

Figure 5

Abundance, phylogeny, and global distribution of the MAST-4 picoeukaryote. (*a*) Whisker plots of cell abundance ($n = 20$) and percentage of heterotrophic flagellates ($n = 17$) in marine sites worldwide. (*b*) Maximum likelihood phylogenetic tree showing five main clades and their bootstrap values (A, *dark blue*; B, *red*; C, *green*; D, *purple*; E, *light blue*). (*c*) Clade affiliation of sequences from the Atlantic Ocean ($n = 33$), Indian Ocean ($n = 22$), Pacific Ocean ($n = 25$), and Mediterranean Sea ($n = 39$). Data from R. Rodríguez-Martínez (unpublished data) and Reference 56.

molecular surveys is that the global distribution does not translate to low diversity, because high OTU numbers are estimated from still undersaturated clone libraries.

Determining the factors controlling the abundance and ecological role of each species is the next critical issue, a titanic task given the large diversity seen. This can be done by

correlating abundance with environmental features or by performing experiments. For instance, the bacterivorous MAST-4 exhibits trophic specialization with respect to other MAST (57). Moreover, it is absent in polar regions, so cold temperatures might set a limit to their ecological success. Similar functional surveys evaluate other picoeukaryotes, such as prasinophytes (16, 51, 68, 86) or picohaptophytes (18, 36, 49). Another important question is whether the five MAST-4 clades are functionally redundant, or whether they exhibit slight ecological differences in their temperature optima, food spectra, or viral susceptibility. A main challenge in microbial ecology is determining the diversity level needed to explain ecosystem functioning.

Community Structure

Oceans, sometimes perceived as homogeneous systems, are composed of distinct water masses that move at different spatial and temporal scales and experience additional external forcing (nutrient inputs, mixing). Picoeukaryote communities are similar in sites with similar oceanographic properties (albeit distant), whereas they change across oceanographic barriers (32). In a given site, sharp vertical stratification and temporal changes are the norm. These studies are limited by sampling oceans at relevant scales, since cruises cover only a snapshot of spatial and temporal variability. Another limitation is that the fingerprinting techniques typically used recover only the most abundant taxa, a concern that will soon be solved by high-throughput sequencing. Further, metagenomics would allow researchers to go beyond the single-gene approach and would provide picoeukaryote functional profiles for comparative purposes.

Microbial surveys show rank abundance curves characterized by a few abundant and many rare taxa, following a well-known community ecology pattern (52). The long tail of rare taxa in molecular microbial surveys, perhaps larger than that in macroorganism studies, has been named rare biosphere and explained by combining evolutionary (high dispersal and low extinction rates) and ecological (feast and famine survival) forces. The rare biosphere could be regarded as a seed bank of less competitive functionally redundant microbes, which may provide an insurance to maintain biogeochemical processes in the face of ecosystem change (10). However, this view derives from prokaryote studies and it is not clear if eukaryotes have similar survival strategies. A deeper sequencing effort (together with careful phylogenetic analysis) is required to determine the extent of the protist rare biosphere.

Rare biosphere: collection of a huge number of low abundant taxa that characterize microbial communities when studied by molecular surveys

Many ecologists claim that a system cannot be understood by studying the parts separately and then putting them together, since important emerging properties appear only with an integral study of the system (48). This bulk approach has been foundational in microbial ecology, by first treating all microbes in a black box and then opening this single box into smaller ones. Recognizing the success of this approach, microbial ecologists are also taking the inverse path, studying populations in detail to link critical functions (including novel mechanisms) to given taxa and to obtain better physiological models. Combining both approaches will surely provide better insights into the evolutionary and ecological significance of marine picoeukaryotes.

SUMMARY POINTS

1. Picoeukaryotes include phototrophic and heterotrophic protists 1–3 μm in size populating surface oceans everywhere, with an estimated global abundance of 10^{26} cells. They contribute significantly to primary production, bacterivory, and parasitism.

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2. Despite being morphologically similar, picoeukaryotes include different organisms. The large diversity detected at all phylogenetic scales is accompanied by the discovery of novel groups, such as MAST, MALV, and picobiliphytes.
3. Phototrophic cells affiliate mostly with haptophytes, chrysophytes, and pelagophytes in the open sea, and with prasinophytes at the coast. Less is known for heterotrophic cells, which may be dominated by MAST, MALV, cercomonads, and chrysophytes.
4. There is no evidence of dispersal barriers in surface oceans, so picoeukaryotes appear globally distributed and constrained by environmental filtering. Therefore, communities appear similarly organized in similar environmental conditions.
5. Picoeukaryote diversity is currently underestimated mostly due to the rare biosphere, which can be partially explained by intragenomic variability and methodological errors.

FUTURE ISSUES

1. There is a need to fully characterize picoeukaryote diversity by high-throughput tag sequencing and -omic approaches. Environmental sequences should be classified into taxonomic groups to assess intragroup diversity and identify novel groups.
2. It is important to understand which degree of environmental sequence variation is evolutionary and ecologically relevant by linking that variation to cells with ecological roles. The final aim is to establish the diversity levels important for ecosystem functions.
3. More attention should be paid to the factors controlling the abundance of particular populations and the rules of community assembly. This should be studied at different spatial and temporal scales.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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6. One of the first efforts to delineate the eukaryotic tree of life into a few supergroups by using multigene phylogeny.

12. Identifies the uncultured MALV-II cells as parasites of dinoflagellates that control host population dynamics and drive relatively fast species substitutions.

20. Provides one of the first molecular surveys of surface marine picoeukaryotes based on rDNA partial sequences, showing high and novel diversity in three distant regions.

30. Infers the existence of infrequent sexual events among *Ostreococcus* strains by analyzing genetic markers of sexual recombination.

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