

# Marine Protistan Diversity

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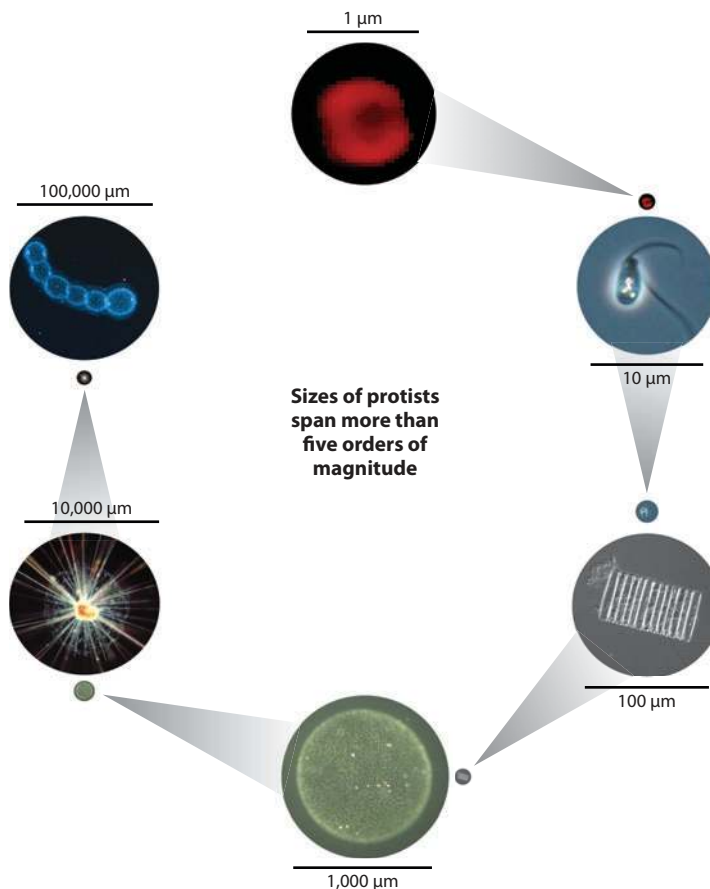
## Abstract

Protists have fascinated microbiologists since their discovery nearly 350 years ago. These single-celled, eukaryotic species span an incredible range of sizes, forms, and functions and, despite their generally diminutive size, constitute much of the genetic diversity within the domain Eukarya. Protists in marine ecosystems play fundamental ecological roles as primary producers, consumers, decomposers, and trophic links in aquatic food webs. Much of our knowledge regarding the diversity and ecological activities of these species has been obtained during the past half century, and only within the past few decades have hypotheses depicting the evolutionary relationships among the major clades of protists attained some degree of consensus. This recent progress is attributable to the development of genetic approaches, which have revealed an unexpectedly large diversity of protists, including cryptic species and previously undescribed clades of protists. New genetic tools now exist for identifying protistan species of interest and for reexamining long-standing debates regarding the biogeography of protists. Studies of protistan diversity provide insight regarding how species richness and community composition contribute to ecosystem function. These activities support the development of predictive models that describe how microbial communities will respond to natural or anthropogenically mediated changes in environmental conditions.

## INTRODUCTION

### Historical Aspects of Protistan Biodiversity

The term protist refers to a diverse array of eukaryotic organisms capable of existence as single cells, although many species form colonies ranging from a few to many cells (**Figure 1**). Eukaryotes have been defined traditionally using morphological criteria, the presence of a membrane-bound nucleus, and the presence of membrane-bound vesicles (most notably chloroplasts in photosynthetic cells and mitochondria). The precise origins of eukaryotes are still unresolved, but putative eukaryotic fossil structures date back to the Proterozoic Era approximately 1.3 to 1.8 billion years



**Figure 1**

The tremendous size range of single-celled eukaryotes. The smallest known protist (a species of the prasinophyte genus *Ostreococcus*) is less than 1 μm in size, whereas the largest noncolonial cells are more than 1 cm in diameter, and colonies of colonial radiolaria can form gelatinous structures that can attain a width of >1 cm and lengths of >1 m. Most species, however, are microscopic or nearly so. Taxa from top, clockwise: the prasinophyte *Ostreococcus tauri* (image is an epifluorescence micrograph showing chlorophyll autofluorescence); *Bodo* sp., a heterotrophic flagellate; a small colony of the diatom *Fragilariopsis* sp.; a colony of the haptophyte *Phaeocystis antarctica*; the planktonic foraminiferan *Hastigerina pelagica*; and a colonial radiolarian (individual central capsules are 100 μm in diameter and too small to see in this photograph).

ago, and chemical signatures of apparent eukaryotes dating back beyond 2 billion years have been proposed (Javaux 2007, Knoll 1992, Knoll et al. 2006, Roger & Hug 2006).

A clear understanding of the magnitude and complexity of protistan diversity was delayed until the latter part of the twentieth century in part because protists were organized into groups based on gross morphology and their various modes of nutrition. Unique approaches and characters were then adopted for taxonomic schemes for these different groups of protists, which often separated phylogenetically related taxa. For example, taxonomic frameworks were constructed independently for photosynthetic taxa (aka microscopic algae or phytoplankton) and heterotrophic protists (aka protozoa). This approach had its roots in the derivation of protistology from botanists and zoologists. Indeed, the first classifications of heterotrophic forms (the Protozoa) in the 1880s did not include photosynthetic forms at all (Bütschli 1880–1889), and the tendency to consider heterotrophic protists separate from the photosynthetic ones persisted for more than a century. Morphological and especially ultrastructural information collected during the late twentieth century, however, clearly indicated phylogenetic links between many chloroplast-bearing and heterotrophic forms.

Early taxonomic schemes also grouped heterotrophic protists into amoeboid forms (species possessing a pseudopod), ciliated forms, and flagellated forms, whereas phototrophic protists were grouped by chlorophyll and accessory pigments types as well as gross morphological characters such as the type and movement of the flagella or the presence of skeletal structures such as a frustule in diatoms. Some of these characterizations were later supported as monophyletic groupings (e.g., ciliated protists, diatoms), but other morphology-based groupings combined taxa that are now believed to be distantly related (e.g., radiolaria, centrohelid heliozoa, and many amoebae were formerly grouped together) or separated taxa that are now believed to have shared significant evolutionary history (e.g., ciliates, dinoflagellates, and apicomplexans are now grouped together) (Adl et al. 2005, Baldauf 2008, Burki et al. 2007, Lane & Archibald 2008).

Phylogenies of single-celled eukaryotes based on plant-like and animal-like nutrition and on gross features of morphology and motility lasted through the latter part of the 1980s, despite mounting information to refute such characterizations. The last of these schemes based on these simplest characteristics (eukaryotic and capable of existence as single cells), placed single-celled eukaryotes into the kingdom Protista, as a transitional evolutionary group between the Monera (prokaryotes) on the one hand and the plant, animal, and fungal kingdoms on the other (Whittaker 1969). However, Protista has been recognized as a pragmatic historical construct rather than a hypothesis regarding the phylogeny of these taxa. This recognition, together with ultrastructural information and rapidly expanding molecular phylogenetic studies, eventually gave way to a rejection of traditional phylogenetic schemes for single-celled eukaryotes. That deconstruction during the 1980s has been followed by a period of reconstruction that has attempted to reconcile ultrastructural information and the continually increasing amount of DNA sequence information that has become available (reviewed in Patterson 2000).

## **CHANGING TIMES, CHANGING PHYLOGENIES: A MODERN OVERVIEW OF PROTISTS**

Classical methods for examining protistan phylogeny and diversity have been augmented tremendously in recent years with genetic information and approaches. Not all aspects of eukaryote phylogeny are well supported or completely accepted, but some of the problems noted above that were present in previous phylogenetic schemes have been addressed (e.g., grouping of photosynthetic and heterotrophic sister taxa), while many remaining unresolved issues are areas of active research at this time.

Protistan taxa are now intermingled with multicellular forms in the domain Eukarya (or Eucarya) according to modern phylogenetic schemes proposed since Woese et al. (1990) redefined the domains of life. There have been several specific schemes proposed for the organization of the major eukaryotic lineages within this domain during the past several years, and many of the higher-level relationships continue to be debated (Baldauf 2008, Burki et al. 2007, Lane & Archibald 2008, Simpson & Roger 2004, Tekle et al. 2009). General consensus has been emerging for six major supergroups within the domain (Adl et al. 2005 Baldauf 2008, Burki et al. 2007, Lane & Archibald 2008, Simpson & Roger 2004), but even the structure of these groups as well as a number (potentially many) of minor issues involving protistan phylogeny are still unsettled (Baurain et al. 2010, Burki et al. 2008, Tekle et al. 2009). The present supergroups include Whittaker's kingdoms of animals, plants, and fungi as minor branches among the eukaryotes, recognizing that (*a*) these former kingdoms have close phylogenetic affinities to specific lineages of unicellular eukaryotes (e.g., choanoflagellates to animals), and (*b*) unicellular eukaryotes (i.e., protists) constitute most of the evolutionary diversity within the domain (**Figure 2**). In fact, the tremendous diversity represented by eukaryotes capable of existence as single cells has led some evolutionists to define protists as eukaryotic organisms that are not true fungi, plants, or animals.

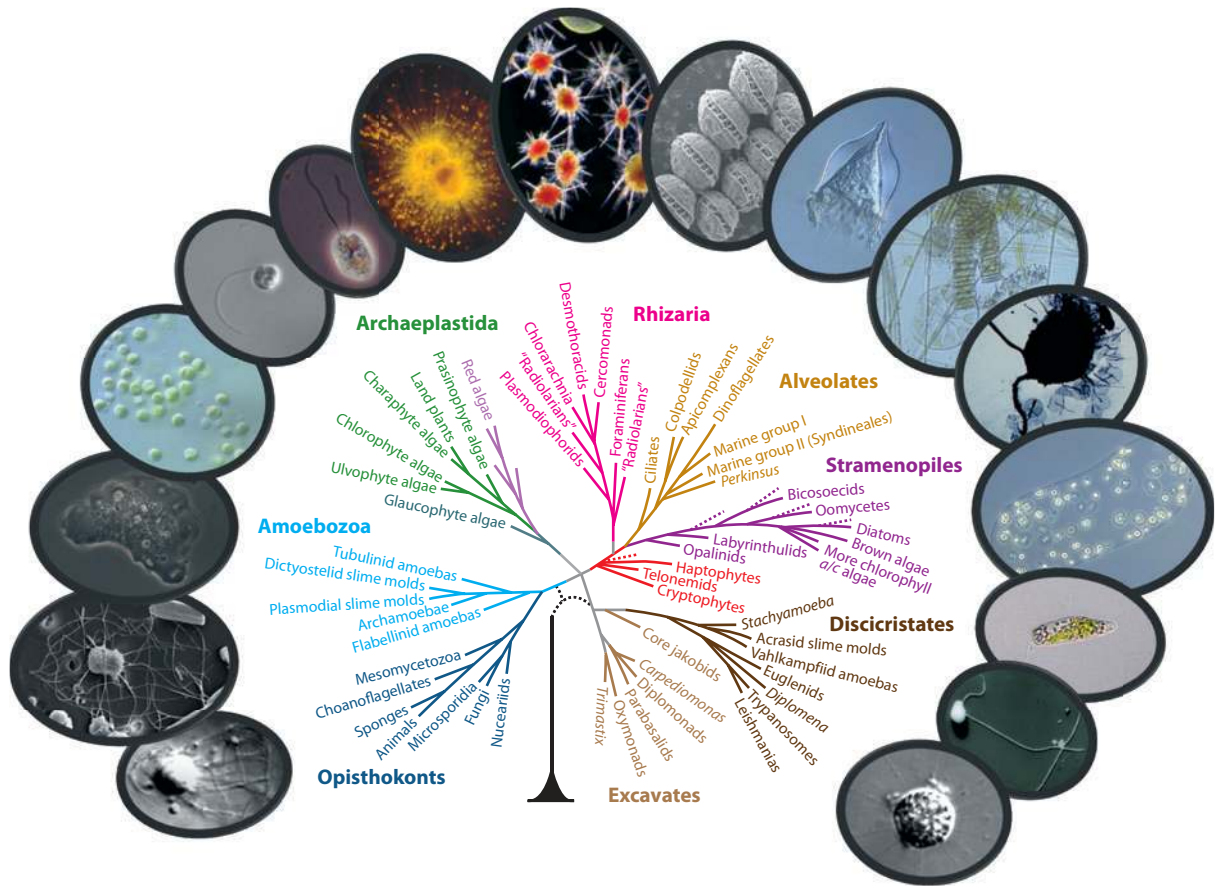
Major revisions in the classification of protists resulting from the incorporation of DNA sequence information have included the consolidation of some photosynthetic and heterotrophic forms that clearly share recent ancestry but differ in their modes of nutrition (e.g., the chloroplast-bearing and heterotrophic chrysophytes) (Cavalier-Smith & Chao 2006), and the separation of some amoeboid forms into several different lineages. The phylogenies of small, heterotrophic flagellates have also undergone significant reorganization as a result of genetic information that has provided new insight into their evolutionary affinities (or lack of affinities) to one another and to other eukaryotes that were previously not apparent from morphological characters alone (Howe et al. 2011, Patterson 1999). A recent phylogenetic scheme proposed for the supergroups of eukaryotic taxa, and the presence of well-known, free-living, marine taxa in those supergroups, is outlined below and in **Figure 2**.

## Opisthokonta

The opisthokonts are a supergroup that is most notable in that it now contains two of the kingdoms of multicellular eukaryotes from the Whittaker (1969) five-kingdom system: the animals and the fungi. This group also contains the nuclearioid amoebae and a clade of small, free-living, heterotrophic flagellates, the choanoflagellates. Choanoflagellates are common in the marine plankton, where they can be important bacterivorous species, particularly in polar ecosystems (Marchant 1985, Thronsen 1970). The choanoflagellates have historically been believed to represent an evolutionary link to the animals in part because of the gross morphological similarity that these species share with the choanocytes (collar cells) of sponges. Indeed, modern molecular phylogenies support this relationship (King et al. 2008). The taxa within the opisthokont supergroup share the morphological feature of a single posterior flagellum on motile cell stages.

## Amoebozoa

Amoebozoans include many of the amoeboid taxa that are extremely common in marine benthic communities, but these species also occur at low abundances on particulate material in the plankton (Butler & Rogerson 1995, Moran et al. 2007). The common lobose amoebae include an array of free-living taxa that have highly flexible cells and use pseudopodia for motility on surfaces and for feeding via phagocytosis. The free-living amoebae were formerly grouped with species of Rhizaria



**Figure 2**

Phylogenetic breadth among protists. Single-celled species (protists) occur in every supergroup within the domain Eukarya, and constitute the entirety of a number of them. Here, a recent phylogenetic scheme by Baldauf (2008), following similar schemes by Adl et al. (2005) and Simpson & Roger (2004), is populated with micrographs of common marine taxa. The pictures depict (clockwise from lower left): two micrographs of choanoflagellates, a free-living lobose amoeba, minute chlorophyte algae, the prasinophyte *Pyramimonas*, the heterotrophic cercozoan flagellate *Cryptobecomonas*, the planktonic foraminiferan *Orbulina*, a mixed natural assemblage of *Acantharia*, the photosynthetic dinoflagellate *Alexandrium*, a tintinnid ciliate, a mixed diatom assemblage, the heterotrophic chrysomonad *Paraphysomonas*, the colonial haptophyte *Phaeocystis*, the euglenid flagellate *Eutreptiella*, a heterotrophic bodonid flagellate, and a heliozoan. *Alexandrium* photo by Carmelo Tomas.

(e.g., foraminifera, radiolaria, acantharia) and the heliozoa based on the presence of pseudopodia in all forms. Molecular phylogenies have separated these taxa (Pawlowski & Burki 2009), and have indicated an affinity of the lobose amoebae with the slime molds common in terrestrial soils as well as the parasitic entamoebae.

## Archaeplastida (Plantae)

As the name implies, this supergroup includes the remaining former multicellular kingdom of the Whittaker scheme, the plants. It also includes ecologically important taxa of the chlorophyte algae. These forms include the macroscopic, multicellular green algae, but also many single-celled and

colonial taxa that are common constituents of marine and freshwater phytoplankton assemblages. Recent microscopical and molecular surveys have reemphasized the diversity and importance of minute chlorophytes in oceanic ecosystems, particularly among the prasinophytes (Guillou et al. 2004, Worden 2006). The red algae are also included in the Archaeplastida supergroup, but there are relatively few single-celled taxa in this clade. These forms are most commonly recognized as multicellular benthic species of neritic ecosystems.

## Chromalveolata

Most molecular phylogenetic analyses now recognize this large, overarching supergroup as the union of two large, related clades, Alveolata and Stramenopila. These lineages constitute a wide variety of extremely conspicuous and ecologically important taxa. The terms Alveolata and Stramenopila refer to morphological (ultrastructural) features of the cells that constitute these groups, and point to the deconstruction and reconstruction of protistan phylogeny that began with the advent of morphological detail provided by electron microscopy before the emergence of DNA sequence information. Alveoli are membrane-bound cavities on the inner surface of the plasma membranes of the cells of these protists. Stramenopiles (or heterokonts) possess two different types of flagella, with one flagellum bearing mastigonemes that have a tripartite structure.

The alveolates include two clades that are extremely well represented in marine ecosystems—the ciliates and the dinoflagellates—in addition to parasitic forms within the apicomplexans that appear sporadically in gene surveys. Additionally, the recent discovery of DNA sequences in environmental samples that have strong affinities to the dinoflagellates but appear to represent novel clades has raised questions regarding the completeness of our knowledge of alveolate diversity (López-García et al. 2001), and led to speculation on the ecological role of these uncultured novel marine alveolate clades (Moreira & López-García 2003). Recent molecular phylogenies place some of these sequences within the dinoflagellate order Syndiniales, which contains a variety of parasitic forms, strengthening the speculation that many of these uncultured forms may be parasites of other marine protists or animals (Guillou et al. 2008).

The dinoflagellate taxa that have been identified by traditional approaches of microscopy and culture, and the ciliates, include an enormous diversity of forms and functions. The speciose nature of these groups and their range of trophies have been well documented historically using microscopy and culture techniques. In addition, the exceptionally large DNA content present in many of these species has resulted in strong representation of these taxa within recent gene surveys of environmental samples.

The stramenopiles within the chromalveolates include several groups of successful marine protists, including the diatoms, brown algae (including the seaweeds, which can represent some of the largest organisms on the planet), golden algae, and bicosoecids. The stramenopiles also include the oomycetes, or water molds, that can be abundant in coastal locations such as mangroves but are not strongly represented in oceanic ecosystems. Highly speciose and ecologically important, the diatoms have a dominant, nonflagellated life stage possessing a siliceous frustule that encloses the cell and is a diagnostic character for this group. The golden algae are common marine (and freshwater) photosynthetic protists, although many of these species are also phagotrophic (Sanders & Porter 1988), and there are numerous heterotrophic forms that are important bacterivores in the plankton and sediments. Bicosoecids are small, heterotrophic forms that are particularly common in the benthos and attached to surfaces.

Recent gene surveys have revealed several new lineages of previously undetected marine stramenopiles (or MAST cells) (Massana et al. 2004) in marine samples. These cells have escaped detection until recently, presumably because they are small, heterotrophic species that possess



relatively few morphologically distinctive features. At least some of these taxa appear to feed on bacteria (Massana et al. 2009).

## Rhizaria

The Rhizaria supergroup is a relatively new phylogenetic construct that includes organisms ranging from minute heterotrophic and photosynthetic forms to some of the largest and most conspicuous solitary and colonial protistan taxa (Moreira et al. 2007, Pawlowski & Burki 2009). The radiolaria, foraminifera, and acantharia produce intricate skeletal structures of silica, calcium carbonate, and strontium sulfate, respectively, which have fascinated biologists since they were the subject of scientist/artist Ernst Haeckel in the nineteenth century. These latter three groups, together with the amoebae, testacea, and heliozoa, were placed into the same group (the sarcodine protozoa) many years ago based on the presence of some form of pseudopod in these taxa. This grouping was practical rather than phylogenetically based, and it has been reexamined as molecular phylogenies have indicated that the amoebae and heliozoa appear to be more closely related to other protistan lineages. Recent molecular analyses indicate that the radiolaria, foraminifera, and acantharia appear to share evolutionary history with the cercozoans (Howe et al. 2011, Karpov et al. 2006), a group of mostly small heterotrophic flagellates that are widely observed in marine ecosystems, and a rather obscure photosynthetic lineage of tiny cells (chlorarachniophytes). Unique insertions within the polyubiquitin protein of these species also indicate a shared phylogeny (Bass et al. 2005).

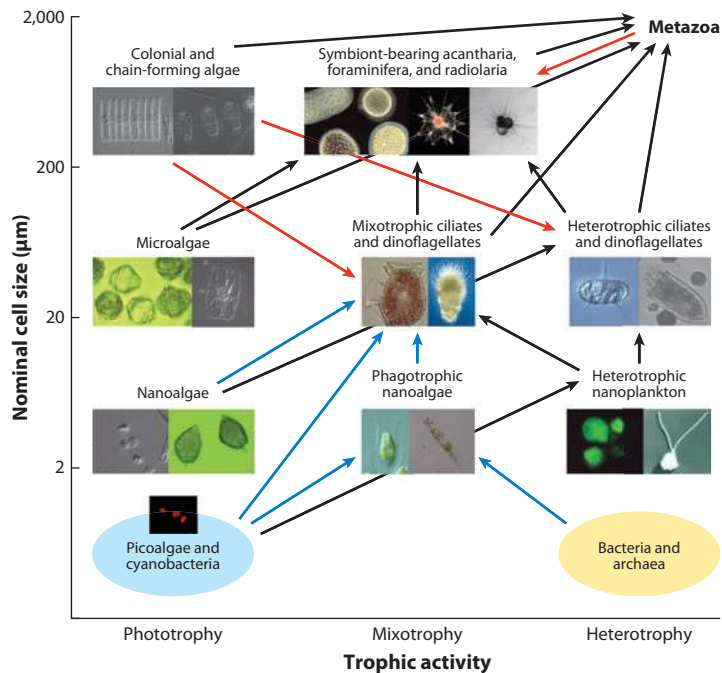
## Excavata (and Discicristates)

Many of these species are parasitic in animals, and the phylogenetic relationships among some candidate groups are still debated (Hampl et al. 2009, Rodríguez-Ezpeleta et al. 2007), but a few clades placed here are well represented as free-living taxa of marine ecosystems. The euglenozoa include protistan species that are photosynthetic, heterotrophic, or mixotrophic flagellates. They are common in shallow planktonic and benthic ecosystems, but also appear to inhabit some deep-sea sediments (Buck et al. 2000). The excavates also include some small, heterotrophic flagellate groups such as the bodonids and jakobids. The latter species can be important bacterivores in planktonic and benthic ecosystems.

## INVESTIGATING PROTISTAN DIVERSITY

### Why Study Protistan Diversity?

Understanding the breadth of biological diversity on our planet, the evolutionary processes that give rise to that diversity, and the manner in which communities of organisms create and maintain ecosystem function and biogeochemical cycles are fundamental goals of biological study. Protists are abundantly and nearly ubiquitously distributed on Earth and thus are an obvious focus for this work. Their countless forms, sizes, and trophic activities constitute a continuum of species ranging from bacterial-sized cells for the smallest known species of chlorophytes to meters in length for the largest colonies of radiolaria (**Figure 1**). The enormous size range of protists, their many nutritional modes, and their rapid metabolic rates result in these species playing pivotal ecological roles as primary producers and consumers at and near the base of marine food webs (**Figure 3**). Modern depictions of protists indicate their presence in (and dominance of) the first several trophic links in marine food webs.



**Figure 3**

A typical box model of organic carbon flow within a planktonic marine food chain, indicating the multiple ecological roles played by protists. Protists play dominant roles in the production and utilization of organic material at the base of marine food webs. Protists in food web models are generally grouped by size (*y axis*) at roughly order-of-magnitude scales, with the assumption that prey are consumed by predators approximately one order of magnitude larger than themselves. They are also grouped by the nutritional modes (*x axis*) of phototrophy, heterotrophy, and mixotrophy (a mixture of phototrophic and heterotrophic growth). Arrows depict trophic interactions: black arrows indicate the direction of carbon flow during the consumption of prey by heterotrophic species, blue arrows indicate carbon flow during the consumption of prey by mixotrophic species, and red arrows indicate the direction of carbon flow when larger prey are consumed by smaller predators.

Roughly half of global primary productivity occurs in the ocean, and a large fraction of that productivity is mediated by photosynthetic protists (Field et al. 1998). Protists are particularly important primary producers in coastal environments and other highly productive ecosystems, where they form the base of food chains that support the world's most important fisheries. Primary production by protists can enter marine food webs at several size categories of herbivores because of the wide size range of photosynthetic protists (left side of **Figure 3**).

Phagotrophic protists dominate the first few trophic levels in pelagic marine food webs and contribute significantly in many benthic ecosystems (right side of **Figure 3**). These species are consumers of bacteria, cyanobacteria, other protists, viruses, and some metazoa. The quantitative importance of phagotrophic protists as consumers of primary production in the ocean has been recognized for decades (Sherr & Sherr 1994), but this fact is still underappreciated because traditional biases of the classical marine food chain (phytoplankton–copepod–fish) still pervade common thought on energy and carbon flow in the ocean. In actuality, copepod predation on heterotrophic dinoflagellates and ciliates constitutes a more important trophic link than the phytoplankton–copepod link in many situations (Calbet & Saiz 2005). Phagotrophic protists are the primary trophic link between minute cyanobacterial and bacterial production and higher



organisms, a realization formalized more than 35 years ago in the microbial food web concept of Pomeroy (1974).

Additionally, some phagotrophic protistan taxa—most notably some heterotrophic dinoflagellates, ciliates, radiolaria, and foraminifera—possess specialized structures for feeding such as tentacles, peduncles, feeding veils, or pseudopodial networks that enable them to consume prey that are larger than themselves (Caron & Swanberg 1990, Jacobson 1999, Sherr & Sherr 2007). Thus, carbon flow through marine food webs is not always toward larger organisms. The amount of photosynthetic production by large chain-forming diatoms that is consumed by smaller heterotrophic dinoflagellates in the ocean is almost certainly underestimated at this time (Sherr & Sherr 2007).

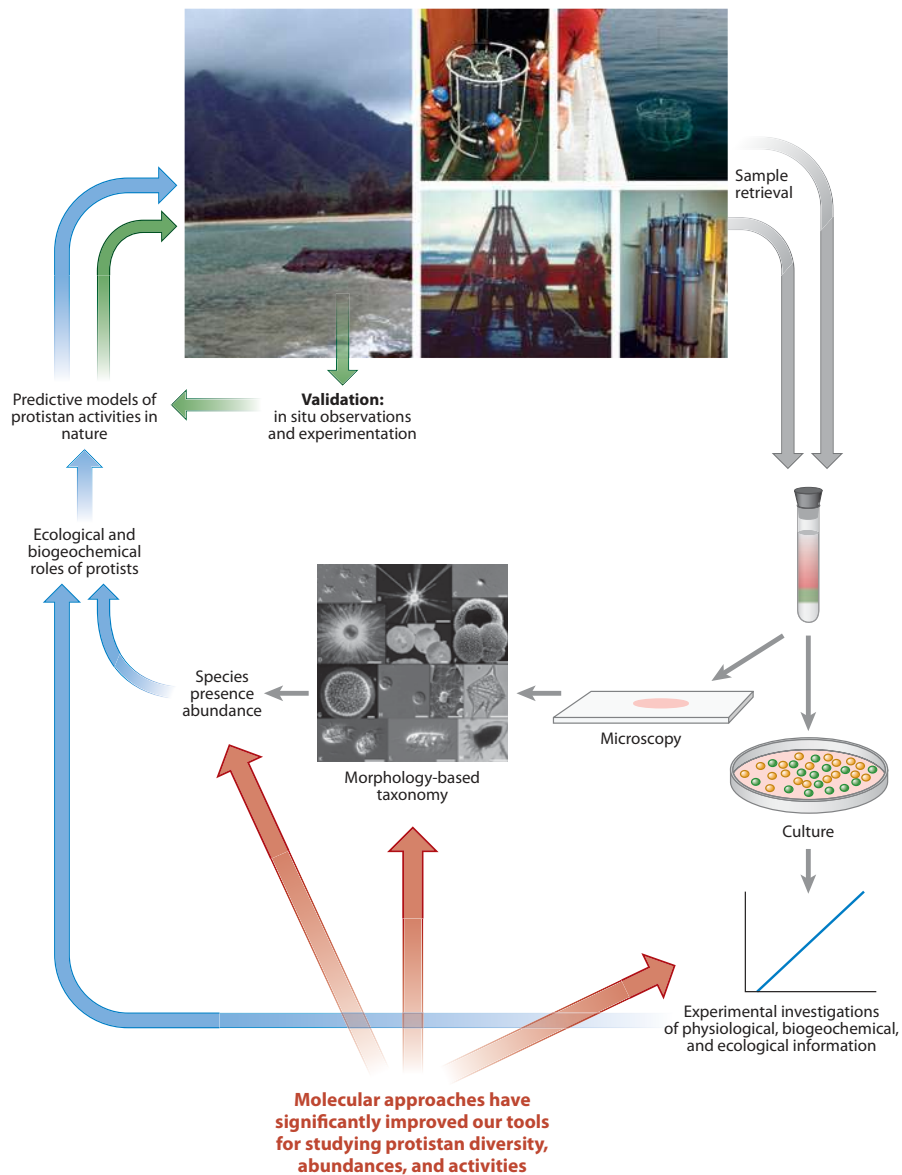
Mixotrophic behavior—the combination of photosynthetic and heterotrophic nutrition in the same cell (central column in **Figure 3**)—also complicates the description of food web structure. Models of carbon and energy flow typically depict organisms as purely phototrophic or purely heterotrophic. However, numerous variations on the theme of mixotrophy exist, including photosynthetic protists that possess phagotrophic ability and heterotrophic species that maintain stolen chloroplasts (kleptoplastidy). The latter behavior is common among ciliates, dinoflagellates, and foraminifera (Stoecker 1999). In addition, numerous symbiotic mutualistic relationships exist between heterotrophic protists and cyanobacteria or phototrophic protists, as do other organism–organism relationships, including parasitism and commensalism (Caron 2000, Gast et al. 2009).

Collectively, the attributes noted above place protists in pivotal ecological roles in marine food webs. Protistan biomass constitutes a substantial portion of the standing stock of living carbon in many marine ecosystems, and metabolic rates of these taxa can be exceptionally rapid, solidifying their importance in biogeochemical cycles in the ocean (Caron et al. 1995, Sherr & Sherr 2002, Sherr et al. 2007). Studies of protistan diversity enable characterization of the many species and their genomic potential for affecting elemental cycling and energy production and utilization in the ocean.

## Roadblocks and New Approaches: Toward a DNA Taxonomy

Characterizing and understanding protistan diversity has been and continues to be an active area of research in marine science for the reasons noted above. Establishing the natural abundances and ecological activities of protists in aquatic ecosystems has involved visualization (historically, protists have been described and identified based on morphological features), culture, and laboratory experimentation to establish basic physiology and behavior, and then extrapolation of that information to nature and verification via field-based observations and experimentation (**Figure 4**). The information garnered from this work has provided fundamental understanding of many of the ecological roles performed by protists in natural communities and allowed for the development of models describing their activities.

Nevertheless, the goal of documenting the wealth of protistan taxa in a sample from a natural community has been hindered greatly by the magnitude of this task (i.e., the tremendous breadth of protistan species richness) and by the difficulties and complexities of protistan taxonomies. These difficulties include multiple fixation and processing procedures as well as the diverse taxonomic characters that must be determined to identify different groups of protists. Even then there are limitations to morphology-based taxonomies. Species with small cell size and/or few morphological features (e.g., many amoebae, and photosynthetic and heterotrophic forms <10  $\mu\text{m}$  in size) present difficulties for rapid and easy identification. Additionally, ecological research often requires the collection and processing of large numbers of samples, making traditional approaches impractical.



**Figure 4**

Molecular methods and approaches have significantly improved our tools for characterizing the diversity, abundances, and activities of protists in natural marine communities. The isolation and culture of protists from natural ecosystems (*pictures at top*) provide specimens for laboratory studies of protistan physiology, biochemistry, trophic activities, etc., while microscopy provides identification and estimates of abundance (*gray arrows*). This information is gathered to understand the ecological and biogeochemical activities of individual taxa of protists, and is used to synthesize predictive models of their activities in nature (*blue arrows*). Iterative testing and reformulation of these models, and our understanding of protistan activities, are accomplished through in situ observations and field-based experimental studies (*green arrows*). The application of molecular approaches and techniques has significantly augmented our ability to identify protists, estimate their abundances in natural samples, and examine the metabolic activities of these species (*red arrows*). Collage of protistan taxa reproduced from Caron et al. (2002).

The incorporation of DNA sequence-based approaches for defining, identifying, and quantifying protistan taxa is rapidly changing the landscape on this issue. These approaches have already begun to enable the development of molecular taxonomies that can be applied using ever-improving genetic technologies to provide taxonomically broad and relatively rapid assessments of protistan diversity (Caron et al. 2009). Genetic information has been useful for identifying cryptic protistan taxa within morphologically defined species (Pfundl et al. 2009) and for providing additional characters to distinguish species with amorphous morphologies, such as lobose amoebae (Nassonova et al. 2010). These findings have prompted work to purposefully incorporate sequence information into protistan species descriptions. Early work in this area used sequence similarity of ribosomal RNA genes to distinguish morphologically similar isolates of the opportunistically pathogenic amoeba genus *Acanthamoeba* (Gast & Byers 1995), but an increasing number of studies have attempted to combine DNA sequence information with morphological and/or physiological information in defining protistan species (Modeo et al. 2003, Takano & Horiguchi 2005).

It is important to note that a DNA taxonomy is not universally accepted at this time (Rubinoff et al. 2006). Critics often argue that genetic differences among strains within a species could overestimate an assessment of the species richness of an assemblage by erroneously equating intraspecific genetic variability to species-level differences, or that the adoption of a DNA taxonomy will result in the loss of understanding of the form and function that epitomizes the species concept. Some have argued that the genetic variations that have been promulgated as evidence of cryptic species are merely accumulated neutral mutations within morphospecies (Fenchel 2005). The presence of pseudogenes and different mutational rates among different genes have also been cited as factors confounding the use of sequence information for defining taxonomy. These criticisms are valid and must be addressed, but the ability to dramatically increase the rate of analysis and decrease the cost required for sequence-based approaches provides a strong impetus for the establishment of molecular taxonomies.

### Approaching Estimates of Total Protistan Diversity

Molecular biology has undeniably begun to provide powerful new tools for assessing diversity of natural protistan assemblages that obviate some of the shortcomings of traditional taxonomies and approaches, as noted above. The general approach for these diversity surveys involves the collection, extraction, and purification of nucleic acids from environmental samples; the application of one or more of a variety of methods for assessing sequence diversity within the sample; and then the interpretation of this genetic diversity in concert with databases that provide taxonomic information.

Two basic genetic approaches have been employed to date: those that use DNA sequences themselves, and those that employ DNA fragment analysis to assess the composition of the most abundant taxa within a community. The latter methods have employed a variety of specific techniques, most commonly denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and automated ribosomal intergenic spacer analysis (ARISA) (Hamilton et al. 2008, Not et al. 2008, Vigil et al. 2009). These methods have as their basic tenet the use of species-specific fragment lengths as genetic signatures of eukaryotic taxa. Fragment analysis is relatively quick and inexpensive and provides a snapshot of a subset of the dominant taxa within a community. However, because different taxa can sometimes yield fragments of the same length, fragment analysis does not readily provide unambiguous taxonomic information. Also, the sensitivity of the approach is limited by the number of fragments that can be detected, and thus these methods are not appropriate for assessing the total species richness of the community (Bent et al. 2007).

DNA sequencing of genetic material from environmental samples has yielded much more information than fragment analyses regarding the total diversity present in natural assemblages of protists and other microbes since the approach gained acceptance in the late 1990s (Pace 1997). The principal target for sequencing efforts thus far has been small subunit ribosomal RNA (SSU rRNA) genes, but different gene targets have been used, and it is probable that a viable molecular taxonomy for protists will employ multiple genes because of the enormous genetic diversity among these taxa.

Sequence information generated by environmental gene surveys is typically converted into information relating to protistan diversity using algorithms that group sequences into operational taxonomic units (OTUs) based on sequence similarity. Optimally for ecological studies, OTUs should represent clusters at the species level, but so far there have been few attempts to define protistan OTUs with this goal in mind (Amaral Zettler et al. 2009, Caron et al. 2009, Nebel et al. 2011). Schloss & Westcott (2011) have recently reviewed the difficulties associated with this process. Using sequence-based approaches, the number of sequences that can be obtained—and thus the depth to which one can characterize the protistan diversity present in a sample—depends on the effort (time and money) allocated for the sequencing. Surveys of sequence diversity within the SSU rRNA genes have been used in studies of protistan diversity for approximately a decade, and sequencing technology has advanced dramatically during that time. These environmental surveys have progressed from cloning and partially sequencing several dozen SSU rRNA genes from a sample to partially sequencing tens of thousands of genes using high-throughput approaches such as pyrosequencing (Amaral Zettler et al. 2009, Sogin et al. 2006, Stoeck et al. 2010).

Practical factors limiting the use of genetic approaches for characterizing protistan diversity at this time relate to extraction efficiencies, primer and polymerase chain reaction (PCR) biases (if the genes are amplified before analysis), sequencing errors, chimera formation, presence of pseudogenes, and variable copy numbers of genes, as well as the cost and time for sample processing. A more vexing issue relates to our limited ability at present to assign species identifications to lists of OTUs generated by sequencing campaigns. Most environmental sequences have not been directly linked to protistan species descriptions that have been defined using traditional (morphological) approaches. Therefore, there is presently no easy way for ecologists to interpret the long lists of OTUs generated in studies of protistan diversity. Debates over the species concept have only exacerbated that problem. It is expected that continued effort will dramatically improve the usefulness of sequence databases and reduce this controversy, improving interpretations of genetic diversity and increasing the use of sequence information in ecological studies.

The general picture emerging from genetic studies of protistan diversity in natural ecosystems is often illustrated in the form of rank abundance curves, which indicate the relative abundances of species (OTUs) within a community (see figure 5 in Caron et al. 2009). The development of genetic approaches for assessing diversity has been instrumental in generating these depictions, because gene surveys are not constrained by the multiple taxonomic approaches traditionally applied to protistan species. Gene-based methods are still in their infancy, but they have already provided new insights into the structure of protistan assemblages and resulted in significant new discoveries for protistan ecology.

One of the most striking features of these rank abundance curves has been the very long tail of “rare” taxa present in virtually all environments that have been sampled extensively. There are several potentially important implications for the presence of this microbial rare biosphere, a term that has been described from studies of marine bacterial communities (Sogin et al. 2006). The existence of an exceptionally large number of rare taxa presently makes it impossible to determine all protistan species present in a given environment or at a given time. Therefore, a variety of indices have been developed to estimate the total species richness of an environment

by extrapolating from the limited sampling that is possible (Bunge 2011, Bunge & Barger 2008, Schloss et al. 2009). The choice of the level of DNA similarity employed to construct OTUs strongly affects the number of OTUs obtained in these studies, the resulting rank abundance or rarefaction curves generated, and thus the total species diversity estimated from the data set. The impact of a similarity value on the number of OTUs generated with a sequence data set can be dramatic (Caron 2009, Sogin et al. 2006), and is indicative of the state of our knowledge on how to interpret genetic data sets of microbial diversity.

A potentially important implication of the demonstration of a protistan rare biosphere is the possibility that these species play important ecological roles in marine communities (Caron & Countway 2009). Theories presently abound on the composition of the rare biosphere. These taxa may include moribund cells (or even dead cells, in the case of DNA-based studies), resting stages (cysts, spores), expatriated cells, active but functionally rare taxa (e.g., taxa conducting keystone activities), taxa that are thriving but occupy rare microenvironments within a much larger ecosystem, or competitively inferior species that somehow avoid complete local extinction (Patterson 2009, Pedrós-Alió 2006). It has been argued that the latter situation is facilitated by the incredibly large population abundances exhibited by most species of protists (Fenchel & Finlay 2004).

It seems probable that many of the rare microbial taxa in an ecosystem contribute to ecological function at one time or another, and that some taxa provide important activities without ever attaining numerical dominance in the community. It also seems conceivable that the rare taxa may act to buffer overall ecosystem processes by ascending to important ecological roles in response to changes in environmental conditions, an idea that has been recently formalized for protists (Caron & Countway 2009). This hypothesis presumes the existence of guilds of protistan taxa in natural communities that conduct similar ecological functions but under different environmental conditions. Guilds may change rapidly and in response to environmental or biological forcing factors, whereas the emergent ecosystem processes of the biological community remain relatively constant.

Several findings lend support to the hypothesis of an important ecological role for at least some of the rare protistan taxa. These include recent studies documenting shifts in the community structure of natural protistan assemblages that were much more rapid than previously known (Countway et al. 2005, Kim et al. 2011, Vigil et al. 2009), and the observation that protistan communities at very different locations in the world ocean can be more similar than communities sampled from nearby locations (Caron & Countway 2009). One might assume that if protistan taxa were widely distributed across the planet, then similar oceanographic conditions in different locales would select for the same or similar groups of protistan species. A recent study of marine microbial taxa in a coastal ocean using a local similarity analysis indicated that guilds of microbial species appeared to share temporal distributions, presumably indicating ecological linkages between these species (Steele et al. 2011). Taken together, these disparate pieces of information may indicate a highly dynamic protistan community in which species (or sets of species) change abundances rapidly and presumably in response to changes in environmental forcing factors. We do not yet understand the rules governing community reassembly, nor the implications of community reassembly for ecosystem function, but documenting the presence of a protistan rare biosphere and its contribution to community structure and ecosystem activities are beginning steps in developing that understanding.

The existence of a protistan rare biosphere has potentially important implications for debates regarding the global diversity and biogeographies of these species. The presence of a vast background of rare taxa in natural communities implies very high local diversity of protistan species, and possibly low global diversity; available niches are filled everywhere in the world by the same

species (Fenchel & Finlay 2004). This finding would also seem to imply relatively easy dispersal of protistan and other microbe taxa around the planet (Finlay 2002, Patterson 2009). An alternative view is that most protistan species exhibit limited geographical distributions and a high degree of endemism, and therefore exhibit exceptionally large global diversity (Foissner 1999, 2006). There is presently information available that appears to support the contention for endemism and ubiquitous dispersal, or at least a model that accommodates both ideals dependent upon the scale of the observation and the genetics and physiology of the group investigated (Bass et al. 2007b). The controversy therefore seems rooted in debates over the species concept applied to protists, our present inability to fully plumb the depths of diversity in natural assemblages, and a lack of knowledge concerning dispersal rates, rates of mutation, and what constitutes an environment for a protistan species (Caron 2009, Fenchel 2005, Finlay 2004). Genetic approaches are providing new tools to address these issues.

### Discovery in the Molecular Age

Another significant outcome of studies of protistan diversity enabled by molecular approaches during the past decade has been the discovery of previously undetected species, groups, or entire clades of protists. These findings have begun to change our views of how marine food webs are structured and how they function (Vaulot et al. 2008).

Chief among these discoveries during the past decade has been the documentation of very high diversity among the minute (<20  $\mu\text{m}$ ) protists in the ocean. The important contribution of minute photosynthetic and heterotrophic protists to total standing stocks of living biomass and trophic activities in marine ecosystems has been well known for decades (Hewes et al. 1983, Malone 1971), and phylogenetic studies have implicated a great wealth of uncharacterized biodiversity among tiny heterotrophic flagellate taxa (Patterson 1999). Yet there has been little detail on the taxonomic diversity of these groups other than for taxa possessing distinctive morphological structures, such as choanoflagellates (Marchant & Perrin 1990). This gap in our knowledge has been greatly altered by genetic studies of microbial eukaryote diversity.

A large number of surveys have appeared in recent years detailing the genetic diversity of microbial eukaryotes in the water column of diverse locations of the world ocean (Cheung et al. 2010; Countway et al. 2007, 2010; Diez et al. 2001; Massana et al. 2011; Medlin et al. 2006; Not et al. 2009; Piganeau et al. 2008; Schnetzer et al. 2011; Shalchian-Tabrizi et al. 2007; Vaulot et al. 2008; Worden 2006). In particular, studies of picoeukaryotes (functionally defined by the sequencing of particulate material that passes through a 3- $\mu\text{m}$  filter) have revealed a wealth of new taxa. The choice to examine these exceptionally small eukaryotes has been partly an attempt to exclude the presence of metazoan taxa, which can be an important component of eukaryote clone libraries when larger size classes are included. Studies of picoeukaryotes have revealed new clades of previously unknown or uncultured protists not represented in extant culture collections (Shi et al. 2009) and the global distribution of taxa previously believed to be limited in their occurrence (Lara et al. 2009). The existence of MAST taxa (Massana et al. 2002), unknown alveolate clades (Guillou et al. 2008), telonemids (Shalchian-Tabrizi et al. 2006), and a variety of photosynthetic picoeukaryotes (Demir-Hilton et al. 2011, Kim et al. 2011, Not et al. 2009) has stimulated ecological studies on the identity and ecological activities of these taxa.

Gene surveys of environmental samples have also brought attention to the presence of previously unknown clades of picoeukaryotes (Kim et al. 2011, Not et al. 2007) and expanded our knowledge of the breadth of diversity and ecological activities in well-known taxa that had previously been underestimated, such as some prasinophytes (Lovejoy et al. 2007, Viprey et al. 2008), ciliates (Doherty et al. 2010), and dinoflagellates (Stern et al. 2010). An example is the



chlorophyte genus *Ostreococcus* (Courties et al. 1994), which has recently been shown to contribute significantly to marine plankton communities (Countway & Caron 2006, Demir-Hilton et al. 2011, Piganeau & Moreau 2007).

## Protistan Diversity in Extreme Environments

Another surprising generality emerging from recent gene surveys of marine protists is that extreme marine environments are not necessarily environments characterized by low protistan diversity. The low environmental temperatures of Arctic and Antarctic ecosystems support robust communities of protists (Caron & Gast 2009, Eddie et al. 2009, Lovejoy et al. 2006) (Figure 5). Traditional studies of microscopy and culture are in agreement with this conclusion, at least for large or morphologically distinguishable taxa in Antarctic environments (Scott & Marchant 2005).

Gene surveys are proving particularly useful for examining environments that are difficult to access by traditional approaches. Ecological studies of protists from deep-sea ecosystems are exceptionally rare owing to obvious difficulties associated with retrieval of samples and the culture of specimens under low temperature and high pressure. However, gene surveys from the water column and the benthos (including hydrothermal vent ecosystems) are rapidly increasing, and depict environments that are replete in a wide variety of heterotrophic protistan taxa (Alexander et al. 2009, Bass et al. 2007a, López-García et al. 2007, Park et al. 2008, Pawlowski et al. 2011, Scheckenbach et al. 2010, Takishita et al. 2007) (Figure 5). One surprising fact is that microscopic fungi have been found to be particularly well represented in deep-sea and coastal marine sediments (Edgcomb et al. 2011, Le Calvez et al. 2009, Singh et al. 2011). These forms are typically found only sporadically in samples collected from the water column, a finding that is presumably reflective of the much higher abundances of photosynthetic taxa in surface waters as well as perhaps a selective advantage for the growth of fungi in the organic-rich environment of sediments. DNA analyses have even been employed to document significant protistan diversity in the surface microlayer of estuarine environments (Cunliffe & Murrell 2010).

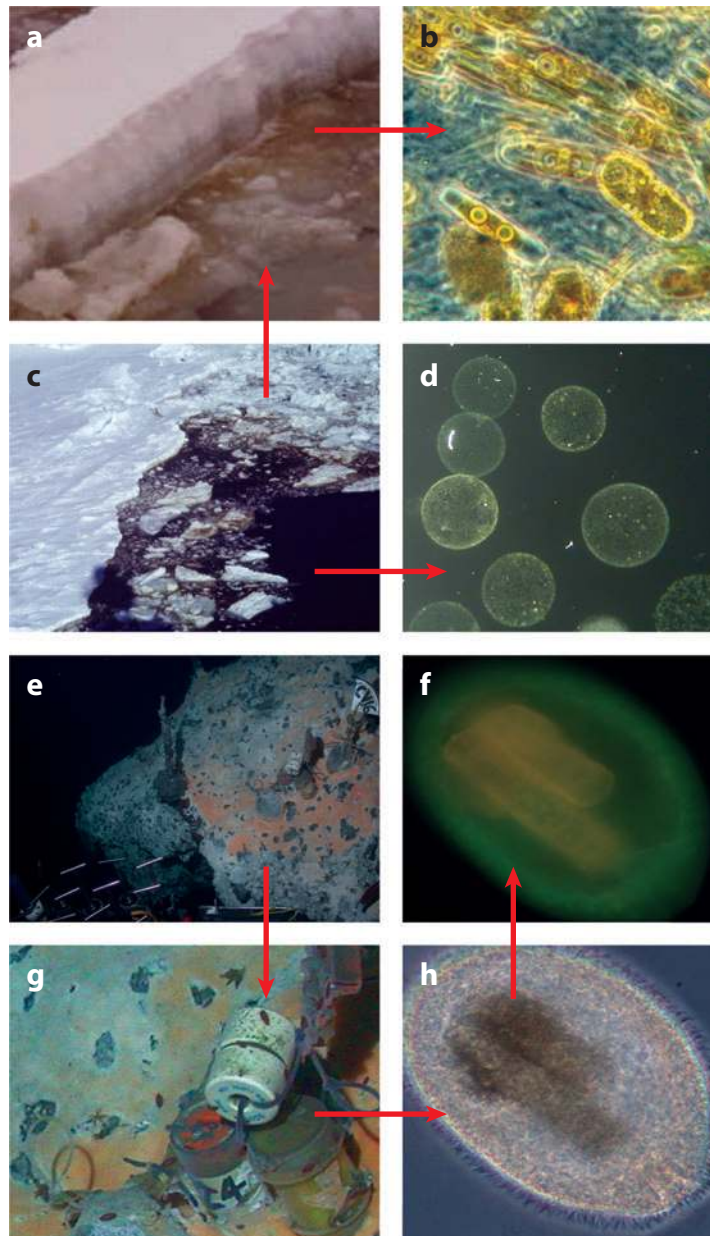
Protistan taxa even abound in anoxic waters and sediments of marine environments, including deep-sea locales (Stoeck et al. 2009, Takishita et al. 2005, Zuendorf et al. 2006). These findings have renewed questions regarding the limits of environmental factors that will support rich protistan communities. Present knowledge of the thermal tolerances of protists indicates that protistan diversity should be greatly curtailed at temperatures above 40°C, but this knowledge is based largely on species isolated from shallow ecosystems, and there is a virtual absence of data on the tolerances of protists inhabiting hydrothermal ecosystems. Reduced protistan diversity might also be expected in some chemically impacted environments, but a wide range of taxa are present and apparently flourish even in such extreme situations as the highly acidic (pH = 2) Río Tinto, Spain (Amaral Zettler et al. 2002, 2003).

## DIRECTIONS FOR FUTURE STUDIES OF PROTISTAN DIVERSITY

Recent molecular studies of protistan diversity have provided tantalizing glimpses of novel taxa, incredibly diverse assemblages, and potentially new ecological roles for protists in marine ecosystems. These studies have also provided fruitful avenues for new research directions.

### Population Biology and Ecology

The availability of rapidly expanding databases of DNA sequence information for protistan taxa has enabled the development of approaches that exploit this information to specifically identify and



**Figure 5**

Protists in fire and ice. Extreme environments on Earth can support diverse and abundant protistan assemblages. Antarctic sea ice in the Ross Sea (panels *a* and *c*) provides the habitat for an incredibly dense assemblage of diatoms (panel *b*) and heterotrophic protists, while seawater  $<0^{\circ}\text{C}$  in this environment supports extensive blooms of the colonial haptophyte *Phaeocystis antarctica* (panel *d*). Extensive bacterial mats at a hydrothermal vent site in Guaymas Basin (panels *e* and *g*) support diverse ciliate assemblages (panel *h*, showing a light micrograph of a large ciliate) that actively ingest bacterial filaments (panel *f*, where the same ciliate examined by epifluorescence microscopy shows autofluorescence of bacterial filaments in food vacuoles).

accurately enumerate species of interest in complex natural microbial communities. Quantitative PCR (qPCR) provides exceptional sensitivity and accuracy for identifying protistan species in natural samples. The basic premise of the method is to employ primers complementary to segments of the DNA that are unique to the target taxon, and that will result in the amplification of short segments of DNA during the PCR reaction (usually 100–400 base pairs). Quantification of the amount of amplified DNA is performed after each cycle of the reaction using a variety of detection approaches. The cost of setup is significant for these approaches, and initial development of an effective qPCR scheme can be laborious, but once developed these approaches can be applied to large numbers of samples in a short period of time, identify a target organism without the need for extensive taxonomic experience, and quantify target DNA over several orders of magnitude.

qPCR has been applied most aggressively to detect and quantify species of harmful and acutely toxic bloom-forming algae, including species of *Pseudo-nitzschia*, *Alexandrium*, *Aureococcus*, *Lingulodinium*, and *Pfiesteria* as well as several raphidophyte algae (Bowers et al. 2006, Coyne et al. 2001, Fitzpatrick et al. 2010, Hosoi-Tanabe & Sako 2005, Lin et al. 2006, Moorthi et al. 2006, Popels et al. 2003). Detection by qPCR can be accomplished at abundances of these taxa that are typically below abundances that bring about ecological damage or present a threat to animal or human health. This capability provides an important new tool for studying the environmental and ecological conditions that lead to harmful algal blooms, rather than simply documenting the existence of harmful events. PCR-based approaches have also been used to facilitate the detection of species whose presence might be difficult or laborious to assess using microscopy or culture alone, and combined with experimental approaches to examine trophic dynamics (Countway & Caron 2006, Demir et al. 2008, Park et al. 2007, Zhu et al. 2005).

Fluorescence in situ hybridization (FISH) is another sequence-based approach that has been applied to gain information on the autecology of specific protistan taxa. FISH uses taxon-specific oligonucleotides as probes to specifically label target cells, but it has the advantage of accomplishing this task in intact cells. It therefore has the ability to provide absolute abundances of target taxa. FISH has been used as a confirmational tool to identify morphologically similar protistan species (Scholin et al. 1996), as a means of distinguishing and identifying taxa with nondescript morphologies (Fuller et al. 2006), and for attempts to provide morphotypes for sequences of uncultured or undocumented taxa known only from genetic surveys (Cuvelier et al. 2008, Kolodziej & Stoeck 2007, Massana et al. 2002, Rodríguez-Martínez et al. 2009). FISH has also proven useful for distinguishing and quantifying taxa that are difficult to differentiate by microscopy in natural samples (Biegala et al. 2003, Fuller et al. 2006), and for examining the trophic activities, life stages, and life cycles of protists (Chambouvet et al. 2008, Jones et al. 2011, Massana et al. 2006).

## Evolutionary Studies of the Eukaryotic State

Marine protists have been and will continue to be a topic for studies of evolution, and studies of protistan diversity will continue to unveil new taxa and therefore provide new fodder for research on evolution. Protistan taxa have served as model systems for understanding the root of the eukaryotic tree (Baldauf 2003), the development of multicellularity among eukaryotes (Grosberg & Strathmann 2007), and the evolution of animals (King et al. 2008).

Most notably, studies leading to a molecular understanding of organelle acquisition and stabilization in eukaryotes have advanced markedly in recent years (Archibald 2005, Chan et al. 2011, Lane & Archibald 2008, Richards & Archibald 2010). This work has benefited from the documentation of the widespread nature of mixed nutrition among protistan taxa, including phagotrophic algae and kleptoplastidic taxa (chloroplast-retaining ciliates, heterotrophic dinoflagellates, and foraminifera). These species provide important model systems for study, and thus provide

information on the mechanisms of genetic interplay between the host genome and the acquired plastid, and the evolutionary transition from harbored stolen organelles to true organelles (Keeling 2004, Kim & Archibald 2010).

### **Physiology and Ecology of Important Protistan Taxa**

Sequence information for protistan (and many other) taxa is beginning to present unique opportunities for characterizing and understanding the physiological and ecological activities of these taxa. Studies of gene expression that monitor the physiology of protists, especially species whose genomes have now been sequenced, have begun to offer insights at the molecular level into how protists respond and adapt to changing environmental conditions. Again, ecologically important or harmful bloom-forming species of algae have been a major target of this work (Allen et al. 2008, Gobler et al. 2011, Lidie et al. 2005, Maheswari et al. 2010, Mock et al. 2008, Van Dolah et al. 2007).

The extrapolation of this information and these approaches to characterize physiological function of protistan species in mixed natural assemblages is a formidable task ahead. Innovative approaches will be needed to decipher the species-specific metabolic responses and activities of protists in nature, and how specific protistan taxa or whole assemblages might respond to changes in environmental forcing factors. These types of approaches might be exemplified by a recent study that employed flow cytometry and transcriptomics to sort cells and examine gene expression of picoplanktonic algae from the subtropical North Atlantic (Cuvelier et al. 2010).

### **In Situ Platforms for Making Sophisticated Measurements**

Hand in hand with the development of sophisticated measurements of protistan community structure and physiological activities will be the development of technologies to deploy these abilities in the ocean. There are presently extremely few in situ sensors or instruments that provide sophisticated biological information. The Environmental Sample Processor (Greenfield et al. 2006, 2008) and the Autonomous Microbial Genosensor (Casper et al. 2007) are two instruments that have begun the daunting task of adapting complex and fastidious genetic approaches to the rigors of the marine environment. These technologies will be essential for making real-time, in situ measurements, a capability that is fundamental for characterizing the activities of microbes in the real world.

Phylochip and gene expression arrays that document the presence and activities of many taxa and/or species of particular significance (e.g., harmful algal bloom species) will aid in capturing the ephemeral nature of marine microbial processes. These latter approaches have been accomplished with protistan species under carefully controlled laboratory conditions (Ahn et al. 2006, Mayali et al. 2010). Adapting them to perform under the conditions of marine environments is a major challenge for the next generation of biological oceanographic sensors. Moreover, coordinating the activity of these instruments into sensor networks will enable measurements of protistan community structure and function in conjunction with measurements of pertinent environmental factors (Porter et al. 2005, Smith et al. 2010).

### **Relating Community Composition to Ecosystem Function**

One overarching, long-term goal of studies of protistan diversity and biological diversity in general is to develop a fundamental understanding of how community composition relates to emergent ecosystem processes (Cardinale 2011, Duffy & Stachowicz 2006, Latta et al. 2011).

This understanding contributes to the design of models that predict how changes in the structure of these communities in response to environmental factors affect ecosystem function. Microbial communities pose excellent, manageable model systems for examining this relationship (Cardinale 2011, McGrady-Steed et al. 1997, Naeem & Shubin 1997). Indeed, microbial processes constitute much of the biogeochemistry of the ocean, and therefore these assemblages are sensible choices for studies of this type.

Observational and experimental approaches to examine the relationship between biodiversity and ecosystem function are still in their infancy. Linking protistan community structure with ecosystem function presupposes the ability to characterize at least the dominant taxa within a community, and to document shifts in the community composition as environmental parameters change. Future work on this topic will entail both observational studies of natural protistan assemblages and how they respond to shifts in environmental factors, as well as manipulative experimental studies in which community compositional changes and the emergent biological processes of the community are characterized in response to specific environmental changes. Understanding the rules of community reassembly in the face of changing environmental forcing factors, and the resulting effects on community-level processes, will allow predictions of possible changes in food web structure in the face of natural and anthropogenic influences.

### SUMMARY POINTS

1. The age of discovery is not over for marine protistology. The extent of protistan diversity is vast and still not fully characterized, but genetic approaches are providing new tools for identifying and quantifying abundances and for delving deeply into the taxonomic composition of natural, complex assemblages. New taxa, and possibly new lineages, continue to be discovered (in particular, small photosynthetic and heterotrophic protists with few readily distinguishable morphological features).
2. Molecular taxonomies, and applications arising from the description of taxon-specific DNA sequences, are enabling the collection of ecological information for protistan taxa at abundances well below the present capabilities of microscopy and culture techniques. This situation is particularly true for the detection of minute taxa that lack distinctive morphological features from among a myriad of minute protistan species in natural, complex assemblages.
3. Dominant taxa within protistan assemblages change often and rapidly, and the rules for community assembly and reassembly are slowly becoming clear, as are the consequences of these shifts in species composition for the emergent ecological processes and biogeochemical cycles of ecosystems.
4. Rare taxa (the protistan rare biosphere) constitute a major source of protistan diversity in natural ecosystems. Some of these taxa may play key roles in providing ecological buffering or a seed bank for new or redundant ecological activities in the face of changing environmental conditions. Understanding the activities of the rare biosphere may be the key to deriving predictive models of protistan community structure and function.

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## Errata

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