REVIEW SUMMARY

ENVIRONMENTAL SCIENCE

Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes

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BACKGROUND: Marine ecosystems are composed of a diverse array of life forms, the majority of which are unicellular—archaea, bacteria, and eukaryotes. The power of these microbes to process carbon, shape Earth's atmosphere, and fuel marine food webs has been established over the past 40 years. The marine biosphere is responsible for approximately half of global primary production, rivaling that of land plants. Unicellular eukaryotes (protists) are major contributors to this ocean productivity. In addition to photosynthetic growth, protists exhibit a range of other trophic modes, including predation, mixotrophy (a combination of photosynthetic and predatory-based nutrition), parasitism, symbiosis, osmotrophy, and saprotrophy (wherein extracellular enzymes break down organic matter to smaller compounds that are then transported into the cell by osmotrophy).

ADVANCES: Sensitive field approaches have illuminated the enormous diversity of protistan life (much of it uncultured) and, coupled with activity measurements, are leading to hypotheses about their ecological roles. In parallel, large-scale sequencing projects are providing fundamental advances in knowledge of genome/gene composition, especially among photosynthetic lineages, many of which are complex amalgams derived from multiple endosymbiotic mergers. Marine protists have yielded insight into basic biology, evolution, and molecular machineries that control organismal responses to the environment. These studies reveal tightly controlled signaling and transcriptional regulation as well as responses to limitation of resources such as iron, nitrogen, and vitamins, and offer understanding of animal and plant evolution. With the formulation of better computational approaches, hypotheses about interactions and trophic exchanges are becoming more exact and modelers more assertive at integrating different data types. At the same time, the impacts of climate change are being reported in multiple systems, of which polar environments are the touchstone of change.

OUTLOOK: Driven by the need to translate the biology of cells into processes at global scales, researchers must bring the conceptual framework of systems biology into bigger "ecosystems biology" models that broadly capture the geochemical activities of interacting plankton networks. Existing data show that protists are major components of marine food webs, but deducing and quantifying their ecosystem linkages and the resulting influences on carbon cycling is difficult. Genome-based functional predictions are complicated by the importance of cellular structures and flexible behaviors in protists, which are inherently more difficult to infer than the biochemical pathways typically studied in prokaryotes. Alongside the plethora of genes of unknown function, manipulable genetic systems are rare for marine protists. The development of genetic systems and gene editing for diverse, ecologically important lineages, as well as innovative tools for preserving microbe-microbe interactions during sampling, for visual observation, and for quantifying biogeochemical transformations, are critical but attainable goals. These must be implemented in both field work and laboratory physiology studies that examine multiple environmental factors. Expanding genome functional predictions to identify the molecular underpinnings of protistan trophic modes and realistically constrain metabolism will position the field to build reliable cell systems biology models and link these to field studies. By factoring in true complexities, we can capture key elements of protistan interactions for assimilation into more predictive global carbon cycle models. ∎
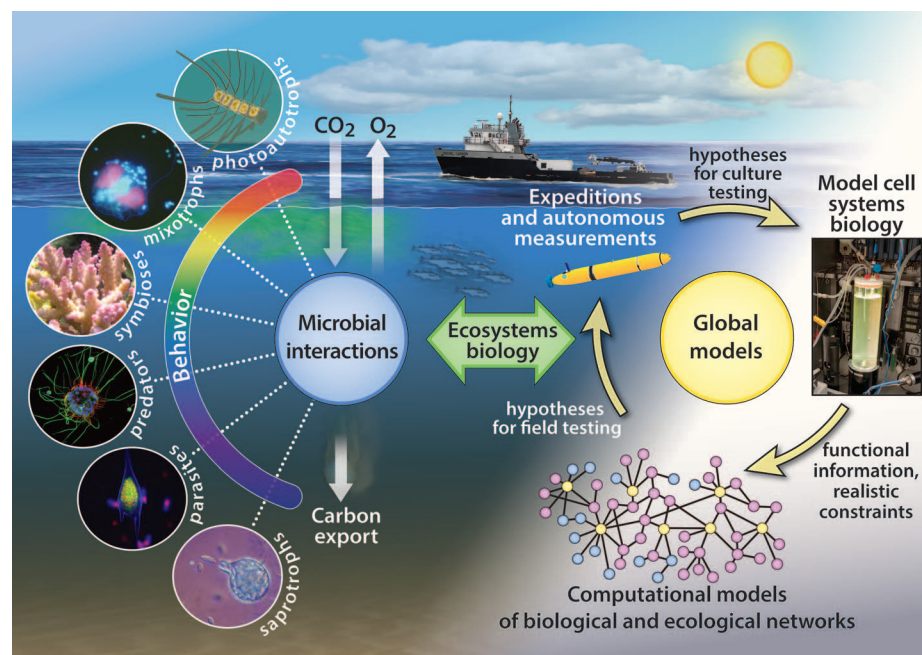
Global biogeochemical and ecological models rely on understanding organismal biology and the interactions occurring in marine microbial food webs. Protists have multifarious roles from the sunlit surface ocean to leagues below. Understanding of protistan behaviors and adaptability lags far behind knowledge of evolutionary processes that have shaped their genomes. As such, microbial mediation of carbon fluxes and specific interactions remain ill-resolved and predictive capabilities are still weak. Strategies to narrow this gap involve iteration between experimental and observational field studies, controlled laboratory experiments, systems biology approaches that preserve cellular structures and behaviors using relevant model taxa, and computational approaches.

REVIEW

Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes

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The profound influence of marine plankton on the global carbon cycle has been recognized for decades, particularly for photosynthetic microbes that form the base of ocean food chains. However, a comprehensive model of the carbon cycle is challenged by unicellular eukaryotes (protists) having evolved complex behavioral strategies and organismal interactions that extend far beyond photosynthetic lifestyles. As is also true for multicellular eukaryotes, these strategies and their associated physiological changes are difficult to deduce from genome sequences or gene repertoires—a problem compounded by numerous unknown function proteins. Here, we explore protistan trophic modes in marine food webs and broader biogeochemical influences. We also evaluate approaches that could resolve their activities, link them to biotic and abiotic factors, and integrate them into an ecosystems biology framework.

The marine carbon cycle plays a critical role in Earth's habitability for humans and other large fauna. At the core of the cycle are interconversions of inorganic and organic carbon forms, which lead to major ecosystem services. Protistan (unicellular eukaryotic) phytoplankton have long been recognized as foundational to fisheries and export of atmospheric CO_2 to the deep ocean (*1–3*). Protists convert CO_2 to organic carbon via photosynthesis, simultaneously altering cycles of other elements linked to carbon by the stoichiometry of cellular composition. Thus, the carbon cycle interacts with biogeochemical cycles of nitrogen, silica, and many other elements (*4–6*). How air-sea exchanges of CO_2, primary productivity, and carbon sequestration to the deep sea will be altered as climate change affects these and other microbes (*7*) is poorly understood. Here, we review current understanding of the diverse functionality and lifestyles of marine protists and how they participate in the carbon cycle.

During the 1970s and 1980s, ideas emerged about microbial connectivity in ocean food webs (*8*) and the microbial loop (*9*), which formalized the importance of bacteria and archaea as hubs of metabolic diversity. The classical diatom-copepod-fish food chain view of ocean productivity was thus revolutionized by the understanding that diverse forms of dissolved organic matter (DOM) derived from algae were a major energy source for heterotrophic bacterial growth (Fig. 1). In turn, predatory protists consumed the bacteria and were themselves preyed upon by larger zooplankton. In this framework, algal primary production had multiple routes to higher trophic levels. The microbial loop was the most inefficient route in terms of respiratory carbon losses (conversion of organic carbon to CO_2) because as algal DOM and particulate organic matter (POM) pass through heterotrophic bacteria and archaea, respiration losses occur alongside important nutrient remineralization. Viral lysis of bacteria was subsequently recognized as another potentially substantial source of DOM (*10*). Protists are now often represented as either photoautotrophs (typically diatoms and coccolithophores) or heterotrophic predators (Fig. 1).

Global-scale simulations of ocean ecosystems and biogeochemical cycles, overlain on circulation models, have become a common tool for carbon cycle and climate sensitivity studies, including Intergovernmental Panel on Climate Change assessments. Such models focus on simulating biogeochemical cycles rather than the organisms that mediate them, and build on the seminal studies of Riley and colleagues (*11*). Plankton populations are described by partial differential equations that represent physical transport, growth, death, and interactions at broad levels. Today, such models resolve a few broad "functional types" of photoautotrophic microbes (e.g., all small phytoplankton described by a single set of parameters) and two predator groups: protistan and metazoan consumers of algae, or "grazers" (*12–14*). If heterotrophic bacteria are included, it is as one homogeneous population. Population growth rates are described using highly idealized relationships to external resources (*15*) or internal stores (*16*), without representation of the highly flexible and adaptive physiology of the organisms concerned. In part, the current parameterization of microbial physiology in models reflects computational limitations, but it also reflects a lag in assimilation of new data and understanding of microbial cell biology. Determining the level of granularity required to accurately simulate and interpret ocean ecosystems, the type of baseline information that would be adequate to assess change, and how to integrate organismal diversity, dynamics, and interactions into large-scale models are not easy tasks.

As reviewed here, protistan biology comprises diverse lifestyles that shape the carbon cycle through elaborate but poorly appreciated food web connections. A repertoire of phagotrophic modes and a penchant for symbioses have led to the evolution of cell architectures that can be orders of magnitude larger and more complex than those of prokaryotes (*17*). Size matters, in part because larger cells (such as the largest coccolithophores) sink faster, altering export of carbon and other elements to the deep ocean on massive scales (*17–20*). Marine microbiologists and modelers alike are becoming acutely aware that phenotypic variation in protistan physiology and behavior is critical to assessing their broader ecosystem roles and future ocean productivity.

Complex factors govern protistan primary production: Phytoplankton

Marine algae account for about 50 Pg C year^{-1} of primary production, rivaling that of terrestrial plants (*1*). In addition to the roles played by larger photosynthetic protists, understanding of the importance of picoplanktonic taxa (diameter ≤2 μm) is growing (*21–23*). In the 1950s, the picoeukaryote *Micromonas pusilla* was shown to dominate in the English Channel (*23*), and subsequent discoveries of the abundant (non-eukaryotic) cyanobacteria *Synechococcus* (*24*) and *Prochlorococcus* (*25*) confirmed the importance of very small phytoplankton to marine systems. However, while larger algae such as diatoms have clear food chain roles and fast, quantifiable sinking rates that result in carbon export to the deep sea, similar knowledge is limited for eukaryotic and prokaryotic picoplankton (*20, 26*). Moreover, photosynthetic protists are extremely diverse; many are difficult to quantify in nature, many remain uncultured, and we lack baseline information on wild populations. "New" algal lineages are still being discovered (*27, 28*). Additionally, picoplanktonic eukaryotes, including uncultured groups, are unexpectedly important contributors to CO_2 fixation in environments where cyanobacteria dominate numerically (*29–32*).

Eukaryotic phytoplankton have evolved from heterotrophic ancestors on multiple independent occasions, resulting in marked differences in genome content and functional capabilities, from which they continue to diversify (Box 1 and Fig. 2). Comparing the cell wall composition of four

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common algal lineages illustrates this diversity and its influence on ocean biogeochemistry: Some stramenopiles have silicate frustules of nanoscale precision (diatoms), whereas others are naked; haptophytes can be covered in ornate calcium carbonate plates (coccolithophores), whereas many picoplanktonic species are naked or have organic scales; dinoflagellates can possess intracellular cellulose plates or be naked; and among the prasinophytes, a few species are naked but most are enveloped by organic scales (4, 33, 34). Consequently, the growth of these algae depends on different elements' input sources, remineralization rates, and crystal structures (e.g., calcium carbonate in the form of aragonite or calcite) and will have correspondingly distinct responses to decreasing pH caused by climate change (35).

Analysis of algae in the context of genomic information is advancing understanding of their physiology. These studies often focus on diatoms or prasinophytes, underscoring highly differentiated responses to iron and nitrogen availability, tightly controlled gene regulatory

programs, and signaling systems conserved with land plants [e.g., (36–41)]. Genomes are available for other marine phytoplankton as well (42–44). Complex relationships between resource availability and adaptive strategies are also being identified using genomes from cultured strains as contextual information and innovative approaches to unraveling interactive effects in the field (45, 46). For example, diatoms in a Pacific Ocean study were highly responsive to iron fertilization, but metatranscriptome analyses suggested continued dependence on iron-free photosynthetic proteins rather than a switch to iron-containing functional equivalents present in their gene repertoire (45). This is hypothesized to allow newly available iron to be used for resource acquisition (rather than for photosynthetic machinery), contributing to frequent diatom success under iron fertilization. These findings would be difficult to deduce in laboratory experiments, because they would not capture the sharp contrasts between diatom responses and those of the broader algal community.

Nonetheless, many aspects of algal physiology remain unclear. This knowledge gap is becoming critical as climate change influences marine food webs and carbon sequestration (47–49). Perturbations typically influence multiple environmental parameters simultaneously, and algal responses appear to be highly regionalized. For example, in the Canadian Arctic, larger photosynthetic protists such as diatoms are reportedly being replaced by photosynthetic picoeukaryotes (50), and similar shifts are expected in other regions as a result of stratification-related causes, altering both the food web and carbon export. In contrast, massive amounts of algal carbon, primarily from diatoms, are sinking to the eastern central Arctic sea floor (51). The interactive effects of perturbations are extensive [e.g., climate-influenced CO_2, pH, and temperature changes; reviewed in (52)], but most laboratory experiments fail to capture their complexity. Cell systems biology experiments involving the controlled study of model organisms in culture are essential for designing field studies that gauge the
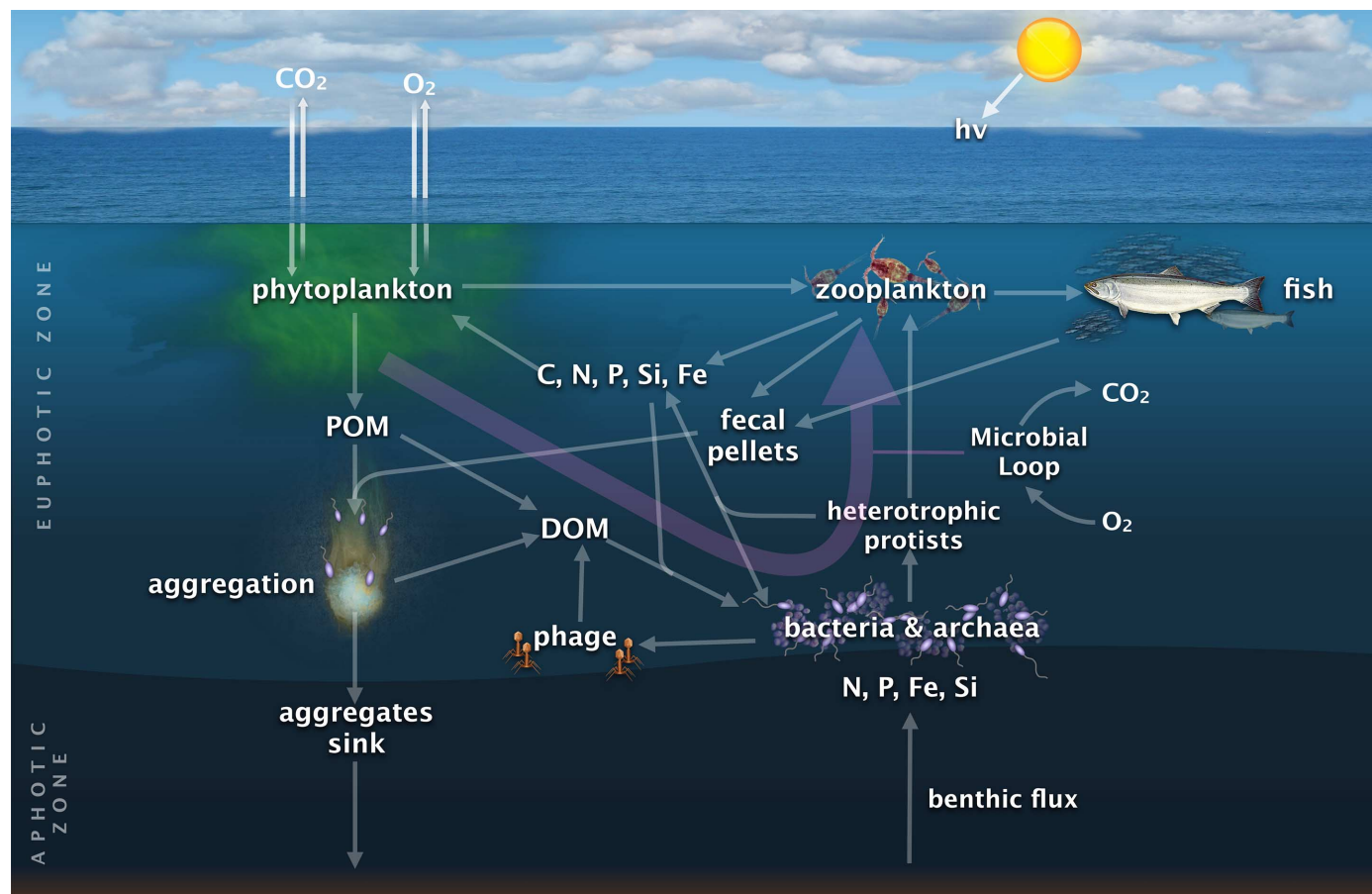

Fig. 1. A common depiction of marine microbial food webs. Dissolved and particulate organic matter (DOM and POM, respectively) from phytoplankton form a basic resource for bacteria and archaea, which respire CO_2 (via the microbial loop, purple arrow), thereby modifying the flow of carbon expected in earlier diatom-copepod-fish food chains (diatoms are algae within the stramenopile lineage, and copepods are multicellular zooplankton in the opisthokont lineage). Most such schemas do not differentiate phytoplankton in different size fractions, although size influences food web linkages. Inorganic nutrients are also important factors shaping community composition. By necessity, processes in the euphotic zone, where photosynthesis occurs, are differentiated from those in the aphotic zone, where sunlight is unavailable. Anthropogenic impacts (not depicted) include acidification, coastal eutrophication, and changes to water column structure induced by warming, such as stronger stratification and reduced nutrient flux into surface waters, as well as increased exposure to high light intensities (7). [Adapted from (105)]

impacts of individual taxa on higher ecosystem-level processes.

Carbon in the balance: Predation, osmotrophy, and parasitism

Carbon cycling and sequestration depend on carbon oxidation rates as much as on photosynthesis. This balance pivots on the wide-ranging nutritional strategies of heterotrophic organisms (Fig. 3). The best-studied heterotrophic mode of marine protists is predation. On average, micro- and nanozooplankton (<200 μm) consume 62% of daily algal production, albeit with high regional and temporal variability (53). These predators are largely protistan, but grazing measurements generally reflect bulk rates without group-specific information. This is problematic because the protists involved are diverse. In productive environments, dinoflagellates can be major grazers of diatom blooms, whereas in more oligotrophic regions, ciliates and diverse flagellates are important consumers of picophytoplankton and bacteria (32, 54–56). Structurally complex choanoflagellates, amoebozoans, dinoflagellates, and rhizarians can also be regionally important predators (57–59). Members of the latter group, which includes Acantharia (part of what used to be called "Radiolaria") and Foraminifera, also feed extensively on hetero-

trophs, including on multicellular zooplankton such as copepods (58). The identity of the taxa responsible for most predation is not known, particularly for heterotrophic flagellates. Novel marine stramenopiles (MASTs) are presumed to dominate predatory flagellates (21), but most MASTs are uncultured (60–62) and correct assignment of trophic modes is complicated because the stramenopiles include algae, saprotrophs, predators, and mixotrophs (Box 2). Some MASTs clearly consume bacteria and picophytoplankton (55, 56), but single-cell and colony isolation studies indicate that more complex associations also exist. For example, the MAST *Solenicola setigera* grows on frustules of the diatom *Leptocylindrus mediterraneus*, sometimes alongside *Synechococcus*, upon which it may also feed (60). These discoveries show the difficulties in discerning the nature of associations in the wild.

The huge variation in protistan predation strategies is not represented in current marine biogeochemical and ecological models because the bases for prey selection, feeding rates, and alternate strategies are not known. Generally predators are larger than their prey; however, protists can ingest prey of equal or larger size than themselves. Examples include the haptophyte *Prymnesium parvum* (63) and the dinoflagellate *Karlodinium armiger*, which immobilizes cope-
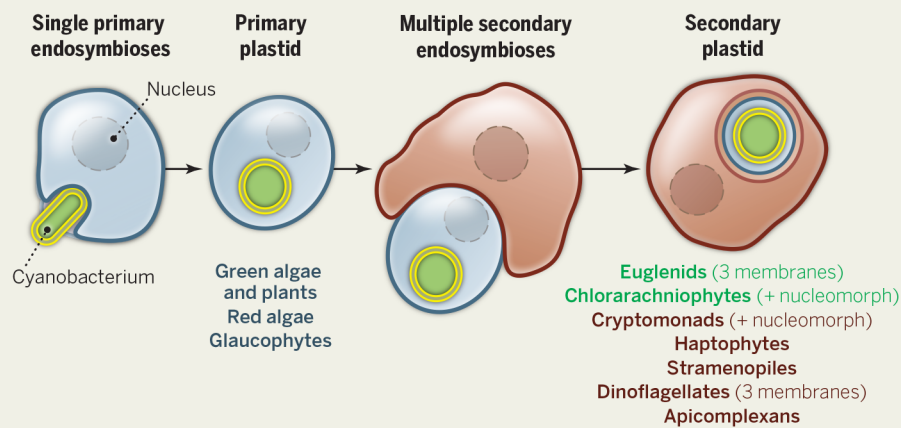
pods that are 50 times its size before consuming them (64). As a consequence, organism size does not necessarily increase with trophic level, allowing for a longer food chain and comparatively greater carbon losses. Although cell size may play a role in prey selection, so too does prey quality, relative abundance, and extracellular characteristics (21, 65–67), which thereby influence the flow of carbon through specific populations. Assimilation efficiencies vary among predators and prey types, as well as with prey quality and quantity. Growth and carbon assimilation by the predatory stramenopile *Picophagus flagellatus* (<2 μm) is very different when fed *Prochlorococcus* versus *Synechococcus*, resulting in differing carbon flows between trophic compartments (65). Additionally, trophic cascades are strengthened at higher temperatures, including grazing control of primary producers, but in a nutrient-dependent fashion (68, 69). The metabolic processes underlying heterotrophy appear to respond more strongly to temperature than does primary production, so that increased temperature results in a shift to more heterotrophic ecosystem metabolism (70).

Predatory activities also contribute to pools of DOM and POM. The marine DOM carbon pool is nearly equal to atmospheric CO_2 and about 200 times that in living marine biomass (71). Both DOM and POM are complex amalgams spanning a gradient of sizes and levels of recalcitrance. The operational definition of DOM—material passing through a 0.2-μm filter—means that it ranges from small molecules that can be transported directly into cells (osmotrophy) to large macromolecules or colloids that may require extracellular digestion (saprotrophy) prior to osmotrophic utilization. Labile forms of organic matter are quickly respired or assimilated by bacteria and protists into living POM (72, 73). Thus, labile DOM accounts for <0.2 Pg C of the 662 Pg C of measurable marine DOM in a recent global survey, and most of the organic carbon pool is resistant to oxidation on time scales from months to millennia (71).

The diversity of marine organic carbon compounds necessitates an elaborate network of heterotrophic strategies to decompose it. Most ocean models assume that DOM and POM are oxidized by a homogeneous class of prokaryotic heterotrophs that live largely by chemistry transacted at the cell surface via enzymes that modify, transport, and/or remineralize organic molecules (i.e., chemoheterotrophs). Ironically, although fungi are considered central to terrestrial decomposition, eukaryotic saprotrophs are not represented in ocean ecosystem models, despite growing evidence of diverse marine fungi (74, 75). Recent reports indicate that correlations exist between fungal populations and total organic carbon, nitrate, sulfide, and dissolved inorganic carbon, in anoxic marine pelagic environments and especially at the sea floor (76, 77). If fungi do contribute to marine organic matter degradation—an idea that has not yet been formally tested—it will be important to learn how flexible they are in this role. To what extent do they and other eukaryotes that function as marine saprotrophs compete for the same substrates? Labyrinthulids and thraustochytrids

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Box 1. Evolutionary history of algal endosymbiosis and putative plastid losses.

Mitochondria and plastids both arose from the endosymbiotic uptake of a bacterium (an alphaproteobacterium and a cyanobacterium, respectively), but the subsequent evolution of plastids has been complicated by additional endosymbiosis events. The original or "primary" plastid that descended directly from the cyanobacterial endosymbiont is still found in the archaeplastids (glaucophytes, red algae, green algae, and plants). But green and red algae have themselves been taken up by other eukaryotic lineages, resulting in "secondary" plastids characterized by the additional membranes and more complex protein-targeting systems present in euglenids, chlorarachniophytes, cryptophytes, haptophytes (also referred to herein as prymnesiophytes), stramenopiles, dinoflagellates, and apicomplexans. Some members of these groups are mixotrophic, and others are purely heterotrophic (predatory, saprotrophic, or even parasitic) because photosynthesis or plastids have been lost. Although green algae are common in marine environments (e.g., picoprasinophytes such as *Bathycoccus, Ostreococcus*, and *Micromonas*), lineages resulting from secondary endosymbiotic partnerships include many other important marine primary producers (e.g., diatoms, pelagophytes, prymnesiophytes, and dinoflagellates) and represent incredible metabolic versatility. It is interesting to speculate that the redundancy and reshuffling of characteristics resulting from mergers of distinct eukaryotic lineages favors new combinations of traits with strong ecological potential.

Single primary endosymbioses — Nucleus — Cyanobacterium

Primary plastid — Green algae and plants, Red algae, Glaucophytes

Multiple secondary endosymbioses

Secondary plastid — Euglenids (3 membranes), Chlorarachniophytes (+ nucleomorph), Cryptomonads (+ nucleomorph), Haptophytes, Stramenopiles, Dinoflagellates (3 membranes), Apicomplexans

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are stramenopiles hypothesized to primarily use terrestrial organic matter present in coastal marine habitats, but they have also been reported in oligotrophic waters (*78*). Preliminary experiments suggest competition with prokaryotes (*79*), but to what extent is this true in nature? Are the activities of eukaryotic saprotrophs largely restricted to sediments, or do they operate in the water column as well?

Eukaryotes also use endocytosis to ingest high–molecular weight and colloidal DOM, rich sources of trace metals (*80*). The Picozoa ingest, then process marine colloids of <150 nm diameter in a vacuole (*81*). These colloids are similar in size to many marine eukaryotic viruses. Interestingly, virus sequences were detected in association with a sorted picozoan cell (*82*) potentially from an

infecting virus or food. Regardless, the discovery of colloid-sized particle ingestion by picozoans has important implications for remineralization rates, because intracellular processing is presumably more efficient than extracellular mechanisms.

Another means by which heterotrophic protists acquire nutrition is parasitism, an efficient strategy once the host is encountered. If life in oceans is anything like that on land, then for every marine animal species there are likely several parasite species. Infective life stages (Fig. 3, inset) allow more constant access to higher concentrations of organic material than are encountered by most free-living microbes. The most abundant sequences in marine protistan diversity surveys are novel uncultured marine alveolates (MALVs) that belong to the Syndiniales (*21*, *83*).

Several Syndiniales groups are known to be parasitic and can control blooms of other marine dinoflagellates or infect ciliates, other protists, and animals (*83–85*). The interplay between the life cycles of one such parasite, *Amoebophrya*, and its dinoflagellate host is complicated, with infection speeding up host cyst formation in a response hypothesized to promote resistance to infection (*84*). Currently, direct evidence that most MALV clades are parasitic is lacking. Associations observed between uncharacterized MALVs and radiolarians (*86*) are equally consistent with a symbiotic relationship, and environmental factors could potentially shift a relationship from commensal to pathogenic. For MALVs that are parasitic, what taxa do they infect and by what mechanism? Is the outcome of infection benign
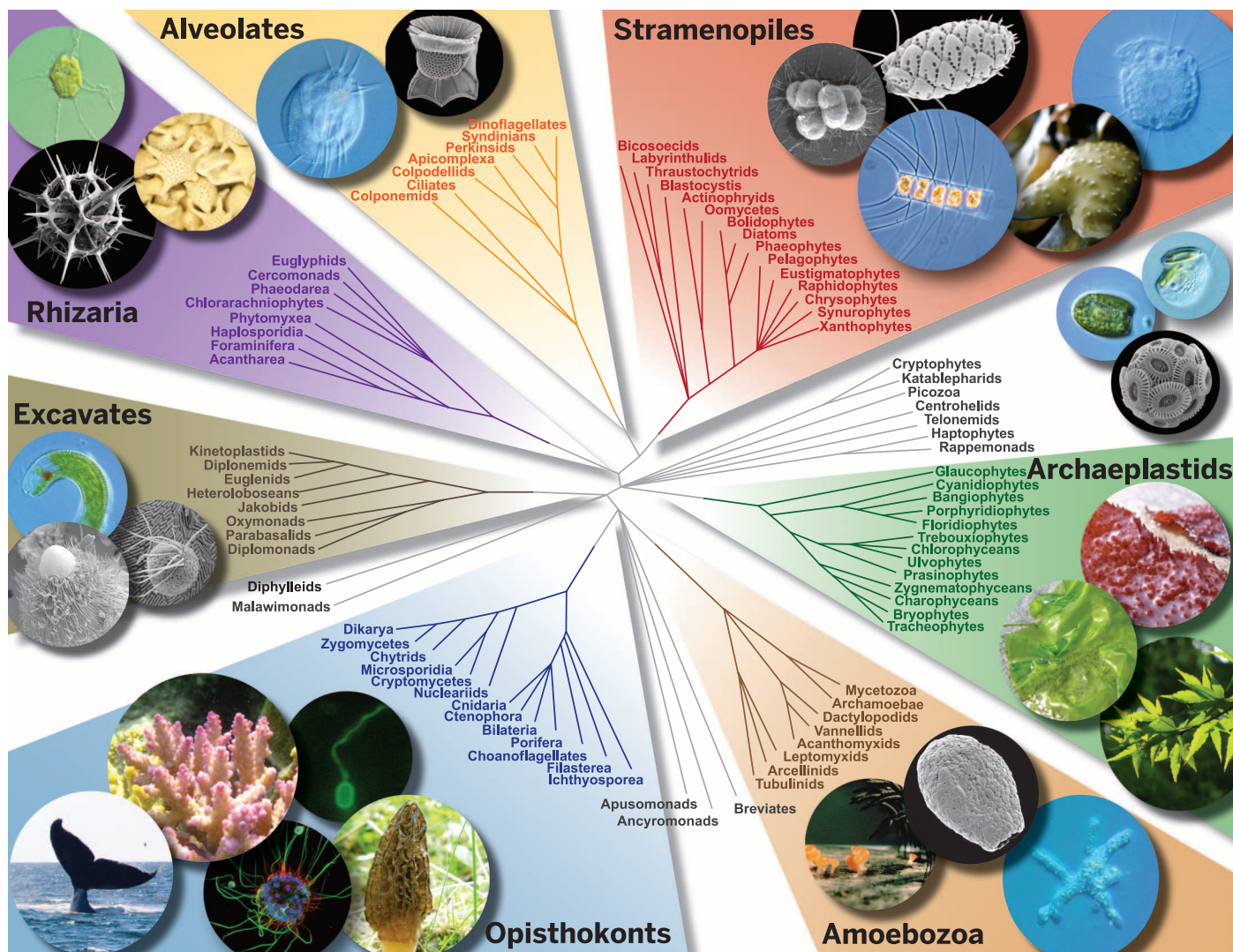

Fig. 2. Protists constitute the majority of lineages across the eukaryotic tree of life. This schematic represents a synthesis of information on morphology, phylogenetic analyses (based on a few genes from a large diversity of organisms), as well as phylogenomic analyses (of many genes from representatives of major lineages). Seven "supergroups" are indicated by colored wedges, all of which contain multiple marine protistan lineages. Relationships of groups listed outside the supergroups remain contentious. Peripheral pictures highlight eukaryotic diversity, both microbial and multicellular. Clockwise from right: archaeplastids (rhodophyte, chlorophyte, streptophyte); amoebozoa (tubulinid, arcellinid, mycetozoan); opisthokonts (fungus, microsporidian, choanoflagellate, cnidarian, bilaterian); excavates (parabasalian, oxymonad, euglenid); rhizaria (acantharian, foraminiferan, chlorarachniophyte); alveolates (ciliate, dinoflagellate); stramenopiles (labyrinthulid, synurophyte, diatom, phaeophyte, actinophryid); unassigned [cryptomonad, katablepharid, haptophyte (referred to here as prymnesiophytes, and to which coccolithophores belong)]. [Phylogenomic analyses adapted from (*158*)]

or pathogenic? Do they have complex life cycles, reservoir species, or active free-living stages?

Parasitism presents challenges for ecosystem modeling, even if it is an easily interpretable nutritional mode. Thus, parasites, like protistan saprotrophs, have not been incorporated into ecosystem and carbon cycle models. What are the tradeoffs between protection against predation, resource acquisition strategies, and costs leveraged by high abundance that increase encounters with parasites and viruses? Some parasites also consume bacteria by phagocytosis. For example, the human parasite *Trichomonas vaginalis* has a predatory mode that remains "unseen" in its genome (*87*). Hence, the complexity of protistan trophic modes underscores the importance of study-

ing organisms in natural or near-natural milieus where sundry interaction possibilities are maintained.

Diverse metabolic pathways facilitate organic matter processing in all domains of life, but in eukaryotes the evolution of phagotrophy and parasitism, in addition to osmotrophy and saprotrophy, renders several carbon oxidation modes difficult to identify (Fig. 3). Phototrophy is easily recognized by huge, highly conserved macromolecular complexes that are found in plastids, the semi-autonomous organelles of photosynthetic protists (Box 1). In contrast, heterotrophy is a diverse amalgam of pathways that overlaps with widely distributed anaplerotic pathways that form intermediates used in both autotrophic and heterotrophic metabolisms. Moreover, the specific

mechanics of most types of protistan heterotrophy are shadowy. Genomic and proteomic studies could provide candidate genes for cell surface properties related to prey sensing (*88*) or host sensing by parasites. Although all aspects of these processes are doubtless driven by proteins encoded in nuclear genomes, these are likely to be among the ~50% of proteins in any given genome that have unknown functions (*89*). Because eukaryotic mechanisms relating to heterotrophy may have evolved in parallel (in contrast to shared ancestry of photosynthetic systems; Box 2), a master list of genes relating to feeding in one predator might have limited predictive power for other predatory lineages. As a result, we lack a catalog of genes responsible for the cellular components
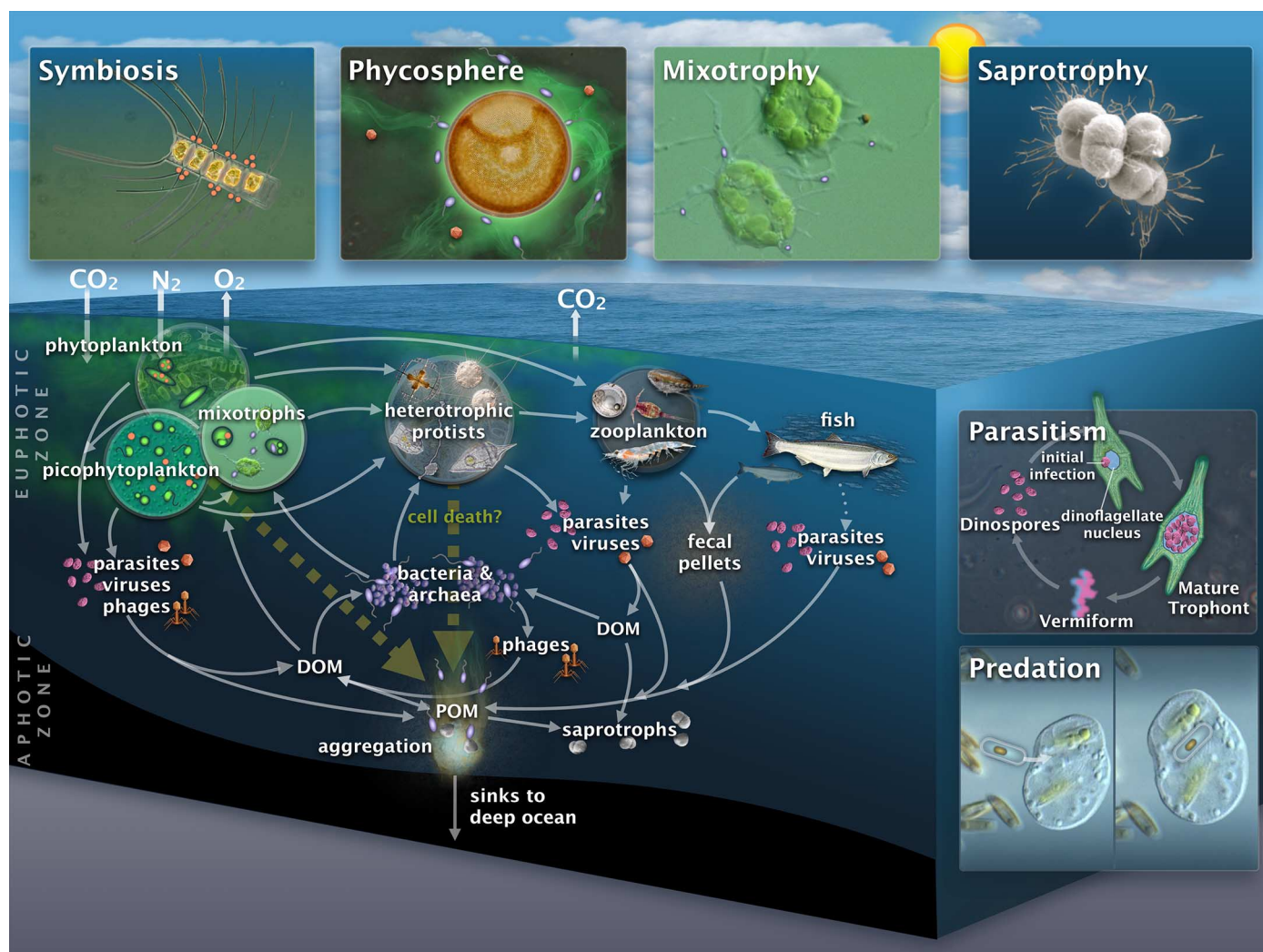
Fig. 3. Microbes from the three domains of life interact to structure ocean ecosystems and the carbon cycle. CO_2 fixation into organic matter is performed by diverse photosynthetic eukaryotes, as well as cyanobacteria, that have different food web linkages. Eukaryotes are involved in many interactions, as depicted in inset boxes. Some widespread phytoplankton consume other microbes (mixotrophs; see Box 2). Eukaryotic parasites are another force of mortality that presumably affects many types of eukaryotes (with host specificity), as do viral infections. Death by parasite or viral infection results in release of a continuum of nondissolvable organic matter (i.e., POM) and DOM, a complex suite of polymers and molecules. Programmed cell death

of protists (apoptosis; background arrow) has also been proposed. Microbes contribute to CO_2 respiration (e.g., archaea, heterotrophic bacteria, saprotrophs, parasites, mixotrophs, and even photosynthetic taxa at night), which can result in CO_2 release to the atmosphere and reduces organic carbon export to the deep ocean. Some eukaryotes grow in low-nitrogen regions by using symbiotic relationships with N_2-fixing bacteria (upper left box). The majority of protistan roles and linkages are not represented in ecosystem and carbon cycle models, and most have not yet been rigorously quantified. Inorganic nutrients and direct release of CO_2 depicted in Fig. 1 have been removed for simplification purposes. [Parasite life cycle adapted from (*159*)]

involved in protistan heterotrophy, such as feeding apparatuses, receptors for specific host or prey recognition factors, or signaling pathways that govern predatory behavior and trophic mode shifts.

Shifting boundaries between trophic modes: Mixotrophy

In addition to the oversimplification inherent in the convenient categories of "producers" (algae) and "consumers" (heterotrophs), a larger problem is that many protists do not even stick to one or the other role. Recognition that mixotrophy—a fusion of phototrophy and heterotrophy—is important arose in 1986, when seminal research on freshwater plankton showed that "…at least some natural phytoplankton are phagotrophic and apparently obtain a substantial fraction of their energy and nutrients by ingesting bacteria at rates very similar to those measured for nonphotosynthetic microflagellates" (*90*). This publication was quickly followed by reports on mixotrophic marine algae (*91*), and more recent shipboard experiments demonstrated that small algae perform 37 to 95% of total bacterivory in the Atlantic Ocean (*32*).

Phagotrophic capabilities in otherwise photosynthetic organisms have exciting ecological implications. Prey ingestion may serve as a source of energy and carbon when photosynthesis is limited by light availability (e.g., deep in the water column or under ice) (*92*). However, in oligotrophic euphotic waters where uncultured mixotrophic prymnesiophytes and chrysophytes (stramenopiles) are prominent (*31, 93, 94*), acquisition of nutrients needed for photosynthetic growth, not carbon, is considered the feeding trigger (*32*). Through prey digestion, demand for scarce inorganic nutrients is lessened because particulate nutrients (from the prey cell) support primary production,

thereby lowering release of remineralized nutrients relative to heterotrophic predation. In primarily heterotrophic mixotrophs, respiratory carbon needs can be met by photosynthesis, making high assimilation efficiencies possible (*95*). If group-specific predation (and photosynthetic inputs to growth) can be accurately estimated, the influences of dissolved inorganic nutrients and remineralization rates can be better assessed.

Little is known about how biotic and abiotic factors influence where a protist resides along the spectrum between heterotrophic and photoautotrophic growth at any given time, or species-level variability. Experiments are difficult, in part because most cultured predators, mixotrophic and otherwise, reside with prey communities and symbionts (if present) that restrict defined experimentation. Moreover, many groups are not represented in culture. Uncultured prymnesiophytes contribute significantly to open-ocean primary production (*30, 31, 94*). Some of these same prymnesiophytes are mixotrophic (*93, 94*) and consume *Prochlorococcus*, the most abundant photosynthetic organism on the planet (and presumably prey upon other picoplankton as well). If such organisms shift trophic modes opportunistically—perhaps to capture resources through predation when that strategy becomes more favorable—then the environmental triggers behind such a shift fundamentally reshape their ecosystem roles and food web dynamics.

Predatory protists, whether mixotrophic or strictly heterotrophic, will likely be affected by ocean change through direct temperature effects and by altered composition of prey. The mixotrophic chrysophyte *Ochromonas* switches to more heterotrophic nutrition under elevated temperatures, although it remains unknown whether higher CO_2 availability reverses this effect (*96*). A shift toward smaller phytoplankton, as observed

with warming in the Arctic and in ocean acidification experiments, favors nanoflagellate grazers over larger predators (*50, 97*). Altered phytoplankton stoichiometry (due to increased CO_2 availability) also affects copepod consumption rates, modifying growth and the fraction of organic carbon converted to CO_2 or excreted as DOM (*98*).

There are multiple further complications. For example, not all phototrophs rely on canonical oxidative photosynthesis. The light-driven proton pump proteorhodopsin, known from some marine bacteria (*99*), occurs in photosynthetic and heterotrophic eukaryotes (*100*). Its distribution and function are still poorly understood, but new copies are being found in additional lineages. Marine proteorhodopsins derived from eukaryotes were recovered in metagenomic surveys but were misinterpreted as being bacterially derived, until they were found and characterized in cultivated protists (*100*). This highlights the power of meta-omic approaches as well as the important role of reference data from cultured taxa in the process of extrapolating function from metagenomic data.

The role of behavior: Ecological capabilities beyond carbon processing

Typically, bacterial and archaeal diversity is manifested at the level of metabolism, whereas eukaryotic diversity is strongly influenced by their cellular structures and, by extension, behavior (*101, 102*). Bacteria and archaea do use sensing and motility (chemotaxis) to optimize their location and communicate by means of pheromones (*103*). Nonetheless, our view of how prokaryotes contribute to the marine carbon cycle (and food webs in general) is dominated by analyzing the sum of the enzymes and biochemical pathways they express, even when microscale heterogeneity (itself influenced by eukaryotic cells' activities, death, and aggregation) is taken into account (*104–106*). Comparative methods can be used to extrapolate these enzymes and pathways from genomic and metagenomic data reasonably well, which in turn allows hypotheses to be generated on their ecological roles, to be generated largely from sequence data.

For eukaryotes, the balance between metabolism and behavior is tipped in the opposite direction: Metabolism may be diverse, but environmental interactions are strongly guided by cellular structures and the behaviors they underpin. Protists can build traps and feeding apparatuses; they not only move about but often actively hunt down specific prey (*57, 107*). They also take actions to evade hunters or to build defenses against them. Protists actively capture other cells, take them up, and digest them or domesticate them (see below). These characteristics emerge from protein functions and interactions that are several degrees further removed from genomic sequence data than are enzymatic pathways. As a result, we are far less adept at reconstructing cellular structures and behavior from sequence data—or, by extension, the role of diverse eukaryotic microbes in the marine carbon cycle.

Of course there are exceptions; many eukaryotes take advantage of metabolic versatility much

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Box 2. Lifestyles and specialized interaction zones.

(i) Primary producer: Generates organic carbon by photosynthesis and CO_2 fixation (the role traditionally played by phytoplankton).

(ii) Osmotrophy: Cells take up organic material from the external environment as small molecules or macromolecules.

(iii) Saprotrophy: A mode of osmotrophy whereby extracellular digestion is involved in processing or recycling organic matter, also often referred to as chemoheterotrophy.

(iv) Predation: Consumption of prey cells, often by phagocytosis.

(v) Mixotrophic predation: A combination of phototrophic and heterotrophic metabolism. In some cases, mixotrophs may alternate between trophic mechanisms (e.g., predation, which can result in release of CO_2, and photosynthesis, resulting in capture of CO_2).

(vi) Parasitism: Defined here as one organism existing in association with another to the detriment of that partner (Fig. 3 inset shows a dinoflagellate infected by *Amoebophrya*).

(vii) Phycosphere: A region surrounding phytoplankton cells that results from the basic physics of the diffusive boundary layer and has a higher concentrations of organic matter (produced by the eukaryote) than local waters. This zone attracts free-living prokaryotes, some of which may attach (and could also include other eukaryotes). The zone of influence in the fluid medium is disproportionately greater for larger phytoplankton cells and is considered akin to the rhizosphere (Fig. 3 inset depicts a diatom phycosphere containing bacteria).

(viii) Symbioses: Defined here as mutualistic relationships where one species lives on or within another species (Fig. 3 inset shows a diatom with N_2-fixing cyanobacteria on its spines).

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as bacteria do (e.g., fungi), and some ecologically important activities are readily interpreted from genomic data (e.g., photosynthesis). But, as we have seen above, we lack the catalog of gene functions needed to recognize many trophic behaviors, or the mechanisms that control shifts between trophic modes. This problem is particularly acute for free-living protistan heterotrophs and mixotrophs, many of which are the most structurally complex but least investigated microbial cells.

Complexity through associations: Phycosphere, symbioses, and viruses

Heterogeneities in carbon pools will also be affected by physical associations, some of which are mutualistic. Bacteria (and potentially archaea and protists) actively detect and take up exuded extracellular products in the zone around phytoplankton known as the phycosphere (Box 2 and Fig. 3) (106, 108–110). The associated bacteria can be attached, or motile and unattached, but so far phycosphere interactions and the molecules exchanged have proven difficult to discern.

Symbiotic associations involving protists are also well known, although overlooked in microbial food web dynamics. Many involve metabolite exchanges, sometimes where the protist is a symbiont within an animal (e.g., algae in corals), or where heterotrophic protists play host to bacterial or eukaryotic algal symbionts, or algal organelles retained by kleptoplastidy (111–114). Thus, mixotrophy is not restricted to phytoplankton capable of phagocytosis, but is also widespread among heterotrophic ciliates, dinoflagellates, foraminifera, and acantharians (59, 115). Obligate N_2-fixing cyanobacterial symbionts are present in some diatoms, dinoflagellates, and prymnesiophytes (116–118), although destructive sampling of the latter initially led to the mistaken conclusion that the symbionts were free-living cells, thus emphasizing the value of visual observation. These symbioses allow goods exchanges, including newly fixed N, that represent alternative resource acquisition strategies for the hosts. They thereby stimulate productivity and influence overall nitrogen availability (119).

Viruses play important but unquantified roles in structuring microbial communities and interrupt flows of carbon and nutrients through the microbial loop (10, 120–122). Labile DOM generated through viral-mediated cell lysis can be rapidly assimilated and remineralized by microbes, promoting nutrient retention in the euphotic zone and potentially decreasing the efficiency of carbon export to the deep sea (121–123). However, infection of photosynthetic protists can also enhance production of exudates, which stimulate particle aggregation and facilitate export (123, 124). Notably, viruses can influence predator populations directly by infection and lysis or by reducing prey availability (122, 125). Indirect effects involve increased bacterial growth on released DOM and POM, resulting in community changes. Moreover, viruses themselves may serve as food particles.

Like their hosts, fundamental differences between eukaryotic viruses and phages necessitate separate empirical methods for characterization and explicit inclusion in marine food webs (Fig. 3). Some eukaryotic viruses appear to exploit mechanisms of programmed cell death during lysis (124), and some may promote sexual cycling in species where ploidy level determines viral susceptibility (126), but this and other resistance mechanisms are not well understood (127). An extreme example of how marine eukaryotic viruses differ from phages is the giant virus that infects a predatory stramenopile alongside a smaller "virophage" that parasitizes the giant virus (128). The diversity of protists and their viruses may preclude broad commonalities for several aspects of interactions (121). Challenges moving forward include linking natural viruses (and viral sequences) to actual hosts, examining viruses as food particles, and incorporating the nuances of species-specific viral interactions in ecosystem models.

Diverse data for a complex problem

An overwhelming volume of sequence data is being collected and analyzed from marine microbes. Its value for characterizing protistan contributions to autotrophic and heterotrophic functionality, and teasing apart their interactions with other microbes and consequent influences on biogeochemical cycles, will be maximized by tools and approaches tailored to the unique biology of protists. Statistical approaches that detect interactions between organisms using co-occurrence networks can help development of hypotheses about the ecology of eukaryotic plankton (129, 130). Massive sequencing of marker genes (or even metagenomic and metatranscriptomic sequences) provides such data, with the caveat that for success large numbers of samples are needed, as are experimental designs that adequately cover key environmental variables and involve rate measurements.

Although rare, studies designed to capture physical associations between cells provide critical avenues for validating ecological interaction networks. For example, genome reconstruction can help to identify traded services, the hallmark of symbioses. Thus, "single cell" sequencing with enough coverage to highlight key metabolic pathways absent from one but present in the other partner can provide evidence of symbiosis. A robust example with relevance to global geochemical models is N_2 fixation by symbionts (116–118). However, in cases involving new and unknown interactions, sequencing alone may not resolve the true nature of interactions, but rather can lead to (mis)interpretation as symbiosis, contamination, predation, presence of osmotrophically degraded material, infection, or even a horizontal gene transfer (131).

A few "rules" that have ensured success in meta-omic studies are not as readily met by protists. These are (i) having a reference genome to help recruit relevant sequences [not the case for most marine protists because only ~20 genomes are available]; (ii) having a small genome and sufficient sequencing depth [genome sizes from free-living protists vary from ~10 MB to much larger than the human genome]; and (iii) having low system diversity (i.e., few taxa present)—a situation sometimes true in extreme environments, but not commonly so. Eukaryotes present other types of genome complexity as well, such as regions of widely varied composition that complicate binning without a reference genome (89) and "mosaic" phylogenetic signals that require deciphering complex evolutionary histories (2, 31, 43, 44, 132). Transcriptome sequencing and assembly has improved protistan reference databases while minimizing genome structural complexity issues (133). Another strategy used to sidestep such issues for wild planktonic protists is cell sorting based on optical signatures, which raises coverage of target organisms by suppressing diversity, making genome assembly more likely (26, 31, 62, 82, 134–136). Microfluidic approaches that sort cells by imaging and thereby link genomes to morphology will further surmount these problems (136). Higher-throughput applications of these technologies are needed to expand genomic representation of protistan complexity.

Biology has much to gain from expanded genomic coverage of protistan diversity, especially as a broader range of trophic strategy variants is investigated. Marine protistan genomes sequenced thus far have resulted in massive strides in understanding the origins of multicellularity (137, 138), intron evolution (139, 140), genome integration and control (43), and even fundamental aspects of methylation, nucleosome positioning, and chromatin compaction (141). Major gains will also be made for marine phytoplankton, given that the evolutionary distance between the two genome-sequenced diatom genera (*Thalassiosira* and *Phaeodactylum*) is greater than that between humans and fish (132), and marker analysis of different clades within the prasinophyte genus *Micromonas* shows divergence equivalent to the separation between corn and rice (139, 142).

Still, genomics has limits. So, as questions about the carbon cycling activities of protists are formulated in greater detail, we need to ask: Where are new strategies needed? One such area involves functional studies on ecologically important model organisms in environmentally relevant conditions. For example, omic data facilitated the discovery that microbes previously considered dependent on exogenous vitamin B_1 grow in its absence, but only after pathway gap theory (143) was implemented together with experiments on cultured species (144, 145). What was thought to represent (incomplete) pathways in the evolutionary process of reduction is actually a noncanonical vitamin pathway dependent on the import of a precursor that can be more abundant in seawater than the vitamin itself. Once understood in a few cultured organisms, the B_1 (thiamine) metabolism of diverse planktonic bacteria and eukaryotic phytoplankton could be reinterpreted (144, 145). These discoveries change "facts" regarding phytoplankton bloom controls and reveal the likely involvement of several proteins of unknown function in thiamine biosynthesis. Such studies illustrate that the power of comparative approaches is contingent on reference data, but more importantly that

the greatest gain is harvested from omic data sets when paired with detailed studies on the biology of strategically chosen marine taxa.

One of the biggest obstacles to interpreting protistan genomes is the dearth of information about the evolution of proteins that control behaviors such as mixotrophy, predation, and parasitism, as well as regulatory strategies—for example, those that control energy allocation (*146*) to optimize growth. The recent discovery of extensive oxidative metabolism for one-carbon and methylated compounds in the hyperabundant Pelagibacterales clade of marine bacteria illustrates how geochemically important information lay within plain view in genomes for a decade before being deciphered (*147*). This discovery focused attention on a neglected but apparently important sector of the carbon cycle: methylated and volatile compounds. This brings us to a second answer to the "Where are new strategies needed?" question. A great deal can be garnered from pathway gap theory, but in the end, studies that infer higher-order function from genomes are founded on experimental proof of protein functions. One of the biggest impediments to systems biology and ecosystems biology (*104*, *148*) approaches is that the majority of important marine protistan groups (even algae) lack representatives with established genetic systems. Robust but relatively low-efficiency manipulation systems exist for two algae with limited marine distributions: the diminutive picoeukaryote *Ostreococcus tauri* (*149*) and the diatom *Phaeodactylum tricornutum* (*150*). New knowledge on marine protistan diversity and distributions calls for concerted initiatives to develop genetic systems that target ecologically relevant lineages from across the eukaryotic tree, and to develop parallel technologies such as CRISPR (clustered regularly interspaced short palindromic repeat) and TALEN (transcription activator–like effector nuclease)–based gene editing. These, as well as sampling that preserves microbe-microbe interactions, improved visualization, and experiments in advanced bioreactors that allow testing of multifactorial impacts, precise modulation of CO_2, light, and nutrients, and real-time measurements of physiological parameters, are key steps forward.

Ecological and biogeochemical modeling

Rapidly advancing bioinformatic and statistical approaches must be complemented by mechanistic models that encapsulate and synthesize understanding of complex systems at cellular, community, and global scales. Marine protistan diversity presents challenges for theory and modeling: Can we understand the rich and flexible ecology of these functionally diverse organisms to interpret and simulate it with mathematical and numerical models? What facets of their biology affect carbon flow and sequestration sufficiently to warrant inclusion in climate and carbon cycle models? Consider mixotrophy: Now that field studies show its prevalence [e.g., (*32*, *94*)], should global carbon cycle models that currently only separate autotrophy and heterotrophy capture this complexity? To date, the costs and ben-

efits of mixotrophic predation have been addressed by few studies (*151–153*). This emphasizes the need to evaluate trophic strategies with data and modeling, explore their biogeochemical importance and food web impacts, and assess how they modify predictions by global models.

Modeling paradigms generally resolve a few coarse phytoplankton phenotypes with highly simplified carbon and nutrient flows between them. Currently, diversity within a few functional types related to cell size or temperature adaptations can also be resolved (*14*). Yet many chemical exchanges occur between protists and their neighbors; are these simplified descriptions sufficient to describe the system? Of particular interest is how diversity and connectivity affect system stability—a question long debated in the context of food webs (*154*). The ability to predict how plankton populations and their biogeochemical roles respond to a changing environment is tightly linked to this question. For example, in a recent five-box model, the relative abundance of fast- and slow-growing phytoplankton taxa controlled community elemental composition and new nitrogen import rates (*155*). In another study, inclusion of contributions from various system members (including microbes) helped to reconcile aphotic zone flux terms that were long out of balance (*156*). These studies highlight the need for improved input data (even relating to the basic elemental stoichiometry of different algae), but also demonstrate that idealized models can be tools for exploring how complex networks behave and for generating hypotheses that can be tested in the lab and field.

The black-box descriptions of physiology used in current ecosystem models neglect the powerful constraints of redox and energy balance that become explicit once cellular metabolism is resolved. Environmental engineers have exploited these constraints using schematic metabolic networks. Flux balance analysis (*157*), a tool from systems biology, extends this approach to provide mechanistic, quantitative predictions of growth rates and efficiencies rooted in genome-scale metabolic reconstructions. Such a goal requires considerable advances in protein function annotations, as well as rigorous testing with model organisms. However, looking forward, the application of flux balance analysis in models of marine ecosystems will enable a vastly richer repertoire of applications and will bring empirical and theoretical perspectives together with a common language.

Conclusions

The complexity of protistan behaviors will test the perspective that mechanistic models of cell functions can be merged with global models to understand marine ecosystems. How scientists will winnow this complexity to find those kernels of protistan biology most important for ocean ecosystem-level processes and carbon cycling remains to be seen. The diverse biology of protists is not yet understood well enough to fully evaluate their whole impact on biogeochemical models, but enough is known to say their roles must be reevaluated. Protistan genomes and behaviors

are innately challenging to decipher but are essential to dissecting trophic connections. Technologies that overcome scale-related issues of sequencing and proteome analyses are helping, as is the application of network theory and modeling to identify ecological links and generate testable hypotheses. As hypothetical links are identified, the challenge is to validate them by characterizing the underlying mechanisms, constrain them by empirical measurements, and parameterize them into global biogeochemical models. The most direct source of data on trophic modes and interactions will come from studying cells at multiple levels, integrating cell and structural biology, physiology, and behavior. Combining investigations of cellular systems with a broader sampling of eukaryotic diversity will empower comparative approaches with the promise of substantial progress in quantifying activities of protists and their impacts on the global carbon cycle.

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Science

Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes

Alexandra Z. Worden, Michael J. Follows, Stephen J. Giovannoni, Susanne Wilken, Amy E. Zimmerman and Patrick J. Keeling

Changing tastes in marine microbe food webs

Protists are single-celled organisms complete with nuclei, organelles, and symbionts, and possess a multiplicity of physiological talents. They are ubiquitous, abundant, and often neglected by science. Worden *et al.* review the challenges of understanding the role protists play in geochemical cycling in the oceans. These organisms can photosynthesize like plants, graze on bacteria and archaea, parasitize each other and bigger creatures, have sex, and sometimes do all these things serially as conditions change. Their activities may have a significant influence on carbon cycling, and research efforts need to be amplified to understand their functional importance in marine ecosystems.
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