Heterotrophic Planktonic Microbes: Virus, Bacteria, Archaea, and Protozoa

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4.2.2

BACKGROUND AND HISTORICAL DEVELOPMENT

Marine Microbial Ecology into the 1970s

Although heterotrophic marine microorganisms in the open sea have been studied since the late 1800s and early 1900s by pioneers like Fischer, Haeckel, and Calkins (1–3), the contribution of these diminutive species to the food webs of oceanographic systems was not fully recognized until nearly a century later. Attention was drawn to the larger and more conspicuous taxa of photosynthetic protists (e.g., diatoms and many dinoflagellates) early in the history of biological oceanography. In contrast, little was known of the abundances or activities of bacteria in the ocean until the past 50 years, and most early studies of marine heterotrophic protists (the protozoa) focused on morphological descriptions and natural history of larger species of these taxa rather than their functional roles in marine food webs. Improvements in microscopy methods used to observe bacteria in seawater during the 1930s to 1950s indicated that bacterial abundances were several orders of magnitude greater than previously believed. However, confusion remained during regarding the ecological significance of marine bacteria because these early abundance estimates were often hundreds of times greater than counts made by cultivation techniques (4).

During the 1960s and 1970s, however, the metabolic activity of aquatic bacterial assemblages was demonstrated by the uptake of radioactive organic compounds in marine and freshwater samples (e.g., [5, 6] and others). These studies demonstrated that organic compounds were readily turned over by microorganisms in aquatic ecosystems, and that microbial communities appeared to be quite dynamic. During that same period, abundant and diverse assemblages of protozoa were demonstrated from a wide array of aquatic ecosystems using more quantitative approaches for the collection, observation, and enumeration of eukaryotes (7–10). Together these observations implied the presence of an active and complex microbial community that might be responsible for much of the metabolic activity in marine ecosystems. This hypothesis stood in contrast to broad oceanographic models of the time that included the bacteria only as a sink for nonliving organic matter on the sea floor and largely ignored the potentially important roles of heterotrophic protists (11).

The Microbial Loop Revolution

Pomeroy's prescient analysis, "The ocean's food web, a changing paradigm" (12) had a significant impact toward transforming the field. This publication is often cited by many microbial ecologists as a turning point in our understanding of the structure and function of marine ecosystems. In it, Pomeroy pointed to lines of evidence that were starting to emerge showing that the smallest members of the food web, including heterotrophic bacteria, cyanobacteria, and small protists (algae and protozoa <20 μm), were probably responsible for a large fraction of important system activities such as overall respiration, photosynthesis, and organic matter turnover (i.e., the ingestion of food particles by protozoa or the uptake of dissolved substances by bacteria). Early studies of bacterial utilization of dissolved organic matter (e.g., 1), and subsequent studies of community metabolism into the 1980s (e.g., 14) indicated that this process accounted for a surprisingly high percentage of total organic matter turnover. Similarly, Fenchel and Jorgensen (15) pointed out that approximately 10–30% of primary productivity might be released as dissolved organic matter, which was then presumed to be taken up by bacteria who respired a portion and passed the rest on to the next trophic level, composed largely of protozoa. In this scenario, protozoa constituted a mechanism by which bacterial biomass reentered the "classical" food web including the metazoan zooplankton and nekton, and also served as a means of remineralizing some of the bacterial biomass back to inorganic nutrients and carbon dioxide for subsequent utilization by primary producers. Although these conclusions were later found to be generally correct, the concepts were a radical departure from the established general biological oceanographic thinking of the time.

The introductions of epifluorescence microscopy of fluorescently stained cells (16) and polycarbonate filters to position the cells all in a single optical plane (17) were significant technological advancements that allowed much more accurate estimates of the total number of microorganisms present in natural water samples. Using this method, it was determined that bacteria are typically present at abundances of 1 million cells per ml in near-surface seawater. This number is surprisingly constant around the world, with most variation falling within a factor of 10 worldwide. Appreciation of the importance of medium to large protozoa (i.e., >20 μm) in oceanic food webs was made possible in the late 1960s and early 1970s largely through the pioneering work of Beers

doi:10.1128/9781555818821.ch4.2.2

et al. (7, 18). However, as with marine bacteria, epifluorescence microscopy facilitated the observation of small single-celled eukaryotes during the 1970s and 1980s and enabled easy discrimination of protists without chloroplasts (protozoa) from those with chloroplasts (algae) based on the autofluorescence of photosynthetic pigments (19–21). The development and refinement of this approach for eukaryotic microorganisms was instrumental in establishing the standing stocks of small protozoa, which typically occur at abundances of tens to thousands per ml in most marine ecosystems. The development of microscopy approaches for larger protozoa such as heterotrophic dinoflagellates and ciliates (10, 22) has been equally important in documenting abundances of these taxa that range up to tens per ml in much of the world's oceans.

Estimates of the overall biomass of various microbial assemblages, and techniques to measure rate processes (e.g., rates of growth, substrate uptake, prey consumption) began to appear in the late 1970s and 1980s, and the refinement of these estimates and measurements continue to the present day. Early attempts to measure bacterial growth rates in seawater involved "indirect" methods such as relating the frequency of dividing cells to rates of division in cultured strains (23). Isotope-uptake based approaches, specifically the incorporation of radioactively labeled thymidine into DNA (24, 25) and/or the incorporation of leucine into protein (26) have become the most commonly used methods (see "Estimating 'Bacterial' Biomass and 'Bacterial Production'"). These methods have indicated that bacterial doubling times can be on the order of one day in coastal temperate waters. Combined with estimates of bacterial biomass, these results led to the conclusion that bacteria must be consuming a substantial proportion—on the order of 50%—of the total system primary productivity. A similar conclusion was reached using direct estimation of microbial respiration by careful measurements of oxygen concentration changes (micro-Winkler method) in seawater that had been prefiltered through 5 μm pore filters to remove animals and many of the protists (14).

During this same period, small protozoa (primarily flagellates and ciliates) were gaining recognition as important consumers of bacteria in the marine plankton and benthos (27–29). An increasing volume of experimental work demonstrated a dominant role for small, bacterivorous protozoa as a mechanism for removing bacterial production and repackaging bacteria into larger particles that might be consumed by metazoan zooplankton. Also, it became recognized around this time that a significant fraction of the phytoplankton biomass and production was consumed directly by herbivorous protozoa rather than by metazoan zooplankton such as copepods (30, 31). Consequently, heterotrophic protists were acknowledged as an important food source for a variety of metazoan zooplankton, and numerous experimental studies subsequently demonstrated this trophic connection (32, 33).

These observations were synthesized in a second benchmark paper (34). The latter publication marked the beginning of the widespread recognition and use of the term "microbial loop" in marine planktonic systems, a concept that emphasizes the remarkable importance of the tiniest organisms as well as dissolved organic matter as an intermediate in material and energy transfer in aquatic ecosystems. An updated illustration of this basic concept is shown in Fig. 1.

Definitions and Concepts

Abundance: the number of individuals in a sample or a population.

Algae: protists that exhibit phototrophic nutrition. Like protozoa, algae span a wide size range (<1 to >200 μm), and have generally been referred to as phototrophic pico-, nano-, or microplankton.

Amensalism: interaction where members of a species inflicts harm to another species without any costs or benefits received by the other.

Autotroph: an organism that uses carbon dioxide as its source of structural carbon.

Biomass: the mass of living organisms within a population, community, or ecosystem.

Chemolithotroph: an organism that uses reduced inorganic molecules as its energy source.

Chemoorganotroph: an organism that uses reduced organic carbon as its energy source.

Commensalism: interaction between species where one benefits from the other but the other is not affected.

Competition: species-species interactions that have a negative effect on both species.

Cyanobacteria: Prokaryotic photosynthetic organisms that contain chlorophyll *a* and generate oxygen during photosynthesis. The free-living ancestors of primary chloroplasts.

Exploitation: species-species interactions that have a negative effect on one species while benefiting the other—can include both parasitism and predation.

Heterotroph: an organism that uses preformed organic carbon as its source of structural carbon, also heterotrophic or heterotrophy when applied to metabolism.

Mixotroph: any of a number of types of organisms that combine (in one organism) multiple metabolic types as described above. For example, a protist that consumes bacteria as prey (heterotroph) but also contains functioning chloroplasts (phototroph) will often be referred to as a mixotroph. Similarly an archaeon that oxidizes ammonia for energy (chemolithotroph) but uses amino acids to build proteins (heterotroph) could be considered a mixotroph.

Mutualism: interaction between species where both benefit from each other.

Phototroph: an organism that uses light as its energy source for production of ATP (or to produce proton gradients in the case of rhodoposin-based phototropy) and sometimes also reducing power from water (in cyanobacteria).

Phytoplankton: the photoautotrophic component of the plankton including cyanobacteria and a large number of eukaryotic phyla that contain chloroplasts.

Protists: eukaryotic species that can exist as a single cell other than a spore, gamete, or zygote (although there are many that form colonies).

Protozoa: protists that exhibit heterotrophic nutrition. Protozoa span a wide size range (≈2 to >200 μm), and have generally been referred to as nano- or microzooplankton.

Relative abundance (and the related term, evenness): the contribution of each species or operational taxonomic unit to a community.

Species diversity: a complex concept composed of species richness and relative abundance.

Species richness: the number of different species or operational taxonomic units present in a sample, habitat, or environment.

Stoichiometry: Studies that involve calculation of the relative quantities of elements or compounds, for example, C:N:P ratios.

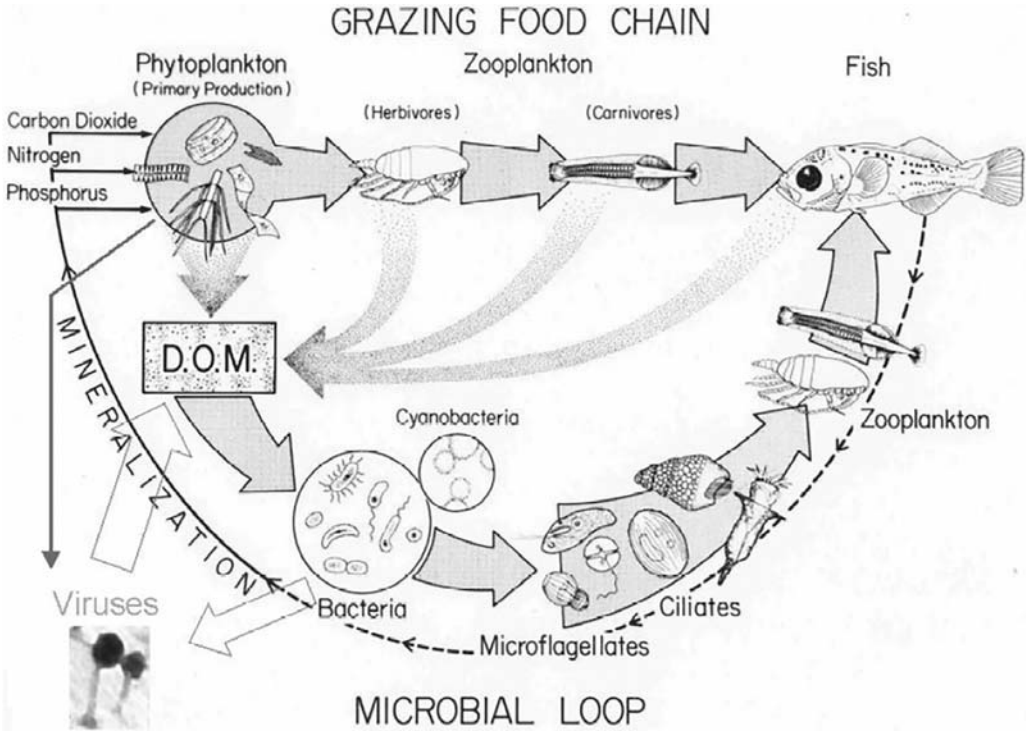

FIGURE 1 An early vision of the "microbial loop" and its connections to the classical grazing food chain via dissolved organic matter (DOM) flux and particulate trophic transfer, with viruses included as a side loop. Modified from (34). Large gray arrows indicate the flow of organic carbon and energy into higher trophic levels of the food web, with recognition of the important roles for heterotrophic microbes (bacteria and protozoa) in this process. Large stippled arrows indicate the production of DOM via excretion and trophic interactions (not all groups are represented). Thin, dotted arrows indicate mineralization of major nutrients contained in organic matter respired by consumers. White arrows indicate bacteria lysis by viruses and DOM released by that process. doi:10.1128/9781555818821.ch4.2.2.f1

Symbiosis: the intimate living together of two kinds of organisms, especially if such an association is of mutual advantage—too vague to be of use in quantitative descriptions of population interactions but very useful in indicating a close association among organisms.

Syntrophy: a metabolic mutualism where one species uses the waste product produced by the other, and in so doing allows both metabolic pathways to be energetically feasible.

Zooplankton: Planktonic eukaryotes that consume other plankton. Includes single-celled organisms (protozoa or protists) and metazoans, and some that are planktonic only as larvae.

Estimating "Bacterial" Biomass and "Bacterial Production": Definitions and Methods

Aquatic microbiologists tend to use the term "bacteria" with a lowercase "b" to describe organisms that appear to be prokaryotic by microscopy—that is, organisms with no membrane-bound nucleus. They include members of the taxonomic domains Bacteria and Archaea (see "Bacteria and Archaea"). Organisms within and between these domains differ in many biochemical and genetic aspects, but they tend to look similar by traditional epifluorescence microscopy. Special methods, such as different versions of fluorescence *in situ* hybridization (FISH) are required to distinguish individual members of these domains microscopically (35–37).

The term "bacterial production" here refers to heterotrophic production of biomass by bacteria. It is meant to include production of nonphotosynthetic bacterial biomass based on the heterotrophic consumption of preformed organic matter (i.e., organic matter in various forms that has been produced primarily by phytoplankton).

Bacterial biomass is usually determined by converting direct counts of bacteria using an estimate of the amount of carbon per cell. Direct counts are most commonly done by epifluorescence microscopy with stains such as acridine orange, 4′,6-diamidino-2-phenylindole or SYBR green I (17, 38, 39). Special procedures are usually applied for sediment samples and samples containing large numbers of bacteria attached to particles (40). SYBR green I also permits direct visualization and counts of viruses in the same preparation. Increasingly, direct bacterial counts in seawater samples have been performed by flow cytometry of fluorochrome-stained cells (41, 42), a method that allows separate counts of cyanobacteria, such as *Synechococcus* and *Prochlorococcus*, which have unique fluorescent signatures due to their photosynthetic pigments, and which can sometimes make up a substantial fraction of the total number of bacteria (43). Flow cytometry is rapid and has a statistical advantage in that it typically observes thousands of prokaryotic and minute photosynthetic eukaryotic cells rather than the hundreds counted microscopically; drawbacks include the cost of the instrument and the fact that cells attached to each other or to other particles are counted as one. Bacterial carbon per cell has been estimated in a variety of ways, most commonly from a determination of cell volume and carbon density per unit volume. These numbers are difficult to obtain accurately for native marine bacteria, which are very small, typically 0.5 μm in

diameter (range is about 0.2–1 μm for free-living unicells). Published estimates of bacterial carbon per cell vary widely and probably constitute the greatest uncertainty with estimating bacterial biomass in natural samples. Typical estimates of the carbon content of a bacterium range from 7 to 50 fg C (1 fg is 10^{-15} g), with most open ocean estimates near 10–20 fg C per cell and coastal ones about double that (44). Thus, in a typical mesotrophic ocean environment with 10^9 bacteria per liter, and an average per cell C content of 15 fg, bacterial biomass would be approximately $10^9 \times 1.5 \times 10^{-14} = 1.5 \times 10^{-5}$ g C per liter, or 15 μg C per liter.

Bacterial production is most often measured by incorporation of tritiated thymidine into DNA (24, 45) or tritiated leucine into protein (26, 46). Thymidine and leucine are intracellular precursors of DNA and protein, respectively, so incorporation of these precursors can be used to estimate the total rates of synthesis of the macromolecules. DNA is synthesized for cell division and protein synthesized roughly in proportion to total biomass, so measuring their rates of synthesis is presumed to track production. Both methods have been calibrated on the basis of theoretical considerations as well as purely empirical approaches, and both methods yield similar results (44). Leucine has a lower detection limit, so it is preferred for slower rates. The results of thymidine incorporation are most often presented as cells produced per liter per hour, which can be converted to a carbon production rate via estimates of carbon per cell as noted. Leucine incorporation may be used to calculate cell production as well as biomass production directly, because protein is a major biomass constituent.

Geographic and Temporal Distributions of Microbial Biomass and Activity

Bacterial abundances by epifluorescence microscopy show that bacteria are present in most marine euphotic zone environments at approximately 10^6 cells per ml. Samples from around the world rarely vary by more than threefold from this typical value (i.e., rarely $<3 \times 10^5$ or $>3 \times 10^6$), which is extraordinary compared to phytoplankton and zooplankton, which may vary by several orders of magnitude over the same spatial scales. However, despite this remarkable general predictability, there is significant variation across both space and time. More nutrient-rich, eutrophic environments tend to have more bacteria (sometimes $>10^7$ per ml; 47), and oligotrophic open ocean environments have less (summarized below). Although bacterial assemblages in warm temperate coastal waters may have doubling times as short as 1 day, this is at the rapid end of the spectrum of *in situ* growth rates. Bacterial assemblages in the open sea, especially in oligotrophic environments, have average generation times typically of a week or perhaps more (see Table 1). These abundances generally apply to the euphotic zone, and bacteria in colder, darker waters have substantially lower abundances and slower growth rates than those of surface waters (48). Benthic bacteria also exhibit fairly constant abundances across wide geographic ranges, but benthic bacteria occur at much higher densities because of the organic-rich and particle-laden nature of the environment (an average of $\approx 10^9$ per ml fluid volume is typical; 49). This value is three orders of magnitude greater than abundances in the water column, implying that the abundances of benthic bacterial assemblages are regulated by a different set of parameters than planktonic assemblages.

Bacterial biomass and productivity vary temporally on a number of scales ranging from diel (50) to seasonal (51) or interannual (52). On time scales of hours, bacterial abundance and production have been shown to often peak in the middle of the day and be low in the middle of the night (50, 53–55). This pattern has been interpreted as a tight coupling between the production of labile organic compounds via photosynthesis and bacterial growth on one hand, and

TABLE 1 Bacterioplankton properties in relation to phytoplankton in the open sea, as compiled by Ducklow (44)

Property	N Atlantic[a]	Eq Pac-Spr[b]	Eq Pac-Fall[c]	Sub N Pac[d]	Arabian[e]	Hawaii[f]	Bermuda[g]	Ross Sea[h]
Euphotic zone m	50	120	120	80	74	175	140	45
Biomass (mg C m^{-2})								
Bacteria	1000	1200	1,467	1,142	1,448	1,500	1,317	217
Phytoplankton	4,500	1,700	1,940	1,274	1,248	447	573	11450
B:P	0.2	0.7	0.75	0.9	1.2	3.6	2.7	0.02
Production (mg C m^{-2} d^{-1})								
Bacteria	275	285	176	56	257	Nd	70	5.5
Phytoplankton	1,083	1,083	1,548	629	1,165	486[i]	465	1248
B:P	0.25	0.26	0.11	0.09	0.22	Nd	0.18	0.04
Growth rates (d^{-1})								
Bacteria	0.3	0.13	0.12	0.05	0.18	Nd	0.05	0.25
Phytoplankton	0.3	0.64	0.8	0.5	0.93	1.1	0.81	0.11
B:P	1	0.2	0.15	0.1	0.19	Nd	0.06	2.3

Notes: All bacterial biomass estimates based on 20 fg C per cell. Data may overestimate heterotrophic bacterial biomass as a consequence of lower C per cell or interference by *Prochlorococcus* and Archaea. Production estimated from 3,000 g C per mole leucine incorporated.

[a] Eastern North Atlantic spring phytoplankton bloom, 47 N 20 W, May 1989, $n = 13$.
[b] Equatorial Pacific, 0 N 140 W, March–April 1992, $n = 8$.
[c] Equatorial Pacific 0 N, 140 W, September–October 1992, $n = 19$.
[d] Subarctic North Pacific, 45 N.
[e] Northwest Arabian Sea 10–20 N, 165 E, January–December 1995, $n = 21$.
[f] Hawaii Ocean Time Series (HOT); 1995–1997; $n = 21$ (http://hahana.soest.hawaii.edu/hot/hot_jgofs.html).
[g] Bermuda Atlantic Time Series (BATS); 1991–1998, $n = 106$ paired comparisons. The ratios are means of the ratios, not ratios of the means. BP calculated from thymidine incorporation (1.6×10^{18} cells per mole incorporated).
[h] Ross Sea, Antarctica, 76 S 180 W, 1994–1997.
[i] 1989–1996; $n = 64$.

bacterial mortality via grazers or viral lysis on the other hand. It is consistent with some measurements of extremely rapid turnover, sometimes several times per hour in rich coastal waters, of labile dissolved organic compounds such as dissolved free amino acids (56) and might also indicate greater predation pressure by protozoa during the night. On longer time scales of weeks to months, bacteria show distinct seasonal patterns. For example, in temperate coastal waters, bacterial biomass and production increase considerably in summer months compared with winter. However, bacteria do not typically show a significant increase during early "spring" blooms in temperate waters when water is still very cold (51). It has been hypothesized that this phenomenon is the result of the suppression of the rate of substrate uptake by temperate bacterial assemblages at low temperature (57). However, while temperature probably has the effect of setting a limit on maximal growth rates (as for phytoplankton [58] and protozoa [59]), temperature alone does not appear to be the main factor controlling growth of marine bacteria under most circumstances (see "Light, Temperature, and Pressure").

It would be overly simplistic to think that all the bacteria and archaea in a sample or habitat have the same level of activity per cell, but it is also easy to think of measured activities as characteristic of all members of a microbial assemblage. So the question arises: are most of the cells active at a similar level, or are some hyperactive while others are completely dead or moribund? This question has been addressed several ways, including microautoradiography, selective staining, "direct viable counts" (where nutrients are added to see what part of the community grows), and *in situ* hybridization (e.g., 60). Based on these contrasting approaches, it appears that a continuum of activity exists within bacterial assemblages from truly dead (cannot be revived) to extremely active. A reasonable interpretation of the existing data is that under typical conditions, a small percentage of the marine bacterial cells, perhaps 10–20%, are generally inactive or dead; the plurality or majority of cells, perhaps 25–75%, are intact and have some moderate level of activity; and a small percentage, perhaps 5–20%, are highly active. It is useful to consider this spectrum, conceptually and numerically, when modeling microbial processes.

Comparisons of bacterial and phytoplankton biomass within planktonic ecosystems show that these are positively correlated across broad scales. Analyses of marine and freshwater samples from several studies (61, 62) have shown that bacterial abundance increases with chlorophyll concentration, at least at the level of a log-log relationship. Similarly, bacterial abundances and the abundances of small protozoa correlate over broad spatial and temporal scales (63). These relationships are sensible in that on the largest scale, primary production is the source of organic material that fuels heterotrophic bacterial activity, and bacteria constitute the prey of many small protozoa. Individual data sets also have sometimes shown strong correlations between bacterial abundance and chlorophyll (e.g., 45), but variability in this relationship over short temporal or spatial scales is to be expected. It would presumably be a consequence of rapid, short-term changes in the rate of substrate supply as well as the normal, oscillatory nature of predator-prey relationships between bacteria and their consumers.

Interestingly, the extrapolation of the positive log-log relationship between bacterial and phytoplankton biomass to environments with very low chlorophyll concentrations (e.g., ultra-oligotrophic oceans) indicates that bacterial biomass may exceed phytoplankton biomass in these situations. While this conclusion is obvious for the deep sea, where there is no photosynthesis, this situation is also often true in oligotrophic surface waters. Measurements made in oligotrophic waters bear out the high bacterial contribution to total biomass (64, 65). Moreover, Cho and Azam (66) confirmed a linear relationship between the log of chlorophyll and log of bacterial abundance, but only at chlorophyll concentrations above approximately 0.5 µg per liter. Below that concentration, bacterial abundance did not correlate significantly with chlorophyll. It should be noted that subsequent analyses have revealed that early epifluorescence measurements of bacterial biomass included the common cyanobacterium *Prochlorococcus*, which can make up to 20% of total bacterial numbers (67). Nonetheless, heterotrophic bacterial biomass is a major fraction of the living biomass of all planktonic ecosystems.

The geographical and temporal distributions of marine protozoa are much more varied than those of the bacteria. As an all-inclusive group, protozoa generally occur in planktonic ecosystems at abundances ranging from 10 s to 1,000 s per ml. Abundances in benthic ecosystems can be one to three orders of magnitude higher, commensurate with the higher abundances of bacteria in those ecosystems. However, it is important to remember that like the term "'bacteria," the term "protozoa" is a rather artificial conglomeration of evolutionarily and ecologically divergent taxa (see "The Changing and Complex World of Eukaryote Phylogeny"). Thus, the abundances of specific lineages of bacteria or protozoa may show spatial (or temporal) variability that is considerably greater than the variability characteristic of these overarching groupings.

The Changing and Complex World of Eukaryote Phylogeny

Not that long ago, textbooks still divided eukaryotic organisms into four major kingdoms (Animalia, Plantae, Fungi, and Protista) while prokaryotic organisms were placed into a single kingdom, the Monera (68). Within this scheme, protists (eukaryotic organisms that can exist as single cells) were divided into two subkingdoms (algae and protozoa) based on their basic nutritional mode, a carryover from the historical distinction between single cells with "animal-like" or "plant-like" nutrition. This distinction presupposed a basic evolutionary divergence among protists into species that retained a heterotrophic, phagocytotic mode of life (protozoa) and those that abandoned phagocytosis for a photosynthetic mode of life (algae). Moreover, the presence/absence of chloroplasts was a feature that could be easily distinguished by early microscopists.

The five-kingdom classification system of Whittaker was recognized as an improvement over previous classification schemes, but it posed a number of problems relating to protists. For example, the distinction between single-celled and multicellular eukaryotes was somewhat arbitrary. More important, the division of protists based on whether they were heterotrophic or photosynthetic was clearly not an appropriate feature if the classification was to recapitulate evolutionary relationships. We now know that chloroplast acquisition and loss has occurred several times in the biological history of our planet (69), giving rise to some closely related protistan taxa that differ largely in the presence or absence of a chloroplast. Further complicating the matter, within many protistan lineages there are species that possess chloroplasts and carry out photosynthesis (phototrophy) but also possess the ability to ingest and digest prey (heterotrophy; 70–72). Some heterotrophic protists even ingest

phytoplankton prey and retain the chloroplasts of their prey in a functional state for a limited amount of time (kleptidoplastidy;73). Various forms and degrees of mixotrophy (mixed phototrophic and heterotrophic nutrition) are common among a number of algal/protozoan lineages (74–77). Under Whittaker's scheme, phytoplankton ecologists studying a lineage of microalgae might have had little familiarity with closely related heterotrophic species, while protozoologists studying a particular protozoan group might have known little about closely related photosynthetic species.

One might expect, given these caveats, that the terms "algae" and "protozoa" are no longer used. In fact, the term "protozoa" is still commonly used (especially by ecologists) to recognize those eukaryotic species that exist as single cells, and whose nutrition is dependent on the uptake of preformed organic substances (primarily via prey ingestion), while protists possessing chloroplasts are still commonly called "algae." Similarly, although the term "protist" has been abandoned as a kingdom designation, it is still widely employed to describe eukaryotic species that are capable of existence as single cells (i.e., algae and protozoa). The term "phagotrophic protist" has also gained popularity in recent years because it recognizes that many protistan species are capable of phagocytosis even though they may also possess their own chloroplasts and thus are technically "algae."

Despite the shortcomings of Whittaker's scheme, it dominated the hierarchical organization of life for approximately a quarter century. During the past few decades, however, this system has given way to a new organizational scheme that recognizes three domains of life (Archaea, Bacteria, Eukarya [or Eucarya]; 78; Fig. 2, upper panel), and is based on what is presently believed to reflect a more realistic view of the evolutionary distances that have developed between organisms in the ≈4 billion years that life has existed on our planet. Within the Eukarya of Woese's scheme, hypotheses regarding the phylogeny of "protists" have changed continuously and rapidly during the past two decades, reflecting new insights into eukaryote evolution provided largely by DNA sequence information (Fig. 2, lower panel; from [79]).

The former protistan phyla of Whittaker's system have now been dispersed among candidate "supergroups" within the domain Eukarya to better reflect hypothesized phylogenetic relationships. For example, the dinoflagellates (which encompass phototrophic, heterotrophic, and mixotrophic species) now form a single group and have been placed together with the ciliates and apicomplexans (sporozoans) in the monophyletic Alveolata (Fig. 2, lower panel). On the other hand, eukaryotic, heterotrophic, single-celled species falling within the general description "protozoa" are now widely distributed among a number of protistan lineages. In short, nutrition has been demoted as a phylogenetic character, and other characters (presumably more indicative of evolutionary relatedness) have ascended to address some long-standing contradictions, although the debate over the relationships among some lineages is still very active at the present time; 79).

Individual protozoan cells range in size from less than 2 μm to greater than 1 cm in diameter (>4 orders of magnitude) (80, 81) with some colonial radiolaria forming cylindrical gelatinous structures a centimeter in diameter and more than a meter in length (82, 83). Because they constitute such a large size range of organisms, protozoa are often divided into size classes that very crudely correlate with their general nutritional preferences. A commonly used convention is that of Sieburth et al. (84), which groups planktonic microorganisms into order-of-magnitude size classes (0.2–2.0 μm = picoplankton; 2.0–20 μm = nanoplankton; 20–200 μm = microplankton; 0.2–2 mm = mesoplankton). Most protozoa fall into the nanoplankton or microplankton size classes. Modeling microbial trophodynamics using this convention assumes that protozoa in one size category generally consume prey one order of magnitude smaller in size (34, 85). Although this approach misses much of the detail and diversity of the trophic activities of individual protozoan taxa, it is a necessary, practical compromise for examining community-scale flows of energy and elements. It also provides a useful mechanism for summarizing and comparing the abundances and biomasses of protozoa from different environments and to other microbial assemblages. Protozoan abundance, summarized in this way, has been shown to contribute significantly to the living biomass of planktonic ecosystems throughout the world ocean (Fig. 3).

Estimates of protozoan biomass, such as those depicted in Fig. 3, typically do not include the contribution of mixotrophic phytoflagellates to heterotrophy. There is presently no easy way to determine the abundances of small phagotrophic phytoflagellates in natural samples, so these species are typically counted as phytoplankton unless specific methods are employed to identify the algae as consumers, such as the use of fluorescently labeled particles (87–90) or through the examination of food vacuole contents (91). On average these species appear to constitute a modest percentage of the phytoplankton assemblage (typically <25%), although they may at times dominate the phototroph assemblages of natural plankton communities. It is important to recognize that their inclusion as functional heterotrophs, rather than phototrophs, could significantly shift the relative contributions of phototrophic and heterotrophic microbial biomass to total biomass within microbial assemblages, and the flow of energy within plankton communities (92).

Heterotrophic protists that harbor photosynthetic protists, or their chloroplasts, within their cytoplasm constitute another complexity for estimating the contribution of protozoa to total microbial biomass. When bulk water samples are analyzed, the contribution of chlorophyll contained within those protozoa is generally assumed to come from free-living phytoplankton. However, studies have shown that chloroplast-bearing ciliates can contribute up to half the total biomass of planktonic ciliates in ecosystems, and chloroplast-retaining ciliates can sporadically dominate the chlorophyll and primary production of some planktonic ecosystems (93–96). The environmental conditions promoting the success of these ciliates are poorly known. Similarly, many species of planktonic foraminifera, polycystine radiolaria, and acantharia harbor large numbers (thousands per protozoan) of endosymbiotic algae within their cytoplasm (97, 98). Caron et al. (99) have demonstrated that primary productivity within these species can contribute significantly to total primary productivity in oceanic ecosystems and can be very important locally in the convergences of Langmuir circulation cells (100).

BACTERIA AND ARCHAEA

"Culturable" versus "Nonculturable" Cells

Most conventional cultivation methods can grow only 1% or less of the bacteria that can be visualized by direct microscopy techniques (e.g., 4). This is true even though most can be shown to be active by techniques such as microautoradiography (25). These readily cultivable organisms appear to represent a group of fast-growing so-called weeds that are adapted to take advantage of rapid growth in rare, organically enriched

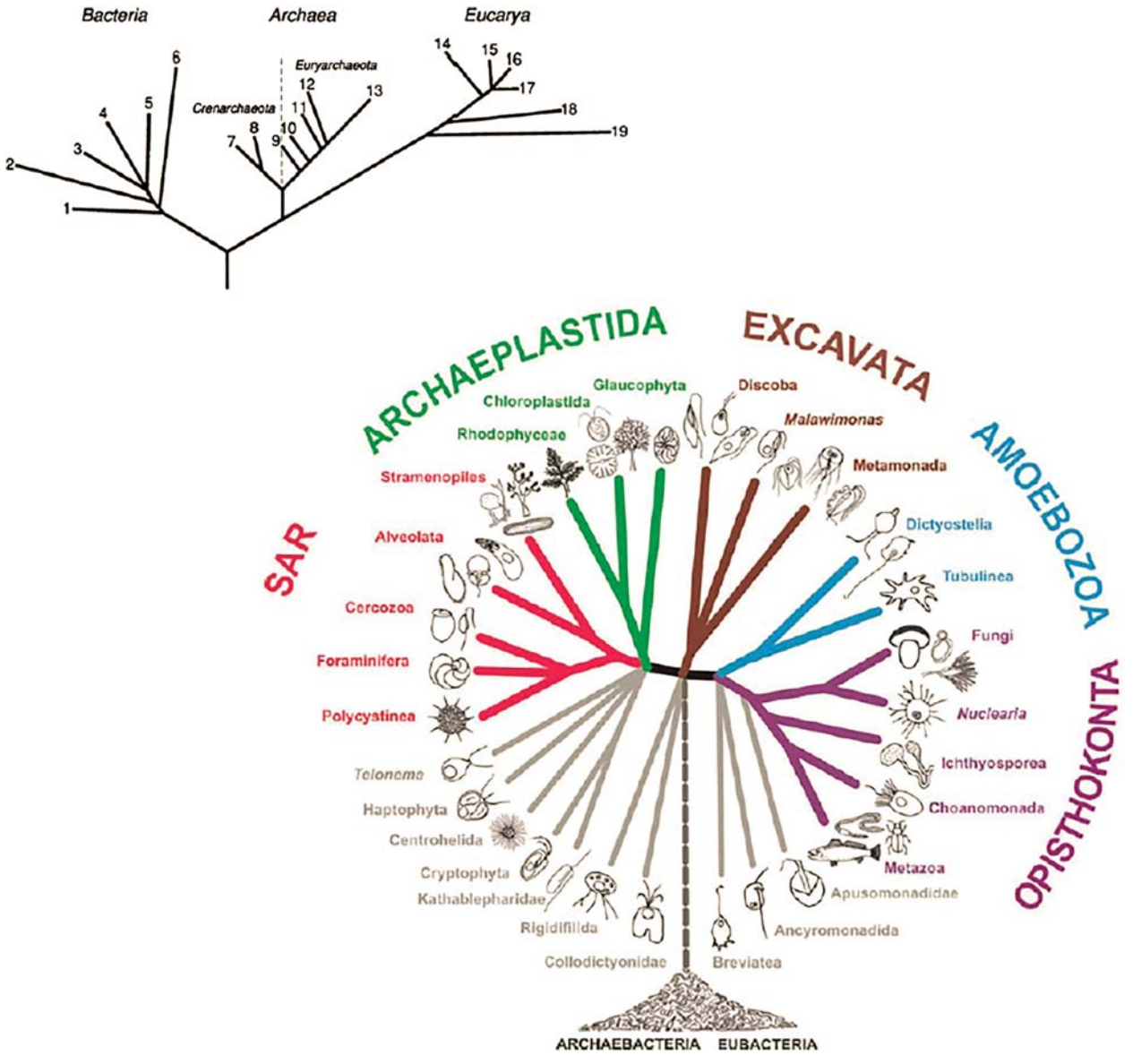

FIGURE 2 The three domains of life (upper left) as proposed by Woese et al. (78), and a recent overview of modifications that have been proposed by Adl et al. (79) to higher-level phylogentic groups within the eukaryotic component of the tree (lower right). Domains figure from Woese et al. (78), eukaryotic tree figure from Adl et al. (79). doi:10.1128/9781555818821.ch4.2.2.f2

environments. This strategy contrasts with the numerically dominant bacteria that are adapted specifically for growth in the dilute nutrient conditions that characterize the vast majority of the volume of the water column.

The most common taxa readily cultured from seawater with standard nutrient broth media include the gamma proteobacterial genera *Vibrio*, *Alteromonas*, *Pseudoalteromonas*, *Marinomonas*, *Oceanospirillum*, *Shewanella* (usually isolated from surfaces such as shellfish and sediments), the alpha proteobacterial genera *Roseobacter*, *Sphingomonas*, members of the family Flavobacteriaceae, and Planctomycetes, as summarized in Giovannoni and Rappé (101) and Fuhrman and Hagstrom (102). The cyanobacteria *Synechococcus and Prochlorococcus* are also now readily culturable, but on low-nutrient inorganic media targeting photosynthetic forms, as opposed to organic media used to cultivate the others listed above.

Because of the low percentage of marine bacteria that can be grown in standard media, organisms that until recently were called "nonculturable" make up the large majority of bacteria in the plankton. Only during the past ~20–25 years have molecular biological methods based on 16S rRNA gene sequences been available to identify these organisms, and these powerful techniques have opened up a large area for exploration (see next section). Similar but more recent studies use 18S rRNA sequences for characterizing protistan diversity, as will be noted below.

Molecular Phylogeny and Metagenomics: Field Applications

Modern phylogeny of microorganisms is based primarily on genetic sequences, the most well-studied gene being the

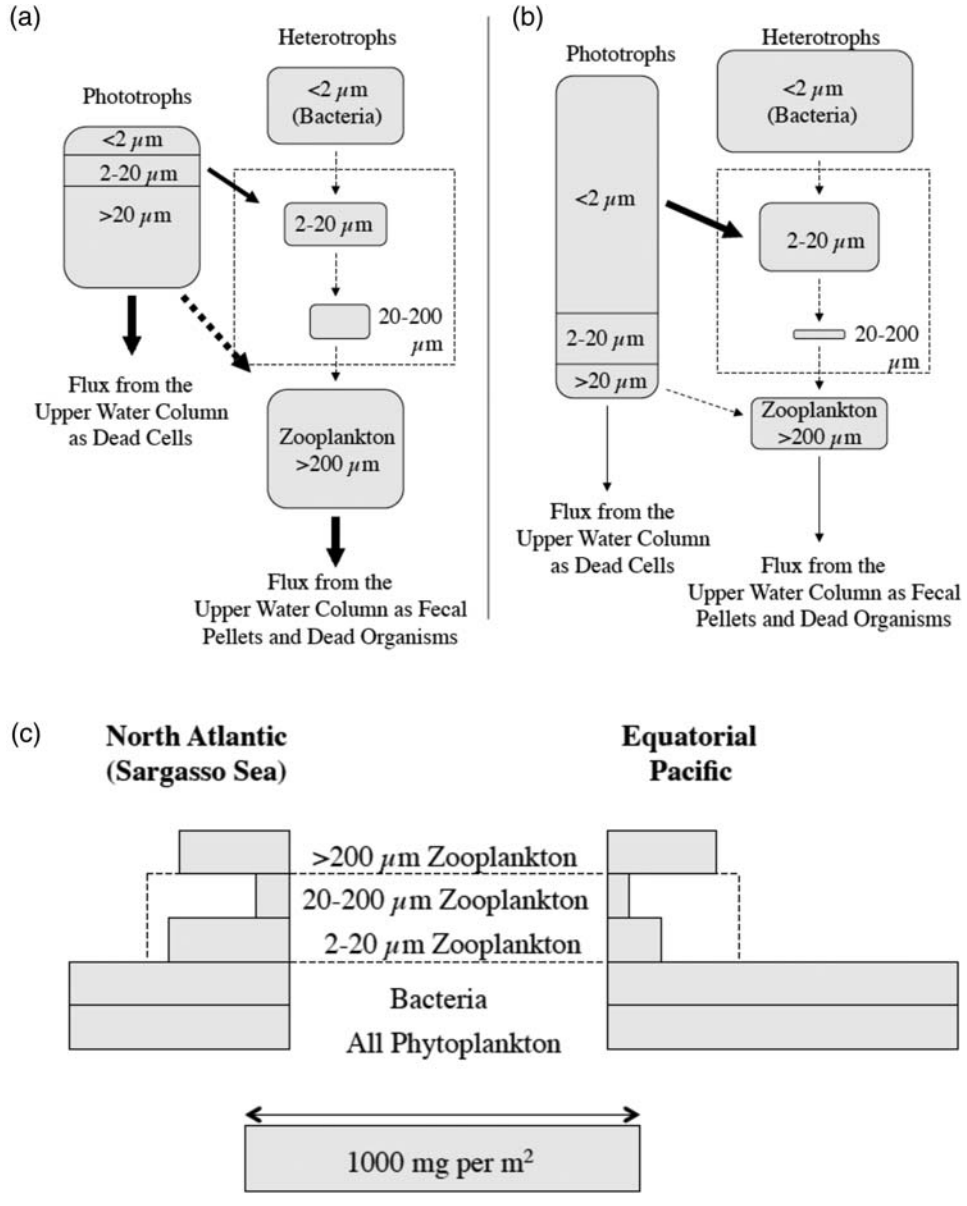

FIGURE 3 (a, b) Plankton biomass in the Arabian Sea during the 1995 southwest monsoon (a) and intermonsoon period (b). Areas of the boxes indicate the relative magnitudes of the biomass in each category. Categories within the dashed boxes in a, b, c are composed of protozoa. Arrows indicate the direction of energy/material flow in the food web, thicker arrows depicting greater flow. Redrawn from (86). (c) Depth-integrated biomass (mg/m^2) in the upper 100 m of the Sargasso Sea near Bermuda, and in the upper 200 m of the equatorial Pacific at 175°E. The width of the bars indicates the biomass in each size category. Heterotrophs have been separated by size class, while phytoplankton have not. Size classes delineated by the dotted box are comprised of protozoa. Redrawn from (65). doi:10.1128/9781555818821.ch4.2.2.f3

small subunit ribosomal RNA gene (16S rRNA in Bacteria and Archaea, and its larger homolog 18S rRNA in eukaryotes). This molecule is strongly conserved over evolutionary time, so this single molecule has been used for constructing phylogenetic trees of all living organisms (http://tolweb.org/tree/phylogeny.html). Analysis of 16S/18S rRNA gene sequences has been used to evaluate deep evolutionary relationships among organisms and was instrumental in pointing out that Archaea, Bacteria, and Eukarya should be considered different Domains of equivalent phylogenetic rank, above kingdoms (78). However, there are sufficient differences in 16S/18S rRNA gene sequences to demonstrate relationships (or make distinctions) at the genus or sometimes species level.

The first phylogenetic studies based on 16S/18S rRNA genes used sequences derived from cultures. However, one does not need cultures to obtain rRNA gene sequences (or any other sequences, for that matter). An idea developed in the lab of Norman Pace in the mid-1980s involved extraction of DNA directly from natural samples, and then cloning and sequencing of the DNA as a means of assaying the microbes present in the samples (103, 104). The original protocols called for cloning by creating what are called "phage libraries" from the natural DNA, but since 1986, PCR has been applied

extensively for cloning and related studies. The target sequence can be almost instantly "identified" to its closest phylogenetic neighbor by what have come to be standard online sequence comparisons.

Beyond the study of targeted genes (like the 16S rRNA gene), shotgun metagenomic studies have examined the entire genetic repertoire of the microbes in a given sample. The metagenome is the collective genome of all organisms in the sample. Initially these studies extracted DNA from all organisms in a sample (usually prefiltered through a filter approximately 1 μm to remove most organisms larger than bacteria), sheared it to produce fragments, and cloned them into standard vectors, either as small (thousands of bases) or large (to hundreds of thousands of bases) inserts, that is, fragments of DNA from the environment now cloned into the vectors in a form suitable for sequencing. The best known early marine study of the former type is the Global Ocean Survey, with initial results published by Venter et al. (105), which generated more than a billion bases of DNA sequence and reported 1.2 million previously unsequenced genes, estimated to come from at least 1,800 different genomic species cumulatively in the many samples they analyzed.

With the advent of next-generation sequencing that generates millions or more sequences in a run (known by a variety of acronyms including 454, Illumina, SOLiD, etc.), cloning of genes has largely been replaced by clone-free sequencing. The extent and power of such sequencing has recently been demonstrated by the ability to construct essentially the entire genome of an uncultivated marine Group II Euryarchaeon that constituted only approximately 2% of a microbial community sample, using SOLiD sequencing, made possible by high coverage and the use of mate pair sequencing of ~3,000 bp fragments (106) (this length is needed to span repeats and highly conserved genes that otherwise make genome construction difficult).

For 16S rRNA gene studies, "tag sequencing" pioneered by Sogin et al. (107) consists of amplifying a suitable sized part of the gene with broadly conserved primers (choice is important—few are truly universal for the groups intended), often "barcoded" to allow multiple samples to be combined into a single run. The amplified products are then sequenced, and sequences processed en masse. Many thousands of partial SSU rRNA gene sequences per sample are economically analyzed this way, though they are usually short (currently a few hundred bases each, depending on the sequencing platform). This way of analyzing the composition of microbial communities is now standard. These approaches provide so much information even about very rare sequences, that the results have led to the important concept of the "rare biosphere," organisms that may be active or dormant and constituting a very small proportion of the community (e.g., often much less than 0.1%), but are potentially important for dispersion, adaptation to changing conditions, or even critical activities like nitrogen fixation or vitamin production (108). However, due to the potential for artifacts like error sequences, this approach requires rigorous application of quality filtering and clustering algorithms to avoid erroneous taxa and overestimation of species richness (109, 110).

Next-generation sequencing approaches have also allowed work to begin metagenomics studies of microbial eukaryotes (111). These studies are still constrained by the much larger genomes of eukaryotes, and therefore the difficulties of obtaining sufficient sequences to reconstruct the genome of a particular species. Along with advances in sequencing ability, studies of eukaryotic metagenomics have been facilitated by advances in single-cell isolation which, when coupled with high-throughput sequencing approaches, reduce the tremendous complexity present in natural, complex eukaryotic communities to a manageable task (112). Such single-cell techniques are also quite valuable in studies of bacteria and archaea, though the amplification technique tends to be very uneven and typically generates less than half the genome of each isolated cell regardless of domain (113, 114).

Additionally, similar to DNA, mRNA is amenable to extraction and sequencing although greater care must be taken during extraction and purification as RNA shows a greater susceptibility to degradation during processing. Copying of mRNA by reverse transcription of RNA into cDNA, followed by DNA sequencing, has allowed insights into the metatranscriptomes of environmental samples. Metatranscriptomic studies provide information on gene expression in an ecosystem, and thereby indicates "activity" of the microbial community rather than simply "potential" represented by the genomic DNA present in the sample, with many applications, from showing which processes are being carried out by which organisms to fine-scaled diel studies (115–118). Nevertheless, given variations in the lifetimes of different transcripts and protein molecules, the transcriptome may not be fully representative of the current activity of an organism.

Limitations of these analyses include sequencing errors, PCR mismatches or biases, clustering and bioinformatics challenges, and chimeras generated during PCR. Also, the phylogenetic resolution of short sequences is limited, given the high conservation of rRNA sequences. Even with clone-free shotgun metagenomics, there may be biases, such as nonrandom losses of DNA during extraction and preparation, or biases (e.g., from G + C content or secondary structure) in the sequencing procedures. Determination of species diversity by these approaches provides an example of these limitations. Shakya et al. (119) working with synthetic communities (purified genomic DNA from 16 Archaea representing 3 phyla and 48 Bacteria representing 16 phyla, remixed to simulate an environmental DNA extract) applied both metagenomic analysis (454 and Illumina platforms) and PCR amplification followed by 454 sequencing of 16S rRNA genes to determine both species richness and relative abundance. They found that PCR amplification/454 sequencing of 16S rRNA genes yielded an accurate measure of species richness (providing that appropriate data processing was applied) but that the relative abundance of up to 94% of the species (depending on domain and variable region amplified) was over- or underestimated by at least 1.5-fold (values ranged from not detected to 10.3-fold overestimation). In contrast, both metagenomic approaches yielded relative abundances that were within the authors' 1.5-fold accuracy cutoff for ~50 of the species. However, they concluded that addressing richness overestimation in metagenomic analyses, that is, distinguishing rare but real OTUs from experimental and computational artifacts, awaits further computational and classification improvements. More recently, Parada et al. (120) used mock communities composed of 16S rRNA clones from 27 common marine taxa (from nine Bacterial and two Archaeal phyla) to show that small differences in PCR primers (and different clustering methods) can yield large differences in apparent relative abundances of reported taxa. However, one primer pair and informatics pipeline they tested, using a particular version of 515F-926R (V4–V5), provided accurate estimates of relative clone abundance ($r^2 = 0.95$) when comparing observed versus expected clone abundance.

Culture-Independent Diversity Studies

The first groups to be identified using cultivation-independent molecular techniques from the marine plankton (121) were the bacterial SAR11 cluster (a group of closely related gene sequences, or phylotypes) and marine picoplanktonic unicellular cyanobacteria *Synechococcus* and *Prochlorococcus*). Of these, the SAR11 cluster was completely unknown, but the cyanobacteria had previously been recognized by their unique pigment fluorescence; Waterbury et al. (122) and Johnson et al. (123) used epifluorescence to observe *Synechococcus*, and Chisholm et al. (124) discovered *Prochlorococcus* using flow cytometry. These cyanobacteria were later isolated and grown in phytoplankton culture media. These two groups are generally common in the euphotic zone, with SAR11 typically comprising one third of the planktonic bacteria (125), and the cyanobacteria common everywhere but polar waters.

Probably the biggest surprise to come from the application of molecular tools was the discovery by Fuhrman et al. (126) of abundant archaea in the deep sea. The archaea were found to be in a unique phylogenetic cluster that was only distantly related to any previously known archaea, but the "closest relatives" (not really close at all) were extreme thermophiles. A subsequent study also found archaea to be present in near-surface coastal waters, albeit relatively rare (<2% relative abundance). This study used a PCR technique specifically targeted archaea, and found "marine Crenarchaea" plus a second group belonging to the phylum Euryarchaea (127).

Up until this time, all known Archaea were thought to be "extremophiles"—adapted for either very high temperatures (thermophiles), extremely salty conditions (halophiles), or strictly anaerobic environments (methanogens). Yet these organisms were present in cold or cool water at ordinary salinity and high oxygen concentrations. Fluorescent *in situ* hybridization (FISH) measurements from deep-sea samples have since showed that the archaea may make up approximately 40% of the total countable prokaryotes, with the percentage reaching to 60% at 200 m depth in the Mediterranean (35, 36). An extensive time series of FISH measurements near Hawaii confirmed that the archaea are indeed very abundant throughout the year from below the photic zone to at least 4,000 m, and typically constituting 30–40% of the total prokaryotes present in waters deeper than a few hundred meters (128), with similar results found elsewhere (129, 130) (Fig. 4). They have been reported from many places, including the Atlantic, Pacific, and Southern Oceans, and are dynamic components of the plankton, the most common type by far being the "marine Crenarcheaea" (128, 129, 131). While the marine archaea have been reported to be dominated by a few major "phylotypes" (132), they also have been shown to have a great deal of microdiversity within these phylotypes, suggesting there are many kinds of close relatives coexisting (133). Interestingly, this group of archaea may likely be the most abundant kind of organism on Earth, given the huge volume of the deep sea and their high abundance there (134). It has recently been proposed that "marine Crenarchaea" be elevated to their own major phylum outside the Crenarchaeota, called the Thaumarchaeota, on the basis of deep phylogenetic branching and fundamental differences between them and the Crenarchaeota (135). The Thaumarchaeota possess the uniquely archaeal membrane lipid crenarchaeol, and are now also known to be abundant in soils.

The physiology of the Thaumarchaeota has been an intriguing area of study. Initially, an autoradiography-FISH combination approach demonstrated that they take up amino acids (130, 136). But subsequent data showed that crenarchaeol was derived largely from CO_2, suggesting autotrophic metabolism (137, 138). An experiment showing uptake of ^{13}C-labeled bicarbonate into these archaeal lipids (139) directly pointed to autotrophy in this group. Chemolithoautotrophy was first hinted at by Venter et al. (105), whose metagenomic analysis showed an apparent archaeal scaffold that contained genes suggestive of ammonia oxidation. Further evidence came from Schleper et al. (140), who detected several ammonia oxidation genes in order directly adjacent to a Thaumarchaeota 16S rRNA gene in a soil-derived metagenomic clone. The issue was directly resolved when Konneke et al. (141) isolated a related marine archaeon from sediment of a marine aquarium, and this organism, *Candidatus Nitrosopumilus maritimus*, was found to have a chemoautotrophic metabolism, quantitatively oxidize ammonia to nitrite, and contain an archaeal ammonia monooxygenase gene (amoA) and interestingly did not grow heterotrophically. Cultivation experiments showed this organism has a high affinity for ammonium, allowing it to outcompete bacterial nitrifiers at low (submicromolar) concentrations as are typical in the sea (142). The complete genome of the organism has shown novel adaptations for nitrification and autotrophy (143).

Even if the Thaumarchaea are primarily chemolithoautotrophs, field data suggest a level of mixotrophy, in that some organic substrates are being incorporated into biomass. A stable isotope study using cells collected at 670 m depth off Hawaii estimated that about 80% of the carbon incorporated into archaea-specific lipids came from inorganic sources and about 20% from organic compounds (144, 145). Interestingly, it has also been claimed that the genomes of deep-sea (>2,000 m depth) members of the Thaumarchaea, as well as those living in equatorial waters, rarely contain the amoA gene and thus may be primarily chemoorganotrophs (48).

Major bacterial groups that have been documented from seawater using 16S rRNA characterization include some that are also known from culture (e.g., *Alteromonas*, *Roseobacter*), and several that are phylogenetically distant from standard cultures. The most common groups are, in rough order of their relative abundance in clone libraries from most to least abundant: SAR11 (relatives of *Pelagibacter ubique*), *Roseobacter*, SAR86, cyanobacteria, SAR116, SAR202, SAR234, and Marine Group A. The SAR designation followed by a number is an arbitrary sequential clone identifier from Sargasso Sea cloning studies done by the Giovannoni lab that did most of the early systematic cataloging of clones. Summaries of the data and phylogenetic relationships of these groups can be found in Fuhrman and Hagstrom (102) and Giovannoni et al. (101).

Molecular Genetic Discoveries in Bacterial and Archaeal Marine Biology

As described earlier, metagenomics is an extension of the ideas used in the 16S rRNA cloning studies, in that *all genes* from the native microorganisms are separated and cloned without having cultivated the organisms. These methods early on started to find unexpected and very interesting results. The best examples involve unexpected marine phototrophy, the first of which is discovery of a nonchlorophyll photosynthetic bacterial pigment, called proteorhodopsin (146). The gene was found on a large environmentally derived fragment of DNA that also had a gene coding for 16S rRNA from the so-called SAR86 group (one of the groups common in seawater). This pigment can act as a light-driven proton pump, thought to permit cells to generate

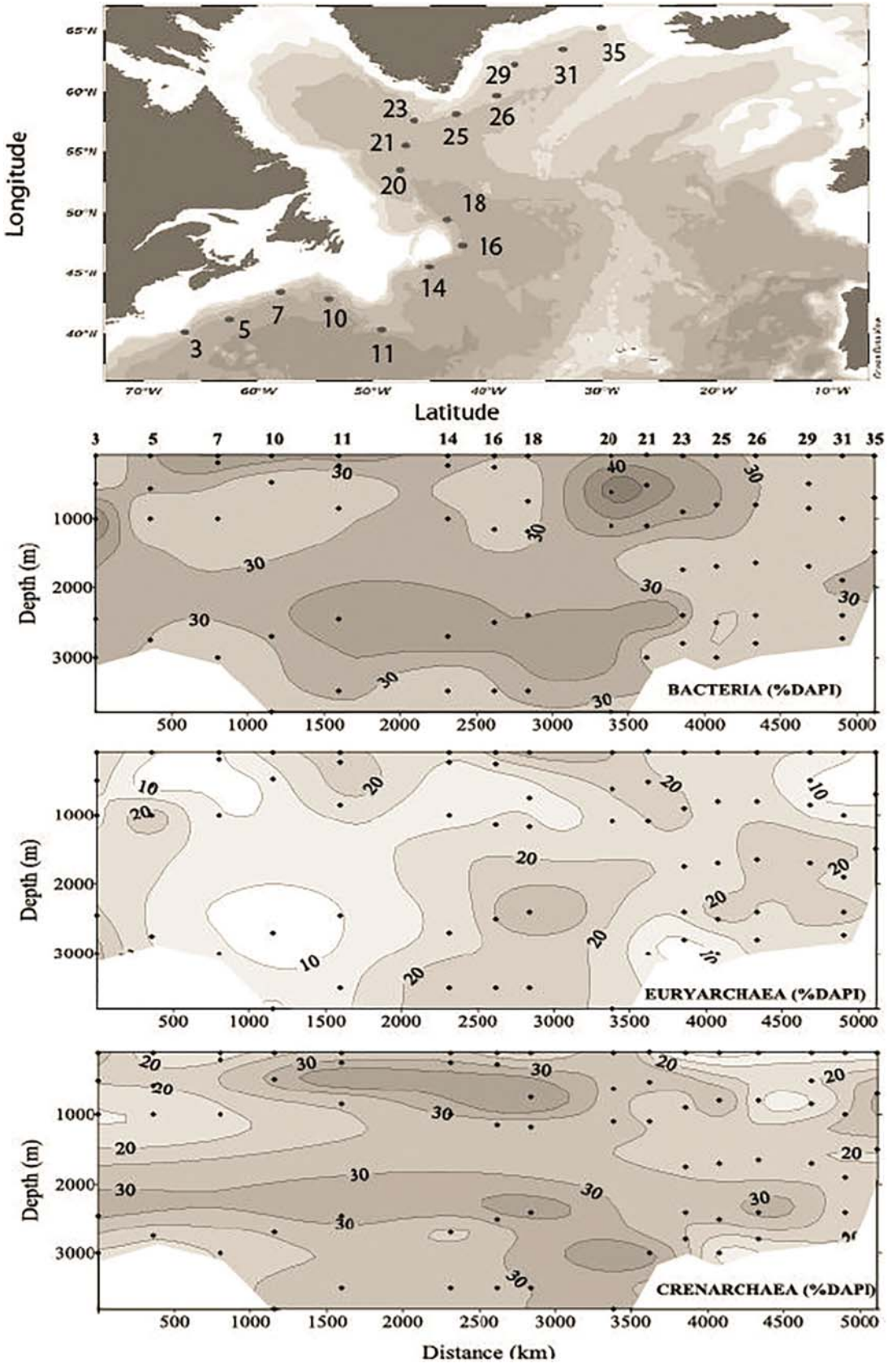

FIGURE 4 Distribution of Bacteria, Euryarchaea, and marine Crenarchaea (recently renamed Thaumarchaea), along a transect in the North Atlantic Ocean, as measured by CARD FISH and expressed as % of total bacteria + archaea counts via DAPI stained epifluorescence, from Teira et al. (130). Top panel shows station locations that are shown on the top of the lower three panels, with the distance in km reported along the transect from lower to higher station numbers. doi:10.1128/9781555818821.ch4.2.2.f4

ATP from sunlight. Interestingly, different versions of this pigment are found at different depths, apparently "tuning" the absorption to match the ambient wavelengths of light as they change with depth (147). The proteorhodopsin genes are very widespread and diverse, perhaps in half or more of all marine bacteria including SAR11 and Euryarchaeota (105, 148–150). However, almost all of several cultured organisms with proteorhodopsin studied to date do not show a growth benefit from light (150), with the exception of a member of the Flavobacteria, *Dokdonia* sp. strain MED134, that grows faster in the light only under moderately low nutrient conditions (151), and a marine *Vibrio* has been shown to survive starvation longer in the light than in darkness (152). These observation may explain the wide phylogenetic distribution and high abundance of this gene in that proteorhodopsin may often assist long-term survival of bacteria under extreme energy-limiting conditions, yet not have much effect on growth during more energy-replete conditions. Interestingly, a recent report suggests the growth benefit from light in *Dokdonia* MED134 is from enhanced uptake of its required growth factor vitamin B_1, which is transported by a tonB-dependent transporter powered by a proton gradient (153). This pigment might also have sensory or other roles not yet well examined (150).

The second kind of phototrophy found by molecular genetic and fluorescence techniques to be unexpectedly important in marine plankton is anoxygenic aerobic bacterial phototrophy, based on the pigment bacteriochlorophyll *a*. Culturable aerobic anoxygenic photosynthetic (AAP) bacteria have been known from seawater for several years (154) and are widespread (155). Initial recent reports of direct counts claimed they represent about 11%, of the total bacterial community in the euphotic zone (156), but those early counts did not correct for the presence of other bacteria, and such correction often yields estimates averaging closer to 2% (157). Direct measurements show the bacteriochlorophyll pigment is relatively rare (158). However studies in various ocean locations, including ocean gyres, report that these AAP bacteria can indeed, on occasion, make up a quarter of the total prokaryotes (159, 160). A recent energetic modeling of AAP and proteorhodopsin-containing bacteria (161) has suggested that proteorhodopsin-containing ones can gain approximately 0.2% as much energy from sunlight as *Synechococcus* (common marine cyanobacterium) and AAP bacteria can gain approximately 1.3% as much energy from sunlight as *Synechococcus*. They concluded the AAP bacteria may gain energy sufficient to meet maintenance costs, but proteorhodopsin-containing bacteria were not expected to do so except at high light intensities and with large numbers of proteorhodopsin molecules per cell. They concluded the ease and low cost of maintaining PR-based phototrophy (a few genes required) may explain the high incidence of proteorhodopsin genes.

Other metagenomic studies have yielded interesting insights about organisms and processes that would otherwise be difficult or impossible to establish. For example, the sequence of an archaeal gene fragment isolated from seawater revealed extensive evidence of genetic exchange with other types of archaea and even bacteria (162). Such genetic exchange was also quite evident from the complete gene sequences of different strains of *Prochlorococcus* and *Synechococcus*, which was attributed in part to virus-mediated gene flow (163–166).

Dilution Cultures of Bacteria and Archaea

Some recent cultivation techniques that permit growth of "typical" oligotrophic marine bacteria in pure culture are based on the simple concept that bacteria living in seawater on dissolved organic matter might best be grown in the laboratory in ordinary filtered seawater. This conclusion follows from similar techniques to grow mixed "seawater cultures" of marine bacteria (167), but the seawater inoculum in this case is diluted so that only one or a few bacteria are added to the initial culture vessel (168). A rapid throughput version of this method has been used to cultivate members of bacterial groups thought to be conventionally uncultivable, such as the SAR11 clade (169, 170), albeit often at very low densities. Such cultures permit focused studies of the properties of those organisms, including genomic analysis (169, 170). This valuable work provides insight into the roles and activities of some of the most common bacterial phylotypes observed in the ocean (171), showing for example that cultivated SAR11 requires a source of reduced sulfur, as well as providing templates aiding the interpretation of metagenomic analysis (172).

PROTOZOA

Diversity and Distribution

Heterotrophic protists have been identified historically from their morphological features that are apparent at the level of the light or electron microscope. Features of cell size, shape, type, and pattern of flagellation/ciliation, skeletal structures, characteristics of the nucleus, and other cellular structures and organelles have been used to differentiate the many thousands of described species. A description of the extent of this diversity of form and function is well beyond the scope of this book (see [79] for more information). However, much of this diversity can be reduced to three basic body plans that dictate the broadest ecological roles of these cells; amoeboid, flagellated, and ciliated forms (Fig. 5). Among the lineages possessing one of these three forms only the last group, the ciliated protists, constitute a monophyletic group within the domain Eukarya.

One of the simplest body plans for protozoa is the amoeboid cell, exemplified by the gymnamoebae, or "naked" amoebae. Motile, nonphotosynthetic cells lacking flagella or cilia occur as life stages in a number of phylogenetically diverse taxa, but for many free-living species of protozoa this form constitutes the only life stage. Motility is largely confined to movement along surfaces by means of pseudopodia that can take on a variety of (species-specific) shapes. Significant abundances of amoebae are largely confined to benthic and epibiotic environments (174) and to suspended particulate material where they can occasionally be highly enriched (27). Most amoebae consume bacteria and other minute prokaryotes and eukaryotes.

Several heterotrophic protistan groups possess complex amoeboid body plans, most notably the foraminifera, polycystine and phaeodarian radiolaria, and the acantharia. Planktonic forms are predominantly oceanic in their distributions (although a large number of benthic species of foraminifera exist). These species are heterotrophic, and many are visible to the naked eye (individual cells can be ≥1 cm, gelatinous colonies can form ribbon-like structures ≥1 m in length). They feed on a wide variety of bacterial, protistan, and metazoan prey using pseudopodial networks, have rather long, complex life cycles for individual cells (weeks to months), and are extensively used in studies of paleoclimatological reconstruction (82, 175). In addition, many of these species possess intracellular symbiotic, usually eukaryotic, algae. The widespread occurrence of algal endosymbiosis among

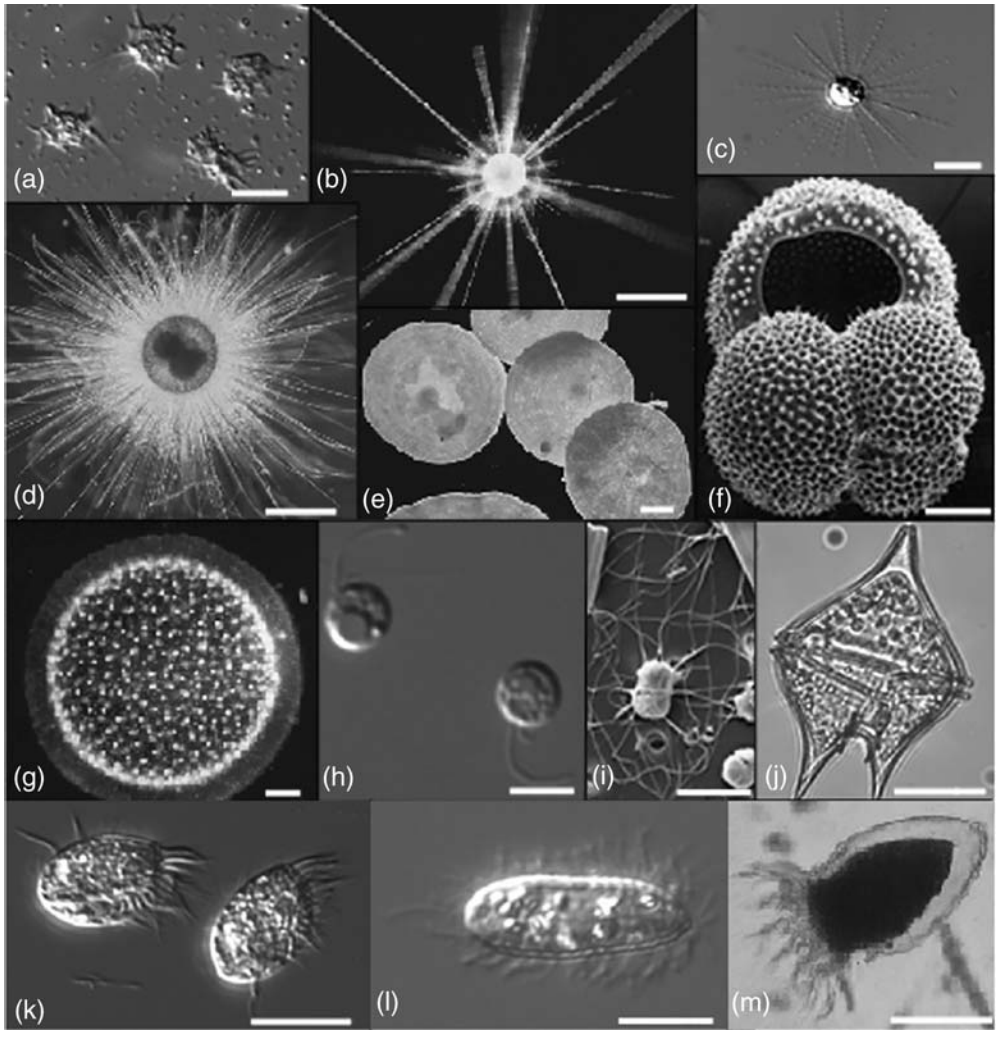

FIGURE 5 Body plans and size ranges of protozoa. These micrographs depict amoeboid (a–g), flagellated (h–j), and ciliated (k–m) forms of protozoa. From Caron et al. (173). Markers bars are 5 (i), 10 (h), 20 (c, j, l), 30 (a), 50 (k, m), 100 (b, f), 500 (d), and 1,000 (e, g) μm.
doi:10.1128/9781555818821.ch4.2.2.f5

these protozoa in oceanic pelagic environments implies that strong selective forces appear to give rise to these associations (97, 98).

Flagellated forms of protozoa exist across many protistan lineages. Eukaryotic flagella come in various sizes, numbers, and forms (e.g., with or without tiny hairs) that are characteristic of the different lineages. Many flagellate species are apparently purely phototrophic or heterotrophic (phagotrophic), but a significant (still poorly known) fraction of flagellates are mixotrophic, combining both phototrophy and phagotrophy (70, 88). Flagella in phagotrophic flagellates are employed for motility and prey capture. Most free-living flagellates possess one to four flagella (typically one or two) that can be many times the length of the cell itself. Although there is tremendous species diversity among flagellated protozoa, many of these species have broadly overlapping ecologies. As a group, flagellates are the most numerically abundant protozoa of both benthic and pelagic ecosystems, and they are fundamentally important as consumers of bacteria, cyanobacteria, and other eukaryotes. Flagellated protozoa within the nanoplankton size class (2–20 μm) are often counted as a single assemblage in plankton studies because morphological details apparent using light microscope are insufficient to distinguish among the many species. This assemblage has been variously referred to by a variety of names and acronyms including heterotrophic nanoplankton (HNAN, HN), heterotrophic flagellates (Hflags), heterotrophic nanoflagellates (HNF), and microflagellates. Heterotrophic nanoplankton is the most accurate term for most methodologies employed to count these cells because it does not require visualization of flagella (which are often lost from these small cells during preparation for microscopy). The confusion over terminology is partly a consequence of the different methodologies that have been used to count these species, and partly a consequence of the fact that these species were first studied prior to the wide acceptance of the size convention of Sieburth et al. (84).

Heterotrophic flagellates in the microplanktonic size class (20–200 μm, mostly dinoflagellates) are important consumers of phytoplankton in pelagic ecosystems. Many of these species are capable of the production of large pseudopodial nets, the pallium, that can engulf prey (particularly diatoms) significantly larger than the diameter of the dinoflagellate theca (176). This behavior and its ecological significance for energy flow in plankton communities have been recognized

only within the past few decades (177). These species are often abundant in waters where diatoms dominate (178, 179). In such situations, heterotrophic dinoflagellate biomass can be similar to that of ciliated protozoa (180).

Ciliates are generally the most recognizable form of protozoa to nonspecialists. Nonetheless, ciliates are a diverse group morphologically as well as ecologically (181, 182). The degree of ciliature on these species can range from uniformly ciliated to totally devoid of cilia during most of their life cycle, to ciliature restricted to specific regions of the cell. In addition, cilia can fuse to form various complex structures (e.g., ciliary membranes or veils, cirri) that assist in locomotion, food capture, or attachment. Ciliates are abundant and ecologically important species in both benthic (8) and pelagic (183) ecosystems and can consume a variety of prokaryotic and eukaryotic prey. Together with the heterotrophic dinoflagellates, these species are the dominant consumers of phytoplankton in many pelagic ecosystems (184), and as a consequence they form an important trophic link to metazoan zooplankton (185, 186).

Diversity and Biogeography of Protists: From Morphology to DNA Sequences

Until relatively recently, the biodiversity of marine protistan assemblages was not generally considered a controversial topic. It was generally accepted that, while all species of protists certainly have not been identified, representatives of most types of algae and protozoa had been observed and described, if not actually brought into culture in the laboratory. Direct sequencing of 18S rRNA genes from environmental samples (as described in "Molecular Phylogeny and Metagenomics: Field Applications") has changed that view. Initial forays into environmental DNA indicated a much greater diversity of protists than previously documented using classical approaches of culture and microscopy (81, 187–191). Numerous publications over the past decades have expanded these findings (see [192] for a recent global analysis), which are highly analogous to discoveries in marine prokaryote research as described already.

Previously uncharacterized protistan diversity has now been documented at virtually every level of eukaryotic organismal classification. Some of these findings could have been expected, but some have been very unexpected. For example, a much greater diversity than noted previously has been observed among small (<10 μm) protists in planktonic ecosystems. These species generally possess few distinctive morphological features, and one could expect that many cryptic species might be present among these small morphotypes. The molecular "discovery" of this eukaryotic diversity has stimulated progress on the isolation and description of new species and genera of minute algae and protozoa (193–197).

Analyses of environmental samples have also indicated the unanticipated existence of novel 18S rRNA gene sequences that imply the presence of novel lineages of eukaryotes in natural protistan communities (198–200). These are sequences for which there are apparently no known, described, or cultured species. The degree to which these sequences differ from sequences of known sequenced eukaryotes implies that some of these lineages may be distinct at the level of phylum (201).

These findings have raised basic questions, and some debate, concerning the true diversity and biogeography of protistan assemblages in natural ecosystems (202–204), the validity of the many novel phylotypes or cryptic species that are being documented (205), the potential importance and significance of the many rare taxa that characterize these communities (206), and the implications of these findings for the ecological/biogeochemical roles that protists play in aquatic ecosystems. For example, it was postulated and subsequently confirmed that some novel alveolate lineages reported from marine ecosystems make up a suite of parasitic protozoa whose ecological importance may have been significantly underestimated in the past (207). Deciphering the identity and significance of these many unknown phylotypes will constitute a significant effort for protistologists in the future (80, 85).

Life Histories and Ecological Strategies of Protozoa

Abundance, prey type, and life histories all vary tremendously among marine protozoa. The smallest species (e.g., many flagellates) tend to be the most abundant and widely distributed in the world ocean. Indeed, many of these species may be globally distributed (208). Most nanoplanktonic flagellates have potentially rapid rates of grazing and growth. Under optimal conditions these species can divide by binary fission several times a day, and thus dramatic increases in their populations can take place in response to favorable conditions within a few days (209). Many of these species are capable of surviving for limited periods of time without food and have developed a variety of physiological or life cycle strategies to cope with these events (209). However, in contrast to some bacteria that may remain viable through long periods of starvation (210), protozoa will expire or encyst in response to low food abundance, and thus "boom-and-bust" population changes are characteristic of small flagellates.

On the other end of the size spectrum from the rather ubiquitous nanoflagellate species, many of the largest protozoan species (polycystine and phaeodarian radiolaria, planktonic foraminifera) are exclusively oceanic (i.e., do not survive in most coastal environments) and/or have specific latitudinal and depth distributions. Thus, the abundances of these latter species may range from undetectable to maximal abundances of $>10^5$ individuals/m^3. Dramatic changes in the abundance of these protozoan taxa also can be related to changes in prey abundance, physical/behavioral aggregation, or to periodicity of life cycle events. For example, the planktonic foraminifer *Hastigerina pelagica* reproduces on a lunar cycle and thus abundances (and life stages) of this species in oceanic waters can vary considerably over the course of a month (211). In general, life cycles for the large amoeboid protozoa are lengthy and complex (for single-celled organisms) with life spans unknown for many species (attempts to culture them in the lab have so far been unsuccessful) but estimated to be on the order of months to perhaps years (175).

The abundance and activities of microplanktonic heterotrophic protists (mostly ciliates and heterotrophic dinoflagellates) tend to be somewhat intermediate to those of nanoplanktonic flagellates and the larger amoeboid forms. These species are present in the majority of marine ecosystems and collectively play an important role in the control of phytoplankton biomass (and probably the abundance of nanoplanktonic protozoa, although there is little information on this topic) in waters throughout the world ocean. Like small flagellates, ciliates and dinoflagellates reproduce primarily by binary fission, but their maximal growth rates are typically slower (one division a day is typical) and their life cycles often include sexual phases that allow for genetic recombination.

VIRUSES

Viral Abundance and General Properties

Viruses are simple biological agents, typically 20–200 nm in diameter, composed of a nucleic acid genome in a protein coat, that infect cells and "commandeer" the cell's machinery to make more viruses, which are released into the environment when the host cell lyses or bursts. A particular virus is thought to be capable of infecting only a narrow range of hosts (usually one species, sometimes a genus, rarely broader). Although some early studies had isolated viruses from the sea, there were no data prior to the 1980s showing such viruses were very abundant, and more significantly, no evidence that infection was occurring in any important part of the plankton community. It wasn't until the late 1980s that electron microscopy with suitable concentration methods showed that viruses are extremely abundant, similar to or even higher than bacterial abundance (212, 213). Interestingly, the most suitable transmission electron microscopy (TEM) approach, used by Bergh et al. (described in detail by [214]) was actually similar to a direct centrifugation method developed in 1949 (215).

TEM studies of viruses permit high-resolution images and observation of viral morphology, showing features such as head diameter, tails, sheaths, and tail fibers (Fig. 6, collage in upper left). Each type of virus has a fixed morphology (unlike bacteria that are potentially more plastic in their appearance) and hence a coarse measure of viral diversity has been possible by cataloging morphologies. Studies that have done so have found dozens or more different morphologies of marine viruses in a given sample (217–219). A large proportion resemble bacteriophages, which are viruses that infect bacteria.

The availability of brightly fluorescent nucleic acid stains and high-porosity fine pore size (0.02 μm) filters made from Al_2O_3 have made it easy to accurately count viruses by epifluorescence microscopy (38, 220, 221). Epifluorescence permits abundance estimates but does not allow observation of viral morphology, as viruses are below the resolution limit of light microscopy; viruses are visible only as sources of light (like stars in the night sky, see Fig. 6, lower micrograph). An extension of manual epifluorescence counts is the use of flow cytometry to count viruses, now commonly employed in some labs (222).

Virus abundance has been found to be closely related to bacterial abundance, with a virus:bacteria ratio typically 10–30:1. A typical oceanic profile of bacterial and viral abundance is shown in (Fig. 6, panel on right). This tight ratio and the strong correlation to bacterial abundance, in relation to weaker correlations to chlorophyll, have been cited as

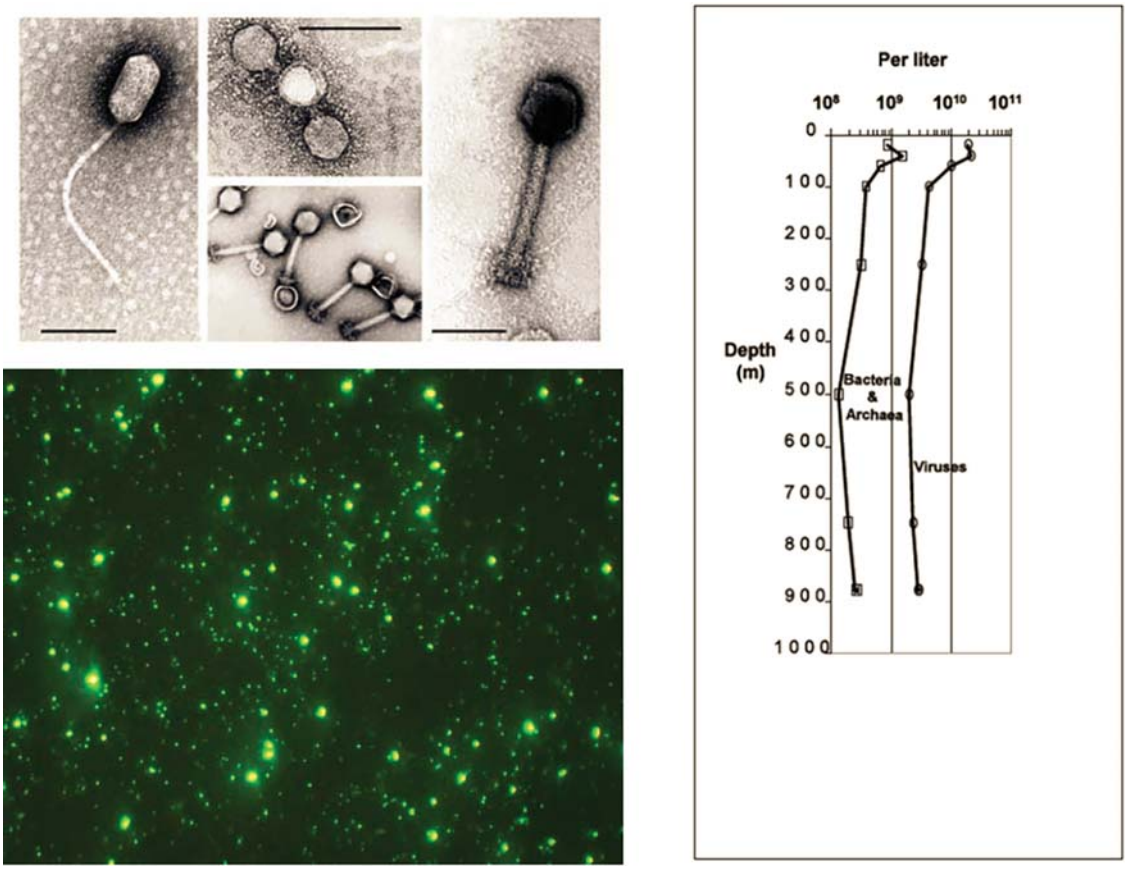

FIGURE 6 Marine viruses (upper left collage) viewed by transmission electron microscopy. These are cyanophages in the groups myoviridae (right and bottom), siphoviridae (left), and podoviridae (top). Scale bars represent 100 nm. From Sullivan (216). Epifluorescence micrograph (lower left) of SYBR green I stained viruses (small fluorescing objects) and bacteria + archaea (large fluorescing objects). Depth distribution of virus and bacteria + archaea abundances, obtained by epifluorescence microscopy of SYBR Green stained cells, in the central San Pedro Basin, California (11 August 2000). doi:10.1128/9781555818821.ch4.2.2.f6

evidence that most marine viruses infect bacteria rather than eukaryotic phytoplankton (217, 218, 223, 224), though there are also many important viruses of phytoplankton and other organisms, with significant impacts on primary productivity, blooms, and ecosystem function (225–227).

The first demonstration of the activity of marine viruses was presented by Proctor and Fuhrman (228), who showed by electron microscopic examination that viruses were actually infecting marine bacteria and cyanobacteria at a measurable rate. Because only the final portion of the virus life cycle is visible by electron microscopy (when the viruses are assembled and ready to lyse the host cell), only a very small fraction of the infected cells can be counted as infected at any given time. Proctor and Fuhrman (228) reported that only a few percent of the total bacteria from coastal waters and the Sargasso Sea were visibly infected, but they interpreted the data with a model that implied the actual fraction of the total community that was infected was much higher. They concluded that the total fraction of bacterial mortality attributable to viruses is roughly 10–40%. Subsequent studies have used refined versions of that same general approach and model (229, 230), and numerous studies have used a variety of alternative approaches to estimate viral activity—all yielding basically the same general conclusion (although the model parameters need to be adjusted for cyanobacteria, which on average seem to be infected less than bacteria). These approaches include (a) calculation of virus turnover from decay estimates (231), (b) measurement of viral DNA synthesis by incorporation of tritiated thymidine (232), (c) observation of effects of added viruses (233, 234), (d) estimation of bacterial mortality in the absence of protists, (e) use of fluorescent viruses to estimate production by an approach analogous to isotope dilution (38), and (f) dilution of viruses in filtered samples to estimate viral production (235). The overall consensus of these studies is that the initial estimates were basically correct, that is, that viruses are responsible for about 10–40% of the bacterial mortality in most marine systems (reviewed by [218, 223, 226, 236, 237]). However, there is still some disagreement about the higher estimates, and it is likely that 40% mortality from viruses alone is not typical for most marine systems.

Most of the foregoing work has focused on the viral infection of bacteria, primarily thought to be heterotrophic, although the original report by Proctor and Fuhrman (228) also noted the occurrence of cyanobacterial infection. Several subsequent studies focused on phytoplankton, including the potential effect of viruses on the termination of phytoplankton blooms of *Emiliania* and *Phaeocystis*, and the likely impact this might have on release of climate-active gases such as dimethyl sulfide (225, 227, 238–247). Although details are beyond the scope of this chapter, viruses are thought to infect virtually all marine organisms, with potentially significant impacts from zooplankton to whales (226). Detailed studies of viruses infecting cyanobacteria such as *Synechococcus* have shown differences in viral host specificity, particularly toward coastal and oceanic host strains, and some occasional high virus abundances (to 10^5/ml), as measured by most probable number (MPN) cultivation techniques, in the Gulf of Mexico near Texas (248, 249). Other virus cultivation studies with *Prochlorococcus* and *Synechococcus* in oligotrophic waters of the Sargasso Sea showed an interesting pattern of cross-infection between these genera by some virus types (suggesting gene flow among these organisms), but generally low MPN estimates of abundance, to 10^3/ml, even when cyanobacterial abundance was near 10^5/ml (216, 250).

Overall, the consensus emerging from direct comparisons of viral-mediated mortality and grazer-mediated (i.e., microzooplankton) mortality have indicated that viral lysis of bacteria constitutes a significant fraction of total mortality of this assemblage, while the mortality of phytoplankton appears to be dominated by microzooplankton in most instances (251, 252).

As mentioned, viruses have significant morphological diversity as observed in TEM studies. It is also possible to investigate aspects of their genetic diversity. In early work on this topic, Wommack et al. (253) and Steward et al. (254) observed the diversity of viral genome lengths in a field sample by pulsed field gel electrophoresis, with viral genomes ranging 25 kb to >300 kb in length. Field results show that the viral community composition is dynamic in space and time, with clear changes in the banding patterns over seasons and locations in Chesapeake Bay (253), between ocean basins and subsequent to dinoflagellate blooms (254), and with depths to 500 m at one location (255).

Although viruses do not all share a set of core genes that allows a universal viral phylogeny (comparable to SSU rRNA gene in cellular organisms), genetic diversity among a single group of viruses can be examined by sequence analysis of shared genes within the group. An example is the g20 gene in T4-like cyanophages, that has shown extremely high diversity even among very closely related viruses and has demonstrated geographic and seasonal variation (256). A second example is g23, which is found broadly in diverse and widespread T4-like phages (257) and can have seasonally repeating patterns (258) as well as short-term rapid dynamics that correlate to those in bacteria (259).

Viral Metagenomics

As with cellular organisms, metagenomics can be used to examine viral diversity and genetics without the many restrictions of cultivation. Viruses can be collected by selective filtration and concentrated by tangential flow filtration or flocculated with iron chloride (260, 261), then their collective metagenome can be extracted, linker amplified, and analyzed by sequencing (262, 263). Viral metagenomic studies are particularly challenging because the large majority of sequences have no annotated matches in any databases, but so far results from marine samples around the world have shown extremely high diversity and variations with depth, location, and time, presumably with highly dispersed types selected by local conditions (262–266). Because metagenomes are best interpreted when there are representative cultures available, the best matches of viral metagenomes originally tended to be to the few viral isolates infecting truly common marine bacteria, like cyanophage infecting *Synechococcus* and *Prochlorococcus* (267). However, the development of dilution-to-extinction cultures representing common heterotrophic marine taxa, like *Peligibacter* (a member of the SAR11 clade) and SAR116, has allowed isolation from seawater of viruses infecting these common organisms, and these isolates have indeed been found to be highly abundant in marine viral metagenomes (268, 269). New approaches to interpret the results include clustering the proteins independent of known proteins to compare samples to each other and try to find environmental factors driving viral community changes (270). Such analysis of a large global data set (Tara Oceans expedition) has shown that extensive sampling has come close to reaching the total diversity in tropical and temperate waters of such viral protein clusters (which essentially represent various viral protein motifs, not all viral protein types), and that viruses appear to be directionally dispersed

"downstream" in major ocean currents, as one may expect from first principles but also supporting the "seed bank" hypothesis of viral biogeography (271).

Viruses and Host Diversity

Viruses are themselves thought to be instrumental in driving increased diversity of their microbial hosts, via a hypothesized process often called "kill the winner." This is because viral infection is host-specific and density-dependent, the latter because viruses diffuse from host to host, so an abundant host is more likely to pass on infection than a rare one. This means that if an organism becomes abundant and blooms, winning the competition for resources, it becomes more susceptible to a viral epidemic. This would benefit the rarer organisms and thus help foster diversity (223, 272, 273). However, bloom scenarios as described above involve systems far from steady state, and the formal theory of Thingstad and Lignell (274) has interesting steady-state solutions where several viruses infect several hosts stably over time (via trade-offs between growth rates and viral susceptibility), which may occur at the strain or species level (274). There is some experimental evidence that viruses have effects on natural marine microbial community composition weaker than the kill the winner (bloom version) hypothesis would suggest (275, 276), so something resembling the steady-state coexistence described in the model may in fact be common. There also appear to be processes that foster coexistence between viruses and hosts, but the mechanisms are largely speculative (223, 277, 278).

Viruses may also be directly involved in host genetic diversity because they can be the agents of genetic exchange between microorganisms (223, 273). This often involves the viral lifestyle known as *lysogeny*, whereby viruses survive within host cells as DNA only, integrated into the host chromosome and being reproduced each time the host divides. A host harboring such a genome is called a lysogen because under conditions of stress to the host cell, a genetic switch may cause the viral genome to initiate the lytic process, producing many progeny viruses and bursting from the host. Lysogeny is a very common property, occurring in a significant part of the bacterial community (279, 280), although the incidence of induction of the lytic phase in nature is apparently low (281). Overall, lysogeny is poorly understood, but thought to have both positive and negative impacts on the microbial community (282). Recent results suggest that benefits of being lysogenic in highly seasonal polar seas leads to fundamental difference between polar and other marine viral communities (283).

Viruses and the Microbial Loop

As part of the food web, viruses occupy a unique position. They infect host cells that are mostly thought to be heterotrophic bacteria, and by doing so they typically burst the hosts to release progeny viruses and cellular debris. But what is the fate of this material? Viruses themselves do not last indefinitely, and a simple steady-state assumption implies that from each burst of viruses (typically 20–100 per lytic event), only one successfully infects another cell. The rest are inactivated and broken down by sunlight (UV and visible exposure) and enzymatic attack (284) or consumed by minute phagotrophic protists (285), thus reentering the food web as substrate for bacteria or food for protistan consumers. Experiments in controlled laboratory systems and field studies with radioactively labeled viral lysis products have supported the conclusion that most of the organic matter released by the viral infection is either taken up by bacteria or respired (286, 287).

Modeling this process as part of the microbial loop shows that viral lysis represent a sort of side loop that has the net effect of remineralizing a significant amount of the carbon and nutrients that enter the bacteria-protist part of the microbial loop (Fig. 7). A theoretical numerical steady-state model comparing a system with no viral activity to one where viruses are responsible for 50% of bacterial mortality showed that the system with viruses had 33% more bacterial production and respiration than the virus-free system, implying that the viruses had the effect of permitting the bacteria to process more of the primary production than they would otherwise (223). Although 50% is a high number unlikely to be common in the sea, this model nevertheless illustrates that viruses can reduce the amount of energy reaching higher trophic levels. The implication is that viruses lead to increased bacterial activity at the expense of the larger organisms.

MAJOR ENVIRONMENTAL CONTROLS

Light, Temperature, and Pressure

Temperature has an important potential influence on biochemical reactions and therefore on biological processes in general. Most ocean waters fall in the range of –2°C to 30°C, with obvious exceptions in hydrothermally heated areas. Temperature has long been known to be a regulating factor for the growth of heterotrophic microbes.

In temperate waters, it has been established that microbial activity is generally much higher in warm summer waters than in winter (55). The relationship is not simple, however, because multiple factors act at the same time. Some controversy still exists regarding the highest and lowest extremes for marine bacterial growth, although there is broad agreement that bacteria grow >100°C at hydrothermal vents and <−5°C in sea ice brines. Pomeroy et al. (57) noted the interesting observation that bacteria seem particularly inhibited near the freezing point of seawater (ca. –2.2°C), compared to eukaryotic phytoplankton. This effect results in polar spring phytoplankton blooms that accumulate organic carbon in advance of the response of the bacterial community and development of the microbial loop, and perhaps lead to enhanced benthic-pelagic coupling (288).

The relationship between temperature and the growth rate of marine phytoplankton was described broadly in a now

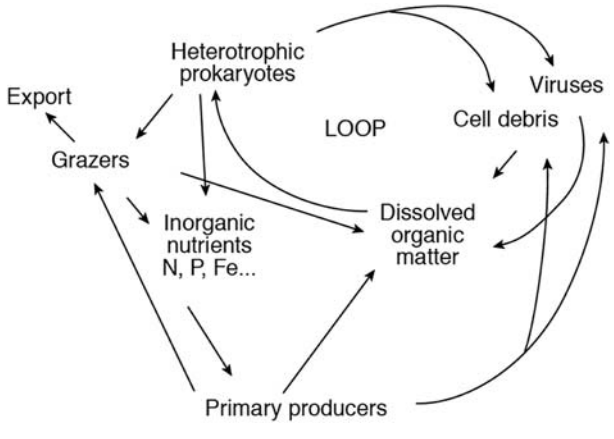

FIGURE 7 Modification of the microbial loop concept that incorporates the functional role of viruses. Export can be via predation or sinking. From Fuhrman (223).
doi:10.1128/9781555818821.ch4.2.2.f7

classic paper by Eppley (58) and later Goldman and Carpenter (289). Temperature was shown to exert a strong and direct effect on the maximal growth rates of these species, with maximal intrinsic growth rates at 0°C generally <1 division/day while growth rates at 30°C may be >4/day. These relationships indicate the maximal rate that might be attained by phytoplankton at these temperatures, but they do not take other factors into account (nutrients, light). While warm temperature ostensibly allows more rapid growth, it creates hydrographic conditions that typically give rise to nutrient limitation of algal growth. Thus, some of the coldest waters in nature witness some of the most massive phytoplankton blooms (290) while warm oceanic gyres represent some of the most oligotrophic areas of the ocean. The multiple and often conflicting effects of temperature on the growth of natural phytoplankton assemblages limit the accuracy of present predictions regarding how primary producers will respond to climate change (291).

An analysis of the effect of temperature on the growth of heterotrophic protists has indicated that the response is qualitatively similar to that of phytoplankton, but with a twist. The growth rates of at least some protozoa can exceed those of phytoplankton at warmer environmental temperatures, but the opposite effect is apparent at very low environmental temperature. This differential effect of temperature on the growth of phototrophic and heterotrophic protists was based on a large meta-analysis of published protistan growth (59). That analysis demonstrated that the maximal growth rates attained by phototrophic protists could exceed the maximal growth rates attained by heterotrophic protists (all other potential growth-limiting factors not considered). Therefore protozoan growth rates may be constrained to a greater degree at low environmental temperature than rates for phytoplankton. If so, then phytoplankton blooms may get a head start on grazers during spring in polar ecosystems. This scenario is consistent with information on seasonal biomass changes and microzooplankton herbivory in the Ross Sea, Antarctica (292, 293) but there are still too few data to fully vet this hypothesis.

The importance of high pressure on bacterial growth gained considerable attention in the late 1960s, when the deep sea submersible *Alvin* was accidentally lost overboard with its hatch open. While no lives were lost in this accident, some workmen's lunches sank to the bottom (~1,500 m) inside the submersible. Alvin was recovered after 10 months and, interestingly, there was a waterlogged lunch containing apples, bologna sandwiches, and broken vacuum bottles with broth that all appeared hardly degraded and tasted palatable. Yet when placed in a refrigerator on the ship, these items degraded relatively quickly. Initially, pressure was thought to be the preserving factor, as the sea floor temperature was similar to the refrigerator temperature and the only major difference would be pressure (294). Following this observation, a series of experiments to measure degradation of various organic materials left in the deep sea for extended periods indicated that degradation was typically significantly reduced, implying that pressure reduces the degradation rates (295). Nonetheless, changes in the protozoan community of natural detrital material sinking to the deep ocean floor indicate that the microbial community can respond relatively quickly in some situations (296). Deep sea microbiology has advanced considerably, yet it is still difficult to interpret results with respect to actual *in situ* rates of naturally occurring organic matter. Deep sea bacteria adapted to high pressures have been isolated that are barophilic (also called piezophilic), meaning that they prefer high pressures and have reduced activity at lower pressures, whereas others are barotolerant (piezotolerant), tolerating but not preferring high pressures, for example, see (297, 298).

Few data are available on barotolerant/barophilic marine protozoa. Protozoa certainly exist and grow at great oceanic depths, but measurements of *in situ* growth rates for these species do not yet exist. Measurable protozoan numbers have been documented in the deep-sea sediments for more than 30 years (299, 300) and viable protozoa have occasionally been cultured from these environments (301–305), but very few direct measurements of the activities of these species *in situ* have been reported (306). A few protozoa have been isolated that will grow at high pressure (302, 303, 307), and protists that appear to be unique to the deep ocean have been observed either directly or through the analysis of DNA sequences (308–311), but possibly the best direct evidence that protozoan activity takes place at the high pressures characteristics of the deep sea are experimental and observational work noting the stimulatory effect that detrital deposition has on some components of the protozoan community (296, 312). These observations indicate a diverse and active protozoan fauna of the deep ocean, although their biogeochemical significance is largely uncharacterized at this time.

Dissolved and Particulate Organic Matter

Bacteria and archaea are thought to be by far the most important organisms with respect to the processing of dissolved organic matter (DOM) and nonliving particulate organic matter (POM; also called detritus) in the ocean. While there may be some uptake of DOM by protists, particularly for growth factors needed in trace amounts such as vitamins (313), the bulk of this material is probably utilized by bacteria and archaea (314), including the smallest cyanobacterium *Prochlorococcus* (315, 316), which thus may be considered a mixotroph. Due to their small size, bacteria have extremely high surface:volume ratios and, combined with their overwhelmingly high abundance, an extremely high integrated surface area. Protozoa tend to obtain the organic materials that they require for growth from their prey rather than through the uptake of DOM. Overall, protozoa tend to be sources of dissolved and detrital organic substances, through the excretion of unassimilated prey biomass in expelled food vacuoles.

Particulate organic matter is not directly available as substrate to bacteria. These substances must first be reduced to small molecules that can be transported into the cell. This is accomplished by the production of extracellular enzymes (note that few if any large polymers are directly taken up by bacteria, with the possible exception of DNA). Hydrolytic enzymes produced by bacteria (and almost certainly archaea) break down polymers like proteins, polysaccharides, and nucleic acids. As with DOM, POM is composed of a complex mixture of compounds that vary in their susceptibility to bacterial degradation and utilization.

Particulate material in the water column serves not only as bacterial substrate but also as substratum. POM occurs in the water column across a huge size spectrum from micrometers up to some detrital aggregates more than 1 m in diameter (317). Much of this particulate material is in a constant state of flux, with colloidal material constantly coalescing and aggregating to form new or larger particles (318) as microbial degradation acts simultaneously to remineralize this material. Detrital particles that attain macroscopic size either by direct formation (317, 319, 320) or via accretion and aggregation (321) and are often called marine snow or macroaggregates. Marine snow particles are readily colonized by bacteria and

many other microorganisms, and their abundances on these aggregates can be orders of magnitude greater than abundances in an equivalent volume of the water surrounding the aggregates (320, 322–324), and their diversity and highly concentrated abundances on detrital aggregates implies that there may be stronger trophic coupling and efficient energy/elemental cycling relative to these processes among free-living microorganisms in the surrounding waters. This speculation has resulted in a number of formalizations of the potential impact of aggregate microbiology on biogeochemical processes in the ocean (325, 326). Most definitions of biofilms include aggregated particulate and these particles are often studied within the biofilm conceptual model (see chapter by Lawrence et al. in this section).

Inorganic Nutrients

Macronutrients (N, P)

Virtually all heterotrophic organisms in the ocean contribute to the pool of available macronutrients via the excretion of metabolic wastes, and protozoa contribute significantly to this process. Macronutrients are often in excess within protozoan prey relative to the consumers' needs because much of the prey carbon is respired to produce energy for metabolism and growth. "Excess" nutrients are eliminated as either organic compounds, or more commonly as ammonium (for N) and phosphate (for P). The importance of the match (or mismatch) between predator and prey stoichiometry has been demonstrated experimentally and repeatedly in marine and freshwater ecosystems and has been elevated to a fundamental ecological principle governing elemental and energy flow in aquatic food webs (327). Based on this reasoning, Caron et al. (328) concluded that bacterivorous protozoa play an important role in nutrient remineralization and release in the ocean. Bacterial biomass is typically rich in N and P relative to other organisms, so bacterial predators experience large excesses of these elements relative to their growth needs. Moreover, bacterivorous protozoa should be disproportionately important in nutrient remineralization among single-celled eukaryotes because, as the smallest protozoa in aquatic ecosystems, they have high weight-specific metabolic rates (329). Thus, both stoichiometric and allometric relationships implicate small, bacterivorous protozoa as important sources of remineralized nutrients in the plankton.

The reasoning also holds true for bacterial utilization of organic compounds, and it has led to some interesting findings and conclusions regarding the role of bacteria in nutrient cycling in the ocean. Bacteria are important sources remineralized nutrients when they are consuming N- or P-rich substrate. Contrary to this traditional view, however, heterotrophic bacteria are often strong competitors for inorganic nutrients under many situations, largely because bacterial biomass is richer in N and P than much of the organic matter they consume in nature, so they require additional N and P to produce their biomass (summarized by [330]). Experimental studies have demonstrated the uptake of ammonium by bacteria other than cyanobacteria using stable isotope ^{15}N (331), and also the short-lived radioisotope ^{13}N (332), often finding that bacteria may be responsible for one third of the N or P uptake. Additionally, there is evidence that bacteria in some oceans, like the Sargasso Sea, are limited by phosphorus (333, 334). Growth limitation of bacteria by phosphorus appears to be a common phenomenon of freshwater ecosystems (335, 336).

N_2 fixation is restricted to prokaryotes, and in the ocean water column it was thought for many years to be done primarily by *Trichodesmium*, a warm-water colonial cyanobacterium that blooms sporadically, and *Richelia*, a cyanobacterial symbiont that lives within certain diatoms (337). Some unicellular cyanobacteria also contribute significantly to global N_2 fixation (338), and molecular biological data suggest that a variety of other bacteria—not phytoplankton—may also be fixing nitrogen in seawater (339). An exciting recent discovery in this field is that a bacterial nitrogen fixer originally known only from nifH sequences, UCYN-A, has had its genome sequenced by parallel pyrosequencing, and was found be a cyanobacterium (*Candidatus Atelocyanobacterium thalassa*) lacking the apparatus to generate oxygen, as well as other pathways (340). Further study showed it to be symbiotic with a marine alga (341).

Micronutrients (Trace Metals, Growth Factors)

Bacterial growth may often be limited by macronutrients as described, and sometimes by trace nutrients, particularly iron, that occur at very low concentrations in seawater (342, 343). The importance of Fe as an element limiting primary production in some oceanic regions has come under close scrutiny and extensive experimental investigation in recent years. Many bacteria have special uptake mechanisms to utilize extremely low concentrations of iron, including siderophores (released compounds that bind iron and are then specifically taken up). Even if bacteria are capable of growth at low Fe concentrations, growth may be less efficient. Kirchman et al. (344) found that Fe limitation strongly reduced bacterial growth efficiency in a cultured marine bacterium (ca. 50% efficiency Fe replete, but <10% when Fe deplete). Thus the effects may be complex. Marine N_2 fixation also may be limited by the availability of Fe because nitrogen fixers have high Fe requirements.

Protozoa tend to be a source rather than a sink for Fe in the ocean, analogous to their role in macronutrient remineralization. Protozoan grazing activity has been shown to release iron from bacterial biomass thereby relieving Fe limitation in co-occurring phytoplankton (345), a mechanistic demonstration of the process using protozoa growing on Fe-replete bacteria. However, it has also been reported that Fe-limited bacteria may contain insufficient Fe for the bacterivorous protozoa that consume them, thus leading to Fe limitation in the protozoan as well (346).

Oxygen

Hypoxic and anoxic regions of the ocean are expected to significantly expand in the coming decades as a consequence of changes in ocean stratification resulting from global climate change (347). As oxygen becomes depleted, bacteria have the capability to utilize a series of alternate electron acceptors. Compositional changes in the bacterial assemblage accompany these changes in metabolic activity, but are beyond the scope of this chapter. Many of the studies on benthic microbiology have focused on redox reactions that occur there. Oxygen is usually consumed by sediment microbial activity much faster than the rate at which it can be replaced by diffusion, so most sediments, especially organically rich ones, are anaerobic below a few mm (fine-grained muds) or cm (coarser sediments) from the sediment/water interface, except where animals ventilate the benthos through tubes. When oxygen is absent, bacteria and archaea use alternate electron acceptors, such as nitrate, nitrite, oxidized Fe or Mn, organic matter, or sulfate. The use of nitrate or nitrite as an electron acceptor, with concomitant production of N_2 gas (denitrification) results in net loss of biologically available nitrogen. Bacteria that perform this reaction are typically

"facultative" organisms that can switch from oxygen to nitrate or nitrite. Organic matter may be reduced by fermentation reactions, in which part of an organic substrate is oxidized while another is reduced; fermenter organisms are also typically facultative. Sulfate is a particularly important alternate electron acceptor in marine systems because it occurs as the second most abundant anion in seawater (behind chloride), typically 28 mM. Its use as an electron acceptor leads to the production of elemental sulfur or sulfide. The use of sulfate as an electron acceptor yields much less energy than oxygen or nitrate, and sulfate-reducing bacteria are strict anaerobes, unable to use oxygen (often strongly inhibited by oxygen). Methane can be produced by methanogenic archaea, which are all strict anaerobes, using CO_2 as an electron acceptor (see Findlay and Battin this section for further discussion).

Reduced compounds, such as sulfide, are produced by the use of these alternate electron acceptors, and they diffuse away from their source. When they diffuse to environments with a strong oxidant, like oxygen, they may be oxidized by chemotrophic bacteria in energy-yielding reactions. Similarly, ammonium can be oxidized to nitrite and then nitrate by chemotrophic nitrifying bacteria, and the nitrate can diffuse into anaerobic layers and become denitrified to N_2. Such coupled oxidation-reduction reactions can lead to accelerated biogeochemical cycling, particularly in sediments.

It was originally believed that relatively few free-living species of protozoa inhabit anaerobic and hypoxic marine environments. This view has changed, based on (largely) molecular analyses of some of these environments (308, 348–351). A significant number of previously undescribed eukaryotic phylotypes have been observed in anoxic environments. High protozoan biodiversity in the absence of molecular oxygen is not completely unexpected because other anaerobic environments such as some gut environments (e.g., cockroach guts and cattle rumen) have notoriously abundant and diverse protozoan fauna, but marine anaerobic environments have been difficult to access and sample properly. Many anaerobic protozoa exist together with symbiotic bacteria (352, 353) or possess unique organelles such as the hydrogenosome (354).

MAJOR BIOTIC CONTROLS

The Trophic Activities of Protozoa: Predation and Top-Down Controls

Predator-prey relationships are fundamental interactions within biological communities, linking production of biomass on the one hand with removal on the other hand. Establishing the trophic relationships among microorganisms and quantifying the rates at which they take place constitute major endeavors within marine microbial ecology. As noted previously, protozoa consume a wide variety of prokaryotic and protistan prey. Numerous laboratory and field measurements have been made throughout the past quarter century to attempt to quantify the rates of bacterial and phytoplankton consumption. The methodology for accomplishing these studies has varied, and a number of approaches are presently employed.

Methodologies for estimating microbial grazing are diverse but generally fall into one of two experimental approaches: perturbation experiments or tracer experiments. Perturbation experiments (size fractionation, metabolic inhibitors, and dilution technique) rely on manipulation of the consumer or prey assemblages in some manner such that the effect is evident from changes in the abundance of the prey population during incubations, relative to control treatments that receive no manipulation. Tracer experiments (radioactively labeled prey, fluorescently labeled prey, stable isotope–labeled pray) attempt to use labeled prey or a prey proxy to quantitatively follow the movement of prey biomass into consumers. For summaries of these methods, see (74, 355).

Perturbation experiments have entailed a variety of mechanisms to "decouple" predators from prey in natural microbial communities. For example, size fractionation uses various size filters to remove an entire size class of grazers from a water sample, leaving the prey to grow in the relative absence of those predators during subsequent incubations. Unfiltered samples (controls) indicate the growth of prey in the presence of predation. Predation impact is obtained by the difference in growth of the prey between these treatments. This method has been used to examine predation on cyanobacteria, heterotrophic bacteria, and minute phototrophic eukaryotes. Alternatively, metabolic inhibitors have been employed to halt predation (or, in some cases, prokaryote growth) instead of physical removal by filtration.

The dilution technique (356) has become a common method for examining grazing mortality of phytoplankton by microzooplankton (which is comprised predominantly of protozoa). This method relies on the dilution of herbivorous zooplankton in a series of subsamples (and concomitant measurements of phytoplankton growth in each subsample) to estimate the grazing mortality of the herbivores.

Tracer experiments have used both radioactively labeled prey and fluorescently labeled prey to examine predation on bacteria and phytoplankton. Radioactive labeling has been accomplished using ^{14}C- or ^{3}H-labeled organic compounds (for bacteria) or ^{14}C-bicarbonate (for phytoplankton). The use of radioisotope approaches has diminished with improvements in the number and types of fluorescent compounds available for this work. Fluorescently labeled prey have been labeled with a variety of compounds that allow the prey to be observed readily (even inside predators) by epifluorescence microscopy (357, 358). Alternatively, a decrease in the total number of fluorescent prey in a sample indicates the rate of removal of the prey assemblage.

Both perturbation experiments and tracer experiments described have inherent advantages and disadvantages. For example, perturbation experiments are fairly straightforward to conduct, but they may introduce artifact by removing potentially important sources of nutrients or organic compounds released by predators (359). On the other hand, the dilution technique is very labor-intensive, but one advantage of this method is that it provides the opportunity to determine both phytoplankton growth and grazing mortality in a single procedure. Most tracer experiments are fairly easy to conduct and generally do not perturb the food web being studied, but feeding selectivity for or against the tracer (relative to natural prey) may produce spurious results. In addition, some of these methods provide a measurement of the activity of the entire grazer assemblage, and others are applicable for examining the activity of specific taxa. For example, size fractionation approaches examine the effect of the removal of an entire size class of organisms (a community-level" measurement) whereas fluorescently labeled prey can be used to examine the ingestion rate of a particular species by observing the uptake of labeled prey into that species (a species-level measurement).

Predation rates of numerous marine protozoa have been examined in the laboratory. From these experiments, a general knowledge of the rates of consumption by these species has been accumulated. Feeding in most protozoa shows a

functional response to the availability of prey that is similar to the response of many organisms (although there are a great deal of species-specific differences). That is, ingestion rate increases rapidly as prey abundance increases until a maximal ingestion rate is attained (360). Many of these curves have the form of a Type II functional response curve (361).

Based on the features of these curves, it is clear that protozoa have the ability to ingest substantial numbers of prokaryote and eukaryote prey in natural ecosystems. Some bacterivorous flagellates can consume bacteria (and cyanobacteria) at rates up to 100 s per individual per hour, whereas some bacterivorous ciliates can consume thousands of bacteria per individual per hour. Rates of consumption of eukaryotic phytoplankton by herbivorous ciliates and large flagellates are considerably more variable and highly dependent of the feeding mechanism (e.g., filtration in ciliates versus pseudopodial net in dinoflagellates) and the size of the prey.

These lab studies demonstrate that rarely are prey sufficiently abundant in nature to support the maximal ingestion rates of which many protozoa are capable. Nevertheless, predation rates of protozoa in nature are sufficient to constitute a major factor controlling the standing stocks of bacteria and phytoplankton (63, 180, 184, 355). For example, these summaries indicate that on average, protozoa consume 20–80% of the standing stock of phytoplankton (measured as chlorophyll) in the world ocean, and 60–75% of daily primary production, a departure from the classical view of copepods as the primary consumers of phytoplankton. This large role of herbivorous protozoa infers a large role for copepods as consumers of planktonic protozoa (185). Similarly, the role of protozoa as major consumers of bacterial biomass is indicated by the relative balance between bacterial production on the one hand and bacterivory on the other (Fig. 8). The approximate one-to-one balance between these measurements over a large number of studies implies that protozoan bacterivory must be a major factor determining the fate of bacteria in marine (and freshwater) plankton ecosystems.

It is interesting to note that some types of bacteria, including marine *Bdellovibrio*, are known to prey on other marine bacteria (362). At the time of this writing, the importance of bacterial predation by other bacteria within the context of oceanographic processes is not known, and presumed to be small. While *Bdellovibrio* may be cultivated from seawater, it can also be found among sequences from cold sediments (363) and the water column. *Bdellovibrio* happens to be the best studied predatory bacterium, but there are no doubt others that we simply have not yet identified (364).

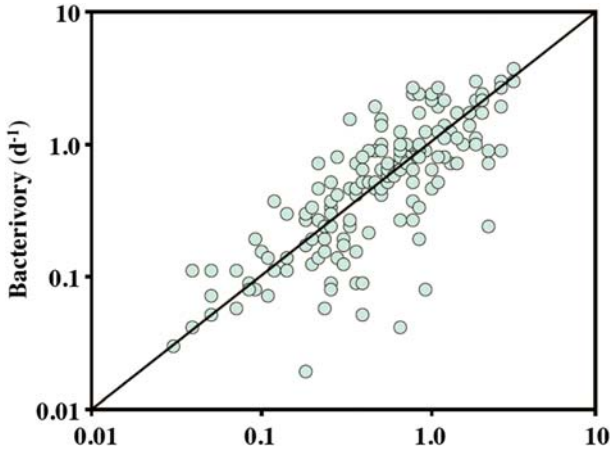

FIGURE 8 Meta-analysis of experimental studies that have compared rates of bacterial productivity (converted to population growth rate, units of d^{-1}), with rates of bacterivory by phagotrophic protists (expressed as equivalent units of bacterial growth rate; d^{-1}). The solid line indicates where measurements of bacterial removal by grazers were equal to measurements of bacterial production. Data redrawn from Sanders et al. (63). doi:10.1128/9781555818821.ch4.2.2.f8

Species-Specific Interactions (Mutualism, Parasitism, Commensalisms)

Interactions between marine microorganisms such as competition, allelopathy, and symbiosis are fundamental aspects controlling community composition and activity, but at present most are poorly characterized in natural marine systems. Bacteria must compete for dissolved and particulate compounds with other bacteria and with protistan taxa, while phagotrophic protists must compete with each other and metazoa for available prey. These interactions manifest themselves in many forms including true competition for available resources, resource partitioning such that some taxa specialize on the utilization of certain substrates or prey while others specialize on different ones (reducing competition), or even the cooperative use of compounds (e.g., one bacterium's waste products being utilized as substrate by another bacterium). These metabolic consortia are probably common in the ocean but can be difficult to identify and characterize. Consortia of bacteria working together to perform complex reactions, especially in anaerobic systems, are well known in microbiology.

Consortia in which there is a very high degree of integration between species (i.e., symbiosis) are very common in the ocean. These associations may be beneficial to both partners (mutualism), beneficial to one part and inconsequential for the other (commensalism), or beneficial to one part and detrimental to the other (parasitism). Many highly visible and well-characterized marine mutualisms exist between chemosynthetic bacteria and animals, such as hydrothermal vent or methane seep tubeworms, clams, mussels, or other invertebrates (365), and also between luminescent bacteria and fish or squids (366). Similarly, many protozoa form mutualistic associations with photosynthetic protists (367). The most conspicuous of these associations involved large, amoeboid protozoa of oceanic ecosystems (planktonic foraminiferans, polycystine radiolaria, and acantharia) which contain large numbers of intracellular algae (74), but associations between some heterotrophic dinoflagellates and cyanobacteria are also common (368). Studies have indicated that efficient nutrient cycling in these protozoan-algal associations confers an ecological advantage for both partners in surface waters of the oligotrophic ocean.

Parasitism is also a common symbiotic relationship among marine protozoa. Protozoan parasites have been reported for a diverse array of protists and animals in the ocean. Heterotrophic dinoflagellates and presently uncultured alveolate clades related to alveolates appear to be particularly successful at evolving this mode of existence (369–371). In a common parasitic group, *Amoebophrya* spp., the parasite invades photosynthetic dinoflagellates where it undergoes reproduction, eventually lysing the host and releasing dozens of infective swarmers. The parasitic protist, *Pirsonia* spp., is a parasite of diatoms (372). The overall importance of these parasitisms in the population dynamics of their hosts is not clearly known but may constitute a significant source of mortality for some species (373, 374). The frequency and diversity of "new"

parasitic phylotypes from environmental gene surveys implies that there are probably many more parasitic relationships yet to be described in the ocean (371).

In addition to these organism-organism associations, there are numerous examples of "chemical warfare" among marine microorganisms. Allelopathy is defined as the production of substances that adversely affect competing species, although substances that are produced to deter grazers is also sometimes included under this rubric. Many marine microorganisms produce substances that adversely affect growth or survival of other species. The most obvious example is the existence of numerous classes of antibiotics produced by marine microorganisms. One study showed that out of 86 marine microbial isolates tested, more than half produced substances that inhibited the growth of other isolates (375). Moreover, it was found that bacteria isolated from marine aggregates were more likely to produce these substances then those isolated from free-living plankton. Thus, species living in close physical proximity to one another may be more likely to produce these substances. Disruption of cell-cell communication, via production or degradation of chemical signals, is currently an area of intensive research within marine ecology. See Roy et al. (376) for a recent review of the chemical ecology of the marine plankton.

Bacteria and phytoplankton in the plankton may compete actively for various macro- and micronutrients, and these competitive interactions appear to be an important reason for the production of many allelopathic compounds (377). In addition, a wide spectrum of protistan species produce secondary metabolites that are believed to play a role in the inhibition of competing species (378, 379) and/or in reducing predation by protozoa and/or metazoa (380–382).

Balancing Microbial Growth and Removal

Microbial communities are highly dynamic. They can exhibit rapid shifts in community composition and significant increases or decreases in biomass of species or whole assemblages. Nevertheless, over reasonably large scales of time and space, these communities are remarkably constant. This observation implies that processes of production and removal must be in overall balance within the ocean.

For heterotrophic bacteria, production is dependent on available substrate for growth and the efficiency of conversion into bacterial biomass. Numerous measurements and estimates have been made of bacterial growth efficiency since the 1980s. These estimates range broadly from approximately 1% to more than 60%, but typically average in the 20–30% range (reviewed by del Giorgio and Cole [383]), indicating that the production of bacterial biomass is a relatively efficient process. Nevertheless, as noted already, abundances of bacteria in the ocean remain remarkably constant, therefore production must be closely coupled to losses from predation (protozoa, viruses) because these cells are sufficiently small that sinking losses must be minimal. The explanation for this tight coupling between bacterial production and loss rates is not completely clear, but may relate to the fact that bacterial predators are capable of rapid increases in abundance. This conclusion is also supported by summaries of large data sets that indicate an overall balance between bacteria production and bacterial mortality across a wide spectrum of environments (Fig. 8).

Protozoan populations exhibit short-term (weeks) to seasonal variances in abundances that exceed the variances typically observed for bacteria, but massive "blooms" of nano- or microzooplankton are not common. This is not the case for phytoplankton assemblages, which exhibit considerably more temporal and spatial variability than bacteria or protozoa. For example, chlorophyll may vary two to three orders of magnitude between rich coastal and oligotrophic open ocean environments, or seasonally in some coastal ecosystems. The less dramatic changes in the standing stock of protozoa cannot be attributed to low growth inefficiency. Typical gross growth efficiencies for these species are quite high, ranging from 30% to 40% (329, 384). It is probably that many metazoan zooplankton prey heavily on protozoa, thereby exerting strong top-down control on these assemblages (and perhaps relieving phytoplankton from grazing pressure exerted by the microzooplankton).

THE MICROBIAL LOOP REVISITED

The original concept of the microbial loop (12, 34) depicted a large fraction of pelagic carbon flux passing from DOM/POM to bacteria, and subsequently bacterial biomass being grazed by protists that enter the classic food web. That depiction of microbial assemblages and energy flow has become more complicated in recent years, with the inclusion of viruses, mixotrophs (both prokaryotic and eukaryotic), archaea, bacterial and archaeal autotrophs, phototrophs like cyanobacteria, and proteorhodopsin-based phototrophy (150). Our views regarding the small-scale distributions of microbes and their substrates have also changed. For example, Azam et al. (325, 385) have pictured the marine bacteria as being embedded in a very loose gel-like matrix composed of these materials, with various local "hot spots" of microbial activity where there may be a particularly rich source of nutrients (leaky organism, degrading particle, etc.). These small-scale gradients and interactions are undoubtedly important for understanding how these systems function (386).

Despite these new wrinkles in our understanding, or perhaps because of them, it is now widely recognized that heterotrophic microbes (viruses, archaea, bacteria, protozoa) play pivotal roles in the ocean as agents of elemental transformation and energy flow (85, 387). Their activities may complicate how we model energy production and utilization and nutrient cycling, but these populations are nonetheless recognized as integral components of global biogeochemical cycles. In the 1980s, a debate began about whether the microbial loop is primarily a link in marine food webs, passing salvaged DOM and POM back up to larger organisms, or a sink, respiring almost all the carbon via microbial trophic interactions. It is clear now that the microbial loop (broadly defined) plays fundamental roles in both trophic transfer of energy and carbon/nutrient remineralization. Which of these roles dominates is highly dependent on environmental conditions and community composition. For example, the remineralization of organic matter by heterotrophic bacteria constitutes a substantial (albeit unavoidable) loss of primary production to higher tropic levels. At the same time, photosynthetic cyanobacteria <2 μm in size make up the largest portion of total primary production in many open ocean ecosystems. The vast majority of this biomass must enter the food web via their consumption by minute protozoa. Thus, the only *link* for this biomass is via the microbial loop.

RESEARCH TRENDS, INCLUDING MICROBIAL ASSOCIATION NETWORKS

Molecular approaches are going beyond just fueling a remarkable "age of microbial discovery" that in many ways has paralleled the level of discovery experienced by biologists studying macroorganisms during the late 1800s and early

1900s. Molecular techniques are being employed to document the presence and abundance of ecologically important or "environmentally relevant" species of microorganisms in natural aquatic communities, to characterize the diversity and community structure of microbial communities, and to begin to understand how microbial diversity extrapolates to the emergent properties of communities (e.g., primary production, respiration, food web structure). The development and application of these tools is enabling extensive and detailed observations and experimental studies of microbes and their interactions. A very recent example is the use of microbial association networks to mathematically examine the co-occurrence of multiple microbial types and environmental parameters to examine possible interactions, typically done in time series studies, and potentially including bacteria, archaea, protists, zooplankton, and viruses (Fig. 9) (388–390). These approaches are uncovering previously unrecognized correlations between taxa (positive, negative, simultaneously, or time-lagged) that hint at functional relationships between them, for example, predator-prey, symbiotic, or other relationships. Such correlations are fodder for hypotheses regarding the nature of these relationships and experimental studies to test them. For example, association network analysis from the Tara Oceans expedition pointed to a specific microbial-animal photosymbiotic interaction, between a flatworm and a green microalga, which was then verified by direct observation (391).

As illustrated throughout this manual, molecular methods are used increasingly to investigate important biogeochemical processes, such as the cycling reactions of N fixation,

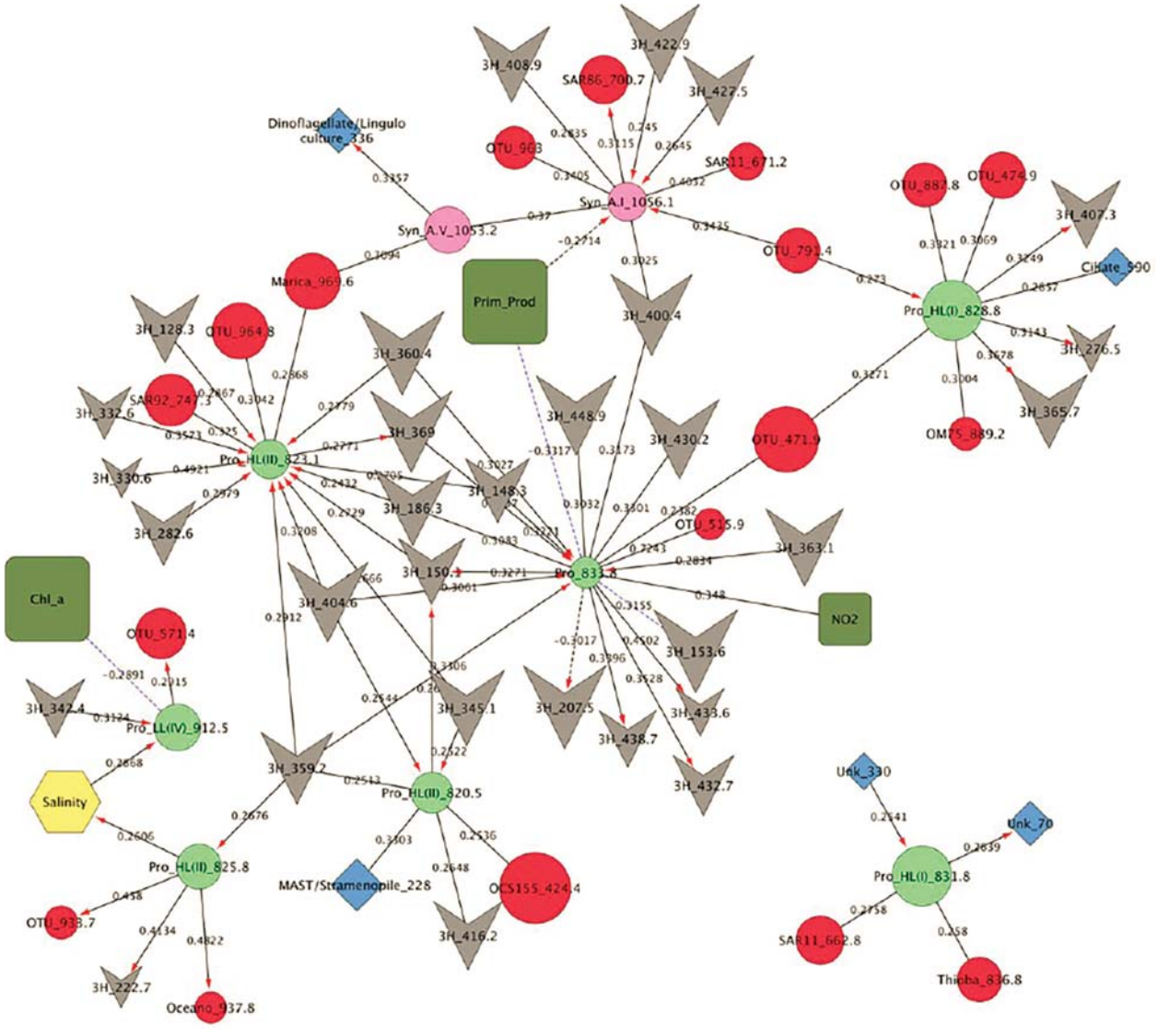

FIGURE 9 Microbial association network, showing co-occurrence patterns of near-surface planktonic microbes at the San Pedro Ocean Time Series, USC Microbial Observatory, sampled monthly for 3 years. This network shows only organisms and parameters directly correlated to cyanobacteria (green, *Prochlorococcus*; pink, *Synechococcus*). Circles, bacteria; V-shape, T4-like myoviruses; blue diamonds, protists; hexagons and squares, environmental parameters and processes (nutrients, salinity, chlorophyll *a* [Chl_a], primary productivity [Prim_Prod]); solid lines, positive correlation as measured by local similarity analysis; dashed line, negative correlation; arrows point to correlations lagged by 1 month; data in Chow et al. 2013 (388). Symbols sized to reflect relative abundances within each group. doi:10.1128/9781555818821.ch4.2.2.f9

nitrification, and denitrification (392, 393). Given the present realization that microbial processes dominate biological productivity, energy utilization, and nutrient cycling in the ocean, these studies will provide fundamental knowledge regarding how biological communities within the ocean are structured, and how they function.

We thank our many students, postdocs, and colleagues for all their contributions over the years, and support from the National Science Foundation including grants OCE 1031743, OCE1136818, OCE0962309, MCB0703159, the National Oceanic and Atmospheric Administration (NA11NOS4780052), and the Gordon and Betty Moore Foundation.

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