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INTRODUCTION: THE EVOLUTION OF MICROBIAL ECOLOGY OF THE OCEAN

JOSEP M. GASOL

Institut de Ciències del Mar, CSIC, Barcelona, Catalunya, Spain

DAVID L. KIRCHMAN

School of Marine Science and Policy, University of Delaware, Lewes, Delaware, USA

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1.1 INTRODUCTION

Microbes are everywhere in large numbers. They were on Earth ca. 4 billion years ago, and they will be on Earth long after multicellular eukaryotes go extinct. In some environments they are the only living beings around because they can thrive in hot and cold environments; they develop inside rocks and can tolerate toxins and ultraviolet (UV) radiation. Covering 3.6×10^{11} km^2 (71% of the Earth surface) and reaching a depth of 3.7 km on average, the marine habitat is likely the largest ecosystem on Earth. It is where all life started. Today, the ocean habitat is teeming with morphologically, genetically, and functionally diverse microbes. Half of the primary production in the planet occurs in the ocean (Field et al. 1998), 90% of which is done by microorganisms (Duarte and Cebrián 1996). They are also responsible for most of the ocean

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respiration and are crucial in most, if not *all*, key transformations in the cycles of nitrogen, phosphorus, sulfur, iron, and other metals.

All these topics and more are part of "microbial ecology of the ocean." The field has maintained momentum since publication of the first edition of this book in 2000 (Kirchman 2000), and arguably its importance has even increased. There is a journal, *Aquatic Microbial Ecology*, specifically devoted to the field (also including other aquatic environtments), and leading general journals such as *Nature* and *Science* have devoted mini-reviews and special issues to it (Rees 2005; DeLong 2007; Ash et al. 2008; Lupp 2009; Bork et al. 2015), as have more specialized journals in oceanography and microbiology (*Nature Reviews Microbiology*, 2007, volume 5; *Oceanography* 2007, volume 20, issue 2). In addition, funding initiatives supported by public (e.g., the US Joint Genome Institute) and private sources (such as the Gordon & Betty Moore Foundation with its Marine Microbiology Initiative, the Agouron Institute, and the Simons Foundation) have sponsored research in marine microbial ecology. A specific Gordon Research Conference on marine microbes has been running since 2004, again reflecting the current dynamism of the field. It has even been the subject of a cultural anthropology book, which analyzes knowledge and practitioners of microbial oceanography (nearly synonymous with marine microbial ecology) in the context of global culture. It claims that microbes in "alien oceans" have come to be an excellent viewpoint for thinking about humans (Helmrich 2009).

One could also claim that marine microbial ecology is a large fraction of general microbial ecology. For example, about 30% of the habitat-specific papers published in the *ISME Journal* during 2015 were partially or totally marine, here including sediments, estuaries, and high-salinity systems. Also microbial research is a large part of marine sciences; about one-third of the papers in *Annual Review of Marine Sciences* between 2009 and 2015 are about microbes.

Marine microbial ecology is one of the most dynamic scientific fields because it is at the crossroads of many disciplines such as oceanography, biogeochemistry, microbiology (including protistology and virology), physiology, evolution, and genomics. Being at a crossroad implies that a current practitioner of the field should have working and conceptual knowledge in all these fields, in addition to practical skills in bioinformatics and statistics (and microscopy). All these fields have advanced greatly in the last few years as have the temporal and spatial scales the researchers study. Microbial ecologists now consider spatial scales ranging from the global ocean, the ocean basins, or a particular ecosystem to the interactions at the microscale or even inside a cell, as is apparent in the different chapters of this book. The temporal scales are also diverse, from the hourly or the diel changes in activity, to the seasonal or interannual scale, including the effects of long-term global change on the dynamics, activity, and diversity of microbes, or even the short- or long-term evolution of marine microbes. The focus of interest also varies. Although the organisms tend to occupy the central point, some studies are more centered on the communities (so-called "microbiomes"), whereas others focus on the environment (how the communities are structured in or within environments, rooted in "hard" ecology). Still other studies tend to skip the cellular level (or the species level) and focus on the gene level. This variety of approaches,

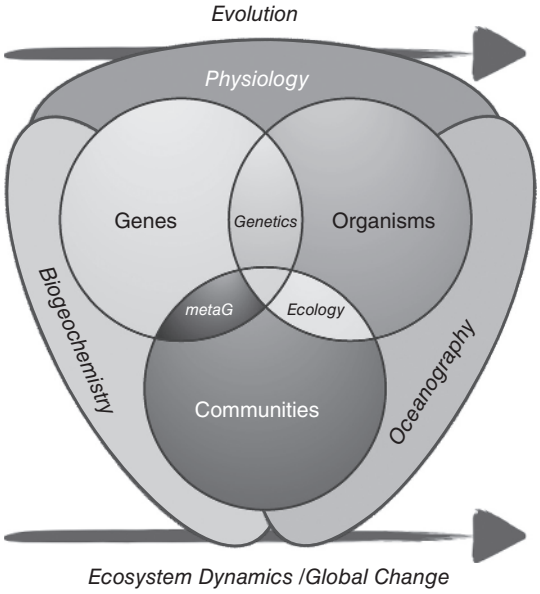

Fig. 1.1 The subject of marine microbial ecology. Organisms and communities are studied in the framework set by oceanography, while genes and organisms determine the biogeochemical effect of microbial communities. Genes and organisms also determine the physiological response to the environment. In addition, genes and organisms evolve with time, and communities and their biogeochemical effects are subject to ecosystem dynamics, most notably those forced by global change. Modified after DeLong (2009). (*See insert for color representation of the figure.*)

this diversity of focuses, is what constitutes the field of marine microbial ecology. For this reason the field has been christened with the motto "from genomes to biomes" (Karl 2007; DeLong 2009) to reflect the wide range of scales and methodological approaches currently used (Fig. 1.1).

This introduction tries to put this book in the context of what has happened in the field in recent years. We evaluate the current state of the field and highlight some approaches or questions that are at its center and end with some predictions of what issues or approaches will dominate in the coming years.

1.2 A BRIEF HISTORY OF MARINE MICROBIAL ECOLOGY

The following historical account highlights some major developments in marine microbial ecology and discusses changes in our ideas about the role of microbes in the biology and ecology of the oceans. The account also discusses the ways in which microbial ecologists have practiced their science over the years (Table 1.1). What follows is focused on carbon (C), nitrogen (N), and phosphorus (P) biogeochemistry and is strongly planktocentric.

TABLE 1.1 History of marine microbial ecology, focusing on the water column

Year	Concept	Key References	Cits.
1959	Early direct count method reveals much larger numbers of bacteria than indicated by traditional plate counts, later termed the "Great Plate Anomaly."	Jannasch and Jones (1959)	262
1966–1970	Incorporation of organic matter in the oceans occurs mostly in the bacterial-size fractions	Williams (1970)	166
1974–1977	More bacteria in the ocean than previously thought	Hobbie et al. (1977)	3856
1978	More active bacteria than suggested by the difference between total plate counts	Meyer-Reil (1978)	169
1979	Large numbers of cyanobacteria (*Synechococcus*) in the ocean	Waterbury et al. (1979) Johnson and Sieburth (1979)	600 430
1980	Bacterial growth and biomass production is substantial	Hagström et al. (1979) Fuhrman and Azam (1980)	345 769
1981	Large fraction of respiration in the oceans is by bacteria	Williams (1981)	117
1982	Bacteria are actively predated particularly by heterotrophic nanoflagellates	Johnson and Sieburth (1982) Fenchel (1982)	216 491
1982	Dilution approach for estimating phytoplankton growth and grazing	Landry and Hassett (1982)	753
1983	Most primary production in oligotrophic oceans is done by picoplankton microbes	Li et al. (1983)	429
1983	Term *microbial loop* is introduced, incorporating bacteria and their grazers into the rest of the food web	Azam et al. (1983), but first outlined by Pomeroy (1974)	2939 747
1986–1988	Flagellates and ciliates can be mixotrophs	Estep et al. (1986)	78
1988	*Prochlorococcus* discovered	Chisholm et al. (1988)	760
1989–1990	High viral abundances in the oceans	Bergh et al. (1989) Proctor and Fuhrman (1990)	835 591
1989–1992	Higher bacterial than phytoplankton biomass in the oligotrophic oceans	Fuhrman et al. (1989) Cho and Azam (1990)	287 313
1990	First marine clone libraries uncover SAR11 and other bacterial groups	Giovannoni et al. (1990)	1056
1992	Archaea are found in marine plankton	DeLong (1992) Fuhrman et al. (1992)	1712 571
1997	Respiration by marine bacteria can be similar as primary production in oligotrophic oceans	del Giorgio et al. (1997)	465
1998	Amplicon sequencing of *nifH* genes discovers many unknown N_2 fixers, including heterotrophic bacteria	Zehr et al. (1998)	228
2000	Photoheterotrophic prokaryotes are abundant in the oceans	Kolber et al. (2000) Béjà et al. (2000a)	194 802

TABLE 1.1 *Continued*

Year	Concept	Key References	Cits.
2000	First marine viral genome sequenced	Rohwer et al. (2000)	106
2000	First edition of *Microbial Ecology of the Ocean*		
2000	Kill the winner hypothesis	Thingstad et al. (2000)	301
2001	New unicellular cyanobacteria that fix nitrogen	Zehr et al. (2001)	447
2001	Large unknown diversity among small eukaryotes	Moon van der Staay et al. (2001) López-García et al. (2001) Díez et al. (2001)	523 495 390
2002	SAR11 is the most abundant oceanic bacteria	Morris et al. (2002)	537
2002	One strain of SAR11 isolated in pure culture	Rappé et al. (2002)	483
2003	*Prochlorococcus* and *Synechococcus* genomes sequenced	Rocap et al. (2003) Dufresne et al. (2003) Palenik et al. (2003)	670 287 413
2003–2004	Viruses of *Prochlorococcus* and diatoms isolated	Sullivan et al. (2003) Nagasaki et al. (2004)	251 74
2003–2004	Photosynthesis genes found in viruses	Mann et al. (2003) Lindell et al. (2004)	205 261
2004	The metagenome of the Sargasso Sea	Venter et al. (2004)	2282
2004	Lithoheterotrophy in a typical coastal marine bacterium (*Silicibacter pomeroyi*)	Moran et al. (2004)	274
2004–2008	Genome of relevant marine eukaryotes (picoeukaryotes, diatoms)	Armbrust et al. (2004) Derelle et al. (2006)	984 422
2005	Marine archaea are chemoautotrophs	Herndl et al. (2005) Könneke et al. (2005)	324 1249
2005	Streamline genome of SAR11	Giovannoni et al. (2005)	522
2005–2008	First marine metatranscriptomes	Poretsky et al. (2005) Frias-Lopez et al. (2008)	117 417
2006	Pyrosequencing and rare biosphere	Sogin et al. (2006)	1731
2007	SAGs of marine bacteria	Stepanauskas and Sieracki (2007)	166
2007	Global Ocean Survey	Rusch et al. (2007)	1095
2007	Proteorhodopsine helps growth in the light	Gómez-Consarnau et al. (2007)	178
2007–2008	High bacterivory by small phytoplankton	Unrein et al. (2007) Zubkov & Tarran (2008)	90 139
2008	Second edition of *Microbial Ecology of the Oceans*		
2008	N_2-fixing cyanobacteria without photosystem II (carbon fixation metabolism)	Zehr et al. (2008)	171

(*Continued*)

TABLE 1.1 *Continued*

Year	Concept	Key References	Cits.
2008	The important role of parasites in controlling planktonic populations	Chambouvet et al. (2008)	110
2009–2011	ICoMM metaanalyses	Galand et al. (2009)	194
		Zinger et al. (2011)	157
2011–2012	Single cell genomics reveals multiple interactions at the protist cell level	Yoon et al. (2011)	121
2012	UCYN symbiosis with an eukaryotic alga	Thompson et al. (2012)	142
2013	Prevalence of genome streamlining in open ocean bacteria	Swan et al. (2013)	100
2013	Discovery of SAR11 viruses—most abundant type in the oceans?	Zhao et al. (2013)	96
2015	Large scale deep sequencing of the ocean microbial genome (TaraOceans)	Sunagawa et al. (2015)	93

Cits: citations according to Thompson Reuters Web of Science, accessed March 13, 2017.

1.2.1 Biological Oceanography and "Black Box" Microbial Ecology

Although there were relevant pioneering efforts as early as the 1800s (Box 1.1), marine microbial ecology can be considered to have started as a discipline in the 1970s, when thanks to improved enumeration methods it was shown that there were many more bacteria in the oceans than previously thought, and that most of respiration and organic matter uptake in the oceans occurs in the bacterial-size fractions (Williams 1970; Azam and Hodson 1977). Before the 1970s, microbes were not even acknowledged in the ecology of the oceans, except as degraders of dead organisms (Steele 1974), usually considered as a "black box" (Box 1.2) with little insight of the structure and function within the box. The seminal papers of Pomeroy (1974), Williams (1981), and Azam et al. (1983) helped also to convince the scientific community that microbes are very relevant in marine primary production, as was highlighted when cells <1 μm were shown to be responsible for up to 60% of primary production in the tropical Pacific (Li et al. 1983).

After the early work by Williams (1981) and others on microbial respiration, the issue of how it compares to primary production has resurfaced several times since then. As an example, del Giorgio et al. (1997) spurred a great deal of discussion between oceanographers and microbial ecologists by arguing that bacterial respiration is higher than primary production in oligotrophic regions of the ocean. Duarte and Agustí (1998) extended the observation to total plankton respiration being higher than primary production. In contrast, geochemical evidence indicated that they are in rough balance. Although short-term incubation experiments seem to confirm heterotrophy of the oligotrophic ocean, other estimates based on geochemical approaches, which average over extensive regions and long times, indicate that the net metabolic state is finely balanced between similar fluxes with large uncertainties (Ducklow and Doney 2013).

Box 1.1 The Pioneers

The German biologist, philosopher, artist, and physician, Ernst Haeckel (1834–1919), was a pioneer in the study of the distribution, abundance, and taxonomy of foraminifera, radiolarians, and diatoms and also attempted an *avant-la-lettre* description of the "Monera." At about the same time, still in the 19th century, Bernard Fischer published his *Die Bakterien des Meeres* (1894), which was probably the birth of microbial oceanography in Central Europe. Russian oceanographers, such as B. L. Issatchenko (or Isachenko) with his monograph in 1914, were among the first to study marine microbes, including protists and bacteriophages. Another book on marine bacteria was published in German by Wilhem Benecke in 1933. In the United States, Haldane Gee was appointed assistant professor of bacteriology in 1928 at Scripps, where other scientists were studying protozoa, diatoms, and dinoflagellates. Around the same time in 1931, Selman A. Waksman (1888–1973), a soil microbiologist, was hired to begin a new program in marine microbiology at Woods Hole. In 1932 Gee was replaced by Claude E. ZoBell (1904–1989), who can be considered the father of marine microbiology. He worked on a wide variety of topics in the field and published a seminal book, *Marine Microbiology*, in 1946. The deep sea work of ZoBell was continued by Holger Jannasch (1927–1998), who bridged the European and American microbial ecology communities; he was a disciple of C. B. van Niel (a Dutch microbial ecologist who worked at Stanford University's Hopkins Marine Station) and also collaborated with ZoBell. Another Russian oceanographer, A. E. Kriss, published a book titled *Marine Microbiology (Deep Sea)* in 1959 in Russian, which was updated and translated into English in 1963. A second book was published in English in 1967 (Kriss et al. 1967). Early efforts at showing the beauty of marine microbes are remarkable. E. J. Ferguson Wood published a marine microbiology book in 1965 that is probably the first aimed at the general public. Later, John McN. Sieburth produced a couple of beautiful books—one an illustrated textbook, *Microbial Seascapes* in 1975, and the second one more of coffee-table book, *Sea Microbes* in 1979, which contains gorgeous images of microbes.

Box 1.2 The Black Box Approach

In science, a Black Box is a physical or conceptual object that has known inputs and outputs, but its internal composition is unknown. Hence "black," to emphasize that the box is opaque to the observer. It is a common concept in computing and engineering, as well as modeling in ecology and oceanography. A relevant example is a box named "Bacteria." The box contains many types of bacteria doing many different things and having different sizes or different growth rates, but the only measured variables are the size of the box (in this case, bacterial abundance or biomass) and the inputs and outputs, such as organic matter uptake and respiration.

Initial measurements showed that the growth rates of the heterotrophic prokaryotes were about as fast as those of the algae (Hagström et al. 1979; van Es and Meyer-Reil 1982), which was confirmed with the development of simpler, more sensitive radiotracer techniques to estimate bacterial production (Fuhrman and Azam 1982; Kirchman et al. 1985). A meta-analysis of studies using ^{3}H-thymidine showed that total heterotrophic bacterial production was a large fraction of primary production (Cole et al. 1988), confirming previous estimates (Derenbach & Williams 1974; Fuhrman and Azam 1980). Bacterial abundance and production were observed to largely track chlorophyll concentrations and primary production (Bird and Kalff 1984; Cole et al. 1988). Other studies indicated that, often, bacterial biomass was larger than that of phytoplankton in some parts of the ocean (Fuhrman et al. 1989; Simon et al. 1992; Li et al. 1992; Buck et al. 1996). Arguable, these have been the most important discoveries in biological oceanography in the 20th century: the identification of the major role that prokaryotes have in carbon and nutrient cycling, including biomass production, in the ocean.

Obviously, if large numbers of bacteria were present with relatively fast (0.5–5 days) growth rates, there is the need for a mechanism to remove prokaryotes at a rate similar to that of growth to maintain abundance at constant levels (Pace 1988). Ciliates and zooplankton were shown not to be the main grazers in most (marine) environments. Pomeroy (1974) had already proposed that nanoplanktonic cells should be responsible for the removal of bacteria, and by the early 1980s heterotrophic nanoflagellates had been identified as the main bacterial predators (Sorokin 1979; Fenchel 1982; Fuhrman and McManus 1984). Development of methodologies for measurement of grazing rates on bacteria (e.g., Sherr et al. 1989) demonstrated that they could indeed balance bacterial growth in some cases—but not in others (Pace 1988; McManus and Furhman 1988)—and potentially become a link by which dissolved organic matter (DOM) is converted to particulate carbon by prokaryotes. This particulate carbon could then reach other trophic levels if these bacterial grazers were in turn eaten by microzooplankton. This pathway for carbon (DOM →bacteria→grazers→higher trophic levels) was called the "microbial loop" (Azam et al. 1983). One question that then became obvious was whether the microbial loop is mainly a "sink" or a "link"; that is, whether most carbon taken up by bacteria is respired and lost from the system or can be channaled through bacteria and protist predators to higher trophic levels. Experimental studies showed that the microbial loop is in fact a sink (Ducklow et al. 1986), although some carbon and nutrients are still transferred up to higher trophic levels as efficiently as other food chains with the same number of trophic steps.

Another parameter relevant to the sink-link debate is the balance between carbon used by bacteria to obtain energy through respiration versus the carbon used for growth (i.e., the bacterial growth efficiency; del Giorgio and Cole 1998, 2000). Initially this efficiency was thought to be high (50% or larger), indicating the microbial loop could be a link, but subsequent work has shown it be low (<20%), consistent with the loop being a sink. The growth efficiency is still one of the least constrained parameters quantifying the role of bacteria in the carbon cycle, mostly because of the uncertainties of the methods for measuring it and because of how it varies over different time scales (del Giorgio et al. 2011).

The cases in which grazing was lower than bacterial growth suggested the existence of another mechanism for bacterial mortality. Viruses were known as early as the mid-1950s to occur in the ocean (Spencer 1955), and an initial study estimated their abundance to be at least 10^4 viruses per mL of seawater (Torrella and Morita 1979), but they were not seriously studied until even higher estimates were published in the late 1980s (Bergh et al. 1989). Subsequent studies demonstrated viruses to be agents of bacterial and cyanobacterial mortality (Proctor and Fuhrman 1990), even potentially affecting primary production (Suttle et al. 1990). They were soon incorporated into models of carbon flux (Bratbak et al. 1992), and their activity was observed to decrease the apparent growth efficiency of the prokaryotes.

Soon after heterotrophic flagellates were identified as the main predators of bacteria, it became evident that some very small protists could ingest bacteria yet had at the same time working chloroplasts (Estep et al. 1986). This type of metabolism is an example of "mixotrophy" (see Chapter 3). We have now come to learn much more about various degrees of mixotrophic behavior in dinoflagellates, ciliates, and small flagellates (Caron 2000; Stoecker et al. 2017). It soon became obvious that the transfer of carbon between bacteria and zooplankton mediated by flagellates assumed in the initial microbial loop model was way too simplistic (Sherr and Sherr 2000) and that viruses and mixotrophic algae among other groups of organisms had to be incorporated into the global picture (Fig. 1.2).

By 1979 it had become apparent that large numbers of unicellular cyanobacteria were present in the ocean (Waterbury et al. 1979; Johnson and Sieburth 1979), and most primary production in oligotrophic regions passes through a 1-μm filter (Li et al. 1983; Platt et al. 1983). The discovery in the early 1980s of *Prochlorococcus*, the most abundant primary producer in the ocean, was a landandmark in the field. These small cells had been observed by transmission electron microscopy (Johnson and Sieburth 1982), but their high abundance and importance were not appreciated until data from flow cytometry became available (Chisholm et al. 1988). The first isolate of *Prochlorococcus* was obtained several years later (Chisholm et al. 1992). That the most abundant organism in the tropical ocean was not identified until the late 1980s demonstrates the delayed development of microbial oceanography as compared to terrestrial ecology. Wouldn't it be a surprise if we learned today that we do not know the most abundant tree in tropical forests?

1.2.2 Opening the Black Box for Variability in Activity and Growth Rates

When prokaryotes were found to be abundant but many were unable to grow on agar plates, some authors considered that a large fraction of oceanic microbes were likely not active, dormant, or simply dead. Some studies used microautoradiography to detect which prokaryotes were active in using organic substrates (Hoppe 1976; Meyer-Reil 1978). These studies found that a relatively large proportion of the community was active, way more than the bacterial fraction growing on plates (ca. 1% of the total). In mid-1990s a series of studies using other methods spurred renewed attention about the proportions of bacteria actively

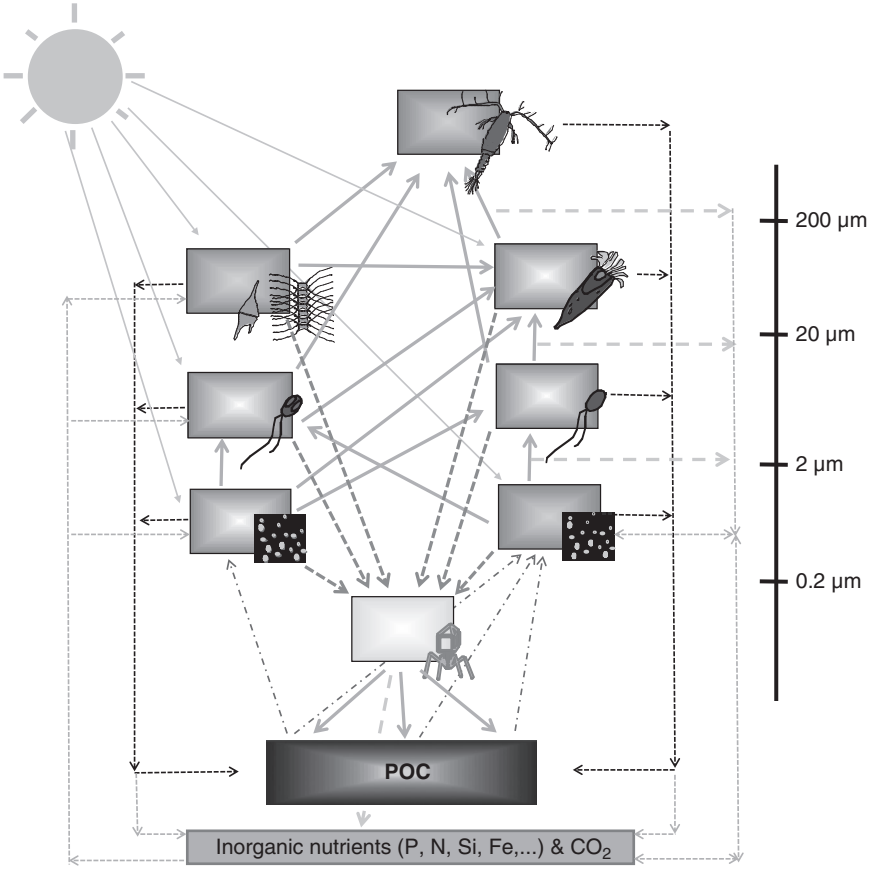

Fig. 1.2 A simplified view of the microbial food web. Mostly heterotrophic organisms on the right side, and mostly autotrophic organisms on the left. Some members of both pico- and nano-phytoplankton can be at least partially heterotrophic. Similarly, some "heterotrophic" bacteria and the ciliates can use light in some way or another. Flows of carbon are depicted as large solid arrows. Use of solar light is in thin arrows. Flows of carbon through viruses as dashed large arrows. Production of dissolved and particulate organic carbon as dashed arrows. Flows of inorganic nutrients in thin dashed arrows. (*See insert for color representation of the figure.*)

growing in the ocean (Zweifel and Hagström 1995; Gasol et al. 1995; Heissenberger et al. 1996). The new methods sometimes gave conflicting results because each one measures a different degree of activity or vitality (del Giorgio and Gasol 2008). Still, they showed that the number of actively growing bacteria is much larger than what grows on plates, and that in fact a large percentage of the cells present in nature are actively growing. It was also shown that the numbers of active cells are below those detected by fluorescent in-situ hybridization (FISH), a method dependent on the levels of ribosomal RNA (rRNA) in cells and, in principle, cellular activity (DeLong et al. 1989).

The FISH method is key to one approach to link up the "black box" approaches examining activity with the work on the diversity of microbial communities discussed later. Microautoradiographic detection of the single-cell use of organic substrates was used, in combination with FISH, by Cottrell and Kirchman (2000b) to describe whether different bacterial groups were equally responsible for the uptake of low- and high-molecular weight substrates. They found *Alphaproteobacteria* to dominate uptake of low-molecular weight substrates and *Gammaproteobacteria* to dominate uptake of high-molecular weight substrates, hinting to clear niche partitioning between the different components of the bacterial community. Using variations of this technique, Ouverney and Fuhrman (2000) showed that archaea could take up amino acids, and deep ocean archaea were later seen to also use inorganic carbon dioxide (CO_2) in addition to organic substrates (Herndl et al. 2005).

Another approach to examine the activity state, if not growth itself, of specific bacterial taxa is to measure the relative content of rRNA as scaled to that of the ribosomal genes (rDNA; e.g., Campbell et al. 2011). The approach is based on classic studies showing that rRNA content increases with growth rate (Lankiewicz et al. 2016) but has some limitations as discussed in Blazewicz et al. (2013). Growth rates can also be estimated in seawater cultures by following the abundance of bacterial groups detected by FISH over time. These approaches have shown that some bacterial groups (e.g., *Alteromonadaceae*) have high rates of growth while others (e.g., SAR11) have relatively low rates of growth (Kirchman 2016). It still remains to be tested how growth rates varies within these groups.

Along with the studies on active prokaryotes, others concentrated on those bacteria apparently not growing actively. Methods detecting these cells are controversial (del Giorgio and Gasol 2008; Blazewicz et al. 2013), but recent work has suggested that dormancy, as a strategy to overcome unfavorable conditions, contributes to the maintenance of microbial diversity (Jones and Lennon 2010) because it generates a "seed bank"–permitting populations to recolonize the environment if conditions allow (Lennon and Jones 2011).

1.2.3 The Molecular Description of Microbial Diversity: rRNA-Based Approaches

Even though it was suspected, evidence that the dominant types of bacteria inhabiting the oceans were very diverse did not come until the 1990s. Using PCR-amplification and sequencing, an approach developed in Norman Pace's lab within the phylogenetic framework postulated by Woese and Fox (1977), Giovannoni et al. (1990) analyzed 16S rRNA gene clone libraries from Sargasso Sea bacterioplankton and discovered that most of the dominant organisms belong to groups really different from isolates maintained in the laboratory. The new dominant groups included the SAR11 bacteria. Giovannoni et al. (1990) showed the magnitude of the ignorance we had about which bacteria were actually most abundant in the oceans. Soon after that study, Fuhrman et al. (1992) and DeLong (1992) showed that archaea are also present in the oceans. This was also a surprise because archaea had been assumed to be present only in anoxic, hot or highly saline environments ("extremophiles") or to be

symbionts of protists. Later FISH-based studies demonstrated that archaea are very abundant in the deep ocean (Karner et al. 2001), whereas SAR11 is likely to be the most abundant bacterium in the surface ocean (Morris et al. 2002).

These findings opened the door to a new view of ocean microbes. Analyses of the identity of the dominant oceanic microbes, their relative abundance, their overall diversity, and the way microbial communities were structured dominated the field (Fig. 1.3). Soon after Giovannoni et al. (1990), a variety of 16S rRNA gene approaches became popular, including clone library studies, more FISH studies, and DNA fingerprinting analyses (Giovanonni and Rappé 2000; Fuhrman and Hagström 2008). Each of these approaches has limitations, and direct comparisons were not particularly rewarding (Cottrell and Kirchman 2000a; Alonso-Sáez et al. 2007), yet combined they identified the large diversity of the microbial communities in the oceans.

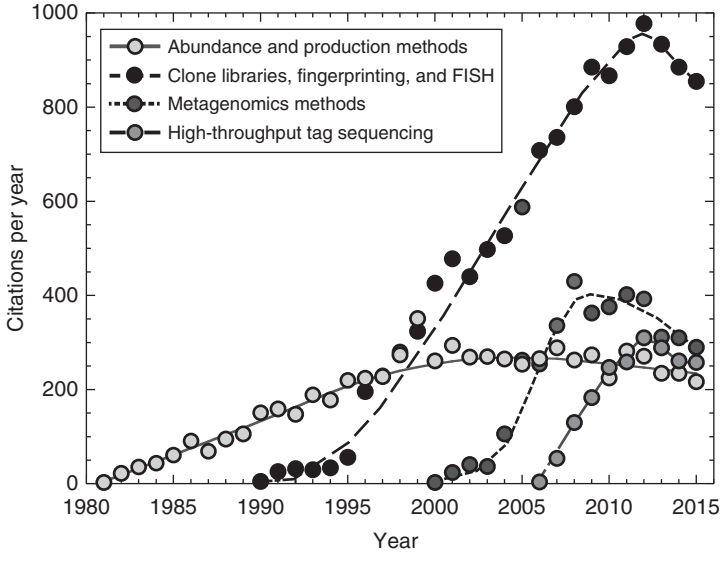

Fig. 1.3 The number of citations to papers describing classical microbial ecology methods (the DAPI method, the thymidine, and leucine methods), 16S rRNA clone sequencing and fingerprinting, metagenomics, and high-throughput sequencing of the 16S rRNA gene. We used the same searches used by Kirchman and Pedrós-Alió (2007). The figure gives the citations to the following papers and methods: for the abundance and production methods, Fuhrman and Azam (1980) (1145 citations), Porter and Feig (1980) (3839 citations), Kirchman et al. (1985) (697 citations), and Simon and Azam (1989) (1297 citations) for a total of 7007 unique citations; for the initial molecular methods, Amann et al. (1990) (5470 citations), Giovannoni et al. (1990), (1047 citations), and Muyzer et al. (1993) (6846 citations) for a total of 13362 unique citations; for metagenomics, Béjà et al. (2000b), (792 citations), Venter et al. (2004) 2250 citations, and Rusch et al. (2007), 1074 citations, a total of 4116 citations; and for high-throughput tag sequencing, Sogin et al. (2006). The analysis was done on November 29, 2016, using the ISI Web of science. There was no effort to discriminate the papers about the oceans from those about other environments. (*See insert for color representation of the figure.*)

They also found that community composition was affected by biotic (e.g., viruses and protists) and abiotic (e.g., temperature and salinity) factors that varied spatially and temporally over many scales (e.g., Schauer et al. 2000; Ghiglione et al. 2005; Fuhrman and Hagström 2008).

If bacteria and archaea are very diverse and respond to ecological changes by changing community composition, it could be expected that small microbial eukaryotes should do the same. Exploring the diversity of microbial eukaryotes, however, is complicated by the large variability in cell size and in rDNA operon content (Zhu et al. 2005), yet by 2001 several studies had shown the large diversity of microbial eukaryotes in the ocean (Moon-van der Staay et al. 2001; López-García et al. 2001; Díez et al. 2001). Many novel, previously unknown lineages were detected, and these were common members of most microbial eukaryotic communities (Massana and Pedrós-Alió, 2008). Recent work confirms the magnitude of protist diversity that might well be larger than prokaryotic diversity (de Vargas et al. 2015), even if prokaryotes have many more lineages (Hug et al. 2016). In part the large diversity of microbial eukaryotes can be traced to the variety of trophic roles that these organisms play (Worden and Not 2008; Worden et al. 2015). Although large eukaryotes were better described than bacteria in the past because they could be distinguished microscopically (e.g., Bachy et al. 2013), molecular surveys still identify new very diverse groups (Flegontova et al. 2016). As an example, the often underappreciated, large *Rhizaria* seem to be abundant and diverse in the world's oceans (Biard et al. 2016). We know much less about the smallest protists. We know that they are very important contributors to primary production (Li 1994; Zubkov 2014) and to grazing on bacteria (Unrein et al. 2007; Zubkov and Tarran 2008; see Chapter 3), but we know little of their diversity and biology, particularly of the heterotrophic flagellates for which there are few cultured representatives (Massana 2011).

Understanding the diversity of both prokaryotes and eukaryotes has been heavily reliant on advances in DNA sequencing technology. The initial approach used for decades was Sanger sequencing, which is slow and expensive. It was replaced by so-called next-generation sequencing methodologies (or high-throughput sequencing [HTS]) that allowed massively parallel analyses, with large throughput at a reduced cost. This dramatically affected microbial oceanography as exemplified by Sogin et al. (2006), the first published study in the field that used HTS of 16S rRNA gene amplicons. Suddenly, instead of obtaining at most 1000 sequences from one sample (e.g., Acinas et al. 2004), microbial ecologists were able to generate several tens of thousands of sequences from several samples at a time. The latests methods yield even more sequences, several million per study (e.g., Salazar et al. 2016). These methods, which are continuously evolving, avoid the need for the cloning required in the Sanger protocols, and the newer methods do not require amplification that might introduce biases. Sequencing a gigabase (Gb or 10^9 bases) of DNA was reduced from many years to hours at a fraction of the cost (Glenn 2011). Although the first HTS approaches yielded many more sequences than the Sanger method, the length of the sequences was much shorter (ca. 100 bp with the initial 454 technologies vs. ca. 1000 bp with Sanger), which led to reduced taxonomic resolution, discrepancies in the analyses, and arguments about the best region of the rRNA gene that should be

sequenced (see Chapter 2). There is a continuous evolution of technologies, and it is now possible to sequence the entire 16S rRNA gene at high throughput (Singer et al. 2016).

The decrease in sequencing costs, the ease of sequencing (let sequencing centers do the work), and the publication of simple tools to process HTS amplicon data all led to the democratization of diversity studies. Virtually any research group can afford and is capable of obtaining rRNA gene data to explore microbial diversity. Consequently, papers on the topic have inundated the literature since the development of HTS technologies. At the start, most studies used the same strategy under the framework of the International Census of Marine Microbes (ICoMM). Several papers were published comparing environments (e.g., Galand et al. 2009; Ghiglione et al. 2012; Zinger et al. 2011), including studies of beta-diversity (i.e., how diversity is structured spatially). One of the most important observations made possible by HTS data was the existence of a "rare biosphere" formed by many different species that had very low abundance (Sogin et al. 2006; Pedrós-Alió 2012; see Chapter 2).

1.2.4 The Molecular Description of Microbial Diversity: Whole Organisms and Genomes

Recognition of the presence, and even dominance, of uncultured and previously unknown microorganisms in the sea prompted efforts to know more about them, to isolate ecologically relevant representatives, and to describe their phylogenetic variability and ecophysiology. As discussed in Chapter 5, isolation allows physiological studies, genome sequencing, and linking the physiological and ecological information to the genome. Remarkable successes in that direction were the isolation of the first *Prochlorococcus* (Chisholm et al. 1992) and of a SAR11 clade member (Rappé et al. 2002). These isolates with their small genomes are representatives of the dominant microbes in the oligotrophic open oceans. Other relevant isolates were a common member of the *Roseobacte*r clade (Moran et al. 2004), the first ammonia-oxidizing chemoautotrophic marine thaumarcheon (Könneke et al. 2005), the gammaproteobacterium NOR5/OM60 (Fuchs et al. 2007), and a proteorhodopsin-containing *Bacteroidetes* (Gómez-Consarnau et al. 2007).

It is curious how long it took before viruses and predators attacking *Pelagibacter* (i.e., SAR11) and *Prochlorococcus* were uncovered. The first *Prochlorococcus* virus was described more than 10 years after the cyanobacterium was initially isolated (Sullivan et al. 2003). Predation on *Prochlorococcus* by flagellates had already been observed in 1999 (Christaki et al. 1999, 2002), and Guillou et al. (2001) identified a tiny (diameter ca. 1.5 μm) chrysophyte that can feed on cultured *Prochlorococcus*. Yet actual identification of the predators was not done until 10 years later when stable isotope DNA probing revealed stramenopiles, haptophytes, and alveolata as grazers (Frias-Lopez et al. 2009; see Chapter 3). Some of these protists appear to be photosynthetic. SAR11 viruses were not isolated until 2013, again more than 10 years after the isolation of *Pelagibacter* (Zhao et al. 2013). Until these viruses were found, the low rates of growth of this organism and its small cell size had supported the hypothesis that they could be immune to viral predation. Instead, the success of

this highly abundant microbial clade seems to be the result of very successful adaptation to compete in a low-resource environment (Giovannoni 2017). Specific predators of SAR11 have not yet been clearly identified, and their measured loss rates are among the lowest of all bacterial groups, commensurate with their relatively low in-situ growth rates (Teira et al. 2009; Ferrera et al. 2011).

Availability of the genomes of the cultured relevant microorganisms facilitated many discoveries with ecological relevance. As an example, the comparative genomics of various cyanobacteria isolated from different oceanic environments (Palenik et al. 2003; Rocap et al. 2003; Dufresne et al. 2003) showed large differences in gene content, particularly between the high-light and the low-light adapted *Prochlorococcus* ecotypes and the presence of genes with obvious roles at increasing fitness in response to the environmental characteristics of the environment from where they were isolated. The *Pelagibacter ubique* HTCC1062 genome was found to be reduced (streamlined) with just 1.3 Mbp thanks to reduced intergenic spacers that made it, at the time, the smallest genome of a free-living organism (Giovannoni et al. 2005). This SAR11 representative has complete biosynthetic pathways for all amino acids, has a proteorhodopsin gene (see below), and other interesting biochemical characteristics related with adaptation to phosphorus starvation and sulfur cycling (Giovannoni 2017).

Model eukaryotes have also been isolated in culture, and their genomes have been sequenced. Some relevant organisms are the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Armbrust et al. 2004; Bowler et al. 2008); *Ostreococcus tauri* and *Micromonas*, the smallest free-living eukaryotic phototrophs (Derelle et al. 2006; Worden et al. 2009); and the ecologically relevant coccolithophorid *Emiliania huxleyi* (Read et al. 2013). In contrast, there are few isolates and genomes of relevant marine heterotrophic protists, with the exception of the choanoflagellate *Monosiga* (King et al. 2008).

The first genome of an isolated marine virus was published in 2000 (Rohwer et al. 2000), but now several other, apparently relevant, viruses exist in culture. In addition to some being RNA, single- or double-stranded, viruses do not have a single gene that can be used to organize and monitor the diversity of the group, analogous to rRNA genes. Consequently, genomic sequencing is necessary. Because most hosts are not in culture, their viruses aren't either, and metagenomic approaches (see below) are needed to advance the understanding of the relevant marine viruses (see Chapter 9).

Similar genomic sequencing methods can be used with the DNA of natural microbial communities. In the first attempts to examine uncultured microbes, cloning and sequencing of large genomic fragments from mixed microbial communities was used to describe rRNA gene sequences, but the authors noted that other genes could be studied as well (Schmidt et al. 1991). This approach of simultaneously examining many genes from many organisms was later called "metagenomics" (Rondon et al. 2000). Subsequent attempts at sequencing genome fragments from a natural community used methods such as the cloning of large (30–100 kb) DNA fragments into bacterial artificial chromosomes (BACs) or fosmids (Stein et al. 1996; Béjà et al. 2000a). The basic strategy was to screen the fragments for phylogenetic information

(i.e., 16S rRNA genes) and to sequence the interesting fragments. This approach allowed assignment of functions to specific microbes defined by 16S rRNA genes or other phylogenetic markers. This large insert approach was later replaced by small-insert (around 1000 kb) shotgun approaches (Venter et al. 2004), and then by short-read HTS, which do not require cloning.

One example illustrating the power of the large insert approach is the discovery of proteorhodopsin. Béjà et al. (2000b) identified a DNA fragment possessing a 16S rRNA gene from the yet-to-be-cultured SAR86 and also a gene for proteorhodopsin (PR), a light-dependent proton pump. This discovery heralded a new type of phototrophy for the ocean. Work by Oded Béjà and others soon showed that this type of pigment existed in many types of bacteria and apparently many surface ocean bacteria had this form of photoheterotrophy, including various *Gamma-* and *Betaproteobacteria*, *Bacteroidete*s, *Archaea*, and the abundant SAR11 (Pinhassi et al. 2016). The known types of PRs in the ocean increased by about 10-fold when Venter et al. (2004) published the first ocean study using whole genome shotgun metagenomics. A total 1 billion (10^9) bp were assembled, corresponding to ca. 1800 genomic species, including ca. 1.2 million unknown genes, along with 782 new rhodopsin-like photoreceptors. The number of known PRs was increased even more when Venter and colleagues launched the Global Ocean Sampling (GOS) Expedition. This project found 2674 proteorhodopsins among 6.3 billion bp from 41 samples. Among the rhodopsin sequences, green-tuned rhodopsins were relatively more abundant in temperate coastal waters, whereas blue-tuned rhodopsins dominated in warmer open ocean water (Rusch et al. 2007) in accordance with the light characteristics of these habitats (Box 1.3).

Although photoheterotrophy is arguable the most important discovery of marine metagenomics, many other new findings about bacterial diversity, phosphorus, sulfur, and nitrogen cycling and eukaryotic diversity arose from GOS and subsequent studies (e.g., DeLong et al. 2006). In 2015 the Tara Oceans consortium published a

Box 1.3 Photoheterotrophy in the Ocean

Proteorhodopsins (PRs) were not the only pigments found at the turn of the century to be used by photoheterotrophic bacteria in the ocean (Karl 2000). Bacteriochlorophyll *a* containing bacteria that could carry out aerobic anoxygenic phototropy (AAP) had been isolated from some coastal sites (Harashima et al. 1978) and were found in a deep hydrothermal vent (Yurkov and Beatty 1998) but remained an exotic group of organisms. With direct measurements of bacteriochlorophyll *a*, infrared fluorometry and infrared fluorescence microscopy, Kolber et al. (2000, 2001) first suggested that AAP bacteria were significant components of the surface ocean bacterioplankton. After some initial discussion about the real abundance of these organisms (Koblížek 2015), AAP bacteria were shown to be less abundant than PR-containing organisms, distributed in fewer types of microbes (mainly in *Alpha-* and *Gammaproteobacteria*), and to have a form of photoheterotrophy energetically more costly than PR (Kirchman and Hanson 2013).

metagenomic overview of the ocean. They sequenced 273 samples from 68 stations and increased the number of nonredundant genes to more than 40 million (the Ocean Microbial Gene Catalog; Sunagawa et al. 2015); only 5–7% of these genes had been captured by GOS or other metagenomic studies. With the newest sequencing technologies, the burden of work now is with the bioinformatics analysis of the huge amount of sequence data that each metagenomic project generates: hundreds of thousands of relatively short sequences that have to be screened for quality and combined into longer fragments to extract useful information.

The logical next step after metagenomics is to target actual functions, through metatranscriptomics (all RNA transcripts from all members of a community), proteomics (all proteins), and metabolomics (all low-molecular weight metabolites). Chapter 4 reviews some of these approaches. The first marine metatranscriptomes were performed by Poretsky et al. (2005) who analyzed clones of cDNA from mRNA, and Gilbert et al. (2008) and Frias-Lopez et al. (2008) who used HTS of cDNA. A challenge facing metatranscriptomic studies is the low and dynamic mRNA reservoir of marine bacteria compared to the rRNA pool. A typical marine bacterium has only about 200 mRNA molecules, which last only a few minutes before they are degraded (Moran et al. 2013). Typical oligotrophic and copiotrophic (Box 1.4) marine bacteria have radically different number of genes being regulated by

Box 1.4 Oligotrophs versus Copiotrophs

Some marine prokaryotes grow better when inorganic nutrients and carbon are plentiful, whereas others do so only in nutrient-depleted areas of the ocean. The former organisms are often called *copiotrophs* (from the Latin "copia" for richness, and the Greek "trophos," meaning food). The cell size of these microbes is large, and they have large genomes (>4 Mb) and diverse metabolisms, including sometimes the capacity for phototrophy with pigments such as bacteriochlorophyll *a* (Lauro et al. 2009; Yooseph et al. 2010). They respond to changes in environmental conditions by controlling the transcription of many genes (Cottrell and Kirchman 2016). In contrast, "oligotrophic" (from the Latin "oligo" for few) bacteria tend to have small (<2 Mb) streamlined genomes, have low transcriptional control, and often lack genes for acquiring essential elements. Some can still use light, but they do it using the simpler and less expensive proteorhodopsin. Typical marine oligotrophic bacteria include *Pelagibacter*, the gammaproteobacterial SAR92, and *Acidimicrobiales*. Typical copiotrophic bacteria are in the orders *Rhodobacterales* and *Alteromonadales*. Most oceanic microbes seem to be oligotrophs (Yooseph et al. 2010; Swan et al. 2013). Copiotrophic bacteria are thought to have an r-strategy (they grow fast and they are subject to higher predation rates), and the oligotrophic bacteria have a k-strategy (they grow more slowly and cannot respond quickly to environmental changes), although these concepts borrowed from general ecology might not fully apply to bacteria. Koch (2001) neatly discusses why oligotrophs have a hard time in rich environments and copiotrophs in nutrient-poor environments.

transcription (Cottrell and Kirchman 2016). The total mRNA pool is also an order of magnitude less abundant than the genes and almost four orders of magnitude less abundant than the proteins, posing many technical challenges. Despite that, and using various strategies to standardize the obtained data, Ottesen et al. (2013, 2014) found diel rhythms of gene transcription, metabolic activity, and behavior in naturally occurring auto- and heterotrophic coastal and open ocean bacteria and archaea. Not only photoautotrophs exhibited pronounced diel periodicity but also so did many gene transcripts from several different heterotrophic bacterioplankton groups. These data suggest population-specific timing of maximal transcription in a variety of metabolic pathways, more conspicuously in the open ocean (Ottesen et al. 2014) than in the coastal region (Ottesen et al. 2013). These studies also showed that it was technically possible to obtain "omic" data using a robotic autonomous system (Preston et al. 2011).

In addition, the coupling of genomics with flow cytometric sorting has enabled enrichment of communities to explore the functional potential of many microbial groups (Palenik et al. 2009). High-throughput techniques have been optimized for isolation of single cells by flow cytometry and genome sequencing of an isolated single cell. The DNA of single sorted cells is first amplified by a process, multiple displacement amplification (MDA), that generates SAGs (single-cell genomes), which are then screened by sequencing PCR amplicons from 16S rRNA genes or from functional genes of interest. Afterward, the entire genomes of interesting SAGs are sequenced. Stepanauskas and Sieracki (2007) demonstrated that the SAG approach could be used to access the genomic information of marine microbes that are abundant but that resist cultivation. Using this technique, Swan et al. (2011) provided evidence that several uncultured *Proteobacteria* lineages that are ubiquitous in the dark oxic ocean possess sulfur oxidation genes and ribulose bisphosphate carboxylase, evidence of potential chemolithoautotrophy. In a subsequent study Swan et al. (2013) found that, compared with bacteria isolated by traditional culturing methods, natural bacterioplankton had smaller genomes and fewer gene duplications, providing strong evidence that genome streamlining and an oligotrophic mode of life are prevalent features among diverse, free-living bacterioplankton.

The single-cell approach has also been applied to protists (Heywood et al. 2011), and several studies revealed multiple interactions among protists, bacteria, and viruses, revealing cell-to-cell predatory, symbiotic or virus-host interactions in uncultivated microbes (Yoon et al. 2011; Martínez-García et al. 2012; Labonté et al. 2015). The method is a powerful addition to metagenomics by providing reference genomes to which the metagenomic reads can be anchored facilitating organism-level interpretation of ecosystem function. It has also been used to provide phylogenetic information for the so-called "microbial dark matter," the yet unexplored fraction of microbial diversity (Rinke et al. 2013).

1.2.5 N_2 Fixation Studies as a Model for Marine Microbial Ecology

Microbes play important, often complex roles in the cycling of inorganic nutrients in the ocean, including those that limit microbial growth and primary production such as N, P, silica, or iron. Several remarkable studies illustrate the complex roles

of prokaryotes in the P cycle (see Chapter 10), which may become even more important to understand because P limitation in the oceans seems to be increasing (Karl et al. 2001). In many oceanic regions with extremely low P concentrations, microbes, most notably cyanobacteria and SAR11 bacteria, reduce their requirements for P by replacing membrane phospholipids with alternative nonphosphorus lipids (van Mooy et al. 2009; Carini et al. 2015; Sebastián et al. 2016). One of the best-studied nutrient cycles, however, is that of N, evident by the many chapters in the two previous editions of this book (Paerl and Zehr 2000; Ward 2000; Capone 2000; Zehr and Paerl 2008).

A review of one step in the N cycle, N_2 fixation, highlights how marine microbial ecology has developed over the years and how it can now operate (Fig. 1.4). Using PCR primers for the *nifH* (nitrogenase) gene, Zehr et al. (1998) discovered sequences similar to heterotrophic N_2 fixers and thus widened the known nitrogen fixers beyond the cyanobacterium *Trichodesmium* (Capone et al. 1997) and the cyanobacterial symbionts of diatoms (Foster and Zehr 2006). The work contributed to solving the apparent imbalance between sources and sinks of N in the global N budget. The 1998 study also described two groups of unicellular cyanobacterial *nifH* sequences. A unicellular, presumably aerobic N_2 fixer was surprising at the time because it was not expected that the fixation process could be done by an oxygenic free-living

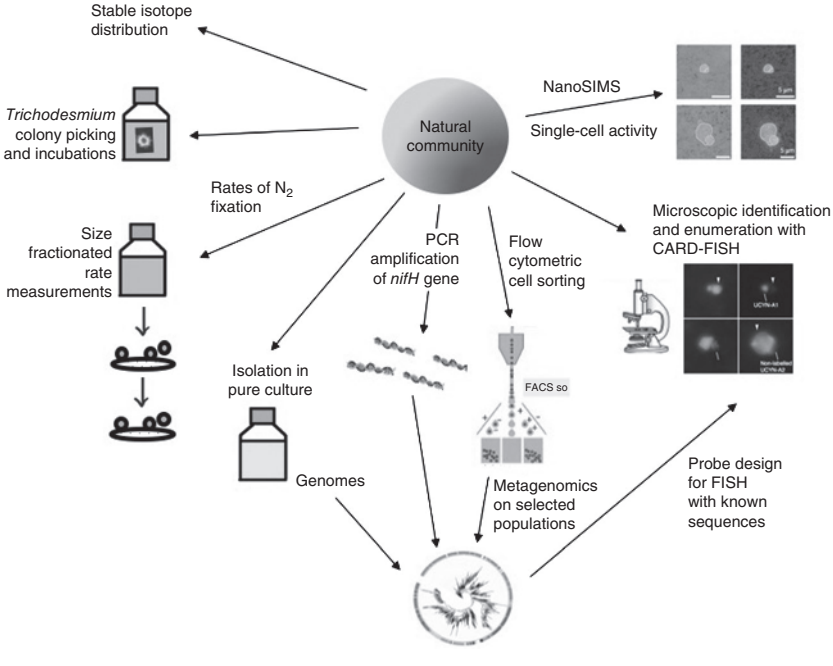

Fig. 1.4 The different approaches used to understand nitrogen fixation in the ocean, as an example of the various methodologies used by microbial oceanographers. Similar processes are used for understanding the P and C cycle. (*See insert for color representation of the figure.*)

organism. One of these two groups corresponded to 3- to 5-μm cyanobacteria (*Crocosphaera*), but the other group, later called UCYN-A, could not be visualized (Zehr et al. 2001). Using a combination of flow cytometry cell sorting and downstream genomics, it was shown that these abundant UCYN-A organisms lack genes for photosystem II and carbon fixation, so they either were photoheterotrophic, had to be symbionts of other organisms, or both things together (Zehr et al. 2008; Tripp et al. 2010). A specific symbiosis with a haptophyte alga was soon proposed in which the haptophyte and the symbiont exchange fixed carbon and nitrogen (Thompson et al. 2012).

We now know that there are several different symbioses between different closely related haptophyte hosts and different UCYN-A symbionts (Cornejo-Castillo et al. 2016; Zehr et al. 2016). Nitrogen fixation by these symbiotic haptophyte-UCYN-A pairs can be as high as by *Trichodesmium*, as shown by size fraction experiments and nanometer-scale secondary ion mass spectrometry (nanoSIMS) analyses (Montoya et al. 2004; Martínez-Pérez et al. 2016).

These studies nicely illustrate the different disciplines involved in aquatic microbial ecology and microbial oceanography: size fraction rate (N_2 fixation) measurements and nutrient budgets, genetic determination of the diversity of organisms performing a particular process, FISH with probes designed from environmentally relevant sequences, microscopy, flow cytometry cell sorting, metagenomics, genome and proteome analysis, and single-cell activity to track nutrient and carbon flow between symbionts and hosts (Fig. 1.4).

1.3 AN ASSESSMENT OF CURRENT MARINE MICROBIAL ECOLOGY

The field of microbial ecology of the ocean has grown exponentially in the last several years, and it encompasses a large range of disciplines as exemplified in Fig. 1.1. Next we assess the current status of the field and speculate about future directions.

As in many, if not all, fields of science, progress in marine microbial ecology has been made possible by leaps in methodology and technology. The influence of methods development is easily spotted in Fig. 1.3 and Table 1.1. It shows the impact of new technologies in attracting the interest of other researchers, evident in the number of citations to the papers reporting new approaches. The field is probably still method-limited, and paradigms will change in the future only after the introduction of a new technology or method.

Very often, the introduced technologies were developed in other scientific fields, in particular the biomedical field. One example is flow cytometry, which was originally developed to enumerate human cells. The instrument was imported into oceanography by Yentsch et al. (1983) and Olson et al. (1983) whose work eventually made the discovery of *Prochlorococcus* possible. Another example is sequencing technology. Advances in sequencing approaches are being driven by the great interest and need for sequence data in many other scientific fields, most noteworthy in

biomedical applications. Flow cytometry and advanced sequencing technologies come together in single-cell analyses, which are also used in the biomedical field (Gawad et al. 2016). Creative inspection of the literature and practices in other fields will help gain knowledge in aquatic microbial ecology.

The introduction of new approaches, however, can create problems in establishing standardized methods for coordinated research, which we believe should be promoted. The history of early "black box" methods offers an example. After several years when different methods were used to measure biomass and production, the Joint Global Ocean Flux Study (JGOFS; Ducklow 1999) program arose as an international initiative to study the ocean carbon cycle using standardized methods. In part because of that standarization, JGOFS studies were successful in advancing our understanding of microbial carbon cycling in the open ocean. We are at a different, earlier stage of development with understanding microbial diversity. There are now a variety of ways to determine the distribution of specific bacterial taxa using 16S rRNA gene tag sequencing (e.g., sequencing technology and depth, primers, and treatment of singletons; see Chapter 2). Comparisons between studies now seem particularly difficult and only possible within consistent sets of data treated in the same way (e.g., data sets from ICoMM, Tara Oceans, or the Ocean Sampling Day [OSD]). The field would advance more effectively and faster if standard, open protocols were used.

The selection of study sites needs more careful consideration because certainly only a small part of the ocean has been explored to date. Most often, the scientist's backyard is the main focus of study, not because it is particularly interesting but for logistic reasons. In the past, when few data were available and the methods were comparable, these backyard studies provided the raw data needed for meta-analyses and data comparisons, some of which helped settle the field (e.g., Cole et al. 1988). Today, unless a specific, novel hypothesis is proposed, unless particularly surprising results are obtained, or unless they come from an informatively designed experiment (or cruise), just another study from another coastal site would contribute little insight into how the microbial ocean works. Hypotheses should not be displaced by accumulation of data (Peters 1991), although data can also stimulate the formulation of new, insightful hypotheses.

Once a new methodology is introduced, a new variable is described, or a new process identified, the studies follow a sequence: (1) Proof of concept and the first measurements. As an example, Giovannoni et al. (1990) examined only 12 clones and nine different sequences, and yet it was an extremely important contribution that opened a new era in microbial ecology of the ocean. (2) Descriptive papers, which contain usually a few samples, sites, experiments per paper. After the initial reports, the descriptive studies make important points and test hypotheses that are derived from the initial study. As an example, Mullins et al. (1995) compared ca. 150 clones from the Atlantic and Pacific Oceans to test for similarities between widely separated oceans. At this point several papers compare the new methodology to older ones, and other papers appear pointing out the biases or problems inherent in the new methodology and strategies to solve them. For example, after Sogin et al. (2006) came Huse et al. (2007, 2008, 2010). (3) A third step corresponds to global analyses using many samples.

For example, Pommier et al. (2007) undertook a global analysis using clone libraries, and Zinger et al. (2011) did the same with pyrosequencing data. Some studies plan specific tests of hypotheses, whereas others are post-hoc data analyses. (4) Well-designed experiments to use the (relatively) new technology to test nontrivial hypotheses. For example, Baltar et al. (2015, 2016) tested the effect of anthropogenic disturbances on the abundant versus rare bacteria and the effects of predator manipulation on bacterial community structure.

The metagenomics literature is full of descriptive studies. However, experimental studies are the future. For example, Mou et al. (2008) coupled immunocapture with sequencing to examine the microbes actively responding to the presence of dissolved organic carbon model substrates. Well-designed experimentation with well-founded hypotheses to test with proper replication is where the field should head. In fact, the lack of replication and proper experimental designs in metagenomics has been identified as a deterrent of progress (Knight et al. 2012). Marine microbial ecology would be stronger if it used more experimental approaches with replication, systematically include internal controls (particularly in metatranscriptomics), and organized data in standardized formats.

The previous discussion raises the question about matching the methodological approach with the hypothesis or with the general objectives of a descriptive study. Within the limits of resources (time, hands, or money), there is a tendency to use the most updated method that generates the most data, simply because the method is new, or because authors fear reviewers will not appreciate results from "old" methods, even if the hypothesis or goals of the study could be reached with an older approach (e.g., Fig. 1.5). As an example, carbon flux studies likely do not require an

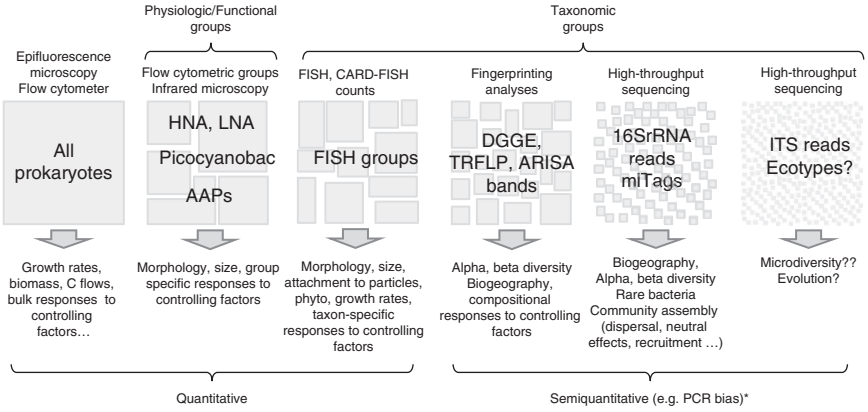

Fig. 1.5 Different approaches to analyze the community of prokaryotes, together with the type of questions that can be addressed by each type of approach. The whole community can be subdivided into physiological or functional groups, large phylogenetic groups, or "species"-level groups (16S rRNA clones, reads, or metagenomic 16S rRNAs) or more detailed ecotype or ITS (internal transcribed spacers)-defined units. *The miTags approach is assumed to be quantitative because it does not use PCR. (*See insert for color representation of the figure.*)

analysis at the ecotype level. Similarly, metagenomic 16S rRNA inventories are still more expensive than PCR-based 16S rRNA tag sequencing and provide less information about less abundant organisms. Each approach has its biases (see Chapter 2). The level of the analysis should be chosen to match the question addressed.

An example of the challenges we face at matching the approach with the question is the response of microorganisms and their activities to global change, particularly to rising temperatures. There is no single experiment that can realistically reproduce what will occur to microbes with rising temperatures. With the 1–2° C expected increase in temperature of the ocean by the end of the century (e.g., Bopp et al. 2013), we should be running 80-year experiments to allow for microbes to meaningfully adapt to the rising temperatures. As long-term experiments show, microbes are known to evolve new capacities and adaptations even in a homogenous lab setup (Tenaillon et al. 2016) and a 2° C change in 100 years is likely equivalent to what will occur in ca. 300,000 bacterial generations. However, because our experiments last only a few generations, one can wonder whether such experiments produce meaningful results (Sarmento et al. 2010). Instead, space-for-time substitutions (i.e., cross-comparisons between natural systems under different temperature regimes) should be considered. They can inform us of how microbes currently respond to changes in temperatures (e.g., Lønborg et al. 2016). Long-term observatories are also needed to resolve the actual changes (e.g., Li et al. 2009; Sarmento et al. 2010; Morán et al. 2010, 2015). Another possibility is to use the metabolic theory of ecology (MTE; Brown et al. 2004), which can provide predictions about the effects of warming on the structure and dynamics of marine ecosystems. As an example, MTE predicts that warming should increase grazing on bacteria more than bacterial production (Sarmento et al. 2010) and stimulate more bacterial carbon consumption than primary production (Hoppe et al. 2002), while primary producers may escape grazing by protists at low temperatures, thus allowing phytoplankton blooms to occur (López-Urrutia 2008). Rigorous analyses, however, must incorporate other factors affecting the relationship between temperature and the microbial processes, such as nutrient and light limitation (López-Urrutia & Morán 2007; Kirchman et al. 2009).

A final challenge is to establish the relationship of omic methods, which are becoming more and more popular (Fig. 1.3), to efforts to understand microbial function occurring in the oceans. One example is whether the presence of a particular phylogenetic group can be taken as indication of a particular ecosystem function (e.g., presence of *Cycloclasticus* indicates hydrocarbon inputs and degradation by microbes). Given the relative simplicity of 16S rRNA studies, there is the temptation to extract functional information from the list of OTUs present in a sample. Methodologies exist that associate each OTU with a genome-sequenced relative and predict functions based on a hypothetical conservation of function (e.g., Picrust, Tax4Fun, Paprica, Faprotax; Langille et al. 2013; Aßhauer et al. 2015; Bowman and Ducklow 2015; Louca et al. 2016), but one can argue that this may lead to erroneous conclusions for environments where most organisms have no sequenced relatives. Empirical work at identifying truly indicator organisms (like *Cycloclasticus* cited previously, e.g., Teira et al. 2007) and more genomic work with relevant marine organisms are needed to obtain really useful tools. In addition, knowing the degree of phylogenetic conservation

(or lack of) of the different functional genes or ecological traits (e.g., Martiny et al. 2013, 2015; Salazar et al. 2015) will facilitate extracting functional information from the species inventories. A different problem faces the metatranscriptomics studies. It is generally assumed that the abundance of specific mRNAs from functional genes is a reliable proxy for those functions in natural environments. Yet, they are not, and neither is the abundance of proteins (Moran et al. 2013; Rocca et al. 2015). However, inventories of genes in the mRNA pool are informative about the relevant ecological processes that are occurring in the environment, and perhaps more importantly, fluctuations in these pools can be used as a sensitive bioassay to detect the response of the microbes to a changing environment.

Finally, the field has been said to lack theoretical grounding (Prosser et al. 2007). Theories should be the bases for the experiments, yet this is hardly ever the case in our field. Established ecological theory should be tested in microbial systems, and theory should guide observations and experimental design. But, equally, data from microbial systems should be used to adapt or modify existing theory, to construct new theory or to understand better the implications of the existing theories. As an example, prokaryotes seem to follow a "superlinear" relationship between cell size and metabolism (slope >1), whereas protists and metazoans have slopes ≤1 (DeLong et al. 2010; García et al. 2016). One explanation is the covariation between cell size and genome content (and thus metabolic potential) in the prokaryotes that then dissipates with larger organism size.

There has been clear progress in the last few years, and now microbial papers are more common in journals such as *Ecology Letters*, in part because the availability of HTS data has been used to test classic ecological theories, such as taxa-area relationships (Horner-Devine et al. 2004), latitudinal patterns in diversity (Fuhrman et al. 2008), and whether bacterial communities are assembled by ecological ("habitat filtering", or niche) versus dispersal or stochastic mechanisms (Hughes Martiny et al. 2006). Further use of theory to determine sampling strategies and experiments would certainly help develop our field.

1.4 THE FUTURE OF MARINE MICROBIAL ECOLOGY

The previous sections have already pointed to some future trends in the subjects that will dominate microbial ecology of the ocean in the following years. With the risk of being wrong (but maybe not as much as in Kirchman and Pedrós-Alió 2007), here are some more thoughts about the future of our field:

1.4.1 Toward Single-Cell Microbial Oceanography

Combining functional and genetic probes has opened the door to simultaneously interrogate single cells for their identity (with, e.g., FISH), physiological status (e.g., membrane probes, del Giorgio and Gasol 2008) or uptake activity (e.g., microautoradiography). Newer methodologies are now expanding the boundaries of this topic. Nanometer-scale secondary ion mass spectrometry (NanoSIMS)

allows the coupling of phylogenetic identity and metabolic function of single cells in the environment, by imaging of isotopic composition of individual cells at the nanoscale. Particularly interesting is the capability to track use of nitrogen forms; practical radioactive tracers are not available for N compounds. NanoSIMS allows various compounds to be tracked simultaneously, and thus, for example, ^{15}N-ammonia or $^{15}N_2$ can be combined with ^{13}C-bicarbonate to detect cells incorporating both inorganic nitrogen and CO_2 (e.g., Musat et al. 2012). Initial studies could identify some individual cells because of their size (e.g., *Trichodesmium*, Finzi-Hart et al. 2009; or *Anabaena*, Popa et al. 2007), but now they can be identified by phylogenetic probing which can be combined with functional probing. Some new phylogenetic probing methods include double imaging FISH (Orphan et al. 2001) and probes labeled with bromine, iodine, or fluorine (HISH-SIMS, for halogen in-situ hybridization), molecules that are not very abundant in microorganisms and that are easily detected by ion mass spectrometry. With this approach Martínez-Pérez et al. (2016) quantified the contribution of *Trichodesmium* and UCYN-A to nitrogen fixation in the Atlantic Ocean and observed that these fixation rates were roughly proportional to carbon fixation except for some *Trichodesmium* cells that were only active in one of the processes. Raman spectroscopy can also be combined with FISH to detect cell-specific incorporation of ^{13}C compounds (Huang et al. 2007) and has been used to probe CO_2 fixation by individual organisms in mixed communities (Li et al. 2012).

We predict that single-cell genomics, already proven to be a powerful approach in marine microbial ecology, will become even more common in the coming years. The approach will continue to evolve as techniques for minimizing DNA contamination improve, the current limitations of DNA amplification by MDA are circumvented, and sequencing technology advances. As sequencing costs continue to decrease, screening of isolated cells by PCR and other approaches may become superfluous, and it will be feasible to address new questions by single-cell genomic approaches. A remarkable example of this is the work with *Prochlorococcus* in which the knowledge gained from various lab isolates has been expanded with ocean uncultured clades (Malmstrom et al. 2013). Large-scale application of the SAG procedures to natural populations of co-occurring *Prochlorococcus* has shown that the community is composed of hundreds of subpopulations with different sets of core gene variants, and a small set of flexible genes (Kashtan et al. 2014) that provides the plasticity needed for fine-tune adaptations to the variety of conditions that occur in the stratified ocean. These data generate questions about how these subpopulations coexist, vary, or are selected while enabling this organism to maintain huge global populations in relatively stable environments. These comparisons will become more and more frequent in the coming years.

In the case of eukaryotes, current single-cell DNA amplification methods generate only a small part of the genome to be sequenced (Roy et al. 2014), a problem that can be overcome by combining the genome fragment information of multiple single-cells (Mangot et al. 2017). Improvements on the amplification side will facilitate further SAG studies with other marine protists. Overall, newer technologies that

allow single-cell visualization, chemical characterization, activity and DNA, RNA, and protein content will allow further progress and will be more common and relevant in the future.

1.4.2 Toward Understanding Cell-Cell Interactions

HTS of metagenomes or 16S rRNA and single-cell interrogation techniques have also facilitated inspection of cell-cell interactions in microbial communities and have recently allowed realization that these cell-cell interactions have implications for whole ecosystem functioning.

The availability of high temporal density data on the abundances of specific prokaryotes, protists, and viruses allows using co-occurrence networks to unveil positive or negative associations between them (Faust and Raes 2012; Fuhrman et al. 2015). The network analyses provide testable hypotheses about, for example, the relationships between a specific prokaryote and a eukaryote, hypotheses that require experimental validation (Lima-Mendez et al. 2015). But determination of network structure can generate hypotheses about long-term system stability depending on the amount of connections and feedbacks present and also test whether external changes may affect these connections and overall system stability.

Associations among organisms detected in these analyses can be used to promote co-culture of otherwise difficult to grow organisms, as is the case for *Prochlorococcus* (Morris et al. 2008) where the "helper" bacteria protect the phototroph from oxidative damage. In other cases, bacterial or algal mediation of essential vitamin synthesis might be the key to interactions (Sañudo-Wilhemy et al. 2014), as can be the production of siderophores that help acquire iron (Amin et al. 2009) or hormone-like molecules that some bacteria can produce to stimulate diatom cell division (Amin et al. 2015).

Many of the unveiled associations in the ocean seem to be parasitic (Lima-Mendez et al. 2015), and likely much of the eukaryotic diversity consists of parasites (de Vargas et al. 2015); some have been observed to terminate dinoflagellate blooms (Chambouvet et al. 2008). Beyond the most common forms of symbiosis, like those involving nitrogen fixers and diatoms and small haptophytes, and those between ciliates and algae, new ones are being described in the plankton, in some cases involving an algal host that is both abundant in the free plankton, as well as in the symbiosis (Decelle et al. 2012). Knowledge about these positive and other negative interactions beyond grazing within the microbial food web will grow in the future and likely will be incorporated into models of the microbial food web such as that of Fig. 1.2.

Finally, communication between microbes is probably important in determining their interactions. Although mediated by chemical quorum sensing processes, communication is visible with specialized microscopes (Malfatti et al. 2010), is known to be the bases of biofilm formation (Flemming et al. 2016), and likely present in bacteria growing on particles (Ganesh et al. 2014). In addition, cell-cell communication (probably including gene transfer) might be (one of) the reason-d'etre of the membrane vesicles described in *Prochlorococcus* and in other marine

bacteria (i.e., Biller et al. 2014). More work will likely be done in this area of cell-cell interactions, cell signaling, and infochemicals as drivers of microbial interactions in the future.

1.4.3 Toward Comprehensive Exploration of All Marine Habitats

Some marine habitats have been studied for many years, and we have a relatively good understanding of how the upper ocean works both in oceanic environments and in coastal sites. This is mostly through use of long-term sampling observatories, such as the Hawaii Ocean Time-series (HOT) or Bermuda Atlantic Time-series Study (BATS), and coastal observatories such as Helgoland, Plymouth L4, San Pedro Basin, Blanes Bay, or the Delaware estuary, to name just a few. The differences between surface coastal and oceanic systems are clear, in the organisms, in the function of the microbial communities, but also in the variations over the year. Other sites that have been studied for long periods of time, such as the Cariaco Basin (Taylor et al. 2012), correspond to another type of marine habitat, the highly stratified, anoxic basins, including the oxygen minimum zones (OMZs). The microbial ecology of this type of environments is relatively well known (see Chapter 7), but of other oceanic habitat types, much less is known.

The deep ocean, probably the largest ecosystem on Earth, has been much less studied than the surface layer (Arístegui et al. 2009). The bathypelagic ocean is particularly interesting because of the presence of many chemoautotrophic archaea and bacteria (Swan et al. 2011; Herndl et al. 2005), because of the large role of particle-attached prokaryotes (Baltar et al. 2010), and because of the paradoxical lack of correspondence between the measured carbon inputs and carbon use by bacteria (Herndl and Reinthaler 2013). It is clear that deep ocean microbial communities work in a fundamentally different way than surface water communities do. More work is needed to solve this apparent imbalance and to understand how carbon is used in the deep ocean. In addition, the difference of pressure between the depth and the surface poses an additional experimental challenge.

Somewhat surprisingly, the fine structure of likely the most remarkable open ocean structuring feature, the deep chlorophyll maxima layer, has also been poorly described. In this layer, opposite gradients of temperature, light, and nutrients define a relatively stable habitat with algae adapted to low light levels. We now know that it structures phototroph communities (Cabello et al. 2016) and is home to particular groups of oceanic bacteria, such as the small *Actinobacteria* (Ghai et al. 2013). The maximum of primary productivity occurs above the chlorophyll maxima, and some authors have observed high bacterial activity right below the chlorophyll maxima (Pedrós-Alió et al. 1999). Still, we know little of whether this open ocean structure, which clearly has different functioning, also has distinct microbial communities. Similarly, the mesopelagic probably holds areas of great microbial interest (Robinson et al. 2010), particularly now that it has been shown that the number of small fish in the mesopelagic "deep-scattering layer" is much larger than previously thought

(Irigoien et al. 2014). These organisms, which feed in other ocean layers, metabolize carbon in the mesopelagic and have a currently unknown effect on microbial diversity and function.

Other environments within the ocean will receive renewed attention, or they should. These include sediments, hydrothermal vents, and hypersaline anoxic basins, but also the microbiomes of pelagic and benthic animals (Thomas et al. 2016). In addition, the linkages between the atmosphere and the sea surface will likely receive renewed interest, either through quantification of the use of volatile or non-volatile atmospheric organic carbon such as acetone and methanol by surface ocean microbes (Dixon et al. 2011; Arrieta et al. 2016), or the bidirectional air-sea flux of microorganisms through the atmosphere (Mayol et al. 2014), which might play a role in microbial dispersal. Comprehensive knowledge of marine microbial ecology will require more research effort on these marine habitats.

1.4.4 Toward Changing Our View of the Fluxes of C and the Role of the Various Microbes

Given the many different trophic roles that have been identified in prokaryotes (chemoheterotrophs, photoautotrophs, photoheterotrophs, chemoautotrophs, etc.), protists (parasites, phagotrophs, mixotrophs, strict phototrophs, symbionts), and even viruses (transferring functional genes, "manipulating" microbial functions; e.g., Worden et al. 2015; Hurwitz et al. 2013; Roux et al. 2016), our thinking about the structure of the microbial food web outlined in Fig. 1.2 will likely change in the future. Mixotrophy seems to be prevalent in the smallest protists and in most prokaryotes, and likely common in all environments (see Chapter 3; Selosse et al. 2017).

Another potentially paradigm-shifting topic is the relationships between the structure of the oceanic organic matter and of the prokaryotic communities interacting with that organic matter (Moran et al. 2016). These relationships and interactions have been elusive until now because of lack of methods to examine both in sufficient detail. This is particularly relevant as the oceanic carbon pool is one of the largest on Earth. Understanding how it is created and maintained is key in the efforts for managing the increasing carbon humans are introducing into the atmosphere. Jiao et al. (2010) proposed that reworking of DOM by bacteria in upper oceanic layers produces recalcitrant DOM (Ogawa et al. 2001) that would not be used by microbes throughout the water column. Recent studies suggest that only a fraction of the DOC that microbes produce is probably recalcitrant (Osterholz et al. 2015), but bacterial use of this carbon is limited because the concentration of each compound in this highly diverse pool of molecules is very low (Arrieta et al. 2015). In any case, given the relevance of oceanic carbon storage, advances toward better understanding of the mechanisms of production and consumption of various molecules, or fractions, of DOC in the ocean, will likely be one of the future highlights of oceanic biogeochemistry and marine microbial ecology. We have methods for characterizing microbial communities in exquisite detail, and analogous approaches for organic material are becoming

widely spread (see Chapter 6). In the future, we will see more studies that combine the two fields of marine science for elucidating direct links between specific molecules and specific microbes.

1.4.5 Toward Describing the Unknown Component of Microbial Diversity in the Oceans

The discovery of new diversity in the ocean will continue to be a major driver of the field. A recent estimate of the number of microbial species on Earth (Locey and Lennon 2016) suggests that we are far from having reached a good knowledge of the various microbial species in the ocean, which are believed to be on the order of a few hundred thousand. Our understanding of genetic diversity is similarly limited; the Tara Oceans metagenomic survey encountered ca. 40% of the orthologous groups of genes of unknown function (Sunagawa et al. 2015). The lack of knowledge of small protists is even greater, and new groups are continuously being discovered (e.g., Kim et al. 2011; Jones et al. 2011). Fungi are increasingly seen to be important in the deep ocean (Pernice et al. 2016; Bochdanski et al. 2017) and in the surface layer, including sea ice (Hassett and Gradinger 2015). In the viruses realm, giant (0.4 μm) viruses infecting eukaryotes (Fischer et al. 2010) seem to be quite prevalent (Hingamp et al. 2013). This type of large viruses was shown to be infected by other phages, something that alters our concept of life. We will continue to see new discoveries, driven by further use of smarter cultivation strategies (see Chapter 5), inspection of undersampled environments, use of single-cell genomics, or more efficient molecular tools, such as metagenomic-assembled genomes (Hugerth et al. 2015).

In the future we will continue to learn from environmentally relevant isolates (see Chapter 5), and more environmentally relevant prokaryotes will be cultured. We especially need new isolates of protists and viruses, because we have no models for most of the groups observed only in environmental sequences, particularly for viruses and protists (del Campo et al. 2014). The power of having isolates is arguably most evident for two abundant clades in the oceans: information from isolates and single-cell genomics demonstrated that *Prochlorococcus* comprises several subgroups with extensive genomic and phenotypic diversity (Biller et al. 2015). Similarly, although all isolates of SAR11 have conserved low GC content and small genomes, they differed in their genes for phosphorus metabolism, glycolysis, and C1 metabolism, suggesting adaptation of the various ecotypes to diverse habitats (Grote et al. 2012). More isolates from other environmentally important microbial groups will be equally illuminating about their biology, ecology, and biogeochemical roles in the oceans.

Another area of great interest where we will likely observe progress is that of the linkages (or lack of) between diversity and ecosystem function. Most marine microbial ecologists would say that their goal is to describe (1) *which microbes are present* (diversity); (2) *how many* and *where they develop* (ecology, biogeography); (3) *what do they do* (function); and (4) *how they interact*. Although we have a relatively good understanding of points (1) and (2), we have much less clear answers on points (3)

and (4) There are still many microbial genes with unknown function. Even if we knew the function, and as argued previously, lists of organisms, genes, or even the full genomes of model organisms do not answer fully the question. In addition, some analyses conclude that the large genomic variability in the ocean does not translate into similarly large taxonomic variability (Louca et al. 2016). That observation seems consistent with the low variability in genes among ocean microbial communities (Sunagawa et al. 2015), possibly because the core set of microbial functions evolved in response to past ocean conditions and spread across many microbial clades by lateral gene transfer or convergent evolution, producing enormous microbial diversity that has large functional redundancy. Targeted research to understand the functional meaning of the genomic variability will be needed in the future. This genomic variability represents a large amount of potentially useful medical and biotechnological resources (Arrieta et al. 2010). Consequently, marine microbial ecology has the potential to lay the basic scientific support for a shift toward a "blue economy" that could create economic value and do it in a sustainable way that preserves and protects the sea's resources and ecosystems.

The chapters assembled in this third edition of *Microbial Ecology of the Ocean* will answer some of the questions posed in this introduction in greater detail. We hope the book overall will stimulate students and practitioners to tackle these issues and to continue progress in our field.

1.5 SUMMARY

1. The field covered by the three editions of *Microbial Ecology of the Ocean* has grown steadily in the last 30 years to become a central part of microbial ecology and of biological oceanography. Special issues in high relevance journals, funding from public and private organizations, and a large share of the papers published in marine science and microbial ecology support this claim.
2. The field started with a "black box" approach, considering all organisms within a "box" to have the same ecology. However, new techniques have allowed the opening of the black boxes to describe the variability in activity, growth rates, and composition of each component.
3. A further leap occurred when it became possible to characterize the genome and potential metabolism of both the community as a whole and of the dominant players. These approaches were applied to natural communities and model organisms cultivated in the lab. Driven by the new powerful molecular techniques, which are now accessible to most laboratories, we are learning much about the physiology, genetics, and ecology of marine microbes.
4. We argue that the subject is still descriptive and that it will benefit from more experimental approaches. It will definitively be stronger with method controls and standardization. We also suggest that the field would benefit from more theoretical grounding.

5. We foresee that future relevant developments in the field will concentrate in the study of cell-cell interactions, single-cell genomics, description of undersampled and understudied marine habitats, and undescribed diversity. Exploring these topics and others will lead to changes in our view of the fluxes of carbon and nutrients and the role the various microbes play in them.

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