

Molecular diversity and ecology of microbial plankton

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The history of microbial evolution in the oceans is probably as old as the history of life itself. In contrast to terrestrial ecosystems, microorganisms are the main form of biomass in the oceans, and form some of the largest populations on the planet. Theory predicts that selection should act more efficiently in large populations. But whether microbial plankton populations harbour organisms that are models of adaptive sophistication remains to be seen. Genome sequence data are piling up, but most of the key microbial plankton clades have no cultivated representatives, and information about their ecological activities is sparse.

Certain characteristics of the ocean environment — the prevailing low-nutrient state of the ocean surface, in particular — mean it is sometimes regarded as an extreme ecosystem. Fixed forms of nitrogen, phosphorus and iron are often at very low or undetectable levels in the ocean's circulatory gyres, which occur in about 70% of the oceans¹. Photosynthesis is the main source of metabolic energy and the basis of the food chain; ocean phytoplankton account for nearly 50% of global carbon fixation, and half of the carbon fixed into organic matter is rapidly respired by heterotrophic microorganisms. Most cells are freely suspended in the mainly oxic water column, but some attach to aggregates. In general, these cells survive either by photosynthesizing or by oxidizing dissolved organic matter (DOM) or inorganic compounds, using oxygen as an electron acceptor.

Microbial cell concentrations are typically about 10^5 cells ml⁻¹ in the ocean surface layer (0–300 m) — thymidine uptake into microbial DNA indicates average growth rates of about 0.15 divisions per day (ref. 2). Efficient nutrient recycling, in which there is intense competition for scarce resources, sustains this growth, with predation by viruses and protozoa keeping populations in check and driving high turnover rates³. Despite this competition, steady-state dissolved organic carbon (DOC) concentrations are many times higher than carbon sequestered in living microbial biomass⁴. However, the average age of the DOC pool in the deep ocean, of about 5,000 years⁵ (determined by isotopic dating), suggests that much of the DOM is refractory to degradation. Although DOM is a huge resource, rivalling atmospheric CO₂ as a carbon pool⁶, chemists have been thwarted by the complexity of DOM and have characterized it only in broad terms⁷.

The paragraphs above capture prominent features of the ocean environment, but leave out the complex patterns of physical, chemical and biological variation that drive the evolution and diversification of microorganisms. For example, members of the genus *Vibrio* — which include some of the most common planktonic bacteria that can be isolated on nutrient agar plates — readily grow anaerobically by fermentation. The life cycles of some *Vibrio* species have been shown to include anoxic stages in association with animal hosts, but the broad picture of their ecology in the oceans has barely been characterized⁸. The story is similar for most of the microbial groups described below: the phylogenetic map is detailed, but the ecological panorama is thinly sketched. New information is rapidly flowing into the field from the

cultivation of key organisms, metagenomics and ongoing biogeochemical studies. It seems very likely that the biology of the dominant microbial plankton groups will be unravelled in the years ahead.

Here we review current knowledge about marine bacterial and archaeal diversity, as inferred from phylogenies of genes recovered from the ocean water column, and consider the implications of microbial diversity for understanding the ecology of the oceans. Although we leave protists out of the discussion, many of the same issues apply to them. Some of the studies we refer to extend to the abyssal ocean, but we focus principally on the surface layer (0–300 m) — the region of highest biological activity.

Phylogenetic diversity in the ocean

Small-subunit ribosomal (rRNA) genes have become universal phylogenetic markers and are the main criteria by which microbial plankton groups are identified and named⁹. Most of the marine microbial groups were first identified by sequencing rRNA genes cloned from seawater^{10–14}, and remain uncultured today. Soon after the first reports came in, it became apparent that less than 20 microbial clades accounted for most of the genes recovered¹⁵. Figure 1 is a schematic illustration of the phylogeny of these major plankton clades. The taxon names marked with asterisks represent groups for which cultured isolates are available.

The recent large-scale shotgun sequencing of seawater DNA is providing much higher resolution 16S rRNA gene phylogenies and biogeographical distributions for marine microbial plankton. Although the main purpose of Venter's *Sorcerer II* expedition is to gather whole-genome shotgun sequence (WGS) data from planktonic microorganisms¹⁶, thousands of water-column rRNA genes are part of the by-catch. The first set of collections, from the Sargasso Sea, have yielded 1,184 16S rRNA gene fragments. These data are shown in Fig. 2, organized by clade structure. Such data are a rich scientific resource for two reasons. First, they are not tainted by polymerase chain reaction (PCR) artefacts; PCR artefacts rarely interfere with the correct placement of genes in phylogenetic categories, but they are a major problem for reconstructing evolutionary patterns at the population level¹⁷. Second, the enormous number of genes provided by the *Sorcerer II* expedition is revealing the distribution patterns and abundance of microbial groups that compose only a small fraction of the

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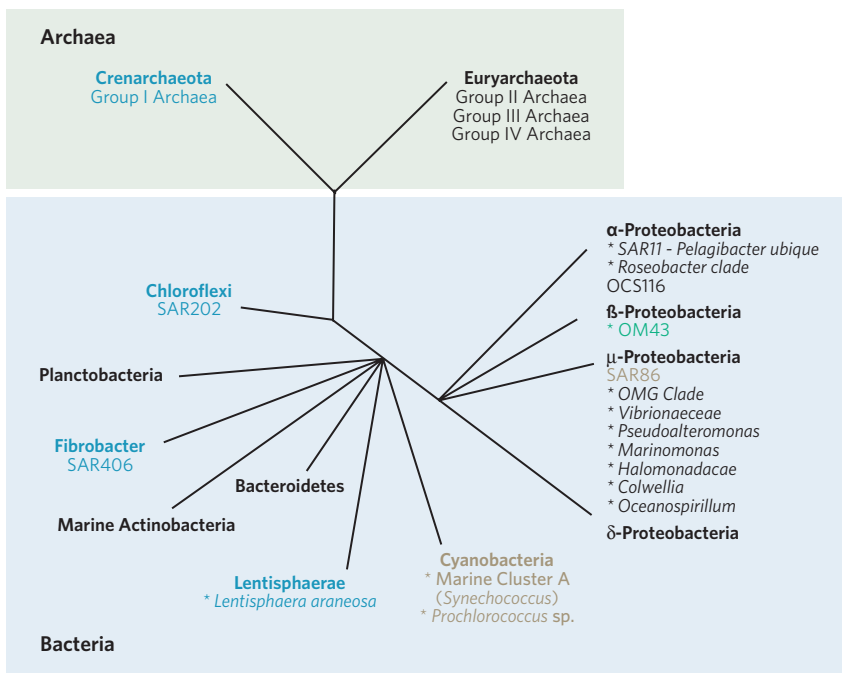


Figure 1 | Schematic illustration of the phylogeny of the major plankton clades. Black letters indicate microbial groups that seem to be ubiquitous in seawater. Gold indicates groups found in the photic zone. Blue indicates groups confined to the mesopelagic and surface waters during polar winters. Green indicates microbial groups associated with coastal ocean ecosystems.

community. As discussed below, some opportunistic strains that exploit transient conditions may fall into this category.

The interpretation of the Sargasso Sea environmental sequence data is already inspiring debate¹⁶. 16S rRNA gene sequence data from the Sargasso Sea WGS data set are shown in Fig. 2 without the sequences of *Burkholderia* and *Shewanella*, which are rare in the Sargasso Sea ecosystem and have been questioned as possible contaminants¹⁶. Naming microbial plankton by clade, as shown in Fig. 2, is a convention used by most oceanographers that is based on evolutionary principles. With few exceptions, the Sargasso Sea data fall into previously named microbial plankton clades. However, Venter has emphasized the new diversity shown by the data, concluding that 1,800 'genomic' species of bacteria and 145 new 'phylotypes' inhabited the samples recovered from the Sargasso Sea¹⁸. To reach this conclusion he applied a rule-of-thumb which assumes that 16S rRNA gene sequences that are less than 97% similar originate from different species. As we discuss further below, the origins of 16S rRNA sequence diversity within the named microbial plankton clades is a hot issue. But, however they are interpreted, the high reliability of raw WGS sequence data will be very useful for understanding the mechanisms of microbial evolution in the oceans.

Although most of the major microbial plankton clades have cosmopolitan distributions, new marine microbial plankton clades continue to emerge from studies that focus on unique hydrographic features. For example, the new Archaea Crenarchaeota and Euryarchaeota were discovered at the brine-seawater interface of the Shaban Deep, in the Red Sea¹⁹.

Patterns in time and space

Molecular biology has filled in some of the blanks about the natural history of marine microbial plankton. As genetic markers became available for ecological studies, it soon emerged that some of the dominant microbial plankton clades are vertically stratified (Fig. 1). Early indications of these patterns came from the distributions of rRNA gene clones among libraries collected from different depths^{20–23}. Although the study of microbial community stratification is far from complete, in many cases (marine unicellular cyanobacteria, SAR11, SAR202, SAR406, SAR324, group I marine Archaea) the vertical stratification of populations has been confirmed by alternative experimental approaches^{21–27}. The obvious interpretation is that many of these groups are specialized to exploit vertical patterns in physical, chemical and biological factors. A clear example is the unicellular marine

cyanobacteria. As obligate phototrophs, these cyanobacteria are confined to the photic zone. A similar pattern is found in the SAR86 clade of γ -Proteobacteria. Proteorhodopsin genes have been found in fragments of SAR86 genomes, suggesting that this clade has the potential for phototrophic metabolism.

Many of the enigmatic microbial groups for which no metabolic strategy has been identified are also stratified. The boundary between the photic zone and the dark upper mesopelagic is particularly striking — below the photic zone the abundance of picophytoplankton and SAR86 declines sharply, and marine group I Archaea, SAR202, SAR406 and SAR324 all assume a prevalent status^{21–24,26,27}. The implications of these observations are clear: the upper mesopelagic community is almost certainly specialized to harvest resources descending from the photic zone. However, with the exception of the marine group I Archaea, very little specific information is available about the individual activities of the upper-mesopelagic groups.

There are also significant differences between coastal and ocean gyre microbial plankton populations (Fig. 1; ref. 28). Typically, continental shelves are far more productive than ocean gyres because physical processes such as upwelling and mixing bring nutrients to the surface. As a result eukaryotic phytoplankton make up a larger fraction of the biomass in coastal seas, and species differ between coastal and ocean populations. Most of the bacterial groups found in gyres also occur in large numbers in coastal seas, but a number of microbial plankton clades, particular members of the β -Proteobacteria, have coastal ecotypes or appear to be predominantly confined to coastal seas²⁸.

One of the most enigmatic microbial groups in the ocean is the marine group I Archaea. Tantalizing geochemical evidence suggests that these organisms are chemoautotrophs²⁹. In the 1990s, DeLong and Fuhrman established that archaea are widely distributed and numerically significant in the marine water column^{11,20,30}. The marine group I Archaea are Crenarchaeotes. They predominantly occur in the mesopelagic, but are found at the surface in the cold waters of the southern ocean during the winter. Fluorescence *in situ* hybridization technology was used to demonstrate that marine group I Archaea populations comprise about 40% of the mesopelagic microbial community over vast expanses of the ocean, making them one of the most abundant organisms on the planet²⁴. All of the marine Archaea remain uncultured.

New data about microbial distributions has provided tantalizing hints about geochemical activity, but most progress on this question

has come either from cultures or from approaches designed to yield information from experiments performed on native populations. Fluorescence-activated cell-sorting and *in situ* hybridization have both been used to separate populations and measure their uptake of radioactive substrates^{31–33}. Testing hypotheses originating from genome sequences, oceanographers were surprised to find that the unicellular marine bacteria, particularly *Prochlorococcus*, can assimilate free amino acids — it had previously been thought that they rely solely on inorganic nitrogen³².

The species question

The question of how to name microbial plankton species is not a trivial matter. For oceanographers the issue is: where should the lines be drawn so that organisms with different properties relevant to geochemistry are given unique names? From an evolutionary perspective, the question might be phrased differently: how does one demarcate cell populations that use the same resources and possess the same suites of adaptations inherited from a common ancestor? Confusion arises from the fact that there is no general agreement about the definition of a microbial ‘species’. The ‘97% rule’ is simple to apply but does not take into account the complex structure of microbial clades. For example, the unicellular marine cyanobacteria form a shallow clade that would constitute a single species by the 97% rule, but all agree that this clade contains several species with distinct phenotypes. Clades such as SAR86 and SAR11 are far more diverse, but can clearly be divided into subclades. One theory is that some of these ‘bushy’ subclades are ecotypes — populations with shared characters and unique niches³⁴.

Acinas and co-workers studied clade structure by using clone libraries prepared using PCR methods that reduced sequence artefacts¹⁷. They concluded that most sequence variation was clustered

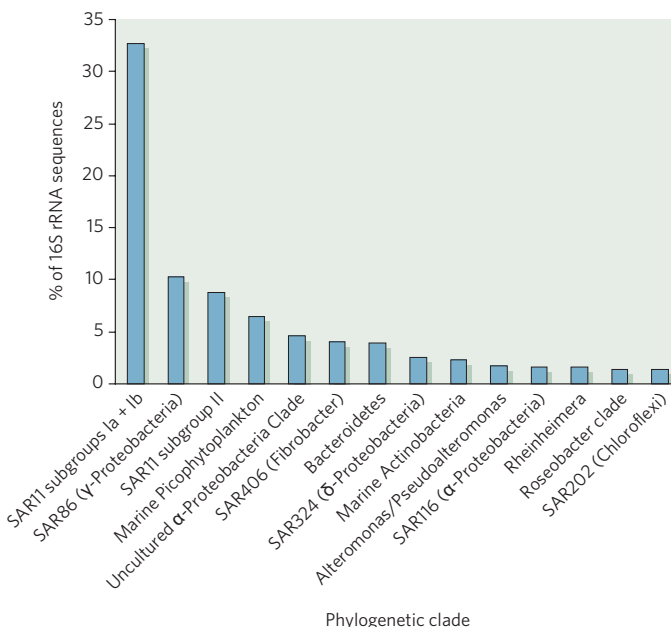


Figure 2 | 16S rRNA genes from the Sargasso Sea metagenome data set, organized by clades. The clades are shown in rank order according to gene abundance. To create the figure, 16S gene fragments were recovered from the data set by the BLAST program using 14 full sequences of different prokaryotic phyla as query. The resulting 934 sequences were phylogenetically analysed using the program package ARB. Venter *et al.* correctly reported more 16S rRNA gene fragments (1,184) because their analysis included smaller fragments that are excluded from the set of 934 sequences used in the analysis shown here. Genes belonging to the genera *Burkholderia* and *Shewanella* were omitted from the analysis because of suggestions that they are contaminants⁷².

near the tips of branches and could be attributed to neutral mutations accumulating in clonal populations. Thompson *et al.* went a step further by examining sequence divergence and genome variability in a set of 232 *Vibrio splendidus* isolates taken from the same coastal location at different times³⁵. The isolates differed by less than 1% in rRNA gene sequences, but showed extensive variation in genome size and allelic diversity. These results could explain why Venter’s group found marine microbial genomes difficult to assemble from shotgun sequence data.

However, Kimura predicted that selection has more opportunity to act on small changes in fitness as population size increases, and therefore very large, stable populations should be more highly perfected by selection³⁶. More specifically, Kimura coined the term ‘effective population size’ to refer to the minimal size reached by a population that is undergoing fluctuations. For these marine microbes, it may be that where large populations have not been through recent episodes of purifying selection, they are able to maintain very large reservoirs of neutral genetic variation. If this hypothesis is correct, then, at least within ecotypes of microbial plankton, one would expect to find a core set of genes conferring relatively conserved phenotype.

The SAR11 clade provided the earliest demonstration that the subclades of environmental gene clusters could be ecotypes²⁶. Probing rRNA revealed the presence of a surface (IA) and a deep sub-clade (II), but failed to identify the niche of a third sub-clade (IB; ref. 26). More recently, the niche of SAR11 subclade IB emerged in a study of the transition between spring-bloom and summer-stratified conditions in the western Sargasso Sea²⁷. Nonmetric multidimensional scaling revealed that the IB subclade occurs throughout the water column in the spring, apparently giving way to the more specialized IA and II subclades when the water column becomes thermally stratified.

The ecotype concept continues to expand with the recognition that many microbial groups can be subdivided according to their distributions in the water column. The unicellular cyanobacteria are by far the best example. Two ecotypes of *Prochlorococcus* can readily be differentiated by their chlorophyll *b*/chlorophyll *a* ratios — a high-light-adapted (high-*b/a*) lineage, and a low-light-adapted (low-*b/a*) lineage. Phylogenetic evidence from internal transcribed spacers (ITS) suggests that the high-*b/a* strains can be differentiated into four genetically distinct lineages. The ITS-based phylogenies indicate that Marine Cluster A *Synechococcus* can be subdivided into six clades, three of which can be associated with adaptively important phenotypic characteristics (motility, chromatic adaptation, and lack of phycoerythrin)^{37,38}. So far, the genome sequences available have provided ample support for the hypothesis that these ecotypes differ in characteristics that affect their ability to compete. Notably, the low-*b/a* strain SS120 has a much smaller genome than the others and can use only ammonium and amino acids for nitrogen sources³⁹. At the other extreme, *Synechococcus* WH8102 can use ammonium, urea, nitrite, nitrate, cyanate, peptides and amino acids as sources of nitrogen. It is interesting to note that Marine Cluster A *Synechococcus* populations seem to prosper during periods of upwelling and vertical mixing — whereby nutrients are supplied but also cause chaotic, transitional conditions. Thus, as observed in the SAR11 clade, there seem to be seasonal specialists and stratification specialists in the marine unicellular cyanobacteria.

The observation that the major microbial plankton clades have diverged into ecotypes is powerful evidence that selection is creating functionally and genetically unique entities, despite the confounding influence of neutral variation, which causes relatively marked divergence in genome sequences. Although the unicellular marine cyanobacteria are a good model for what the future may hold, the debate about diversity is far from over.

Old paradigms challenged by new forms of phototrophy

The new millennium arrived in tandem with discoveries of new forms of phototrophy in the ocean surface, which in turn fundamentally changed perspectives on microbial food webs. Béja *et al.* reported the

presence of bacteriorhodopsin gene homologues that were linked to SAR86 rRNA genes on bacterial artificial chromosome (BAC) clones from Monterey Bay⁴⁰. And Kölber *et al.* used fast repetition rate infrared fluorescence spectroscopy to identify transient fluorescence signatures emanating from bacteriochlorophyll *a* in seawater⁴¹. Neither of these photosynthetic systems was biochemically novel; both had been previously discovered in obscure microbial groups. The two analogous systems use energy from light to pump protons out of cells, and are believed to function by charging transmembrane potentials and supporting uphill reactions, such as active transport and adenine dinucleotide phosphate (ADP) phosphorylation. What was new was the idea that bacterioplankton might be using these strategies on a vast scale to channel energy into cells that had been ostensibly regarded as respiratory heterotrophs. The details of which species have these systems, when they are expressed and what functions they support are obscure.

The very high abundance and diversity of bacteriorhodopsin homologues in the surface waters of the Sargasso Sea suggests that these genes are important¹⁸. Venter *et al.* recovered 743 'proteorhodopsin-like' sequences and 1,164 rRNA sequences from the Sargasso Sea — a ratio of 0.7. But, so far, only one report provides direct evidence that proteorhodopsins are active in seawater⁴²; another shows that incubating seawater in the dark has little impact on microbial populations, although this does inhibit the growth of the unicellular marine cyanobacteria^{43,44}. A key question is whether the proteorhodopsin gene family serves other functions as well — homologues of proteorhodopsin are also known to function as sensory receptors and to pump ions other than protons.

Kölber's report of the widespread presence of photosynthetic electron-transport systems based on bacteriochlorophyll *a* in the oceans was validated, but recent reports indicate that the quantitative significance of this process is less than was originally indicated⁴³.

Adaptive strategies

Some evidence suggests that bacteria can be divided between two general ecological strategies: those that exploit patchiness in the environment, and those that compete effectively at ambient nutrient background levels^{45,46}. Most of the strains studied by pioneering marine microbiologists were heterotrophic γ -Proteobacteria that grew very rapidly on marine nutrient agar. These organisms are rarely observed in 16S rRNA gene diversity surveys, but they frequently dominate experiments in which natural microbial plankton communities are manipulated^{47,48}.

Challenged with the problem of obtaining biologically relevant cultures, marine microbiologists have increasingly focused their efforts on new heterotrophic isolates that replicate effectively at very low nutrient concentrations. These organisms, referred to as oligotrophs, often do not respond well to high nutrient concentrations. One theory is that oligotrophs lack the complex regulatory responses and alternative metabolic pathways that enable their more robust counterparts to grow rapidly in high-nutrient media. The first isolate of SAR11, *Pelagibacter ubique*, grows slowly on seawater, doubling its population size every 29 hours, and does not increase its growth rate in response to the addition of organic carbon nutrients⁴⁹. Its genome is small — 1.3 million base pairs. There is also a suggestion that fast- and slow-growing bacteria may differ fundamentally in the rate of translation per ribosome⁵⁰.

Metabolic modelling has not 'arrived' in marine microbiology yet, but is looming on the horizon. Flux models for geochemical processes at the ecosystem level are historically one of the central themes of biological oceanography. It seems only a matter of time before the post-genomic effort to create *in silico* models for cells merges with the ecosystem flux models. In an intriguing foray into theory, Button *et al.* developed a mathematical model for oligotrophic cells that predicts their properties from basic principles⁵¹. The central concept in this theory is that evolution has acted on oligotrophic cell design to maxi-

mize a term called the specific affinity constant. The specific affinity constant takes into account the cell's overall ability to transform nutrients into biomass by considering the number of transporters and the surface area of the cell, in addition to transporter substrate affinities. This theory predicts that selection acts to reduce the size of oligotrophs so that the capacity of the cytoplasm to process substrate will not exceed the capacity of transport systems to supply substrate.

The concepts outlined above are general and do not take into account specialized adaptations that allow organisms to exploit specific features of the ecosystem. For example, most marine bacterioplankton seem to be lone cells that drift in the water column, but there is a subset of marine microbial groups that specialize in the colonization of particles. These cells have a special geochemical significance because particles sink, carrying carbon and other nutrients from the surface into the mesopelagic. This is the essence of the 'biological pump', which effectively reduces the partial pressure of carbon dioxide in the ocean surface, thereby increasing the rate at which atmospheric CO₂ dissolves in seawater⁵². A particularly important class of particles are macroscopic aggregates known as marine snow, which tend to form in the lower photic zone and disappear as the particles sink into the upper mesopelagic. DeLong *et al.* investigated organisms associated with marine snow and found little overlap between these organisms and the species freely suspended in the water column⁵³. Members of the Planctobacteria and Bacteroidetes phyla are particularly abundant on marine snow particles. Most Planctobacteria have specialized holdfast structures for attachment to surfaces. Bacteroidetes members frequently use gliding motility to traverse surfaces, and have been implicated in degradation of biopolymers associated with detritus⁵⁴. The recently discovered phylum Lentisphaerae has an unusual phenotype that suggests it might participate in particle formation⁵⁵. Lentisphaerae form a three-dimensional polysaccharide net that makes water viscous. A function for this net has not been proven, but the hypothesis that it serves to trap sinking particles fits the location of this organism, which resides in the upper mesopelagic, just beneath the photic zone.

Viral mortality and predator-prey relationships dominate current thinking about interactions between microbial plankton species⁵⁶, but it is difficult to believe that the water column has not spawned more insidious biological devices for gaining on the competition. A number of recent reports have focused on interactions between bacterioplankton and phytoplankton⁵⁷. Several strains of algicidal bacteria that produce algicidal peptides, antifouling agents or antimicrobial peptides have been described^{58,59}. The common genus *Pseudoalteromonas* crops up repeatedly in this context. But, for now, the passive 'microbial loop' model, in which heterotrophic microbial plankton acquire organic carbon that is lost from phytoplankton by leakage or lysis, reflects the prevailing view.

Rare species can be important

Some rare organisms that do not often show up in gene clone libraries may contribute significantly to oceanic geochemical processes. For example, the rate of nitrogen fixation is low in the oceans because fixed nitrogen is recycled efficiently in the photic zone, and other limiting nutrients prevent the expansion of plankton communities. However, nitrogen fixation plays a critical role in supporting productivity by replacing fixed nitrogen that is lost from the ocean surface. Zehr and colleagues identified diverse nitrogenase genes in seawater by culture-independent approaches^{60,61}. On the basis of Zehr's evidence, Montoya *et al.* identified new unicellular cyanobacteria that seem to contribute significantly to nitrogen fixation in the oceans⁶².

Cultivating key species

New microorganisms in culture are big news if they represent microbial groups that play important roles in the environment. Heightened attention to cultivation is producing a steady flow of new isolates from several research groups. The value of cultures has been underscored by

the problems that Venter's group encountered when reconstructing genomes from the Sargasso Sea metagenome data set. Complete genomes from cultures are now perceived as crucial reference points for assembling metagenome data. More importantly, strains in culture provide the means to study cell physiology and test hypotheses emerging from genome sequence data. Once again, the unicellular marine oxygenic phototrophs provide the best example. The cultivation of marine *Synechococcus* and *Prochlorococcus* species led to rapid advances in understanding the biology of these organisms (see above). Another group that has been studied in culture is the *Roseobacter* clade. One *Roseobacter* clade member, *Sulfitobacter*, has been shown to use dimethylsulphoniopropionate and can produce metabolic energy by sulphide oxidation⁶³. Another cyanobacterium that defied cultivation for decades, *Trichodesmium*, is now in culture in labs around the world⁶⁴.

Isolating cells by dilution (extinction culturing) into natural seawater was pioneered by Button *et al.*⁶⁵ and was later used to obtain *Sphingopyxis alaskensis*⁶⁶ and *Marinobacter arcticus*, which became model organisms for studying the properties of oligotrophic cells^{51,67}. High-throughput versions of extinction culturing led to the isolation of many more oligotrophic species^{55,68–70}, most of which replicate poorly or not at all on marine nutrient agar. Some of the important isolates to emerge from this approach are *Pelagibacter ubique*⁴⁹, the first cultured member of the SAR11 clade, as well as OM43, *Lentisphaera*⁵⁵ and the Oligotrophic Marine γ -Proteobacteria group⁶⁸. However, many of the dominant marine plankton groups, including the SAR86 cluster, the SAR202 cluster, the SAR324 cluster, the marine Actinobacteria, SAR406, and the group II marine Archaea, have not yet been cultivated. Given the number of replications of extinction cultures from seawater that have now been examined, it is clear that some of the important clades that remain uncultivated are not going to grow without further advances in cultivation technology.

Future prospects

Historically, biological oceanography has focused on developing quantitative models of food-web dynamics. With the emergence of the 'microbial loop' hypothesis in 1983 (ref. 71), bacteria assumed a major role in food-web models, but were treated as a 'black box' for the purpose of creating tractable models for geochemical fluxes. At a conceptual level the microbial black box has been repeatedly transformed by discoveries of new photosynthetic microorganisms and new forms of photoheterotrophy. If the traditions of oceanography eventually merge with current trends in microbiology, then the future metabolic models for the major bacterioplankton clades may become elements in larger models that predict geochemical fluxes in the ocean water column. ■

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