



A Sea of Microbes: What's So Special about Marine Microbiology

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Abstract

This chapter investigates what justifies marine microbiology as a discipline in its own right. Do marine microorganisms really exist? And if so, what distinguishes them from freshwater- or terrestrial microorganisms, or from microorganisms living in any other specialized habitat? The marine environment—particularly the ocean—is the largest continuous habitat on Earth. This makes the ocean different in terms of scale and sharing space and nutrients, as well as in terms of the distribution, dispersal, and encounters of microorganisms that inhabit it. The ocean comprises a large variety of confined sub-habitats. It has an impact on the function of the planet Earth as a whole. A critical property of seawater is that it contains a large amount of salt and that this requires microorganisms that live in it are able to adjust their osmotic pressure. The marine microbiome is composed of the three domains of life: bacteria, archaea, and eukarya, as well as viruses, all of which in dazzling numbers and diversity. All of the known microbial lineages are represented and many are exclusively found in the ocean and there is little doubt that life originated in the ocean.

Keywords

Marine microbiology · Milestones of marine microbiology · Nitrogen fixation · Ocean · Redfield ratio · Salinity

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1.1 Introduction

Nowhere else where life is possible, probably in no other place in the Universe except another ocean, are so many conditions so stable and so enduring.

Baas Becking (1934) put this quote from Henderson's book "*The Fitness of the Environment*" (Henderson 1913) above Chap. 9 "De Zee" ("The Sea") of his book "*Geobiologie of inleiding tot de milieukunde*" (Fig. 1.1). But immediately, Baas Becking put this quote in perspective by stating that it might refer only to the physicochemical characteristics, because the ocean is considered to be stable because it is composed mainly of water. He continues by stating that when viewing it from a biological perspective no environment has such a diversity as the ocean with its adjacent seas, bays, estuaries, and coasts, with their currents and variations in illumination, temperature, salinity, hydrostatic pressure, and a variety of chemical components and organisms. Baas Becking's book, although written in Dutch (an English translation appeared in Canfield 2015, published by Wiley, and edited by Don Canfield), became famous by the quote: ". . .alles is overal: maar het milieu

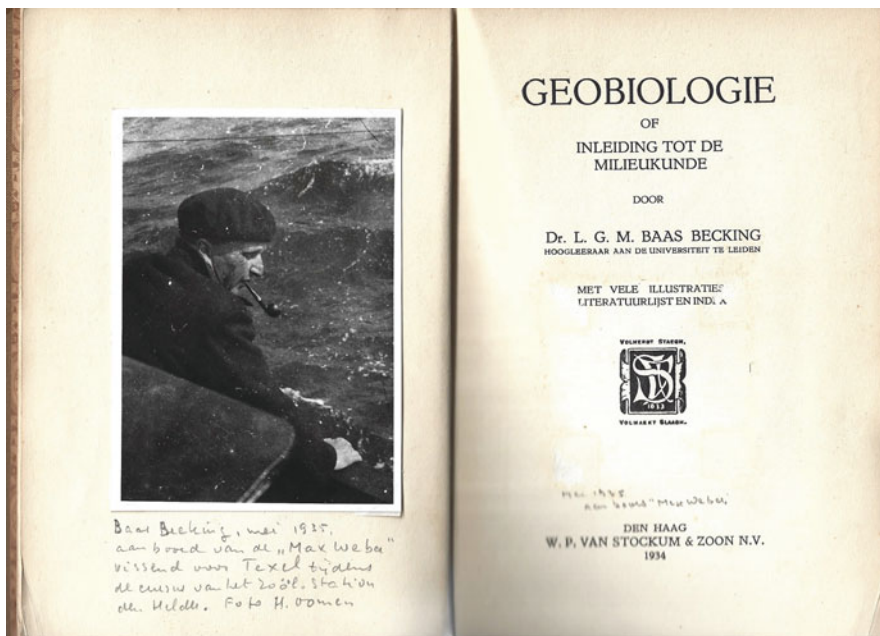


Fig. 1.1 Scan of the title page of Baas Becking's book "*Geobiologie of inleiding tot de milieukunde*" with a photo of the author on board of the RV "*Max Weber*" of the Zoological Station Den Helder (predecessor of the Netherlands Institute for Sea Research, NIOZ) while doing research in the waters around the island of Texel in the Netherlands in 1935. The photo is an original copy which was made by Dr. H. Oomen, who also was the original owner of this copy of the book which he obtained in 1935 during the same cruise on board of the RV "*Max Weber*." The book is now in possession of L.J. Stal

Fig. 1.2 Scan of the text of Baas Becking's book "*Geobiologie of inleiding tot de milieukunde*" with his famous quote: "...everything is everywhere: *but* the environment selects." Underlining presumably by Dr. H. Oomen, the first owner of this copy of the book

leven te werken door een geschikt milieu voor hen te scheppen, en als wij een „scherp antwoord”, in de zin van Beyerinck, wenschen moet dit milieu scherp worden gedefinieerd. Het moet worden tot een selectief milieu. En zoo kan men toevoegen aan den eersten hier gestelde regel *alles is overal*: maar *het milieu selecteert*.

Zoowel in de natuur als in het laboratorium. Hoe het milieu selecteert zal verder onderwerp van deze voordracht zijn. Men voelt dat, wanneer de natuur of de onderzoeker zeer definiete condities scheidt, de kans op een specifiek antwoord ook grooter zal zijn. Een warme bron

selecteert" (p. 15; italics and roman are from Baas Becking) (Fig. 1.2) ("... *everything is everywhere: but the environment selects*"). This quote has been cited often wrong, e.g., by leaving out "but" or replacing it by "and," both of which fundamentally change the meaning as nicely explained by De Wit and Bouvier (2006). Does this physicochemically stable ocean select for microorganisms that can be considered as truly marine?

"A Sea of Microbes" was the title of a special issue of "Oceanography" published in 2007 and edited by Proctor and Karl (2007). It was also the title of a book review (Karl 2001) of Kirchman's edited publication "Microbial Ecology of the Oceans" (2000) (second edition published in 2008) (Kirchman 2000, 2008). I took the translation in Dutch as the title for my inaugural lecture for celebrating the (first) chair of "Marine Microbiology" in the Netherlands at the University of Amsterdam on 11 April 2008 in Amsterdam: "*Een Zee van Bacteriën*." It is a double entendre in both languages meaning "a lot of microbes" as well as "a sea owned by the microbes." Here, I borrowed it again as the title for this introductory chapter, which tries to examine the question why marine microbiology would be a discipline in its own right.

1.2 Planet Ocean

The planet Earth is covered for more than 70% of its surface by the ocean and because water preferentially absorbs red light it leaves the complementary blue color. Therefore, "Ocean" would be a more appropriate name for this planet. The ocean has an average depth of 3700 m and its deepest point is the Challenger Deep in the Mariana Trench with ~11,000 m. The ocean contains 1.35 billion cubic kilometers of water (97% of Earth's water, Table 1.1) and is distributed over 5 named ocean basins: the Pacific Ocean, the Indian Ocean, the Atlantic Ocean, the Arctic Ocean, and the Southern (Antarctic) Ocean (Table 1.2). These oceans are all interconnected and altogether there is one world ocean. Five main currents called

Table 1.1 Distribution of water on Earth

Water body	%
Ocean, seas, bays	96.5
Ice caps, glaciers, permanent snow	1.74
Saline ground water	0.94
Fresh ground water	0.76
Ground ice and permafrost	0.022
Fresh water lakes	0.007
Saline lakes	0.006
Soil moisture	0.001
Atmosphere	0.001
Swamp water	0.0008
Rivers	0.0002
Biological water	0.0001

https://en.wikipedia.org/wiki/Water_distribution_on_Earth

Table 1.2 Distribution of water between the main oceanic basins (% of total water on Earth)

Ocean	%
Pacific	48.30
Atlantic	22.40
Indian	19.00
Southern	5.18
Arctic	1.35

https://en.wikipedia.org/wiki/Water_distribution_on_Earth

gyres connect the oceanic basins: the North Pacific Gyre, the South Pacific Gyre, the Indian Ocean Gyre, the North Atlantic Gyre, and the South Atlantic Gyre. These currents are formed by wind and the rotation of the Earth (Coriolis effect). It is estimated that to complete one circulation may take up to 1000 years. This means that oceanic microorganisms are globally distributed by these currents and that this one ocean, therefore, represents the largest habitat in the biosphere. Coastal areas where surface water is replaced by cold, nutrient-rich, deep water are called upwelling zones that are characterized by high productivity. The surface water is heated in the tropics and cooled down in the arctic. Freezing rises the salinity and together with the decrease in temperature the density of the seawater increases and seawater sinks and flows to the equatorial regions where it emerges to the surface. These gyres altogether form the thermohaline circulation, a global water transport system that is also known as “conveyor belt.”

1.2.1 Salinity

A remarkable property of seawater is that it contains salt. More precisely, it contains on average 35 ppt (parts per thousand) or 35 g salts per kilogram of water (‰) (Millero et al. 2008). Note that salinity is dimensionless. The quantitative most important components (solutes) of seawater are listed in Table 1.3. The salinity is

Table 1.3 Composition of standard seawater compared to a hypersaline sea, a freshwater lake, and a brackish sea

	Standard Seawater ^a (35‰)	Dead Sea ^b (230‰)	Lake Michigan ^c (0.1‰)	Baltic Sea (Bornholm Sea) ^d (~10‰)
Solute	g kg ⁻¹			
Na ⁺	10.781	30.156	0.016	~3.080
Mg ²⁺	1.284	31.156	0.015	~0.367
Ca ²⁺	0.412	13.001	0.016	~0.118
K ⁺	0.399	5.616	0.013	~0.114
Sr ²⁺	0.008	0.231	n.r.	~0.002
Li ⁺	n.r. ^e	0.015	n.r.	
Cl ⁻	19.353	163.397	0.025	~5.529
SO ₄ ²⁻	2.712	0.385	0.013	~0.775
HCO ₃ ⁻	0.105	0.154	0.009	
Br ⁻	0.067	3.923	n.r.	
CO ₃ ²⁻	0.014	n.r.	n.r.	
B (OH) ₄ ⁻	0.008	n.r.	n.r.	
F ⁻	0.00004	n.r.	n.r.	
B (OH) ₃	0.0006	n.r.	n.r.	
CO ₂	0.0004	n.r.	n.r.	

The salinity and precise composition of seawater depend on the exact location (geographic; depth) from which the seawater is considered. Here, the standardized seawater as defined by Millero et al. (2008) is listed as a good average. The Dead Sea is taken as just one example. Depending on the hypersaline system considered, the composition will be totally different. The same is true for freshwater systems, which may have greatly different composition in different lakes or rivers. The Baltic Sea is the largest brackish sea but characterized by a salinity gradient from the North Sea (almost full seawater salinity) to the Gulf of Bothnia (almost freshwater). Also, there is a pycnocline because the deeper waters are more saline (North Sea water entering the Baltic Sea dives underneath the lighter less saline Baltic Sea water). Here, the brackish Bornholm Sea is taken as an example. The salinity of the Baltic Sea does not show anomalies and the composition shown is just derived from standard seawater by dividing by 3.5

^aMillero et al. (2008)

^bNissenbaum (1975)

^cChapra et al. (2012)

^dGrasshoff and Voipio (1981)

^eNot reported

not everywhere the same and not homogeneously distributed in the ocean and its adjacent waters. For instance, the Mediterranean Sea salinity is higher (38–40 ppt) because of its limited exchange with the Atlantic Ocean and higher evaporation. The (coastal) North Sea has a slightly lower salinity (30–34 ppt) because of river discharge. The Baltic Sea has a salinity gradient from the North Sea to almost freshwater in the Bothnian Gulf and an average of 10 ppt in the Bornholm Sea. This sea has also a stable halocline because the saltier and therefore denser North Sea water dives underneath the less dense surface water and does not mix. Deep

hypersaline anoxic basins (DHABs) are found on the bottom of the Mediterranean Sea, Red Sea, the Black Sea, and the Gulf of Mexico. They are formed by dissolution of ancient evaporites and due to their high salinity do not mix with the overlying seawater (Merlino et al. 2018; Wallmann et al. 1997). Also, partial enclosed bays or lagoons in tropical areas where high evaporation occurs and no freshwater discharges, may exhibit above seawater salinity such as is the case in Hamelin Pool (Shark Bay, Australia) (Edgcomb et al. 2014). Estuaries are typically exhibiting a salinity gradient decreasing from the sea to the river. In some cases, the denser seawater dives underneath the discharging fresh river water (salt wedge estuary) (Heip et al. 1995; Watanabe et al. 2014). Upstream some tidal estuaries even experience tides of freshwater. Solar lakes in tropical regions are separated from the sea by a sand bar through which seawater seeps through. The water in this lake evaporates due to the high temperature and is heated by the high solar irradiation. It becomes denser due to the increasing salinity and the hot surface water sinks producing a thermocline with hot (65 °C) water below (Cohen et al. 1977). There is a variety of hypersaline systems. Natural or man-made solar salterns that evaporate seawater to produce sodium chloride (table salt) or magnesium salts (bitters) are made of a series of evaporation ponds with increasing salinity until saturation (Oren 2009). There are many examples of salt lakes in the world that are not connected to the sea such as the Dead Sea in the middle east (Table 1.3) or Great Salt Lake in the USA, which are different in their chemical composition. They are formed by evaporation of the river water that is discharged in them and their chemical composition is a function of their watersheds.

These hypersaline lakes, ponds, and lagoons are considered as extreme environments that are the habitats of specialized microorganisms. Microbiologists working in those hypersaline environments laugh about regular seawater salinity, which they consider not too much different from freshwater (see Table 1.3). In fact, seawater is probably less extreme than freshwater as it more closely equilibrates the concentrated cytoplasm. Most likely, seawater was the medium in which life evolved.

The sea receives salts via river discharge and run-off. This water contains minerals from rock dissolution by the acidic rain in the riverine catchment area. Rain dilutes seawater and this dilution is counteracted by evaporation keeping salinity in equilibrium. Also, melting sea ice contributes to the freshwater input into the ocean, which is enhanced by global warming. Whether or not this will cause a different equilibrium of the seawater salinity is an open question. Because the chemical composition of seawater did not change during the last 100 million years minerals must have been removed, which among other may have been accomplished by processes such as mineral precipitation and deposition (e.g., calcification), sulfur emission to the atmosphere (dimethylsulfide, DMS), organic matter decomposition, -deposition, -burial, and mineral removal. These are fast processes and without any of these mineral removal components the ocean could have reached its present salinity in only a few million years. It would also mean that seawater salinity is not in equilibrium and that the ocean would become more saline. However, there is

evidence that the ocean became saline during its formation more than 4 billion years ago, i.e. before life evolved.

1.2.2 Origin of Salinity and Early Ocean

Knauth (2005) estimated that the salinity of the Archean ocean was 1.5–2 times the present value. This high salinity remained until continental cratons developed that sequestered halite and when brines formed that derived from evaporating seawater. Knauth (2005) also estimated that the surface of the Archaean ocean had a temperature of 55–85 °C and that only 1.2 Ga bp ocean surface temperature cooled down to present levels. The high salinity and temperature of the seawater prevented the dissolution of oxygen (O₂) and as a consequence the ocean stayed anoxic even when the atmosphere contained O₂ to ~70% of its modern value and hence the ocean remained a domain for anaerobic microorganisms (Knauth 2005). Yet, modern hypersaline environments have largely an aerobic microbiota. Multicellular eukaryotic O₂-dependent organisms may have, therefore, evolved on land and moved to the sea after its surface became oxygenated. Gaucher et al. (2003) predicted on the basis of the phylogeny of resurrected proteins that deep-rooted bacteria must have been thermophiles with a temperature optimum of 55–65 °C and not meso- or hyperthermophiles. Hence, that would have allowed bacterial life emerging globally rather than in isolated spots around hyperthermal vents as have been hypothesized previously (Weiss et al. 2016) (but see Schreiber and Mayer 2020).

While chlorine (Cl⁻) is the most abundant anion in seawater, sulfate (SO₄²⁻) is quantitatively the second-most important anion with about 28 mM. This high concentration guarantees that sulfur is never a limiting nutrient for microorganisms thriving in the sea (which may be sometimes the case in freshwater systems) and that when oxygen is in short supply sulfate may take over as electron acceptor and reduced sulfur compounds (such as sulfide: H₂S, HS⁻, S²⁻) may dominate as, for instance, is the case in the so-called sulfureta (Overmann and van Gemerden 2000). Assimilatory sulfate reduction is used by (aerobic) microorganisms to satisfy their sulfur demand. Dissimilatory sulfate reduction is a form of anaerobic respiration that becomes important when oxygen is at low supply such as in marine aggregates (marine snow), oxygen minimum zones, intertidal sediment, or other anoxic marine or saline environments. Sulfate is also the main oxidant of deep-sea cold seeps of methane, which is achieved by aggregates of a methanogenic archaea and a sulfate-reducing bacteria, in which the former reversed its metabolism to methane oxidation rather than methanogenesis (Boetius et al. 2000).

1.2.3 Microorganisms in the Ocean

Bacteria have been around for almost 4 billion years and probably evolved in the ocean. Marine microorganisms comprise all three domains of life: bacteria, archaea, and eukarya, as well as the viruses as another important biological entity. They are

the engines and engineers of marine ecosystems and are at the basis of the marine foodweb (Kirchman 2000, 2008). Marine microorganisms are responsible for the primary and secondary production and recycle nutrients and organic matter. Many biogeochemical processes are carried out exclusively by microorganisms and without them an ecosystem would not function. With their vast numbers, untold diversity, and high reproduction rates they truly represent the unseen majority (Whitman et al. 1998). Microorganisms represent 90% of the biomass in the oceans. There are 10^6 bacterial and archaeal cells and 10^7 viral particles in 1 ml of seawater. The total of microorganisms in the open ocean amounts to $>10^{29}$ (Whitman et al. 1998). These incredibly large numbers of individual cells are statistically separated by 100–200 cell-lengths (Moran 2015; Zehr et al. 2017).

Smith and Baker (1982) showed that ocean surface chlorophyll concentrations could be obtained by using satellite data and that this opened the possibility to obtain such data on a scale that is impossible to obtain from shipboard measurements. These satellite data were subsequently used for the estimation of primary production at the same (global) scale (Behrenfeld and Falkowski 1997).

The primary production in the ocean by marine phytoplankton accounts for roughly half of the global primary production (Field et al. 1998). A quarter of this production has been attributed to only two genera of cyanobacteria, the picocyanobacteria *Prochlorococcus* and *Synechococcus* (Flombaum et al. 2013). *Prochlorococcus* is exclusively found in the ocean, while *Synechococcus* has also brackish and freshwater lineages.

Marine phytoplankton and to a substantial extent also chemoautotrophic bacterioplankton fix an enormous amount of CO_2 of which a small but important amount escapes degradation and is transported to the deep-sea where it is buried and turned over on geological time-scales (carbon pump). This is a crucial process because it serves as a sink for the increasing emission of CO_2 because of anthropogenic activities, namely the burning of fossil fuel. The carbon pump counteracts global warming. The bulk of the autotrophically fixed CO_2 is immediately respired by heterotrophic microorganisms although there are seasonal variations in the ratio of production to respiration (Sherr and Sherr 1996). These authors noted a ratio > 1 during 3–5 months in winter and spring while during the rest of the year it was < 1 . It is generally assumed that during its voyage to the deep-sea the most labile dissolved organic carbon (DOC) is respired first leaving the more refractory DOC for the greater depths. Arrieta et al. (2015) proposed an alternative explanation. They hypothesized that despite its lability the concentration of organic matter in the deep ocean is too low to yield net energy upon consumption by heterotrophic bacteria.

1.2.4 The Oceanic Habitat

The ocean is probably the largest habitat and cohesive ecosystem in the biosphere. It is not just the vast amount of water but it is also a habitat that is composed of a vast diversity of sub-habitats. Just some of them are mentioned here. The sea surface–

atmosphere boundary is an important but often ignored habitat. The pelagic habitat is subdivided into five major realms. The epipelagic extends to a depth of 200 m and is defined as the layer that receives enough light for photosynthesis. This is followed by the mesopelagic that extends to 1000 m and is also known as the twilight or disphotic zone and the bathypelagic covers the depth between 1000–4000 m. Everything deeper is called the abyssal or abyssopelagic. The ocean floor represents a vast surface because of a complex landscape of ridges, mountains, and valleys that covers an area much larger than the 70% of the surface of the Earth that is covered by the ocean. The ocean floor also contains brine lakes and hydrothermal vents and cold seeps each with their special environmental conditions. Thousands of meters below the ocean floor in what is called the deep subsurface is a habitat of vast numbers (3.5×10^{30} ; Whitman et al. 1998) of specialized microorganisms, barophilic species that grow extremely slow. Then there are the adjacent seas, bays, lagunas, coral reefs, intertidal areas, and estuaries. Last but not least are epiphytic habitats on macroalgae and seagrasses as well as associations with all marine animals (both inside and outside). The atmosphere is another important component that drives the transport of microorganisms. It has been estimated that 4.3×10^{21} microbes are airborne of which 33–68% have a marine origin and are transported over large distances (Mayol et al. 2017). Dust is also transported through the atmosphere and may supply the ocean with nutrients such as iron, which is thought to limit primary production in large areas (Rijkenberg et al. 2008). Diazotrophs are known to have a high iron demand and may depend on such dust depositions (Langlois et al. 2012).

Temperature has often been assigned a critical role in determining the composition and activity of microbial communities (Logares et al. 2020; Sunagawa et al. 2015). However, Gilbert et al. (2012) on the contrary concluded that day-length is a major force for the composition of microbial communities. This conclusion was also reached by Stal and Walsby (2000) who showed that blooms of diazotrophic cyanobacteria in the Baltic Sea would develop independent on temperature but determined by day-length (i.e., the total irradiance received by the system). Temperature may affect the size of the total standing stock biomass because it affects respiration and production differently. Another important effect of temperature is that it determines the density of the water and together with wind force may or may not lead to stratification of the water column. A stratified or mixed water column in the euphotic layer determines the amount of light that is received by the phytoplankton and hence their production.

1.3 What Is a Marine Microorganism?

1.3.1 What Is a Microorganism?

A microorganism is by definition small, too small to be seen by the naked eye, which means smaller than 0.1 mm. Microorganisms comprise the three domains of life we know: bacteria, archaea, and eukarya. The former two are also jointly known as “prokaryotes” because they do not possess a nucleus (karyon; from Greek *káruon*,

which means “nut” or “kernel,” while the prefix “pro” means “before”) (or a cell organelle such as a mitochondrion or chloroplast), while eukaryotes (means with a true (“eu-”) nucleus) do possess a nucleus (and cell organelles). The opposite of the Greek “eu-” would be “dys-,” but introducing the term “diskaryote” would not make much sense. Grouping two domains of life based on lacking a property is probably not a good idea (Pace 2006, 2009). Moreover, phylogenetically, archaea and eukarya are more closely related than each of them to bacteria (Imachi et al. 2020) and this is another argument against the term “prokaryote” for binning the two domains. The term “prokaryote” has been used from the days that it was synonymous to bacteria as being microorganisms without possessing a nucleus and that that property distinguished them from those cells that did possess a nucleus (Stanier and Van Niel 1962). There were only two domains of life: prokaryotes and eukaryotes. With the discovery of archaea and their phylogenetic position in the tree of life, the term “prokaryote” is in fact obsolete. Moreover, the meaning “before the nucleus” is also confusing because neither bacteria nor archaea possess or developed one. Nevertheless, it is still commonly used; not seldom in a sloppy way when only bacteria are referred to and excluding archaea. When archaea were first recognized as a domain, they were initially termed “archaeobacteria” because they were assumed to have evolved first in the extreme conditions of the early Earth. The bacteria were then termed “eubacteria” (meaning “true” bacteria as opposed to the untrue bacteria, the archaeobacteria). Obviously, the term “eubacteria” is also obsolete and should not be used.

Microorganisms are unicellular although some may form multicellular forms but with few exceptions these are all composed of the same undifferentiated cell. Macroorganisms all belong to the eukarya but by far most eukarya are in fact microorganisms. A few bacteria are big, i.e. bigger than 0.1 mm (Schulz and Jørgensen 2001). They are nevertheless considered as microorganisms just because they are bacteria. Viruses are not a domain of life because these biological entities do not multiply independently, which is said to be a property of life. Nevertheless, viruses are important because they are agents that help exchanging genetic material between cells of the same species and even interdomain exchanges have been reported. They also keep in check too successful organisms (killing the winner) and aid the recycling of nutrients in the ecosystem (Thingstad and Lignell 1997). With the discovery of giant viruses with large genomes and with the size of small bacteria as well as with the introduction of advanced sequencing techniques, the border between viruses and cells is fading away (Harris and Hill 2021). Hence, viruses fulfill essential roles in any ecosystem including in the marine environment and in the ocean where they occur in a dazzling high number of up to 10 billion viruses per liter. The marine microbiome cannot be understood without considering viruses.

1.3.2 Do Marine Microorganisms Exist?

Throughout the history of (non-medical) microbiological studies terrestrial (soil) and aquatic microbiology have been treated almost as different disciplines. This has been largely due to historical determined developments in the scientific approach, methodology, and the problem focus (e.g., agriculture). Such a difference did not develop between freshwater- and marine microbiology and therefore it is less obvious to define each of them as a discipline. Microorganisms growing in freshwater may also be found and grow in sea water and vice versa. Many microorganisms are found and may grow in a wide range of aquatic environments. Even terrestrial microorganisms may end up in the ocean through run-off and through air transport (Mayol et al. 2017). For a long time, it was thought that marine fungi do not exist and those that were found were considered to be accidentally deposited there from a terrestrial source and non-growing in the marine environment. There is no doubt that fungi inhabit and grow in the marine environment and fulfill the same ecological role as terrestrial fungi do in terrestrial environments (see Chap. 5). Hence, what is a marine microorganism? MacLeod (1965) stated “. . . the only one which clearly distinguishes them (marine bacteria, sic) from bacteria in other habitats is a capacity to survive and grow in the sea.” Both ZoBell and Rittenberg (1938) and Stanier (1941) considered bacteria marine when obtained from a marine environment and degrade typical marine derived substrates such as chitin and agar-agar, respectively. The former is a major compound of Arthropoda and other marine animals and the latter is derived from marine red algae.

Many marine bacteria require elevated Na^+ for transporting substrates into their cells or to retain intracellular solutes (osmotic balance). However, this seems not to be uniquely the case for marine bacteria because all cells require Na^+ and marine bacteria exhibit a wide variety of tolerances for elevated salt concentrations (MacLeod 1985, 1986). ZoBell (1946) writes: *“Although there are no infallible criteria for the differentiation of marine from non-marine bacteria, most of the bacteria which occur in the open ocean differ in certain respects from those found in non-marine habitats. That is probably because adventitious organisms either fail to perpetuate themselves in the marine environment or else lose their identity in becoming acclimatized thereto.”* Can we agree simply on any microorganism that occurs and thrives in a marine or saline environment and their adjacent environments such as brackish seas, estuaries, bays, and lagunas as MacLeod (1985) proposed.

Most of the ocean is an extremely oligotrophic environment. The concentration of inorganic nutrients as well as of organic matter is low. Many marine pelagic microorganisms (plankton) have adapted to this environment by their small size (Ghai et al. 2013; Schut et al. 1995). The small size provides these microorganisms with a large cell surface area to cell volume ratio which enhances their affinity for the uptake of nutrients and substrate. The most abundant bacteria in the ocean are *Prochlorococcus* and *Pelagibacter* and are among the smallest microbes that exist. These microorganisms encounter their substrate by Brownian diffusion and that explains why these marine microorganisms are in general non-motile because theory predicts that swimming to a nutrient hotspot would be inefficient (Zehr et al. 2017).

However, when motile, marine bacteria swim much faster than freshwater species which can be attributed to the shallow gradients in the sea (see Chap. 2). During diatom blooms and their subsequent collapse chemotactic motile bacteria outcompete the non-motile species (Smriga et al. 2016).

1.3.3 How Many Species of Marine Microorganisms Exist?

In average there may be a billion of microorganisms in a liter of seawater but how many species are there? The number of species in seawater has been estimated from ~1500 (Hagström et al. 2002) to ~20,000 (Sogin et al. 2006) to one million (Curtis et al. 2002) or even tenths or hundreds of millions (Dykhuizen 1998). These large numbers may refer to sequence diversity but that does not make them species (Riley and Lizotte-Waniewski 2009). This makes clear that the number of species is in fact not known nor much is known about the role of this microbial diversity in the ocean. The species concept in microbiology is still a matter of controversy. It is usually based on the level of sequence identity of the 16S rRNA gene as well as a number of phenotypic characteristics. Rosselló-Mora and Amann (2001) wrote in the abstract of their review on the species concept for prokaryotes (sic) “*a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property.*” They refer to it as the “phylogenetic” species concept. However, what to say of 2 phenotypically different strains of picocyanobacteria (*Synechococcus*) of which one is red because of the possession of phycoerythrin and the other is blue-green because it does not produce the red pigment (Haverkamp et al. 2009). The red strain is several times bigger than the green strain. Also, the green strain grows much faster and there are more differences. Would you consider these as two different species? But these two strains have 100% identical 16S ribosomal RNA gene sequences and differ only 2 base pairs in ~1000 bp long internally transcribed spacer (ITS). And there are several other similar cases known from the literature. Obviously, phenotypic characteristics are important and should not be ignored (Margulis 1992). It shows the problem of defining a microbial species only by their 16S rRNA gene sequence, even though it is true that organisms with <98.7% 16S rRNA gene sequence identity can be considered to be different species (Achtman and Wagner 2008). An alternative for delineating microbial species is to compare average nucleotide identity (ANI) and a cut-off of 95% ANI to define a species. Using this criterion would require the whole genome of an organism. Riley and Lizotte-Waniewski (2009) adopted the core genome which encodes the essential housekeeping genes as the basis to define bacterial species. This would still not solve the question of the concept of a microbial species nor allows to answer the question whether it exists or not (Achtman and Wagner 2008). Wilkins (2006) argues that there is a continuum of recombination of genetic information from viruses and bacteria to the sexual recombination of 50% of the two parents as in metazoans and metaphytes. However, the question is whether there is such a continuum because evolutionary forces would prevent this (Achtman

and Wagner 2008). Wilkins (2006), therefore, proposes to combine the recombination and the ecological species concept. Whether this will solve the problem of recognition of a microbial species remains to be seen.

Approximately 10,000 “species” of marine microorganisms have been isolated in culture and described to some extent. It is said that the vast majority (“90–99%”) of species cannot be cultured using standard media and growth conditions. For many years it was known that counting marine microorganisms on agar plates yielded only a fraction of the number that was directly microscopically counted (Jannasch and Jones 1959) or by using fluorescent microscopy of stained bacteria fixed on nucleopore filters (Hobbie et al. 1977). This difference between “culturable” and total bacterial count has become known as “The Great Plate Count Anomaly” (Staley and Konopka 1985). Hence, this majority is only known by their DNA sequence or at best as microscopic images using techniques such as Fluorescent In Situ Hybridization (FISH) and its derived methods. The description, identification, and the growth and metabolic characteristics of these uncultured microorganisms will remain difficult if not impossible unless brought into culture. Similarly, the circumnavigating cruises such as the Global Ocean Sampling (GOS) and TARA Oceans Expedition have revealed many sequences of open reading frames (ORF) that could not be linked to any known gene or function. This again points to a huge hidden diversity and metabolic potential.

1.4 (Some) Milestones of Marine Microbiology

Karl and Proctor (2007) wrote a nice overview on the “Foundations of Microbial Oceanography” and I refer those who would like to have a more extended account on the subject to that paper and the references therein.

Fischer (1894) wrote one of the several monographs that appeared at the end of nineteenth century in several countries and in their own languages that focused specifically on marine microorganisms. This author made the following remark in the introduction of his book written in German “Die Bakterien des Meeres” (Fig. 1.3) (The Bacteria of the Sea) “*Nun war aber, als die Expedition unternommen wurde, über die Bakterien des Meeres noch so gut wie gar nichts bekannt geworden, wenigstens fehlte es damals und fehlt es auch heute noch in der Literatur an systematischen Untersuchungen über die Bakterien des Meeres, ja es finden sich selbst bis auf den heutigen Tag noch nicht einmal Angaben darüber, ob auf hoher See Bakterien überhaupt vorkommen.*” (Fischer 1894). Translated by me: “However, almost nothing was known about marine bacteria when the expedition (1889 plankton expedition in the Atlantic Ocean of the Humboldt Foundation, added by me) took place, at least at that time as well as of today systematic investigations about marine bacteria were lacking in the literature, and yes even of today no reports exist whether or not bacteria are present on the high seas.”

Fischer (1894) reported counts of bacterial colonies on gelatin (supplemented with agar-agar when high temperatures prevented the use of gelatin). The medium was supplemented with NaCl and fish extract. Fischer concluded that salt

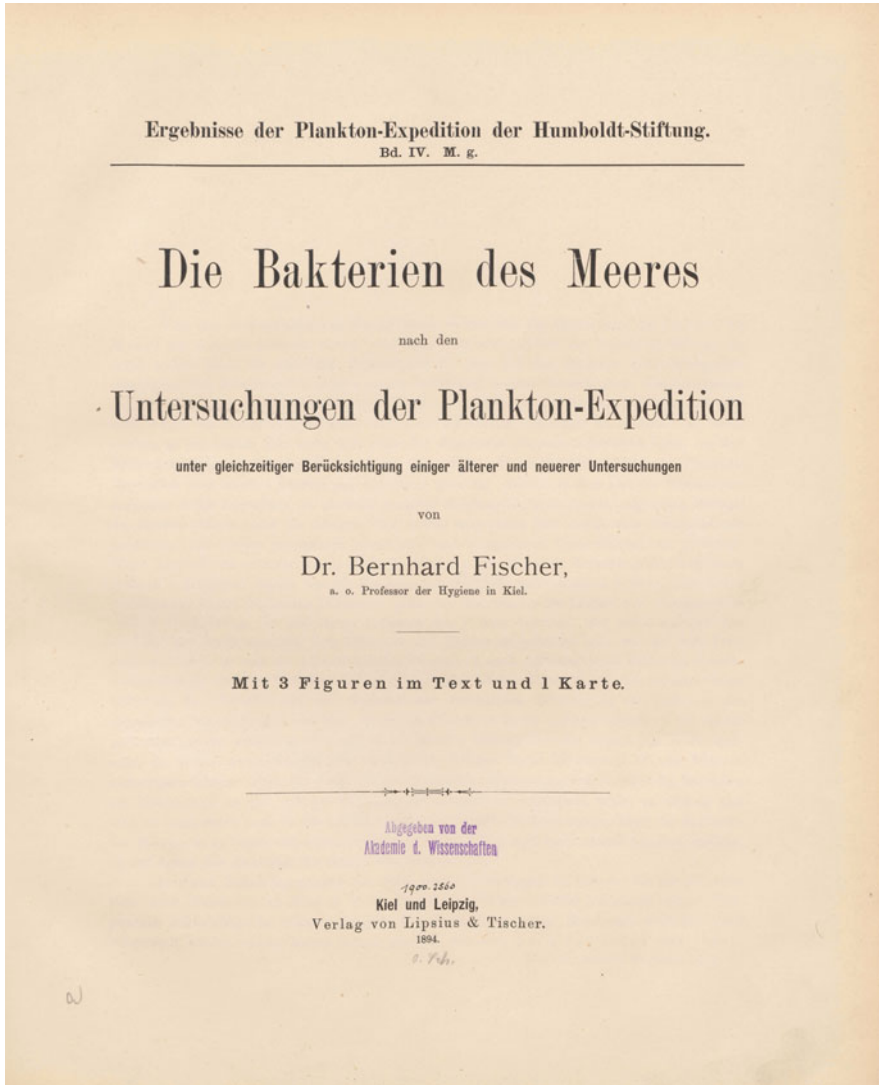


Fig. 1.3 Scan of the title page of B. Fischer's "Die Bakterien des Meeres" of 1894, describing the results of a Plankton expedition in the Atlantic Ocean of the Humboldt foundation in 1889

requirement was a distinguishing characteristic for growth of marine bacteria. Water samples were obtained from the surface, deep water, and from different geographical locations and seasons. The numbers he found were low and rarely exceeded 1000 ml^{-1} . Colony counts from deep water samples were rarely higher than 10 ml^{-1} and not seldom zero, which Fischer attributed to the hydrostatic pressure. He also isolated some marine bacteria into pure culture and frequently reported specifically bioluminescent bacteria. He concluded that marine bacteria exist

everywhere on high seas but that much is unknown and advocated further studies on this subject.

In 1934 A.C. Redfield published a remarkable finding of a constant ratio of N:P of 20 in a wide range of samples taken from different geographical regions of the Atlantic Ocean and from different depths as well as from other oceans (Redfield 1934). It reflected the stoichiometry in marine plankton and its degradation would consequently also be recovered from seawater. Redfield concluded that oceanic plankton was remarkably uniform in their chemical composition. He noted that this composition may be different, for instance, in coastal regions. Redfield also noted the possibility of N₂-binding plankton introducing new nitrogen but only at the expense of phosphorus: “. . . the quantity of nitrate in the sea may be regulated by biological agencies and its absolute value determined by the quantity of phosphate present.” This number of N:P was later adjusted to 16 and became known as the Redfield ratio. The constant stoichiometry was subsequently extended to other major elements and adjustments have been made for specific cases. But ever since the Redfield ratio remained intact and continues to stay an important keystone for biological oceanography.

While “Die Bakterien des Meeres” may be considered as the hesitantly beginning of “Marine Microbiology,” it became the subject of many studies and part of (biological) oceanographic cruises and research institutes during the years to follow and culminating in Claude ZoBell’s seminal monograph “Marine Microbiology” published in 1946 (ZoBell 1946) (Fig. 1.4). As the subtitle of the book indicates, ZoBell’s monograph focused on bacteria and left out all other microorganisms. Between the lines ZoBell was criticized for this even in the foreword of his book written by Selman Waksman and by other contemporaries. Nevertheless, his book is still remarkably actual as a basic text on the subject of marine microorganisms, even though much new knowledge has been obtained since, the basics as presented by Claude ZoBell did not see many fundamental changes and so it stands as an important keystone of marine microbiology.

The binding (“fixation”) of atmospheric dinitrogen (N₂) in the marine cyanobacterium *Trichodesmium thiebautii* in the Sargasso Sea was reported in 1961 by Richard Dugdale and colleagues (Dugdale et al. 1961). Until then, N₂ was considered unimportant in the ocean, even though Redfield (1934) pointed out that the binding of N₂ could contribute to maintain the N:P ratio in marine plankton. Later, this author, therefore, concluded that oceanic primary production could not be limited by nitrogen (Redfield 1958) (which was re-emphasized by Tyrrell in 1999). Dugdale’s report showed for the first time that *Trichodesmium* was able to incorporate the stable isotope ¹⁵N from ¹⁵N₂ and that this process depended on light. Now it is known that the ocean is responsible for ~50% of the global N₂ binding (excluding the contribution of the chemical Haber–Bosch process for the production of fertilizer). Richard Dugdale, David Menzel, and John Ryther’s discovery was the start of an extensive worldwide research of oceanic N₂ binding, which is still ongoing today and that resulted in many exciting discoveries (see Zehr and Capone 2021).

MARINE MICROBIOLOGY

A MONOGRAPH ON HYDROBACTERIOLOGY

BY

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Foreword by SELMAN A. WAKSMAN

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1946

WALTHAM, MASS., U.S.A.

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Fig. 1.4 Scan of the title page of C. ZoBell's "Marine Microbiology." (first print 1946 possessed by L.J. Stal)

In 1974, Pomeroy published a keystone publication in which data from more than a decade of plankton studies were put together and a synthesis was made. Pomeroy (1974) understood that the classical picture of the oceanic food web needed revision. Marine microorganisms produce and recycle organic matter many times before it enters higher trophic levels. This part of the oceanic food web that recycles energy has later been named the “microbial loop” (Azam et al. 1983).

Waterbury et al. (1979) reported the widespread occurrence of small *Synechococcus* spp. containing the red pigment phycoerythrin. Due to the small size (<2 μm) of these chroococcalean cells they were later addressed as picoplankton or picocyanobacteria in case it concerned cyanobacteria. These tiny organisms carry out most of the primary production in the ocean (Platt et al. 1983).

A symposium series on marine microbiology was initiated in Europe since the beginning of the 1980s. The seventh European Marine Microbiology Symposium (EMMS) took place from 17–22 September 2000 in Noordwijkerhout in the Netherlands and was combined with the seventh International Workshop on the Measurement of Microbial Activities in the Carbon Cycle in Aquatic Environments. During this joint meeting it was decided to continue as a combined meeting under the new name “Symposium on Aquatic Microbial Ecology” (SAME) (but keeping the count as by coincidence both meetings were at the SAME sequence). An important reason for this merge was that both meeting series attracted largely the SAME group of scientists. This emphasized that the marine aspect was not a distinguishing characteristic for this group of scientists. They use largely the SAME methods and focus on the SAME fundamental research questions.

Ducklow (1983) argued that small heterotrophic protists actively predate on bacteria and microalgae in the ocean. Subsequently, Hagström et al. (1988) quantified the predation of bacteria in the oligotrophic marine environment by these nanoflagellates. These authors also showed that the carbon that is excreted by the nanoflagellates is returned to the heterotrophic bacteria thereby completing the “microbial loop.”

Waterbury and colleagues discovered in 1985 a marine unicellular cyanobacterium belonging to *Synechococcus* that is capable of swimming (Waterbury et al. 1985). Until then the only motility known among cyanobacteria was gliding, which was used mainly by filamentous species on a solid substrate or along other cyanobacterial trichomes. Swimming appeared to be in the absence of flagella and therefore unique among bacteria. Although the precise mechanism of motility has not yet been elucidated, several genes involved in it have been identified in the genome of this organism and particularly in modification of the cell surface that might play a role in motility of this cyanobacterium (Palenik et al. 2003). This research also revealed marked difference with typical freshwater representatives of the same genus, particularly with regard to transporters and nutrient sources (N, P, Fe) and the authors considered this *Synechococcus* an ecological generalist for which strategy motility may be advantageous.

Shipboard flow cytometry discovered in 1988 the highly abundant cyanobacterium *Prochlorococcus* (Chisholm et al. 1988). This organism was later considered to be the most abundant photosynthetic organism on Earth. Originally, it was thought

to be related to *Prochloron* based on its remarkable pigment composition, which is unusual for cyanobacteria. It contained a divinyl derivative of chlorophyll *a* as well as chlorophyll *b*, the latter typical for the chloroplasts of higher plants and a few other cyanobacteria which were at the time collectively assigned to as prochlorophytes. Another remarkable property of *Prochlorococcus* is the absence of phycoerythrin, which is characteristic for the oceanic picoplanktonic *Synechococcus*, which was discovered a decade earlier.

Since 1989, as the result of a new methodology, it became clear that ocean water contained high numbers of viral particles, while until then viruses were considered to be low in number and importance in aquatic environments (Bergh et al. 1989). Subsequent research revealed that viral abundances could reach as high as ten million per milliliter of seawater and established their role in the recycling of nutrients, in controlling successful microorganisms (“killing the winner”), and generate diversity by horizontal gene transfer (Suttle 2005).

Analysis of the sequences of clone libraries of 16S rRNA was introduced in 1990 in order to investigate the diversity of marine bacterioplankton allowing the culturing-independent study of marine microbial communities (Fuhrman et al. 1992; Giovannoni et al. 1990; Schmidt et al. 1991). Using this approach, Giovannoni and colleagues discovered the highly abundant bacterium SAR11, which became later known as *Pelagibacter* while Fuhrman and colleagues discovered meanwhile abundant marine archaea as component of the oceanic plankton even in cold and well-oxygenated waters. Until then archaea were rather considered to be associated with more extreme environments. Very soon after these important discoveries, phylogenetic stains were developed and used to microscopically visualize marine bacteria in natural seawater samples (Lee et al. 1993).

In 1992, the German Max Planck Society founded the Institute for Marine Microbiology (MPIMM) in Bremen, Germany (founding director was Prof. Bo Barker Jørgensen and co-director Prof. Friedrich Widdel). It may have been the first research institute that is fully dedicated to the study of marine microorganisms. Remarkably, MPIMM is located in the city of Bremen (Germany) rather than directly at the coast. This recalls MacLeod who said in 1985 that marine microbiology can be done far from the sea (MacLeod 1985). At MPIMM marine microbiological research is strongly internationally oriented. Since its foundation, research at MPIMM contributed importantly to new knowledge in the field of marine microbiology.

The European Commission launched the Marine Science and Technology (MAST) Program in 1997. Under the umbrella of MAST a wide variety of research projects were funded many of which focused on the biotechnological use of marine microorganisms.

In 1997, it was recognized that the oligotrophic unproductive ocean behaved as a source of CO₂ because bacterial respiration seemed to exceed primary production in such areas emphasizing the importance of bacterial activity (del Giorgio et al. 1997). However, this conclusion has been challenged by Richard Geider (Geider 1997 and replied by del Giorgio and Cole).

In 2000, metagenomic techniques such as the cloning and sequencing of bacterial artificial chromosomes (BACs) revealed the existence of proteorhodopsin hinting to the widespread existence of photoheterotrophy in the sea (Béjà et al. 2000). Kolber et al. (2001) discovered the widespread presence of bacteriochlorophyll *a* and demonstrated aerobic anoxygenic photosynthesis in the ocean. Subsequently, a variety of aerobic anoxygenic phototrophic bacteria were discovered and isolated and their ecology investigated (Rathgeber et al. 2004).

New investigations in 2002 and sequencing of 18S ribosomal genes of tiny protists (eukaryotic microorganisms) in the ocean revealed a much larger diversity among this neglected group of organisms (Massana et al. 2002).

The Global Ocean Sampling (GOS) expedition (Fig. 1.5) took place from 2004 to 2006, after a successful pilot in the Sargasso Sea in 2003 of which the metagenome was constructed from shotgun sequencing. This revealed a large number of unknown microbial functions in the ocean (Venter et al. 2004). The new high-throughput sequencing technologies that were applied to the GOS samples revealed a much higher microbial diversity than expected at the time and that the ocean contains an extreme large number of microbial genes of which most of them with unknown function. Another Sorcerer II expedition took place from 2007 to 2008 sampling special environments such as polar ice, deep-sea thermal vents, and high saline ponds. In 2009–2010, a Sorcerer expedition took place focusing on the Baltic Sea, the Black Sea, and the Mediterranean Sea, large seas that are mostly disconnected from the ocean. These expeditions circumnavigated the globe and took water samples that were size-fractionated by filtration in order to obtain DNA from different size classes and eventually from viruses and from free DNA in the filtrate. The filters were shipped to the laboratory where DNA was extracted and sequenced and the genetic diversity of the microorganisms was analyzed. Genes and genomes were partly assembled from the fragments using advanced computer algorithms. The genomic databases were enriched with millions of novel genes and genomes most of which from uncultured and hitherto unknown microorganisms of each of three domains of life as well as of marine viruses. This was unprecedented at the time and boosted the knowledge of marine microbiological diversity and its global distribution (Kannan et al. 2007; Rusch et al. 2007; Yooseph et al. 2007). Most of the sequences could not be assigned. A considerable part (15%) of the recruited sequence reads belong to only three groups of widely abundant marine microorganisms: *Pelagibacter*, *Prochlorococcus*, and *Synechococcus*. Much of the discoveries from the GOS databases hint to life strategies and adaptations typical for the oceanic and marine environment. It should be noted that GOS sampled only surface waters and it only unveiled the tip of the iceberg of marine microbial diversity.

The Census of Marine Life (CoML) was a 10-year initiative involving a global network of scientists. It started in 2000 and received initial funding from the Sloan Foundation. Although there were several “field projects” within CoML that focused in part on marine microorganisms, the International Census of Marine Microbes (ICoMM) that was launched as a CoML field project in 2004 was the only one that aimed at making an inventory of all diversity of bacteria, archaea, protists, and

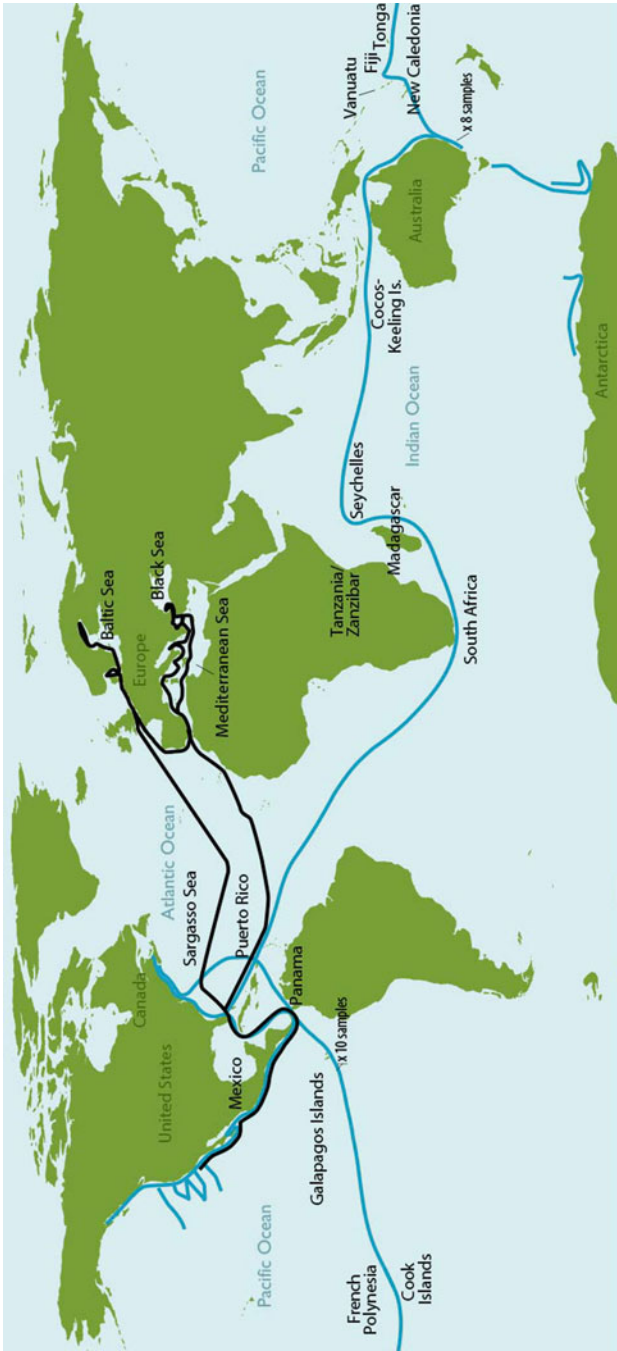


Fig. 1.5 Global Ocean Sampling (GOS) Sorcerer II Expeditions during the years 2003–2010 by the J. Craig Venter Institute. Graph taken from <https://www.jvvi.org/research/gos/#past-voyages> (public domain)

viruses in the ocean. It was the first attempt to sequence massively the microorganisms in a wide variety of marine environments using at the time novel parallel tag sequencing (Sogin et al. 2006). This work revealed a far higher diversity than was anticipated and shed light at the so-called rare biosphere, microorganisms that are present in low abundance (Pedrós-Alió 2006). The technique was subsequently applied on a wide range of samples from a variety of environments (Amaral-Zettler et al. 2010).

Until 2001, it was thought that *Trichodesmium* was the only quantitatively important diazotroph (N_2 binding; living on N_2 as source of nitrogen) in the ocean. This changed with the discovery of Zehr and colleagues that unicellular cyanobacteria were equally important as diazotrophs (Zehr et al. 2001). This discovery was subsequently confirmed and extended by many reports of phylogenetically different groups of unicellular diazotrophs, including obligate symbiotic organisms.

In 2002, it became clear that SAR11 (*Pelagibacter ubique*) dominates the ocean surface waters (Morris et al. 2002) and this organism was brought in culture using a dilution to extinction method, which was an important breakthrough (Rappé et al. 2002). At the same time innovative methods became available to isolate marine microorganisms among others by encapsulating them in gel microdroplets followed by flow cytometric detection and sorting (Zengler et al. 2002). During the years following 2002, many exciting discoveries were made of new marine microorganisms and metabolic pathways. Anaerobic ammonium-oxidizing (Anammox) bacteria belonging to the Planctomycetes were discovered in the Black Sea and from marine sediments (Jetten et al. 2003; Kuypers et al. 2003; Thamdrup and Dalsgaard 2002) and a meso- to psychrophilic marine aerobic ammonium-oxidizing archaea was isolated (Könneke et al. 2005), which subsequently became known as Thaumarchaeota and are widespread in the ocean (Brochier-Armanet et al. 2008; Wuchter et al. 2006).

The Gordon and Betty Moore Foundation launched in 2004 the Marine Microbiology Initiative, which supported an enormous amount of scientific research. It ended in 2021 and has been one of the longest research programs in marine microbiology with an investment of more than USD250 million. This program also supported the sequencing of the genomes of a large number of marine microorganisms that were isolated and brought into culture in the laboratory, including several cyanobacteria.

The Tara Oceans Expedition project was launched in 2009 and collected 35,000 plankton samples from all major oceans and at depths up to 2000 m (Sunagawa et al. 2020; Zhang and Ning 2015). The project was a true milestone in marine microbiology. Tara Oceans was led by the European Molecular Biology Laboratory (EMBL) and ended in 2013. The analysis of the genomics data resulted in a wealth of new knowledge on the functioning of the marine microbiome. Among many other discoveries Tara Oceans Expedition data revealed that marine microbial community composition seemed to be driven by temperature and the remarkable finding that three quarters of the ocean microbial core functionality is shared with that of the human gut (Logares et al. 2020; Sunagawa et al. 2015, 2020) (see Chap. 8). How special is the marine microbiome?

The 8 months lasting Spanish Malaspina expedition took place from December 2010 to July 2011 and circumvented the globe with the *RV Hespérides*. This expedition was named after the famous late eighteenth century scientific expedition led by Alejandro Malaspina. The 2010 Malaspina expedition included also microorganisms and particularly focused on deep water.

The European Commission funded the project “Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications” (MaCuMBA) (FP7 grant agreement 311,975), which ran from 2012 to 2016 and comprised a total budget of more than 12 million euros and joined 23 participants from 11 countries. This project was designed by the awareness that marine microorganisms comprise an incredible diversity that is mostly unknown. These microorganisms form an almost untapped resource of biotechnological potential that may help mitigate climate change, control disease, and generate alternative energy sources. However, to access this resource culturing of these microorganisms is essential. The challenge of this project was to increase the success rate of isolating and culturing novel marine microorganisms. The project developed automated high-throughput culturing techniques (robotics) and increased the efficiency of growth by introducing co-culturing and nature-mimicking techniques as well as by genetic strategies. MaCuMBA noted the importance of culture collections in which isolated microorganisms are identified, stored, and maintained, and searchable by the public. This is still an underestimated challenge. The German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) plays an important role in the collection of marine microorganisms. MaCuMBA led the initiative for the first edition of “The Marine Microbiome” (Stal and Cretoiu 2016). MaCuMBA formed a strategic alliance with two other large EU projects PharmaSea (which used the marine microbiome to discover new pharmaceuticals; see Chap. 17) and MicroB3.

Ocean sampling day (OSD) (Kopf et al. 2015) was an initiative of the European Union project Micro B3 (Marine Microbial Biodiversity, Bioinformatics, Biotechnology) and started in 2014. In 2016, after the Micro B3 project ended OSD was taken over by the Institute of Marine Biology, Biotechnology, and Aquaculture of the Hellenic Center for Marine Research in Heraklion, Crete. The idea of this sampling day was to obtain a worldwide inventory of the marine microbial biodiversity by extracting and sequencing nucleic acids from the samples taken at one single day (solstice, 21st July) together with their metadata. A worldwide consortium was put into place to coordinate this sampling which is still going on. An important aspect on this sampling effort was that sampling, sample handling, metadata reporting, and data processing were done according to a compulsory protocol resulting in a true standardized dataset.

1.5 Selected Aspects of the Marine Microbial System

1.5.1 The Redfield Ratio

Redfield observed a constant average ratio of C:N:P of 106:16:1 in the ocean (Redfield 1934, 1958). The Redfield ratio works for a large system as the ocean but less well for small lakes (Hecky et al. 1993). This has been attributed to the low water residence times and a greater contribution of continental watershed (They et al. 2017). The water residence time of the ocean is ~ 1000 years and exceeds the residence times of nitrate and phosphate which are an order of magnitude shorter (Falkowski and Davis 2004). This explains that the ratio of nitrate and phosphate in the ocean (Fig. 1.6) equals more or less the average composition of the

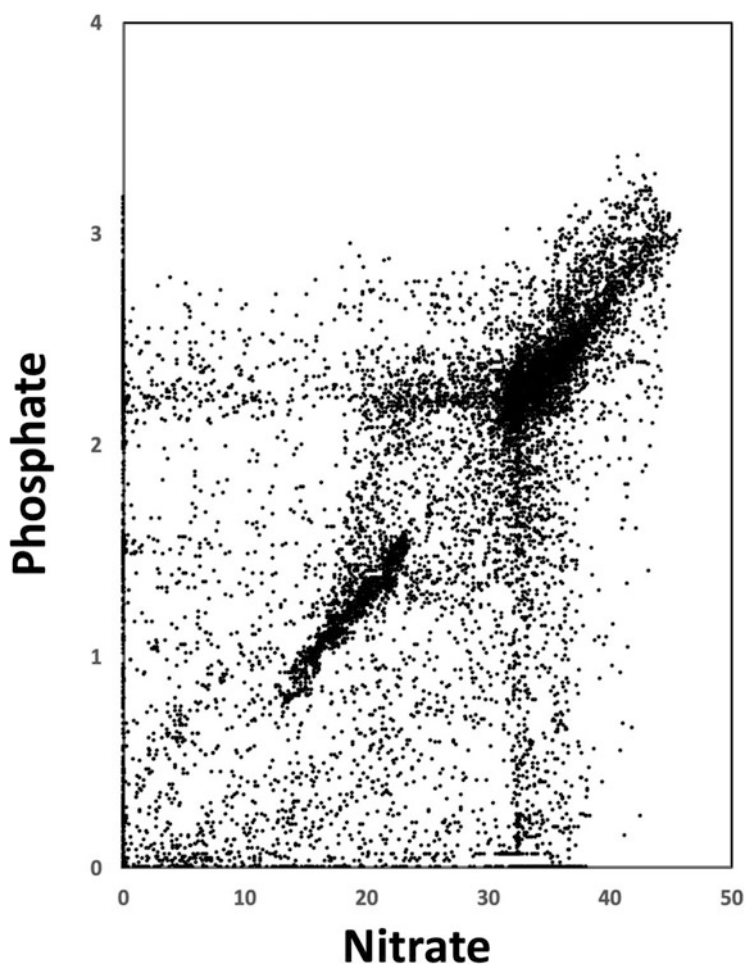


Fig. 1.6 $[\text{NO}_3^-]$ versus $[\text{PO}_4^{3-}]$ ($\mu\text{mol kg}^{-1}$) scatter plot using data from GEOSECS global data set (<https://iridl.ldeo.columbia.edu/SOURCES/.GEOSECS/>)

phytoplankton. The smaller the system the greater is the deviation from the Redfield ratio. Therefore, an important aspect of marine microbiology is a matter of scale: the ocean is the largest continuous (eco)system. Also, in the ocean there is variability of the elemental ratios depending on the latitude. Martiny et al. (2013) reported C:N:P ratios varying from 195:28:1, 137:18:1, to 78:13:1 in the warm nutrient-depleted low-latitude gyres, warm nutrient-rich upwelling zones, and cold nutrient-rich high latitudes, respectively. A low N:P ratio has been taken as an indicator for the loss of nitrogen through denitrification, while a high ratio represents the binding of dinitrogen (N_2) (“nitrogen fixation”) (Gruber and Sarmiento 1997). These processes can be quantified on an ocean-based scale by the tracer N^* (Gruber and Sarmiento 1997). Using a modeling study, Tyrrell (1999) found that phosphate would eventually be the ultimate limiting factor for primary production in the ocean because the binding of dinitrogen by diazotrophic plankton would always alleviate nitrogen limitation. Falkowski (1997) noted that on a geological scale, phosphate could not be responsible for the variations of primary production due to its more or less constant supply by continental weathering and fluvial discharge. He argues that iron availability controls nitrogen fixation and that small changes in the ratio of N_2 fixation to denitrification may control primary production and carbon export which is limited by nitrate.

There are various processes that disobey Redfield. It is known that fast-growing organisms have lower N:P ratios than slow-growing organisms (Koeve and Kähler 2010). This is because fast-growing organisms need a higher number of the phosphate-rich ribosomes. Using an ecosystem model, Mills and Arrigo (2010) showed that fast-growing organisms depleted the phosphate thereby decreasing the phosphate available for diazotrophs. For the same reason, slow-growing organisms stimulated nitrogen fixation. If these non-Redfield uptake processes are ignored, estimates of oceanic nitrogen fixation may be wrong. Mills and Arrigo (2010) conceive that the relative composition of fast- and slow-growing phytoplankton controls nitrogen fixation in the ocean. Moreover, while, on the one hand, cyanobacteria may exhibit N:P ratios that vary over one order of magnitude, diatoms, on the other hand, are characterized by low N:P ratios (Planavsky 2014). Hence, the phytoplankton composition controls the global ratio of the nitrate to phosphate concentration in the ocean explaining that locally and temporally large deviations from the Redfield ratio are observed.

Staal et al. (2007) measured N_2 fixation activity along a transect following the West-African coastline in the southern hemisphere from latitude N4.698; longitude E-18.497 to latitude N-19.200; longitude E5.744. North of latitude N-10.972; longitude E1.321 nitrogenase activity was detected and south of it not. Along the whole transect N:P ratios were low and decreasing from north to south. The average N:P in the area where nitrogen fixation took place was 5.5 (with the highest value 14.1) and in the area negative for nitrogen fixation this ratio was on average 1. The phototrophic biomass (measured as chlorophyll *a*) in the area positive for nitrogen fixation (average 0.187 mg L^{-1}) was twice as high as in the area without nitrogen fixation (average 0.098 mg L^{-1}). The phosphate concentration in that area was on average 196 nmol L^{-1} and about 4 times higher than in the area positive for

nitrogen fixation (47 nmol L^{-1}). The concentration of silicate behaved opposite and was twice as high in the area positive for than nitrogen fixation compared to the area negative for it (1209 vs 707 nmol L^{-1}). The concentrations of combined nitrogen were equally low throughout the transect. These data were interpreted as that primary production was nitrogen limited in the southern part of the transect where no nitrogen fixation took place. The draw-down of silicate hints to diatoms as an important component of the phytoplankton. It is known that areas dominated by diatoms are characterized by low N:P ratios (Planavsky 2014). Also, the low levels of combined nitrogen made that a substantial part of the phosphate could not be used resulting in the observed low N:P ratios. The limitation in the northern part of the transect where nitrogen fixation took place cannot be determined with certainty. Most likely it was phosphate but it might also have been the availability of iron, which could have limited nitrogen fixation (and thus primary production still nitrogen limited) or the lower temperature might have limited nitrogen fixation (Stal 2009). The average surface water temperature in the area positive for nitrogen fixation was $27.9 \text{ }^\circ\text{C}$, while it was $24.9 \text{ }^\circ\text{C}$ in the area negative for nitrogen fixation. Temperature may be a crucial factor for nitrogen fixation in the ocean (Stal 2009).

1.5.2 Nitrogen Fixation

Nitrogen is after carbon quantitatively the second-most abundant element in structural cell material where it occurs in its reduced (amino) form and represents about 10% of the dry weight biomass. Organisms obtain nitrogen mostly as organic nitrogen, ammonium, or nitrate and nitrite, which are commonly referred to as “bioavailable” nitrogen. However, the largest source of nitrogen is dinitrogen gas (N_2), which is in fact “bioavailable” as well, but for a limited group of selected bacteria and a few archaea (and not eukarya!, which can use it only in symbiosis with a specialized bacterium, when ignoring *Homo sapiens*, who with its industrial production of fertilizer—by the Haber–Bosch process—is the main nitrogen fixer and responsible for about 50% of the total global nitrogen fixation!). All these microorganisms capable of using N_2 as a source of nitrogen possess nitrogenase, a highly oxygen-sensitive enzyme, which is capable of breaking and reducing the strong double bond between the two nitrogen atoms. Leaving out the Haber–Bosch process, about half of the global nitrogen binding occurs in the ocean (Canfield et al. 2010), where it supplies “new” nitrogen to drive primary production. It counteracts the loss of combined “bioavailable” nitrogen by denitrification although it is unclear whether these two processes are in balance. Estimates indicate that the loss may exceed the gain, which has led to the idea that the modern ocean might be losing nitrogen (ocean is a “black hole” for nitrogen). However, the application of the N^* tracer (Gruber and Sarmiento 1997) indicated that the fixation of N_2 had to be higher than estimated based on measurements of the known diazotrophs. Subsequently, many new diazotrophs were discovered in the ocean and estimates of “new” nitrogen input are going up (Zehr and Capone 2021).

Until 1961, N₂ fixation was thought to be unimportant in the ocean. Dugdale et al. (1961) discovered that *Trichodesmium thiebautii* fixed dinitrogen in the Sargasso Sea. *Trichodesmium* is a filamentous non-heterocystous cyanobacterium that forms surface blooms in the tropical and subtropical ocean. Subsequently, a variety of unicellular diazotrophic cyanobacteria were found (Moisander et al. 2010; Zehr et al. 2001). A wide range of diazotrophic cyanobacteria live in symbiosis with microalgae, seagrasses, and corals, including the remarkable anoxygenic, photosystem-2-lacking, and non-CO₂ fixing *Candidatus Atelocyanobacterium thalassa* (Foster and Zehr 2019; Thompson et al. 2012; Zehr et al. 2008, 2016). There are several reports of marine diazotrophs other than cyanobacteria but it is uncertain to what extent they contribute to the import of “new” nitrogen in the ocean (Farnelid et al. 2011; Langlois et al. 2015; Moisander et al. 2014). The detection of *nif* genes belonging to chemotrophic microorganisms and even their expression does not necessarily translate into N₂ fixation. Moreover, such genes have even been found in a cyanobacterium (Bolhuis et al. 2010)!

Cyanobacteria are responsible for the import of the “new” nitrogen into the ocean and thereby driving primary production. Hence, cyanobacteria support the ocean food web and are also key to CO₂ fixation and crucial for climate and global change. Cyanobacteria are oxygenic photoautotrophs and therefore the diazotrophs among them must have ways to prevent the inactivation of the oxygen-sensitive nitrogenase. Terrestrial and freshwater diazotrophic cyanobacteria are filamentous and differentiate special cells, the heterocyst, which is the site of nitrogen fixation in those organisms. In the ocean there are no free-living heterocystous cyanobacteria. They appear in some symbiotic associations or on sandy or rocky shores and beaches, which will not be considered here. The heterocyst lacks the oxygenic photosystem 2 (quite like *Candidatus Atelocyanobacterium thalassa*) and possesses a special cell wall that serves as a gas diffusion barrier and limits the rate by which oxygen enters the cell. This oxygen is scavenged by the respiratory system and keeps the intracellular oxygen partial pressure sufficient low to prevent nitrogenase inactivation. The gas diffusion is a trade-off between sufficient entry of N₂ to satisfy nitrogen fixation and the highest entry of O₂ that still can be scavenged by respiration. The solubility of O₂ in water decreases with increasing temperature with approximately the same quotient as gas diffusion rate increases, which therefore counteracts each other. However, metabolic processes vary with temperature according to a $Q_{10} \sim 2$, which means a doubling or halving of the rate with each 10 °C increase or decrease, respectively. That means that in cold water the heterocyst cell wall must be a stronger diffusion barrier (“thicker” cell wall) because respiration will be slower and less O₂ can be scavenged while in warm water the opposite is the case. Staal et al. (2003) showed that this was the case and demonstrated that *Trichodesmium* would not need such a special cell wall, also aided by the lower solubility of O₂ in the warm (sub)tropical ocean. Because *Trichodesmium* fixes N₂ during the daytime it still needs anoxygenic cells. These cells exist and have been termed diazocytes (Bergman et al. 2013). The unicellular diazotrophic cyanobacteria solved this problem of the incompatibility of oxygenic photosynthesis and nitrogen fixation by carrying out the latter during the night. The gas diffusion and respiratory capacity

still requires a minimum cell size ($\sim 5 \mu\text{m}$) in order to keep the intracellular level of O_2 sufficient low. *Candidatus Atelocyanobacterium thalassa* is smaller and fixes N_2 during the day but does so in symbiosis which apparently makes the situation much different. Also, benthic systems such as marine cyanobacterial mats that occur worldwide in coastal intertidal areas and all of which are diazotrophic behave different from the oceanic planktonic system (Severin and Stal 2008). While chemotrophic bacteria are all too small to be able to keep their cells close to anoxic in a fully oxygenated environment, they are, therefore, unlikely to be able to fix N_2 . However, this might be different in oxygen minimum zones (OMZs) or in aggregates (e.g., marine snow) where chemotrophic diazotrophs may indeed contribute to the binding of N_2 (Paulmier and Ruiz-Pino 2009; Schunck et al. 2013; Shanks and Reeder 1993).

While there is a reasonably good explanation why nitrogen fixation seems to be restricted to the euphotic surface waters of the warmer areas of the ocean it is an enigma why heterocystous cyanobacteria do not seem to have this function in the temperate or cold ocean. Heterocystous cyanobacteria form blooms in freshwater lakes irrespective of their geographic location. The brackish Baltic Sea is exhibiting massive blooms of heterocystous cyanobacteria, notably *Nodularia*, *Aphanizomenon*, and *Dolichospermum* (formerly *Anabaena*). The Baltic Sea exhibits a salinity gradient from freshwater in the northern Bothnian Gulf to full seawater salinity in the Kattegat and Skagerrak toward the North Sea. Diazotrophic blooms of the heterocystous cyanobacteria suddenly stop in the Bornholm Sea at a salinity of $\sim 9\text{‰}$ (Stal et al. 1999). These authors speculated that the high sulfate concentration may prevent nitrogen fixation rather than NaCl, e.g., by depleting reducing equivalents through sulfate reduction. Although this may be part of the explanation the absence of heterocystous cyanobacteria in the temperate and cold ocean is still an unresolved question.

1.5.3 Adaptation to Salt

While NaCl is the most important component that contributes to the salinity of seawater, it might not be the most important one that determines the microbiology of seawater compared to freshwater. In terms of salinity, freshwater and seawater are not too much different (compared to hypersaline waters) and a whole range of salinities exists between freshwater and full-salinity seawater (or even a bit higher such as in the Mediterranean) but the relative ionic composition is the same in that range. The difference of salinity would probably not pose a serious problem for many microorganisms. Seawater may even be closer to a physiological solution than freshwater. Environments with salinities far beyond seawater are called hypersaline and are really challenging environments for any organism. Culturing hypersaline microorganisms is challenging. It is not just the concentration of NaCl. The composition varies greatly among the different hypersaline ecosystems worldwide and the actual ionic composition of a particular saline water may be critical for the growth of a microorganism.

Cells need a certain turgor (osmotic or hydrostatic pressure) to be able to carry out their cellular functions. Water can freely pass the cytoplasmic membrane and the cell has no means to actively control this water transport in order to regulate the turgor pressure. The only way is to regulate the cytoplasmic solute concentration in response to hyper- or hypoosmotic stress (Bremer and Krämer 2019). When the salt concentration in the surrounding environment increases the intracellular solute concentration must go up. In order to counteract the osmotic value of the surroundings, the cell will accumulate the so-called osmo-protectants or osmolytes (and several other synonyms). These are small molecules that need to be highly soluble in order to reach sufficient high concentrations. Another important requirement of an osmolyte is that they do not interfere with the cellular metabolism, i.e. they must be compatible. Therefore, they are also called “compatible solutes.” There is a whole range of such compounds in microorganisms and they are generally involved in any situation of low water potential (high salinity, drought) and (low) temperature. An important role is to help folding of proteins and improve their stability and hydration level to allow their function. Microorganisms may employ a “salt-in” or “salt-out” strategy. The first is used by some halophilic microorganisms, who are adapted to high intracellular concentrations of salt (KCl). Most microorganisms pump the salt out of the cell while synthesizing or taking up from the environment organic compatible solutes. In the constant pelagic oceanic environment microorganisms are not exposed to great salinity changes and therefore do not need to respond to them. This is different, for instance, in coastal tidal mudflats or sandy beaches, where microorganisms are exposed to large fluctuations of salinity. For instance, while osmotically adjusted to seawater a benthic microorganism inhabiting a tidal environment needs to respond quickly to a rain shower by releasing or degrading its osmoprotectant otherwise the cell may lyse due to the influx of water (Kirsch et al. 2019). It also needs to take up or synthesize osmoprotectant if the opposite situation takes place. Upon a hyperosmotic shock, Na^+ that enters the cell is actively pumped out by an antiporter transporter in the cytoplasmic membrane and subsequently replaced by K^+ , which is subsequently replaced by the organic osmolyte that is synthesized in response of the salt shock. Organic osmolytes in many microorganisms may also be used as a storage compound and metabolized in times of energy shortage. They may temporarily be exchanged for inorganic ions.

Compatible solutes that serve as osmoprotectant can be subdivided into three major groups: sugars (such as the disaccharides sucrose and trehalose), polyols (e.g., glycerol), heterosides (e.g., glucosyl glycerol), and amino acids and derivatives thereof (e.g., ectoine, betaine, proline, glutamate) (Bremer and Krämer 2019; Kirsch et al. 2019). Sucrose is often found as a compatible solute at low salinities while another disaccharide, trehalose, is more frequently found at intermediate salinities and glucosyl glycerol is typical for full marine salinity. At salinities above normal seawater glycine betaine, ectoine, and glycerol are found. There are many other derivatives of these compounds and amino acids (proline) that are used as compatible solutes in microorganisms (Bremer and Krämer 2019; Kirsch et al. 2019; Kumar et al. 2020). Certain marine microalgae produce dimethyl sulfoniopropionate

(DMSP), which upon release is converted to dimethylsulfide (DMS) that has been thought to play a role in a climate feedback.

1.5.4 Sulfate

An important component of seawater is sulfate (SO_4^{2-}) that is present in a concentration of 28 mM. This high concentration has several consequences. Even though sulfur is not a major constituent of cellular biomass, it is a component for some essential amino acids without which proteins cannot be synthesized. In the marine environment sulfur will not become limiting as is sometimes the case in freshwater. Under low oxygen conditions such as in oxygen minimum zones (OMZs), marine aggregates (marine snow), marine sediments, and cold seeps, sulfate may become the preferred terminal electron acceptor by sulfate-reducing bacteria. These bacteria reduce sulfate to the extremely toxic sulfide that also reacts with oxygen and leads to hypoxia, which has led to the so-called dead-zones in coastal waters and sediments (Middelburg and Levin 2009). They are not really “dead” because they are teeming with anaerobic microorganisms. The anaerobic respiration by sulfate-reducing bacteria is especially important in marine systems whereas methanogenic archaea fulfill this role in anoxic freshwater systems. Nevertheless, the latter are certainly also present in selected marine systems thriving on substrates not used by sulfate-reducing bacteria. The oxidation of methane emitted from cold seeps on the ocean floor is carried out by aggregates of methanogenic archaea and sulfate-reducing bacteria (Boetius et al. 2000).

Sulfur may also be incorporated in certain phytoplankton species in the form of dimethyl sulfoniopropionate (DMSP) where it serves a role as osmoprotectant. Upon release (e.g., through lysis or grazing), DMSP is converted into the gas dimethyl sulfide (DMS) which may be transported to the atmosphere and conceived to play a role in climate feedback but also transports sulfur to the continents.

The molybdate ion is a structural analog of sulfate and a co-factor in certain enzymes such as nitrogenase and nitrate reductase. It has been hypothesized that the high sulfate concentration in seawater would complicate the uptake of molybdate and thereby the processes depending on it. However, in the light of the abundant presence of diazotrophs and phytoplankton depending on nitrate as the source of nitrogen, this scenario does not seem plausible. Also, molybdate may be taken up by designated transporters that do not compete with sulfate. The high sulfate concentration may nevertheless prevent the proliferation of free-living heterocystous cyanobacteria in the ocean (Howarth and Cole 1985; Stal 2009; Stal et al. 1999).

1.5.5 Freshwater- and Marine Microbiomes: What Are the Boundaries?

The limiting nutrient in temperate marine waters is often nitrogen, while in freshwater lakes is often phosphate. Blomqvist et al. (2005) attributed this difference to

salinity, more precisely to the high sulfate concentration of seawater (28 mM). Sulfide produced by sulfate-reducing bacteria scavenges iron, which will precipitate as FeS. Upon oxygenation the ferric iron will combine with phosphate. While in anoxic marine systems the ratio of dissolved iron and phosphate is such that there will be phosphate left after the iron phosphate precipitated, this is not the case in freshwater. The residual phosphate in the marine system results in a nitrogen limitation while in a freshwater system phosphate will be the limiting nutrient for primary production.

Dupont et al. (2014) concluded that the differences between the freshwater- and marine microbiomes can be found in the core metabolic functions and pathways of microorganisms. These authors observed large differences in a variety of central metabolic processes. The genomic information of these central metabolic pathways hint at an early differentiation of freshwater and marine microorganisms. These adaptations determined whether or not a microorganism is able to thrive at a certain salinity. Salinity is, therefore, the main driver of microbial community composition. Other environmental factors determining the microbial community composition and distinguishing freshwater- and marine microbiomes were N:P ratio and total phosphorus. The boundary between freshwater- and marine environments appears to be strict and difficult to cross despite the high rates of dispersal and large population sizes of their microbial inhabitants (Logares et al. 2009). These observations describe the boundary between freshwater- and marine environments and the phylogenetic split between their microbiomes but do not explain them. Why does it seem so difficult to migrate and adapt in either direction?

1.6 On a Personal Note: How Did I Become a Marine Microbiologist

At the time of writing this I have been retired from science for more than 3 years after working in the field for approximately 40 years. Thus, I thought it might be a good idea to also add a bit of a personal account on what I think is so special about this discipline of Marine Microbiology and how I got involved in it.

I studied Biology at the University of Groningen in The Netherlands from 1972 to 1978 and specialized in Microbiology and Molecular Genetics. I was taught microbiology by Prof. Hans Veldkamp (1923–2002) who was the founder and first Head of the Department of Microbiology at the University of Groningen in 1963. He belonged to the Delft School of Microbiology where he studied with Prof. Albert Jan Kluyver and later worked with Prof. Cornelis Bernardus van Niel at Hopkins Marine Station at Pacific Grove in California. Prof. Veldkamp established the field of general microbiology at Groningen University in the spirit of these founding fathers of microbial ecology. During those days the Department of Microbiology at Groningen University had three specialized working groups: Microbial Ecology (led by Prof. Veldkamp), Microbial Physiology (led by Prof. Wim Harder (1939–2008)), and Molecular Microbiology (led by Prof. Wil Konings (1937–2014)). In the second year of my biology studies, I decided to take the course

General Microbiology with a series of morning lectures that covered the field of Microbiology into great depth and were backed up by Veldkamp's lecture notes and two text books: Prof. Hans Schlegel's *Allgemeine Mikrobiologie* (in German; later translated in English: *General Microbiology*) and Prof. Roger Stanier's *General Microbiology* (together with Doudoroff and Adelberg). In the afternoon, there were hands-on practical lessons. These were focused on the enrichment of various microorganisms from natural samples, their isolation and purification to pure cultures as well as carrying out a range of physiological tests in order to identify the organism. This was well before the time of DNA-based identification of microorganisms. In the third year, I decided to take an extended course in Microbial Ecology which was offered by Prof. Veldkamp and two other staff: Dr. Gijs Kuenen (became later professor microbiology at Delft University) and Dr. Hans van Gernerden (1936–2006). This course was offered for the first time and was strongly modeled to the scientific research of the Microbial Ecology group. Their research was focused on the growth and behavior of microorganisms under sub-maximum growth rates and their competition with other microorganisms. At the time most of the microbiological research was done using the so-called batch cultures. In batch cultures all media components are present in excess. Microorganisms inoculated in this medium would, after a so-called lag phase, in which growth is sub-optimal, grow exponentially, i.e., at its maximum growth rate under the given conditions, until it runs out of its growth substrate and/or when the accumulation of metabolites exuded into the medium would inhibit further growth. The culture is said to enter its stationary phase, which eventually will be followed by a collapse of the culture and the death of the microorganisms in it. The research of the Microbial Ecology group was based on the idea that growth as it happens in a batch culture would rarely occur under natural conditions. Under natural conditions there would be a growth-limiting substrate and its concentration would be low and not supporting the maximum specific growth rate of the organism (compare the Michaelis–Menten model). In order to overcome the limitation of batch cultivation, the Microbial Ecology group adopted the chemostat as their culturing device and even as a device to enrich for particular microorganisms. This culturing technique was at the time quite revolutionary, even though its theory was already described in the fifties of the previous century by the scientists Monod (Monod 1949) and its application by Herbert (Herbert et al. 1956). Instead of a closed culture system such as batch culturing, the chemostat is an open continuous culture system. Sterile growth medium in which one component is the growth-limiting substrate (e.g., the source of energy or a nutrient) is pumped at a constant rate in the growth vessel. The concentration of the growth-limiting substrate will determine the eventual cell number or microbial biomass in the growth container. The volume of the growth container is kept constant by means of an overflow of culture into a waste container. The culture in the growth container needs to be well mixed. Often the pH has to be maintained at a specified value by means of an automatic titration system and depending on the cultured organism and required conditions, other conditions (e.g., the level of dissolved oxygen) need to be controlled and maintained at certain set of values. Theory predicts that at steady state the growth rate of the

microorganisms equals the dilution rate imposed by the medium pump and all conditions (chemical composition of the culture; biomass/cell number) stay constant (chemostat). Hence, by simply changing the pumping rate of the medium supply, the growth rate of the cultured microorganism could be imposed from very low growth rates to close to the maximum specific growth rate. When using the chemostat as a device to enrich and isolate a microorganism it is inoculated with a natural sample. The theory predicts that the organism with the best affinity for the limiting growth substrate of the medium will become dominant and outcompete all other microorganisms in the chemostat at steady state (usually considered to be reached after ~5 volume changes). Of course, the reality was often less simple but nevertheless many microorganisms have been isolated into pure culture using this technique, while batch enrichments would always have led to the isolation of the organism with the highest growth rate in that medium and set of conditions. It was said that the continuous culture was named after the continuous attention that was required by its operator and that this has scared off many and kept the batch culture as a popular technique in microbiology.

The technique of the continuous culture was also used for the study of phototrophic microorganisms. Dr. Hans van Gernerden used it for the growth of anoxygenic purple and green sulfur bacteria. In those cultures, the source of energy (light) was supplied independently from the medium pump but the growth-limiting substrate could be a component of the growth medium such as the electron donor sulfide. Prof. Luuc Mur (University of Amsterdam) introduced the continuous culture of oxygenic phototrophs such as the eukaryotic microalgae and cyanobacteria. By choosing light as the growth-limiting factor, the chemostat was turned into a photostat (in which the average photon flux density in the culture vessel was kept constant), while the growth rate was still imposed by the medium pump rate. In steady state, the density of the culture is determined by the average photon density in the culture. However, these oxygenic phototrophs could also be grown as a chemostat by choosing nutrients such as phosphate or nitrate as the growth-limiting substrate. Apart from studying the behavior of these microorganisms at sub-maximum growth rates and at different growth limitations, these continuous cultures were also excellent tools to study the competition of different species of microorganisms. Prof. Jef Huisman (the successor of Prof. Mur at the University of Amsterdam) has brought this type of investigations to a higher level and increased our understanding of competition, collaboration, and co-existence of different microorganisms tremendously. In particular, these experiments shed light into the “paradox of the plankton” as was formulated by Hutchinson (1961). The paradox is that so many phytoplankton species co-exist in seemingly homogeneous aquatic environments freshwater as well as marine, while under such conditions one would expect that only one or a few would outcompete all others.

After finishing my Master’s in Biology, I was offered the possibility to move to marine microbiology by joining the geomicrobiologist Prof. Wolfgang E. Krumbein (1937–2021) at the Carl von Ossietzky University in Oldenburg, Germany. With him I started a project on dinitrogen fixation by marine benthic cyanobacteria that built microbial mats in coastal intertidal sediments. Through his inspiring

enthusiasm I became interested in geology, geomicrobiology, and biogeochemistry and learned that microorganisms and their activities are instrumental in many geological processes throughout the history of planet Earth. Sadly, Wolfgang Krumbein passed away on 4th April 2021.

While working on my research project on dinitrogen fixation in microbial mats of the desert island Mellum, belonging to the barrier islands of the southern North Sea, I was attracted to the wonderful layered structure of those microbial mats that developed on the intertidal sand flats, characterized by a sediment of fine sand and some silt. They showed a nicely lamination of the green cyanobacteria and purple sulfur bacteria underneath. This was known from the German literature as “Farbstreifen-Sandwatt,” which means so much as colored striped sand. (“Watt” is not translated in English because it is a proper name of that sea (Wattenmeer in German, Waddensee in Dutch, Vadehavet in Danish) and the German word “Watt” refers to the intertidal sand- or mudflats that are exposed at low tide. Despite several efforts, the German concept of “Farbstreifen-Sandwatt” did not make it in the English literature, nor did any English synonym such as color-striped sand and this and similar systems are generally addressed to as microbial mats.

I went to my former Microbiology Department at Groningen University and told them enthusiastically about this unique microbial ecosystem. Dr. Hans van Gernerden was a microbial ecologist studying purple sulfur bacteria and other anoxygenic phototrophs doing competition experiments in chemostats. His model organisms until then were derived from freshwater lakes. He became enthusiastic about the microbial mat as a model system and I invited him on our next field trip to the desert North Sea island Mellum (while we would normally organize a boat trip or get a lift of the research vessel “Senckenberg” of the same-named institute in Wilhelmshaven, on that particular expedition we could reach the island only by helicopter). Hans van Gernerden had prepared lots of tubes with sterile enrichment medium in order to isolate anoxygenic phototrophs. When I asked him about his medium and whether he realizes that we are working in a marine environment he admitted that he completely forgot about that. While we had to work with what was available in the only house on the island, we took a letter scale and kitchen salt and amended every single tube with salt to seawater salinity. It worked very well!! Unfortunately, we forgot about a control to check whether the salt addition made any difference.

In 1988, I joined the Department of Aquatic Microbiology of Luuc Mur at the University of Amsterdam and developed marine microbiology as a sub-discipline, with a continued focus on phototrophic microorganisms, mainly cyanobacteria and the eukaryotic diatoms.

In 1996, I founded the Department of Marine Microbiology in the Netherlands Institute of Ecology (NIOO). It was the first scientific department in the Netherlands devoted to this research field. Ten years later, the University of Amsterdam founded a chair in Marine Microbiology for which I was appointed until my retirement in 2017, when I was followed up by Prof. Linda Amaral Zettler who is currently holding the chair. The chair of Marine Microbiology at the University of Amsterdam was the first and still the only one in the Netherlands. This is remarkable considering

the long history of and internationally highly recognized field of microbiology as well as the long tradition of marine and maritime research in the Netherlands.

For a long time, pure (axenic) cultures were the golden standard in microbiology. Anything that could not be isolated and grown in the laboratory as a pure culture was basically ignored and could not be formally described as a (new) species (genus, or other taxonomic level). Culturing meant in the first place that it demanded that the organism could be grown in the laboratory on more or less defined growth media and secondly that it could be separated from other types of organisms (the other microorganisms were referred to as contaminants). Isolation was usually done by starting an enrichment culture. The growth medium composition and the culture conditions were chosen such that it would specifically benefit the target organism. This implicated that the microbiologist already knew what the main properties of the target organism would be. After one or several rounds of enriching the culture for the target microbe, it was separated by dilution in growth medium supplemented with agar-agar gel that was cooled down just before it would solidify or streaked by an inoculation loop on the surface of agar-agar solidified growth medium. The idea was that the spatially isolated cell of the target organism would grow out to a colony visible by the naked eye. Such colony was picked up and re-diluted or re-streaked in or on agar-agar and this procedure was repeated at least one more time in order to be sure that the colony was formed by only cells of the target organism, which was in the past checked among other by microscopy and by now more regularly by DNA sequencing. Many microbes do not tolerate agar-agar while others, particularly marine microorganisms, degrade it. Even when highly purified agar-agar is used or in some cases agarose, certain microbes refuse to grow on it. In some cases, other gelling agents (gelatin, silica gel) could be applied as an alternative but they usually do not have the properties of agar-agar that makes it so suitable for microbiological purposes. Moreover, liquid enrichment media are often not suitable for microbes which live attached to surfaces such as in biofilms.

Although this technique has been used throughout the history of microbiology and is still used, there are many limitations. It starts with the design of the growth medium and culture conditions which is a prejudication of the scientist, mainly a precedent of what the scientist already knows, rather than aiming at the unknown. It selects for organisms that grow in that specific medium and conditions. Moreover, using batch enrichment cultures it selects for the fastest growing organism and in chemostats for the organism with the highest affinity for the growth-limiting substrate at the chosen dilution (= specific growth rate). Another technique that allows to isolate the most abundant organism(s) in a sample is by dilution to extinction. This prevents that abundant species are overgrown by rare species in the sample.

If a single species is brought into culture, it means that this particular microorganism is capable of growing in isolation. However, many species of microorganisms require one or more other types of organisms in order to be able to carry out the trophic reaction that allows them to proliferate. They will only form a colony when in very close proximity. The classical case of *Methanobacillus omelianskii* is what was thought to be an axenic culture that could ferment ethanol to methane and that later was found to be in fact a co-culture of two different

organisms that could perform the fermentation only jointly (Barker 1939; Bryant et al. 1967). This way of life is known as syntrophy (feeding together) (Morris et al. 2013). Another good example is the methane-oxidizing consortium of a methanogenic archaea and a sulfate-reducing bacterium (Boetius et al. 2000).

Pure cultures were critical for the classical work on the cause of tuberculosis and other infectious diseases by R. Koch in the late nineteenth century (Koch 1884). Koch's postulates required the isolation of the bacteria (or viruses, fungi/yeasts, parasites, as well as bacteria contaminated with viruses that are the cause of disease) from a person suffering the disease. Koch's postulates have been used in a simplified form to solve scientific questions in microbial ecology and biogeochemistry (Grimes 2006). These simplified Koch's postulates can also be applied to mixed cultures, which overcomes the problem of the isolation of pure cultures, but still requires culturability of environmental microorganisms or more or less defined mixtures of such microorganisms.

The stringent procedures that were advocated by many traditional and hard-core microbiologists have led to the ignorance of an enormous diversity of microorganisms that could not be isolated and grown in axenic culture and led to experimentation with "lab rats." Those studies have revealed an enormous amount of knowledge on the physiology of microorganisms and their application in some major biotechnological processes. However, it has put back the development of microbial ecology enormously (see, e.g., Dubos 1974). Many environmental microbiologists made important observations by microscopy and laboratory and field experiments but as they were unable to isolate the microbes they observed in pure culture, their observations were treated lightly and considered by many as soft science. This has changed completely after nucleic acid-based studies were introduced in microbial ecology. The concept of pure cultures as the only standard in microbiology has been abandoned. Now, microbial ecologists use defined mixed cultures with 2 or more different types of microorganisms or even undefined natural communities as their study objects and investigate the interactions or communication between the microbial components of the system. This discipline has also become known as microbial systems biology.

One of those hard-core molecular microbiologists who always had looked down on microbial ecology entrusted me shortly before his retirement that he would have become a microbial ecologist if he could do it all over again.

1.7 Concluding Remarks

Much of the modern research on marine microbiology can be summarized as taking samples as many as possible from as many as possible oceanic and marine areas, including from the deep-sea, measure the physicochemical and other oceanographic parameters for metadata, filter the (size-fractionated) water samples, extract the nucleic acids, sequence them, and put the sequences in public databases. Bioinformatic tools are used to assemble genes and (partial) genomes and analyze them. What is often missing are (1) extensive metadata from the samples and the areas and

times they were taken, (2) rate measurements of relevant microbiological processes, (3) biogeochemical processes, and (4) biochemical identification of predicted genes. To go out at sea, filter water, extract nucleic acids and have them sequenced is nowadays easy and does not require much scientific originality. Much of this research is not scientific research question driven and trusts that, once the sequence reads are in the data bases, interesting questions may come up and may be answered. This may be the case and in fact many interesting discoveries have been made this way. But the approach is taken just because it can be done and perhaps not in the last place because of its public outreach impact. This aspect is not unimportant, for instance, because of fund raising and public awareness. When the microbial and biogeochemical processes become linked with the genetic information it will become possible to have a system's view of the oceanic ecosystem and understand how it is regulated. Such approach is urgently needed to address the questions of global change (increasing temperature, acidification, sea level rise, plastisphere).

In the near future marine microbiology will rely less on expensive and inefficient research vessels. Many data may be obtained from automatic systems and sensors, either operating autonomous or carried by ships of opportunity (ferries, merchant ships) (Paul et al. 2007). Examples are among others the autonomous Argo Floats (Claustre et al. 2020), remote sensing (Coles et al. 2004; De Monte et al. 2013; Wurl et al. 2018), autonomous measurement of gene expression using an environmental sample processor connected to a drifting buoy for Lagrangian sampling (Birch 2018; Ottensen et al. 2013), autonomous tracking instruments (Zhang et al. 2020), and in situ flow cytometry and plankton analyzers. Other important projects are the so-called time series, where at a certain location sampling is done at regular time intervals and a large number of biological, chemical, and physical parameters are measured using strict and standardized analytical protocols. Some of these time series are running for several decades. While many marine research institutes run their own long-term samplings often off their own docks, others are only reached by ship. Among them are the Hawaiian Ocean Time Series (HOT) in the Pacific Ocean, the Bermuda Atlantic Time Series (BATS) in the western Atlantic Ocean, and the Cape Verde Ocean Observatory in the east Atlantic Ocean.

There may be many answers to the question of what is so special about marine microbiology but it depends in the first place on the devotion with which scientists that might call themselves marine microbiologists try to understand the life and role of microorganisms in the marine environment. And with the words of Louis Pasteur:

Messieurs, c'est les microbes qui auront le dernier mot.
(Gentlemen, it is the microbes who will have the last word)

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