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An integrate ecogenetic study of minimal ecosystems: The microbial mats of Ebro Delta and the Camargue (Western Mediterranean)§

Ricardo Guerrero,[1] Mercedes Berlanga[2]

[1]University of Barcelona and Institute for Catalan Studies, Barcelona, Catalonia.
[2]Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Barcelona, Barcelona, Catalonia

Correspondence:
M. Berlanga
Department of Microbiology and Parasitology
Faculty of Pharmacy, University of Barcelona
Av. Joan XXIII, s/n.
08028 Barcelona, Spain
Tel. +34-934024497
Fax +34-934024498
E-mail: mberlanga@ub.edu

§This paper is dedicated to Lynn Margulis, unforgettable teacher of one of us, unforgettable partner of the other.

Summary. Microbial mats are vertically stratified microbial communities that develop in the physical-chemical microgradients established at the interfaces of water and solid substrates. They form laminated multilayered biofilms, which as a result of their metabolism notably alter those microgradients. As highly diverse, physically and chemically active systems, microbial mats stabilize the sediment surface and prevent erosion of the surfaces where they are established. Lithified remains of microbial mats, known as stromatolites, may be very old. In fact, the oldest known microfossils are stromatolites that date back from more than 3500 million years ago. Therefore, microbial mats are considered to have constituted early ecosystems, probably the earliest ones. Although they now reach high degrees of complexity, during the Archean Eon they must have been very simple and thus fit well with the concept of a minimal ecosystem. Microorganisms in mats or in complex biofilms form coordinated functional communities that are much more efficient than mixed populations of floating planktonic organisms. Microbial mats resemble tissues formed by animals and plants in both their physiological cooperativity and in the extent to which they protect the "organism" from variations in environmental conditions, by a kind of homeostasis provided by the matrix or the boundaries of the mat. The survival value of this strategy in the milieu of the early Earth can be considered the main clue to the resilience of life against adverse environmental conditions. Furthermore, the "invention" of the ecosystem has promoted recycling of the scarce and limited chemical elements on the surface of our planet, thus allowing the evolution of other, more diverse forms of life and the persistence of life as a planetary phenomenon.

Keywords: microbial mats · minimal ecosystems · earliest ecosystems · structured biocenoses · populations diversity and dynamics · prokaryotic diversity

Resum. Els tapissos microbians són comunitats microbianes estratificades verticalment que es desenvolupen en microgradients físico-químics establerts en les interfícies d'aigua i substrats sòlids. Formen biopel·lícules (biofilms) amb diverses capes laminades, les quals, a causa del seu metabolisme, alteren intensament els microgradients. Aquests sistemes actius tan diversos físicament i química estabilitzen la superfície del sediment i prevenen l'erosió de les superfícies dels llocs on s'han establert. Les restes litificades dels tapissos microbians, conegudes com estromatòlits, poden ser molt antigues. De fet, els estromatòlits són els microfòsils més antics coneguts, i daten de fa més de 3500 milions d'anys. Per tant, els tapissos microbians han estat considerats com ecosistemes primerencs, probablement els més primitius. Tot i que en l'actualitat poden assolir un alt grau de complexitat, en l'Eó Arqueà haurien estat sistemes senzills i això encaixa amb el concepte d'ecosistema mínim. Els microorganismes en els tapissos o en bio-

films complexos constitueixen comunitats funcionals coordinades molt més eficients que les poblacions mixtes d'organismes planctònics que neden lliurament en l'aigua. Els tapissos microbians s'assemblen als teixits formats per animals i plantes tant en la seva cooperació fisiològica com en el fet de protegir "l'organisme" de les variacions en les condicions ambientals, mitjançant una espècie d'homeòstasi proporcionada per la matriu del biofilm o els límits del tapís. El valor de supervivència d'aquesta estratègia a la Terra primerenca pot considerar-se la principal raó de la resiliència de la vida davant les condicions ambientals adverses. A més, podem proposar que la "invenció" de l'ecosistema ha permès el reciclatge dels elements químics, escassos i limitats, sobre la superfície del nostre planeta, cosa que ha permès l'evolució d'altres i més diverses formes de vida, i la persistència de la vida com a un fenomen planetari.

Paraules clau: tapissos microbians · ecosistemes mínims · ecosistemes primitius · biocenosis estructurades · diversitat i dinàmica de poblacions · diversitat procariota

MICROORGANISMS PROVIDE THE BEST EVIDENCE of the enormous versatility of life and its ability to adapt itself to the variety of conditions found on Earth. The Earth's habitats present complex gradients of environmental conditions that include extreme variations in temperature, light, pH, pressure, salinity, and both inorganic and organic compounds. Each geochemical scenario features its own set of resources that can be physiologically exploited by microorganisms (for example, peat lands, deep-sea hydrothermal vents, soil, and deep subsurface sediments). *Bacteria* and *Archaea* have an essential role in the Earth's systems. They are ubiquitous, have enormous metabolic and physiological versatility, and are essential to virtually all biogeochemical cycling processes. Although the number of bacterial species described thus far is low (less than 7000), the total number of bacterial species that inhabit the Earth is estimated to be several million [53].

The approaches used in the enrichment and isolation of bacterial species establish artificial conditions under which only the "fittest" microorganisms can successfully compete. Furthermore, the conditions that allow microbial growth in culture are not easily determined, and their identification requires not only ability and persistence on the part of the researcher but also a fair amount of luck. Thus, an alternative strategy was proposed, discussed in two articles published in 1986 that set the tone for a new era in microbial ecology: one by Olsen et al. [*Annu Rev Microbiol* 40:337-365] and the other by Pace et al. [*Adv Microbial Ecol* 9:1-55]. Those authors independently described a framework around which the study of microbial diversity and community structure could proceed outside the confines of the agar plate. The central tenet was that all cellular organisms could be detected and potentially identified *in situ* based solely on their rRNA, even organisms that have yet to be isolated in axenic culture. As a result of these two papers, over the past four decades there has been an exponential increase in the amount of environmental sequence data that have become available.

Microbial mats: a model study of microbial ecosystems

The development of cultivation-independent molecular techniques such as genomics, metagenomics, transcriptomics, and proteomics has generated a plethora of new and more comprehensive observations of microorganisms in nature. But they have also left many questions unanswered: How many microbial species exist? What is the real cause of this diversity? What does all that microbial diversity do? How do ecosystems ultimately work? The first, albeit very preliminary, step in tackling these questions is to identify the different components of the various microbial communities. The resulting information must then be complemented with data on community function (metabolism, lifestyle), interactions, spatial and temporal dynamics, and the response to environmental variables. Molecular analysis of different habitats has thus far revealed different levels of microbial diversity. For instance, in agricultural soils worldwide, 20 bacterial phyla have been detected; approximately 12 phyla are present in the waters of the Sargasso Sea (North Atlantic); the adult human gastrointestinal tract has eight phyla, and healthy human skin eight phyla as well. But these are the environments that contain the lowest number of phyla, although each one is highly diverse at the strain and species levels [29]. Understanding the ecology of microorganisms is inarguably one of the most compelling intellectual challenges facing contemporary ecology.

In most, but not all ecosystems, light is the primary energy source. Consequently, biological communities are usually stratified horizontally, because of light extinction with depth. Tropical forests, planktonic communities, stratified lakes, and microbial mats can be considered as analogous forms at different scales. The photosynthetic layer expands for many meters in tropical forests; from a few meters to a few centimeters in multilayered planktonic microbial com-

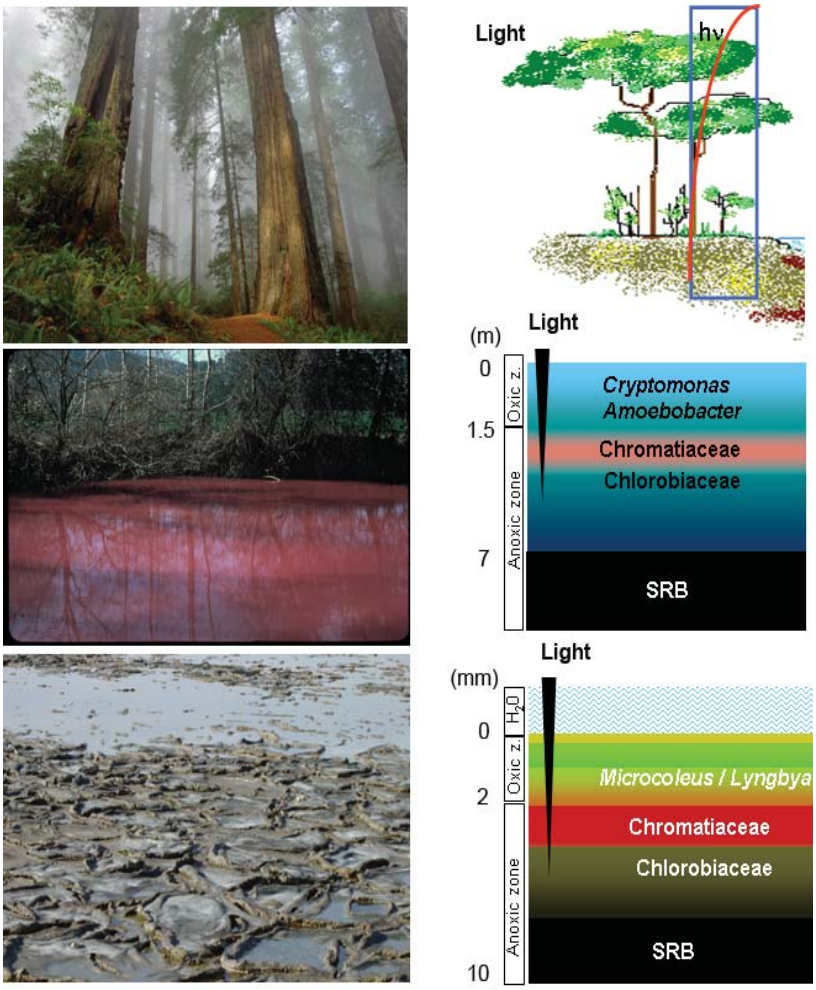

Fig. 1. Vertical structuring in several ecosystems in which light is the primary energy source results in the same "ecological theater" but one in which different actors play their roles at different scales, e.g., the forest ecosystem, the planktonic microbial community in a lake, and the microbial mat [27]. SRB: sulfate-reducing bacteria. (Photographs and sketches by R. Guerrero.)

munities; and for a few millimeters in microbial mats (Fig. 1). Autotrophic and heterotrophic organisms are major components of ecosystems. Autotrophs are producers. They fix energy either from light (phototrophs) or from light-independent chemical reactions (chemotrophs) and obtain nutrients from simple inorganic substances such as water, carbon dioxide, and nitrates. Heterotrophic organisms are consumers. They use, rearrange, and decompose the compounds synthesized by autotrophs but are unable to produce their own nutrients, instead obtaining them by consuming preformed organic matter.

Microbial mats are layered microbial communities made up of accretionary, cohesive microbial populations that grow at sediment–water (occasionally sediment–air) interfaces [27]. Their occurrence is confined to several habitats such as coastal zones with an intermittent input of seawater [13,15,23,30], thalassic wetlands [19], diverse geothermal environments [32], and in polar regions [70]. Mats develop in the physical-chemical microgradients established at the interfaces of water and solid substrates, forming laminated multilayered biofilms whose metabolism notably alters those microgradients. As highly diverse, physically and chemically active systems, microbial mats stabilize the sediment surface and prevent erosion of the surfaces where they are established. They can be several millimeters to a few centimeters thick, and develop along a variety of microgradients established between water and sediments. Lamination within the mats is evident, even macroscopically. It is the result of a light gradient along the vertical axis and of physicochemical microgradients due to the metabolism of different prokaryotic populations.

In Spain, microbial mats have been described in several different environments: Ebro Delta, Tarragona (temporarily inundated sand flat); Salinas Bonmatí, Santa Pola, Alicante; Lagunas and Salinas de Cabo de Gata, Almería; Salinas de San Rafael, Almería; Fuente de Piedra, Málaga (hypersaline lagoon); Sanguijuela, Albacete (endorheic lagoon); Saladar, Albacete (hypersaline lagoon); Alcahozo, Ciudad Real (hypersaline lagoon); Cerro Mesado, Ciudad Real (hypersaline lagoon); Las Yeguas, Ciudad Real (lagoon rich in Mg^{2+}); Laguna de Tirez, Toledo (hypersaline lagoon); Gallocanta, Zara-

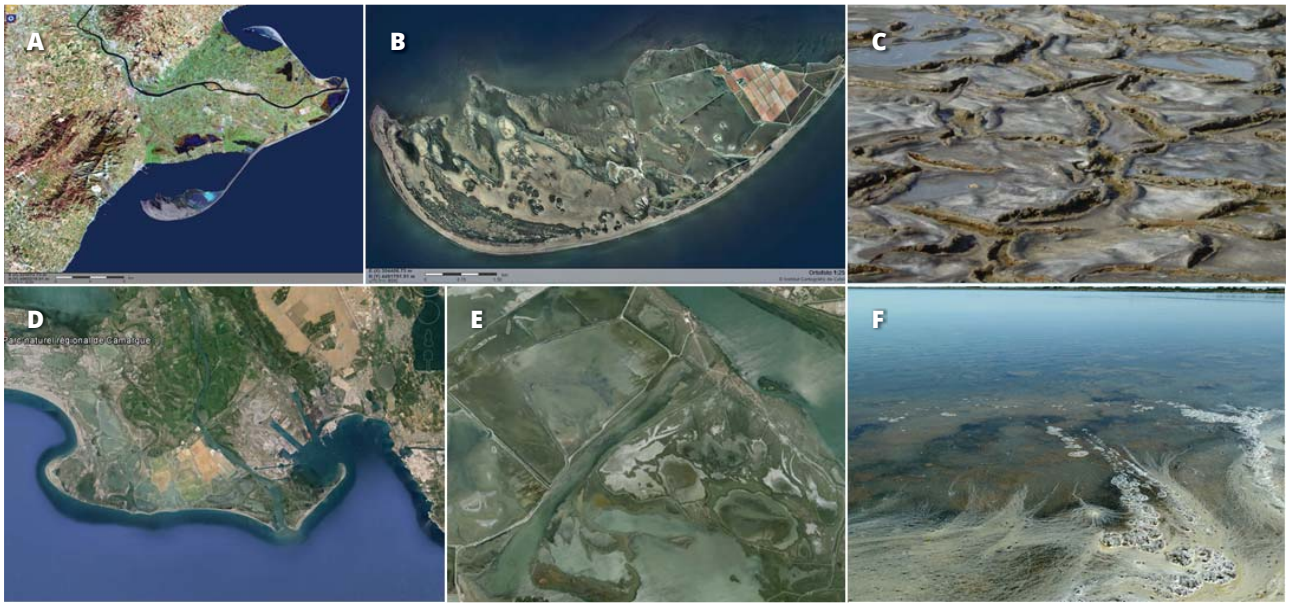

Fig. 2. (**A**,**B**) Sampling location in the Ebro Delta (images from the Cartographic and Geological Institute of Catalonia). (**C**) Macroscopic aspect of a microbial mat; area covered: ***ca***. 1 m^2 (photo by M. Berlanga). (**D**,**E**) Sampling location at the Camargue, Rhone Delta (images from Google Earth), (**F**) Macroscopic aspect; area covered: *ca.* 1 m^2 (photo by M. Berlanga).

goza (endorheic and hypersaline lagoon); Chiprana, Zaragoza (hypersaline lagoon); Carravalseca, Álava (endorheic hypersaline lagoon); Font de la Puda, Banyoles, Girona (sulfurous spring); Fumarolas del Teide, Santa Cruz de Tenerife (hot springs); El Charco de la Mareta, El Médano, Santa Cruz de Tenerife (hypersaline pond); Playas de Sotavento, Península de Jandía, Fuerteventura (sand flats) [18].

Exhaustive investigations have been carried out in the Ebro Delta (NE Spain) and in the Camargue (Rhone Delta, southern France). The Ebro Delta, the third largest delta in the Mediterranean (320 km^2), began to develop after the glaciation and has expanded seaward since the Holocene period. A delta is a sedimentary body that marks the transition between continent and sea, where the morphology of the coastline can be modified very rapidly in response to fluvial and marine changes. In the Ebro Delta, the tendency of the coastline in the last few decades has been generally regressive. This is a result of the decrease in sediment input from the drainage basin, due to the construction of large water reservoirs (mainly during the 1960s) that retain much of the coarser material before it reaches the delta.

Microbial mats in the Ebro Delta occur all along the coast, in the narrow ephemeral ponds of the backshore, on the flanks of the storm inlets, and, most commonly, on the sand flats and channels of La Banya spit (40° 35′ N, 0° 40′ E). Microbial mats are situated 1–7 cm below the water surface during flooded periods. The water covering these microbial mats ranges in temperature from 12 to 30 °C, with conductivity in the range of 59–105 $mS \cdot cm^{-1}$, salinity from 40 to 75‰, and pH from 7.5 to 9.0 [47].

The microbial mats in the area of Salins-de-Giraud, in the Camargue (La Camargue, southern France, 04° 11′ E to 04° 57′ E; 43° 40′ N to 44° 40′ N) are located inside commercial salterns, which are nowadays actively exploited. These salterns are a succession of water concentration ponds at the final part of the main "bouche" of the Rhone River. In the first series, seawater is concentrated to about 50–130‰ total salinity and is used for the storage of pre-concentrated seawater. The pond has an area of about 10 km^2, with the depth of the water column never exceeding 20 cm. The underlying sediment is mostly composed of a mixture of sand and clay. In the second series, salinities are in the range of 130–300‰, while in the final series of ponds the salinity increases to 340–350‰ (Fig. 2).

Microbial mats and lithification: an ancient strategy of life

The formation of microbial mats is an extremely ancient biological phenomenon, as the early Earth was probably covered by communities of different types of prokaryotes. Microbial mats must have dominated Archean landscapes. Their presence is best documented in the fossil record in laminated sedimentary rock structures called stromatolites. These organo-sedimentary structures are produced by trapping, binding, and/or precipitation as a result of the growth

and metabolic activity of microorganisms [53,54,65]. The persistence and abundance of stromatolites throughout most of geological time attest to the evolutionary success of microbial mat ecosystems. Stromatolites are found in rocks as old as 3500 million years from the Warrawoona Group of Western Australia [40] and Buck Reef Chert, South Africa [68].

Depending on the prevailing environmental conditions and the activities of indigenous microbial populations, individual cells can facilitate the crystal nucleation of different minerals. Mineral precipitation may be promoted by: (i) changes in microenvironmental chemical conditions and hence saturation state as a result of microbial metabolic processes, or (ii) nucleation on the surfaces of microorganisms (e.g., the cell envelope is very important for calcification) or on microbial products. Visscher et al. 2000 [78] correlated sulfate-reduction activity with high zones of $CaCO_3$ precipitation in modern marine stromatolites, although the biogeochemical processes of accretion and mineralization (i.e., lithification) in mat systems are poorly understood. Modern microbial mats are usually viewed as analogues of ancient stromatolites, but a major difference between them is that the latter have lithified laminae that form domal or columnar structures. Why ancient stromatolites and "modern living stromatolites," such as those from Shark Bay in Western Australia and the Exuma Sound in Bahamas, form lithified laminae, whereas other microbial mats do not is still unresolved. Microbial mats from the Ebro Delta undergo accretion and partial lithification but do not form lithified laminae [80]. Figure 3D is an impressive electron micrograph showing the progressive and rapid conversion of active living matter (the cyanobacterium *Microcoleus* actively reproducing in the top layer) to lithified organic matter at a depth in the mat of only 0.6 mm.

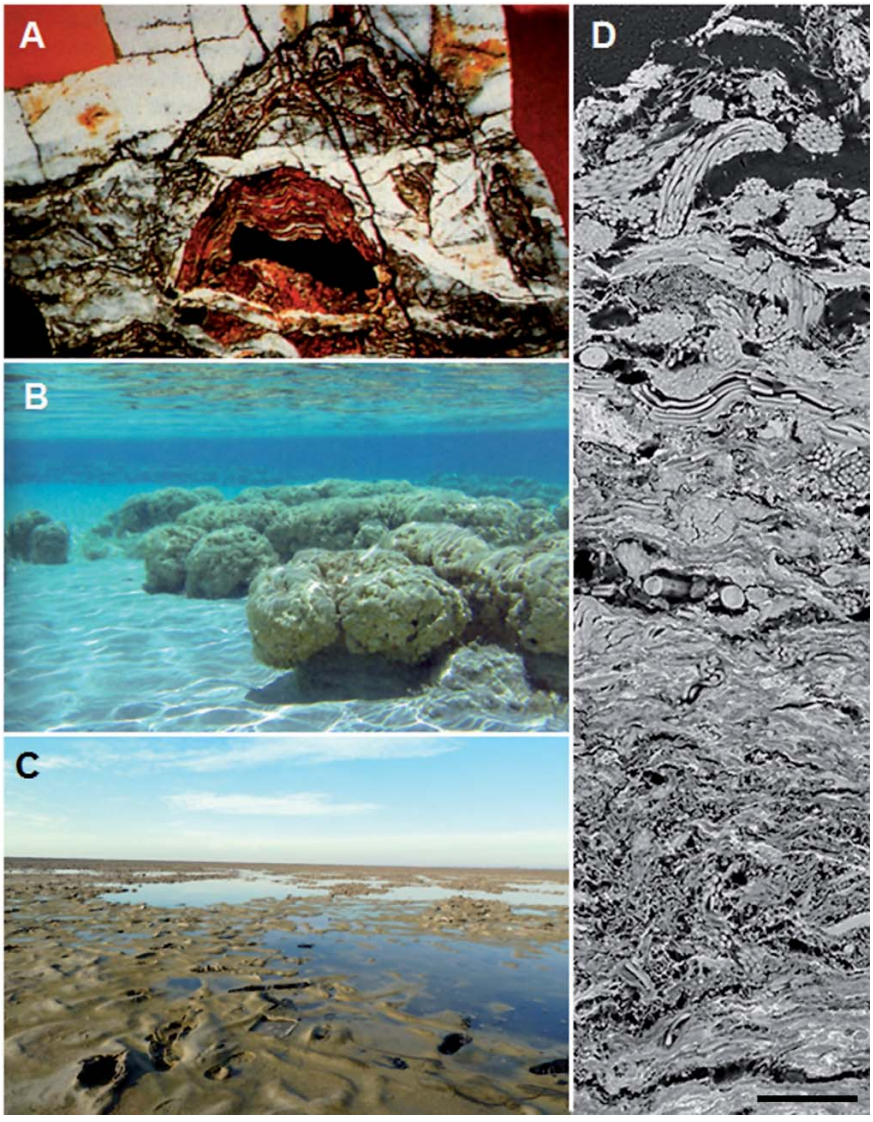

Fig. 3. (**A**) A 3500-million-year-old stromatolite from Warrawoona, Western Australia, (Smithsonian National Museum of Natural History, Washington, DC, USA). (**B**) Modern living stromatolites from Shark Bay, Western Australia. (**C**) Microbial mats landscape in the Camargue (photo by M. Berlanga). (**D**) Composite scanning electron micrographs of a microbial mat sample from the Ebro Delta, obtained in the backscattering electron mode. The vertical cross-section is 0.6 mm thick (photo by J. Wierzchos) [80].

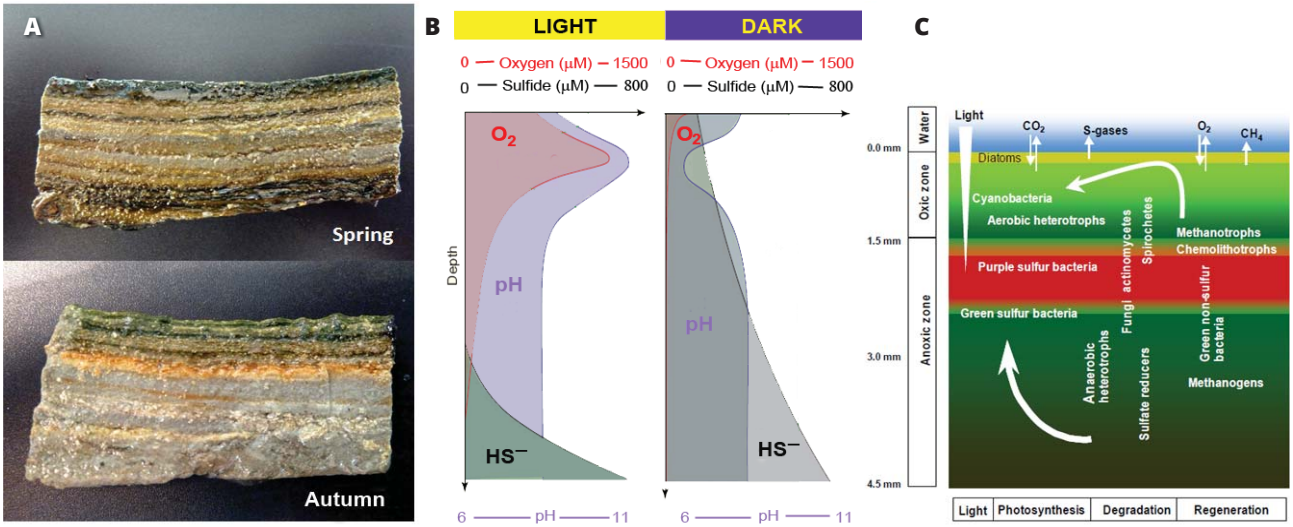

Fig. 4. (**A**) Cross section of microbial matsfrom the Camargue sampled during two seasons, showing the different layers. (**B**) Day-night gradient of oxygen, sulfide, and pH. (**C**) Major bacterial groups and matter cycling occurring in the microbial mat.

We are only beginning to appreciate the intimate interdependence of minerals and microorganisms, in particular the essential role that several chemical elements must have played in the first stages of life. Microorganisms in sediments contribute to the immobilization of metals through a continuum of sorption and precipitation reactions. Depending on the prevailing environmental conditions and the activity of indigenous microbial populations, individual cells can facilitate the nucleation and growth of crystal forms of distinct minerals. The uptake and release of chemicals by living organisms are necessary conditions of life and cause perceptible geochemical changes in the surrounding environment.

Presently, current rates of organic matter sedimentation from the open ocean environment to near-shore and lagoon environments vary between 20 and 10,000 g/m^2 per year. Under these conditions, and assuming a calcium carbonate yield of 0.5, induced carbonate precipitation by bacteria may produce a layer ranging from 4 µm to 2 mm in thickness. Thus, over one million years, bacterial carbonatogenesis could form a limestone layer 4–2000 m thick. As Vladimir Ivanovitch Vernadsky (1863–1945) stated in his seminal book *The Biosphere* [72], "If life were to cease the great chemical processes connected with it would disappear, both from the biosphere and probably also from the crust. All minerals in the upper crust—free alumino–silicious acids (clays), the carbonates (limestones and dolomites), the hydrated oxides of iron and aluminum (limonites and bauxites)—as well as hundreds of others, are continuously created by the influence of life. The biosphere is not only the face of Earth but is the global dynamic system transforming our planet since the beginning of biogeological time" [page 56, *The Biosphere*].

Microbial mats: the first structured habitats

Microbial mats exemplify functionally integrated, self-sustaining, laminated microbial consortial systems. They exhibit a remarkably high degree of biodiversity compressed into a few millimeters, with dense horizontal arrays of different functional groups of *Bacteria* and *Archaea*. The microbial functional groups commonly present in mats are photosynthetic bacteria, aerobic heterotrophs, fermenters, anaerobic heterotrophs (notably sulfate-reducing species) and chemolithotrophs (especially, sulfur-oxidizing species) [27].

The consumption of resources and the generation of metabolic products by microbial populations are the driving forces in the formation of gradients [12]. Gradients are particularly evident in physically structured habitats, producing new spatial heterogeneity and diversity that can select for a particular genetic variant among other genotypes. There is a principal difference between the gradients of compounds used for biomass synthesis and those needed for energy conservation, such as oxygen. While nutrient limitation leads mainly to a decrease or cessation of metabolic activity, the lack of energy substrates forces an organism to switch to a different type of metabolism, or may even cause a shift in the composition of the microbial community. As a result, gradients enable microbiota to diversify metabolically and lead to more complete nutrient cycling and community-level interactions over a range of temporal and spatial scales [12,16,50].

Chemical properties within mats fluctuate daily and seasonally. During the day, oxygenic photosynthesis operates in the uppermost layers. At night, however, the mats become anoxic and high in hydrogen sulfide concentrations, a conse-

quence of ongoing sulfate reduction in the absence of photosynthesis. Gradients produce a different microniche for every population in the community. Populations can also develop metabolically integrated consortia that can adopt specific spatial configurations. Microbial mats, with their rich diversity of organisms, are sites of complex elemental transformations [28,64]. High O_2 consumption in the mat leads to a low O_2 concentration which together with low O_2 penetration *in situ* during the night confines the oxic zone to the top 0.2 mm of the mat. At noon, high rates of oxygenic photosynthesis result in a strong increase in O_2 concentrations both in the overlying water and within the mat. The O_2 penetration depth in the mat increases to 2 mm.

Primary producers, e.g., cyanobacteria, excrete hydrogen and small organic acids, which serve as substrates for the growth of a broad array of microorganisms. Bacterial production of low-molecular-weight nitrogen and sulfur compounds is also important. All these substrates are involved in energy and electron flow in anaerobic ecosystems and thus form the potential basis for microbial interactions, such as those between phototrophic green sulfur bacteria and chemolithotrophic, sulfur-reducing bacteria, in which sulfur compounds are exchanged between the partners, and syntrophic associations between fermentative bacteria and methanogenic archaea or sulfate-reducing bacteria [59] (Fig. 4).

Two Western Mediterranean microbial mats: populations diversity and spatial distributions

Microbial populations rarely occur alone in nature; rather, they interact with each other to form complex communities [27], that is, heterogeneous microbial assemblages living together at a given place or habitat. Population identification is the first step in understanding the relationship between the community as a whole and the various assemblages that give rise to it. The study of microbial communities has raised questions about their composition, structure, and stability but also about the activities and functions of their individual inhabitants.

The microorganisms from microbial mats have been characterized using a variety of observational techniques, such as light microscopy and scanning or transmission electron microscopy [17,61]. However, they can also be detected based on their molecular markers, by employing molecular biological techniques such as lipids analysis (especially fatty acids) and 16S rRNA, both of which can reveal microbial diversity and the structure of microbial communities [14,66,77]. By combining the different methodologies, a more representative picture of the distribution and abundance of microorganisms in complex communities is obtained.

Physico-chemical gradients and microbial diversity. There are three main chemical zones in mats of temperate environments, such as those in the Western Mediterranean (Ebro Delta and Camargue): the photo/oxic (*ca*. 0–2 mm depth) zone, the low sulfide (*ca*. 2–4 mm depth) zone, and the high sulfide (*ca*. 5 mm and deeper) zone. The photo/oxic zone is typically dominated by oxygenic cyanobacteria and eukaryotic algae. Cyanobacteria are usually the most important primary producers in this kind of environment. Due to their photosynthetic metabolism, they not only generate oxygen, which can diffuse a few millimeters into the mat, but they also synthesize organic carbon compounds that are available to the rest of the microbial populations by active excretion or cell lysis. Among all cyanobacteria evaluated, *Microcoleus chthonoplastes* is the most abundant, reaching 61.2% of the total photosynthetic biomass in the photic/oxic zones of the studied mats, whereas *Lyngbya aestuarii* and coccoid cyanobacteria represent only 20.6% and 6.4%, respectively. Oxygenic phototrophs constitute about 58% of total photosynthetic biomass, measured as biovolume, in the photic zone of Ebro Delta microbial mats. In addition to cyanobacteria, oxygenic phototrophs include diatoms, which account for 11.8%, with the genera *Amphora*, *Navicula*, and *Nitzschia* as the most abundant [45]. *L. aestuarii* and diatoms, together with different coccoid cyanobacteria and some filaments of *M. chthonoplastes*, coat the surface of the mat. Below the oxic layer but still in the photic zone are anoxygenic phototrophic bacteria (mainly purple and green sulfur bacteria), which contribute the remaining 42% of total photosynthetic biomass. Thus, the photo/oxic zone also supports a rich community of anerobic/aerobic facultative and fermentative heterotrophs.

In several Camargue ponds, where the salinity of the water ranges from 70 ‰ to 150 ‰, *M. chthonoplastes* and *Halomicronema excentricum* are the dominant filamentous cyanobacteria, but *Oscillatoria* and *Leptolyngbya* strains have been observed as well. Unicellular types affiliated with the genera *Microcystis*, *Chroococcus*, *Gloeocapsa*, and, especially, *Synechocystis* account for *ca*. 27% of the cyanobacterial population. Although diverse anoxygenic phototrophic bacteria such as *Rhodobacter* and *Ectothiorhodospira* inhabit Camargue mats, *Halochromatium salexigens* and *Roseospira marina* predominate in surface and deep zones, respectively [22]. The major heterotrophs in the photic zone of Ebro Delta mats belong to the Alpha- and Gammaproteobacteria. By contrast, in the cyanobacterial layer of hypersaline Camargue mats, Cytophaga–Flavobacterium–Bacteroides (CFB) is the predominant group, together with Alphaproteobacteria [22,73].

In Ebro Delta mats, both the ratios of the different cyanobacteria and the presence and thickness of the layers of anoxygenic sulfur phototrophic bacteria depend on the moisture content, system stability, and age of the microbial mat. *Lyngbya, Oscillatoria,* and *Spirulina* are the first cyanobacteria able to colonize the bare sediment. *Lyngbya* dominates in young microbial mats and in mats exposed to frequent desic-

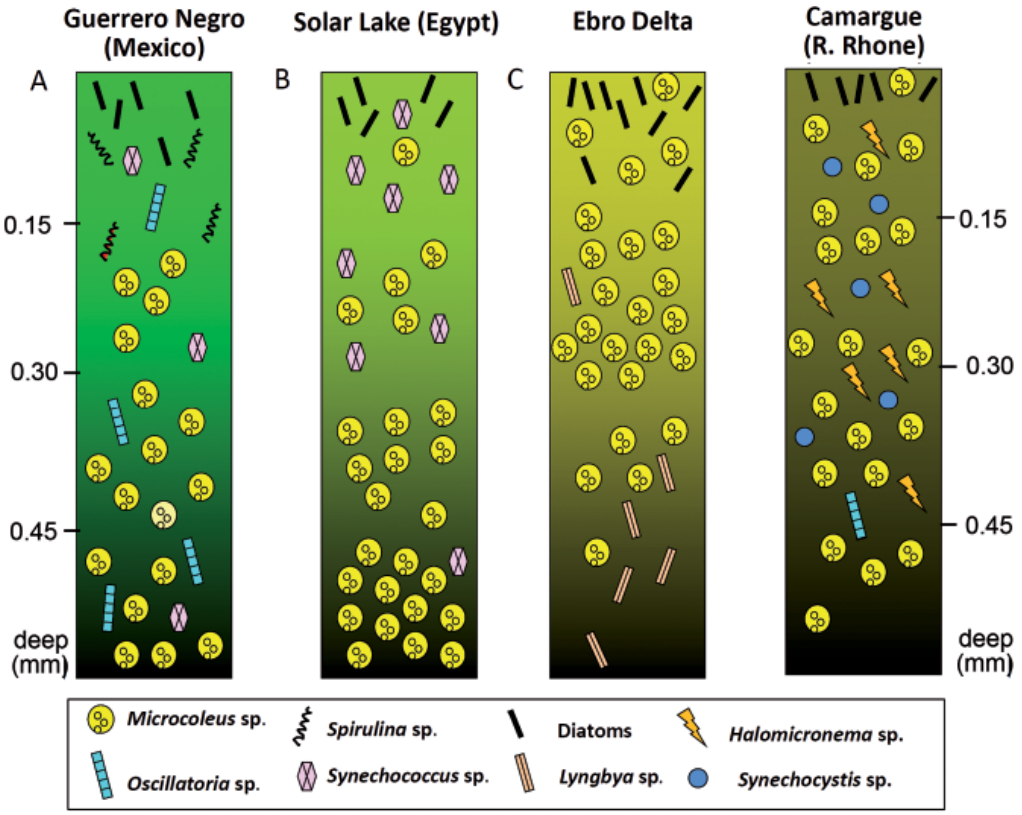

Fig. 5. Vertical distribution of aerobic-oxygenic phototrophs through the top 0.6 mm of several geographically different microbial mats.

cation. *Microcoleus* is the second most important colonist in the microbial succession; it thrives in the alternating submergence and re-emergence of the mat [23]. A schematic summary of the vertical distribution of aerobic phototrophic microorganisms through the top 0.6 mm of several highly geographically separated microbial mats is shown in Fig. 5. The filamentous forms of *M. chthonoplastes* are dominant, along with *Lyngbya* sp., and *Synechococcus* sp. These mat-forming taxa are embedded in matrices of extracellular polymeric substances, which hold large amounts of water and thus serve as a protective mechanism against the osmotic stress that is particularly high in shallow surface brines and during periods of desiccation. The comparison shown in Fig. 5 demonstrates the universal presence of these communities in microbial mats regardless of their geographic locations.

Light becomes more diffuse past a depth of 3 mm but it is sufficient to drive anoxygenic photosynthesis in groups such as purple sulfur bacteria and green sulfur bacteria. In Ebro Delta microbial mats, different strains have been isolated and cultured in axenic culture, such as *Chromatium* sp., *Thiocapsa* sp., *Lamprobacter* sp., and *Ectothiorhodospira* sp. Also isolated from these microbial mats are green sulfur bacteria belonging to the genus *Prosthecochloris* [45]. At depths greater than 5 mm, light is absent and photosynthesis does not occur. Here, the microbial community primarily consists of anaerobic sulfate reducers.

Microbial diversity by detecting 16S rRNA. Metagenomic shotgun and targeted-gene amplicon sequencing are two complementary, culture-independent approaches to assess microbial biodiversity. The former offers a relatively unbiased view of the suite of genomic information in an environmental sample, based on adequate sequencing depth and assembly. A high-throughput 454 pyrosequencing approach to 16S rRNA was used to study the composition and diversity of Camargue microbial mat communities. The detected sequences represented more than 20 phyla, with important contributions by only eight phyla, although each phylum-level group is represented by broad intra-phylum diversity (Fig. 6). In laminated microbial mats from Guerrero Negro ("Exportadora de Sal" Saltworks, Baja California Sur, Mexico), where 42 bacterial phyla were recorded, molecular surveys determined the ratios of bacterial, archaeal, and eukaryotic 16S:18S rRNA of 90%, 9%, and 1%, respectively [20,30,37,55]. In these mats, maximum archaeal diversity occurs within the oxic zone

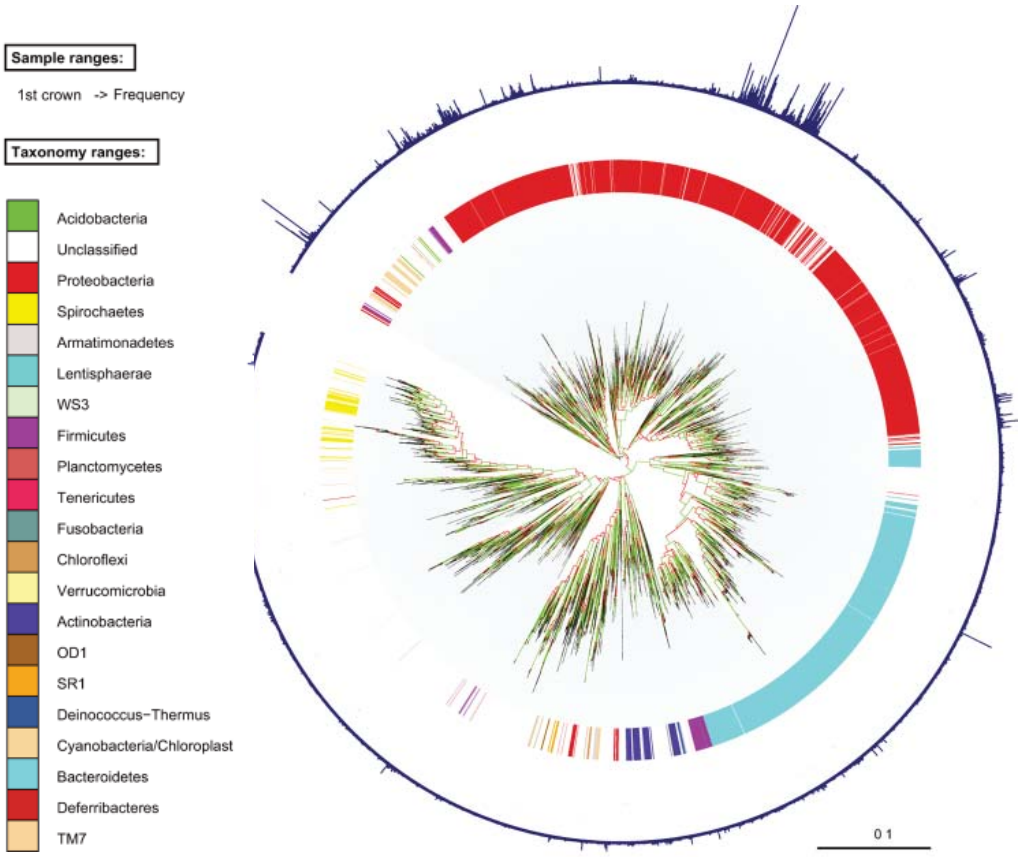

Fig. 6. Phylogenetic tree of the bacteria associated with a Camargue microbial mat and their GenBank relatives of the most prevalent operational taxonomic units (OTUs; with a distance threshold of 0.03), as generated by 454-pyrosequencing. The tree was obtained using the Interactive Tree of Life, a web-based tool [http://itol.embl.de].

and consists mainly of members of the Euryarchaeota. Euryarchaeotes also dominate the archaeal diversity to a depth of *ca.* 26 mm, i.e., into the anoxic, high-hydrogen sulfide zone of the mat. In contrast, few crenarchaeal sequences are found above 2 mm, but their numbers increase with depth to become the most numerous and diverse archaeal sequences in the mat below *ca.* 27 mm [55]. The eukaryotic diversity of the Guerrero Negro mat is surprisingly sparse considering the vast bacterial diversity in the same setting. Bacteria collectively have broad metabolic capabilities and can occupy many chemical niches, whereas the metabolic versatility of eukaryotes seems more limited, even though they are capable of survival under high sulfide, fermentative, anoxic conditions. The dominant eukaryotic organisms in the mat are bacterivorous nematodes. Although nematodes represent only a small fraction of the total biomass in Guerrero Negro mats, they may still play a significant role in the community, by imparting churning action and microbial/chemical transport within the mat [20].

Population changes and physiological status. Microbial mats are characterized by cyclical and seasonal fluctuations of flooding and desiccation and by diel fluctuations in the concentrations of oxygen and sulfide and other chemical compounds. Diversity is generally thought to be desirable for ecosystem stability; that is, more complex systems are more robust than simpler ones and thus less vulnerable to environmental changes. This robustness is based in part on redundancy, in which multiple units perform the same or very similar functions inside the system. If several individuals are lost after a challenge, many other almost-functionally identical individuals are available to replace them, thus repairing the system [5,8].

Analyses of lipid biomarkers provide a quantitative means of measuring viable microorganisms, microbial community composition, and community nutritional/physiological status. Gram-negative bacteria are evidenced by the presence of monoenoic phospholipid fatty acids (PLFA), and Gram-positive bacteria by terminally branched saturated fatty acids. Branched monosaturated and mid-chain-branched saturated fatty acids are representative lipids of anaerobic microorganisms, while polysaturated fatty acids identify cyanobacteria and eukaryotic microorganisms. Others compounds, such as plasmalogen-derived dimethyl acetals (DMA), and sphingoid bases, provide additional clues as to mat community composition [49,74,75,77].

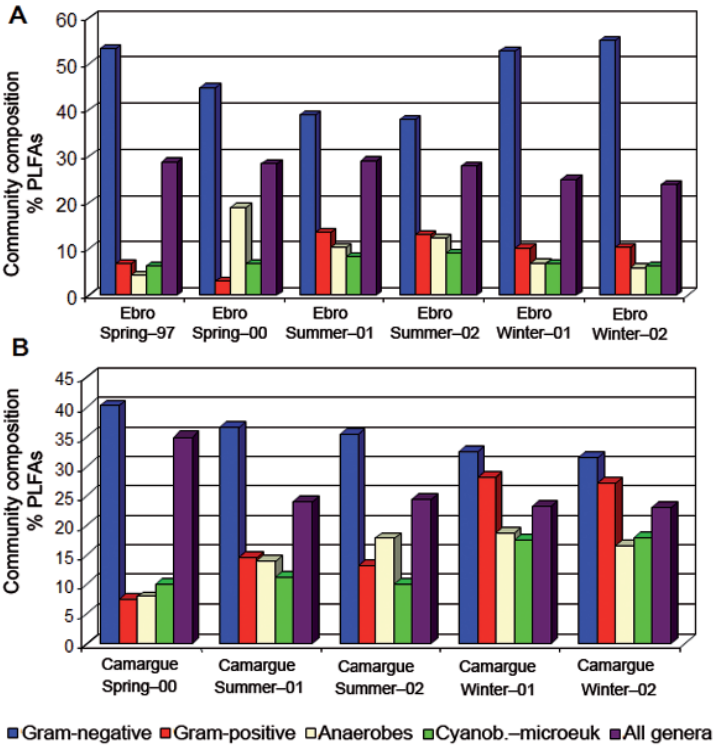

Fig. 7. Community composition of microbial mat samples from (**A**) the Ebro Delta and (**B**) the Camargue, as determined by % PLFA analysis.

Physiological stress within microbial communities can be measured by the ratios of cyclopropane to monoenoic PLFA and *trans*-monoenoic to *cis*-monoenoic PLFA. The former ratio is increased in response to changes in environmental conditions. Monoenoic fatty acids (16:1v7c and 18:1v7c) are converted to cyclopropyl (cy 17:0 and cy 19:0), such that the ratio is usually within the range of 0.05 (exponential phase) to 2.5 or higher (stationary phase). Microbes grow slowly when carbon source(s) and terminal electron acceptors are present but the supply of some essential nutrients is limited. Under these conditions of "unbalanced growth," bacteria can accumulate biopolymers (such as poly-β-hydroxyalkanoates). In response to metabolic stress (e.g., toxicity, starvation), bacteria produce *trans*-monounsaturated fatty acids. *Trans/cis* ratios higher than 0.1 indicate starvation in bacterial isolates. This value is usually 0.05 or less in healthy, non-stressed populations [49,73].

Differences in community composition may reflect seasonal environmental parameters, principally temperature (Fig. 7). In mat samples collected during the summer and winter of 2001 and 2002, anaerobes were more abundant in summer. Higher temperatures increase respiratory activity, which favors the anoxic conditions that predominate in the mat. During winter, ambient temperatures are considerably lower and the daily temperature variations (day-night) are less pronounced than in summer. During the spring and summer, the mats were desiccated, the consequence of high temperatures and irradiance levels and increased salinity.

The community composition of Ebro Delta microbial mats during a day-night cycle was characterized based on the PLFA pattern [74]. In that study, the responses of individual populations to changing solar illumination was variable, with some responding stably throughout the diel cycle, while others significantly shifted their location within the mat (Fig. 8). The community consisted mainly of Gram-negative bacteria (especially Proteobacteria) at 12:00 h and 15:00 h, as indicated by the presence of a high percentage of monoenoic PLFA. At all sampling times, the abundance of lipids representative of microeukaryotes (polyenoic PLFAs) and Gram-positive bacteria (terminally branched saturated PLFAs) remained stable. Among ubiquinones, the Q-10 percentage was highest at 12:00 h, 15:00 h, and 21:00 h; at all other times Q-8 predominated. The%mol of Q-9 was similar at all times. Based on chemotaxonomic studies, Beta-, Gamma-, and Alphaproteobacteria were considered as major sources of Q-8, Q-9, and Q-10, respectively. Among the quinones, the highest%mol was that of menaquinone (MK)-9, which can be found in members of the Firmicutes, Actinobacteria, and Bacteroides. MK-6, MK-7, MK-8, and MK-10 were also present in important relative percentages in all samples. MK-6 has been detected in Actinobacteria, Cytophaga-Flavobacteria, and Deltaproteobacteria; MK-7 and MK-8 in Euryarchaeota; and MK-10, in green nonsulfur bacteria.

Changes in population distribution may correspond to the night-time decrease in oxygen and increase in sulfide. In

afternoon samples, oxygenic photosynthesis contributed to oxygen supersaturation in the water column above the mat, where a maximum concentration of 1.04 mM was reached. From the changes in sulfide concentrations under light-dark cycles, the rate of H_2S production was estimated to be 6.2 µmol H_2S cm^{-3} day^{-1} at 2.6 mm and 7.6 µmol H_2S cm^{-3} day^{-1} at 6 mm. Sulfide consumption was also assessed, yielding rates of 0.04, 0.13, and 0.005 mmol l^{-1} of sulfide oxidized at depths of 2.6, 3, and 6 mm, respectively [47]. An increase in the abundance of *Microcoleus* near the surface during the day confirmed that these gliding filamentous cyanobacteria indeed migrate, likely in order to modulate their irradiance levels [71].

In summary, microbial mat diversity is apparently stable over a period of hours during the daily cycle, with the exception of those microorganisms that migrate vertically or undergo changes in abundance, especially after events of intense photosynthetic activity. The final result is the stratification of the community [74].

Enrichment and isolation of several bacterial groups from Ebro Delta and the Camargue microbial mats

Significant insights into microbial physiology have been gained by studying the small number of prokaryote species already cultured. However, despite these numerous breakthroughs, cultivation is still a limited approach to the study of the mat microbial community. As noted above, only slightly more than 7000 prokaryotic species have been thus far described (and validated by the International Committee on Systematics of Prokaryotes), although 130 years have passed since the invention of the Petri dish. The lack of an extensive and accurate picture of microbial diversity is partly due to a deficiency in technical advances in the field of microbial cultivation, given the prevailing absence of knowledge about the targeted species. The techniques used in the enrichment and isolation of microorganisms establish artificial environmental conditions that allow the development of only a few microorganisms, i.e., those most able to thrive in the conditions of the manufactured culture environment. Given that these conditions are the result of the researcher's skill, persistence, and, to a large extent, luck, it should not be surprising that the vast immensity of the microbial world remains uncultured. Therefore, the following description of bacterial groups from microbial mats consists only of those that we have been able to enrich, as we have yet to achieve their growth on Petri dish media.

Spirochetes. Spirochetes are a group of helical, motile, Gram-negative bacteria that are widely distributed in nature. They constitute a monophyletic phylum characterized phenotypically by a special cellular ultrastructure (periplasmatic flagella) and a form of motility that is unique among members of the Domain *Bacteria*. Fifteen species of *Spirochaeta* are presently known [36,51]. There is also an immotile spirochete with a coccoid morphology, strain SPN1. Although it was first classified in the genus *Spirochaeta* it was later reclassified as a novel species in the genus *Sphaerochaeta*, a sister group of the *Spirochaeta.* This strain is of interest because it may play an important role in the digestion of breakdown products from cellulose and hemicellulose in the termite gut [2].

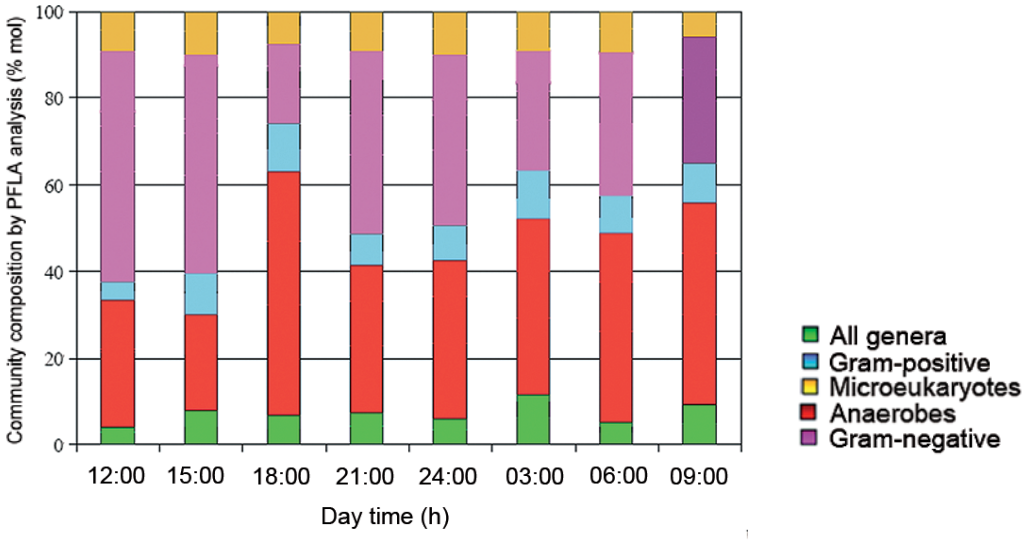

Fig. 8. Community composition of an Ebro Delta microbial mat during a day-night cycle, as determined by % PLFA analysis [74].

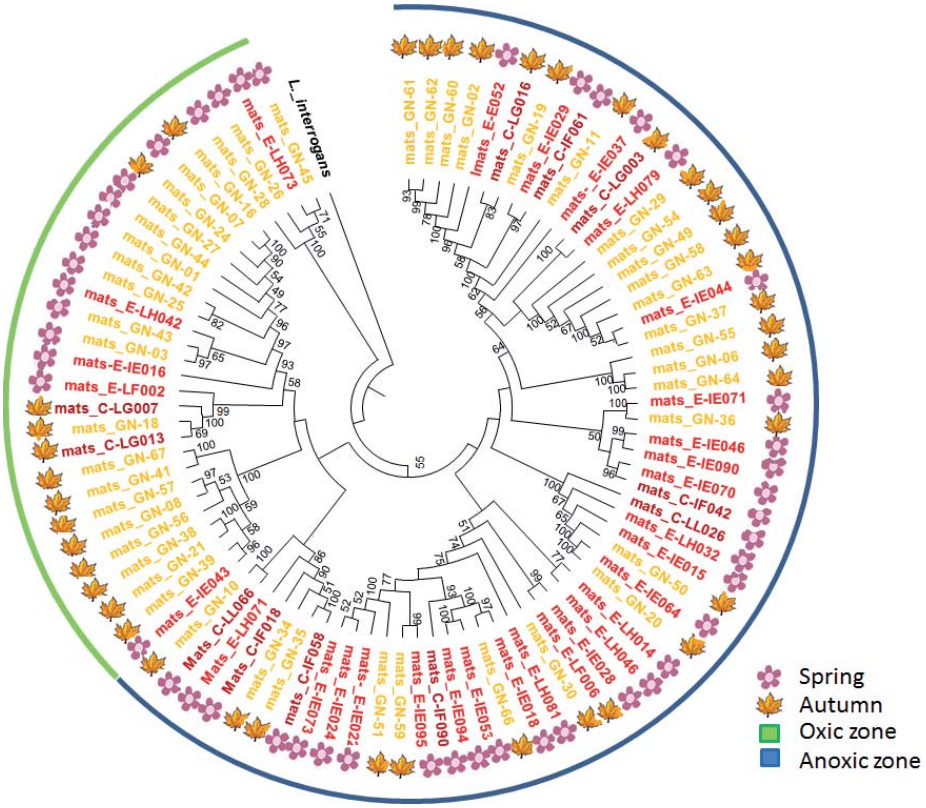

Fig. 9. A comparison of *Spirochaeta* phylotypes (16S rRNA) from Ebro Delta (E) and the Camargue (C) mats with *Spirochaeta* phylotypes from Guerrero Negro mats (GN). One-thousand bootstrap trees were generated; bootstrap confidence levels, expressed as percentages (only values >50%), are shown at tree nodes.

Free-living spirochetes of the genus *Spirochaeta* are one of the bacterial groups often observed in the hydrogen-sulfide-rich layers of microbial mat habitats. In several studies, spirochetes comprised 1–4% of the bacteria total 16S rRNA sequences analyzed per sample [3,7,37]. Novel *Spirochaeta* phylotypes, which have not yet been cultivated in vitro, have been identified in samples from hypersaline ponds in Western Mediterranean salterns [7,48]. However, relatively few spirochetes from mats have been isolated and characterized [42,67,79].

Phylogenetic analysis based on 16S rRNA genes was used to investigate spirochetal diversity in Ebro Delta and Camargue mats. Samples from each location were collected in the spring and winter over a period of 2 years. Novel phylotypes of not-yet-cultivated spirochetes belonging to the genus *Spirochaeta* were detected. None of the phylotypes were identified as known culturable species of *Spirochaeta* or as previously identified phylotypes cloned from other hypersaline microbial mats, such as those of Guerrero Negro, México. Ebro Delta and Camargue phylotypes, like phylotypes from Guerrero Negro, grouped according to the vertical gradient of oxygen and sulfide in the mat, and not by sampling season (Fig. 9). The presence of spirochetes in microbial mats of different locations suggests that they would constitute very diverse and stable populations involved in a well-integrated metabolic symbiosis (i.e., permanent physiological cooperation) with other specialized populations in the mats, where they would maintain a coordinated functional and stable community [7].

Thus, spirochetes are major constituents of the biota in microbial mats. The main compounds produced by spirochetes are acetate, H_2, and CO_2, all of which are usually consumed by sulfate-reducing bacteria and by methanogens (two groups highly represented in microbial mats). Spirochetes have metabolic capabilities unrecognized for many years, such as nitrogen fixation [38] and the reduction of thiosulfate and elemental sulfur, but not sulfate, to sulfide [41]. In conclusion, spirochetes form a dynamic population involved in maintaining stable ecosystem functioning, by supplying carbon sources and electron donors to other members of the mat community. They are a ubiquitous component of the oxic-anoxic gradient of microbial mats, where they compete effectively with other heterotrophic organisms for soluble sugars [7].

Spirosymplokos deltaeiberi is a spirochete first described in Ebro Delta microbial mats [24,43], and later in samples from the Sippewissett salt marsh (Woods Hole, Massachusetts,

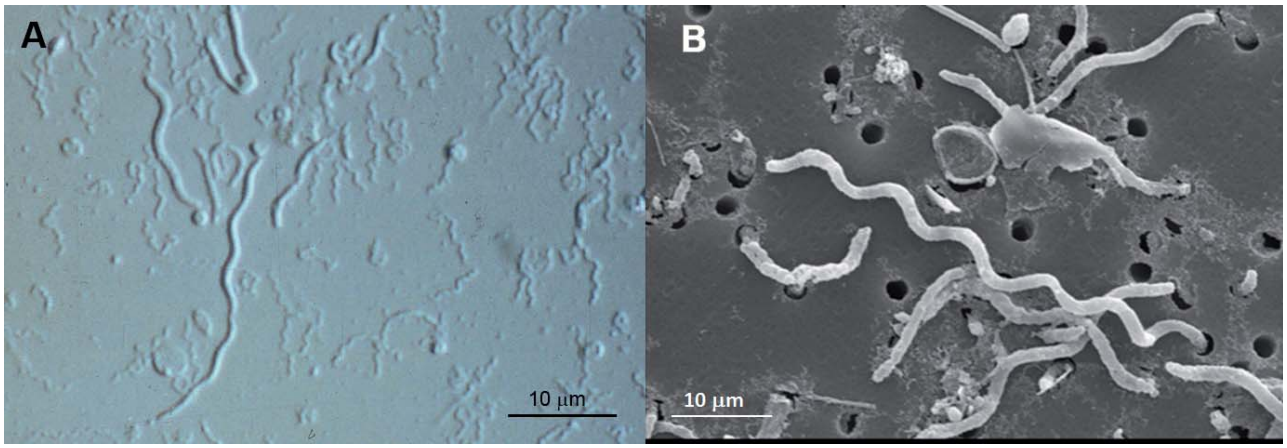

Fig. 10. Spirochetes from an Ebro Delta microbial mat. (**A**) *Spirosymplokos deltaeiberi* (photo by L. Margulis). (**B**) Scanning electron micrographs of spirochete-like bacteria (recovered from a bloom) filtered onto a 3-μm-pore-size polycarbonate filter (photo by L. Villanueva).

USA) and in microbial mats at North Pond of Laguna Figueroa (Baja California Norte, México). The identity of these spirochetes was confirmed by electron microscopy [44]. *Spirosymplokos* is a large (up to 100 μm long, average of 70 μm), loosely coiled, free-living spirochete with variable diameters (from 0.4 to 3 μm along the same cell), and containing 3–6 periplasmic flagella (Fig. 10). This spirochete has been observed in mud water and enrichment media, associated with *Microcoleus chthonoplastes,* but not in muds lacking clearly laminated, brightly colored, overlying sediments. Light microscopy confirmed phototaxis in this large spirochete.

Spirosymplokos deltaeiberi was enriched from samples of microbial mats of the *Microcoleus/Thiocapsa* type. Enrichment assays consisted of placing mat pieces from the original field samples in tubes containing cellobiose and rifampicin. After incubation of the tubes for 1–2 weeks in the light at 25 °C, populations of the large spirochete developed but they could not be transferred to fresh medium [24] because they swelled on exposure to air. Within a few hours, while they continued to move, one to four refractile bodies formed along nearly all the cells. These became visible after the protoplasmic cylinders were withdrawn. The refractile, membranous, round bodies (RBs; also referred to as reproductive propagules, coccoid bodies, globular bodies, spherical bodies, granules, or cysts) provide a morphological basis for the oxygen and desiccation resistance of *Spirosymplokos*. Mud-dwelling spirochetes are anaerobic and aerotolerant chemoheterotrophs that survive in changing intertidal environments, and they are probably among the most ancient mat inhabitants [43].

Spirochetal RBs are a cell form induced by environmental conditions unfavorable for growth. Following the resumption of growth, RBs reversibly convert into motile cells. Reversible pleiomorphy has been recorded in at least five spirochete genera (*Borrelia*, *Spirosymplokos* and three ectosymbiont spirochetes from termite protists such as the protist *Mastotermes*). This phenomenon could explain chronic spirochetoses in humans and the reappearance of motile bacteria after long dormancy periods. Although in both cases there must also be an immunological contribution, that symptom reappearance is related to spirochete differentiation merits consideration. Persistence of tissue spirochetes of *Borrelia burgdorferi* as helices and RBs could likewise explain many erythema-Lyme disease symptoms [11] as well as changes in the course of syphilis, which is caused by the tightly-coiled small (3 μm × 0.3 μm) spirochete *Treponema pallidum*. The only known habitat of *T. pallidum* is the human body; the bacterium cannot be cultured in axenic media. Based on the main clinical and pathological manifestations of syphilis, it has been divided into three classical stages. A primary stage, with development of the typical chancre; a generalized secondary stage, reflecting hematogenous dissemination of the spirochetes; and a late, chronic or tertiary stage (neurosyphilis), which can appear months, years, or even decades following the primary infection. During the "latent" period (between secondary and tertiary stages) *T. pallidum* cannot be detected in host tissues [46]. It is in this stage that *Treponema* probably forms RBs, similar to those of *Borrelia* and *Spirosymplokos* [24]. The detection of *T. pallidum* in the brains of patients with general paresis established a direct link between persisting infection and the manifestations of (tertiary) neurosyphilis.

Magnetotactic bacteria from the Ebro Delta and the Camargue. The term "magnetotactic bacteria" (MTB) has no taxonomic significance but instead describes a heterogeneous group of *Bacteria* displaying many different cellular morphologies, including coccoid, rod-shaped, vibrioid, spirilloid (helical), and even multicellular (aggregates), but sharing an ability to passively align and actively swim along

the Earth's geomagnetic and local magnetic field lines. MTB are motile Gram-negative prokaryotes that are ubiquitous in aquatic habitats, including freshwater and marine sediments. They are cosmopolitan in their distribution and most abundant at the oxic-anoxic transition zone (OATZ; also referred to as the redoxocline or microaerobic zone). The capacity for magnetotaxis is due to the presence of magnetosomes, intracellular membrane-bound crystals of magnetic iron minerals, such as magnetite (Fe_3O_4), or greigite (Fe_3S_4) [57]. The function of magnetotaxis in bacteria is to facilitate their finding and maintaining a favorable position in vertical chemical gradients in stratified environments. Despite their ubiquity and abundance in many marine and freshwater habitats, only a small number of magnetotactic strains have been isolated in pure culture [33–35]. The MTB identified thus far are associated with several phyla: the Alpha-, Gamma- and Deltaproteobacteria classes of Proteobacteria, the Nitrospirae, the candidate division OP3, and part of the Planctomycetes–Verrucomicrobia–Chlamydiae bacterial superphylum [35].

MTB have been observed both in fresh samples and in microcosms prepared from the Ebro Delta and Camargue. The microcosms were established in bottles containing two-thirds sediment mats, overlain with one-third sample water. The loosely covered bottles were incubated at room temperature in dim light without agitation. Five days before isolation analysis, they were stored in complete darkness to prevent photosynthesis. Ebro Delta fresh samples (from the Encanyissada Lagoon) typically contained various characteristic MTB morphotypes, including cocci, spirilla, rods, and vibrios. The communities in the microcosms underwent a characteristic succession within several weeks that resulted in an apparent loss of diversity. After prolonged incubation, they were ultimately dominated by magnetotactic cocci (no other morphotypes were observed by microscopy). In one microcosm containing a sample from the Camargue, only one morphotype was detected, multicellular MTB or aggregates thereof that rapidly disappeared upon laboratory incubation (Fig. 11). These magnetotactic multicellular organisms (10–20 cells, each) were highly motile, with a complex swimming behavior consisting of a forward movement in the direction of the magnetic field and a backward movement in the opposite direction, indicating that flagellar movement in the whole organism is coordinated. Disaggregation of the cells resulted in their loss of motility. These magnetotactic multicellular organisms were spherical in shape and grew by an increase in cell size, but not in cell number. The cells divided synchronously. These organisms and their behavior were similar to those later described by Abreu et al., [1] in Brazil.

Major factors determining the distribution of bacteria in a stratified habitat include the location and width of the chemocline as well as the proximity and concentrations of electron donors and acceptors. The assumption of a sulfide-oxidizing metabolism is reasonable for MTB populations in microcosms. Changes in magnetotactic diversity over time might be determined by different optima in sulfide and oxygen gradients present in the microcosm [21]. Lin & Pan (2010) [39] showed that the phylogenetic discrepancy in MTB communities between two microcosms is more prominent than that of the same microcosm at different times, implying adaptation of MTB phylogenetic lineages to specific microenvironments. Among the physico-chemical variables measured, nitrate availability was found to strongly correlate with the main genetic variability of MTB communities, indicating that nitrate influences the occurrence of MTB phyloge-

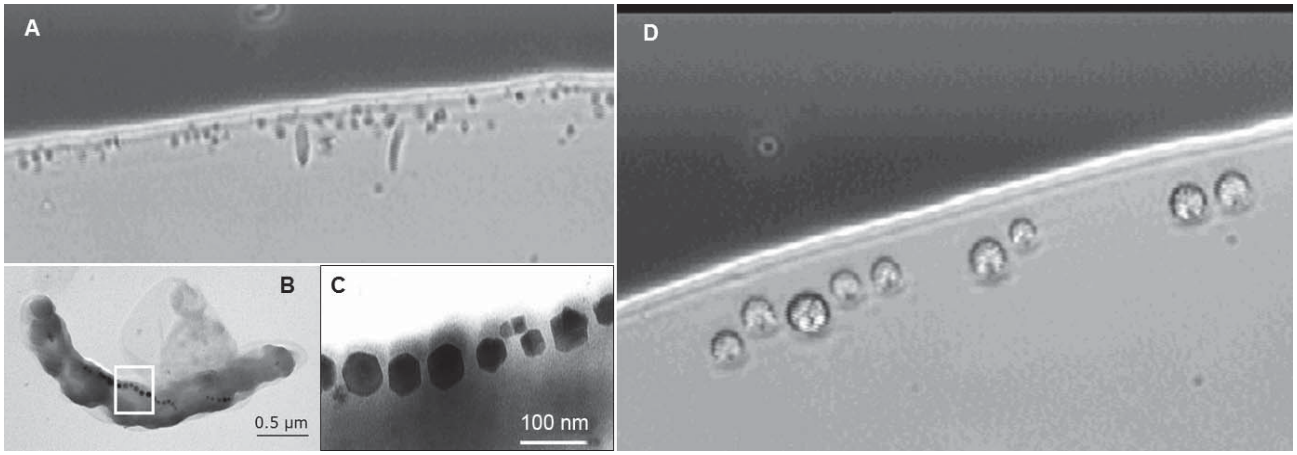

Fig. 11. (**A**) Contrast phase optic microscopy showing the morphological diversity of magnetotactic bacteria from Ebro Delta microbial mats (photo by M. Berlanga). (**B**) Transmission electron micrograph showing a magnetotactic bacterium. (**C**) A detail of a magnetosome chain (photo by J. Wierzchos). (**D**) Contrast phase optic micrography showing magnetotactic aggregates from a Camargue sample (photo by M. Berlanga).

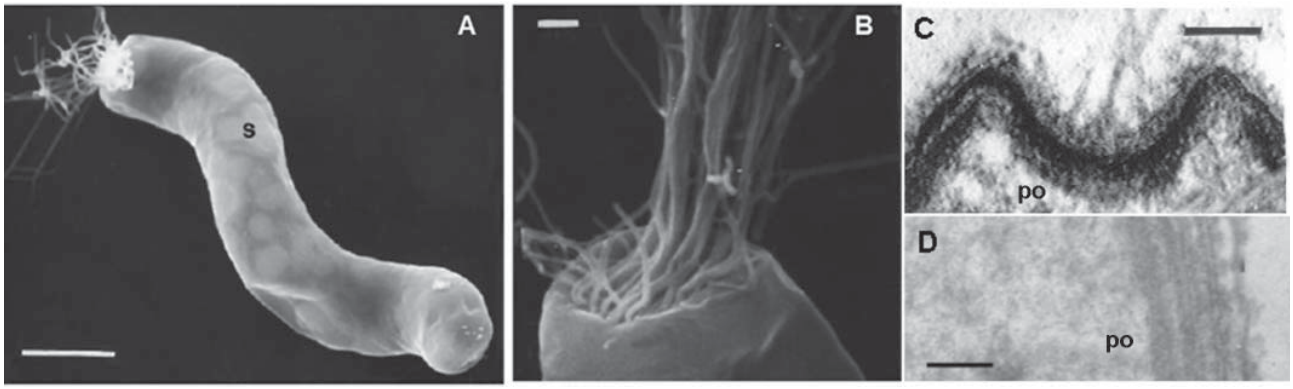

Fig. 12. (**A**) Scanning electron micrograph of a large spirillum in which only one pole has retained its flagella. Sulfur globules are visible through the cell wall (bar equals 5 µm). (**B**) Scanning electron micrograph of the cell terminus shows one vaulted end with its residual flagella and the indentation coated by the polar organelle (bar equals 0.5 µm). (**C**) The polar organelle (po) underlies the indented terminus (bar equals 1 µm). (**D**) Polar organelles lie proximal to at least nine layers of wall material at the cell termini (bar equals 0.25 µm) [26].

netic lineages in natural environments. Other works have pointed out that microaerophilic, magnetite-producing, coccoid sulfide oxidizers are present at the top of the chemocline, while greigite-magnetosomes occur at its base [60]. The magnetotactic cocci observed in Ebro Delta microcosms were microaerophilic sulfide oxidizers and were probably representative of those that accumulate at the oxygen-sulfide interface. Aggregates from the Camargue only appeared when the sulfide concentration in the microcosm medium was high (detected by odor), suggesting that within vertical gradients different species differ in their positional preferences.

***Titanospirillum velox:* A large and fast sulfur-storing spirillum from Ebro Delta microbial mats.** This microorganism was first observed in microcosms constructed in the University of Massachusetts-Amherst from the Sippewissett mats (Woods Hole, MA, USA). The bacterium is 20–30 µm long and 3–5 µm wide, with elemental sulfur globules irregularly distributed throughout the cytoplasm. Unique cell termini were observed in scanning-electron and transmission-electron micrographs. A polar organelle underlies bundles of greater than 60 flagella at each indented terminus. These Gram-negative bacteria bend, flex, and swim in a spiral fashion, moving at speeds greater than 10 body lengths per second [26] (Fig. 12A).

Unlike *Rhodospirillum* or *Thiospirillum*, these large spirilla are not phototrophs, because they survive and grow no differently in light or darkness; rather, they are sulfur-rich heterotrophs. They are far larger and differ morphologically from *Oceanospirillum*, and fail to grow on medium that supports the growth of that genus. *Titanospirillum velox* from a microbial-mat bacterium from the Ebro Delta was grown in mixed culture by dropwise addition of an inoculum of a suspension to tubes with a large airspace and containing a 1-cm^3 piece of microbial-mat inoculum from the original Ebro Delta site in 1–2 ml of filtered, but not sterilized, natural sea water. Enormous populations of small spirilla developed in 2 or 3 days, followed 3–6 days later by the appearance of the huge spirillum. Populations of other bacteria (e.g., small rods and spirilla) and protists (e.g., ciliates and diatoms) developed in all tubes. After 3–4 weeks, the numbers of huge spirilla spontaneously declined. Later, unless transferred to fresh seawater, both spirilla morphotypes disappeared.

The unique characteristics of the polar section of *T. velox* cells merits a detailed description: at each cell pole there is a polar organelle. This proteinaceous, ribbon-like, submembranous structure spans portions of the periphery of different but always flagellated bacteria. Polar organelles underlie the flagellated portion of the cell wall during the developmental cycle, when flagella are present. They are associated with ATPase activity in *Campylobacter* and *Sphaerotilus natans* and presumably function in the release of energy to power the flagella. The presence of polar organelles correlates with motility in bacteria such as *Aquaspirillum* and spirochetes [26]. In Fig. 12B, note the inverse dome in the pole. Under each raised-rim indented cell terminus (Fig. 12C) is a polar organelle, a densely staining line of globular units 5–6 nm in diameter, and a unique conspicuous space (Fig. 12C,D). Bundles of flagella, containing more than 30 flagella rotating in unison, emerge from these vaulted unique cell ends. A similar structure in any other kind of cell, either prokaryotic or eukaryotic, has yet to be identified.

Potential uses of microbial mats in biotechnology

Carbon cycling is closely related to the dynamics of PHAs in microbial mat communities [56,74]. The intracellular storage of these ester carbon polymers is a strategy that increases cell survival in changing environments [9,62,69]. PHAs serve as

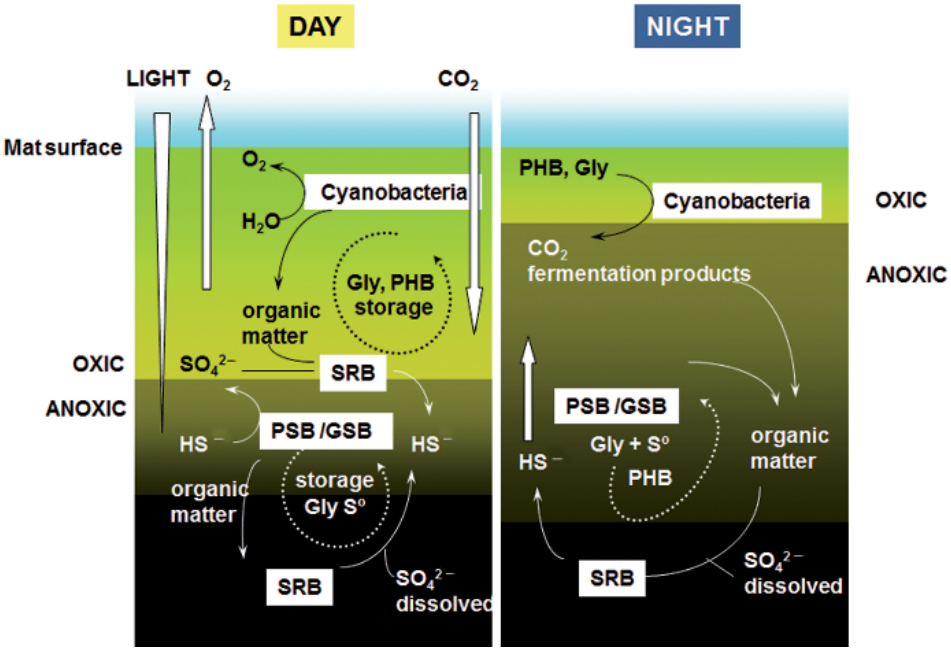

Fig. 13. Ecophysiology of the day-night carbon and sulfide cycles carried out by different populations of a typical marine microbial mat community (Ebro Delta). PHB : poly-β-hydroxybutyrate, Gly: glycogen, SRB: sulfate-reducing bacteria, PSB/GSB, purple/green sulfur bacteria (anoxygenic phototrophs) [27].

an endogenous source of carbon and energy during starvation. They accumulate when a carbon source is provided in excess and another nutrient (such as nitrogen, sulfur, phosphate, iron, magnesium, potassium, or oxygen) is limiting [31]. Since poly-β-hydroxybutyrate (PHB), one of the most abundant PHAs, was first described in *Bacillus megaterium* by Lemoigne in 1926, several studies have demonstrated PHA production by a wide variety of prokaryotes. PHA granules are coated with a monolayer of phospholipids and proteins. The latter play a major role in the synthesis and degradation of PHAs and in PHA granule formation.

Polyhydroxyalkanoate (PHAs) polymers in mats. Microbial mats, as highly diverse and productive systems, accumulate large quantities of PHA under natural conditions and in the community as a whole [56,74]. During the day, oxygenic and anoxygenic photosynthesis predominates over aerobic respiration and sulfatoreduction in the microbial mat ecosystem. Excess carbon not used for growth accumulate in the cells as biopolymers. At the end of the day, when the light intensity decreases drastically, and during the night, respiratory and sulfate reduction activity predominate because photosynthesis is stopped. Anoxic conditions during the night may also trigger the growth of sulfate-reducing bacteria, which may become very active by using the organic matter (reduced products) generated during the day as electron donors for sulfate reduction. Under these conditions, the sulfide concentration inside the mat increases whereas oxygen and readily assimilated organic carbon are quickly depleted (Fig. 13) [27].

Bacteria can synthesize a wide range of biopolymers that serve diverse biological functions but whose material properties also make them suitable for numerous industrial and medical applications. The use of laboratory culture techniques is appropriate in: (i) procuring organisms for fundamental biochemical, physiological, genetic, or developmental studies in which growth in vitro is a prerequisite for the provision of adequate biomass, ensuring the purity of the sample, and thus as a means of study; and (ii) screening and isolating organisms for potential biotechnological application. Most of the PHA-producing strains isolated from Ebro Delta mats belong to the genus *Halomonas* [6,10,76]. Members of the Halomonadaceae are Gram-negative Gammaproteobacteria, chemo-organotrophic, aerobic or facultative anaerobic, moderately halophilic, haloalkaliphilic, halotolerant, or non-halophilic. *Halomonas* strain isolates, such as *H. alkaliphila* (MAT-7, -13, -16), *H. neptunia* (MAT-17), and *H. venusta* (MAT-28), accumulate PHAs in amounts of up 40–60% of the total dry weight. These three different species have been used to determine the influence of different growth modes, i.e., planktonic cells and artificial biofilms [10], on PHA accumulation.

Artificial biofilms consist of cells immobilized by encapsulation on alginate beads. Commercial alginates are produced mainly by brown algae (*Laminaria hyperborea*, *Macrocystis pyrifera*, and *Ascophyllum nodosum*). Alginate is a polymer of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid residues varying in proportions and sequence and yielding com-

pounds of different molecular weights. The ionic interaction of multivalent cations (usually Ca^{2+}) with the blocks of guluronic residues present in alginate results in gelation [58]. Depending on the characteristics of the alginate beads, bacteria growing on their surfaces are able to form microcolony-like cellular aggregates that can be easily detached and released into the surrounding medium (Fig. 14A).

PHA accumulation in cells detached from alginate beads has been compared with that by their planktonic counterparts to determine whether bacterial immobilization enhanced PHA production [10]. In the three strains assayed in that work, i.e., *H.alkaliphila* (MAT-16), *H. neptunia* (MAT-17), and *H. venusta* (MAT-28), PHA accumulation, measured as the relative fluorescence intensity after 48 h of incubation at 30 ºC with an excess of glucose, was higher in cells growing on alginate beads than in planktonic cells (Fig.14B). Thus, to obtain high PHA concentrations, the use of immobilized cells may be a good alternative to bacteria growing in the classical, planktonic mode [10].

Biosurfactant polymers. Other polymers of interest produced by microbial mat bacteria are surface-active biosurfactants (BSs). These compounds allow microorganisms to change their own surface or interfacial properties or those of their surroundings. Surfactants are amphiphilic compounds that can be classified into two groups, low- and high-molecular-weight. Low-molecular-weight BSs are generally glycolipids or lipopeptides and they are more effective at lowering interfacial and surface tension. High-molecular-weight BSs, which are mostly amphipathic polysaccharides, proteins, lipopolysaccharides and lipoproteins, are effective stabilizers of oil-in-water emulsions [4]. In complex systems such as microbial mats, BSs may alter the physical and chemical conditions of the local environment, thus modulating microbial interactions with interfaces.

Using a cultivation step, we obtained heterotrophs from Ebro Delta microbial mats. Colonies grown on TSA and having distinguishing morphologies were selected. According to 16S rRNA analyses, we identified the four strains as *Bacillus licheniformis* (Fig.15). One of the classes of surfactants produced by *B. licheniformis* is lichenysin. These compounds are the most potent anionic cyclic lipoheptapeptide BSs produced when glucose is used as the carbon source. Their secretion by *B. licheniformis* is stimulated by the presence of excess glucose, as was the case in the isolation conditions, in which 10% glucose was added [4]. All four strains had similar capacities to lower the surface tension of the culture medium from 65 to 35 mN/m.

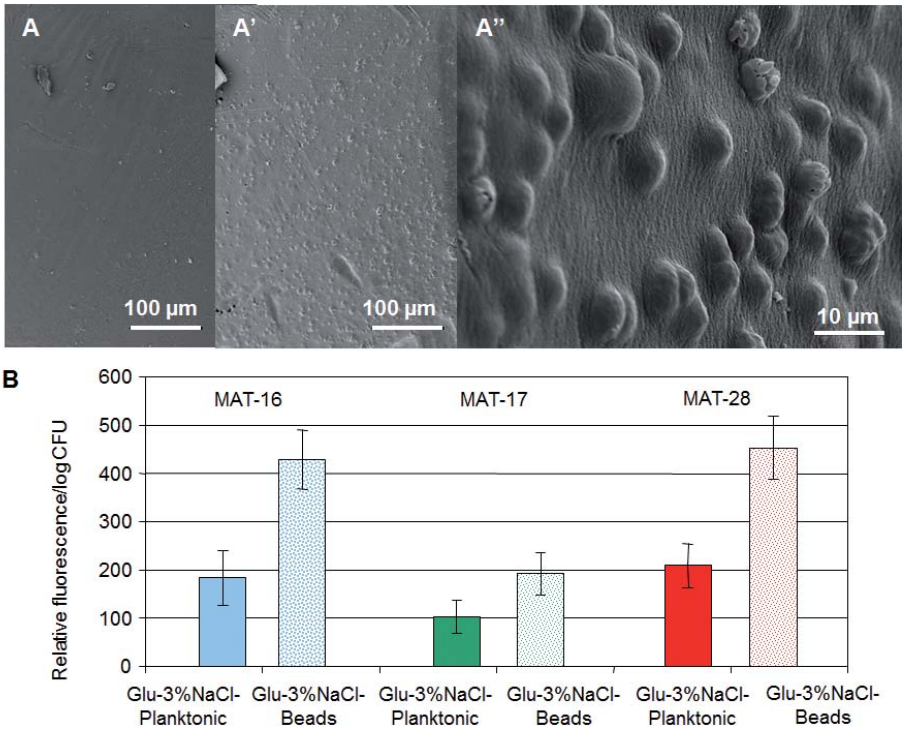

Fig. 14. Scanning electron micrograph of immobilized cells of *Halomonas* strain MAT-28 at time 0 (**A**), and after 48 h of incubation (**A'**). (**A''**) Microcolonies formed at the surface of a bead and about to detach. Note that several individual cells are protruding from the bumps produced by the presence of the microcolonies (arrows). (**B**) PHA accumulation in *Halomonas* spp. was measured spectrofluorometrically after 48 h of incubation at 30 ºC in minimal medium with glucose (Glu) and 3 % NaCl; and in two growth modes: planktonic or artificial biofilm (alginate beads). Data shown are the average of the results of four independent experiments [10].

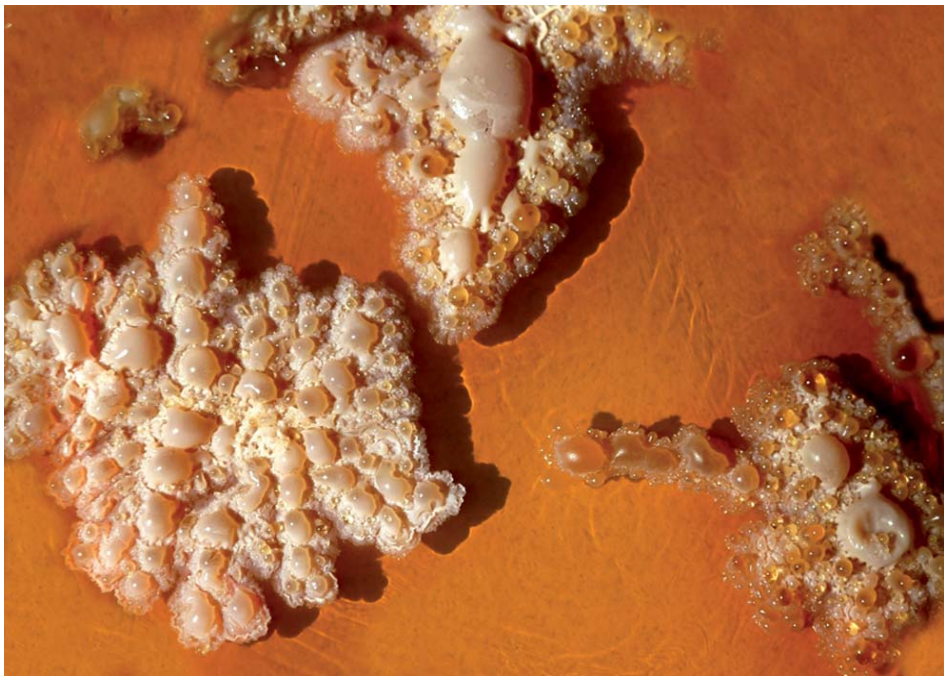

Fig. 15. Three *Bacillus licheniformis* strains growing in MRS medium (named after its inventors: de Man, Rogosa, and Sharpeh).

Marine microbes have been scarcely explored for their ability to produce polymers such as PHAs and BSs. Microbial mats constitute a potential source for the isolation of new polymer-producing strains, although culture conditions must be optimized to obtain polymer concentrations that are high enough to be industrially and commercially exploited.

Life on and beyond the Earth

The origin and evolution of life on Earth are the result of three major events that have modified our planet, making it completely different from Venus and Mars: biopoiesis (the origin of life), ecopoiesis (the origin of ecosystems, initially represented by stromatolites and microbial mats), and eukaryopoiesis (or eukaryosis, the origin of nucleated cells) [25].

The Earth became independent of the proto-planetary solar disk about 4550 ± 20 million years ago. Biopoiesis might have taken place on our planet as early as 3850 ± 50 million years ago. While it is possible that the initial features of our two neighbors in the solar system were conducive to the appearance of life, most likely, it could not have been maintained on either planet. Probably, on the young Earth (early Archaea Eon) life appeared several times in different manners. But life is a non-stochastic phenomenon that contradicts (at least locally and temporally) the second law of thermodynamics: that entropy always increases. Although there was a certain probability that life would appear, the probability that it would be destroyed was much higher.

The minimal unit of life that we know is the cell. Cells exploit the medium in which they live and multiply. The residues of their metabolism cannot serve as their own further nutrients. Thus, the cellular environment is eventually depleted of food and no longer able to sustain life. However, cells have also evolved to use other foods, which, again, will be eventually exhausted. If the succession of cell types is sufficiently long, with each one able to feed on the residues of previous ones, then a closed food web becomes possible, with the residue of the last cell type able to feed the first cell type. This cycle of metabolic products, the product of ecopoiesis, happened on the Earth, at least once, and allowed life to persist: it is evidenced in the earliest ecoystem among prokaryotes. Thus, the stromatolites of Warrawoona (3500 million years old) can be considered as one of the first known and most primitive ecosystem and their existence implies that ecopoiesis happened on Earth when life was very young, only 300 million years old, at the most. In the absence of ecopoiesis, all life on Earth would be extinguished, as all available chemical elements would eventually be depleted.

In general, the growth of each individual population can be expressed by the adaptation of the Monod's equation: $dP / dt = \mu P$; where P is the population density in a given time (t) and μ is the specific growth rate of the population. The value of μ depends on both favorable conditions (K), such as nutrients, water, light, pH and temperature, and negative or deleterious conditions (ω), such as outflow, predation, lysis and sedimentation, which reduce the numbers of cells in the population. If $K > \omega$,

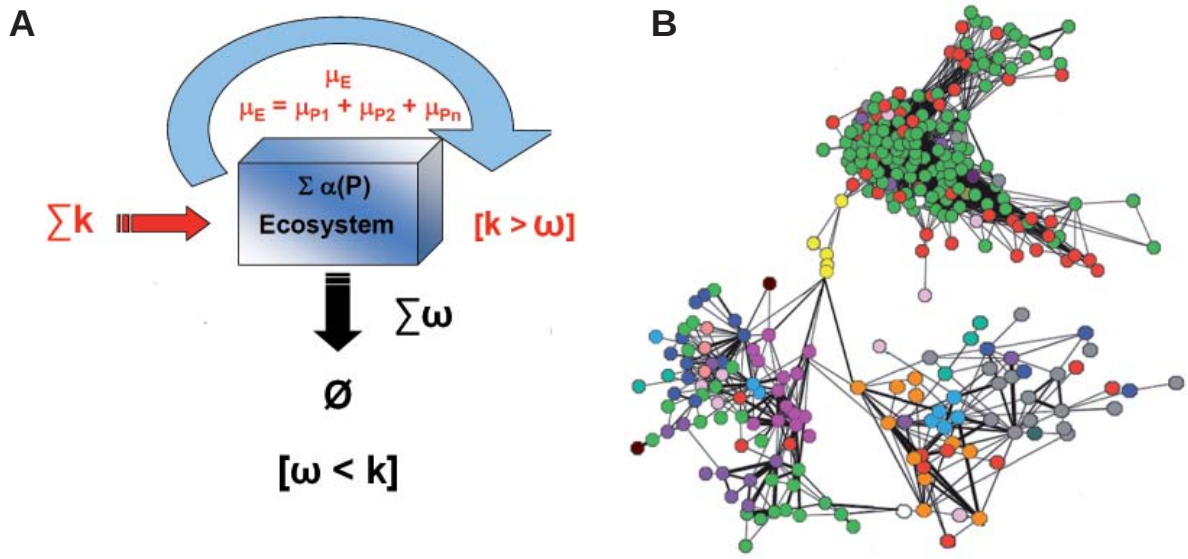

Fig. 16. (**A**) The growth of a bacterial population follows Monod's equation, in which the number of individuals in each moment (and therefore the probability of being in stationary, exponential or death phases) depends on the initial number and the specific growth rate (μ). The specific growth rate, in its turn, depends on favorable (K) and unfavorable (ω) conditions for growth. (**B**) Interactions among different populations in an ecosystem. The prediction of pairwise relationships were inferred from operational taxonomic units (OTU). Each node represents an OTU, and each edge represents a significant pairwise association between them.

then $\mu > 0$, and the population increases, whereas if $K < \omega$, then $\mu < 0$, and the population decreases. In the second case, if ω is much higher than K, then $\mu << 0$, leading to the death of the population. The growth of a community depends on the growth of each population. If there is energy flux and matter recycling, the ecosystem will persist [27] (Fig. 16).

Prokaryotes experience their environment and respond as individual cells to specific environmental challenges; but they also act cooperatively, carrying out activities as a community [29,63]. In many microbial ecosystems, the functionally active unit is not a single species or population (clonal descendants of the same bacterium) but a consortium of two or more types of cells living in close symbiotic association. Microbial mats can be considered to be extant early ecosystems (see Fig. 3, Warrawoona). Although present-day mats can reach high degrees of complexity, initially they must have been very simple. They fit well with the concept of a minimal ecosystem, whose five basic components are: (i) properties, which are state variables; (ii) forces, which are outside energy sources or casual forces that drive the system; (iii) flow pathways, which are energy or material transfers that connect properties with one another and with forces; (iv) interactions, which are functions by which forces and properties modify, amplify, or control flows; and (v) feedback loops, which are circuits through which matter or energy flows and influences an "upper stream" component or flow [27].

The initial conditions on Earth was different, as not only was there a flow of energy (as is, inescapably, the case today), there was also a flow of matter. While energy comes mainly from the sun, matter is restricted to that retained when our planet accreted material and became independent of the protoplanetary solar disk. Thus, the Earth's matter is limited.

An ongoing example of the process is the biocenoses around deep-sea vents. The source of energy is the sulfide emanating from the vents. Sulfide is used by sulfur bacteria as a source of energy, and the multiplication and metabolism of those bacteria yield nutrients that feed a diversity of animals, including long pogonophora (*Riftia*), giant clams (*Calyptogena*), and strange types of crabs.

In 1986, one of us (RG), together with Lynn Margulis and James Lovelock, visited the volcanic caldera of Kilauea, in Hawaii Island. On the walls, near sulfarola emissions, we observed patches of biofilms (50 cm to 3 m wide) with laminated layers not described before (Fig. 17). The top layer was covered by cyanobacteria with dark brown sheaths, probably belonging to the genera *Fischerella/Mastigocladus*. The dark pigmentation of the cyanobacterial cells was indicative of the stressful conditions under which these organisms were living (high temperatures and ultraviolet irradiation). Below, there was a yellowish-transparent layer similar to gelatin and perhaps composed of sulfur chemolithotrophic bacteria and several populations of heterotrophs. However, the typical black layer observed in microbial mats or sediments in which the dominant microbiota are sulfate-reducing bacteria was absent. (The black color is due to the formation of pyrites as a consequence of sulfate reduction.) In this system there are inputs of energy, solar radia-

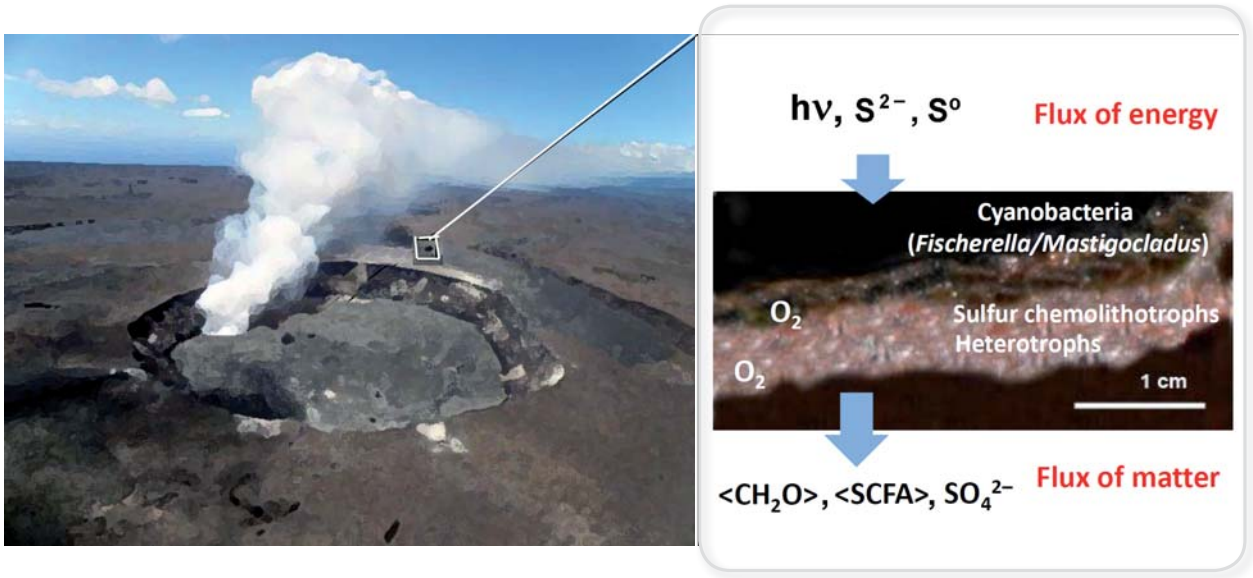

Fig. 17. Illustration of volcanic caldera in Hawaii and biofilm systems in which there were a constant input of energy (solar and reduced compounds), and a not complete cycling of the matter. hν, radiant energy (usually, light); <CH_2O>, organic matter; SCFA, small carbon fatty acids (acetate, butyrate, etc.)

tion, and reduced sulfur compounds, but not a complete matter cycle. If the sulfarola becomes extinct, this microbial system will disappear. Early ecosystems (microbial mats), unlike this example in Hawaii, may have had a constant input of chemical elements. But this situation, which prevented the depletion of biogenic elements on the surface of the planet, would last a maximum of 200 or 300 million years, after which primitive life would be extinct (see Fig. 16).

Bacteria were the earliest life form to evolve. Their unprecedented activities in photosynthetic and chemical production, cell reproduction, genetic recombination, networking, architecture, emission of gases, and other environmental changes altered the Earth's surface long before the evolution of a single eukaryote. The first 2000 million years of prokaryotic evolution saw the development of almost all metabolic strategies. Symbiotic associations among prokaryotes gave rise to the ancestors of all the complex and varied biological forms that followed and that now exist on Earth. Prokaryotes were the basis on which all other forms of life arose. They served as the origin of the eukaryotic cell, or eukarypoiesis, in the form of protists (i.e., protozoans, unicellular algae, etc.), which were the first eukaryotic-celled organisms. Indeed, all such organisms emerged within a prokaryotic world and have retained intimate connections with, and dependency upon, prokaryotes.

"Our sensibilities come directly from the world of bacteria. The vast numbers of incessantly moving but mute bacterial denizens ignore us as they eat, grow, and reproduce, as we ignore them. Very few, only the "freaks," poison us or directly feed on us in ways that injure us. It is the notorious self-centeredness of our species that leads to the manic claim that all bacteria are killer germs to be eradicated from our lives. We should not malign our own ancestors with harsh words. Our intolerant slogans denigrate the non-human life with which we share the planet. The bacterial patina more likely will rid this planet of us, the voluble, ignorant ape, far sooner than we will cause any type of bacteria to disappear. No matter how we protest and what we proclaim, they most likely will thrive, frolicking to grow and reproduce in their own way, long after *Homo sapiens* extinguishes" (L. Margulis, 2006. In: Bernasconi et al., (eds) Questioni di natura e cultura: non solo DNA. Cellule e genomi - V corso).

The Gaia concept recognizes a planet-wide physiological control system at the Earth's surface. Temperature, atmospheric gas composition, acidity-alkalinity, and oceanic salinity are among the factors regulated by metabolism, growth, interaction, extinction, and other processes characteristic of life. Interstellar space is not empty, but overflows with organic and inorganic material. It is estimated that in the volume determined by a parsec (i.e., a cubic parsec) there is enough matter to form more than 200 Earths. Note that a parsec is about 3,000,000,000,000,000,000 cm, corresponding to more than 3 light-years. From the cosmic point of view, the Earth is only a small planet, ranking third in order of distance from its star, the Sun, which in turn is an average star in an average galaxy, located in a not-special place in the intergalactic void. The origin and development of the Earth and the evolution of life have been contingent phenomena whose occurrence was never certain. Thus, life could have developed either one way or another. A single line of events that actually

took place gave origin to the planet, to life, to ecosystems, to eukaryopoiesis, and eventually to our species. Despite both the certainty of life, as a necessary continuity of the laws of physics, and its very possible ubiquity, we might ask whether somewhere else in the universe, in the cosmic vastness, either a long time ago, at present, or in the future, there could be exactly the same vital phenomena that have emerged on this inconspicuous planet that we call Earth.

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