Microbial Diversity and Biosignatures: An Icy Moons Perspective

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Abstract :

The icy moons of the outer Solar System harbor potentially habitable environments for life, however, compared to the terrestrial biosphere, these environments are characterized by extremes in temperature, pressure, pH, and other physico-chemical conditions. Therefore, the search for life on these icy worlds is anchored on the study of terrestrial extreme environments (termed “analogue sites”), which harbor microorganisms at the frontiers of polyextremophily. These so-called extremophiles have been found in areas previously considered sterile: hot springs, hydrothermal vents, acidic or alkaline lakes, hypersaline environments, deep sea sediments, glaciers, and arid areas, amongst others. Such model systems and communities in extreme terrestrial environments may provide important information relevant to the astrobiology of icy bodies, including the composition of potential biological communities and the identification of biosignatures that they may produce.

Extremophiles can use either sunlight (phototrophs) or chemical energy (chemotrophs) as energy sources, and different chemical compounds as electron donors or acceptors. Aerobic microorganisms use oxygen (O_2) as a terminal electron acceptor, whereas anaerobic microorganisms may use nitrate (NO^{-}_{3}), sulfate (SO^{2-}_{4}), carbon dioxide (CO_2), Fe(III), or other organic or inorganic molecules during respiration. The phylogenetic diversity of extremophiles is very high, leading to their broad dispersal across the phylogenetic tree of life together with a wide variety in metabolic diversity.

Some metabolisms are specific to archaea, for example, methanogenesis, an anaerobic respiration during which methane (CH_4) is produced. Also sulfur-reduction performed by some bacteria and archaea is considered as a primitive metabolism which is restricted to anoxic sulfur-rich habitats in nature.

1 Introduction

Most definitions of a "living" process or system (Cleland and Chyba 2002) refer to three distinct properties: the ability to self-organize complex macromolecular structures, the ability to harness the energy necessary to maintain separate organization from the environment ("metabolism"), and an ability to replicate the self and to proliferate more or less identically. Thus, self-organization, metabolism and self-replication are the characteristic "cornerstones" of any living entity. This conceptual definition is the result of a long historical process, which has forced biologists of subsequent eras to redefine their understanding of what is, and is not, alive.

Regardless, a general and consensual definition of "living" is a matter of concern to many fields—physicists, chemists and astrobiologists—all of whom seek to recreate life-like behaviors, or to identify its signatures. A challenge is the continual confrontation only with terrestrial biology composed of a remarkably common base of molecular components (nucleic acids, proteins, lipids), each of which is dedicated to a main specific function: the conservation and handling of information for nucleic acids, structural organization and biochemical catalysis for proteins, and spatial delineation of compartments for lipids. On Earth, the co-occurrence of these building blocks indicates an affiliation with the "living" world.

The issue of the origin of life is usually addressed from two different perspectives: the first examines the conditions under which the basic building blocks and macromolecules significant for life may have emerged on the early Earth, the second explores the origin of functional subsystems (metabolism, replication) and basic structural organization (i.e., the cell) of what is recognized as "alive".

Experiments from the 1920s onward (Oparin 1924; Haldane 1929; Urey 1951; Miller 1953) led to the hypothesis of multiple "possible" scenarios of the origin of life (on Earth), often conflicting and irreconcilable, and typically almost mythological "stories" of physically and/or chemically possible processes, of which only small parts had an empirical basis. Despite their value in the inception of a debate on the origins of life, these hypotheses were often based on what we now know to be inaccurate or incomplete data (early Earth atmospheric composition, the genesis and dynamics of the Solar System, etc.). Early reports on the diversity of life (Woese 1979) also failed to consider the incredible metabolic sophistication that microbial life has shown in acquiring energy from its environment, even under the most inhospitable and extreme conditions. Clarification of the co-evolution of the

biosphere and geosphere has led to an increasing recognition of the fact that the two are intimiately associated and likely constrained the development of the other (Lovelock and Margulis, **?**lm74; Williams and Fraústo Da Silva 2003). <ref:lm74?>

The Earth is generally thought of as a world inhabited by plants, animals and microorganisms able to grow under conditions compatible with life as it is found in most terrestrial and marine ecosystems (temperature: 10–40 °C, pH $\sim$ 7, pressure: 1 atm, water availability, minimal ionizing radiation level, etc.). Extremophiles have succeeded in inhabiting environmental niches with physicochemical parameters outside this comfort zone.

Extreme environments are characterized by environmental parameters at the boundaries of conditions that sustain and shape life in its various forms; whether terrestrial, oceanic, cryospheric or deep endolithic, they are widespread on our planet. Far from being marginal areas, they (especially the deep ocean and polar regions) represent, in terms of biomass volume, the most important part of the Earth biosphere.

In these extreme environments, dominated by prokaryotic microorganisms (Bacteria and Archaea), some organisms thrive under conditions that are at the limits of their physiological and energy potential, whereas others have highly adapted genetic features that result in acrucial requirement of such conditions. When classifying microorganisms as extremophiles, the concept of a "normal" environment is used as a reference. In assuming this anthropocentric view, it should not be forgotten that "extreme" environments, which today seem so hostile, appear to have predominated when the first life forms appeared on Earth. Nowadays, these environments are still colonized by highly diverse microbial communities.

Depending on the prevailing physico-chemical parameters of the environment, extremophiles can be subdivided into different categories: hyperthermophiles ($T_{opt} \geq 80$ °C) e.g. *Methanopyrus kandleri*, () the archaeon with the highest temperature life record); psychrophiles ($T_{opt} \leq 15$ °C) such as the bacterium *Psychrobacter fulvigenes* (Romanenko et al. 2009) capable of growing at temperatures as low as -5 °C; acidophiles ($pH_{opt} \leq 3$) including *Picrophilus oshimae* (Schleper et al. 1995), an archaeon that has shown optimal growth at pH = 0.7; alkaliphiles (pHopt $\geq$ 9) such as *Bacillus pseudofirmus* (Nielson et al. **?**ni95), <ref:ni95?> capable of growing at pH 11, halophiles such as the archaeon *Halobacterium salinarum* (Ventosa and Oren 1996), which can survive in the presence of 5.5 M (32%) NaCl (its saturation limit); and piezophiles, e.g. *Thermococcus piezophilus* the archaeon that holds the record for withstanding the highest hydrostatic pressure (130 MPa, i.e. 1300 times atmospheric pressure) (Dalmasso et al. 2016).

Extremophiles expand our understanding of biodiversity on Earth and our knowledge of the limits of life. Deducing the mechanisms that enable extremophiles to persist under harsh conditions not only provides a thorough knowledge of the functioning of living cells but can also lead to interesting applications in biotechnology, particularly the economic utility of extremophiles. Understanding the uncommon properties of extremophiles has led to questions about their origin (have these organisms recently adapted to the extreme conditions of their environment or are they relics of organisms that existed on the Early Earth and that had to face even harsher environmental conditions?). Understanding the limits of life on Earth can provide hints of the diversity of potential extraterrestrial life (past or present). It is therefore not surprising that astrobiology studies the properties of life in Earth's extreme environments.

This review is dedicated to describing the state of the art and raising questions about taxonomic and metabolic diversity and the evolution of microorganisms (archaea and bacteria), notably extremophiles, and their biosignatures with an astrobiological perspective.

2 Extremophiles: Diversity, Adaptation and Biosignatures

Over the past forty years, research has dramatically altered our understanding of the limits of life in terms of its physical and chemical constraints. Organisms, mainly prokaryotes, have been found to live optimally at very high or very low temperatures, in hyperacid or alkaline environments, or in salt-saturated environments, for example. Other organisms are able to live or survive under conditions of extreme stress, for instance a lack of water, the presence of high concentrations of heavy metals, or exposure to significant radiation doses or extreme pressures. In the following, we review and offer perspectives on extremotolerances to hypersaline and high hydrostatic pressure environments that are of particular relevance to the icy oceanic bodies of the outer Solar System.

2.1 Hypersaline Biotopes

Habitats with salinities higher than average seawater (i.e. 3.5% total dissolved salts) are considered hypersaline. Many of these habitats result directly from the evaporation of sea water, and thus have similar relative proportions of ions; for example, they are dominated by sodium and chloride. Marine hypersaline environments are termed thalassohaline, in contrast to athalassohaline environments, which have non-marine ionic compositions and are associated with non-coastal water bodies (DasSarma and Arora 2001; Rodríguez-Valera 1988).

A profusion of hypersaline biotopes, distributed across the Earth, can be found in arid, coastal and even deep-sea settings (e.g. Antunes et al. 2011; DasSarma and Arora 2001; Oren 2002a, 2002b). In coastal regions, seawater often penetrates through seepage or narrow inlets creating small evaporation ponds. Well-known examples of such ponds are Solar Lake and Gavish Sabkha near the Red Sea coast, Guerrero Negro on the Baja California peninsula (Mexico), Lake Sivash near the Black Sea (Crimea), and Shark Bay in Western Australia. Such hypersaline evaporation ponds have also been found in Antarctica (e.g. Deep Lake, Organic Lake and Lake Suribati). Elevated salinities are usually found in natural inland hypersaline lakes such as the Dead Sea (Middle East) and the Great Salt Lake (USA), the two largest and best-studied such environments. A number of alkaline hypersaline soda brines also exist, including the Wadi Natrum lakes of Egypt, Lake Magadi in Kenya, the Great Basin lakes of the western United States (Mono Lake, Owens Lake, Searles Lake and Big Soda Lake), and several series throughout China and India.

The number of hypersaline sites is further increased by the numerous artificial solar salterns constructed for the production of sea salt, by subterranean brines and evaporite deposits and by the existence of several brine-filled deep-sea basins. Another type of hypersaline biotope is presented by the often-overlooked saline soils. These include desolate areas in such places as Death Valley (California, USA), Alicante (Spain), Iraq and even the Dry Valleys in Antarctica, amongst others (Ventosa et al. 1998).

Anoxic hypersaline basins, or deep-sea anoxic brines, are very special and rare environments in the oceans. They are formed as a result of tectonic activity and exposure of ancient salt deposits, existing under layers of sediments and originated from evaporated ancient seas (e.g. Antunes et al. 2011; Antunes 2017). The interaction of seawater with the underlying salt leads to the formation of brines which are 4 to 5 times more concentrated in salt than the surrounding seawater, creating highly saline “lakes” on the sea floor (Camerlenghi 1990). The presence of such basins often coincides with the presence of cold seep zones or, more rarely, hydrothermal vents, resulting in the release of methane, hydrogen sulphide and hydrocarbons. One of the characteristics of anoxic hypersaline basins is the

presence of multiple gradients, particularly at the interface between seawater and the hypersaline zone (brine), including salinity, temperature, free O_2, density and pH (Antunes et al. 2018). These physico-chemical gradients provide highly variable and specific environments of interest for the growth of microorganisms. In addition, the density gradient formed at the seawater/hypersaline zone interface acts as an organic and inorganic particle trap, providing the significant amount of nutrients necessary for cell growth (Daffonchio et al. 2006). It is possible to distinguish differences between the known brine lakes of different seas; in the Mediterranean Sea, concentrations of Mg^{2+}, SO_4^{2-} and K^+ are high, whereas in the Red Sea, concentrations of Ca^{2+} and Mn^{2+} are higher. On the contrary, lower ionic concentrations, particularly Mg^{2+} and K^+, exist in the Gulf of Mexico (Antunes et al. 2011).

The relevance of deep-sea brines in the context of the exploration of the oceans of the icy moons the outer solar system is particularly worth highlighting as they have been recently proposed as potential terrestrial analogues to conditions in such exooceans (Antunes et al., accepted).

2.2 Biodiversity in Hypersaline Environments

Despite being considered extreme, hypersaline environments host a diverse variety of organisms, including representatives from all three domains of life. In fact, microbial densities can be so high in these locations that the thriving communities of pigmented halophilic microorganisms (which includes a few bacteria but is composed mostly of halophilic archaea and/or the β-carotene-rich green alga *Dunaliella*) often give the water characteristic pinkish or even reddish hues. The inhabitants of saline environments range from higher organisms to unicellular eukaryotic microorganisms, and a heterogeneous group of prokaryotes, which constitute the predominant microflora (Rodríguez-Valera 1988; Ventosa and Nieto 1995).

2.2.1 Eukarya in Saline Environments

Within the domain Eukarya, halophiles are scarce, and mostly restricted to unicellular forms (Oren 2002b; Trüper and Galinski **?**tg86). A variety of plants (e.g. *Atriplex halimus*) can survive in moderately high saline soils, although apparently no vertebrate has ever been reported at salinities higher than 1 M NaCl (DasSarma and Arora 2001; Ollivier et al. 1994). The most common multicellular eukaryotes in hypersaline environments are invertebrates, with reported species including rotifers, tubellarian worms, copepods, ostracods, and insects. Noteworthy among the insects are the well-known brine flies (*Ephydra hians* and *E. gracilis*) and brine shrimp (*Artemia franciscana* and *A. salina*), with the latter playing an important role in the nutrition of the pink flamingo and other birds (DasSarma and Arora 2001; Ventosa and Nieto 1995). <ref:tg86?>

Dense populations of unicellular green algae can be observed at moderately high salinities, with most being moderate halophiles and only very few examples observed at higher salinity level (e.g. *Dunaliella salina* and *Asteromonas gracilis*). The several species of the genus *Dunaliella* are almost ubiquitous in hypersaline environments, being often the main or only primary producer and serving as main food source for brine shrimps and larvae of brine flies, while representatives of diatoms are also frequently found but rarely abundant (DasSarma and Arora 2001; Oren 2002a).

Other eukaryotic representatives include a large variety of protozoa (e.g. *Porodon utahensis*) as well as yeasts and other fungi (DasSarma and Arora 2001). These groups of organisms are very often overlooked when looking at the microbiology of high salinity

environment but our knowledge about their diversity has been getting increased attention (excellently reviewed by e.g. Gunde-Cimerman et al. 2009; Hardy and Simpson **?**hs17; Zajc et al. 2017). <ref:hs17?>

2.2.2 Archaea in Saline Environments

Extreme halophiles are traditionally associated with the euryarchaeal class *Halobacteria*, which was recently reorganized (Gupta et al. 2015) and split into 3 different orders—*Halobacteriales*, *Haloferacales*, and *Natrialbales*—and is still undergoing taxonomic restructuring based on phylogenomic data (e.g. Gupta et al. 2016). As of September 2019, this family of aerobic euryarchaeotes currently comprises 259 species with validly published names, placed in 63 genera (Table 2). An interesting member of the *Halobacteriaceae* is the more recently isolated first representative of the square haloarchaea of Walsby, *Haloquadratum walsbyi* (Bolhuis et al. 2004; Burns et al. 2004). This intriguing group of microorganisms was first reported by Walsby (1980) but remained elusive despite numerous cultivations attempts and well-known widespread and abundant occurrence.

Extremely halophilic archaea are less common outside the *Halobacteria* but can also be found within some euryarchaeal genera namely within the class *Methanomicrobia* (e.g. *Methanosalsum*, *Methanohalobium*, *Methanohalophilus*, within the family *Methanosarcinaceae* and *Methanocalculus*, within an unassigned family of the order *Methanomicrobiales*, and the recently described genus *Methanonatronarchaeum* of the class *Methanonatronarchaeia*; Oren 2014; Ventosa et al. 2012; Sorokin et al. 2018). In addition to these, a few other methanogenic genera are also known to include moderately halophilic species (Ventosa et al. 1998). Aside from these methanogens, and the *Halobacteria*, no other archaeal halophiles have been identified outside the Euryarchaea.

2.2.3 Bacteria in Saline Environments

Overall, halophilic bacteria are a very diverse and heterogeneous group. Phylogenetically they are included in at least seven phyla: *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, and *Thermotogae* (Ventosa et al. 2012).

Compared to the Archaea, fewer examples of extreme halophily are currently known in Bacteria but their numbers have increased rapidly in the last few years. Some examples of this wide diversity include the actinomycete *Actinopolyspora halophila*, several gammaproteobacteria of the genus *Halorhodospira*, and *Salinibacter ruber*, which is a member of the *Cytophaga-Flavobacterium-Bacteroides* group (Antón et al. 2000, 2002; Kamekura 1998). *Salinibacter* is especially interesting due to its significant contribution to the biota of NaCl-saturated saltern crystallizer ponds. The surprisingly numerous similarities with the haloarchaea, specifically in osmotic adaptation strategy, point to a possible process of convergent evolution (Antón et al. 2002; Oren 2004).

Moderately halophilic bacteria, however, are much more diverse, being present in many of the major bacterial phylogenetic groups. The vast majority of the validly described moderately halophilic bacteria are members of the Proteobacteria, with the gamma-subgroup, namely the genera *Salinivibrio, Marinobacter*, and *Arhodomonas*, as well as members of the family *Halomonadaceae*, being especially preponderant. The *Halomonadaceae* includes some of the most versatile prokaryotes regarding their adaptability to a wide range of salinities (Oren 2000; Ventosa et al. 1998). *Rhodospirillum salinarum*, an anaerobic phototroph, and *Desulfovibrio halophilus* and *Desulfohalobium retbaense*, both anaerobic sulphate reducers, are further examples of organisms with wide ranges of salinity tolerances within

the alpha- and delta-Proteobacteria, respectively (Galinski and Trüper 1994; Ollivier et al. 1994).

The *Halanaerobiales*, an order within the low G+C branch of the Gram-positive bacteria includes the families *Halobacteroidaceae* and *Halanaerobiaceae*, other very important and numerous groups of moderately halophilic bacteria (Rainey et al. **?**ra95). Further representatives are found in the low G+C and high G+C Gram-positive bacteria, the cyanobacterial branch, the *Cytophaga-Flavobacterium-Bacteroides* branch, and also within the spirochetes and the actinomycetes (Ventosa et al. 1998). <ref:ra95?>

2.2.4 Physiological Adaptations to High Salinity

Life at high salinity is not without its burdens. Increased salinity leads to a decrease in water activity (i.e. the amount of water that is thermodynamically available) which, in accordance with the natural tendency of systems to attain and maintain equilibrium and the permeability of the cytoplasmic membrane to water, afflicts cells with osmotic stress (Brown 1990; Csonka 1989; Vreeland 1987). Indeed, an unadapted organism placed in a saline environment (i.e. hyperosmotic conditions) will rapidly lose water, leading to decreased cell volume and/or turgor pressure and ultimately affecting its metabolism and macromolecules (da Costa et al. 1998; Poolman and Glaasker 1998). Failure to adjust to these new conditions results in cessation of growth, possibly due to molecular crowding and a consequent reduction in diffusion rates of proteins and metabolites, which may eventually result in cellular death (Kunte et al. 2002). Evolution has provided life with two different approaches to deal with osmotic stress:

The salt-in-cytoplasm strategy. Using this strategy, the necessary thermodynamic adjustment of the cell is achieved through an increase in cytoplasmic salt concentration (normally through an increased intake of K^+ and Cl^-). The resulting increase in intracellular ionic strength requires several changes in cellular function, most markedly at the level of the enzymatic machinery, resulting in a characteristic excess of acidic amino acids and small amounts of hydrophobic amino acids (da Costa et al. 1998; Oren 1999). The predominance of charged amino acids on the surface of enzymes and ribosomes stabilizes their hydration shells under high ionic conditions. Moreover, most of these enzymes are only functional at increased ionic levels (da Costa et al. 1998; Galinski and Trüper 1994). The permanent character of these cellular modifications restricts organisms that use this strategy to highly saline environments.

This salt-in-cytoplasm strategy was first discovered in aerobic, extremely halophilic archaea of the order *Halobacteriales* and is considered the typical archaeal strategy of osmoadaptation (Kunte et al. 2002). This strategy is also used by anaerobic halophilic bacteria of the order *Halanaerobiales* and the aerobic halophilic bacteria *Salinibacter ruber* (Oren 2000).

The organic-osmolyte strategy. This strategy relies on an increase in external salinity being counteracted by the accumulation (either by *de novo* synthesis or uptake from the environment) of uncharged, highly water-soluble, organic solutes (Kempf and Bremer 1998) (Fig. 1). These osmolytes do not disrupt metabolic processes and include sugars (e.g. trehalose), polyols (e.g. glycerol) and their derivatives, free amino acids (e.g. glutamate) and their derivatives, betaines, and ectoines (Csonka 1989; da Costa et al. 1998; DasSarma and Arora 2001; Galinski and Trüper 1994). This strategy allows organisms to keep their cytoplasm free of NaCl, to a large extent, while avoiding the need for major changes in cellular machinery, thus providing a higher physiological flexibility. This explains the characteristically wide salt tolerance ranges associated with the use of this type of osmotic adaptation.

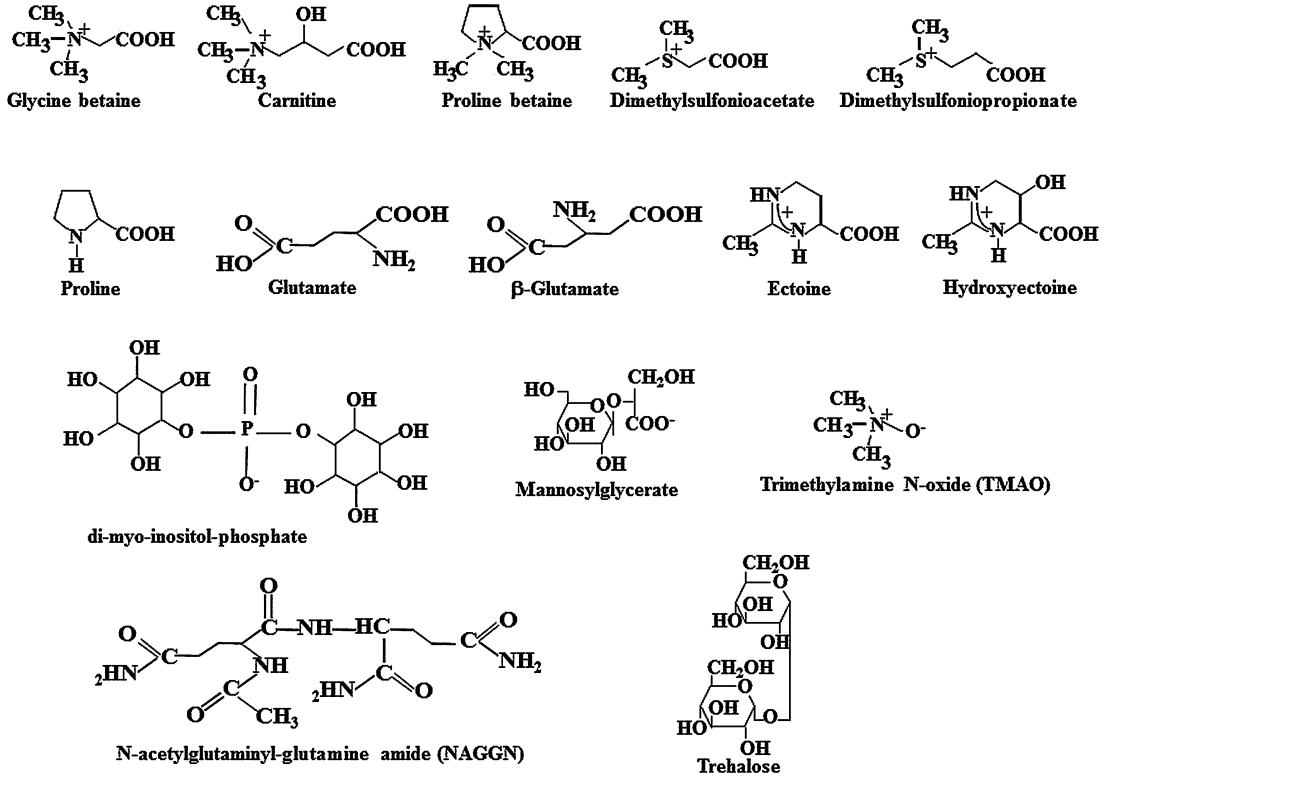

Fig. 1 Chemical structures of selected compatible solutes or osmolytes

The organic-osmolyte strategy is widespread among Bacteria, Eukarya and some Archaea. Indeed, some methanogenic along with some haloalkaliphilic Archaea are known to use a combination of both strategies (Desmarais et al. 1997).

2.3 High Hydrostatic Pressures Biotopes

The Twentieth Century was marked by technological and scientific breakthroughs that have drastically modified the way we understand life on our planet. It was demonstrated that unicellular prokaryotic life forms are able to inhabit virtually any environment on Earth, and that they constitute life's largest diversity reservoir. The domain Archaea was created to accommodate newly isolated prokaryotic organisms with specific features that make them more similar to eukaryotes. Recent estimates also suggest that life dwells mostly underground (Reith 2011; Colwell and D'Hondt 2013; Colman et al. 2017) and that this deep biosphere, located in the continental subsurface and in the oceans below 1000 m in depth, could represent up to 70% of all cells on Earth, and up to 50% of (Oger and Jebbar 2010) the primary production of biomass. Most of these biotopes are oligotrophic in nature and characterized by high hydrostatic pressures (HHP). Although the deep biosphere represents the largest ecosystem on Earth, however, it is still poorly characterized in terms of diversity and its mechanisms of adaptation to HHP.

Amongst deep-biosphere biotopes, hydrothermal vents may be the most intriguing. Discovered in 1979, they were shown, despite being hot oligotrophic and HHP environments, to harbor abundant primary productivity and diversity (Corliss et al. 1979). Primary production, in these environments, is based exclusively on the anaerobic chemical harvest of the energy of the geologically sourced fluids seeping through the ocean floor. Because of this, they are the only ecosystems on Earth not linked to photosynthesis, or photosynthesis-derived products such as O_2. It has been postulated that deep-sea hydrothermal vent systems were the birth sites of life on Earth (e.g., Martin and Russell 2003; Russell et al. 2010) and this item is described below in more details in Part 4 of this review.

HHPs are ubiquitous in deep environments. Hydrostatic pressure increases with depth at an approximate rate of 10 MPa (∼100 atmospheres or 100 bar) per km in the water column and 30 MPa per km in the crust. The definition of the deep biosphere is conveniently and arbitrarily defined as water depths of 1000 m and more (Jannasch and Taylor 1984). Consequently, all environments above 10 MPa qualify as high-pressure biotopes. HHP waters encompass 88% of the volume of the oceans—which have an average depth of 3800 m—and thus an average hydrostatic pressure of ca. 38 MPa, but reach 110 MPa in the trenches. In contrast, the average geothermal gradient in the continental system is ca. 25 °C km^{-1} (Oger and Jebbar 2010). The current temperature limit for life, 122 °C (Takai et al. 2008), would thus place the "deep" limit for the putative continental biosphere at ca. 5 km below ground on average, under maximal pressures of 150 MPa. Most of the Earth's prokaryotes live in these subsurface oceanic and terrestrial environments. From current knowledge of the deep-biosphere their cell number is estimated at 3.5×10^{30} and ca. $2–6 \times 10^{29}$ respectively i.e. about 10 times that estimated for surface environments (Whitman et al. 1998; Magnabosco et al. 2018). Thus, even though the maximal productivity of the high-pressure continental or marine biosphere is orders of magnitude lower than that of the surface biotopes, due to their extremely large volume, these high-pressure biotopes contribute significantly to the production and recycling of organic carbon (Fig. 2) (Magnabosco et al. 2018).

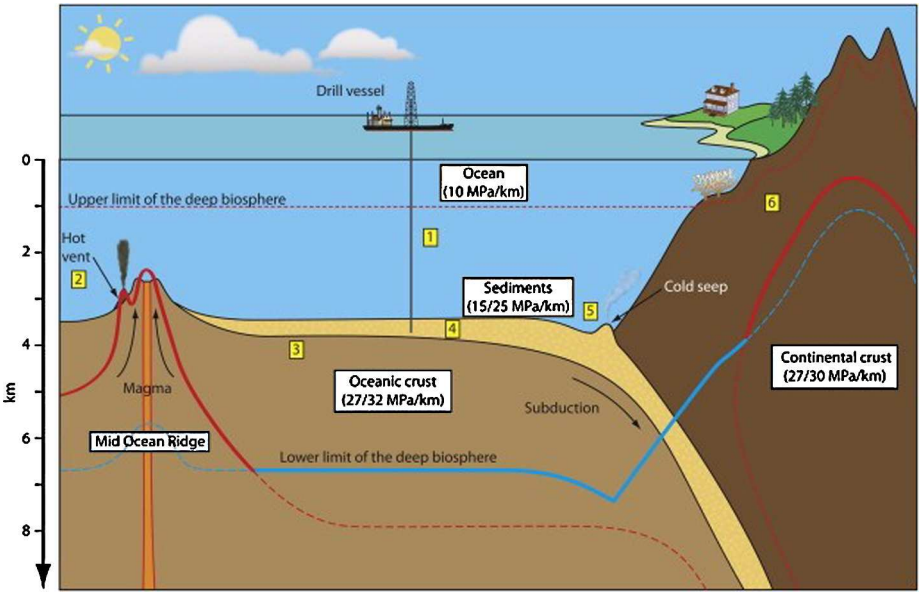

Fig. 2 Schematic transversal section of the Earth highlighting the numerous settings of the deep biosphere. 1: deep-sea; 2: deep-sea hydrothermal vents; 3: deep oceanic crust; 4: sedimentary sub-seafloor; 5: deep-sea cold seep; 6: continental deep biosphere. The red and blue lines represent the currently known temperature and pressure limits for life, respectively. Solid lines highlight the parameter which limits the depth of the deep biosphere. The upper dashed red line symbolizes the arbitrary 10 MPa upper limit of the deep biosphere (Oger and Jebbar 2010)

2.3.1 Physical Characteristics of High-Pressure Biotopes

The deep ocean is characterized by HHP, darkness, a stable average temperature of ca. 2 °C, low organic carbon and a relative constant oxygen concentration. It is estimated that, at present, ca. 1% of the carbon fixed by photosynthesis on the ocean surface eventually reaches the ocean floor, thus the major nutritional potential of the deep-sea is defined by a relatively low input of organic carbon (Oger and Jebbar 2010). As a corollary, adaptations to oligotrophy (life with limited access to nutrients) and psychrophily (optimal life at low temperature) are common in these environments. In contrast to the deep-sea biosphere, the deep-continental biosphere is considerably more diverse.

2.3.2 Diversity of HHP-Adapted Microorganisms

The field of piezomicrobiology has suffered largely from a requirement for expensive high-pressure retention sample containment and culturing laboratory equipments. The first HHP-adapted prokaryotes were bacteria isolated from deep-sea sediments by Zobell and Johnson (1949). The first obligate piezophiles, e.g. organisms that cannot develop at ambient pressure and temperature, were isolated in 1981 (Yayanos et al. 1981). The diversity of piezophiles in the deep-sea is largely dominated by five genera of psychrophilic, heterotrophic bacteria (*Colwellia*, *Moritella*, *Shewanella*, *Psychromonas*, and *Photobacterium*) from the gamma-Proteobacteria (Fig. 3). In contrast, the diversity of prokaryotes isolated from hydrothermal environments is dominated by archaeal and bacterial hyperthermophilic chemolithotrophs, i.e., those capable of gaining energy from the chemical transformation of dissolved minerals

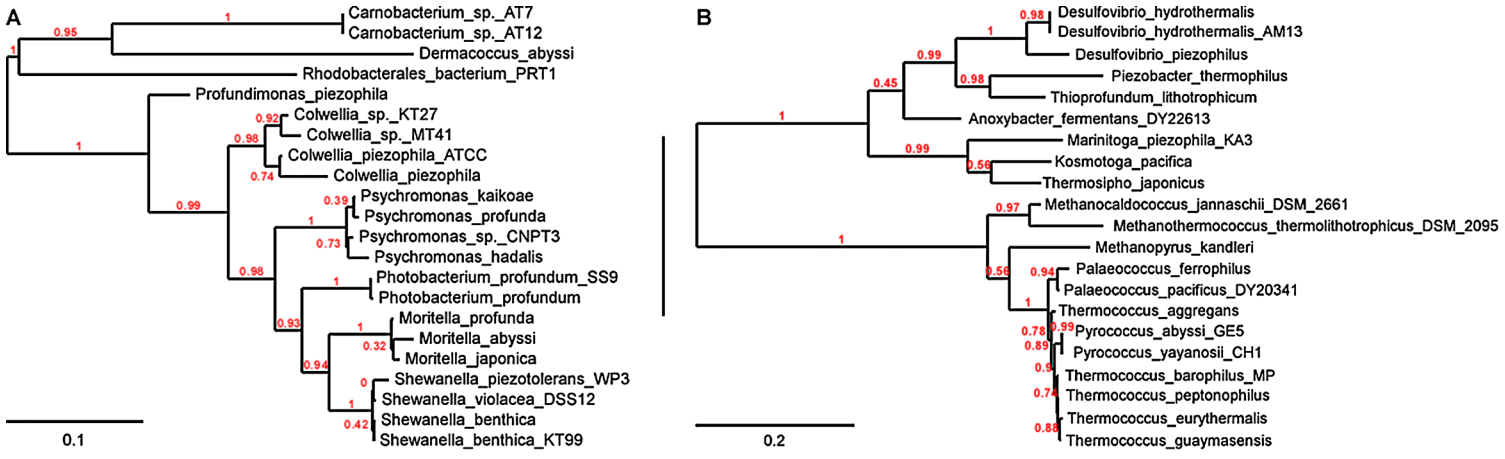

Fig. 3 Phylogenetic trees of (**A**) psychropiles and (**B**) mesophiles and (hyper)thermophilic piezophiles. The trees were created using the SILVA database (Quast et al. 2013) and the Phylogeny.fr website with default parameters (Dereeper et al. 2008). The bar represents 0.1 or 0.2 nucleotide substitutions per position. The numbers above the branches are tree support values generated by PhyML using the aLRT statistical test

and able to fix dissolved carbonates into organic molecules (Jebbar et al. 2015). Discoveries of abundant life in diverse high-pressure environments, including the deep oceans, hydrothermal vents, and crustal rocks, supports the existence of an adaptation of life to HHP, and is consistent with the significance of HHP in the prebiotic synthesis of key biomolecules and the origin of life on Earth (Hazen et al. 2002).

2.3.3 Effect of HHP on Biomolecules

Pressure affects both chemical equilibrium and reaction rates, depending upon the reaction (ΔV) and activation ($\Delta V \neq$) volumes involved. The behaviour of systems under high pressures is governed by Le Châtelier's principle, which states that the application of pressure shifts equilibrium toward the state that occupies the smallest volume. It accelerates a process in which the transition state has a smaller volume than that of the ground state, for example, if the volume of a protein is smaller in its unfolded form, this protein will be denatured by the application of HHP.

At HHP of greater than 400 MPa, most proteins tend to unfold (Aertsen et al. 2009). Exposure to mild HHP ($\sim$200 MPa) often affects only the quaternary structure, leading to the dissociation of oligomeric proteins. As a consequence, HHP modulates the activity of enzymes. The enzymatic activities of proteins isolated from HHP-adapted organisms tend to be less affected by HHP than those of surface organisms (Aertsen et al. 2009), however, the true structure-function relationships underlying the pressure stability of proteins are still unknown.

2.3.4 Effect of HHP on Biological Systems

Biological membranes play a fundamental role in the adaptation of microbes to their environment. The membrane acts as a physical barrier to regulate influx and efflux activities, it plays a central role in energy storage and processing *via* ion gradients, and it provides a template for environmental sensing, multicomponent uptake and signaling pathways and motility. Thus, maintaining optimal membrane biological function is crucial for any organism. Temperature-, pH-, salinity- or hydrostatic pressure-induced shortcomings in membrane organization are a serious threat to the cell. Archaeal and bacterial membranes have significant structural differences in spite of the fact that they perform identical functions. The mechanisms used by these membranes to cope with harsh conditions and shifting environments are quite similar. Bacterial polar lipids, with only a few rare exceptions, are based on straight chain hydrocarbons linked by ester bonds on the sn-1 and sn-2 positions of glycerol. Archaeal polar lipids are composed of isoprenoid hydrocarbon chains bound by ether bonds to the sn-2 and sn-3 positions of glycerol (Fig. 4). Polar headgroups consist of phosphodiester-linked polar groups or sugar moieties on the sn-1 (archaea) or sn-3 (bacteria) positions of the glycerol backbone (sn-glycerol-1-phosphate, or G-1-P, structure and sn-glycerol-3-phosphate, or G-3-P, structure).

Following the observation that the lipids in the membrane of *E. coli* cells grown under temperatures of 43 °C and 15 °C were different (Marr and Ingraham 1962; Sinensky 1971), yet the corresponding membranes had similar physical characteristics at their respective growth temperatures, Sinensky simulated the homeoviscous adaptation basis (Sinensky 1974; Oger and Cario 2013). According to this approach, organisms adjust the lipid composition of their membrane to facilitate the preservation of the appropriate membrane fluidity in order to work optimally. This concept—in a broader sense—is understood to encompass adaptation to proton/water permeability and the dynamic character of plasmic membranes

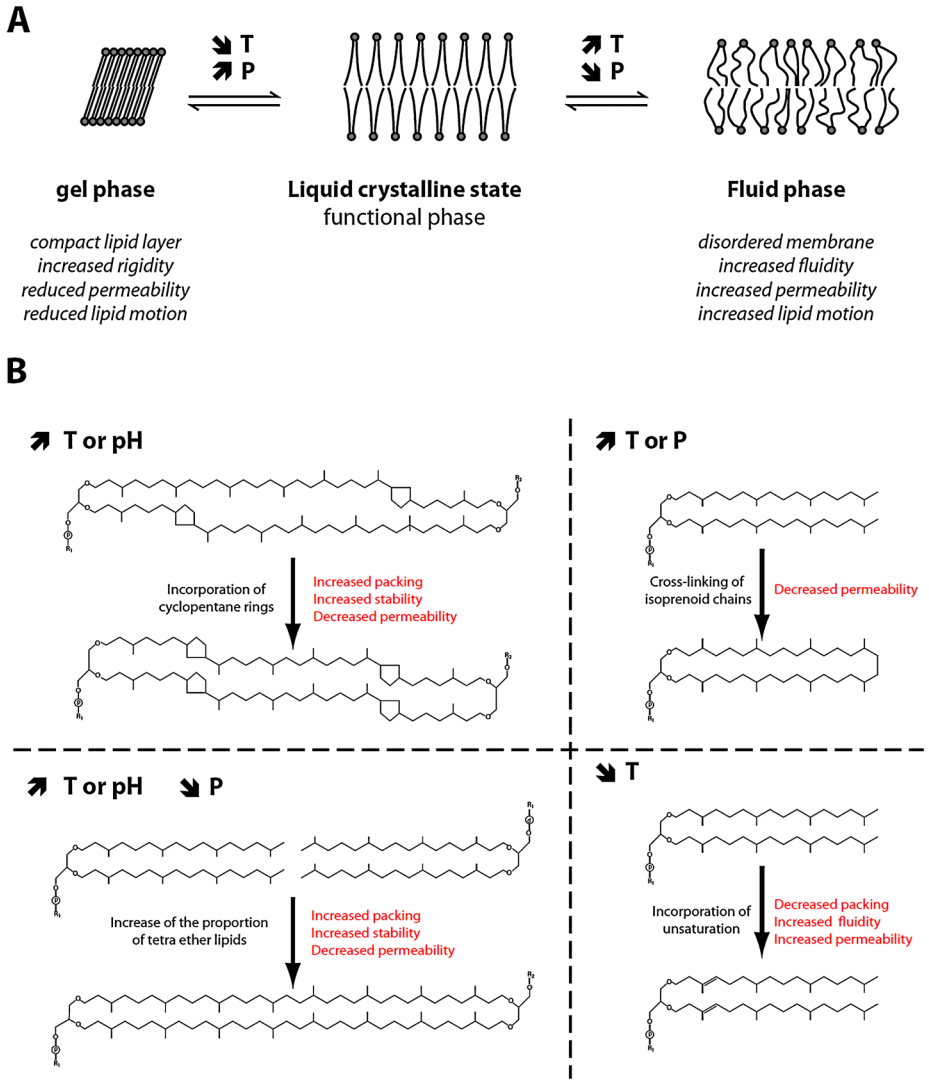

Fig. 4 Homeoviscous adaptation in Archaea. (**A**) In its functional state, the membrane is in a liquid crystalline state. Upon increasing temperature or decreasing hydrostatic pressure, lipid motion increases and the membrane enters the fluid phase. Conversely, when temperature drops or hydrostatic pressure increases, the lipid molecules pack more tightly and enter a gel phase. Membranes in both gel and fluid phases have impaired membrane function. (**B**) Known mechanisms of membrane lipid composition adaptation in Archaea (Oger and Cario 2013; Cario et al. **?**ca15; Jebbar et al. 2015) <ref:ca15?>

(Oger and Cario 2013). Homeoviscous adjustment should also be regarded as a manner of adapting the composition, and therefore the functionality, of the membrane to abrupt shifts in the environment, or to stresses, including those of temperature, salinity, osmotic stress, pressure and pH. Under optimal physiological conditions, membranes are rather fluid and formed of disordered liquid crystalline phases. As temperature decreases or hydrostatic pressure increases, lipids in the membrane may undergo a transition from fluid to gel phase. If the

temperature is higher or if the pressure is lower than the optimal physiological conditions, the movement rate of lipids in the membrane is greater, and this can affect the membrane's stability and its inherent permeability (Fig. 4). As one might expect, the disruption of the lipid phase state has a significant impact on the structure and function of the membrane (Lee 2003, 2004). The shift to the gel phase can lead to the aggregation of membrane proteins, which are excluded *de facto* from the gel phase areas, thereby limiting the diffusion and activity of proteins in the membrane and slowing down the flow of transported solutes, but enhancing the permeability of cations and water.

The adjustment of the characteristics of the bacterial membrane is made according to four main processes: (1) the change in acyl chain length, i.e., an increase in the length of the two-carbon chain causes an increase in the lipid phase transition temperature from 10 °C to 20 °C, and decreases membrane permeability to protons and water (Winter 2002); (2) the build-up of unsaturated fatty acids, since the introduction of a single unsaturation can shift the fluid/gel transition from 10 °C to 20 °C (Russell and Nichols 1999; Winter 2002); (3) the accumulation of specific polar groups such as phosphatidylcholine (PC) or phosphatidylglycerol (PG) instead of phosphatidylethanolamine (PE), indeed, the presence of PC as a polar head group results in a significant change in the fluid/gel transition temperature (Yano et al. 1998; Winter 2002; Mangelsdorf et al. 2005; Winter and Jeworrek 2009), partially due to the diminished hydration and stearic volume of ethanolamine compared to choline, and partially to the capacity of PE and the failure of the PC groups to form hydrogen bonds; and (4) the buildup of branched-chain fatty acids.

Archaeal lipid membranes usually have a considerably lower phase transition temperature than bacterial acyl fatty ester lipids (Yamauchi et al. 1993). The adaptation of the archaeal membrane to extreme environments may be attributed in part to the specific structure of its lipids. Although membranes consisting of fatty acyl ester lipids are in the gel phase or liquid crystal phase according to their fatty acid composition, it is presumed that Archaeal polar lipid membranes of archaeol and caldarchaeol are in the liquid crystal phase over a wide temperature range of 0–100 °C (Stewart et al. 1990; Dannenmuller et al. 2000).

The adaptability of the archaeal membrane is very similar in its physics to that of the bacterial membrane, albeit using slightly different mechanisms to attain the same effects. There exist several different routes, as follows. (1) The incorporation of cyclopentane rings along the isoprenoid chain as a function of fluctuating temperature (De Rosa et al. 1980a, 1980b; Ernst et al. 1998; Uda et al. 2001, 2004) or pH (Shimada et al. 2008) increases the packing efficiency of the membrane lipids (Gliozzi et al. 1983), which increases membrane stability as a function of increasing temperature or salinity and decreasing pressure or pH, and consequently lowers membrane permeability (Chong et al. 2012). (2) The regulation of the tetraether-to-diether lipid ratio (Sprott et al. 1991; Lai et al. 2008; Matsuno et al. 2009; Baumann et al. 2018; Taubner et al. under review; Taubner et al. (under review)), since increasing tetraether lipids will stabilize membranes by forming monolayer-type membranes or domains in the membrane, and consequently helping to regulate the flux of solutes and protons across the membrane. (3) The crosslinking of the two acyl-chains of the lipids yields macrocyclic archaeol or caldarchaeol derivatives by a covalent bond between the isoprenoid chains, which also reduces molecular motion to create a more closely packed structure and increases membrane stability, creating an efficient barrier against water, proton and solute leakage (Dannenmuller et al. 2000; Mathai et al. 2001). (4) The increase in unsaturation along the isoprenoid chains of the lipids as a function of temperature (Nichols et al. 2004) or salinity (Dawson et al. **?**da12); although this has, to date only been described in the psychrophilic methanogen <ref:da12?>

Methanococcoides burtonii (Franzmann et al. 1992; Nichols et al. 2004), unsaturated lipids have been characterized in several species of hyperthermophiles (Hafenbradl et al. 1993; Gonthier et al. 2001), which might indicate the occurrence of a similar adaptive strategy in deep-sea hydrothermal vent organisms.

The adaptation of bacterial and archaeal membranes to harsh environments is clearly visible in the most common lipids, however, responding to variations in environmental stressors might involve only a fraction of the adaptive traits mentioned above. Indeed, in order to be effective, the membrane composition adaptation response needs to be very rapid. The routes described require different timeframes, thus certain adaptive mechanisms will prevail over others. For example, the increasing unsaturation of membrane lipids will decrease the gel/fluid transition temperature to the same extent as the shortening of an acyl chain or the substitution of a phosphatidylcholine by a phosphatidylethanolamine polar head, but will be quicker because it is performed inside the cytoplasmic membrane on existing lipids by a membrane protein (Kasai et al. 1976; Cybulski et al. 2002; Aguilar and de Mendoza 2006; Beranova et al. 2008), whereas the other actions would require *de novo* lipid synthesis.

2.3.5 Adaptations to HHP in Piezophiles

DeLong and Yayanos (1985) showed that deep-sea organisms harbor an unusually high proportion of mono- and poly-unsaturated fatty acids. This leads to highly disordered phospholipid bilayers that are less permeable to water molecules and are proposed to maintain the plasma membrane in a functional fluid state despite the rigidification effect of pressure. The genes responsible for the synthesis of these unsaturated lipids have been shown to be up-regulated by HHP in the moderate piezophile *Photobacterium profundum* strain SS9, and are induced by HHP as part of the HHP-induced stress response in yeast (Allen et al. 1999; Abe 2015). These results have led workers to propose that adaptation to HHP involves the expression of HP-specific genes. This view is supported by genome-wide comparisons of gene expression in piezophile and piezosensitive strains of the *Photobacterium* complex (Campanaro et al. 2005).

In *P. profundum* SS9, transporters are mainly up-regulated at sub-optimal growth pressure, e.g. 0.1 MPa in comparison to the pressure optimum of 28 MPa. Bartlett and colleagues (Lauro et al. 2008) speculated that SS9 transporters evolved a novel protein structure to adapt to elevated pressures, and that their up-regulation at 0.1 MPa could compensate for a reduction of functionality at lower pressures. Kasahara et al. (2009) first demonstrated a weak HHP adaptation in the 3-isopropylmalate dehydrogenase of piezophilic *Shewanella* strains. Thus, adaptation to HHP may result from an evolution of proteins towards an optimal activity under HHP. The observation of the growth of *T. piezophilus* at 130 MPa, and that of the dissociation of ribosomes in *E. coli* at ca. 30 MPa, clearly supports the necessity for HHP-adapted ribosomes in the piezophilic strain.

Piezophilic *Shewanella* express a specific cytochrome protein complex under HHP (Tamegai et al. 1997). The importance of specific piezo-adaptation in the respiratory chain is further suggested by the presence of three complete sets of cbb3 cytochrome oxidase genes in the *P. profundum* SS9 genome (Vezzi et al. 2005). A large-scale transposon mutagenesis of *P. profundum* revealed several HHP-specific loci, most of which are involved in chromosomal partitioning and ribosomal function (Lauro et al. 2008). Therefore, adaptation to HHP may require specific genes.

In the deepest parts of the oceans and, if present, on ocean worlds, hydrothermal vent ecosystems are characterized by large fluctuations in salinity and temperature, from 0.1 to twice the salinity of seawater and from fluid temperatures as high as 350 °C at the heart of the

vent, to 2 °C, the average temperature of the surrounding deep ocean waters. Hydrothermal vent environments in the deep sea are also subject to extremely high hydrostatic pressures up to 50 MPa, i.e., 500 times the atmospheric pressure, based on values measured at the deepest known hydrothermal vent field in the Cayman Trough of the Caribbean Sea (Dalmasso et al. 2016).

Deep sea hydrothermal vents are among the ecosystems on Earth where polyextremophilic conditions or multi-stress situations are encountered by living organisms, such as high or low temperature, high salinity, high hydrostatic pressure and nutrient starvation within the same environment. Organisms, whether eukaryotes or prokaryotes, thriving in these areas have evolved mechanisms to adapt to these harsh conditions. It is known that an increase in hydrostatic pressure affects many cell functions involving macromolecules, including growth, cell division and protein synthesis (Bartlett 2002). High salinity and high and low temperatures have in common that they may trigger a cell dehydration effect and the loss of internal water, thus compromising the ability of the cell to survive. An increase in hydrostatic pressure does not result in changes in the pressure differential across the cell membrane, whereas increased salinity may trigger an increase in osmotic pressure outside the cell that provokes a change in turgor pressure. To maintain the appropriate cell turgor and restore the cell volume, organisms accumulate low-molecular-weight osmolytes that are mainly organic solutes. These organic solutes are also accumulated by many organisms in cold and heat stresses, and possibly under high hydrostatic pressure (Martin et al. 2002; Yancey 2005). The solutes are amino acids and derivatives, polyols, sugars and derivatives, methylamines, and methylsulfonium compounds (Fig. 1). Organic osmolytes fall into several chemical categories: amino acids (glycine, alanine, proline, α-glutamate, β-glutamate, and N-acetyl-β-lysine), and derivative N-methyl-substituted amino acids (e.g., glycine betaine, homobetaine, carnitine, proline betaine, trimethylamine oxide), ectoine and hydroxyectoine, methylsulfonium solutes (dimethylsulfoniopropionate and dimethylsulfonioacetate), and small carbohydrates including monosaccharides (glucose), disaccharides (trehalose, sucrose, mannosucrose), sugar derivatives (glucosyglycerol, mannosylglycerate, glucosylglycerate), polyols (glycerol, inositol, sorbitol), and cyclitols (di-myo-inositol-phosphate) (Empadinhas and da Costa 2006; Neves et al. 2005; Wood et al. 2001; Jebbar et al. 1992; Essendoubi et al. 2007; Yancey 2005; Kempf and Bremer 1998). Some solutes are widespread, for example glycine betaine, which is found in all domains of the tree of life, and carbohydrate osmolytes that occur in bacteria, archaea, fungi, algae, plants, mammalian kidneys and possibly deep-sea invertebrates. Other solutes are restricted to a small number of organisms, for example those thriving in hot environments (Empadinhas and da Costa 2006). Most organic osmolytes are neutral (either zwitterionic or lacking charges) at optimal physiological pH, although some (i.e. mannosylglycerate and di-myo-inositol-phosphate in hyperthermophilic prokaryotes) are negatively charged and must be paired with potassium to achieve neutrality.

These solutes are often called “compatible solutes”, a term that refers to compounds that can accumulate at very high levels without perturbing cell metabolism or enzyme activity (Brown 1976). Many such solutes have protective properties, such as cell metabolic protection, and serve as antioxidants that scavenge free radicals and reactive oxygen species generated under stress treatments (Cushman 2001; Sunda et al. 2002; Yancey 2005). They can also stabilize macromolecular structures (proteins, membranes) only when stresses such as high salinity, high temperature, freezing and high hydrostatic pressure are present and directly destabilize cell components (Singer and Lindquist **?**sl98; Story and Story **?**ss96; Rudolph and Crowe **?**rc85; Santos and da Costa **?**sc02; Kelly and Yancey 1999). <ref:sl98?> <ref:ss96?> <ref:rc85?> <ref:sc02?>

In many bacteria and archaea, it has been demonstrated that a number of compatible solutes are accumulated by the cell in response not only to salt stress but also as a means

to counteract the destabilizing effects of heat and chill stresses on cell macromolecules (Kuhlmann et al. 2008; Empadinhas and da Costa 2006; Holtmann and Bremer 2004). Despite this, the compatible solute counteraction of the destabilizing effect of high hydrostatic pressure on macromolecules is not obvious and poorly demonstrated, particularly in prokaryotic cells. Yancey and coworkers have shown that the organic osmolyte trimethylamine oxide (TMAO) occurs at high levels in many deep-sea animals in comparison to related shallow-water species (Gillett et al. 1997, Kelly and Yancey 1999). Since hydrostatic pressure is the only physico-chemical parameter that is linear with depth, these authors suggested that TMAO might counteract the effects of high hydrostatic pressure. In the deep-sea bacterium *P. profundum* strain SS9, cells accumulate mainly glutamate and glycine betaine at atmospheric pressure (0.1 MPa), whereas at optimal growth pressure (28 MPa), cells preferentially increase intracellular concentrations of β-hydroxybutyrate and β-hydroxybutyrate oligomers termed "piezolytes" for solutes that are accumulated at high hydrostatic pressures (Martin et al. 2002). In addition, another study on marine bacteria has shown that adaptation to high salinity synergistically enhances cell survival at high hydrostatic pressures, which suggests the involvement of osmolytes in counteracting both stresses in these prokaryotes (Kaye and Baross **?**kb04). <ref:kb04?>

In hyperthermophilic piezophiles, it is evidenced that adaptation to HHP involves a global change in the expression of genes in some metabolic pathways (amino acid biosynthesis, hydrogen metabolism), rather than the expression of a stress response *per se* (Vannier et al. 2015). In *Thermococcus barophilus*, for example, adaptation to HHP involves osmolyte accumulation to maintain proper protein folding and activity (Cario et al. 2016). Mannosylglycerate (MG) is primarily accumulated as a compatible solute in response to salinity stress, but in contrast to other Thermococcales, MG also accumulates in response to thermal stresses, and its accumulation peaked in the case of combined stresses. The accumulation of MG has been found to drastically increase under sub-optimal hydrostatic pressure conditions, demonstrating that low pressures are perceived as a form of stress in this piezophile, and that the proteome of *T. barophilus* is sensitive to low-pressures. MG accumulation is strongly reduced under supra-optimal pressure conditions, clearly demonstrating the structural adaptation of this proteome to high hydrostatic pressure. There is direct and indirect evidence for the structural adaptation of the proteome to HHP, although the specific signature of this adaptation at the genome level remains elusive.

This section provided an in-depth overview of the biodiversity of micro-organisms in extreme hypersaline environments and also where high hydrostatic pressure prevails. Molecular signatures and cellular and physiological responses to extreme salinity and high hydrostatic pressures were also examined. Among the microorganisms associated with these extreme environments described above are the methanogenic archaea that have successfully colonized all of the earth's ecosystems. Methanogenesis and methanogens are described in more detail in the following paragraph.

3 Methanogens as Model Organisms for Icy Moon Related Cultivation: Adaptation to Extreme Conditions

McKay et al. (2008, 2012) determined that only three microbial ecosystems on Earth could serve as analogues for a potential ecosystem on an icy moon. These ecosystems do not rely on photosynthesis, on any by-product of photosynthetic metabolism, nor are they dependent on O_2. One of these ecosystems is based on sulfur-reducing bacteria, and the other two are based on methanogenic archaea (methanogens). In the following section, we will focus on

methanogens and their adaption to extreme conditions. A more detailed review about that topic can be found in Taubner et al. (2015).

Besides Earth, methane (CH_4) has been detected on every planet of the Solar System, on the dwarf planets Pluto, Makemake, and Eris (Formisano et al. 2004, Mumma et al. 2009, Webster et al. 2015), and on the icy moons Titan (Niemann et al. 2005) and Enceladus (Waite et al. 2009, 2014, 2017). Most of the CH_4 found on Earth is of biogenic origin (Liu et al. 2008). Methanogens are the overwhelmingly dominant producers of CH_4 as metabolic end products of their carbon- and energy-yielding reactions (Thauer et al. 2008; Liu et al. 2008; Taubner et al. 2015; Rittmann et al. 2015), however, some (aerobic) marine microorganisms were also shown to produce CH_4 from methylphosphonic acid (Karl et al. 2008; Metcalf et al. 2012; Carini et al. 2014). Methanogens are a phylogenetically and metabolically diverse group of prokaryotic organisms from the domain Archaea. Within the domain Archaea, methanogens belong exclusively to the phylum Euryarchaeota. All characterized methanogens are known to be obligate anaerobic chemolithoheterotrophs or chemolithoautotrophs. Moreover, methanogens might resemble amongst the oldest life forms that emerged on Earth (Grassineau et al. **?**gr06; Ueno et al. 2006; Martin et al. 2008), <ref:gr06?> but this is still under discussion (Brochier-Armanet et al. 2011; Blank 2009). Methanogens are used as astrobiological study objects because of both their metabolic versatility and ability to withstand extreme environmental conditions (Cavicchioli 2006; Huber et al. 1989; Taubner et al. 2018); they are further characterized by a variety of unusual morphological and ecophysiological features. In this subsection, we review and discuss methanogens with respect to temperature, pressure, pH and osmolarity, and highlight recent studies performed with methanogens in an astrobiological context.

3.1 Adaptions to Temperature

Individual methanogenic strains are viable within a temperature window for growth of approximately 45 °C, however, the biochemical pathways of methanogenesis *per se* are not restricted to a certain temperature, but are generally functional at temperatures from below 0 °C (Cavicchioli 2006) up to 122 °C. This allows individual strains of methanogens to grow from psychrophilic to hyperthermophilic growth conditions (Nakamura et al. **?**na13; <ref:na13?> Ma et al. 2006; Lü and Lu 2012; L'Haridon et al. 2003; Jones et al. 1983a; Jiang et al. 2005; Jeanthon et al. 1998; Jeanthon et al. 1999; Cheng et al. 2007; Parshina et al. 2014; von Klein et al. 2002; Franzmann et al. 1997; Wagner et al. 2013; Schirmack et al. 2014; Takai et al. 2008). Metabolic reactions occurring at the highest temperatures were observed for *M. kandleri* strain 116 when grown at 122 °C (Takai et al. 2008).

Bodies of the outer Solar System, which could possibly support methanogenic life, fall within the temperature range of psychrophilic methanogens. Recent results advocate the possibility that hydrothermal vents might exist on icy moons, such as Enceladus (Hsu et al. 2015) or Europa (e.g., Zolotov and Kargel 2009), which would widen the growth temperature range for methanogens in the subsurface water reservoirs of these bodies. However, as these potential warm to hot spots at the bottom of the subsurface oceans are most likely locally restricted, we will focus on psychrophilic methanogens in the following.

To distinguish different levels of psychrophily, psychrophilic methanogenic strains were classified according to their temperature niche adaptation, which can be narrow or wide (Cavicchioli 2006; Dong and Chen 2012), respectively denoted as "stenopsychrophile" and "eurypsychrophile" organisms (Cavicchioli 2006; Siddiqui et al. 2006; Feller and Gerday 2003). Stenopsychrophiles are considered true psychrophiles and are only able to grow within a narrow temperature range. Compared to stenopsychrophiles, eurypsychrophiles can tolerate a larger temperature interval, tolerate a higher mean optimum

Table 1 Summary of presently known psychrophilic strains and their main temperature and pH features

Strain	T [°C]			pH			Ref.
	min	opt	max	min	opt	max	
Methanospirillum psychrodurum	4	25	32	6.5	7	8	Zhou et al. (2014)
Methanosarcina baltica	3	21	28	6.3	7.2	7.5	von Klein et al. (2002)
Methanosarcina lacustris	1	25	35	4.5	7	8.5	Simankova et al. (2001)
Methanolobus psychrophilus	0	18	25	6	7–7.2	8	Zhang et al. (2008)
Methanogenium marinum	5	25	25	5.5	6–6.6	7.7	Chong et al. (2002)
Methanogenium frigidum	0	15	17	6.3	7.5–7.9	8	Franzmann et al. (1997)
Methanohalobium evestigatum	50	n.a.	n.a.	n.a.	7.4	n.a.	Zhilina and Zavarzin (1987)
Methanogenium cariaci	15	20–25	35	6	6.8–7.2	7.5	Romesser et al. (1979)
Methanogenium boonei	5	19.4	25.6	6.4	n.a.	7.8	Brauer et al. (**?**br11)
Methanoculleus marisnigri	15	20–25	48	6	6.2–6.6	7.6	Maestrojuán et al. (1990)
Methanoculleus chikugoensis	15	25	40	6.7	6.7–7.2	8	Dianou et al. (2001)
Methanococcoides alaskense	2.3	23.6	28.4	6.3	n.a.	7.5	Singh et al. (2005)
Methanococcoides burtonii	1.7	23.4	29.5	6.8	n.a.	8.2	Franzmann et al. (1992)
Methanospirillum stamsii	5	20–30	37	6	7.0–7.5	10	Parshina et al. (2014)
Methanosarcina soligelidi	0	28	54	4.8	7.8	9.9	Wagner et al. (2013)

<ref:br11?>

growth temperature, and can (sometimes) be cultivated when exposed to elevated temperatures. Psychrophilic methanogens have been used in many research ventures, as they are important organisms in cold habitats on Earth (Cavicchioli 2006; Dong and Chen 2012). A list of psychrophilic methanogenic strains and their respective temperature niche can be found in Table 1.

The temperature adaptation mechanisms of methanogens were identified at different levels. At the protein level, cold adaptation mechanisms were examined in *Methanococcoides burtonii*. Here, the archaeal elongation factor 2 (EF2) proteins were found to be active at low growth temperatures but unstable at high growth temperatures (Siddiqui et al. 2002; Thomas and Cavicchioli 2000; Thomas et al. 2001). Moreover, proteins interacting with EF2 of *M. burtonii*, but also compatible solutes, are involved in activating as well as stabilizing protein machinery under low growth temperatures (Thomas et al. 2001). Another study showed that in *M. burtonii*, a putative DEAD box RNA helicase gene (deaD) was abundantly expressed at 4 °C (Lim et al. 2000). Additional characteristics for cold adaptation in methanogens include the increased presence of dihydrouridine in tRNAs of *M. burtonii* compared to the presence of dihydrouridine in other archaeal strains (Noon et al. **?**no03). Unlike adaptations to cold in thermophiles, *M. burtonii* did not show decreased modification of its tRNAs, but exhibited few modifications (comparable to bacteria), in particular dihydrouridine incorporation into tRNA.

<ref:no03?>

A genome comparison of the psychrophilic methanogens, *M. burtonii* and *Methanogenium frigidum* was performed to identify characteristics which distinguish cold adaption mechanisms in these organisms from other archaea. Predicted and modelled proteins from *M. burtonii* and *M. frigidum* comprise a higher quantity of non-charged polar amino acids present in the solvent-accessible area of proteins. Specifically, glutamine and threonine were detected in higher abundance. Moreover, a lower content of hydrophobic amino acids, in particular leucine, were noted. Finally, two hypothetical proteins with CSD-folds and a unique winged helix DNA-binding domain protein were identified in *M. burtonii*, together with a

cold shock domain (CSD) protein (homologue of CspA) in *M. frigidum* (Saunders et al. 2003). In another study, a proteomics approach was taken to analyze the functional characteristics of *Methanosarcina barkeri* during a low-temperature down shock response (from 37 °C to 15 °C) and for its low-temperature adaptation strategies at 15 °C. In a combined approach using growth studies and proteomics insights into the low-temperature adaptation capacity of *M. barkeri* could be obtained (Gunnigle et al. 2013). Astrobiologically oriented experiments have been performed to examine the temperature-dependent starvation features of selected *Methanosarcina* species including *M. solegelidi* SMA-21, finding that this methanogen tolerated freezing with a survival of 98.5% in comparison to, e.g., *Methanobacterium* sp. MC-20, which exhibited only 1% survival under the same conditions (Morozova and Wagner 2007). *M. soligelidi* SMA-21 showed a high survival potential at 4 °C and at 28 °C compared to other methanogens tested (Morozova and Wagner 2007).

Methanogens possess other physiological adaption mechanisms to changes of growth temperature, for instance the ability to modify cytoplasmic membrane lipids to maintain membrane fluidity. A prerequisite is that the lipid membrane of organisms must be kept in the liquid crystalline phase in order to stay functional, which was found in methanogens over the temperature range between 0 and 100 °C (Koga 2012).

Membrane fluidity maintenance in psychrophilic methanogens is achieved through growth temperature-mediated lipid saturation instead of the unsaturation mechanisms that occur in bacteria (Nichols et al. 2004). In methanogens, lipid unsaturation is performed by geranylgeranyl reductase. Notwithstanding, the cytoplasmic lipid composition in general, and its unsaturation properties of methanogens in particular, are unclear indicators as to whether a methanogen is adapted to a psychrophilic or a thermophilic lifestyle (Koga 2012).

The core lipids of *M. thermoautotrophicus* growing at its optimal growth temperature of 65 °C are composed of archaeol and caldarchaeol, whereas the core membrane lipids of *M. kandleri*, growing at 90 °C, are archaeol (Koga 2012). The core lipids of *Methanocaldococcus villosus* and *Methanothermococcus okinawensis* are archaeol and macrocycle (with minute abundances of tetraether lipids) (Baumann et al. 2018) but, upon increasing the growth temperature of *Methanocaldococcus jannaschii* from 45 °C to 65 °C, the lipid membrane composition changes from mainly archaeol to macrocycle as well as caldarchaeol (Koga 2012; Sprott et al. 1991). Moreover, the presence of double bonds in isoprenoid chains is not indicative of adaptation to lower growth temperatures (Koga 2012).

The above-mentioned results indicate that mechanisms at the genome level (e.g. the expression of deaD at suboptimal growth temperature), at the proteome level (e.g. activity of EF2), and in the lipid membrane composition distinguishes the adaptations of stenopsychrophilic, eurypsychrophilic and thermophilic methanogens. Other described physiological characteristics of methanogens to cope with adaptations to psychrophilic cultivation conditions include the uptake of compatible solutes (Dong and Chen 2012; Cavicchioli 2006, Grochowski et al. **?**gr08). A discussion on the role of compatible solutes as osmoprotective compounds is given below. <ref:gr08?>

3.2 Adaptions to Pressure

Methanogens are known to grow under low- and high-pressure conditions. The cultivation of methanogens under high-pressure conditions offers an opportunity for astrobiological studies, for example, the investigation of physiological responses and metabolic adaptations, and for investigating the ecology of hydrothermal vent systems proposed for ocean worlds. Methanogens are known to grow at more than 20 MPa of pressure (Jeanthon et al. 1998, 1999, 2012). The cultivation of methanogens under high-pressure conditions of up to 300

kPa can be easily performed in closed batch cultivation in either serum bottles (Taubner et al.?ta16) or in sophisticated cultivation devices such as bioreactors (Nishimura et al. 1992; Seifert et al. 2014). The cultivation of methanogens under low-pressure conditions and at pressures beyond 300 kPa requires special equipment (Kral et al. 2011; Kral and Altheide 2013; Park and Clark 2002; Miller et al. 1988; Boonyaratanakornkit et al. 2006; Taubner et al. 2018). Low-pressure experiments are relevant for astrobiology, to represent the lower above ground pressure present on Mars and other Solar System bodies. <ref:ta16?>

With respect to growth, substrate uptake, and CH_4 production kinetics, two methanogens (KN-15 and *M. marburgensis*) examined under moderate high-pressure conditions in fed-batch or continuous culture mode in bioreactors (Nishimura et al. 1992; Seifert et al. 2013; Seifert et al. 2014) have shown that the point at which growth kinetics changed from exponential growth to linear growth (and the specific growth rate (μ)) of strain KN-15 increased with increasing pressure (Nishimura et al. 1992). The results obtained for *M. marburgensis* showed that CH_4 production is gas-limited and, although applying high-pressure conditions, the maximum physiological capacity of the organism to produce CH_4 was not reached (Seifert et al. 2014).

M. jannaschii was cultivated under gas-limited conditions and it was found that the tested strain exhibited a stress response under both high-pressure and low-pressure cultivation conditions at the transcriptional level (Boonyaratanakornkit et al. 2006). High-pressure and decompression experiments were also performed using *M. jannaschii*, employing a high-pressure bioreactor. When rapid decompression from approximately 26 MPa to atmospheric pressure was performed, the cell envelopes of *M. jannaschii* ruptured, however, when the decompression time was increased from 1 s to 5 min, the rupture of *M. jannaschii* cell envelopes decreased significantly (Park and Clark 2002). In another study, *M. jannaschii* was used to investigate growth and CH_4 production kinetics at high-pressure conditions and at different temperatures, in the presence of He or Ar in addition to H_2/CO_2. It was found that the high-temperature limit for CH_4 production kinetics of *M. jannaschii* increased with increasing pressure (Miller et al. 1988).

Additional high-pressure and high-temperature investigations using *Methanococcus thermolithotrophicus* were accomplished in 10 mL nickel tubes in series of connected autoclaves. This experimental setup was used to expose the organism of choice to temperature and high-pressure changes of 400 °C and 400 MPa over 10 min to investigate optimum pressure levels (Bernhardt et al. 1987). A pressure of 50 MPa was found to be optimal for the growth of *M. thermolithotrophicus*, whereas applying overpressure of >75 MPa resulted in increased cell lysis and in changes of morphology and in changes of growth kinetics (Bernhardt et al. 1987).

In a recent study mimicking the concentrations of gaseous and liquid inhibitors as well as high-pressure conditions on Enceladus (Taubner et al. 2018), different pressure conditions with and without gaseous inhibitors were applied to evaluate the viability of methanogens in these environments. It was shown that the methanogenic strain *M. okinawensis* produced CH_4 at pressures up to 9 MPa, but only in the presence of molecular nitrogen in the gas phase. Using a H_2/CO_2 gas phase at this high pressure results in a high CO_2 partial pressure which significantly lowers the pH of the medium. Under putative Enceladus-like conditions including potential gaseous and liquid inhibitors like crabon monoxide (CO), ethene (C_2H_4), formaldehyde (CH_2O), or methanol (CH_3OH), CH_4 production was observed up to 5 MPa.. The CH_4 production kinetics did not change due to the presence of gaseous and liquid inhibitors during experiments between 300 kPa to 5 MPa (Taubner et al. 2018). A simultaneous bioreactor system (SBRS) was developed, consisting of four identical tabletop bioreactors that are suitable for performing gas conversion and gas production kinetics at

pressures up to 50 bar and temperatures up to 145 °C. *M. marburgensis*, *M. palustre*, and *M. thermaggregans* were successfully cultivated at 1 MPa and/or 5 MPa and differences in the CH_4 production kinetics of these organisms were detected (Pappenreiter et al. 2019). The SBRS system facilitates throughput high-pressure astrobiological research, which is of timely relevance in assessing the possibility of high-pressure habitats on outer Solar systems bodies.

3.3 Adapations to pH

Most of the >150 characterized methanogens grow at neutral pH values (Taubner et al. 2015). Methanogens such as *M. okinawensis* (Takai et al. 2002; Taubner et al. 2018) and *M. marburgensis* (Bernacchi et al. 2014) tolerate a broader pH range down to values of 3.5 and 4.5, respectively. Furthermore, there are also other methanogens known to be able to grow under acidic pH conditions (Bräuer et al. 2011; Cadillo-Quiroz et al. 2009; Ver Eecke et al. 2013). However, from an astrobiological viewpoint, at least Enceladus' subsurface ocean is rather alkaline (Glein et al. 2015). Currently six alkaliphilic methanogens have been characterized and are available in pure culture (Table 2). The most alkaliphilic methanogens are *Methanocalculus natronophilus* (Zhilina et al. 2013), *Methanocalculus alkaliphilus*, and *Methanosalsum natronophilum* (Sorokin et al. 2015). *M. natronophilus* was isolated from the sediments of a collector in the vicinity of a soda lake. The strain utilizes CO_2 and H_2 or formate as an energy source and acetate as a carbon source. *M. alkaliphilus* and *M. natronophilum* were enriched from hypersaline soda lake sediments at pH 10. All three alkaliphilic methangogens grow at pH between 8.2 and 10.0 and optimally around pH 9.0–9.5. *M. alkaliphilus* utilizes formate or H_2 as an electron donor and acetate as a carbon source, whereas *M. natronophilum* metabolizes methanol, methylamines, and dimethyl sulfide. Another alkaliphilic and slightly thermophilic methanogen, *Methanonatronarchaeum thermophilum* was recently characterized. This methanogen comprises a new euryarchaeal class, the *Methanonatronarchaea*. This organism grows between pH values of 8.2–10.2 and optimally between pH 9.5–9.7. *M. thermophilum* utilizes methanol, methylamines and dimethylsulfide as electron acceptors and formate or H_2 as electron donors (Sorokin et al. 2018). A list of methanogens cultivable in either acidic or alkaline conditions is shown in Table 2.

3.4 Adaption to Osmolarity

All known methanogens depend on low intracellular salt concentrations to maintain cellular integrity and the functioning of homeostatic processes. For the maintenance of cellular functions at higher extracellular concentrations of salt, some methanogens are known to accumulate compatible solutes to reduce the difference of osmotic potentials between the cytoplasm and the environment. Compatible solutes are osmoprotective molecules (Fig. 1) and do not alter the metabolic and cellular processes, even when accumulated in high concentrations (Jones et al. 1983a, 1983b).

Trimethylglycine (glycine betaine) and β-glutamate were shown to act as compatible solutes in methanogens, whereby the former can be assimilated by some methanogens from the growth medium (Grochowski et al. **?**gr08; Robertson et al. 1990; Lai et al. 1991). Additionally, an adenosine derivate was proposed to act as a compatible solute in *Methanolobus psychrophilus* R15. Furthermore, it was suggested that some of the compatible solutes described for methanogens could possibly possess cryoprotective functions (Dong and Chen 2012). The main compatible solute utilized by methanogens is trimethylglycine (Robertson

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Table 2 Summary of currently known methanogens cultivable in either very acidic or alkaline conditions

Strain	T [°C]			pH			Ref.
	min	opt	max	min	opt	max	
Methanospirillum stamsii	5	20–30	37	6	7–7.5	10	Parshina et al. (2014)
Methanocalculus natronophilus	14	30–37	45	8	9–9.5	10.2	Zhilina et al. (2013)
Methanospirillum hungatei	20	37–45	50	6.5	7–9	10	Iino et al. (2013)
Methanobrevibacter millerae	33	36–42	43	5.5	7–8	10	Rea et al. (2007)
Methanobrevibacter olleyae	28	28–42	42	6	7.5	10	Rea et al. (2007)
Methanotorris igneus	45	88	91	5	5.7	7.5	Burggraf et al. (1990)
Methanosphaerula palustris	14	30	35	4.8	5.5	6.4	Cadillo-Quiroz et al. (2009)
Methanoregula boonei	10	35–37	40	4.5	5.1	5.5	Bräuer et al. (2011)
Methanothermococcus okinawensis	40	60–65	75	3.5	6–7	8.5	Takai et al. (2002), Taubner et al. (2018)
Methanonatronarchaeum thermophilum	30	50	60	8.2	9.5–9.7	10.2	Sorokin et al. (2018)

et al. 1990), which is used by e.g. *Methanosarcina thermophila* TM-1 (Proctor et al. 1997) and can be accumulated through an uptake system composed of a single, high-affinity H^+- and/or Na^+-driven transporters (Proctor et al. 1997). *M. thermophila* TM-1 can adapt to different osmolarities by synthesizing α-glutamate and N-ε-acetyl-β-lysine, or by accumulating trimethylglycine or K^+. In *Methanohalophilus portucalensis* FDF1, the compatible solutes α-glutamate, β-glutamine, and N-ε-acetyl-β-lysine were described as osmoprotectives (Lai et al. 1991, 2000), whereas trimethylglycine was preferentially taken up from the medium as an osmoprotective compound instead of being produced *de novo* (Lai et al. 2000).

Many experiments examining the effect of osmolarity have used *Methanosarcinales*. NaCl concentrations from 0.05 to 1.0 mol L^{-1} were used to examine the effect of osmolarity on growth kinetics and changes of morphology in *Methanosarcina* spp. (Sowers et al. 1993) and NaCl concentrations between 0.4 to 1.0 mol L^{-1} disintegrated the methanochondroitin and sheath, which resulted in growth of *Methanosarcina* spp. as single cells. Furthermore, all tested *Methanosarcina* spp., which were encapsulated by a methanochondroitin layer, exhibited enhanced stability to <0.2 mol L^{-1} NaCl osmolarity and grew at higher temperatures compared to the control group (Sowers et al. 1993).

An adaptation to high salt concentrations was shown with *Methanosarcina mazei* Gö1. The strain was able to tolerate up to 1 mol L^{-1} salt through the uptake and accumulation of trimethylglycine from the growth medium. The osmoprotectant transporter A (OpuA) was involved in trimethylglycine uptake from the medium and its expression was demonstrated to be salt-induced (Roeßler et al. 2002).

Methanohalophilus spp. strains grown at different NaCl concentrations between 0.7 to 3.4 mol L^{-1} demonstrated that the strains accumulated K^+, however, the osmoprotective β-glutamate was detected when the strains were grown at NaCl concentrations of <1.5 mol L^{-1} (Lai et al. 1991).

The alkaliphilic methanogens *M. natronophilus* (Zhilina et al. 2013), *M. alkaliphilus* and *M. natronophilum* (Sorokin et al. 2015), and *M. thermophilum* (Sorokin et al. 2018)

are slightly halophilic, extreme halotolerant, and extreme halophilic, respectively, i.e., they exhibit polyextremophily. Optimal growth of *M. natronophilus* requires carbonate concentrations of 0.7–0.9 mol L^{-1} and Na^+ at concentrations of 1.4–1.9 mol L^{-1}. *M. alkaliphilus* is characterized as a moderately salt-tolerant strain within the range from 0.2 to 1.5 mol L^{-1} total Na^+ in carbonate buffer at a pH of 9.5. *M. natronophilum* is highly salt-tolerant in a range from 0.5 to 3.5 mol L^{-1} total Na^+ growing also in carbonate buffer at a pH of 9.5. The recently described *M. thermophilum* is an extremely halophilic organism growing at total Na^+ concentrations between 3 and 4.8 mol L^{-1} with an optimum at 4 mol L^{-1}, and its cells lyse at a Na^+ concentration below 2 mol L^{-1}. *M. thermophilum* accumulates K^+ as its main compatible solute.

The above described characteristics and adaptations towards low- and high-pressure conditions, psychrophily and (hyper)thermophily, acidiphily and alkaliphily and osmolarity reveal that methanogens thrive under a variety of extreme growth conditions, but also during multi-factorial stress conditions (e.g. simulataneous multivariate concentrations of gaseous and liquid inhibitors, low pH, and pressure influences) and they respond to environmental disturbances in numerous ways (Kral et al. 2011; Taubner et al. 2015, 2018). Further, as already mentioned in Sect. 2.1.1 of this review, no other archaeal halophiles than methanogens have been identified outside the Euryarchaea. Only one recently characterized halophilic methanogen, *M. thermophilum*, is uses the salt-in-cytoplasm strategy for osmoprotection and other methanogens employ the organic-osmolyte strategy to deal with osmotic stress. Hence, with respect to their potential to adapt to changing and extreme environmental conditions, methanogens are considered to be among the ideal candidates for further astrobiological studies.

4 Origins of Life and Biosignatures on Icy Worlds

4.1 Ocean World Settings for the Origins of Life

In any origins of life scenario, prebiological chemical complexification would have necessitated the confined or compartmentalized reaction of ions and molecules within an aqueous geological setting characterized by gradients and disequilibria (Russell et al. 2010). Over time, prebiotic complexification would have driven the system closer to obtaining life-like characteristics (e.g., metabolism, replication). The step(s) linking the immediate life-like precursor to the first living entity (i.e., a pioneer organism) are the most contentious (e.g. Wächtershäuser **?**wa88; Martin and Russell 2003; Russell et al. 2010), and will not be covered in this review. A number of geological settings for the origins of life have been proposed. These settings are generally hydrothermal (submarine or subaerial hot springs) or hydrothermally influenced (the hydrothermal-sedimentary reactor hypothesis), though others are passive and rely on providing naturally reactive mineral-rich environments as "reactor flasks" for organic molecules sourced from elsewhere, for example, the pumice raft hypothesis (Brasier et al. **?**br11). Further possibilities, as yet incompletely explored, are the geodynamic nuclear reactor hypothesis (Ebisuzaki and Maruyama **?**em17) or the hydrodynamically driven volcanic-hosted splash pool hypothesis (Fox and Strasdeit **?**fs13). A review of all of these hypotheses, culminating in a suggestion that the cycling of organic-rich fluids through the volcanogenic sediments in the vicinity of hydrothermal fields (the hydrothermal-sedimentary scenario for the origin of life), is given in Westall et al. (2018).

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Despite the wide range of propositions behind the mechanics of the origins of life, most hypotheses for the geological setting of this process focus on either submarine or subaerial

hydrothermal environments. Such settings are supported by both the top-down and bottom-up approaches to the origins of life. From the viewpoints of organic chemistry and geochemistry, hydrothermal settings produce, and have the potential to concentrate within geological (mineral) edifices, the range of simple organic monomers and polymers whose gradual complexification theoretically leads to 'protocells' and cellular life (Russell et al. 2010; Lane and Martin 2012; Westall et al. 2018). From the biological viewpoint, estimations of the nature and metabolism of the earliest life invariably find a root in thermophilic, metal-rich settings (Nisbet and Fowler **?**nf96; Williams and Fraústo Da Silva 2003; Gaucher et al. **?**ga10; Lane and Martin 2012). Whether life originated in the submarine or subaerial arena is a topic of contention, focused on the specific characteristics and parameters of the organic chemistry and geochemistry possible in these settings that may lead to prebiotic chemical complexification.

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<ref:ga10?>

Certainly, it is necessary that the geological environment of the origin of life was able to naturally produce or directly receive a wide range of organic molecules. Chemical complexification requires that this production is harnessed by a combination of gradient-driven compartmentalized or confined milieu composed of mineral surfaces, preferably chiral (Martin and Russell 2003; Hazen and Sverjensky 2010; Dass et al. 2018). Gradients in temperature, salinity, redox state, and pH are natural disequilibrium drivers that are implicated in chemical evolution, and mineral surfaces are considered equally necessary for abiogenesis, given their ability to chelate and determine the conformation of molecules concentrated at their surfaces (Hazen and Sverjensky 2010). Whether mineral phases act purely as the catalytic forces of conformation, or have morphologies at the microscale that drive the concentration of organic molecules and favor forward reaction dynamics (e.g. Parsons et al. **?**pa98), the role of minerals in systems chemistry models for the origins of life is irrefutable. These three factors—organic molecule production, reaction concentration and substrate availability—are necessary prerequisites for an environment to be considered as a potential theatre for the origins of life (Westall et al. 2018).

<ref:pa98?>

Further constraints, such as whether the fluid dynamics of the environment are appropriate for long-term turbid mixing of molecule-mineral mixtures, and whether temperatures favor forward complexification or backward molecular simplification reactions are yet to be fully assessed (Westall et al. 2018). The timescales involved in the environmental and organic chemistry processes and the lifetime of the environment itself are further parameters to be considered. In this regard, subaerial hot spring systems are less compelling than their submarine equivalents; however, hydrothermal fields of all types may endure for over several million years (Martin and Russell 2007; Westall et al. 2018; Cavalazzi et al. 2019). On the early Earth, geological longevity of subaerial environments would have been limited by periodic destruction by impactors, which were incident upon the Earth at a frequency up to hundreds or thousands of times higher than at present (Koeberl 2006; Sleep 2018; Pearce et al. 2018). Notwithstanding, the recent re-evaluation of the severity of the Late Heavy Bombardment means that planet-sterilizing impact events may have been very uncommon (Zellner **?**za17). Recent schemes for long-term chemical evolution in subaerial hot springs (Van Kranendonk et al. 2017) would, however, be significantly limited by such temporal constraints, since environments that were not protected by an oceanic covering would have been susceptible to irrevocable alteration and destruction on potentially short-term periodic cycles. For this reason, ocean worlds deserve recognition as hosting habitable environments that may have allowed the origin and proliferation of life (Lammer et al. **?**la09; Barge and White **?**bw17). Enceladus is the ideal test case, given that the proposed conditions at its ocean floor or in its plumes may be simulated in the laboratory as part of experiments with astrobiological application (Barge and White **?**bw17; Taubner et al. 2018).

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Both Enceladus and Europa are thought to have an internal structure that includes a contact zone between the ocean layer and the underlying silicate crust (Kargel et al. **?**ka00; Chyba and Phillips **?**cp01; McKay et al. **?**mc18). Devolatilization of the rocky crust or mantle of these moons would conceivably lead to hydrothermal effluent generation, which could have produced local oceanic conditions conducive to habitability and prebiotic chemistry through providing an aqueous environment with an adequate energy source, the production of bio-essential elements and organic monomers and oligomers, and disequilibrium conditions in the form of temperature and pressure gradients (Kargel et al. **?**ka00; Lammer et al. **?**la09). Ocean worlds are therefore potential localities for a second, possibly independent, origin of life in the Solar System. Assuming that life could have arisen on the icy moons of the outer Solar System, the question for palaeobiologists becomes one of the nature of traces of life that might be preserved and detectable. This poses a range of challenges very different to the extant life discussed thus far.

<ref:ka00?> <ref:cp01?> <ref:ka00?> <ref:la09?>

4.2 Ancient Traces of Life and Their Lessons for Biosignature Detection on Ocean Worlds

Robust evidence for life on Earth dates back to at least 3.481 Ga, based on critical studies of the stromatolites of the Dresser Formation (Walter et al. 1980; Van Kranendonk et al. 2006; Hickman-Lewis et al. **?**hl19). The Dresser Formation stromatolites do not preserve unambiguous evidence of the microfossil architects themselves, for which further detailed studies beyond current carbon isotope work (Ueno et al. **?**ue01, 2006) are required, but rather lamination characteristics in the organo-sedimentary structure that are demonstrated to have biological morphogenesis (Hickman-Lewis et al. **?**hl19). Nonetheless, it is highly probable that these stromatolites are photosynthetic in origin, and are thus not of direct relevance to the habitable realms of ocean worlds, which demand chemosynthetic metabolic networks. Aside from the Dresser Formation, the oldest generally accepted fossiliferous horizons—those that have undergone and resisted some level of scientific criticism—are the 3.44 Ga Kitty's Gap Chert (Westall et al. 2006, 2011), the ∼3.43 Ga Strelley Pool Formation (Hofmann et al. 1999; Allwood et al. 2007) and the 3.42 Ga Buck Reef Chert (Tice and Lowe 2004; Tice 2009; Greco et al. 2018), although the first two examples are not without their critics (Lowe 1994; Lindsay et al. 2005; Wacey 2009). Comparably ancient Palaeoarchaean fossiliferous material has more recently been described from the 3.47 Ga Middle Marker horizon (Hickman-Lewis et al. 2018), the 3.46 Ga stratiform Apex chert (Hickman-Lewis et al. 2016) and the 3.27 Ga Mendon Formation (Trower and Lowe 2016). With continued study at ever higher resolutions and ever more careful scrutiny, these and other examples may or may not emerge as widely accepted biosignatures. At the limit of the geological record, proposed Eoarchaean biosignatures have been described from the >3.7 Ga Isua supracrustal belt of Greenland (Rosing 1999; Nutman et al. 2016; Hassenkam et al. 2017), and the >3.7 Ga Nuvuagittuq greenstone belt (Dodd et al. 2017) and >3.8 Ga Saglek Block (Tashiro et al. 2017) of Canada, but these are considerably more controversial. The iron-rich filament-like structures in hydrothermal deposits described by Dodd et al. (2017) have been reconsidered as volcanic glass (Wacey et al. **?**wa18), whereas the putative stromatolites described by Nutman et al. (2016) have been found to more closely resemble metamorphosed carbonate sedimentary textures resulting from compression (van Zuilen **?**zu18; Allwood et al. **?**al18). The carbon isotopes described by Rosing (1999) in Greenlandic sediments are, however, distinctly more robust, since the average value, $\delta 13C = -19‰$, is consistent with the simple Chloroflexus-like microbial consortia coupled with archaeal methanogens, i.e., an ecosystem dominated by the

<ref:hl19?> <ref:ue01?> <ref:hl19?> <ref:wa18?>

acetyl-CoA or propionyl-CoA pathway, that is suspected to dominated the primary productivity on Earth prior to the advent of oxygenic photosynthesis (Nisbet and Fowler 1999).

In all cases, putative fossil biosignatures must meet three benchmark criteria: (i) they should possess features or signatures (morphological, structural and geochemical) consistent with biology; (ii) they should be syngenetic with their host rock; and (iii) the host rock should evidence a geological setting consistent with habitability. The order in which this assessment is made is of little consequence to proposing a biosignature, since failure to meet even one of the three criteria is sufficient to preclude a feature being adjudged of palaeobiological significance. Having been submitted to several billion years of processes capable of changing them beyond recognition, the identification and analysis of the most ancient biosignatures can be fraught with difficulties. On Earth, the fundamental dichotomy between Eoarchaean and Palaeoarchaean-Mesoarchaean fossil-like remains is the difference in metamorphic grade between the host rocks, which can be approximately summarized as "up to amphibolite facies" and "up to greenschist facies", respectively. Consequently, the stratigraphic, geochronological and eventual biogeochemical constraints able to be placed upon Eoarchaean traces of life are far less robust than those for the Palaeoarchaean (Whitehouse et al. 2019). This has resulted in a range of putative Eoarchaean biosignatures postulated based on geochemistry and geochronology, and for which biogeochemistry is criticized subjectively, contrasted with a range of Palaeoarchaean biosignatures evaluated by microbial palaeontology and biogeochemistry, and whose fossiliferous nature can be assessed objectively. In all biosignatures assessment, the first task is to determine whether the purported signature is truly of biogenic origin and not an abiotic look-alike (biomorph) or artefact. Microbial structures and constructs, both macroscopic and microscopic, often have very simple shapes that can be imitated by abiotic processes (Garcia-Ruiz et al. **?**gr03; Westall et al. 2006; Rouillard et al. **?**ro18): spheroidal microfossils may be easily confused with spheroidal mineral precipitates, such as silica, while a sheet-like concentration of abiotic organic material could, without microscopic assessment, superficially resemble a biofilm. Disseminated organic matter in ancient sediments, especially when significantly degraded, needs to be distinguished from abiotic organic matter of hydrothermal or other origin. A noteworthy case study of controversial biogenicity is presented by the microfossil-like objects of the 3.46 Ga "Apex Chert," Western Australia. Although initially interpreted as organisms with a cyanobacterial affinity (Schopf and Packer **?**sp87; Schopf **?**sc92), later studies of the same material gradually unravelled the case for their biogenicity (Brasier et al. **?**br02, **?**br05, **?**br06; Wacey et al. **?**wa16). Although of superficially microfossil-like morphology (filamentous, apparently septate), high-resolution FIB-SEM work demonstrated that this morphology results from aluminous clay minerals onto which carbon had become fortuitously adhered (Brasier et al. **?**br15; Wacey et al. **?**wa16). Recent isotopic studies suggesting morphotype-specific carbon isotope fractionation indicative of a mixed methanogen-methanotroph community (Schopf et al. **?**sh17) mean that this particular controversy is ongoing. Such cases of controversial or mistaken biogenicity plague biosignatures of all sizes, up to and including stromatolites. A famous (albeit extreme) example thereof is the "Taylor stromatolite," a complex laminar-domical structure closely resembling modern stromatolites but having been created by coincidence during paint spraying in the mid-Twentieth Century. Similar supposed abiological examples are known from the geological record, and especially ancient stromatolitic occurrences, such as the 3.481 Ga Dresser Formation and 3.43 Ga Strelley Pool Formation stromatolites, have been routinely subject to strong criticism (Lowe 1994; Lindsay et al. 2005) in spite of bearing many biological characteristics (Walter et al. 1980; Van Kranendonk **?**kr07; Hickman-Lewis et al. 2016, **?**hl19). At the time of writing, scientific consensus on these stromatolites suggests that their origin is biological.

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<ref:sp87?>
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Having established the biogenicity of the feature, the second task is to establish its syngenicity with the host rock. Microbes may infiltrate cracks and fissures in rocks of various ages (as chasmoliths or endoliths) and can become fossilised in their endo-/chasmolithic habitats. Westall and Folk (**?**wf03), for example, demonstrated that organisms previously considered syngenetic within ∼3.8 Ga rocks from the Isua supracrustal belt are in fact Holocene endolithic cyanobacteria. The case for syngenicity in carbonaceous microfossils on Earth is often strengthened by Raman spectroscopy demonstrating that the carbonaceous material and its host rock have equivalent thermal histories (e.g. van Zuilen et al. **?**zu07; Marshall et al. **?**ma07)

The third, governing consideration in biogenicity is the environment of formation, i.e., does the purported biosignature occur in a geological context consistent microbial habitability? Most such proof in ancient successions relies on a combination of sedimentology and trace and rare earth element geochemistry (e.g., Lowe and Byerly **?**lb99; Hofmann and Bolhar **?**hb07) and shows that early Earth environments were strongly influenced by volcanogenic inputs and hydrothermal fluids that are manifested as silicification zones in basalts beneath chert horizons.

4.3 Fossil Microbial Biosignatures Relevant to Ocean Worlds

As highlighted in the earlier sections of this review, the diversity of microbial biosignatures of relevance to ocean worlds is vastly reduced when compared to that of Earth due to the fact that all habitable environments on ocean worlds, particularly those at the seafloor, many tens of kilometres beneath the outer icy covering, would have been polyextremophilic. Accordingly, 'highly evolved' Palaeoarchaean microbial mat communities may reflect a degree of biological complexity beyond that possible on Enceladus or Europa. Proposed primitive, uncomplicated biofilm communities that may be evidenced in the Eoarchaean fossil record may be of more relevance, reflecting hyperthermophile, non-photosynthetic autotrophic communities (Nisbet and Fowler **?**nf96, 1999; Rosing 1999). Methanogens are among the proposed earliest independent lineages in the tree of life, diverging from Euryarchaeota before 3.51 Ga and perhaps as early as 3.8–4.1 Ga (Battistuzzi et al. **?**ba04; Wolfe and Fournier **?**wf18). Having numerous extremotolerances that make them suitable candidate organisms for ocean world biomes (Taubner et al. 2015, 2018), understanding biosignatures of methanogenic life in the fossil record may be informative for their detection on ocean worlds. The obvious caveat to this section of the review is that it would be extremely challenging to access and analyse an extinct biosphere within the crust of either Europa or Enceladus.

Most evidence for methanogenesis in the fossil record relies upon carbon isotope ratios measured by in situ secondary ion mass spectrometry, since methanogenesis is characterized by a range of δ13C values mostly falling between -5 and $-41‰$, i.e., with values slightly to significantly more negative than other major metabolic pathways—rubisco-mediated photosynthesis, sulphate reduction, photoferrotrophy—evidenced in the fossil record at the same time (Schidlowski **?**sc88; Vieth and Wilkes **?**vw09). Their more negative carbon isotope fractionations can thus be used to indicate the presence of both Bacteria and Archaea in fossilized biomass (Hayes **?**ha94; Nisbet and Fowler 1999). The carbon isotope record therefore provides independent support for the molecular clock estimations of methanogenesis as early as 3.8 Ga by virtue of highly 13C depleted carbonaceous material in Greenlandic rocks (Grassineau et al. **?**gr05). Extreme depletions of up to $-60‰$ in carbonaceous material from Palaeoarchaean horizons have been used as implicit evidence for coupled methanogenesis and methanotrophy in widespread microbial ecosystems (Hayes **?**ha94; Schopf et al. **?**sc17; Lepot et al. **?**le19).

<ref:wf03?> <ref:zu07?> <ref:ma07?> <ref:lb99?> <ref:hb07?> <ref:nf96?> <ref:ba04?> <ref:wf18?> <ref:sc88?> <ref:ha94?> <ref:gr05?> <ref:sc17?> <ref:le19?>

For ocean worlds, traces of planktonic life might also be among the key biosignatures for a fossil biosphere. Prior to the advent of oxygenic photosynthesis, planktonic life seems essentially limited to the hypothesised modes of life of large spheroidal and lenticular microfossils described from numerous horizons in the East Pilbara terrane and the Barberton greenstone belt, although this is likely a function of preservational potential. Indeed, planktonic life away from hydrothermal vents is considered to have had the opportunity to proliferate once organisms had adapted to oligotrophy (e.g., Nisbet and Fowler 1999; Brasier et al. **?**br06). In Archaean metasediments, spheroidal and lenticular microfossils <ref:br06?> up to several hundred microns in size and with interpreted robust cellular morphologies (thickened, spore-like cell walls) span more than 400 Ma of geological history, from the 3.4 Ga Strelley Pool Formation (Sugitani et al. 2015) and Kromberg Formation (Walsh 1992; Oehler et al. 2017) to the 3.0 Ga Farrell Quartzite (Sugitani et al. 2007). These microfossils are characterised by strongly negative carbon isotope fractionation ($\delta 13C = -30$ to $-45‰$), consistent with biological origin and sufficiently restricted in range as to preclude origin in abiogenic chemical reactions such as the Fischer–Tropsch type processes (House et al. 2013; Oehler et al. 2017). The highly negative depletions may also be consistent with methane cycling, but this has yet to be unambiguously demonstrated (Oehler et al. 2017). Particularly enigmatic amongst these microfossil-like objects are the lenticular microfossils. The near-equant morphologies of lenticular microfossils, together with the flange-like appendages that characterise their equatorial regions, have been used as specific evidence for their having a planktonic stage in their life cycle (Sugitani et al. 2007, 2015; Oehler et al. 2017). Fluid dynamic modelling of virtual flanged cells has demonstrated both that the presence of the flange reduces sedimentation velocity and enlarges cell volume, two factors increasing their propensity for suspension and dispersion as part of a planktonic lifestyle (Kozawa et al. 2018). Dispersion may further be inferred from the widespread distribution of these fossils in space, i.e. across two Archaean landmasses (the Pilbara and Kaapvaal regions). Although many of these microfossils are solitary occurrences, some pairs, clusters and chains of lenticular objects have been described, particularly in the examples from Western Australia, strongly increasing the case for their biogenicity (see Sugitani 2018).

In contrast to coccoidal and filamentous microfossils from the same formations (Walsh 1992; Walsh and Lowe 1999; Westall et al. 2001), lenticular and large spheroidal microfossils typically show no strict association with stromatolitic or mat-like laminations, which imply that they are not involved in the mat-building process. Although this can be seen as implicit support for a planktonic lifestyle, instances of lenticular carbonaceous objects from the Middle Marker horizon do indeed occur within microbial mats (Hickman-Lewis et al. 2018). While this does not argue against their biogenic origin, their evident simultaneous formation with microbial mats in this unique case warrants further investigation.

The thick cell walls that characterize these organisms have been argued to be beneficial to open ocean modes of life. Oehler et al. (2017) interpreted that such thick walls may have enabled the cells to withstand high levels of UV radiation, metal toxicity, or sudden evapotranspiration events and associated salt stress that may have characterised early Earth habitats (see Lowe et al. 2014; Lowe and Byerly 2015). The potential for dispersion and longevity may also have permitted robust, lenticular cell-like objects to withstand local-scale environmental stresses inherent to ocean worlds in ways that more fragile organisms with thinner cell walls could not. The application of the microfossil record to ocean worlds remains very much an open topic; indeed, limited, if any, discourse on the subject had been attempted before this review.

The true challenge of a correlative microscopy approach in palaeobiology applied to the putative biomes of an oceanic celestial body such as Europa and Enceladus lies in the dif-

ficulty inherent in accessing samples. At present, no mission objectives involve the assessment of the crust of an ocean world due to the near-insurmountable challenge of reaching the required localities. This section of the review may therefore be little more than intellectual discourse. Nonetheless, one can state that deducing the geology and geochemistry of putative hydrothermal vent deposits on Europa or Enceladus would open up the possibility to appraise the habitable niches of ocean worlds and consider the likelihood of a fossilised biosphere of purely chemotrophic life. Such a biosphere may be an excellent—and indeed a truly pristine—analogue for the most primitive (hyper)thermophile biospheres on the Hadean-Eoarchaean Earth.

5 Conclusions

Life, especially in the form of microorganisms, has achieved colonization of almost all areas on Earth, even the most hostile and extreme parts of the planet. Organisms have adapted by tailoring their cellular constituents to operate also at the boundaries and limits of life. Microorganisms have been successful in diversifying their metabolisms and taking benefit of the resources available in environments which might be low in the levels of nutrients or extreme in its physical conditions. In these harsh environments they manage to generate enough energy to ensure a minimum of maintenance of cellular constituents and even to proceed reproduction. This metabolic adaptation and diversity of microorganisms has been illustrated by the ability of microorganisms to produce energy from different types of substrates, to produce different types of molecules, such as those that make membranes more robust or those that are characteristic in the response to different stresses whose harmful effects normally cause the denaturation of most cellular components and finally leads to cellular death.

The extent of microbial diversity on Earth is far from being fully elucidated, particularly in remote and extreme environments such as deep sea and subsurface sediments. Organisms living in extreme environments and in particular microorganisms have, over the evolutionary process, developed a large variety of adaptive strategies. As a result, they present a repertoire of original metabolic pathways and biomolecules that allow them not only to survive in extreme conditions, but often to grow in an optimal way in extreme ecological niches. Metabolic markers such as membrane lipids (saturated and polyunsaturated fatty acids, archeaol and caldarcheaol, etc.), compatible solutes (amino acids and derivatives, sugars and derivatives, polyols) or gas production (e. g. methane), witnesses of biological activity, have been detected in increasingly improbable environments previously considered sterile: thermal springs, hydrothermal vents, acidic lakes, alkaline lakes, hypersalines, deep marine sediments, oil reservoirs, glaciers, etc. The physico-chemical and energetic characteristics of some extreme terrestrial environments are analogous to those of other planets and icy moons in the Solar System, which raises the question of the past or present existence of life on these planets and icy moons, or the fulfilment of all the conditions for another origin of life. Biomolecules or biosignatures such as those listed in this chapter can be traced to detect early clues to potential extraterrestrial biological activity.

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