

# Exploring Deep-Sea Brines as Potential Terrestrial Analogues of Oceans in the Icy Moons of the Outer Solar System

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## Abstract

Several icy moons of the outer solar system have been receiving considerable attention and are currently seen as major targets for astrobiological research and the search for life beyond our planet. Despite the limited amount of data on the oceans of these moons, we expect them to be composed of brines with variable chemistry, some degree of hydrothermal input, and be under high pressure conditions. The combination of these different conditions significantly limits the number of extreme locations, which can be used as terrestrial analogues. Here we propose the use of deep-sea brines as potential terrestrial analogues to the oceans in the outer solar system. We provide an overview of what is currently known about the conditions on the icy moons of the outer solar system and their oceans as well as on deep-sea brines of the Red Sea and the Mediterranean and their microbiology. We also identify several threads of future research, which would be particularly useful in the context of future exploration of these extra-terrestrial oceans.

## The icy moons of the Outer Solar System

Icy moons are natural satellites that are characterised by a surface that is composed predominantly of ice, which may contain a sub-surface ocean,

and possibly a rocky core of silicate or metallic rocks. In the outer Solar System, icy moons orbit the gaseous planets, Jupiter and Saturn. The icy moons of Jupiter — Io, Europa, Ganymede and Callisto — were discovered by Galileo Galilei in 1610 and are frequently referred to as the Galilean moons of Jupiter (Showman and Malhotra, 1999); whilst Saturn is orbited by numerous icy moons, which include Enceladus, Mimas, Tethys, Dione, and Titan.

The icy moons are of extensive interest for future exploration due to their potential sub-surface oceans. For example, Europa (Kivelson et al., 2000), Ganymede (Kivelson et al., 2002), Callisto (Khurana et al., 1998), and Enceladus (Spencer and Nimmo, 2013) present evidence of a briny ocean under an icy surface. As our understanding of these icy moons evolves so does interest from an astrobiological point of view. The following sections will discuss two of these icy moons, Enceladus and Europa, in more detail.

### *Enceladus*

#### Surface

Enceladus is covered with a thick icy shell that varies in depth dependant on latitude. Based on data from Cassini, modelling has predicted that the ice shell is approximately 18 to 22 km thick on average, but less than 5 km at the South Pole region (Cadek et al., 2016). The composition is believed to be almost pure water with spectroscopy data suggesting that it also contains small amounts of carbon dioxide, hydrogen peroxide, light organics, and perhaps ammonium (Brown et al., 2006, Emery et al., 2005). The temperature at the surface at low latitude varies from approximately -223°C at night to -193°C during the day (Howett et al., 2010). The surface has a particularly high albedo (Howett et al., 2010) and contains several regions of cratered and smooth terrain, which may denote differences in their respective age (Squyres et al., 1983a).

In the South Polar Region unique surface features called "tiger stripes" are observed, which are central to present-day geological activity (Spencer and Nimmo, 2013; Spencer et al., 2006). The stripes are the source of thermal activity and associated plume emissions venting from the surface (Squyres et al., 1983a), which were observed by the Cassini flyby mission. The presence of these plumes suggests the existence of a sub-surface ocean consisting of liquid water (Waite et al., 2017) and analysis of the icy grains in the plumes imply that they are rich in sodium (Postberg et al., 2011). The salt-composition is similar to that expected for water that has equilibrated with Enceladus's presumed silicate core (Zolotov, 2007). The plumes may be caused by hydrothermal activity at the ocean floor (Hsu et al., 2015; Waite et al., 2017), which is supported by data from the Cassini mission that shows tidal dissipation in the rocky core.

### Sub-surface ocean

Contrary to previous assumptions, the ocean of Enceladus is believed to have a global distribution rather than being restricted to polar regions (Patthoff and Kattenhorn, 2011). The energy required to maintain liquid water most likely originates from the dissipation of tidal energy from the friction of the sub-surface ocean with the internal silicate interior (Nimmo et al., 2007).

Information regarding the composition of the ocean has been obtained from plume analysis, which showed that particle emissions are dominated by water ice and is rich in sodium and potassium salts (0.5 to 2 % by mass), but also contains sodium bicarbonate/ sodium carbonate (Postberg et al., 2011). The ocean is expected to contain low concentrations of ammonia, methane, carbon dioxide and molecular hydrogen (Waite et al., 2017). These elements are potentially produced as a result of geochemical reactions occurring at the interface of the chondrite-like core and the ocean at temperatures below 100°C (Waite et al., 2017). The circulation of the water would drive the chemical evolution of both the rock material and the ocean water, producing a chemical gradient, which could be used by microorganisms to generate energy (Barge and White, 2017). The presence of silica nanoparticles in the plumes is also evidence for hydrothermal reactions occurring in the interior, which may be the source of molecular hydrogen (Sekine et al., 2015).

Based on the chemistry of the plumes, the pH of the ocean is predicted to be alkaline, with current estimates placing it between 8.5 and 13 (e.g. Postberg et al., 2009; Hsu et al., 2015). These values have been calculated by either modelling the equilibrium reactions at the water-rock interface or by the concentrations of salts in the plumes (Zolotov, 2007; Postberg et al., 2009). The temperature varies within the sub-surface oceans. The estimated temperature at the ocean-ice interface is approximately -0.15°C (Glein et al., 2015); whereas SiO<sub>2</sub> nanoparticles in the plumes suggest a minimal localised temperature of 90°C at the water-rock interface. Gravimetric measurements have suggested that that rock-ocean and ice-oceans interfaces are at depths of 50 km (~ 5.3 MPa) and 35-40 km (3.6-4.2 MPa) (less et al., 2014). However, values have been estimated to be as high at 10 MPa at the rock-ocean interface (Zolotov, 2007) and 8 MPa at the ice-ocean interface (Hsu et al., 2015). It should be noted that these pressures are lower than that on Earth due to the gravitational force associated with the icy moons.

### *Europa*

#### Surface

Europa is covered by an icy shell, with a highly debated thickness that is estimated to range between a few km to over 30 km (Billings and Kattenhorn, 2005; Quick and Marsh, 2015). The surface of Europa is

dominated by ridges and chaotic terrain, which is thought to be relatively young, or still geologically active (Squyres et al., 1983b; Carr et al., 1998). These regions indicate locations where resurfacing of material from the sub-surface ocean may have occurred (Hand and Carlson, 2015). The surface is a distinct yellow-brown colour, which has been postulated to result from sulfur chemistry, either from an exogenous or endogenous source (Geissler et al., 1998; Carlson et al., 2009). However, Hand and Carlson suggested that the discolouration might be due to sodium chloride from the sub-surface, which yields a yellow-brown colour when exposed to the radiation conditions present at the surface of Europa (Hand and Carlson, 2015). Observations of the thermal emissions by the Galileo mission showed low latitude diurnal brightness temperatures between  $-194^{\circ}\text{C}$  in the Polar Regions and approximately  $-143^{\circ}\text{C}$  at the equatorial regions (Spencer et al., 1999). Images from the Hubble Space Telescope suggest the presence of water-vapour plumes, appearing to rise 200 km above the disk of Europa's solid body, which would indicate transport of material from the interior ocean to the surface (e.g. Ross and Schubert, 1987; Roth et al., 2014; Sparks et al., 2016; Jia et al., 2018). Furthermore, tidal stress has been suggested as playing a role in the opening and closing of fractures at the surface to allow water vapour to be released from the sub-surface ocean (Roth et al., 2014).

#### Sub-surface ocean

Magnetometer measurements suggest that Europa has a global-scale ocean (Ross and Schubert, 1987; Kivelson et al., 2000). The formation of this liquid ocean is thought to be due to tidal heating, tidal flexing, and/or to radioactive decay of the silicate core (Ross and Schubert, 1987; Han and Showman, 2010). Based on the expected salinity of the ocean brine the temperature will be at a minimum,  $-13^{\circ}\text{C}$  (Zolotov and Kargel 2009), with the maximum temperature occurring at the ocean floor where hydrothermal activity may occur ( $\sim 90^{\circ}\text{C}$ ) (Kargel et al., 2000; Zolotov and Kargel, 2009). Therefore, temperatures that are significantly higher than freezing should only exist in porous channels below the seafloor, or close to hydrothermally active regions (Vance and Goodman, 2009). At the ocean floor the pressure has been modelled to be approximately 110 MPa (Hand et al., 2009).

Due to lack of direct measurements of the sub-surface ocean, the chemical composition remains unknown. However, initial experiments and theoretical simulations strongly suggest that the composition of the ice surface is a direct result of the ocean material from below and could thus be used to infer its composition (Kargel et al., 2000). Yet, more recently the chemical species present at the surface (e.g. sulfates) were suggested to be a result of radiation (Hand and Carlson, 2015). Therefore, geochemical models have been used to predict the composition of the salty brine, which results in widely varying compositions. For example, modelling of the initial chondritic composition leads to a magnesium and sulfur-rich ocean (Kargel

et al., 2000); whereas modelling of the water-to-rock cycling at the silicate seafloor leads to a chloride-rich ocean (Glein and Shock, 2010). The chemical composition of the ocean would influence the pH, which limits our ability to make conclusive predictions in this regard. For example, a brine dominated by sulfate would be acidic and a brine rich in chloride would be basic or neutral (Zolotov, 2007; 2009). Due to surface irradiation and tidal forces associated with Europa it has been postulated that a metastable dynamic state would occur, which could support microbial metabolism. For example, the water-rock interaction at the ocean floor would produce electron-rich energy sources, while oxidants would be produced due to radiolysis at the ice surface (Hand et al., 2004; Russell et al., 2017).

### *Icy moon analogues*

Terrestrial analogue environments exhibit conditions that are similar to those of planetary bodies and moons in the Solar System (Martins et al., 2017). Historically, the majority of terrestrial analogue sites have been selected for studying Mars. More recently, however, attention has focused on the icy moons with a number of studies using sulfate lakes (e.g. Prieto-Ballesteros et al., 2003), cold-springs (e.g. Gleeson et al., 2012) and sub-glacial lakes as analogues for the sub-surface oceans (Garcia-Lopez and Cid, 2017). For example, the hypersaline subglacial lake beneath the Devon ice cap in the Canadian Arctic has been discovered recently and considered as an analogue for the sub-surface ocean of Europa (Rutishauser et al., 2018). However, to fully understand the processes that occur, and the potential life, in the sub-surface oceans of the icy moons an ideal analogue would be terrestrial deep seas. These environments are among the last earthly frontiers for discovery. Many areas of the ocean floor remain inadequately mapped, and new geomorphological features, and several new life forms have been isolated. The discovery and exploration of deep-hypersaline anoxic basins is particularly relevant from an astrobiological perspective, as it shows striking parallels with the oceans of the icy moons of the Outer Solar System.

While detailed information on the exact physical-chemical conditions present in the exooceans of the icy moons of the outer Solar system is still quite limited, we now know that these are brine oceans and likely associated with localized hydrothermal input. Salinity estimates vary but most seem to point to a somewhat lower value than the Earth's oceans, although these have been reported as having a high degree of uncertainty of two orders of magnitude (Lunine, 2017). Furthermore, several authors propose mechanisms that would lead to expected localized significant increases in salinity. Indeed, increase in salinity might arise from brine exclusion during freezing at the interface between the ice crust and the underlying ocean (equivalent to the formation of brine inclusions on marine ice on Earth) or interactions between seawater and rocks at the bottom of these exooceans (Hendrix et al., 2019). Furthermore, strong double diffusive convection processes have been predicted for Europa (Vance and

Goodman, 2009; Elsenousy et al., 2015), leading us to expect density gradients driven by temperature and salinity. According to some of the possible scenarios proposed by these authors, Europa might have a stratified ocean and precipitation from rising plumes could cause "snowing" effects with precipitation of salt, which would increase the salinity closer to the bottom and might even lead to thick layers of salt deposits.

Deep-hypersaline anoxic basins provide an interesting proxy for several of these conditions and their wide range of physical-chemical conditions allow us to partly circumvent the wide variability of predictions on the physical-chemical settings in these exooceans. The following section will focus on these unusual deep-sea extreme environments and their microbes, with the discussion being centred on the Red Sea and the Mediterranean.

### **Deep-hypersaline anoxic basins (DHABs)**

#### *Origin and main characteristics*

DHABs are very unusual environments, which combine a unique range of environmental extremes and are regarded as one of the most extreme environments on our planet (e.g. Antunes, 2017; Merlino et al., 2018; Antunes et al., 2019). They were accidentally discovered in the axial region of the Red Sea after the collection of unexpectedly warm and salty deep-sea water during an expedition of the RV Albatross in 1947/48 (Bruneau et al., 1953). Further studies led to the identification and description of the first DHAB in the Red Sea (Miller, 1964) and wider surveys identified several additional ones scattered across the central axis of the Red Sea. Similar environments were later detected in the Mediterranean Sea (Cita et al., 1985) and in the Gulf of Mexico (Shokes et al., 1977), with on-going exploration regularly increasing the number of identified DHABs (e.g. Ehrhardt et al., 2005; Yakimov et al., 2013, 2015).

All of these DHABs seem to be associated with tectonic activity in areas of the globe with deeper saline strata formed *via* evaporation of ancient seas. Such tectonic activity promotes the formation of topographical depressions and exposes these evaporite strata to dissolution and leaching events and formation of brines that accumulate in several of these depressions as a result of their significantly higher density. The sheltered location of the brines together with their higher densities, and very weak deep-sea currents, contribute to very limited mixing with overlying seawater and lead to stable brine bodies with very sharp brine-seawater interfaces, which display drastic transitions in physical-chemical conditions often occurring within the span of a few meters (De Lange et al., 1990a; Eder et al., 2001; van der Wielen et al., 2005; Borin et al., 2009; Antunes et al., 2011d).

The brine-seawater interfaces are very complex environments, with significant shifts in salinity, O<sub>2</sub> concentration, pH, and temperature over a relatively small vertical scale. Such transitions provide a variety of

environmental niches and ideal conditions for the establishment of local redox cycles, which involve biotic input. Furthermore, the density difference occurring at the brine-seawater interface leads to increased particle load by trapping sinking particles and mineral precipitates produced in local redox reactions. The trapping of organic matter combines with the redox gradient to create a variety of electron donor-acceptor couplings that are favourable for microbial generation of energy, making this compartment of the brines the one with the highest activity.

There is extensive discussion about the extent to which these DHABs were formed by leaching of tectonically exhumed halite or by release of relic brines trapped within the evaporites (Vengosh et al., 1998; Camerlenghi, 1990; Cita, 2006), or supercritical heating at great depth of seawater resulting in hydrothermally formed salts and brines (Hovland et al., 2018), with the diverse sources of the brines resulting in distinct chemistries (Table 1).

The primary difference between the DHABs of the Red Sea and the eastern Mediterranean Sea is that those in the Mediterranean are generally cooler (~15°C), which reflects the inherently lower temperature of the deep Eastern Mediterranean (~13.5°C; Tsimplis and Baker, 2000) compared with that of the Red Sea (~21.5°C; Yao and Hoteit, 2018), as well as a lesser contribution by hydrothermal fluids in the Mediterranean (Cita, 2006). They are also the deepest DHABs at more than 3000 mbsl (Cita, 2006). Overall, the Red Sea DHABs are, perhaps, geochemically less diverse than those of the Mediterranean (based on dissolved ion concentrations) but have more variable temperatures and generally higher concentrations of heavy metals (Antunes et al., 2011d).

#### *Red Sea DHABs*

The DHABs of the Red Sea were formed as a result of the tectonic split of the Arabian and African tectonic plates and re-dissolution of evaporites from the Miocene (mostly composed of halite and anhydrite).

The Red Sea has the highest number of known DHABs, which include: Albatross, Atlantis II, Chain, Conrad, Discovery, Erba, Kebrit, Nereus, Oceanographer, Port Sudan, Shaban, Shagara, Suakin, Valdivia, and Wando Basin (Bruneau et al., 1953; Backer and Schoell, 1972; Pautot et al., 1984; Cochran et al., 1986). Based on their geochemical properties (Table 1), a recent cluster analysis by Schmidt et al. (2015) split the DHABs of the Red Sea into two groups, which could be correlated with the local sedimentary and tectonic setting of the individual deeps.

#### *Red Sea Type 1 Brines – Oceanographer and Kebrit DHABs*

Type 1 brines have high salinity, low pH, low temperature, and low trace-metal concentrations which are probably linked with their high H<sub>2</sub>S content (e.g. Weber and Gurskii 1982; Schmidt et al., 2003). They are located

**Table 1.** Overview of major physical-chemical characteristics of the DHABs of the Red Sea and the Mediterranean Sea.

	Na <sup>+</sup>	Cl <sup>-</sup>	Mg <sup>2+</sup>	K <sup>+</sup>	Ca <sup>2+</sup>	SO <sub>4</sub> <sup>2-</sup>	pH	T (°C)
<b>Red Sea</b>								
<b>Albatross</b>	4344.88	4608.75	48.84	55.96	111.98	14.89	6.8	23.9
<b>Atlantis II</b>	4677.73	5189.97	35.42	86.88	148.71	10.74	5.21	68.2
<b>Chain</b>	4800	5200	36.8	66.8	143.3	8.5	6.48	53.2
<b>Conrad</b>	4295.43	4619.67	101.01	67.7	24.63	48.77	6.1	23.0
<b>Discovery</b>	4635.53	5021.75	36.78	84.86	144.87	10.66	6.2	44.8
<b>Erba</b>	2621.99	2678.14	71.43	30.92	29.57	47.15	7.4	26.1
<b>Kebrit</b>	4805.74	5135.67	121.13	36.7	56.56	20.08	5.5	23.3
<b>Nereus</b>	3556.62	4131.47	64.06	77.42	230.8	11.25	7.43	30.2
<b>Oceanographer</b>	4353.37	4716.86	280.64	77.09	86.21	10.32	5.6	24.9
<b>Port Sudan</b>	3784.68	3855.22	70.89	46.5	35.63	47.77	6.43	36.2
<b>Shaban (N)</b>	4784.51	4900.52	97.96	49.29	19.74	51.57	6.0	25.3
<b>Suakin</b>	2416.77	2612.84	62.87	35.42	57.14	36.05	7.85	24.6
<b>Valdivia</b>	4100	4600	95.3	52.0	25.0	72.0	6.21	29.8
<b>Mediterranean Sea</b>								
<b>Bannock</b>	4200	5378	643.9	126.3	16.3	135.2	6.5	15.1
<b>Discovery</b>	840.17	10154.29	5142.97	89.52	1	110.35	4.5	14.5
<b>Hephaestus</b>	93	9120	4720	28	2	203	5.0	15.3
<b>Kryos</b>	1236.32	9054.24	4402.39	84.4	1	322.71	5.4	14.5
<b>L'Atalante</b>	4654.24	5302.8	658.3	368.31	7.49	333.13	NA	14.3
<b>Medee</b>	4178	5259	788	471	2.8	201	6.7	15.4
<b>Thetis</b>	4760	5300	604	230	9	265	NA	15.1
<b>Tyro</b>	5300	5350	71.1	19.2	35.4	52.7	6.75	14.2
<b>Urania</b>	3505	3730	315	122	31.6	107	6.8	18.3

All chemical composition data provided in mM. NA: Not Available. Data compiled from Antunes et al., 2011d; De Lange et al., 1990b; La Cono et al., 2011; 2019; Merlino et al., 2018; Sass et al., 2001; Schmidt et al., 2015; Van der Wielen et al., 2005; Yakimov et al., 2013; 2015; and references cited therein.

slightly off-axis and controlled by evaporite dissolution rather than contributions from sediment alteration with hydrothermal fluid derived from water/volcanic rock interaction, which appear to be of minor importance.

#### Red Sea Type 2 Brines – Suakin, Port Sudan, Erba, Albatross, Discovery, Atlantis II, Nereus, Shaban, and Conrad DHABs

These brines are influenced by hydrothermal activity and variable contributions from volcanic/ magmatic rock alteration, which explain e.g. the high concentration of Mn, Fe, and Zn and low concentrations of Mg and



sulfate (Seyfried, 1987). This hydrothermal influence is strongest in Atlantis II, which has a multi-layered brine and is also the DHAB with highest recorded temperature.

#### *Mediterranean Sea DHABs*

The DHABs of the Mediterranean share clear similarities with those in the Red Sea, although they occur under a different tectonic setting (convergent plate boundary). The source of the brine is underlying evaporites (deposited in the Mediterranean basin during the Messinian Salinity Crisis 5.97 to 5.32 million years ago), mostly from the acme evaporitic stage (5.60 to 5.55 MYA), which has thick beds of halite with associated minerals (Roveri et al., 2004). However, hydrothermally-derived salt and brine has also been proposed as an alternative source (Hovland et al., 2018).

The Mediterranean DHABs are (in order of discovery): Tyro (Jongsma et al., 1983; De Lange et al., 1990b), Bannock (Scientific staff of Cruise Bannock 1984-12, 1985; De Lange et al., 1990b), Urania (Medriff Consortium, 1995), L'Atalante (Medriff Consortium, 1995), Discovery (Medriff Consortium, 1995), Thetis (La Cono et al., 2011), Medee (Yakimov et al., 2013), Kryos (Yakimov et al., 2015), and Hephaestus (La Cono et al., 2019). Their chemical differences (outlined in Table 1) allow us to split them into three groups.

#### **Mediterranean Type 1 Brines – Urania DHAB**

Urania DHAB is >100 m deep, filled with NaCl-rich brine, similar to type 2 brines but with lower salinity (about six times seawater salinity). Additional features include a second stable but less pronounced chemocline (in addition to the brine-seawater interface), as well as very high sulfide concentrations of up to 16 mM (van der Wielen et al., 2005; Borin et al., 2009). This makes Urania brine among the most sulfidic water body on Earth.

An intriguing feature of the deeper western part of the horse-shoe shaped Urania DHAB is the high-temperature bubbling mud vent (45°C; 10% salinity) beneath the relatively cold Urania brine (16°C; 27% salinity) resulting in a reverse halocline (Yakimov et al., 2007a).

**Mediterranean Type 2 Brines – Bannock, L'Atalante, Medee, Thetis DHABs**  
Mediterranean Type 2 Brines are dominated by Na<sup>+</sup> and Cl<sup>-</sup> ions, have an ionic composition that largely reflects concentrated seawater, and have a salinity that is about eight times higher than that of seawater, which differentiates them from Mediterranean Type 1 Brines. The proximity of the different basins seems to have little effect on chemical composition. For example, L'Atalante basin (Type 2) is close to Urania basin (Type 1) and Discovery basin (Type 3), whereas it is more distant from other Type-2 brines (Merlino et al., 2018).

Mediterranean Type 3 Brines – Discovery, Hephaestus and Kryos DHABs  
DHABs within this group are perhaps the most intriguing of all owing to the exceptionally high  $\text{MgCl}_2$  concentration of their brines (~5 M), which are presumably derived from bischofite ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) that forms in the very latest stages of seawater evaporitic cycles (see La Cono et al., 2019). This high concentration of the divalent ion  $\text{Mg}^{2+}$  coupled with  $\text{Cl}^-$  results in brines that are below the currently accepted water activity ( $A_w$ ) limit of life, which has recently decreased from the long-established value of 0.605 (Stevenson et al., 2015) to 0.585 (Stevenson et al., 2016), as well as brines that are extremely destabilising towards biological macromolecules (i.e. chaotropic) (Hallsworth et al., 2007). The presence of life in such hostile brines would alter our perception of life's limits on Earth and elsewhere.

### *Life in Deep Hypersaline Anoxic Basins*

#### Microbial isolates

The Red Sea DHABs were originally declared sterile in the 1960s (Watson and Waterbury, 1969) and left mostly unexplored for several decades. Later studies, particularly based on molecular-based methodologies, have shown them to be teeming with life.

Work in the DHABs of the Red Sea has proven particularly fruitful in the isolation of microbes representing novel higher taxa. These include *Flexistipes sinuarabici* (the first representative of the phylum Deferribacteres; Fiala et al., 1990; Garrity et al., 2001), *Salinisphaera shabanensis* (representing the new family *Salinisphaeraceae* within the Gammaproteobacteria; Antunes et al., 2003; Vetriani et al., 2014), and *Haloplasma contractile* (the first representative of the order *Haloplasmatales* and likely representing a novel bacterial phylum; Antunes et al., 2008a). Further fully described taxa include several novel species and a new genus, and have been often associated with whole-genome sequencing (e.g. Antunes et al., 2008b; 2011b; Zhang et al., 2017a, 2017b; 2017c; see Table 2 and Table 3 for a full overview). Several additional studies reported the isolation of further microbial strains, unfortunately never fully described but still providing us with further insights into the cultivated microbial diversity of these locations. (e.g. Eder et al., 2001; Sagar et al., 2013a, 2013b; Zhang et al., 2016a, 2016b; see Table 2 and Table 3 for a full overview).

The exploration of the DHABs of the Mediterranean has led to the description of one new species: the archaeon *Natrinema salaciae* (Albuquerque et al., 2012). Studies on the Mediterranean have frequently focused on cultivation-independent approaches or have reported on the isolation of microbial strains that have not been fully described. Enrichment campaigns by Daffonchio et al. (2006) isolated fermentative halophiles such as *Halanaerobiales*, a *Halothiobacillus* that aerobically oxidised thiosulfate with  $\text{CO}_2$  as sole carbon source over a NaCl range of 0.5–23%,

**Table 2.** Proteobacteria isolated from DHABs of the Red Sea and the Mediterranean Sea.

Class Family	Genus/Species/Strain	Origin			Description	Genome	Ref	
<b>Alphaproteobacteria</b>								
<i>Rhodobacteraceae</i>	strain GMDJE10F1	Med	Bannock	BSI	-	-	1	
	<i>Ponticoccus marisrubri</i>	RS	Erba	BSI	new species	+	2	
	<i>Ruegeria marisrubri</i>	RS	Erba	BSI	new species	+	3	
	<i>Ruegeria profundii</i>	RS	Erba	BSI	new species	+	3	
	<i>Roseobacter</i> sp.	Med	Urania	BSI	-	-	4	
	<i>Sediminimonas</i> sp.	RS	Kebrit	BSI	-	-	5	
	<i>Sulfitobacter</i> sp.	RS	Kebrit	BSI	-	-	5	
<i>Rhodospirillaceae</i>	ND	Med	Urania	BSI	-	-	4	
<i>Erythrobacteraceae</i>	<i>Erythrobacter</i> sp.	Med	Urania	BSI	-	-	4	
<b>Betaproteobacteria</b>								
<i>Nitrosomonadaceae</i>	<i>Nitrosovibrio</i> sp.	Med	Bannock	Sed	-	-	6	
<b>Gammaproteobacteria</b>								
<i>Alcanivoracaceae</i>	<i>Alcanivorax</i> sp.	Med	Bannock	BSI	-	-	1	
	<i>Fundibacter</i> sp.	Med	Urania	BSI	-	-	4	
<i>Alteromonadaceae</i>	<i>Alteromonas</i> spp.	Med	Bannock	BSI	-	-	1	
			Urania	BSI	-	-	4	
			L'Atalante	BSI	-	-	7	
			Discovery	BSI	-	-	7	
	<i>Marinobacter</i> spp.	Med	Bannock	BSI	-	-	1	
			Urania	BSI	-	-	4	
			RS	Erba	BSI	-	-	5
			RS	Nereus	BSI	-	-	8
	<i>Marinobacter salsuginis</i>	RS	Shaban	BSI	new species	-	9	
<i>Colwelliaceae</i>	Isolate S11L1B	RS	Shaban	BSI	-	-	10	
<i>Enterobacteriaceae</i>	<i>Salmonella</i> sp.	Med	Bannock	BSI	-	-	1	
<i>Halomonadaceae</i>	<i>Halomonas</i> spp.	Med	Bannock	BSI	-	-	1, 7	
			Urania	BSI	-	-	4, 7	
		RS	Erba	BSI	-	-	8	
			Kebrit	BSI	-	-	5, 8	
			Nereus	BSI	-	-	5, 8	
	<i>Chromohalobacter</i> sp.	RS	Atlantis II	BSI	-	-	5	
			Discovery	BSI	-	-	5, 8	
<i>Halothiobacillaceae</i>	<i>Halothiobacillus</i> spp.	Med	Bannock	BSI	-	-	1	
			Urania	BSI	-	-	7	
			Urania	Sed	-	-	11	

<i>Idiomarinaceae</i>	<i>Idiomarina</i> spp.	RS	Discovery	BSI	-	-	5
		RS	Erba	BSI	-	-	5, 8
		RS	Kebrit	BSI	-	-	5
		RS	Nereus	BSI	-	-	5
		RS	Shaban	BSI	-	-	12
		Med	Bannock	BSI	-	-	1
<i>Oceanospirillaceae</i>	<i>Oceanospirillum</i> sp.	Med	Urania	BSI	-	-	4
<i>Pseudoalteromonadaceae</i>	<i>Pseudoalteromonas</i> sp.	Med	Bannock	BSI	-	-	1
<i>Pseudomonadaceae</i>	<i>Pseudomonas</i> spp.	RS	Suakin	Sed	-	-	13
		Med	Bannock	BSI	-	-	1
			Urania	BSI	-	-	4, 14
			Bannock	Sed	-	-	6
<i>Salinisphaeraceae</i>	<i>Salinisphaera shabanensis</i>	RS	Shaban	BSI	new order	+	15
<i>Vibrionaceae</i>	<i>Vibrio</i> sp.	Med	Bannock	BSI	-	-	1
<b><i>Deltaproteobacteria</i></b>							
<i>Desulfovibrionaceae</i>	<i>Desulfovibrio</i> sp.	RS	Atlantis II	BSI	-	-	16
<i>Cystobacteraceae</i>	ND	Med	Urania	BSI	-	-	4
<b><i>Epsilonproteobacteria</i></b>							
<i>Campylobacteraceae</i>	<i>Sulfurospirillum</i> sp.	Med	Bannock	BSI	-	-	1

Med, Mediterranean; RS, Red Sea; BSI, Brine-seawater interface; Sed, Sediment.; ND: Not determined; Data from: 1, Daffonchio et al., 2006; 2, Zhang et al., 2017a; 3, Zhang et al., 2017b; 4, Sass et al., 2001; 5, Sagar et al., 2013b; 6, Rodondi et al., 1996; 7, Borin et al., 2008; 8, Sagar et al., 2013a; 9, Antunes et al., 2007; 10, Eder et al., 2002; 11, Sorokin et al., 2006; 12, A. Antunes, M. Taborda and M.S. da Costa (unpublished); 13, Heitzer and Ottow, 1976; 14, Brusa et al., 2001; 15, Antunes et al., 2003; 16, Trüper, 1969.

as well as members of the *Epsilonproteobacteria* and the *Bacteroidetes*. A later study by Sass et al. (2008) reports on a large collection of over 80 strains obtained from several Mediterranean DHABs and including *Bacillus*-like isolates, and a few *Halomonas* and *Alteromonas*. More recent, noteworthy isolation and description results include: i) a strain of *Halanaeroarchaeum sulfurireducens*, an unusual sulfur-reducing and acetate-utilising haloarchaeon obtained from Medee DHAB, ii) a strain closely related to the Red Sea DHAB *Halorhabdus tiamatea* (Werner et al., 2014), and iii) the description of a three-component microbial consortium from Thetis DHAB (consisting of *Halobacteroides*, *Methanohalophilus*, and *Halanaerobium*), which linked anaerobic glycine betaine degradation with methanogenesis (La Cono et al., 2015).

#### Molecular-based studies

The original misconception of DHABs as sterile environments was drastically revised after the pioneering 16S rRNA gene phylogenetic studies by Eder et al., (1999, 2001, 2002). These studies uncovered thriving microbial communities and detected several bacterial and archaeal sequence groups which were completely new to Science, which were later

**Table 3.** Non-proteobacterial prokaryotes isolated from DHABs of the Red Sea and the Mediterranean Sea.

Class Family	Genus/Species/Strain	Origin			Description	Genome	Ref		
<b>Actinobacteria</b>									
<i>Microbacteriaceae</i>	<i>Microbacterium</i> sp.	Med	Urania	BSI	-	-	1		
<i>Micrococcaceae</i>	<i>Arthrobacter</i> sp.	Med	Urania	BSI	-	-	1		
<b>Bacilli</b>									
<i>Staphylococcaceae</i>	<i>Staphylococcus</i> sp.	Med	Bannock	Sed	-	-	2		
	<i>Staphylococcus</i> sp.	RS	Kebrit	Sed	-	-	3		
<i>Bacillaceae</i>	<i>Bacillus</i> spp.	Med	L'Atalante	Sed	-	-	4		
				Bannock	Sed	-	-	4	
				BSI	-	-	5, 6		
				Urania	Sed	-	-	4	
				Discovery	BSI	-	-	5	
				Sed	-	-	4		
	<i>Halobacillus</i> spp.	RS	Kebrit	Sed	-	-	7		
				Med	L'Atalante	BSI	-	-	5
				Urania	Sed	-	-	4	
	<i>Pontibacillus</i> sp.	Med	L'Atalante	Sed	-	-	4		
	<i>Pontibacillus</i> sp.	Med	Urania	Sed	-	-	4		
<i>Thalassobacillus</i> sp.	Med	Bannock	Sed	-	-	4			
<i>Virgibacillus</i> sp.	Med	Bannock	Sed	-	-	4			
			RS	Nereus	B	-	-	3	
<b>Clostridia</b>									
<i>Halanaerobiaceae</i>	<i>Halanaerobium</i> spp.	RS	Kebrit	BSI	-	-	8		
				Shaban	BSI	-	-	9	
		Med	Bannock	BSI	-	-	6		
<b>Cytophagia</b>									
<i>Cytophagaceae</i>	<i>Cytophaga</i> sp.	Med	Bannock	BSI	-	-	6		
<b>Deferribacteres</b>									
<i>Deferribacteraceae</i>	<i>Flexistipes sinusarabici</i>	RS	Atlantis II	BSI	new phylum	+	10		
<b>Firmicutes/ Mollicutes</b>									
<i>Haloplasmataceae</i>	<i>Haloplasma contractile</i>	RS	Shaban	BSedl	new order	+	11		
<b>Flavobacteria</b>									
<i>Flavobacteriaceae</i>	ND	Med	Urania	BSI	-	-	1		
				RS	Atlantis II	BSI	-	-	3
<b>Halobacteria</b>									
<i>Halobacteriaceae</i>	<i>Halorhabdus tiamatea</i>	RS	Shaban	BSedl	new species	+	12		
<i>Haloferracaceae</i>	<i>Halanaeroarchaeum</i> sp.	Med	Medee	B	-	+	13		
				RS	Discovery	BSI	new species	+	14
				RS	Discovery	BSI	new species	+	14
				RS	Discovery	BSI	new genus	+	15
<i>Natrialbaeaceae</i>	<i>Natrinema salaciae</i>	Med	Medee	B	new species	+	16		

Med, Mediterranean; RS, Red Sea; BSI, Brine-seawater interface; B, Brine; BSedl, Brine-sediment interface; Sed, Sediment; ND, Not determined; Data from: 1, Sass et al., 2001; 2, Rodondi et al., 1996; 3, Sagar et al., 2013b; 4, Sass et al., 2008; 5, Borin et al., 2008; 6, Daffonchio et al., 2006; 7, Sagar et al., 2013a; 8, Eder et al., 2001; 9, Eder et al., 2002; 10, Fiala et al., 1990; 11, Antunes et al., 2008a; 12, Antunes et al., 2008b; 13, Messina et al., 2016; 14, Zhang et al., submitted; 15, Zhang et al., 2017c; 16, Albuquerque et al., 2012.

further expanded by similar studies in the Mediterranean DHABs (e.g. van der Wielen et al., 2005; Daffonchio et al., 2006). Some of these new sequence groups seemed to be specific to individual brines, while others (e.g., KB1, MSBL1) were detected in multiple DHABs both in the Red Sea and the Mediterranean (reviewed in Antunes et al., 2011d). Such a widespread distribution pointed to a high degree of adaptation of these new groups to life in such unique settings.

More recent years generated a considerable amount of data linked with further molecular-based studies, whole-genome sequencing, single-cell

genomics, and metagenomics. These studies have confirmed the vertical stratification of microbial and viral communities across the brine-seawater interface and offered important insights into the taxonomic and physiological diversity of the brine-seawater interface and the sediments of DHABs (e.g. Antunes et al., 2015; Bougouffa et al., 2013). Significant contributions were made in the clarification of microbial groups involved in sulfate reduction, methanotrophy, methanogenesis and in several aspects of the Carbon, and the Nitrogen cycle (e.g. Yakimov et al., 2007b; La Cono et al., 2011; Siam et al., 2012; Abdallah et al., 2014; Pachiadaki et al., 2014; Guan et al., 2015; Ngugi et al., 2015, 2016; Sorokin et al., 2016).

Whole-genomic sequencing projects have provided some much-needed data on isolated strains from several of the Red Sea DHABs (Table 2 and Table 3), while single-cell genomics efforts based both on Red Sea and Mediterranean samples provided relevant insights into non-cultivated strains from the bacterial KB1 group and the euryarchaeal MSBL1 and SA1 groups (Yakimov et al., 2013; La Cono et al., 2015; Mwirichia et al., 2016; Nigro et al., 2016; Ngugi and Stingl, 2018), which helped to clarify their physiology and might assist in their cultivation (see sections below).

#### Further insights into the Life in DHABs

Life in the DHABs is increasingly seen as more complex than anticipated, extending past its prokaryotic communities, with first insights available both on their viral and eukaryote communities.

Metagenomic-based analysis on samples from the Red Sea DHABs gave us some clues on viral diversity and community structure, and revealed that viral communities are stratified along the brine-seawater interface (Antunes et al., 2015), similarly to what had been previously reported for prokaryotes.

In the Mediterranean DHAB, sediments were shown to have viral abundances similar to oxic deep-sea sediments (Danovaro et al., 2005), and had high levels of viral infection, resulting in the release of large amounts of prokaryote DNA (Corinaldesi et al., 2014). Viral lysis, rather than grazing by eukaryotes, was proposed to be the main top-down control of prokaryotes in DHAB sediments, as well as a major means of recycling nutrients (Corinaldesi et al., 2014).

The uncovering of eukaryotic life associated with DHABs started with the molecular-based detection of an incredibly diverse and novel range of protists (mostly composed of dinoflagellates, ciliates and other alveolates, as well as fungi) present in Bannock and Discovery, in the Mediterranean Sea (Edgcomb et al., 2009). Danovaro et al. (2010) reported three novel species of the animal phylum *Loricifera* in L'Atalante DHAB sediment, proposing that they were active and thus the first described metazoans seemingly completing their life cycle in permanently anoxic conditions

(Danovaro et al., 2016). Our knowledge of eukaryotes associated with DHABs has further escalated with the discovery of macrofauna in a few DHABs in the Red Sea. These findings include: the description of a new genus of bivalve (Oliver et al., 2015), the detection of enriched zooplankton and discovery of fish apparently feeding on the thick particle load present at the brine-seawater interface (Kaartvedt et al., 2016; Vestheim and Kaartvedt, 2016). This rich macrofauna associated with some of the investigated DHABs is in contrast with the extremely poor benthos usually reported for the Red Sea and has been suggested to be linked with increased microbial load and activity in the brine-seawater interfaces (Vestheim and Kaartvedt, 2016).

Despite these recent advances, DHAB research into these two fields is still in its infancy. Further efforts are essential to understand the likely grazing effects and complex trophic interactions between the viral, prokaryotic, and the eukaryotic components of these ecosystems.

#### Microbiology and geochemistry of the DHABs

The link between local geochemistry of the brines and their microbiota often predated the first data from microbial studies on several DHABs. Indeed, geochemical data hinted at biogenic sulfate reduction in sulfide-forming processes as well as biotic methane oxidation occurring at the brine-seawater interface (Blum and Puchelt, 1991; Faber et al., 1998). Complex redox cycles involving iron and manganese were also postulated to occur in the brine-seawater interface of several DHABs of the Red Sea (Stoffers et al., 1998).

In recent years we have accumulated increasing support for the existence of microbes associated with sulfate reduction, acetogenesis, methanogenesis, autotrophic, and heterotrophic activity, as well as different steps of the nitrogen cycle, including AnAmmOx by *Planctomyces* (Borin et al., 2009; 2013; Speth et al., 2017) and ammonia oxidation by *Thaumarchaeota* (Ngugi et al., 2015). These processes have been recently reviewed by Merlino et al. (2018) and Antunes et al. (2019), and are outside the direct scope of this review. The topic of methanogenesis in DHABs has been reviewed by McGenity and Sorokin (2018), but will be discussed briefly in the following section, as methane and other volatile compounds could be detected remotely to inform on the possibility of life in icy moons, as is currently being done with the NOMAD detector on the Mars Trace Gas Orbiter (Vandaele et al., 2015).

#### *Methanogenesis in the DHABs*

Early investigators of the Red Sea DHABs, such as the Kebrit and Shaban deeps, suspected the presence of methanogens based on archaeal biomarkers (Michaelis et al., 1990) and euryarchaeal 16S rRNA gene amplicons (Eder et al., 2002), both of which could derive from non-methanogenic Archaea. There is also evidence for methylotrophic

methanogenesis in Mediterranean DHABs due to the common, but inconsistent, detection or isolation of *Methanohalophilus* sequences (Hallsworth et al., 2007; Yakimov et al., 2007b; Yakimov et al., 2015; La Cono et al., 2015; McGenity and Sorokin, 2018), as well as demonstration of the osmolyte and compatible solute, glycine betaine, fuelling methane production *via* fermentation to methylamines (Yakimov et al., 2013; La Cono et al., 2015). In the Mediterranean DHABs methane production rates ( $\mu\text{M CH}_4 \text{ d}^{-1}$ ) of 85.8 (Urania), 16.9 (l'Atalante), 4.2 (Bannock) and 2.6 (Discovery) were detected (van der Wielen et al., 2005; Borin et al., 2009). The most abundant uncultivated archaeal clones (termed MSBL-1) in most of these four basins (van der Wielen et al., 2005), the Urania hydrothermal mud vent (Yakimov et al., 2007a), Thetis DHAB (La Cono et al., 2011), Medee DHAB (Yakimov et al., 2013) and Kryos DHAB (Yakimov et al., 2015), are phylogenetically related to methanogens. This observation, coupled with coincident methane production and/or detection of *mcrA* transcripts, together with the occasional absence of known methanogenic taxa (Yakimov et al., 2013), led to the suggestion that Candidate Division MSBL-1 Archaea may be methanogens. However, this supposition is not supported by a single-cell genomics analysis of MSBL-1, where the core methanogenesis genes were not detected, and pathway analysis suggest a mixotrophic lifestyle, fermenting glucose or, in the absence of organic carbon, fixing  $\text{CO}_2$  (Mwirichia et al., 2016).

So, which microbes are carrying out methanogenesis (in addition to *Methanohalophilus* when present)? Several studies from the Red Sea now report the presence of methanogenesis and methanogens in the brines and the brine-seawater interface (Antunes et al., 2011d). Guan et al. (2015), for example, detected *mcrA* genes in several of the interfaces, finding typical halophilic methylotrophic genera *Methanohalophilus* and *Methanococcoides*, as well as phylotypes similar to *Methanomassiliicoccales*, which were also detected in Kryos DHAB (Yakimov et al., 2015). *Methanomassiliicoccales* is a methanogenic order in the *Thermoplasmata* that can reduce methylamines and methanol with hydrogen ("methyl reduction"; Borrel et al., 2014), although, currently, only non-halophilic members of this order have been characterized. Arguably the most interesting group of halophilic methanogens is the Methanonatronarchaeia (SA1), which were isolated from anoxic hypersaline environments and shown to carry out methanogenesis by methyl reduction as with the *Methanomassiliicoccales* (Sorokin et al., 2017). The methyl-reduction pathway is distinguished from methylotrophic methanogenesis, as the  $\text{C}_1$  methylated compounds are used only as electron acceptors while  $\text{H}_2$  is the external electron donor (Sorokin et al., 2017). Comparison of sequences from Archaea in the DHABs revealed that the uncultivated Candidate Division Shaban Archaea (SA1), which is commonly detected in Red Sea and Mediterranean DHABs (Eder et al., 2002; Yakimov et al., 2013; Merlino et al., 2018; McGenity and Sorokin, 2018), is closely related to Methanonatronarchaeia. Thus, this type of methanogenesis may be



quantitatively important in the DHABs. Ngugi and Stingl (2018), however, added a note of caution when they carried out single-cell genomic analysis on SA1 cells, and revealed no core methanogenesis genes. However, there is a lot of diversity in the SA1 group, and the genome-sequenced lineage had only 93% identity in 16S rRNA sequence to Methanonatronarchaea (Ngugi and Stingl, 2018). Further cultivation-dependent and -independent explorations of SA1 group Archaea are required to understand the metabolic diversity within this lineage.

In the context of detecting methane from subterranean hypersaline oceans on icy moons it is important to consider that a high proportion may be microbially oxidised before entering the atmosphere. There is evidence for anaerobic oxidation of methane (AOM) in the DHABs, primarily via detection of sequences from uncultured Anaerobic Methane oxidizers Group 1 (ANME-1) (van der Wielen et al., 2005; Daffonchio et al., 2006; Lloyd et al., 2006; Yakimov et al., 2007a, b; La Cono et al., 2011; Pachiadaki et al., 2014). Indeed ANME-1 seem to be the main group carrying out AOM in a wide range of anoxic hypersaline environments (McGenity and Sorokin, 2018). The methane-oxygen counter gradients in DHAB brine-seawater interfaces are conducive to aerobic methane oxidation, and carbon isotope analyses suggest its occurrence in Atlantis II, Discovery and Kebrit DHABs from the Red Sea (Faber et al., 1998; Schmidt et al., 2003), and Type-I methanotrophs (and a small proportion of Type-II methanotrophs) were detected in the brine-seawater interfaces of the aforementioned DHABs (Abdallah et al., 2014).

Further discussions on the feasibility of specific types of microbial metabolism are obviously hampered by the limited amount of actual data on the physical-chemical conditions on these exoceans and is outside the scope of this paper. Nonetheless, it is worth noting that a few authors have been looking into the possibility of microbial activity occurring under predicted exocean conditions. A relevant example is the study by Taubner et al. (2018), where methane production by the methanogenic archaeal species *Methanothermococcus okinawensis* under putative Enceladus-like conditions was tested and confirmed.

## Future research

### *The limits of life*

Studying the limits of life is one of the main pillars of Astrobiology. The DHABs provide an opportunity to explore the impact of multiple extremes on microbes, especially the interactions of high pressure and high salinity, including both kosmotropic and chaotropic salts. It is well known that microbial activities are influenced by high pressure (Tamburini et al., 2013) and high salinity (Oren, 2011), but synergistic and antagonistic effects on microbes of multiple environmental stressors, despite being the norm, requires much more in-depth investigation in order to predict biological

processes on Earth and elsewhere (Harrison et al., 2013; Robinson and Mikucki, 2018). The classic experiments by Yayanos (1986) investigated the interaction of temperature and pressure on the growth rate of isolates, while Kaye and Baross (2004) showed improved growth of *Halomonas* strains under high-pressure at intermediate, compared with low, salinities. However, there have been few systematic studies investigating polyextremophily, and only modest mechanistic understanding has been obtained, e.g. compatible solutes have been proposed to protect cellular macromolecules from both high salt and pressure stress (Yancey, 2004). In order to understand the interacting effects of those multiple extremes that are most relevant to DHABs and icy-moon brines, a much broader range of microbes and communities must be investigated, especially those that are native to the DHABs. To date, no microbes from the DHABs have been isolated under high hydrostatic pressure, despite evidence from metagenomic expression of esterases, which exhibited optimal function at high hydrostatic pressure and salinity, indicating that they probably derived from piezophiles (Ferrer et al., 2005).

It is known that chaotrope-induced cellular damage can be offset (Hallsworth et al., 2007) and growth enhanced (Zajc et al., 2014; Lima de Alves et al., 2015) by adding kosmotropic solutes to chaotropic solutions/media, which reduces the water activity but counterbalances the chaotropicity, thus demonstrating that chaotropic-solute-induced destabilisation of macromolecules is a key growth-limiting factor. Despite their hostile chemistry, there is some evidence of microbial activity in both Discovery (van der Wielen et al., 2005) and Kryos DHABs (Yakimov et al., 2015; Steinle et al., 2018), but no evidence to date of life in the youngest (700-year-old) MgCl<sub>2</sub>-dominated Hephaestus DHAB beyond 2.97 M MgCl<sub>2</sub> (La Cono et al., 2019). Therefore, it will be important to understand which microbes may be performing these activities and how they protect themselves against extremely chaotropic brines. Equally, an improved understanding of the compensatory effects of chaotropic and kosmotropic salts (Hallsworth et al., 2007), and differential microbial adaptations to both types of salts, are needed to predict where life will occur, particularly given the uncertainty of the ionic compositions of icy-moon brines (Marion et al., 2003). Ultimately, the ionic composition (balance of kosmotropes and chaotropes) as well as the overall ionic strength of brines elsewhere in the solar system, together with the physical conditions (pressure, temperature etc.) and microbial adaptations to counter these effects, will be key to determining brine habitability (Hallsworth et al., 2007; Fox-Powell et al., 2016). To this end the Mediterranean type-3 DHABs, rich in MgCl<sub>2</sub>, provide ideal environments to explore the water activity and chaotropicity limits of life, and potentially to find obligate chaophiles.

#### *Source of microbial strains for exposure experiments*

The use of exposure experiments and testing the resilience of several microbial strains to stressful conditions (equivalent to those found outside

our planet) has been a strong focus of recent research as highlighted by several dedicated large-scale research projects (e.g. BIOMEX-Biology and Mars experiment, MEXEM- Mars exposed extremophile mixture, IceXpose-icy exposure of microorganisms) and discussed by Martins et al. (2017). These experiments are a vital source of data for discussions in Astrobiology, namely on the feasibility of Life outside our planet and on Planetary Protection.

The testing of microbes obtained from extreme terrestrial analogues has been suggested as particularly advantageous as they were hypothesized to have highly effective cellular and molecular adaptations and repair mechanisms which would allow them to better withstand conditions present in their extra-terrestrial counterparts (currently being tested as part of the MEXEM project). In this context, microbes isolated from DHABs (Table 2 and Table 3) constitute a rich source of interesting new targets for exposure experiments and should be prioritized in future studies. The availability of microbial strains representing novel higher-ranked taxonomic groups would significantly extend the range of tested taxa and constitute a relevant source of new data. Furthermore, DHABs' strains belonging to previously known taxa would provide an interesting target for comparative studies which would: a) pair them with closely-related strains isolated from different environments and b) look into potential differences in their resilience when exposed to stress. The existence of genomic data for many of the strains isolated from DHABs (Table 2 and Table 3) would further facilitate a more in-depth analysis.

#### *Long-term viability and preservation: microbes and biomolecules*

As is frequently the case when surveying previously uncharted extreme environments, the microbial exploration of DHABs has uncovered several novel phylogenetic groups. Surprisingly, several of these novel groups seem to be DHAB-specific and have a very wide-spread distribution, being consistently detected in brines of the Red Sea and the Mediterranean (e.g. KB1, MSBL1, and SA2 phylogenetic groups), despite the isolation imposed by their geographical location and by the nature of the brines. Previous studies on haloarchaea, the most extreme of halophiles, pointed to their apparent capability of global dispersal across isolated coastal hypersaline environments (Clark et al., 2017), but the mode of dispersal is most likely via birds and wind, where they may be protected inside halite crystals (Clark et al., 2017; Kemp et al., 2018), especially given that many haloarchaea lyse in seawater (Torreblanca et al., 1986). No such vectors or protection would be available for transporting haloarchaea to the deep sea (though our understanding of dispersal mechanisms is limited). Dispersal of other DHAB-specific novel groups may be facilitated by overlying seawater. Haloclines do not represent an absolute barrier for penetration by motile microbes in the micrometre range (Doostmohammadi et al., 2012), but any DHAB-adapted microbe would presumably have to be actively moving while in the deep-sea water (or induced to reactivate from a dormant state

upon reaching the halocline), and then able to overcome the rapid increase in viscosity and salinity as it returns to its preferred habitat. Nevertheless, to date, DHAB-specific taxa have not been detected in seawater.

An alternative explanation for this apparent paradox is microbes from these groups could be trapped in the salt crystals and/or brine inclusions, present in the possibly linked salt deposits under the Red Sea and Eastern Mediterranean, and released during the formation of the brines where they would thrive (Antunes et al., 2011d). Such long-term preservation and continued viability of microbes in ancient salt deposits is in line with several previous studies (e.g. Grant et al., 1998; McGenity et al., 2000; Mormile et al., 2003). Indeed, evidence is mounting in support of survival over millions of years (Røy et al., 2012), specifically in halite (McGenity et al., 2000; Schubert et al., 2010; Gramain et al., 2011; Jaakkola et al., 2016), and the interconnection between the geosphere and the biosphere, specifically in the context of the DHABs (biosphere) and the Messinian evaporites (geosphere) was discussed by McGenity et al. (2008). Collection and analysis of sediment cores of the evaporite layers would allow us to confirm the presence of such microbial groups and help clarify the process involved in the microbial colonization of the DHABs. Such studies would be very relevant for the future exploration of the icy moons of the outer Solar System.

An equally relevant and under-studied aspect regarding long-term preservation is the effect of such brines in the stability of bio-molecules. Previous reports pointed to the preserving effects of samples from the DHABs of the Mediterranean on DNA (Borin et al., 2008). One should note that this preservation would be affected by the type of salts present in the brine. In this regard, one particularly interesting location is the  $MgCl_2$ -saturated Discovery lake. Chaotropic  $MgCl_2$  destabilises macromolecules and would therefore significantly restrict any microbial/enzyme activity (Hallsworth et al., 2007). Therefore, Discovery DHAB, together with the other known  $MgCl_2$ -rich brines, Lake Kryos and Hephaestus, would provide excellent locations for preserving biomolecules (Hallsworth et al., 2007; Sass et al., 2008).

The wide-range of chemical conditions present in the DHABs provide an ideal testing ground for looking into the effects of brine on microbes and bio-molecules, which would be easily linked to the astrobiological study of biosignatures under high-salinity conditions and lead to technical improvements. As an example of such an improvement, the discussion on this brine-preserving effect and detrimental impact on the reliability of results obtained from standard molecular-based analysis led to a switch to alternative RNA-based approaches in several subsequent studies.

*Technology development and testing: Fine-scale sampling and laboratorial replication of complex environments*

One of the major benefits of working with terrestrial analogues is the opportunity to use such sites as a basis for developing, testing and fine-tuning different technologies and equipment in preparation for future space missions. Relevant examples of this approach include e.g. the use of Boulby mine in the MINAR- Mining and Analogue Research project (e.g. Payler et al., 2017; Cockell et al., 2018) or the current testing of prototype ice-melting probes in glaciers and regions with ice-sheets across the globe (e.g. Dachwald et al., 2014; Funke and Horneck, 2018). The exploration of deep-sea terrestrial locations and access to water bodies below thick ice layers are seen as vital for the future exploration of Mars and the icy moons of the outer Solar System so such inputs are an invaluable source of information.

The complex nature of DHABs and the drastic transitions observed at their brine-seawater interfaces also provide remarkable technical challenges that are relevant for Astrobiology. We believe that they also provide unique opportunities and ideal settings to develop and study new equipment for fine-scale sampling which would be relevant for exploring equivalent settings in the oceans of the icy moons of the Outer Solar System. The development of MODUS (Mobile Docker for Underwater Sciences), a Remotely Operated Vehicle designed to improve precision of sampling technologies and facilitate finer-scale sampling in such challenging environments was tested *in situ* in the DHABs of the Mediterranean (Malinverno et al., 2006) and was seen as a very relevant advance. The attempt to deploy the recently developed Microbial Sampler-Submersible Incubation Device (MS-SID) at the brine-seawater interface of Urania DHAB is also a welcome advance, notwithstanding the technical issues that affected its use (Pachiadaki et al., 2016). Despite these improvements, there is a clear need for further technical developments and improvements in this field.

Another technical issue to keep in mind is our limited capability to successfully replicate gradient-rich environments in the lab, a bottleneck which significantly hampers research in this field. Recent advances making use of gel-stabilised gradient plates (More et al., submitted) or the use of simulation chambers (e.g. Herschy et al., 2014) for the simulation of chemical gradients in liquid media, should help to fill this technical gap and are welcome additions to our current range of tools.

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