

## Minireview

# Current state of athalassohaline deep-sea hypersaline anoxic basin research—recommendations for future work and relevance to astrobiology

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## Summary

**Deep-sea hypersaline anoxic basins (DHABs) are uniquely stratified polyextreme environments generally found in enclosed seas. These environments select for elusive and widely uncharacterized microbes that may be living below the currently recognized window of life on Earth. Still, there is strong evidence of highly specialized active microbial communities in the Kryos, Discovery, and Hephaestus basins located in the Eastern Mediterranean Sea; the only known athalassohaline DHABs. Life is further constrained in these DHABs as near-saturated concentrations of magnesium chloride significantly**

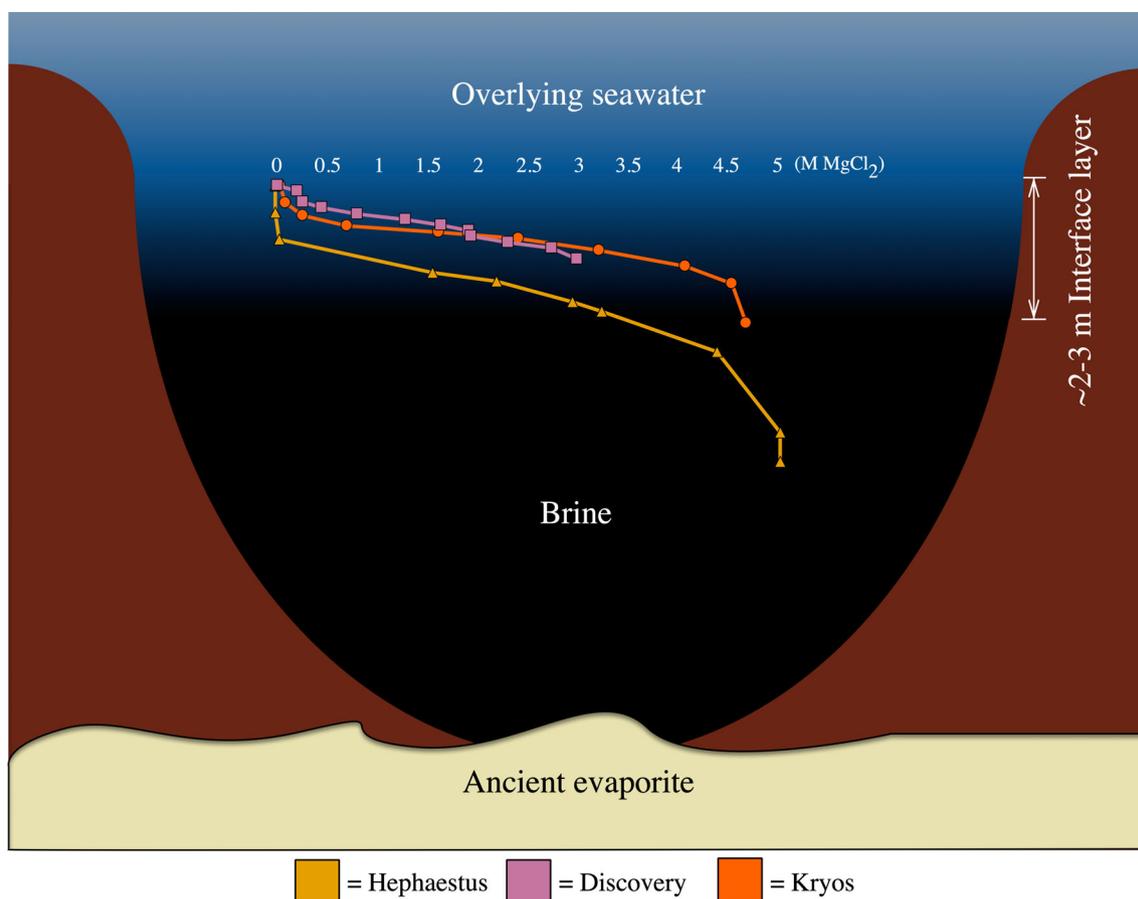
**reduces water activity ( $a_w$ ) and exerts extreme chaotropic stress, the tendency of a solution to disorder biomolecules. In this review, we provide an overview of microbial adaptations to polyextremes focusing primarily on chaotropicity, summarize current evidence of microbial life within athalassohaline DHABs and describe the difficulties of life detection approaches and sampling within these environments. We also reveal inconsistent measurements of chaotropic activity in the literature highlighting the need for a new methodology. Finally, we generate recommendations for future investigations and discuss the importance of athalassohaline DHAB research to help inform extraterrestrial life detection missions.**

## Introduction

Deep-sea hypersaline anoxic basins (DHABs) present a combination of high osmotic stress, anoxia, elevated hydrostatic pressure and low water activity, making them one of Earth's most extreme environments for life. These multiple stressors are unique to DHABs and distinguish them from other hypersaline environments such as the Dead Sea, the Great Salt Lake, or solar salterns. DHABs form via dissolution of ancient evaporites creating brine that collects in deep-sea basins (Fig. 1). A 2–3 m interface layer, sometimes referred to as the seawater–brine interface or SBI, forms above the brine, inhibiting mixing, resulting in steep gradients of oxidants, reductants, and captured organic matter, capable of supporting microbial life (Antunes *et al.*, 2020). To date, a few dozen DHABs have been discovered in close proximity to ancient salt formations in tectonic/hydrothermal regions in the Gulf of Mexico, the Eastern Mediterranean Sea, the Red Sea and the Black Sea (Merlino *et al.*, 2018).

Eastern Mediterranean Sea DHABs, the focus of this review, were formed as tectonic activity in the subduction zone of the Mediterranean ridge exposed 5–6 million-year-old Messinian Salinity Crisis evaporites to seawater

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**Fig. 1.** Basic diagram of a deep-sea hypersaline anoxic basin (DHAB). Eastern Mediterranean Sea DHABs are about 3300 mbsl with approximate brine depths ranging from 50 to 160 m and surface areas of 1.7–25 km<sup>2</sup> (La Cono *et al.*, 2019). The plot shows the concentration of MgCl<sub>2</sub> in Molar with depth in meters within the Hephaestus, Discovery and Kryos basins. Values based on published data from La Cono *et al.* (2019), Hallsworth *et al.* (2007) and Yakimov *et al.* (2015). Data were depth corrected for better comparison of chemoclines. Note: the figure is not to scale. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

(Camerlenghi, 1990; Roveri *et al.*, 2014; Merlino *et al.*, 2018). Biologically, DHABs can be thought of as ‘isolated islands of evolution’ due to their distinct chemical compositions (Stoeck *et al.*, 2014); moreover, new DHABs are continually being discovered, suggesting the potential existence of as yet unknown novel environments and/or taxa. The majority of DHAB environments are NaCl dominated and are described as thalassohaline, characterized as brine maintaining ionic ratios nearly equal to that of seawater, but notable exceptions exist. For example, the Kryos, Discovery and Hephaestus basins located ~200 km west of the island of Crete are MgCl<sub>2</sub> dominated, presumably due to the dissolution of ancient bischofite (MgCl<sub>2</sub> · 6H<sub>2</sub>O) deposits, which precipitate in the final evaporative stages of seawater. These resulting brines are characterized as athalassohaline (Antunes *et al.*, 2020), which deviate significantly from seawater compositions, instead of having near-saturated concentrations of salts such as MgCl<sub>2</sub>, CaCl<sub>2</sub>, or MgSO<sub>4</sub>. While the seawater–brine *interface layers* of both

thalassohaline and athalassohaline DHABs teem with microbial life, the deeper athalassohaline DHAB *brine* endmembers are considered by many to be sterile due to extreme chaotropic stress and low water activity (Hallsworth *et al.*, 2007; Cray *et al.*, 2013; La Cono *et al.*, 2019).

### The many extremes in athalassohaline DHABs

#### *Adaptations to hypersaline environments*

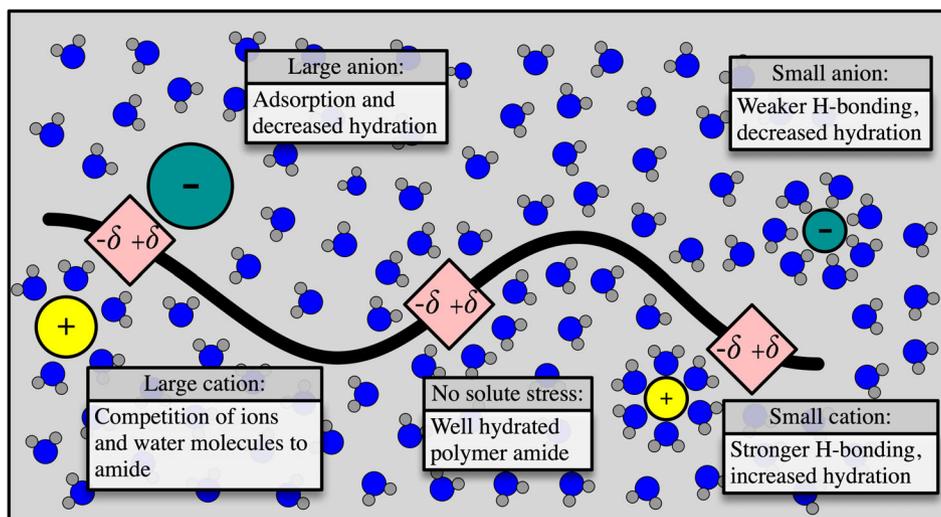
To survive in DHAB environments, prokaryotes must resist extreme desiccation and osmotic stress in order to sustain activity and growth (Boetius and Joye, 2009). To cope with high ionic strength water, these halophilic prokaryotes must remain isosmotic with their surrounding environment using the salt-in or salt-out strategy (Oren and Litchfield, 1999). Salt-in adaptation involves accumulation of inorganic salts (K<sup>+</sup> and Cl<sup>-</sup>) within the cytoplasm to become isosmotic with their surroundings. This mechanism requires considerable evolutionary innovation to

enable the intracellular machinery to function under elevated KCl concentrations, notably the presence of highly acidic proteomes. The resulting charges on protein surfaces organize a hydrated ion network that promotes overall polymer structure, thus preventing protein precipitation at high osmolarity (Matarredona *et al.*, 2020). Salt-in adaptation is thermodynamically less costly than the more common salt-out strategy (DasSarma *et al.*, 2006). The salt-out strategy requires the uptake or synthesis of compatible solutes, commonly alcohols and carbohydrates, free amino acids and their derivatives, methylamines, and other molecules that are generally uncharged or zwitterionic (Oren, 2008). Cells employing this strategy export salts but remain isosmotic with their environment by manufacturing or importing organic osmolytes that do not interfere with enzyme function, thus requiring less proteome modification. Both strategies are used by prokaryotes in DHABs to help them cope with extreme osmotic pressures (Merlino *et al.*, 2018). These mechanisms are well described for thalassohaline environments (Oren, 2008); however, molecular and physiological mechanisms to cope in athalassohaline brines, that is, under highly chaotropic  $\text{MgCl}_2$  stress, are virtually unknown.

#### Living in chaotropic brines

The architecture of life as we know it is based upon water. Yet, the effect of chaotropes on macromolecules can render some aqueous environments sterile (Hallsworth *et al.*, 2007; Cray *et al.*, 2013). Chaotropicity

is defined as a disordering/disrupting effect on biomolecules and is less easily constrained, versus specific chemical parameters such as pH. Chaotropic agents, or chaotropes, are generally large ions with a low charge density that cause a disruption to the ordering of water molecules, and act to destabilize proteins, inhibit enzymes, solubilize membranes, and at high concentrations can lead to cell death (Supplementary Fig. 3). Chaotropes increase solution entropy, allowing for more translational and rotational freedom of water molecules which are normally constrained by strong hydrogen bonds (Collins, 1997; Timson, 2020). Chaotropes also affect the binding of peptides to cell membranes and the kinetics of membrane interactions (Sachs and Woolf, 2003). Weak chaotropes such as  $\text{Cl}^-$  adsorb to amides and cationic nitrogen-based amino acid chains that are normally stabilized in aqueous solutions due to their inherent hydrophobic properties, further decreasing protein hydration and stability (Fig. 2; Collins, 1997). Although  $\text{Cl}^-$  binds far more weakly compared with the most chaotropic anions ( $\text{SCN}^-$  and  $\text{I}^-$ ), the large concentrations of  $\text{Cl}^-$  in athalassohaline brines (upwards of 10 M) completely inhibits enzyme and cellular function (Supplementary Table 1; Hallsworth *et al.*, 2007; Rembert *et al.*, 2012). Chaotropes can be balanced by kosmotropes (stabilizing ions) such as sulfate, trimethylamine-oxide, ectoine and sodium. These ions and biomolecules have a higher charge to size density compared with chaotropes and conversely stabilize proteins (Collins, 1997; Hallsworth *et al.*, 2003a; Hallsworth *et al.*, 2003b; Hallsworth *et al.*, 2007; Yakimov



**Fig. 2.** Proposed molecular interactions between solutes and polymer amide dipoles (pink diamonds). From left to right, adsorption of a large anion, e.g.  $\text{Cl}^-$  (large teal circle), to the polymer dipole (shown as  $\pm\delta$ ) decreases hydration and protein stability; below, competition occurs between a large cation (yellow circle) and water molecules over polymer dipole. Middle amide: a stable hydrated polymer dipole under no solute stress. Rightmost amide: a well-hydrated small anion (teal circle) is repelled from the polymer dipole decreasing overall hydrogen bonding and protein stability; a small cation (yellow circle) promotes protein hydration and stability. This figure was based on work from Von Hippel *et al.* (1973), Oh and Markley (1990), Collins (1995, 1997) and Shechter *et al.* (2010). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

*et al.*, 2015). In high enough concentration, kosmotropic  $Mg^{2+}$  ions can make biomolecules too rigid. This constrains the conformational changes of DNA and proteins thereby inhibiting enzyme and cellular function. For example, concentrations of magnesium above 600 mM have been shown to induce hexagonal Z-DNA duplex packing (Supplementary Fig. 3B, Chatake and Sunami, 2013). Many mechanisms have been proposed regarding how chaotropes and kosmotropes interact to affect cellular function and habitability, but because there is no singular universal effect of chaotropes on biomolecules, quantifying the effects of chaotropy is exceedingly difficult (Ball and Hallsworth, 2015; Timson, 2020).

Microbial life in athalassohaline brines is also limited by very low water activity; where water activity ( $a_w$ ) is a thermodynamic measurement of water availability in the system (Litchfield, 1998; Grant, 2004; Steinle *et al.*, 2018). The activity of pure water is 1 (ionic strength = 0), that of seawater is ~0.98, and the generally accepted  $a_w$  limit of life is 0.585 (Stevenson *et al.*, 2017). Water activity in athalassohaline DHABs is remarkably low ( $a_w \sim 0.4$ ) compared with thalassohaline DHABs ( $a_w \sim 0.75$ ) due to the high solubility of  $MgCl_2$  leading to high ionic strength waters. This also leads to increased osmotic stress and an elevated concentration of high charge-to-size ratio  $Mg^{2+}$  ions. Small divalent cations, such as  $Mg^{2+}$ , form strong hydration shells in solution leaving less available water for cellular processes (Supplementary Table 1; Supplementary Fig. 1). Most organisms are unable to multiply below an  $a_w$  of 0.9, and cell division has never been observed lower than 0.585, a current record held by the fungus *Aspergillus penicillioides* (Grant, 2004; Stevenson *et al.*, 2017). Therefore, any confirmation of cell division in athalassohaline DHAB brines ( $a_w \sim 0.4$ ) would greatly expand the limit of life on Earth.

#### Effects of high pressure on microbial processes

The high hydrostatic pressures of Eastern Mediterranean Sea DHABs, located ~3300 m below sea level, is approximately 33 MPa, sufficient to impact cellular function and select for moderately piezophilic ('pressure loving') microorganisms (Kato and Qureshi, 1999). Known physiological impacts of pressure include disruption of protein structure and function, DNA replication, DNA-protein interactions and oxidative stress, as a result of decreasing system volumes (Bartlett, 2002; Marietou *et al.*, 2015). Furthermore, pressure can have confounding counterintuitive effects, such as inducing the production of cold and heat shock proteins in *E. coli* (Bartlett, 2002). Microbes adapt to changes in pressure in part by adjusting the physical state of the membrane, needed for cell integrity, and nutrient, waste, and ion transport. Lipids

are an order of magnitude more compressible than proteins which make cell membranes highly sensitive to changes in pressure (Bartlett, 2002). Increased pressure leads to the lateral compression of hydrocarbon chains, and adaptation to these effects in deep-sea bacteria is accompanied by increased proportion of unsaturated fatty acids, including in some cases high levels of omega-3 polyunsaturated fatty acids modifying membrane fluidity and phase (Bartlett, 2002).

#### Interplay of poly-extremes and DHAB habitability

A major knowledge gap to understanding the biological potential of DHABs is in characterizing the microbial response to polyextremes, largely due to unintuitive and complex effects of multiple extremes on one another. Remarkably, multiple extremes such as pH, salinity, temperature, anoxia and pressure in unison can in some cases widen the habitable zone via largely unknown microbial adaptations and complex biochemical interactions (Harrison *et al.*, 2013; Merino *et al.*, 2019). Chaotropes have been shown to lower growth temperature optima and osmotic stress has been shown to greatly increase pressure tolerance in some microorganisms (Tanaka *et al.*, 2001; Harrison *et al.*, 2013). Furthermore, the presence of highly chaotropic perchlorate anions under elevated hydrostatic pressure increases enzyme activity, suggesting that pressure may broaden cellular chaotropic tolerance (Gault *et al.*, 2020). Therefore, life in athalassohaline DHAB brines could be possible due to the interplay of chaotropy, osmotic stress, abundance of  $Mg^{2+}$  ions, anoxia and high pressure (Chilukuri *et al.*, 1997; Smolin and Winter, 2004; Lee *et al.*, 2019).

#### Preservation of biomarkers within athalassohaline DHABs

Free genetic material in most marine environments is chemically unstable and is further degraded by DNases/RNases, microorganisms and other physical/chemical reactions. Conversely, genetic material in athalassohaline brines is preserved over longer periods of time by  $MgCl_2$  (Hallsworth *et al.*, 2007; Borin *et al.*, 2008). Preservation may be attributed to the complete inhibition of DNases/RNases by abundant  $Cl^-$  ions and DNA stabilization by  $Mg^{2+}$  cations as they are strongly attracted to the phosphate backbone of DNA (Luck and Zimmer, 1972; Chatake and Sunami, 2013; Serec *et al.*, 2016). The ability of  $MgCl_2$  to preserve other relevant biomarkers such as lipids and ATP has been previously noted, but to-date has been poorly described (Tuovila *et al.*, 1987; Turich and Freeman, 2011). Studying the preservation of biomarkers and microbial activities in the Kryos, Discovery

and Hephaestus basins would be a beneficial and strategically relevant pursuit for future planetary science missions.

Hallsworth *et al.* (2007) showed that free DNA and 16S ribosomal RNA (rRNA) from *Marinobacter* sp. D-5, isolated from the upper interface of the Discovery basin, can be preserved in athalassohaline brine conditions (~5 M MgCl<sub>2</sub>) with minimal degradation for upwards of 45 days while mRNA completely degraded within an hour of exposure. Borin *et al.* (2008) did not investigate mRNA degradation but showed that naked plasmid DNA from seven relevant isolates is well preserved under DHAB conditions for upwards of 32 days. Since DNA and 16S rRNA are well preserved within athalassohaline DHABs, a significant proportion of 16S rRNA reads, especially in the lower interface and brine, could represent material derived from overlying layers, rather than from active microbes within the brines. This hypothesis is supported because no brine-specific groups have been discovered. Therefore, it should be noted that analyses of mRNA rather than DNA/rRNA may be better suited for the purposes of life detection, as well as a more confident determination of the biologically habitable zone in athalassohaline DHABs.

#### *Evidence and prospects of microbial life in the Kryos, Discovery and Hephaestus basins*

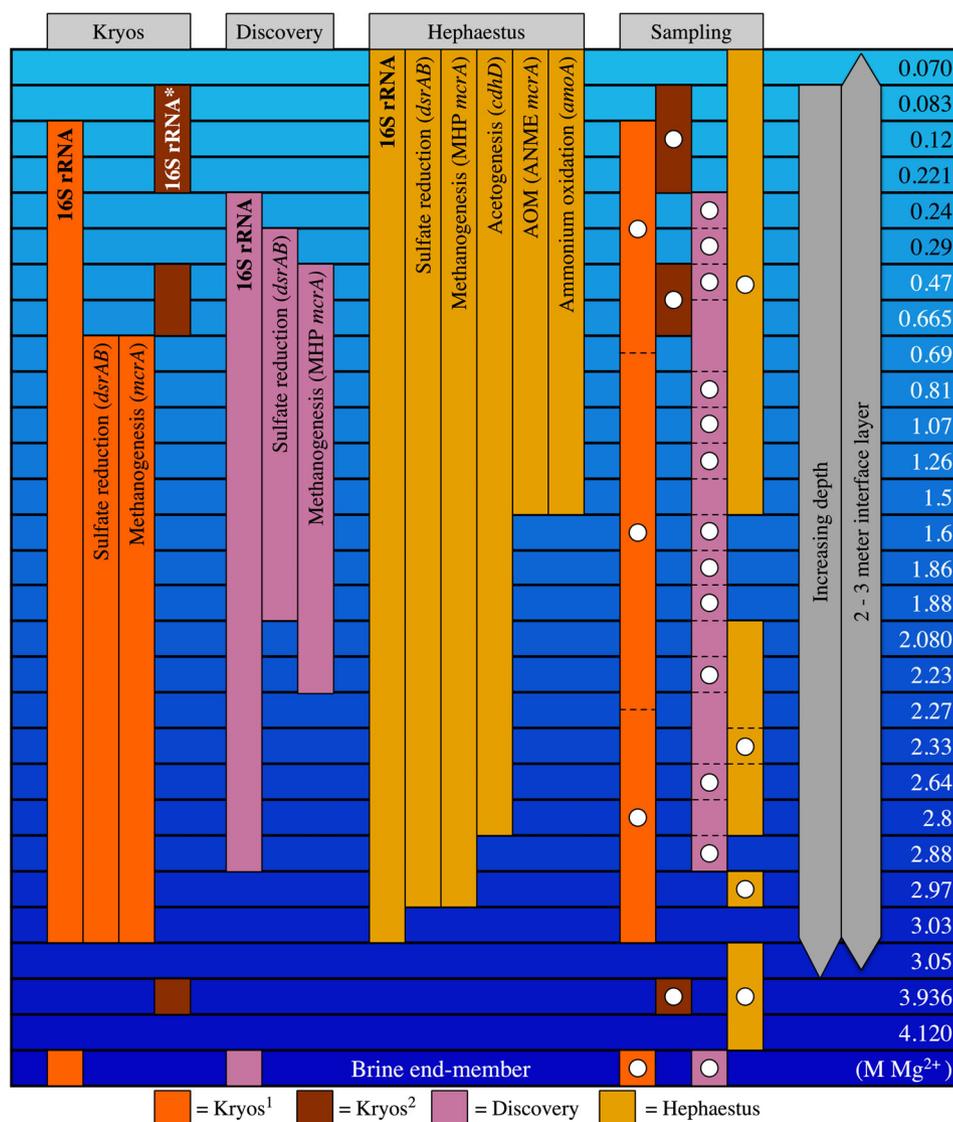
There is evidence that highly specialized and stratified microbial communities inhabit the interface of the Discovery, Hephaestus and Kryos basins. Yet, few studies have analysed the microbial communities within these environments, highlighting the lack of athalassohaline DHAB research (van der Wielen *et al.*, 2005; Hallsworth *et al.*, 2007; Polymenakou *et al.*, 2007; Borin *et al.*, 2009; Yakimov *et al.*, 2015; Edgcomb *et al.*, 2016; Steinle *et al.*, 2018; La Cono *et al.*, 2019). One goal of such research has been to identify extremely halophilic 'chaophiles' that may be living below the currently recognized chaotropic limit of life.

The Discovery basin interface contains Marine Group I Crenarchaeotes, *Desulfobacteraceae*, *Desulfohalobiaceae* and active methylotrophic methanogens inhabiting lower layers (Hallsworth *et al.*, 2007). Work on deeper, more chaotropic layers, shows an abundance of 16S rRNA associated with the candidate division MSBL1 (Mediterranean Sea Brine Lake), *Halobacteria* (specifically *Halorhabdus utahensis*), *Planctomycetes* and *Proteobacteria* (van der Wielen *et al.*, 2005). Only one study thus far has characterized microbial life in the Hephaestus basin showing an abundance of *Acetothermia*, *Bacteroidetes*, *Epsilonproteobacteria*, *Methanohalophilus*, *Nanohaloarchaeota* and *Halobacteriaceae* in lower interface layers based on total

extracted RNA (La Cono *et al.*, 2019). The vast majority of archaeal reads were associated with Marine Group I *Thaumarchaeota* within all layers and were most abundant within a highly chaotropic 2.97 M Mg<sup>2+</sup> layer. Based on 16S rRNA gene amplicon sequencing, the most dominant groups detected within the lower interface and brine of the Kryos basin mostly include *Proteobacteria*, *Planctomycetes*, *Acetothermia*, *Euryarchaeota* and *Thaumarchaeota* (Steinle *et al.*, 2018). *Sulfurovum* and *Sulfurimonas*, sulfur-oxidizing and nitrate-reducing members of *Epsilonproteobacteria*, accounted for approximately 29% and 8% respectively of total bacterial reads within the brine (3.936 M MgCl<sub>2</sub>, *a<sub>w</sub>* ~ 0.4), and 15% and 16% within the lower interface (0.665 M MgCl<sub>2</sub>) respectively.

Messenger RNA (mRNA), generally considered a proxy for active microbial life in DHAB literature, has been detected in up to 3.03 M Mg<sup>2+</sup> (*a<sub>w</sub>* = 0.631) in the Kryos basin, 2.97 M Mg<sup>2+</sup> (*a<sub>w</sub>* = 0.653) in the Hephaestus basin and 2.23 M Mg<sup>2+</sup> (*a<sub>w</sub>* = 0.801) in the Discovery basin (Hallsworth *et al.*, 2007; Yakimov *et al.*, 2015; Steinle *et al.*, 2018; La Cono *et al.*, 2019). Only sulfur respiration (*dsrA/B* transcripts) and methanogenesis (*mcrA* transcripts) were targeted in the Kryos and Discovery basin, while transcripts for acetogenesis (*cdhD*), anaerobic oxidation of methane or AOM (*mcrA*), and ammonium oxidation (*amoA*) were targeted in the Hephaestus basin (Fig. 3). AOM transcripts were detected in less chaotropic layers (0.1–1.5 M Mg<sup>2+</sup>) while transcripts for sulfur respiration and methanogenesis extended further to 2.97 M Mg<sup>2+</sup>.

Recovered mRNA from these DHABs suggests the potential presence of active, yet to be cultivated methanogens related to *Methanohalophilus* and sulfur reducers including but not limited to *Desulfobacteraceae* and *Desulfohalobiaceae* (both members of *Deltaproteobacteria*) inhabiting the deeper more chaotropic interface layers in athalassohaline DHABs (Hallsworth *et al.*, 2007; Yakimov *et al.*, 2015; Steinle *et al.*, 2018; La Cono *et al.*, 2019). There are few cultured isolates from the lower brine interfaces and no representatives of the DHAB-relevant proposed candidate phylum *Acetothermia*; which are likely anaerobes capable of producing acetate, formate and hydrogen via fermentation of amino acids, peptides and sugars (Hao *et al.*, 2018). Although few athalassohaline DHAB isolates exist, relevant well-characterized taxa, such as *Methanohalophilus* species, are available in public culture collections: these species, however, are largely isolated from surficial hypersaline environments such as solar salterns, bitterns and soda lakes and may be evolutionarily distant and physiologically distinct. Recent isolation and whole-genome sequencing of a *Virgibacillus dokdonensis* strain from the Discovery interface layer revealed significant MgCl<sub>2</sub> tolerance (1.8 M) and proteome adaptation to DHAB conditions, such as



**Fig. 3.** Extent of community 16S rRNA, targeted mRNA detection, and sampling regions within the interface layer and brine of the Hephaestus (tan), Discovery (purple), and Kryos (orange/brown) basins. Data were compiled from La Cono *et al.* (2019); Hallsworth *et al.* (2007); <sup>1</sup>Yakimov *et al.* (2015) and <sup>2</sup>Steinle *et al.* (2018) respectively. On the right under 'Sampling', bars separated by dashed lines and white dots represent discrete samples that were used for biomolecular analysis. Values at right in blue slabs refer to molar concentrations of Mg<sup>2+</sup>. For concentration of Mg<sup>2+</sup> in 'Brine' endmember, see Supplementary - Table 1. The grey bars show increasing depth from top to bottom and scale context of the narrow interface. Note, DNA, but no mRNA work was conducted in Steinle *et al.* (2018) (brown). Figure style adapted from Hallsworth *et al.* (2007). \*16S rRNA (brown, from Steinle *et al.*, 2018) is based on DNA amplification, not RNA. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

osmoregulation, and the ability to use diverse carbon sources (Zeaiter *et al.*, 2019). It should be noted that fungal species are also relevant to athallassohaline DHABs as some can grow at extremely low water activities and high concentrations of magnesium chloride and should be targeted for future DHAB culture work (Zajc *et al.*, 2014). For a detailed list of cultured isolates from DHABs, see Antunes *et al.* (2020).

Measurements indicating the occurrence of active sulfate reduction and methanogenesis at  $a_w \sim 0.4$  within the Kryos and Discovery brines have been previously described in the literature (van der Wielen *et al.*, 2005; Steinle *et al.*, 2018). However, while intriguing, these results require further investigation and would benefit from paired analyses of brine mRNA transcripts that have yet to be detected. To date, the cellular division has not been observed below 0.585  $a_w$ . Moreover, extremely

chaotolerant and xerophilic lab isolates do not grow above  $\sim 2.1$  M MgCl<sub>2</sub>, far lower than  $\sim 5$  M MgCl<sub>2</sub> athallassohaline DHAB brines, and isolates have yet to be obtained and grown at *in situ* Kryos, Discovery, or Hephaestus brine conditions (Zajc *et al.*, 2014).

#### Measuring chaotropic activity

Life in athallassohaline DHABs is thought to be limited by extreme chaotropicity rather than low water activity (Hallsworth *et al.*, 2007). Commercially available meters can accurately measure the water activity of a liquid/solid sample. However, defining and measuring chaotropic activity remain difficult. Chaotropic activity can be measured spectrophotometrically by determining the gelation temperature of a water sample amended with agar. Gelation occurs at the point of bulk hydrogen bonding,

coincident with the formation of other non-covalent bonds between polymer chains. As these are inhibited by chaotropes and promoted by kosmotropes, chaotropic solutions thus gelate at lower temperatures. Gelation temperature depression of a given sample is multiplied by the heat capacity of a 1.5% agar solution to report chaotropic activity in  $\text{kJ kg}^{-1}$  or  $\text{kJ kg}^{-1} \text{mol}^{-1}$ . This method, developed by Cray *et al.* (2013), is the first standardized assay to measure chaotropic activity. Using this method to spectrophotometrically measure the gelation temperature of an agar amended athalassohaline DHAB brine sample is exceedingly difficult, if not impossible, due to its extreme chaotropic activity which depresses gelation temperature, if extrapolated to DHAB brine molarities, too far below  $0^\circ\text{C}$ .

Currently, the chaotropic activities in athalassohaline DHABs have inconsistencies. Based on mRNA recovery, the chaotropic limit of life in the Kryos basin is thought to be  $296 \text{ kJ kg}^{-1}$ ; equivalent to a  $3.03 \text{ M MgCl}_2$  *in situ* sample (Yakimov *et al.*, 2015). This finding increased the previously presumed chaotropic limit of life of  $2.3 \text{ M MgCl}_2$  in the absence of compensating kosmotropes (Hallsworth *et al.*, 2007). The reported chaotropic activity for the Kryos lower interface ( $3.03 \text{ M MgCl}_2$ ) is  $296 \text{ kJ kg}^{-1}$ , which is surprisingly *greater* than the reported chaotropic activity of pure, saturated ( $5 \text{ M}$ )  $\text{MgCl}_2$  established by Hallsworth *et al.* (2007) and Cray *et al.* (2013) which is 212 and  $270 \text{ kJ kg}^{-1}$  respectively. Moreover, Cray *et al.* (2013) suggest that chaotropic activity increases by  $+54 \text{ kJ kg}^{-1}$  per mole  $\text{MgCl}_2$  added to pure solution (meaning a pure  $5 \text{ M MgCl}_2$  solution would have a chaotropy of  $270 \text{ kJ kg}^{-1}$ ), whereas other data suggest an increase of approximately  $+15\text{--}20 \text{ kJ kg}^{-1}$  per mole  $\text{MgCl}_2$  added (Yakimov *et al.*, 2015). The extreme chaotropic effect exerted by high concentrations of  $\text{MgCl}_2$  means gelation temperature could theoretically be as low as  $-115^\circ\text{C}$  at  $5 \text{ M MgCl}_2$ , assuming a second-order polynomial regression, which cannot be measured spectrophotometrically (Yakimov *et al.*, 2015). Therefore, the chaotropic activity of DHAB brine samples must be extrapolated. This is difficult as chaotropy is not additive, meaning mixing solutions of chaotropy A and B do not equal  $A + B$ , and gel-point temperature does not decrease linearly with  $\text{MgCl}_2$  concentration (Supplementary Fig. 2). This lack of linearity raises questions about currently published extrapolations, as no empirical measurements of gelation temperature beyond  $2.5 \text{ M MgCl}_2$  have been obtained. All athalassohaline DHAB studies report the chaotropic limit of life corresponding to the concentration of  $\text{MgCl}_2$  or  $\text{Mg}^{2+}$ , which is a useful proxy for chaotropic activity. However, the concentration of  $\text{MgCl}_2/\text{Mg}^{2+}$  is not fully comparable between DHABs as the chaotropic effect is amended by the differing concentrations of compensating kosmotropes

between brines (Supplementary Table 1). While these small differences can offset the chaotropic effect of  $\text{MgCl}_2$ , they are insufficient to explain inconsistencies in the literature and require further investigation.

#### Future directions

To better understand finely stratified microbial communities in DHABs, sampling techniques and sub-sample fractionation require careful consideration during collection, downstream analysis and data interpretation (Supplementary Fig. 4). Samples also undergo decompression upon recovery leading to changes in microbial morphology, inactivation and alterations of microbial communities which may explain why culture collections lack DHAB representatives (Yayanos and Dietz, 1982). Future work should include the use of pressure-retaining samplers and high-pressure culturing equipment to minimize decompression effects and more closely represent *in situ* conditions (Peoples *et al.*, 2019).

Earlier work in the Discovery basin showed that community composition can change significantly at a  $10 \text{ cm}$  resolution (Hallsworth *et al.*, 2007). This fine-scale stratification may be disrupted during the mixing of brine within Niskin bottles, the most commonly used DHAB sampling device, thereby exaggerating microbial distributions and gene expression patterns. The error can be mitigated by lowering the sampling device (usually a rosette) well below the interface, allowing the RV to drift  $50\text{--}100 \text{ m}$ , then slowly ascending the rosette through the interface while closing Niskin bottles within the interface layer. Sub-sampling can then be conducted upon recovery (La Cono *et al.*, 2019). Some investigators have solved this issue through the development of a 'brine sampler' which eliminates mixing risks by compartmentalizing samples (Supplementary Fig. 4C; Joye *et al.*, 2005).

The complexity of solute-biomolecule interactions and the inability to spectrophotometrically measure gelation of agar amended athalassohaline DHAB brine calls for the development of new methods to measure chaotropy. First, a precise and accurate novel assay of measuring chaotropic activity must be established which could later be developed into specialized instruments, probes, or sensors. Instrumental data could be used to create *in silico* machine learning models to predict chaotropic activity based upon biogeochemical parameters, which could inform development of an improved theoretical understanding of chaotropy. Nevertheless, reporting ion ratios of chaotropes ( $\text{Cl}^-$ ), to compensating kosmotropes ( $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{SO}_4^{2-}$ ) factoring in their relative chao/kosmotropic strength, based on past work from Cray *et al.* (2013), may be a comparable proxy for chaotropic activity between DHABs.

To better understand the microbial activity, cell counts using CARD-FISH analysis have also been conducted in DHABs showing upwards of sixfold increases in prokaryotic cell counts from overlying seawater into the upper interface layer, followed by a decrease in deeper layers (Yakimov *et al.*, 2015). However, cell counts, particularly of fungi and Gram-positive bacteria that have thick cell walls, must be carefully considered as they likely represent dead ‘micromummies’ that retain cell wall ultrastructure and preserved genetic material as a result of chaotropic stress (Duda *et al.*, 2004; Yakimov *et al.*, 2015). A combination of fine-scale NanoSIMS (nanoscale secondary ion mass spectrometry), CARD-FISH and BONCAT analyses could be practiced to determine taxonomic activity at the single-cell level (Dekas and Orphan, 2011). Future research of the Kryos, Discovery and Hephaestus basins should consider incorporating these and other next-generation physiology techniques such as SIP/SAP (qualitative stable isotope probing/substrate analogue probing) to carefully analyse community interactions and biogeochemical cycling. Using these state-of-the-art techniques may support existing evidence of active athalassohaline brine communities and will benefit life detection approaches in hypersaline environments. For a detailed review of next-generation physiology approaches, see Hatzenpichler *et al.* (2020).

#### Implications for astrobiology

Deep ocean environments have been considered analogs to sub-surface oceans of the ice-covered oceans moons of the outer solar system. Having a range of compositions, elevated ionic strength, chaotropicity and high hydrostatic pressure (Preston and Dartnell, 2014), DHABs comprise physical properties analogous with potential environments within other ocean worlds. The icy moons of Saturn (Enceladus and Titan) and Jupiter (Europa, Callisto, and Ganymede) likely harbour oceans underneath thick surface ice (e.g. Kivelson *et al.*, 2000; Postberg *et al.*, 2009). The ice shells and oceans of these moons could be conducive to life and favour the preservation of organic material (Hoover and Gilchinsky, 2001; Marion *et al.*, 2003; Preston and Dartnell, 2014). Furthermore, the presence of surface salts, especially on Europa, including magnesium and sulfur-rich compounds in high concentrations (Carlson *et al.*, 2009) points to a need to understand microbial habitability and preservation in exotic brines.

#### Concluding remarks

Athalassohaline DHABs offer an opportunity to explore the edge of life on Earth. Evidence of active prokaryotes in the seawater brine interfaces within the Kryos,

Discovery and recently discovered Hephaestus basin exist. These active communities likely consist of highly specialized chaotolerant extremely halophilic methanogens such as *Methanohalophilus*, anaerobic fermentative *Acetothermia* and sulfur-respiring taxa within *Proteobacteria*. Ambiguous evidence supports the presence of active life within the MgCl<sub>2</sub> brine beyond the currently accepted chaotropic and  $a_w$  limits of life. Athalassohaline DHAB research is notoriously difficult and confounded by the poorly understood effects of MgCl<sub>2</sub> on biomolecules. Life detection strategies require nuanced analysis of various unstable biomarkers such as mRNA and fine-scale vertical sampling of highly stratified communities to avoid false positives of life. The application of advanced sampling technologies and next-generation physiology approaches will be essential for future work, along with the need for more extensive measurements of chaotropicity. Further investigation of athalassohaline DHABs could expand the known limits of life on Earth, enable the discovery of novel metabolic pathways and provide new insights and tools in the search for life elsewhere.

#### Acknowledgements

This work was funded as part of the Oceans Across Space and Time program of the NASA Astrobiology Program, grant 80NSSC18K1301, PI B.E. Schmidt. More information about OAST and its investigators can be found at <https://oast.eas.gatech.edu>.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1:** Supporting information