NaCl-saturated brines are thermodynamically moderate, rather than extreme, microbial habitats


Published in:
FEMS microbiology reviews

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
Copyright 2018 Wiley. This work is made available online in accordance with the publisher’s policies. Please refer to any applicable terms of use of the publisher.

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Download date:04. Aug. 2019
NaCl-saturated brines are thermodynamically moderate, rather than extreme, microbial habitats

One-sentence summary:

Although NaCl-saturated brines are thought to represent a limit-for-life on Earth, they are biomass-dense, display broad biological and metabolic diversity, and (despite their reduced water activity of ~0.755) some halophiles thrive here indicating that these brines are thermodynamically mid-range rather than extreme microbial habitats.

Callum J. D. Lee¹, Phillip E. McMullan¹, Callum J. O’Kane¹, Andrew Stevenson¹, Inês C. Santos², Chayan Roy³, Wriddhiman Ghosh³, Rocco L. Mancinelli⁴, Melanie R. Mormile⁵, Geoffrey McMullan¹, Horia L. Banciu⁶, Mario A. Fares⁷⁹, Kathleen C. Benison¹⁰, Aharon Oren¹¹, Mike L. Dyall-Smith¹² and John E. Hallsworth¹,∗

¹Institute for Global Food Security, School of Biological Sciences, MBC, Queen’s University Belfast, Belfast, BT9 7BL, Northern Ireland; ²Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, TX 76019, USA; ³Department of Microbiology, Bose Institute, P-1/12 CIT Scheme VII M, Kolkata, 700054, India; ⁴BAER Institute, Mail Stop 239-4, NASA Ames Research Center, Moffett Field, CA 94035, USA; ⁵Department of Biological Sciences, Missouri University of Science and Technology, Rolla, MO 65401, USA; ⁶Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeș-Bolyai University, 400006 Cluj-Napoca, Romania; ⁷Department of Abiotic Stress, Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-Universidad Politécnica de Valencia, Valencia, 46022, Spain; ⁸Institute for Integrative Systems Biology (I2SysBio), Consejo Superior de Investigaciones Científicas-Universitat de Valencia (CSIC-UV), Valencia, 46980, Spain; ⁹Department of Genetics, Smurfit Institute of Genetics, University of Dublin, Trinity College, Dublin 2, Dublin, Ireland; ¹⁰Department of Geology and Geography, West Virginia University, Morgantown, West Virginia 26506-6300, USA; ¹¹Department of Plant & Environmental Sciences, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Edmond J. Safra Campus, Givat-Ram, Jerusalem 91904, Israel; and ¹²Faculty of Veterinary and Agricultural Science, The University of Melbourne, Parkville, VIC, 3010, Australia

Correspondence: John E. Hallsworth, Institute for Global Food Security, School of Biological Sciences, MBC, Queen’s University Belfast, 97 Lisburn Road, Belfast, BT9 7BL, Northern Ireland. Tel: +44-2890972314; fax: +44-2890972570; e-mail: j.hallsworth@qub.ac.uk
Running head: NaCl-saturated brines are moderate microbial habitats

Abstract

NaCl-saturated brines such as saltern crystallizer ponds, inland salt-lakes, deep-sea brines, and liquids-of-deliquescence on halite are commonly regarded as a paradigm for the limit-of-life on Earth. There are, however, other habitats that are thermodynamically more extreme. Typically, NaCl-saturated environments contain all domains-of-life and perform complete biogeochemical cycling. Despite their reduced water activity, ~0.755 at 5 M NaCl, some halophiles belonging to the Archaea and Bacteria exhibit optimum growth/metabolism in these brines. Furthermore, the recognized water-activity limit for microbial function, ~0.585 for some strains of fungi, lies far below 0.755. Other biophysical constraints on the microbial biosphere (temperatures of >121°C; pH >12; and high chaotropicity; e.g. ethanol at >18.9% w/v (24% v/v) and MgCl₂ at >3.03 M) can prevent all ecosystem function and cellular metabolism. By contrast, NaCl-saturated environments contain biomass-dense, metabolically-diverse, highly-active, and complex microbial ecosystems which underscores their moderate character. Here, we survey the evidence that NaCl-saturated brines are biologically permissive, fertile habitats that are thermodynamically mid-range rather than extreme. Indeed, were NaCl sufficiently soluble, some halophiles might grow at 8 M. The finite solubility of NaCl may have stabilized the genetic composition of halophile populations and limited the action of natural selection in driving the halophile evolution towards greater xerophilicity. Implications are also considered for the origin(s)-of-life and other aspects of astrobiology.

Keywords: Dunaliella salina; extreme halophiles; habitability of Mars; hypersaline brines; limits-of-life on Earth; solar salterns

Introduction

Various types of NaCl-saturated environment occur throughout Earth’s biosphere, spanning a wide range of scales from solar salterns, inland salt-lakes, and deep-sea brines to fluid inclusions of NaCl crystals and liquids-of-deliquescence on halite¹. They also include ephemeral habitats such as those created by the sea spray which lands on rock surfaces or saline aerosols which circulate within the atmosphere. Marine solar salterns, which are of human construction and include NaCl-saturated crystallizer ponds, occur along the coasts of many

¹ At specific relative humidities and temperatures, salts absorb water from the vapour phase and liquefy to produce saturated brine (Gough et al., 2011; Rummel et al., 2014; Stevenson et al., 2015b).
countries with warm sunny climates, such as Australia, China, Indonesia, Japan and the USA (Antón et al., 2000; Zhaxybayeva et al., 2013; Cui and Qiu, 2014; Mori et al., 2016), while inland solar salterns are often situated where natural saline waters and high evaporation rates occur, such as Australia, India, Spain, Peru and the USA (Maturrano et al., 2006; Zafrilla et al., 2010; Jose and Jebakumar, 2013). Large-scale inland bodies of water, including the Dead Sea (Jordan, Rift Valley, Middle East) and the Great Salt Lake (Utah, USA), vary in their solute composition, but their NaCl component is at times saturated or oversaturated. In the Mediterranean Sea there is a cluster of deep-sea basins (e.g. L’Atalante and Bannock), most of which contain NaCl-saturated brines that are known amongst microbiologists as ‘deep-sea hypersaline anoxic brine lakes’. NaCl crystals can form in these, as well as other, hypersaline systems. Ancient salt crystals make up evaporite sedimentary rocks that underlie some of Africa, Asia, Australia, Europe, and North and South America (Warren, 2016); and halite rock is also present in above-ground locations and has been well-studied, for example, in the Atacama Desert in Chile (Benison et al., 2007) and Death Valley in California (Lowenstein and Hardie, 1985). Many of these systems are geochemically complex, containing other types of ions such as Mg$^{2+}$ and SO$_4^{2-}$ (e.g. Yakimov et al., 2015). Shallow saline lakes in particular are characterized by fluctuating salinity due to cycles of flooding, evapoconcentration and desiccation (Lowenstein and Hardie, 1985). For instance, the shallow acid-brine lakes of Western Australia can cycle between approximately 5% and 35% w/v total salts and so can be either saline waters or hypersaline brines (Benison et al., 2007). Natural brines tend to be enriched in many elements besides Na and Cl; these most commonly include S, Mg, Ca, K, and Br, though brines with unusually high or low pH also may have elevated dissolved concentrations of Al, Fe, and Si (Bowen and Benison, 2009)$^2$. All brines are characterized by low water activity, that can be a potent stress parameter for microbial cells. Indeed, water activity (i.e., the mole fraction of water) acts as determinant for life processes and habitability of hostile environments (Grant, 2004; Rummel et al., 2014; Stevenson et al., 2015a; 2015b). Water activity, defined by Raoult’s Law, is dependent on temperature and pressure. In relation to cellular stress biology, and microbial ecology, this thermodynamic parameter interacts with other factors such as temperature, pH and chaotropicity to determine the rates and windows for metabolic activity and growth of microbial systems (e.g. Williams and Hallsworth, 2009; Harrison et al., 2013; Alves et al., 2015).

Here, we focus on NaCl-dominated brines that are at or close to saturation with respect to halite, either all or some of the time. These systems are commonly regarded as representing

---

$^2$ Whereas such elements are often present at trace levels, they can also reach concentrations which are stressful and/or toxic in some brines (see below).
the dry extreme for life – i.e. a limit of the Earth’s biosphere – or to view them as a model for studies of water-constrained ecosystems and habitability of hostile terrestrial environments in the context of astrobiology (e.g. Brown, 1990; Grant, 2004; Harrison et al., 2013; Crits-Christoph et al., 2016). In addition, there has been an assumption that the water-activity limit for the cells and ecosystems of the most extremely halophilic microbes is 0.755, i.e. the value corresponding to water that is saturated with NaCl (Anderson, 1954; Brown, 1976; Grant, 2004; Kminek et al., 2010; Rummel et al., 2014. However, recent studies have revealed active cell division of halophilic Archaea and Bacteria in the range 0.748-0.635 water activity, with theoretically determined water-activity minima as low as 0.611 (Stevenson et al., 2015a). One study, of Aspergillus penicillioides (a xerophilic ascomycete capable of growing at high concentrations of various solutes including NaCl, sugars and glycerol), established a new water-activity limit for microbial differentiation and cell division: 0.585, with a theoretically determined limit of 0.565 (Fig. 1a; Stevenson et al., 2017a;b;c). Collectively, these studies suggest that some extreme bacterial and archaeal halophiles may grow even when water activity is as low as 0.565. Furthermore, it is plausible that microbial populations which subsist under extreme conditions may undergo cell divisions over long timescales (Rummel et al., 2014), yet to be quantified, at even lower water-activity values. Whereas the most active microbes present in NaCl-saturated environments are known as halophilic extremophiles, the water activity of NaCl-saturated brine falls far short of the limit for extant halophilic life or the theoretical limit to which microbes could plausibly adapt (see below). This article reviews the evidence that microbially diverse, biomass-rich, and highly productive ecosystems occur at 5 M NaCl, and that they are functioning under moderate, rather than thermodynamically extreme, conditions.

There is no microbial ecology near the biophysical limits for life

In this article, we use the term ‘extreme’ to refer to conditions which impose biophysical constraints on cellular life and, as such, represent the thermodynamic extremity of microbial life on Earth. For instance, the water-activity window for the microbial biosphere spans from 1 to 0.585, i.e. 0.415 units. Only five or six types of microbe are known to function in the range 0.635 to 0.585; this range represents the most extreme 12% of the known window for microbial functionality (Fig. 1; Stevenson et al., 2015a;b). Diverse parameters are known to limit life on Earth, either singly or in combination. For example, extremes of temperature and/or pressure;
pH, chaotropicity\(^3\) and ionic strength and low water-activity can prevent microbial cell function. This occurs via biophysical mechanisms which operate at the levels of the biomacromolecule, whole cell, ecosystem and, ultimately, the microbial biosphere (Kashefi and Lovley, 2003; Hallsworth et al., 2007; Chin et al., 2010; Cray et al., 2015; Santos et al., 2015; Stevenson et al., 2015a,b; Yakimov et al., 2015; Fox-Powell et al., 2016; Roy et al., 2017). For such parameters, the failure points for cell division of the most resilient microbial systems occur close to, for example, 18.9% w/v (24% v/v) ethanol, 121°C, -18°C, and 0.585 water activity (Fig. 1). In relation to water activity, Pitt and Christian (1968) stated that germination of *Xeromyces bisporus* (FRR 0025) aleurospores occurred at 0.605; a finding which has neither been surpassed nor repeated for this species (Fig. 1a; Williams and Hallsworth, 2009; Stevenson and Hallsworth, 2014; Stevenson et al., 2015a,b; 2017a,b). At this water-activity limit biotic activity was exceptionally slow; i.e. germination was recorded only after a period of three months. The study of *A. penicillioides* strain JH06THJ, which germinated under some conditions at water activities as low as 0.585, revealed more rapid growth even at water activities of < 0.605 (Fig. 1a; Stevenson et al., 2017c). At 0.585 water activity, after 4 days conidia had swollen by >40% on a volume basis and were in a pre-germination phase; by 11.5 days conidia had differentiated to form polarized, tapered germination structures, and between 36 and 57 days fully formed separate germings had been produced (Stevenson et al., 2017c).

In relation to low temperatures, there are few reliable reports of microbial growth in the range -5 to -15°C (Fig. 1d; Chin et al., 2010 and Rummel et al., 2014). Beyond this range, there is a single report of microbial reproduction, for *Rhodotorula glutinis*, at -18°C, although it took 17-18 weeks for cell numbers to double, and the yeast has an optimum temperature for growth between 20 and 30°C (Fig. 1d; Collins and Buick, 1989; Martínez, 2006). Ethanol, which is produced in bulk by *Saccharomyces cerevisiae* and other microbes and acts as a potent antimicrobial agent, reduces water activity and acts as a chaotropic stressor for cellular systems (Hallsworth et al., 1998; 2003; Cray et al., 2013a; 2015). There are very few studies reporting microbial tolerance to ethanol at concentrations of > 12.5% w/v (15.8% v/v) (Fig. 1c). However, one study demonstrated metabolic activity and ethanol production in *S. cerevisiae* at 18.9% w/v ethanol (Fig. 1c; Thomas et al., 1993), which corresponds to a water activity of 0.901 and chaotropicity of 24.31 kJ kg\(^{-1}\) (Hallsworth and Nomura, 1999; Cray et al., 2013b). Reduced temperatures and kosmotropic substances\(^4\), such as the compatible solute proline, can mitigate

\(^3\) Chaotropic substances are those which entropically disorder biomacromolecular systems, and includes compounds such as ethanol, phenol, urea, LiCl, MgCl\(_2\) and guanidine hydrochloride (Hamaguchi and Geiduschek, 1962; Cray et al., 2013b; 2015a; Ball and Hallsworth, 2015).

\(^4\) Kosmotropic substances are those which entropically order or stabilize biomacromolecular systems (Collins, 1997; Cray et al., 2013b; Ball and Hallsworth, 2015).
against and, in some cases, prevent the chaotrope-induced stress associated with ethanol and mechanistically comparable stressors (Hallsworth, 1998; Hallsworth et al., 2007; Bhaganna et al., 2010; Bell et al., 2013; Alves et al., 2015; Cray et al., 2015). Recent studies were carried out based on genome analysis, hybridization, and subsequent strain selection to optimize ethanol tolerance and product yield from S. cerevisiae strains used for bioethanol fermentations (Fig. 1c; Swinnen et al., 2012; Pais et al., 2013). Whereas these were successful, the ethanol concentrations obtained fall well below the 18.9% w/v value reported previously. In relation to each of these extremes, the mechanisms, history and biology will be detailed.

At or close to each of these thermodynamic boundaries for life, there is no evidence of any functional microbial ecosystem. Furthermore, there are no reports of microbial metabolism below 0.585 water activity or above 18.9% w/v ethanol (Fig. 1a), and only sporadic reports of limited metabolic activities in a handful of microbial species below -18°C (Fig. 1d; Kminek et al., 2010; Rummel et al., 2014). For instance, studies of the lichen Pleopsidium chlorophanum that were performed in a Mars Simulation Chamber indicated significant photosynthetic activity at -40°C (Fig. 1d; de Vera et al., 2014). In natural habitats such as sea ice and permafrost, however, there is no evidence of ecosystem function at temperatures below -18°C, hence the standard (-20°C) temperature of freezers used for food storage. The data pertaining to the lower temperature limits for microbial life have been discussed elsewhere (Chin et al., 2010; Kminek et al., 2010; Rummel et al., 2014) so will not be discussed at length here. There is no evidence of microbial fermentation at ethanol concentrations > 18.9% w/v from culture-based studies; similarly, no anthropogenic systems or microbial habitats which are sugar-rich are known to facilitate ecosystem function above such ethanol concentrations according to either culture-dependent or -independent studies (Fig. 1c; Cray et al., 2015; Lievens et al., 2015).

Likewise, the chaotropicity of MgCl₂ limits the biotic activity of all microbial systems at concentrations ≥ 2.40 M (Hallsworth et al., 2007), though activity for some halophile systems has been reported in the range 2.50 to 3.03 M when kosmotropic ions are present (Hallsworth et al., 2007; Yakimov et al., 2015). Whereas extrapolations made using data of fungal germination at low water-activity suggest theoretical water-activity minima for germination in the range 0.575 to 0.565 (Fig. 1a; Stevenson et al., 2017b;c), there is no empirical evidence for metabolic activity or cell division of xerophilic fungi at such values. The few microbes known to be capable of biotic activity at < 0.690 water activity inhabit diverse substrates and environments in nature and so are unlikely to occur together or form an active community under these conditions (Fig. 1a; Stevenson et al., 2015a;b; 2017c). In other words, conditions or habitats of < 0.690 might be regarded as ‘extreme’ and are characterized by low biodiversity and a negligible biomass of functional microbes. Each of the findings which demonstrated microbial activity at 18.9% w/v (24% v/v) ethanol, -18°C or 0.585 water activity was reported in
an individual published study and for a single microbial system (Fig. 1; Collins and Buick, 1989; Thomas et al., 1993; Stevenson et al., 2017c). Chaotropic environments containing high concentrations of MgCl₂ or ethanol are effectively sterile (Hallsworth et al., 2007; Cray et al., 2015; Yakimov et al., 2015), hence the value of such substances as food preservatives or biocides.

**Rich and active ecosystems in NaCl-saturated brines**

NaCl-saturated systems are highly permissive for microbial life (Table 1) and, accordingly, there is an active international community of scientists working on these ecosystems. This is illustrated by the recent and well-attended 13th International Conference on Halophilic Microorganisms of the International Society for Salt Lake Research (2017, in Ulan-Ude, Russia with 122 delegates). There are 2569 papers published on halophiles and their hypersaline habitats, according to the Thomson Reuters Web-of-Science database (as of 28 May 2018)⁵.

**Biomass density and richness**

In both natural and anthropogenic systems (such as industrial salterns), NaCl-saturated brines can have an extraordinarily high microbial biomass. For instance, NaCl-saturated solar salterns have $10^6$ to $10^8$ microbial cells ml⁻¹ (Table 1), and this is equal to the yeast-cell densities attained in wine- and cider fermentations (Cocolin et al., 2000; Herrero et al., 2006; Taniasuri et al., 2016). Even within the hypersaline fluid inclusions of a single NaCl crystal, cell densities greater than $4 \times 10^8$ ml⁻¹ have been observed (Table 1)⁶. Furthermore, NaCl-saturated systems frequently exhibit a high abundance and diversity of microbial types (Table 1). The Shannon Indices in one marine saltern in Tunisia, for instance, were 0.98 for Bacteria and 3.04 for Archaea (Table 1; Baati et al., 2008), values comparable to those for microbial diversity within bioethanol fermentations of corn, i.e. up to 3.23 (Li et al., 2016). The salt-saturated (~ 35% total salinity) bottom water layer of two inland permanently stratified (meromictic) salt lakes situated in Romania revealed unusually high alpha-diversity for both Bacteria (SI 3.5 and 5.4; 330 and 459 operational taxonomic units – OTUs₀,₀₃ – defined as ≥97% 16S rRNA gene sequence similarity clusters) and Archaea (SI 4.0 and 3.3; 144 and 116 OTUs₀,₀₃). In these lakes, the microbial diversity

---

⁵ The searches “(halophil* or halotoler* or microbial-community or microbial-ecology or microbiology or Haloquadratum or Dunaliella or Salinibacter or Actinopolyspora or Halorhodospira) and (saltern* or NaCl-crystal* or halite or brine-lake* or deep-sea-lake* or deep-sea-basin* or deep-sea-brine* or fluid-inclus* or crystallizer-pond* or crystalliser-pond* or Dead-Sea or hypersalin* or NaCl-saturat* or salt-saturat* or saturated-NaCl or saturated-salt* or 5-M-NaCl or 5-molar-NaCl)* and “extreme-halophil* or obligate-halophil*” were carried out, and then combined, to derive this number.

⁶ These communities can remain active; survive within the crystal over long time-periods (possibly geological timescales); can be released upon dissolution of crystals or crystal deposits; and collectively constitute enormous microbial ecosystems which extend across considerable regions of the Earth’s surface and subsurface (Grant, 2004; Sankaranarayanan et al., 2014).
increased along the salinity gradient reaching maximum at highest salt concentration (Andrei et al., 2015). In Mediterranean deep-sea brines, despite the lack of phototrophs, more than 110 OTUs (the criterion ≥98% was used for 16S rRNA gene sequence similarity) were present which encompassed microbes from each domain of life (Table 1; Ferrer et al., 2012; Stock et al., 2012).

The liquids-of-deliquescence on halite of the Atacama Desert can contain cell densities of 2-10 x 10⁷ cells g⁻¹ (Table 1), and the NaCl-saturated north arm of the Great Salt Lake, and saltern crystallizer ponds (in diverse locations) contain 10⁷-10⁸ microbial cells ml⁻¹, a density that gives rise to the distinctive red colour, and opacity of these brines (Table 1; Oren, 2009). Most other aquatic systems are characterized by microbial cell densities that are significantly lower than this. For example, a study of tropical freshwater reservoirs in Sri Lanka reported up to 4.73 x 10⁶ bacterial cells ml⁻¹ (Peduzzi and Scheimer, 2004) and a study of coral atolls reported from 7.2 x 10⁴ to 8.4 x 10⁵ microbial cells ml⁻¹ seawater, depending on the sample site (Dinsdale et al., 2008). A number of studies show that cell number increases as NaCl concentration increases (e.g. Benlloch et al., 1996; Daffonchio et al., 2006). There are various reasons which, when combined, help to explain why cell densities are so high in hypersaline environments; these include:

1. salterns, and other NaCl-saturated habitats, can have a plentiful supply of nutrients (including the glycerol leaked by algae; Elevi Bardavid and Oren, 2008; Elevi Bardavid et al., 2008; Oren, 2017) that can become further concentrated by evaporative processes,

2. saturated NaCl is not thermodynamically extreme, e.g. daytime temperatures (as well as water activity) are often close to the optimum, i.e. for haloarchaea (see below; Oren, 1994),

3. the most-extreme halophiles are able to fully adapt to hypersaline conditions (Cray et al., 2013a; Oren and Hallsworth, 2014), and so their high growth rates and biomass are a reflection of high nutrient levels, and

4. the losses due to predation/grazing are relatively low (Guixa-Boixareu et al., 1996; Pedrós-Alió et al., 2000a; 2000b).

Halophile function at extremely low water activity
The water activity of NaCl-saturated brines (at ca. 0.755) is hostile to many types of microbe. At least 100s-1000s of microbial species are nevertheless all capable of growth in the water-activity range 0.755-0.701, but below this range there is a more restricted number of microbial systems able to function (Fig. 1a; Williams and Hallsworth, 2009; Stevenson et al., 2015a;b; 2017c). Even so, some extremely halophilic prokaryotes – which can be active down to 0.635-0.611 – exhibit high or optimum rates of growth at saturated NaCl (Figs. 1a; 1b; 2; Stevenson et al., 2015a;b). For instance, the extremely halophilic archaeal strains GN-2 and GN-5 (both presumed to be Halobacterium), Halobacterium strain 004.1, Haloquadratum walsbyi (DSM...
16790), and *Halorhabdus utahensis* (DSM 12940<sup>T</sup>), and the bacteria *Actinopolyspora halophila* (ATCC 27976<sup>T</sup>), *Halorhodospira halophila* (DSM 244<sup>T</sup>), *Pontibacillus* strain AS2, and *Salinicola* strain LC26 exhibited very high or optimum growth rates at saturated NaCl (or at water-activity values equivalent to that of saturated NaCl; Fig. 2; Stevenson et al., 2015a). Furthermore, their growth windows extend down to ∼0.600 water activity; so more than one third of the water-activity window for growth lies beyond the water-activity value for saturated NaCl (Figs. 1a; 1b; 2a-e). Similarly, for *A. penicillioides*, growth rates of germlings are remarkably high at ∼0.755 water activity on media supplemented with NaCl and glycerol, and 42% of the water-activity window is < 0.755 (Stevenson et al., 2017a;b;c). This finding is pertinent to the ecophysiology of halophiles because *A. penicillioides*, along with extreme halophilic fungi such as *Hortaea* and *Wallemia* species and halophilic algae such as *Dunaliella* species (all capable of growth at or close to 5 M NaCl, see Stevenson et al., 2015a), are exposed to molar concentrations of glycerol which they accumulate in the cytosol at high salt concentrations.

Some experimental settings, as well as studies of bittern brines, represent a transition between Mg-rich hostile environments that have no microbial activity, and densely-populated NaCl-saturated brine (Javor, 1984; Baati et al., 2011; Stevenson et al., 2015a; Yakimov et al., 2015). For halophiles cultured at very low water-activities, biotic activity was thereby revealed at < 0.755. However, the environmentally relevant salts which reduce water activity to this level are typically chaotropic (see above), and so chaotropicity cuts in as a stress parameter to limit life before water activity can do so (Hallsworth et al., 2007; Alves et al., 2015; Stevenson et al., 2015a;b; Yakimov et al., 2015). Other substances, such as glycerol, are more permissive such that NaCl+glycerol mixtures enable microbial activity at < 0.600 water activity (see above).

**Ecosystem activity and complexity**

The microbial ecology of biomass-dense NaCl-saturated habitats is both complex and dynamic (Table 1). Rates of primary production (via photosynthesis) of 120 to 220 µg carbon l<sup>−1</sup> d<sup>−1</sup> have been reported in crystallizer-pond brines (Elevi Bardavid et al., 2008), and the alga *Dunaliella salina* produces (and releases) sufficient amounts of glycerol to be able to sustain communities of heterotrophs (Hart and Gilmour, 1991; Elevi Bardavid and Oren, 2008; Elevi Bardavid et al., 2008; Oren, 2017; Williams et al., 2017). Heterotrophic microbes are abundant in hypersaline systems, including the haloarchaeal genera *Halobacterium* and *Halococcus* (Table 1), and the bacterium *Salinibacter ruber* which is commonly found in some NaCl-saturated habitats (Antón et al., 2008; Cray et al., 2013a). For instance, *S. ruber* can utilize glycerol, converting some to dihydroxyacetone (glycerone) which is excreted, and may be utilized by halophilic *Archaea*.
such as *Hqr. walsbyi*\(^7\) (Sher *et al*., 2004; Elevi Bardavid and Oren, 2008; Elevi Bardavid *et al*., 2008). A recent review of the various roles of glycerol in the context of the ecological complexity of hypersaline habitats has been carried out by Oren (2017). Halophiles are known to be highly proficient at the symbiotic utilization of various metabolites excreted by the primary producers, and the necromass produced by microbial cell turnover. This is exemplified both by the utilization of glycerol leaked from microalgae as well as some deep-sea NaCl-saturated systems, where communities can be sustained by trophic networks which ultimately rely on the sinking/migration of organic matter from the overlaying seawater (Daffonchio *et al*., 2006; Yakimov *et al*., 2013).

The various halophiles found in hypersaline brines exhibit a wide range of metabolic capabilities. For instance, inland hypersaline alkaline lakes located in Egypt contain microalgae, cyanobacteria, anoxygenic phototrophic bacteria, denitrifying bacteria and (in sediments at least) sulfate reducers (Table 1; Imhoff *et al*., 1978; Oren, 2011). Microalgae (such as algae of the *Mamiellaceae* family and *Halothec* cyanobacteria), as well as halophilic bacteria and archaea (e.g. *Halorhabdus* spp. and *Halococcus* spp.), inhabit the liquids-of-deliquescence according to studies of halite rock in the Atacama Desert (Robinson *et al*., 2015; Crits-Cristoph *et al*., 2016). Another study of halite rock demonstrated carbon fixation in the absence of light, with ammonia used as an electron donor, further exemplifying the diversity of microbial activities which take place in the communities inhabiting these liquids-of-deliquescence (Davila *et al*., 2015).

Sulfate-reducing bacteria, such as *Desulfobacter*, *Desulfovibrio*, and *Desulfococcus*, can be active at NaCl saturation in the clay, silt, and sand sediments of salters (Kerkar and Loka Bharathi, 2007; Mani *et al*., 2012); sulfur-oxidizing heterotrophs of the family *Ectothiorhodospiraceae* are abundant in environments with NaCl ranging between 1.7 and 4.3 M (León *et al*., 2014). Together, these findings indicate active bacterial networks for the redox cycling of various sulfur species. Anaerobic iron metabolism has been demonstrated in Lake Kasin (Russia) and Lake Chaka (China) (Jiang *et al*., 2006; Emmerich *et al*., 2012), while archaeal methanogenesis coupled with active sulfur cycling (by epsilonproteobacterial sulfur-oxidizers and deltaproteobacterial sulfate reducers) has been reported to sustain biomass-rich complex microbial communities in deep-sea brine lakes\(^8\) such as those in the Urania basin of the Mediterranean (Borin *et al*., 2009). Active networking between sulfur-oxidizing

\(^7\) Genera are abbreviated according to the accepted three-letter acronym for *Halobacteria* genera (Oren and Ventosa, 2016).

\(^8\) In microbiology, the convention has arisen to refer to the bodies of hypersaline water located in deep-sea basins as *brine lakes*, whereas geologists use the term *stratified marine brines* when referring to a body of brine which is overlaid by seawater.
chemolithoautotrophs similar to the endosymbiont of the deep-sea geothermal vent tube worm *Riftia pachyptila*, and sulfate reducers, methanotrophs, and nitrogen fixers, has been indicated by metagenomic and metatranscriptomic analyses across the chemoclines of the deep Mediterranean Sea hypersaline anoxic lake Thetis (Ferrer *et al*., 2012; Pachiadaki *et al*., 2014). Indeed, various studies have focused on characterizing the geochemical and biological complexity of microbial ecosystems, including biogeochemical cycling in NaCl-saturated deep-sea brines (Table 1; Daffonchio *et al*., 2006; Alexander *et al*., 2009; Edgcomb *et al*., 2009; Danovaro *et al*., 2010; Ferrer *et al*., 2012).

Inland saline alkaline lakes mostly result from the evaporative concentration of Na$_2$CO$_3$/NaHCO$_3$/NaCl (‗trona‘)-enriched water. Alkaline hypersaline (haloalkaline) waters such as those in Wadi An Natrun (Egypt), Lake Sambhar (Rajasthan, India), or Salton Sea (California, USA) have high NaCl contents but low alkalinity. In this way, their water chemistry differs from that of soda lakes found in arid or semi-arid zones such as North America (e.g., Mono, Big Soda, and Soap lakes), Central Asia (e.g., in the SE Siberian steppe and Inner Mongolia), as well as in the East African Rift Valley (e.g., Magadi, Bogoria, and Natron lakes) (Sorokin *et al*., 2014). These soda lakes are characterized by an elevated HCO$_3^-$/CO$_3^{2-}$ concentration. The haloalkaline Lake Sambhar is a large, inland, NaCl- and bicarbonate-dominated system where salt concentration fluctuates depending on season and local rainfall. Total salt content within can reach up to 30% (w/v), with Na$^+$ at 118.1 g l$^{-1}$, Cl$^-$ at 147.12 g l$^{-1}$, and HCO$_3^-$ 41.5 g l$^{-1}$. The remaining ions include other metallic ions Ca$^{2+}$ (0.002 g l$^{-1}$), Mg$^{2+}$ (0.018 g l$^{-1}$), K$^+$ (1.96 g l$^{-1}$), Fe (0.076 g l$^{-1}$), and Mn (0.0052 g l$^{-1}$) and anions NO$_3^-$ (1.07 g l$^{-1}$) and CO$_3^{2-}$ (33.12 g l$^{-1}$) (Pathak and Cherekar, 2015). The pH varies depending on the salinity, and is typically ranges from 8.18-11 (Yadav *et al*., 2007; Pathak and Cherekar, 2015). One study revealed 64 morphologically distinct isolates in 26% w/v NaCl samples taken from Lake Sambhar, including the extreme halophile *Natronomonas* that is known to grow up to 32% w/v NaCl (Pathak and Cherekar, 2015). It is populated by haloalkaliphilic *Archaebacteria* (Upasani and Desai, 1990), *Actinobacteria* (Jose and Jebakumar, 2013), several members of the phylum *Firmicutes* (Paul *et al*., 2015), *Proteobacteria* (Cherekar and Pathak, 2015), and also a number of halophilic or halotolerant bacteria (Bachani *et al*., 2016), as well as phytoplankton that exhibit a range of salt tolerances (Jakher *et al*., 1990).

Despite their multiple stress parameters (salinity; low water activity; pH >9.5), both alkaline hypersaline lakes and soda lakes are among the most productive ecosystems; up to 7 g C·m$^{-2}$·day$^{-1}$ (Melack and Kilham, 1974). Furthermore, they are inhabited by biodiverse communities of microorganisms which drive full C-, N-, P-, and S cycles (Jones *et al*., 1998; Sorokin *et al*., 2014; Sorokin *et al*., 2015b). It is not surprising, therefore, that soda lakes have become a rich source for novel microbes during bioprospecting studies. Some of the microbes isolated from haloalkaline brines are capable of energy generation (e.g., via elemental sulfur disproportionation, acetoclastic and hydrogenotrophic methanogenesis, syntrophic methanogenesis) (Sorokin *et al*., 2015a; Sorokin *et al*., 2016a; Sorokin and Chernyh, 2017) and degradation of recalcitrant polymers (e.g., cellulose; chitin and pectin) (Zhilina *et al*., 2005, Sorokin *et al*., 2012a and 2012b, respectively) regardless of the...
cellular inhibition imposed by the *in-situ* stress parameters. This said, there has been little work done on the water activity of haloalkaline environments\(^1\). At *in-situ* concentrations, sodium carbonate behaves as a weak electrolyte, and organisms inhabiting such environments require less energy for osmotic protection than organisms in pH-neutral NaCl environments (Sorokin *et al.*, 2015b; 2016a). Furthermore, the osmotic pressures of synthetic analogues for waters from soda lakes differ from those of pH-neutral saline lakes at equivalent Na\(^+\) concentrations because carbonate/bicarbonate ions have a less effects than Cl\(^-\) ions (Banciu *et al.*, 2004). Collectively, these data suggest that the water activities of soda lakes, and many haloalkaline lakes, can be considerably higher than 0.755.

The microbial communities discussed above (see also Table 1) are sufficiently diverse in terms of phylogeny and ecophysiological functions to form sustainable ecosystems. Their primary producers can ultimately support heterotrophic, and other trophic, networks without requiring inputs from outside the NaCl-saturated milieu. Furthermore, culture-independent studies indicate the presence of genes that are, collectively, capable of completing the biogeochemical cycles that would enable the microbial ecosystem to function independently (Allen and Banfield, 2005). However, the ecological interactions of halophilic microbes within and beyond their NaCl-saturated habitats are neither limited to other microorganisms nor to the immediate locality. For instance, *Dunaliella* and *Asteromonas* (Oren, 2017) and other microbial biomass supports high densities of *Artemia* (brine shrimp) in saltmarshes, salterns, and brine lakes (Triantaphyllidis *et al.*, 1998; Clegg and Trotman, 2002) which, in turn, may sustain populations of aquatic insects, and birds such as flamingos, gulls, and waders (Ayadi *et al.*, 2004; Torrentera and Dodson, 2004). Such avian species by nature have a wide home range and/or are migratory, and so microbial halophiles can ultimately impact biological systems located far from their hypersaline habitat. This exemplifies how the ecosystems found within many types of NaCl-saturated environment are not closed systems, and may contribute to other, remote and/or non-saline, ecosystems.

**NaCl-saturated brines as a hotspot for life**

A NaCl-saturated body of water can act as a hotspot for life within in an otherwise low-biomass, low-biodiversity environment. Recent studies of NaCl-dominated lakes in Western Australia, for instance, exemplify this well; these lakes are located in arid, nutrient-poor regions characterized by sandflats and sand dunes. Even those lakes with a low pH (that are hostile due to their acidity and other ions that can be toxic to life) contain microbial life and are surrounded by plants (including two species of *Eucalyptus* and *Enchylaena tomentosa*, the ruby saltbush), sometimes with signs of animal life nearby (Benison, 2006). Moreover, the pH-neutral lakes (even those at NaCl saturation) are extremely rich in microbial biomass and diversity, with some also containing dense populations of *Artemia* as well as *Coxiella* gastropods and other invertebrates, and are surrounded by an abundance of plant and animal life including insects and spiders, snakes, emus and other birds, and mammals such as dingoes and kangaroos (Benison, 2006). The NaCl-saturated brines of Death Valley also support a biodiverse
ecosystem, including plants of the genus Salicornia, some of which can grow while their roots are in NaCl-saturated brine, and gastropods such as the Badwater snail, Assiminea infirma. Incredibly, a fish Cyprinodon salinus (the pupfish) can also survive at molar concentrations of NaCl (Schoenherr, 1992). In addition, many mammal, reptile, bird, and insect species live near and interact with the saline waters within the valley (Bryan and Tucker-Bryan, 2009). In terms of microbiology and, in many cases flora and fauna, many other types of NaCl-saturated brines act as oases for life in regions which are generally barren (Table 1; Daffonchio et al., 2006; Davila et al., 2015; Crits-Christoph et al., 2015; Robinson et al., 2015; Çınar and Mutlu, 2016).

Species dominance within the open habitats created by NaCl-saturated brines
In some NaCl-rich environments, there is a shift in microbial habitability – a selection for extreme halophiles – at approximately 3.4 M NaCl. This is evidenced, for instance, by shifts in community structure in solar salterns, as inferred from the relative percentages of the various phylum- or class-level taxa present (Ventosa et al., 2015, and references therein). In a study of a series of saltern ponds at Santa Pola (Spain), those with lower NaCl concentrations were populated by taxa including Euryarchaeota (~46% of the community), Alphaproteobacteria, Actinobacteria, Verrucomicrobia, Deltaproteobacteria, and Firmicutes (Ventosa et al., 2015). Those salterns with 3.6-5 M NaCl were populated primarily by members of the Euryarchaeota (80-90% of the community), and Bacteroidetes (9-10%). Via what syntrophic synergies the two dominant phyla manage to sustain viable ecosystems is a topic worthy of future exploration. Shifts in microbial community composition from moderate- (< 3.5 M) to high-salt (> 3.5 M) conditions might be governed by a combination of several factors such as increase in salinity, the availability of light and, perhaps, oxygen, that simultaneously select for dominance of aerobic or microaerophilic haloarchaea and Bacteroidetes in hypersaline Santa Pola salterns. A reverse trend was noted along the salinity gradient in a meromictic hypersaline lake in Romania. Euryarchaeota dominated (~90% relative abundance) the communities dwelling in the photic, oxic, and saline (20-25% total salinity) upper water layer whereas the aphotic, anoxic, and hypersaline bottom brine (~35% total salinity) was the niche preferred by anaerobic halophilic bacteria (Deltaproteobacteria, Clostridia, Spirochaetes, Bacteroidetes and Actinobacteria) in an overall abundance of ~75%. Under such conditions, Archaea (mostly represented by microaerophilic Halorhabdus spp.) became secondary (~20%) (Andrei et al., 2015). The structuring of microbial diversity at a global scale, however, may primarily driven by salinity, and also by habitat type (water versus sediment) and lifestyle (free-living versus host-dependent) (Lozupone and Knight, 2007; Auguet et al., 2010), and hypersaline sediments are amongst highest phylogenetically-diverse habitat on Earth (Table 1; Daffonchio et al., 2006; Lozupone and Knight, 2007).
Some types of NaCl-saturated environments, such as salterns, represent open habitats\(^9\) that are characterized by high levels of competition between different microbes (Table 1; Hallsworth, 1998; Cray \textit{et al.}, 2013a; Oren and Hallsworth, 2014; Lievens \textit{et al.}, 2015). In addition, hypersaline systems in which NaCl concentration varies (due to processes such as ingress of freshwater through rivers or streams, precipitation, and solar evaporation) are also open habitats for microbes during their transitions between different salt concentrations. Such transitions can promote competition (Řeháková \textit{et al.}, 2009) and, under these conditions, some halophile species can reach high cell densities and therefore become very dominant within the community; such species are known as ‘microbial weeds’ (Cray \textit{et al.}, 2013a; Oren and Hallsworth, 2014). In solar salterns, weed species include \textit{D. salina}, \textit{S. ruber} and \textit{Hqr. walsbyi} (Cray \textit{et al.}, 2013a); and can even dominate in low-temperature hypersaline systems such as Deep Lake in the Antarctic (DeMaere \textit{et al.}, 2013). In this environment, the mean temperature is approximately -20°C and yet haloarchaea, such as \textit{Halohasta litchfieldiae} strain tADL which comprises 44\% of the population, are still able to out-compete and out-grow potential competitors (Williams \textit{et al.}, 2014). Strangely, the halophilic \textit{Archaea} isolated from this lake are all mesophiles, with optimum growth temperatures of around 30°C. \textit{Haloferax mediterranei} also has many characteristics of a weed species and, whereas it does not dominate microbial communities in saltern brines (Oren and Hallsworth, 2014), it can do so in high-NaCl sediments (Youssef \textit{et al.}, 2012). Microbial weed species, therefore, may simultaneously cause some reduction in species diversity and an increase overall cell density (due to their own abundance). Whereas specific microbial weeds within the community can in this way act as ‘mavericks’, the ecology of NaCl-saturated systems typically function in both a self-regulating and sustainable manner.

\textbf{Cellular adaptations that enable high rates of biotic activity at 5 M NaCl}

\textit{Strategies to overcome high concentrations of salts}

Halophile systems are readily able to tolerate high concentrations of NaCl because the water activity is not extreme, and have many adaptations to enable this, which underscores the moderate character of such habitats. These adaptations are detailed in reviews by Grant (2004), Balashov \textit{et al.} (2005), Cray \textit{et al.} (2013a), Oren (2013), and Lebre \textit{et al.} (2017). Adaptation of halophiles to high salinity in the extracellular milieu has involved the evolution of several mechanisms to balance the osmotic stress induced by such conditions. Indeed, for

\(^9\) Open habitats are resource rich, available for colonization, and are habitable by, a large diversity of microbes; they tend to promote intense competition between species. For a more detailed definition of open habitats, see Cray \textit{et al.} (2013a).
some halophilic microbes, NaCl-saturated brines may not significantly stress the cellular system (Stevenson et al., 2015a; Hallsworth, 2018). A number of strategies adopted by halophiles to balance osmotic stress caused by high salt concentrations have been investigated, which allow classifying halophiles into either being ‘salt-in’, including halophilic archaia of the class Halobacteria and a small number of halophilic bacteria, or ‘salt-out’ represented by halophilic methanogenic archaia, most halotolerant/philic bacteria and all halophilic eukaryotes. Both these strategies involve a number of molecular adaptations that make it possible to counterbalance the stress imposed by the low water-activity conditions of saline environments. Halophiles adopting salt-in strategies balance the low water-activity of the extracellular milieu by accumulating compensatory amounts of K+ sometimes accompanied by minor amounts of Na+ ions (Oren, 2008). In contrast, the salt-out strategy employed by most halophilic bacteria involves mechanisms for excluding salt from the cellular environment or the synthesis of compatible solutes, such as glycine betaine (i.e. N-trimethylated glycine) for bacteria and glycerol for eukaryotes (Roberts, 2005). Arguably, many extreme halophiles accumulate ions rather than organic compatible solutes for this purpose thereby conserving energy (Oren, 1999).

In hypersaline environments, most proteins from non-halophilic microbes are inhibited due to the low water-activity and/or high ionic strength, and are typically also denatured and aggregate and/or precipitate (e.g. Baldwin, 1984; Li et al., 2012; Alves et al., 2015). However, the extracellular and cytosolic enzyme systems of some halophiles can work optimally at NaCl concentrations as high as 5 M and osmotically equivalent K+ concentrations, respectively. Intracellular proteins of salt-in species exhibit increased hydrophilicity and charge allowing them to work at very high salt concentrations. Several studies, including those that first sequenced Halobacterium sp. NRC-1 (Reistad, 1970; Ng et al., 2000) and those that resolved the structure of halophilic proteins (Dym et al., 1995; Kennedy et al., 2001; DasSarma et al., 2006) revealed that, for some halophiles, their proteins contain few basic amino acids (relative to their orthologs in non-halophilic proteins), but are enriched in the acidic amino acids, glutamic acid and aspartic acid. They also revealed that halophilic proteins of ‘salt-in’ strategists (Bolhuis, et al., 2008; Capes et al., 2012), have more negative charges on their surfaces than comparable proteins from non-halophiles. Structural studies have also revealed that increasing solvation for halophilic enzymes makes them capable of performing their activities at (or below) 0.755 water activity (Karan et al., 2012).

Indeed, some enzyme systems remain functional close to a water-activity value of zero (from microbes as well as other organisms; e.g. Dunn and Daniel, 2004; Kurkal et al., 2005; Lopez et al., 2010). Indeed, it is the norm in biology that different components of the cellular or organismal system exhibit optimal performance, and have different maximum and minima for function, in relation to water activity and other biophysical parameters (Hallsworth, 2018). The increase of hydrogen bonds between protein-surface acidic amino acids and water molecules, and the reduction of hydrophobic patches, enhance the enzyme structure and function at high salt concentrations (Dym et al., 1995; Britton et al.,...
The dynamics of protein folding determined through incoherent neutron spectroscopy of the extreme halophile *Halobacterium salinarum* demonstrated that a large proportion of the proteins in the proteome displays a strong dependency on high salt concentrations, with cellular vitality becoming compromised at K⁺ concentrations below 2.2 M, with many proteins misfolding, precipitating and forming aggregates (Vauclare *et al.*, 2015).

In general, the acidity of their proteins correlates with the salinity of the environment in which halophiles live. For example, the genomes of the extreme halophiles *Acetohalobium arabaticum* and *S. ruber* exhibit extremely acidic proteomes (DasSarma and DasSarma, 2015). In general, acidic proteomes are found in salt-in strategists, and the most-extreme halophiles typically use the salt-in strategy, which is less energy expensive and correlated with a more acidic proteome. Highly halotolerant and moderate halophiles (e.g. representatives of *Halomonadaceae* family) are salt-out strategists and usually have a less acidic proteome (Coquelle *et al.*, 2010; Schwibbert *et al.*, 2011; Oren, 2013). For salt-in halophiles, the presence of acidic amino acids at protein surfaces correlates with cellular tolerance to salinity. This enables the use of mutations in genes which encode protein surface amino acids, resulting in the conversion of a halophilic enzyme into a non-halophilic one, demonstrated empirically with the DNA ligase of *Haloferax volcanii* (Tadeo *et al.*, 2009), *Halobacterium* diphosphate kinase (Ishibashi *et al.*, 2012), protL from *Streptococcus magnus* (Qvist *et al.*, 2012), and more recently in the protein β-trefoil (Longo *et al.*, 2015). In conclusion, the ability of halophiles to function in conditions with moderately low water-activities (e.g. ~0.755) is based on the evolution of enzymes that are sufficiently stable, and work optimally, in these conditions. Such hyper-stability has evolved through specific modifications of the surface of the enzymes to establish stable hydrogen bonds with water molecules and reduce the exposed hydrophobic patches (Winter *et al.*, 2009).

At saturation, NaCl is permissive for the activity of extracellular enzyme systems of extreme halophiles, as evidenced by the functional diversity of enzymes observed in hypersaline environments and the known adaptations of these proteins (Ferrer *et al.*, 2005; Borin *et al.*, 2009; Ferrer *et al.*, 2012; Pachiadaki *et al.*, 2014). For instance, an extracellular protease from a saltern bacterium was found to be dependant on NaCl for its functionality, and exhibited optimum catalytic activity at 4.5 M NaCl (Vidyasagar *et al.*, 2009). Another protease, from *Haloferax lucentense* (formerly *Haloferax lucentensis*), exhibited a similar NaCl optimum, and also retained high activity at NaCl saturation (Manikandan *et al.*, 2009); *Halobacterium* strains are also known to produce extracellular proteases which function optimally close to NaCl saturation (Norberg and Hofsten, 1968). It is no surprise that halophilic communities have been recognized as a potential source for industrially important enzymes which are able to function
under various extremes (Ferrer et al., 2005; Kumar et al., 2012; Alsafadi and Paradisi, 2013; Moreno et al., 2013).

The genes for these highly adapted proteins do not appear to be isolated to individual strains and species. Genome sequencing has revealed high levels of horizontal gene transfer occur between members of natural consortia of halophilic Archaea (DeMaere, et al., 2013), and the transfer of large (310-530 kb) DNA segments between distinct species has been analysed in the laboratory (Naor et al., 2012). Recently, for example, a membrane vesicle-mediated DNA-transfer mechanism has been discovered in Halorubrum lacusprofundi (Erdmann et al., 2017). This means that genes for advantageous halophilic proteins do not need to evolve by the relatively slow process of mutation and selection, in situ, but can spread readily between diverse members of a microbial community.

**Other adaptations of halophiles**

In addition to osmoadaptive strategy, other survival mechanisms are featured by extreme halophiles. For example, sequestration of resources via intracellular storage improves competitive ability and enables tolerance of prolonged periods without carbon substrate, phosphate, etc (Cray et al., 2013a; Oren and Hallsworth, 2014). Some members of the Halobacteria store polyhydroxyalkanoates in the form of intracellular granules (Saponetti et al., 2011), while Dunaliella species can accumulate triacylglycerols as an overflow product of photosynthesis (Chen et al., 2011). Extreme halophiles can also have adaptations which enhance the generation of cell-available energy such as light-activated proton pumps, that use light to generate a proton motive force, and have been found in species such as S. ruber and Hqr. walsbyi (Balashov et al., 2005; Cray et al., 2013a). Some species such as Hqr. walsbyi and Hfx. mediterranei utilize gas vesicles, which facilitate buoyancy and may also maintain the horizontal orientation of the cell thereby optimizing light capture; this may also enhance oxygen acquisition by maintaining the cell’s position within the oxygenated regions of stratified brines (Englert et al., 1990; Bolhuis et al., 2006; Cray et al., 2013a, Oren and Hallsworth, 2014). Halophiles can be exposed to high levels of ultraviolet light, and some species such as D. salina, S. ruber and most haloarchaea synthesize and accumulate high concentrations of carotenoids, thereby protecting their macromolecular systems from ultraviolet radiation (Antón et al., 2002; Chen and Jiang, 2009). A number of recent studies have utilized (culture-independent) analytical techniques to identify metabolites of halophilic microbes, including mass spectrometry coupled with gas- or liquid chromatography and/or capillary electrophoresis (e.g. Kido Soule et al. 2015; Johnson et al., 2017). The comprehensive power of these technologies should in future reveal as-yet-unidentified compounds or strategies underlying the cellular phenotype of extreme halophiles.
Evolutionary biology of halophiles; implications of the finite solubility of NaCl

For all types of environments (saline or not), habitability by microbes is constrained or prevented by one or more factors; nutritional, physicochemical, and/or biophysical. Environmental parameters – including temperature, water activity, irradiance, ultraviolet radiation, pH, chaotropicity, and availability of electron donors or nitrogen substrates – determine biodiversity, abundance, overall biomass, and ecosystem function. To put it simply, ecosystems (and ultimately, the entire biosphere) are restricted and shaped by available resources and prevailing conditions. And yet, there are few microbial ecosystems in aquatic environments which appear to outperform NaCl-rich microbial habitats. Just as tropical rainforest is generally considered the pinnacle of biomass and biodiversity when compared with the other plant biomes (Pan et al., 2013), the halophile communities of solar salterns and deep-sea brines may represent the greatest attainment of aquatic ecosystems in relation to collective microbial metabolism and cell densities. By contrast, in ethanol-producing fermentations, the microbial community is biomass- and diversity-limited by the chaotropicity of ethanol, low pH, and low water-activity (Cray et al., 2015). Whereas NaCl-saturated environments clearly select for halophiles, the growth rates, cell densities and microbial abundance of these inhabitants are remarkably high (Table 1, Fig. 2a-e). This is because the NaCl concentration-limit is not determined by biology (as discussed above, 5 M NaCl is not the theoretical maximum concentration at which the cellular systems of extreme halophiles can function), but by a quirk of chemistry. The dipoles of water molecules attract ions more than Na and Cl ions attract each other; and these ions attract water more than water molecules attract each other, hence the kosmotropicity of NaCl (Cray et al., 2013b; Alves et al., 2015). At the saturation point, however, most of the water is interacting with these ions so it becomes more thermodynamically favourable for additional ions to bond with each other; i.e. precipitation of NaCl occurs (Fig. 2f).

The empirically determined and theoretical water-activity limits for fungal cell division or metabolism and those for growth limits for halophilic prokaryotes (0.585 and 0.565, and 0.635 and 0.611 respectively) are equivalent to a theoretical NaCl concentration of up to 8 M (equivalent to 47%, w/v). This is consistent with the thesis that 5 M NaCl provides mid-range (i.e. moderate) conditions for numerous types of halophile (Fig. 2a-e; Stevenson et al., 2015a). Whereas specific temperatures or ethanol or MgCl₂ concentrations act as barriers to life (see above), there is no threshold value at which NaCl prevents microbial habitation (Figs. 1a; 1b; 2). For extreme halophiles, therefore, there is considerable thermodynamic leeway before the theoretical failure of the cellular system is reached.

Whereas the finite solubility of NaCl does not curtail ecosystem development, the biology of some saturated brines may be constrained by the interactive effects of other limiting factors. These include nutrient deficiency (e.g. scarcity of phosphate in hypersaline brines can restrict the growth of...
phototrophs such as *Dunaliella*; Gasol et al., 2004; Oren, 2017); high pressure and extremes of pH or temperature (Harrison et al., 2013); high irradiance (Wieland and Kuhl, 2000); or high concentrations of chaotropic ions (see above) or toxic metals, such as arsenic, though there are some halophile communities that function in arsenic-containing alkaline brines (Oremland and Stolz, 2003; Oremland et al., 2005). Populations of *Bacteria* and *Archaea* can be constrained by the activities of viruses and/or grazers; for instance, bacteriophage-induced lysis and nanoflagellate grazing have been identified as key factors reducing prokaryote abundance in Spanish crystallizer ponds and Korean salterns, respectively (Guixa-Boixareu et al., 1996; Park et al., 2003; Cray et al., 2013a). The ecological importance of viruses within halophile communities was highlighted in a study which successfully assembled several viral genomes from the metagenome of crystallizer pond CR30 of Santa Pola salterns (Garcia-Heredia et al., 2012). On the basis of shared CRISPR spacer sequences, some of these viruses were found to prey upon *Hqr. walsbyi*. Those salterns with 3.6-5 M NaCl were populated primarily by members of the Euryarchaeota or Nanohaloarchaeota (making up to 80-90% of the community and mostly represented by *Hqr. walsbyi* and *Nanohaloarchaea*) and Bacteroidetes (9-10%; mainly *Salinibacter ruber*) (Garcia-Heredia et al., 2012), so their phage-associations further underscored that viruses are ubiquitous within, and central to the ecologies of, NaCl-saturated habitats.

Novel halophilic methanogenic Archaea have been isolated from the anaerobic sediments of alkaline hypersaline lakes, soda lakes and pH-neutral hypersaline lakes (Sorokin et al., 2017a). The candidate ‘Methanohaloarchaea’ (HMET) and ‘Methanonatronoarchaea’ (AMET) strains were capable of methylotrophic methanogenesis at slightly thermophilic (50-60°C) and either saturated NaCl (pH 7) or soda (pH 9.6) conditions, respectively. These strains belong to ‘Methanonatronoarchaeia’ (former SA1 clade), a distinct class-level deep phylogenetic group within suggest that a common halophilic, methanogenic ancestor might have diverged into ‘Methanphaloarchaeia’ (by gene loss; Sorokin et al., 2017a; Spang et al., 2017) and Halobacteria (by massive inter-domain gene transfer from aerobic bacteria; Nelson-Sathi et al., 2012; Nelson-Sathi et al., 2015; Sousa et al., 2016). Although the Halobacteria appear to be comprised primarily of aerobic and chemoorganoheterotrophs with halophilic lifestyle, a few are facultatively anaerobic (Hattori et al., 2016) or fermenting (Antunes et al., 2008). However, recent studies allude to greater metabolic capabilities within this group. For example, *Halanaeroarchaeum sulfurireducens* is a strictly anaerobic, sulfidogenic haloarchaeon that utilizes acetate and pyruvate while reducing elemental sulfur (Sorokin et al., 2016b, 2016c) whereas *Halodesulfurarchaeum formicicum* is the first evidenced lithotrophic Halobacteria member as it uses H₂ or formate as electron donor and reduced sulfur compounds (thiosulfate, dimethylsulfides and S⁰) as electron acceptors (Sorokin et al., 2017b).

The finite solubility of NaCl raises a number of questions in relation to the evolutionary biology of halophiles. In particular, it remains to be investigated how evolvable halophiles are in their natural environments. Because of the strong selective constraints imposed by the need of highly acidic proteins, the fixation rate of mutations in halophiles is expected to be low. In fact, the mutation rate itself of some halophiles, such as *Hfx. volcanii* is lower than other mesophilic microbes (Mackwan et al., 2007; Busch
and DiRuggiero, 2010). Since the fixation rate of mutations is much lower than the mutation rate, it is reasonable to assume a lower fixation rate of mutations for halophiles than for most other microbes. Mutation rates in thermophiles are also reported to be lower than in mesophiles (Friedmen et al., 2004; Kissling et al., 2013), and similar arguments have been proposed, i.e. that the functional constraints are much higher for thermophilic than for mesophilic proteins. The greater than expected genetic diversity seen in *Halobacteria*, such as the genera *Halorubrum* and *Haloarcula*, is produced mainly by horizontal gene transfer and homologous recombination (Mohan et al., 2014; DeMaere et al., 2013; Naor et al., 2012). This genome-level diversity could in principle generate a reservoir of genomic variants that can be maintained phenotypically cryptic in the population, but with enormous potential to originate adaptations to other environmental perturbations. In NaCl-saturated environments, selection becomes stronger against many halotolerant (and some halophilic) microbes allowing the persistence of only those genetic variants able to function optimally at 0.755 water activity. However, NaCl is not sufficiently soluble to reduce water-activity values to between 0.755 and 0.585; a range experienced by other extremophiles. Those salts which are sufficiently soluble (e.g. MgCl₂) are so chaotropic that the cellular systems of most halophiles are inhibited by their chaotropicity rather than reduced water activity. Even if genetic variants arose that could grow at water activities below 0.755, even to 0.585, they are not likely to be any fitter than existing species in natural hypersaline environments, and without positive selection are unlikely to maintain or increase their frequency. At the water activity for life, molecular crowding within the cytoplasm and/or inadequate hydration or flexibility of macromolecular systems may act to prevent cellular function (Fields, 2001; Zaccai, 2004; Miermont et al., 2013; Parry et al., 2014). It has yet to be demonstrated that such factors are also limiting at in NaCl-saturated brines at 0.755 water activity.

**Implications for astrobiology and concluding remarks**

The likelihood that early life was energized by Na⁺ electrochemical gradients has been discussed by Price et al. (2017) who revealed steep natural Na⁺ gradients (i.e., 4-52 mM Na⁺ in the venting liquids versus 468 mM Na in the surrounding sea water) across thin mineral layers (figuratively termed ‘membranes’) within the pores of serpentines located in shallow sea water. The cell-sized serpentine pores percolated by the alkaline, reducing (H₂- and CH₄-rich) fluids which contain Na⁺ (at concentrations one to two orders of magnitude less than in sea water) may have acted as incubators for the development of proto-cells on the bottom of the (slightly acidic) Hadean oceans (Sojo et al., 2016; Price et al., 2017).

There are very specific combinations/concentrations of NaCl-MgCl₂ or NaCl-MgCl₂-glycerol mixtures that permit the proliferation of halophiles below 0.755 water activity (Stevenson et al., 2015a,b). The highest concentrations of NaCl that can be experienced by the cells of halophiles, 5 M, enables optimum rates of metabolism and growth for some populations, and facilitates the development of biomass-dense and highly active ecosystems. Therefore, we conclude that the environments with salt concentrations of 5 M NaCl are not thermodynamically
limiting and, thus, this concentration does not represent a biophysical limit for life on Earth. Given the high cell densities and broad functional diversity of many hypersaline brine systems, it is intriguing to ask why their microbial diversity is not even higher. Can this be explained as a product of dominance by a small number of halophile weed species, or are there other factors at play? The solution chemistry of NaCl raises additional questions pertinent to the field of astrobiology:

i. why are there no truly psychrophilic haloarchaea?,

ii. whereas chaotropic brines may be more readily habitable at sub-zero temperatures (Ball and Hallsworth, 2015), would the evolutionary trajectories of terrestrial halophiles (which are not usually exposed to habitable brines with water activities much below 0.755) make them unable to function in chaotropic brines on Mars or Europa (Hallsworth et al., 2007; Chin et al., 2010; Rummel et al., 2014)?

iii. would the diverse solution chemistries found on different planetary bodies (e.g. Muñoz-Iglesias et al., 2013; Fox-Powell et al., 2016) result in qualitative differences in the evolution of terrestrial halophiles and their ability to tolerate multiple extremes not found on Earth, and could terrestrial, extreme halophiles tolerate the high ionic strength which characterizes some martian brines (Fox-Powell et al., 2016)?

iv. diverse NaCl stoichiometries have been reported at high pressures and temperatures (i.e. 200 000 atmospheres and 2 000 K), resulting in the formation of stable salts such as NaCl$_3$ (Zhang et al., 2013). Whereas such salts may only be formed during accretion and deep within a planetary or moon body, only very small amounts would be present at or near the surface. More importantly, they may not exist at high concentrations under conditions which are pertinent to (known forms of) life. Nevertheless, this finding raises the question: do sodium chlorides, that are more soluble than NaCl, reduce water activity to a lower value and exhibit chaotropic activity?, and

v. given that saturated NaCl is not stressful for some halophile systems, could these species act as pioneers in the event that NaCl-rich sites on other planetary bodies are contaminated during space exploration missions?

The evolution of life on early Earth is thought to have taken place in extreme aqueous environments such as hydrothermal vents and acid-brine oceans. Hypersaline brines have remained a stable habitat throughout the planet’s geological history (Warren, 2010). Buick and Dunlop (1990) describe evaporite deposits from 3.5 Ga Warrawoona Group of Western Australia, proving the presence of brine surface environments at the time of some of the first life on Earth. Diverse lines of evidence suggest that terrestrial life may inhabited, and could have even arisen, at high-NaCl, KCl, and/or MgCl$_2$ concentration (e.g. Dundas, 1998; Stevenson et al., 2015b; Matveev, 2017). Water activity and chaotropicity are likely to have been key determinants for both habitability and the evolution of early cells in relation to early life on Earth (Stevenson et al., 2015b).
In relation to astrobiology, the occurrence of halophily throughout the phylogenetic tree suggests that it is relatively easy to evolve and evolved more than once. If life evolved in locations beyond Earth, and where salty brines occur, then there would almost certainly be the evolution of halophilic organisms (Mancinelli, 2005a;b). Strong evidence exists for the past existence of acid brines on Mars (Squyres et al., 2004; Benison and Bowen, 2006). Mars hosts abundant saline minerals, including chlorides (likely halite) and hydrated calcium sulfates (likely gypsum) in lake deposits and lake-derived eolian deposits (Glotch et al., 2010). The interpreted acid and brine nature of past Mars surface waters have led to speculation that Mars would be inhospitable to life (Tosca et al., 2008; Rummel et al., 2014). However, in light of the findings of this study, halophilies may potentially be able to evolve the ability to proliferate at, or below, 0.585 water activity and so the possibility that such microbes may potentially contaminate saline milieu present on Mars in this water activity range should not be discounted. The findings reported in the current article also expose a need for new experimental and theoretical approaches, which circumvent the apparent limits for life which are imposed merely by solution chemistry, to identify and characterize the thermodynamic constraints of halophilic life.

Funding

This work was supported by Natural Environment Research Council (NERC), United Kingdom (grant number NE/E016804/1).

Acknowledgements

Useful discussion was provided by Josefa Antón (University of Alicante, Spain), Philip Ball (East Dulwich, UK), Catherine Conley (NASA HQ, USA), Terry J. McGenity (University of Essex, UK), Beatriz Sabater-Muñoz (Instituto de Biología Molecular y Celular de Plantas [CSIC-UPV], Spain), Sohini Banerjee (Visvabharati University, India), R. Thane Papke (University of Connecticut, CT, USA), and John D. Rummel (SETI Institute, USA).

References


Matveev, V.V. (2017) Comparison of fundamental physical properties of the model cells (protocells) and the living cells reveals the need in protophysiology. Astrobiology 16: 97–104.


Fig. 1. The most extreme values thus far reported for microbial cell division, based on empirical data (black values); microbial-cell metabolism, based on empirical data (grey values, normal font); and microbial cell division, based on theoretically determined limits (grey values, italics) in relation to: (a and...
(b) low water-activity (turquoise scale-bars), (c) sub-zero temperatures (denoted using a blue scale-bar), and (b) ethanol concentration (pink scale-bar). The scale bars span -2.5 to -40°C, 12.5 to 20% w/v ethanol, 0.710 to 0.560 water activity, and 0.950 to 0.560 water activity, respectively. Red boxes indicate the empirically determined limits for microbial cell division of the most extremely stress tolerant microbial system in relation to each stress. For (a), the microbial systems are: (i) Aspergillus penicillioides JH06THJ, (ii) Xeromyces bisporus FRR 0025, (iii) A. penicillioides JH06THJ, (iv) Xerochromium xerophilum FRR 0530, (v) halarchaeal strain GN-5, (vi) haloarchaeal strain GN-2, (vii) Aspergillus echinulatus, (viii) Zygosaccharomyces rouxii (ix) Halorhodospira halophila DSM 244T (x) Eurotium halophilicum FRR 2471, (xi) X. bisporus FRR 3443, (xii) X. bisporus FRR 1522 (xiii) X. bisporus FRR 2347 (xiv) Halorhabdus utahensis DSM 12940T (xv) Eurotium amstelodami FRR 2792 and FRR 0475, (xvi) Eurotium chevalieri JH06THI, (xvii) Halobacterium strain 004.1, (xviii) X. xerophilum CBS 153.67T, (xix) Actinopolyaspera halophila ATCC 27976T, (xx) Eurotium repens JH06JPD, (xxi) A. penicillioides, (xxii) Halanaerobium lacusrosei DSM 10165T, (xxiii) Halorhodospira halochloris 0.680, (xxiv) mixed halophile community, (xxv) Bettsia fastidia FRR 77, (xxvi) Natrinema pallidum NCIMB 777T, (xxvii) Halobacterium noricense DSM 15987T, (xxviii) Wallemia sebi FRR 1473, (xxix) Halococcus saltitodinae DSM 13046, (xxx) Eurotium rubrum FRR 0326, (xxxi) Polyphaecilium pisce, (xxxii) Eurotium repens FRR 382, (xxxiii) X. xerophilum FRR 3921, (xxxiv) Aspergillus conicus, (xxxv) A. penicillioides FRR 3722, (xxxvi) Eremascus albus (Williams and Hallsworth, 2009; Pitt et al., 2013; Stevenson et al., 2015a; 2017b; von Schelhorn, 1950). For (b), the limit of NaCl solubility is indicated (at 0.755 water activity) and the theoretical solubility limit of NaCl is indicated at the 0.585 water-activity limit for microbial growth; see (a). For (c), the microbial systems are: (i) Pleosporium chlorophanum, (ii) permafrost microbes including Leucosporidium and Geomyces, (iii) Paenisorosporicarina, (iv) Chryseobacterium sp. V3519-10, (v) Nitrosomonas cryotolerans, (vi) Planococcus halocryophilus Or1 (vii) Cladonia foliacea, (viii) Colwellia psychrerythraea, (ix) permafrost microbes, (x) permafrost microbes, (xi) Rhodotorula glutinis, (xii) permafrost microbes, (xiii) Psychrobacter frigidicola, (xiv) Umbilicaria aprina, (xv) Planococcus halocryophilus, (xvi) Colwellia psychrerythraea, (xvii) genetically modified Escherichia coli, (xviii) Psychromonas ingrahamii, (xix) Psychrobacter urativorans, (xx) permafrost microbes, (xxi) Nitzschia frigida, (xxii) Mrakia frigida, (xxiii) Mrakia nivalis, (xxiv) Thalassosira antarctica, (xxv) Chlamydomonas sp., (xxvi) Thylphila shikariensis, (xxvii) Thylphila incarnata, (xxviii) Thylphila phacorhiza, (xxix) Methanogenium frigidum, (xxx) Humicola marvinii, and (xxxi) Methanococoides burtonii (Lange and Bertsch, 1965; Schroeter et al., 1994; Chin et al. 2010; Rummel et al., 2014). For (d), the microbial systems are: (i) Saccharomyces cerevisiae CCY 21-4-13, (ii) S. cerevisiae NCYC 1327, (iii) S. cerevisiae, (iv) S. cerevisiae (shochu strain), (v) S. cerevisiae IFO 2347, (vi) S. cerevisiae (baker’s strain), (vii) S. cerevisiae, (viii) S. cerevisiae (strains PE-2 and CA1185), (ix) S. cerevisiae (brewer’s strain), (x) S. cerevisiae (brewer strain), (xi) S. cerevisiae (brewer’s strain), (xii) S. cerevisiae (BAW 6), (xiii) S. cerevisiae, and (xiv) Zymomonas mobilis (Hallsworth, 1998; Cray et al., 2015).
Fig. 2. In relation to the water activity of saturated NaCl, i.e. 0.755 (pink dashed line), (a-e) plots are shown of doubling times, dry mass, or optical density measurements - i.e. turbidity - for extreme halophiles and (f) salt concentrations for NaCl (blue line) and MgCl₂ (black line; Hallsworth et al., 2007 at
25°C). Growth curves for (a) the bacterium *Halorhodospira halophila* (strain DSM 244^T^, cultured in a defined medium supplemented with NaCl), (b) the bacterium *Actinopolyspora halophila* (strain ATCC 27976^T^, cultured in a complex medium supplemented with NaCl at 37°C), (c) the archaeon *Halorhabdus utahensis* (strain DSM 12940^T^ in a defined medium supplemented with NaCl at 30°C), (d) Haloarchaeal strains GN-2 and GN-5 shown in red and black, respectively (cultured in bitter brines supplemented with peptone at 37°C) and (e) the archaeon *Halobacterium* strain 004.1 (in a synthetic seawater medium supplemented with NaCl, MgCl\_2, Na_2SO_4 and KCl) were reproduced from Stevenson *et al.* (2015a); original data were obtained from Javor (1984); Yoshida *et al.* (1991); Wainø *et al.* (2000); and Deole *et al.* (2013). Extrapolations of growth curves (dotted lines) were carried out using Excel or Sigmaplot as described by Stevenson *et al.* (2015a). Plots of water activity versus salts concentrations (f) were modified from Hallsworth *et al.* (2007). The water-activity versus concentration curve for NaCl was extrapolated using polynomial order 2 in Excel (blue dotted line).

### Table 1. Microbiology of diverse, NaCl-saturated habitats

<table>
<thead>
<tr>
<th>Habitat</th>
<th>Microbial biomass</th>
<th>Phylogenetic diversity</th>
<th>Typical members of the community</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal lizer ponds of marine solar salterns</td>
<td>Compared with most other aquatic microbial habitats, NaCl-saturated solar salterns are biomass-rich. Even at ~5 M NaCl, the high cell-density of halophilic microbes, and the high intracellular concentrations of their pigments, results in highly coloured turbid brines (Oren, 2009). Cell densities are typically in the range 10^6 to 10^8 cells ml(^{-1}) (Guixa-Boixareu <em>et al.</em>, 1996; Çinar and Mutlu, 2016), which is orders of magnitude higher than those observed in a study of coral atolls in the Marine, NaCl-saturated solar salterns are characterized by an exceptionally high microbial diversity, and contain taxa from each domain of life as well as diverse types of viruses. One study, which used both culture-based and culture-independent methods (Burns <em>et al.</em>, 2004) revealed that an individual solar saltern (NaCl-saturated, coast of Australia) contained 57-66 identifiable types of Archaea and Bacteria. A 16S rRNA gene clone-library study of a</td>
<td>Archaeal genera commonly present include <em>Haloarcula</em>, <em>Halobacterium</em>, <em>Halobaculum</em>, <em>Halococcus</em>, <em>Halofex</em>, <em>Halobacterium</em>, <em>Halorubrum</em>, and <em>Halococcus</em> and <em>Haloquadratum</em>, and bacterial genera include <em>Halomonas</em>, <em>Halovibrio</em>, <em>Salicola</em>, and <em>Salinibacter</em> spp. (Manikandan <em>et al.</em>, 2009; Sabet <em>et al.</em> 2009; Cray <em>et al.</em>, 2013a).</td>
<td></td>
</tr>
</tbody>
</table>
Northern Line Islands (Dinsdale et al., 2008). Indeed, the cell densities of hypersaline crystallizer ponds can also surpass, or are comparable to, those of other fertile anthropogenic systems such as aquaculture waters, fishponds, and even ethanol-producing fermentations (Cocolin et al., 2000; Herrero et al., 2006; Kalcheva et al., 2008; Taniasuri et al., 2016; Wada et al., 2016).

Halite rock is commonly found in terrestrial surface- and subsurface environments, and, under specific conditions, deliquesces to produce hypersaline NaCl-brines (Rummel et al., 2014). These brines, typically exposed to the air, are commonly inhabited by microbes. Cell densities reported for liquids-of-deliquescence on halite rocks of the Atacama Desert in the range 2-10 \times 10^7 \text{ cells g}^{-1}, a Tunisian saltern (S5 pond, 31% total salinity), carried out by Baati et al. (2008), revealed eight operational taxonomic units (OTUs) pertaining to Bacteria (Bacteroidetes and Proteobacteria) and 39 OTUs assigned to Euryarchaeota (Halobacteria). For prokaryotes from this saltern, the Shannon Index (Spellerberg and Fedor, 2003) was 0.98 for Bacteria and 3.04 for Archaea (Baati et al., 2008). Another study retrieved 86 isolates of 27 fungal species from the waters of the Cabo Rojo salterns on the coast of Puerto Rico (Cantrell et al., 2006), and a NaCl-saturated crystallizer pond (Spanish coast), using a fosmid metagenome library, revealed the presence of 42 viral genomes (Garcia-Heredia et al., 2012).

The phylogenetically diverse communities present include microbes from each domain of life, from photosynthetic Bacteria and algae to heterotrophic Archaea and Bacteria, including both aerobes and the anaerobic fermentative bacteria of Halanaerobiales (Robinson et al., 2016). The most common algae are Dunaliella spp., which are also the most xerophilic of the microalgae (Cifuentes et al., 2001; Oren, 2014; Stevenson et al., 2015a). The nanoflagellates Euplaesiobystra, Halocafeteria and Pleurostomum spp. are the most xerophilic protists known, and have also been found inhabiting NaCl-saturated salterns (Park et al., 2009; Stevenson et al., 2015a). Amongst the microbes present in marine solar salterns, there are several species which recurrently dominate halophile communities, most notably Dunaliella salina, Salinibacter ruber, and Haloquadratum walsbyi (Cray et al., 2013a; Oren and Hallsworth, 2014).

Microbial taxa found in liquids-of-deliquescence include all domains of life but archaeal species are typically the most prevalent (Robinson et al., 2015). Of the DNA extracted and analyzed, 71% of metagenomic reads were assigned to Archaea (the majority of which were Halobacteria), 27% to Bacteria.
microbial biomass which is three orders of magnitude greater than that of the surrounding soil (Robinson et al., 2015). The microbial diversity of liquids-of-deliqescence can be extraordinarily high, the brine communities on halite of the Atacama Desert have Shannon Indices as high as 6.25, which is in excess of those usually associated with soils (Fierer and Jackson, 2006). At least 30 distinct viruses were identified within the cellular biomass found at one sample site; most of these are thought to occur within cells of the Halobacteria (Crits-Christoph et al., 2016)

Deep hypersaline anoxic basins (DHABs) NaCl-saturated, deep-sea brine lakes are characterized by a considerable, highly active biomass (Daffonchio et al., 2006). The halocline at the interface of the deep-sea Lake Bannock (located beneath the Mediterranean Sea) and the open ocean has a NaCl-concentration ranging from that of the overlying sea water (690 mM), where there are 4.32 x 10⁴ cells ml⁻¹, to approximately 4.5 M which has 4.39 x 10⁵ cells ml⁻¹ (Daffonchio et al., 2006). There is, therefore, an order-of-magnitude increase in microbial biomass as NaCl concentration increases, a phenomenon reported within other deep-sea-basin systems, such as the NaCl-saturated Lake L’Atalante (Mediterranean al., 2015; Crits-Christoph et al., 2016). The most prevalent Archaea were, in descending order, Natronomonas, Halococcus, Haloarcula, Halorhabdus, Salinarchaeum, Halobacterium, and Halomicrobium, and the most prevalent bacteria were Salinibacter. Cyanobacteria of the genera Chroococcidiopsis and Halothece carry out the majority of primary production in halite ecosystems (Wierzchos et al., 2006; Robinson et al., 2015)

Insights into composition (and function) of microbial assemblages inhabiting the deep-sea brine lakes have been gained mostly by metagenomic analyses. The most common Archaea in the brine layer of the Mediterranean deep-sea hypersaline lakes are MSBL1 and Halophilic Cluster 1 (HC1) candidate divisions (Daffonchio et al., 2006; La Cono et al., 2011; Yakimov et al., 2013;). The vast majority of the bacteria inhabiting deep-sea brine lakes was assigned to Alpha-, Delta-, Epsilon-, and Gammaproteobacteria and
Inland salt-lakes (Sea) where a cell number of 1.07 x 10^5 ml^{-1} has been recorded (i.e. more than that for the overlying sea water which has 3.57 x 10^4 ml^{-1}) (Yakimov et al., 2007)

Of the large-scale water bodies located inland, it is estimated that 45% are saline (Wetzel, 2001), a value which is likely to rise due to global warming (Williams, 2002). Lake Kasin, located in Russia, is a shallow, NaCl-saturated water body containing sufficient iron to support specialist, iron-metabolizing microbes. Fe(II) ions are weak electron donors compared with organic substrates for instance, and can limit the efficiency with which cell-available energy is generated, yet living at 5 M NaCl imposes a high energetic cost to the cell. Nevertheless, the NaCl-saturated sediment of Lake Kasin contains ≥ 1 x 10^4 iron-metabolizing microbial cells g^{-1} (and 1.1 x10^6 to 6.7 x 10^7 total microbial cells g^{-1}) (Emmerich et al., 2012). Lake Chaka which is located at 3200 m above sea-level on the Tibetan Plateau, is 2-3 cm deep and NaCl-saturated. Despite sequences corresponding to Archaea and bacteria (in a ratio of more or less 1:1) in Lake Thetis (Ferrer et al., 2012). Methane oxidizers, methylothrophs, sulfate reducers, sulfur oxidizers, and/or microaerophiles are found in Lakes L’Atlante, Bannock, and Thetis (Daffonchio et al., 2006; Yakimov et al., 2007; La Cono et al., 2011)

Both Lakes Chaka and Kasin are dominated by Archaea belonging to Halobacteria. Whereas the genera Haloarcula and Halosimplex prevailed in Lake Chaka, Halobaculum spp. seemed to be abundant in Lake Kasin (Jiang et al., 2006; Emmerich et al., 2012). The dominant bacterial classes in Lake Kasin were Bacilli and Clostridia, with Halothiobacillus as the most common genus (Jiang et al., 2006). Common bacteria in Lake Chaka include Salinibacter ruber, and those KB1 candidate group (Yakimov et al., 2007; 2013; La Cono et al., 2011; Pachiaiaki et al., 2014). Bacteria from the Desulfobulbaceae and Sphingobacteria have been identified in Lake Bannock (Daffonchio et al., 2006). The most common protistan genera in Lake Thetis include Pleuronema, Strombidium and Trinyma, while the predominant fungal RNA gene sequences are closely related to those of the genera Aspergillus, Cladosporium, Malassezia and Rhodotorula (Stock et al., 2012; Bernhard et al., 2014)
the high altitude and relative isolation from other high-NaCl habitats, the Lake Chaka brine contains $4.8 \times 10^6$ cells ml$^{-1}$ and the lake sediment contains $4.2 \times 10^7$ cells ml$^{-1}$ (Jiang et al., 2006). The NaCl-saturated brine of Aran-Bidgol Lake (Iran) has been shown to contain $3.4 \times 10^2$ cells ml$^{-1}$ (Makhdoumi-Kakhki et al., 2012). Numerous salt lakes with appreciable depths (> 10 m) were reported in Central Romania (Baricz et al., 2014; Baricz et al., 2015; Andrei et al., 2015; Andrei et al., 2016). These permanently stratified bodies of water feature steady physicochemical gradients resulting in sharp niche partitioning and distinctive microbial communities, respectively. As a general characteristic, a less saline (6-20% total salinity) surface layer overlies a NaCl-saturated bottom stratum in such lakes. Estimation of prokaryotic cell densities by qPCR and DAPI indicated values ranging between $10^5$ - $10^6$ cells ml$^{-1}$ for Bacteria and $10^7$-$10^8$ cells ml$^{-1}$ for Archaea, with the densest microbial population recorded in most saline lakes (Andrei et al., 2015; Andrei et al., 2016). The concentration and composition of salts within large-scale inland bodies of water, including the Dead Sea (Middle East) and the Great Salt Lake (Utah, USA) can vary. The Dead Sea contains relatively high levels of chaotropic MgCl$_2$ (Hallsworth et al., 2007), but is nevertheless characterized communities within, and between, the lakes (Mesbah et al., 2007). High microbial diversity is also prevalent in Lake Aran-Bidgol which has been found to contain 37 OTUs corresponding to Archaea and Bacteria; 63% of these were unrelated to any taxa thus far identified (Makhdoumi-Kakhki et al., 2012). Andrei et al. (2015) reported an increasing prokaryotic diversity along the salinity gradient in two meromictic salt lakes of Central Romania. Highest OTU numbers were retrieved from the NaCl-saturated layers of Fara Fund (144 archaeal and 330 bacterial OTUs) and Ursu lakes (116 archaeal and 459 bacterial OTUs). Phototrophs (cyanobacteria and Dunaliella sp.) were also found in these stratified lakes (Máthé et al., 2014; Andrei et al., 2015).

One hundred genera of Archaea and Bacteria were isolated from the NaCl-saturated northern arm of the Great Salt Lake (Weimer et al., 2009). An analysis of 16S rRNA gene clone libraries recovered from brine samples taken from Lake Kasin identified 290 sequences for bacteria and 231 sequences within the genera Bacillus and Halanaerobium (Emmerich et al., 2012). In the Wadi An Natrun Valley, members of the bacterial genus Haloarchaea dominated six hypersaline alkaline lakes, and other prevalent microbes within these lakes included the archaeal genus Halobacterium, the prokobacterium Chromatium, and the cyanobacterial genera Spirulina and Synechococcus (Imhoff et al., 1978). This has been corroborated by a metagenomic study which also detected members of the bacterial genera Rhodobaca and Roseinatronobacter and the cyanobacterial genus Euhalothecce (Mesbah et al., 2007). The study on permanently stratified salt lakes in Romania pointed to clear niche-based differentiation of microbial communities inhabiting the water column. The aphytic, anoxic and hypersaline zone of investigated lakes (~35% total salinity), Bacteria dominated the microbial communities, being represented by anaerobes classified within the genera Desulfovermiculus, Acetohalobium, and Halanaerobium. In such
Fluid inclusions within individual NaCl- evaporite crystals by high levels of microbial biomass during periodic blooming events (Oren, 1985; Oren and Gurevich, 1994). Biomass-rich microbial communities are also active in the Great Salt Lake, even when NaCl concentrations approach saturation (Post, 1980).

Each year, many 100s millions tons NaCl crystals precipitate from evaporating brines located in both natural halophile habitats and industrial salterns. Halophiles are readily entrapped during crystal precipitation and remain in NaCl-saturated brine inclusions within the crystal (Norton and Grant, 1988). The concentrations of cells in these fluid inclusions can be equal to or greater than those found in the evaporate brines from which they came; and can exceed 4 x 10^8 cells g^-1 (Norton and Grant, 1988).

A culture-independent study by Baati et al., (2010) identified 21 bacterial and 44 archaeal OTUs in a sample taken from the fluid inclusions of NaCl crystals from the benthic surface of a solar saltern in Sfax, Tunisia (on the Mediterranean coast).

Microbes from each domain of life have been found within hypersaline inclusions of NaCl crystals, including Dunaliella spp., Archaea and Bacteria (Conner and Benison, 2013; Lowenstein et al., 2011)

for Euryarchaeota and other Archaea (Emmerich et al., 2012)

conditions, Archaea were less abundant and mostly represented by Halobacteria (Halorhabdus sp. and Halorubrum sp.). It should be noted that substantial number of reads retrieved in this study related to candidate OP1 and OD1 bacterial clades (Andrei et al., 2015). The most common eukaryotic species in many inland salt lakes is Dunaliella salina, among other Dunaliella species (Imhoff et al., 1978; Weimer et al., 2009).

It may be that any of the microbes which inhabit supersaturated saline systems could potentially become entrapped within NaCl crystals. Amongst the taxa thus far identified within fluid inclusions of NaCl crystals are Dunaliella salina, Archaea such as Haloarcula, Halobacterium, Haloferax, Halonotius, Halorubrum, Haloquadratum, and Natronococcus, and the bacteria Salinibacter and Halobacillus (Norton and Grant 1988; Mormile et al., 2003; Conner and Benison, 2013; Henriet et al., 2014)
Footnote a. In some environments, these habitats/communities may occur simultaneously in the same location.

The NaCl concentration of hypersaline alkaline lakes can reach up to 5 M NaCl (e.g., in Wadi An Natrun) whereas it is 0.5-1 M in soda lakes, that contain more carbonates (approximately 2 M Na₂CO₃ and NaHCO₃ in total).

This may be due to the albedo effect that can influence how much solar radiation is retained by shallow waters, according the colour of the underlying sediment. If the water is on a white salt, for example, the light is readily reflected by the sediment. For waters that are underlain by darker materials, such as a black mud or dark microbial biomass, more light is absorbed and so is retained within the water body. Abundant chemical precipitation of minerals can also make water turbid. For example, if one salt lake is precipitating clay minerals or iron oxides (which happens in Western Australian acid brine lakes), then the water gets cloudy temporarily, so light would not penetrate the water column as well.

It should be noted that some stresses experienced by, and cellular adaptations of, halophiles are not unique to these microbes. Indeed, cellular stress is the norm for all microbial systems and many sources of stress can induce the stress via the same mechanism regardless of microbial species or habitat type (Hallsworth, 2018).