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Minireview

Multiplication of microbes below 0.690 water activity: implications for terrestrial and extraterrestrial life

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Summary

Since a key requirement of known life forms is available water (water activity; a_w), recent searches for signatures of past life in terrestrial and extraterrestrial environments have targeted places known to have contained significant quantities of biologically available water. However, early life on Earth inhabited high-salt environments, suggesting an ability to withstand low water-activity. The lower limit of water activity that enables cell division appears to be ~0.605 which, until now, was only known to be exhibited by a single eukaryote, the sugar-tolerant, fungal xerophile *Xeromyces bisporus*. The first forms of life on Earth were, though, prokaryotic. Recent evidence now indicates that some halophilic Archaea and Bacteria have water-activity limits more or less equal to those of *X. bisporus*. We discuss water activity in relation to the limits of Earth's present-day biosphere; the possibility of microbial multiplication by utilizing water from thin, aqueous films or non-liquid sources; whether prokaryotes were the first organisms able to multiply close to the 0.605-a_w limit; and whether extraterrestrial aqueous milieux of ≥ 0.605 a_w can resemble fertile microbial habitats found on Earth.

Introduction

Given the fact that water is one of the principal ingredients of cellular life (Daniel *et al.*, 2004), insights into the minimum water requirements of cells are imperative to understanding both the functionality of living systems (at every level, from biomacromolecule to biosphere) and the origins of life itself. The generally held opinion is that life appeared independently on Earth and, possibly, elsewhere in the Solar System (Clancy *et al.*, 2005), though one other explanation for the presence of life on Earth is that it appeared on another planet and was transported here in the form of prokaryotes or their ancestors (an idea known as panspermia; Thomson, 1871). Until recently, eukaryotic microbes have held the record for life under water-constrained conditions, as some species are

capable of cell division down to a water activity (a_w)[1] of 0.605 at high sugar concentrations (Pitt and Christian, 1968; Williams and Hallsworth, 2009). Whereas such data have formed the basis for international policy for planetary protection in relation to space exploration missions (see below), sugar-rich substrates have very limited applicability to those extraterrestrial habitats with which we are familiar. Historically, the accepted limit for cell division of prokaryotic microbes has been 0.755 a_w (for a small fraction of halophilic species at high salt concentrations, see Grant, 2004). However, both culture-based and culture-independent studies provide evidence for multiplication and metabolic activity of halophilic Archaea and Bacteria down to 0.611 a_w, both in their natural habitats *in situ* and *in vitro* (Javor, 1984; Yakimov *et al*., 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted).[2] Whereas the vast majority of yeasts and fungi are active somewhere within the range 1 to 0.900 a_w (or within a segment of this range; for examples, see Brown, 1976; Hallsworth and Magan, 1994; Kashangura *et al*., 2006), only ~12 species have been observed to grow and/or germinate at < 0.700 a_w (Williams and Hallsworth, 2009; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). Here, we discuss the evidence for microbial activity in habitats at or below 0.690 a_w which represent the very fringe of the functional biosphere on Earth. Low water-activity environments are also discussed in relation to early life on Earth, the plausibility of cell division in extraterrestrial environments which contain biologically available water and a series of unanswered scientific questions.

Water activity at the fringes of the microbial biosphere

The primary physical determinants of the habitable space on Earth are temperature and water activity; these parameters are also used to designate the 'special regions' of Mars in which microbial cell division might feasibly take place (Beaty *et al*., 2006; Kminek *et al*., 2010).[3] The temperature window over which microbes are, collectively, capable of cell division (i.e. from −18°C to +122°C; Takai *et al*., 2008; Chin *et al*., 2010) spans ≤ 40% of the entire range of temperatures to which life forms on Earth can be exposed (i.e. from approximately −90°C to ≥ 250°C for some hydrothermal vents and the deep subsurface; Fig. 1A). By contrast, environmental water-activity values range from 1 to ~ 0, and the functional biosphere exists between 1 and ~ 0.60 a_w. Furthermore, most cellular systems of known life forms on Earth are only active within the range, or a segment of the range, 1 to 0.900 a_w (Fig. 1B; Brown, 1976; Grant, 2004); for example, there is a drop-off in the measurable activity of soil microbiota at < 0.890 a_w (Manzoni *et al*., 2012; Moyano *et al*., 2013; Stevenson and Hallsworth, 2014). However, metabolic activity and cell division has been reported below 0.900 a_w for a great number of xerotolerant/philic and halotolerant/philic microbes (Brown, 1976; Grant, 2004), and even below 0.755 a_w for both eukaryotic and prokaryotic species (Javor, 1984; Williams and Hallsworth, 2009; Yakimov *et al*., 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). Of the microbes known to multiply below 0.720, the majority (unlike *Xeromyces bisporus*) are not obligate osmophiles that are only

[1]Water activity, the mole fraction of water, is defined by an equation (water activity = vapour pressure of the solution/vapour pressure of the water) which is derived from Raoult's Law; this parameter and its derivation are discussed in detail by Brown (1990) and Grant (2004).

[2]This finding has implications for planetary protection in relation to the potential contamination of other planetary bodies with such halophilic prokaryotes sent as accidental passengers on spacecraft from Earth (see also Footnote 3).

[3]See also J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished. Planetary-protection policy, in relation to space missions, aims to protect those planets where spacecraft are landed, as well as Earth, from accidental contamination with non-native life forms (Kminek *et al*., 2010; 2014). Mars special regions have been defined according to the activities of the NASA Mars Exploration Program Analysis Group's (MEPAG)'s Special Regions-Scientific Analysis Group 1 (SR-SAG1) and the Committee on Space Research (COSPAR) which is part of the International Council for Science. Both these committees conservatively recommended 0.500 a_w as the limit beyond which no known terrestrial microorganism is capable of multiplication, implying that Martian environments with a water activity of >0.500 may potentially enable proliferation of xerophilic microbes if they happened to arrive as accidental passengers on spacecraft sent from Earth (Fig. 1; Beaty *et al*., 2006; Kminek *et al*., 2010). Thus, the safety margin used for planetary protection purposes in relation to water activity (i.e. approximately 0.100 a_w units below the established limit for microbial cell division) is more conservative than that used for temperature (i.e. approximately 10°C below the established temperature limit for cell division, and within the range for metabolic activity) (Fig. 1). A revised analysis of Mars special regions is currently underway by the MEPAG SR-SAG2 (J.D. Rummel *et al*., unpublished).

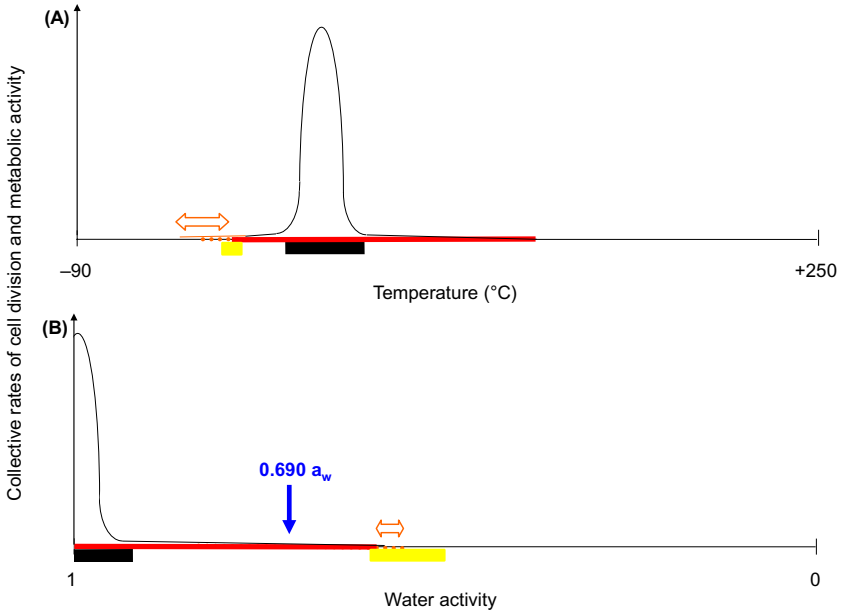

Fig. 1. Diagrammatic representation of collective activity (compound rates cell division and metabolic activity) for microbes on Earth in relation to prevailing environmental (A) temperatures and (B) water activities. Red bars indicate the known range for cell division of microbes (–18°C to +122°C, and 1 to 0.605 a_w), and orange dotted lines indicate for (A) the established limit for cellular metabolism (33°C), and (B) the suspected limit for physiological function of DNA (down to 0.530 a_w). The blue arrow indicates the water-activity value (0.690 a_w) below which the material of this review focuses on. Black bars indicate the range in which the overwhelming majority of microbial activity takes place, and curves represent collective biotic activity of microbes on Earth. Yellow bars indicate safety margins used for the designation of 'special regions' on Mars (down to –25°C and 0.500 a_w; Kminek *et al.*, 2010) in relation to international policy on planetary protection. Horizontal orange arrows indicate zones in which cell division may take place over extended timescales (tens to thousands years) though there is a paucity of data on this topic; this zone for temperature extends considerably below –33°C because of the possibility that chaotropic substances can enhance flexibility of macromolecular systems sufficiently to reduce the temperature minima for microbial activity by a further 10°C to 20°C (Chin *et al.*, 2010). Although the chao-kosmotropic activity of hydrazine, which is used as spacecraft fuel to launch back to Earth, has not been quantified to date, this antimicrobial substance (Kane and Williamson, 1983) has a number of chemical and behavioral properties of chaotropic compounds (Cray *et al.*, 2013b). In the event of a spacecraft collision, if both microbial cells and fuel were released into the Mars regolith, the hydrazine could potentially enhance macromolecular flexibility of cellular membranes, proteins, etc (Bhaganna *et al.*, 2010).

capable of inhabiting sugar-rich substrates. These include halophilic prokaryotes and xerophilic fungi such as *Aspergillus penicillioides* and *Eurotium herbariorum* (Samson and van der Lustgraaf, 1978; Williams and Hallsworth, 2009; Yakimov *et al.*, 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted).[4] Even for the most xerophilic microbes thus far characterized, rates of cell division typically decrease by an order of magnitude between 0.870 and 0.770 a_w, and by a further order of magnitude between 0.770 and 0.670 a_w (Stevenson and Hallsworth, 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted; see also Yakimov *et al.*, 2014). Whereas all of these species are obligately xerophilic eukaryotes or obligately halophilic prokaryotes, which have low rates of cell division – or are incapable of growth – close to a water activity of 1, the ultimate limit for multiplication of even the most resilient strains appears to be ~0.61 a_w (Pitt and Christian, 1968; Williams and

[4]This has implications for preventing contamination of other planetary bodies which, as far as we know, lack sugar-rich environments.

Hallsworth, 2009; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). For microbes on Earth, therefore, biotic activity spans approximately 40% of the available water-activity range, thus emphasizing the potency of water as a determinant of the functional biosphere. The overwhelming majority of microbial systems are metabolically active somewhere within the ranges 5°C to 40°C, and 1 to 0.900 a_w, which represent even smaller portions of the environmentally pertinent temperature and water-activity ranges, i.e. only 10% in each case (Fig. 1). Of the microbial systems characterized thus far, the 20 to 30 known to be active at ≤ 0.690 a_w represent the most extreme forms of life to have penetrated low water-activity, hostile environments (Fig. 1).[5]

Some reports have alluded to the possibility of microbial growth and metabolism at the otherwise unprecedented water-activity values of 0.382 (for deep-sea halophiles in $MgCl_2$-saturated brine; van der Wielen *et al*., 2005), < 0.450 (for halophiles in the $CaCl_2$-rich, Antarctic Don Juan Pond; Siegel *et al*., 1979), 0.500 (Actinobacteria isolated from algal mats and cultured in soil-based substrates; Doroshenko *et al*., 2005; Doroshenko *et al*., 2006; Zvyagintsev *et al*., 2009; Zvyagintsev *et al*., 2012), 0.570 (for halophiles in acidic, saline lakes; Mormile *et al*., 2009), 0.600 [for germination of *Wallemia sebi* (a xerophilic basidiomycete) on high-sugar substrates; Frank and Hess, 1941] and 0.600 [reported value for optimum growth of halophiles (Jaenicke and Bohm, 1998), and biotic activity in salt lakes (Cobucci-Ponzano *et al*., 2006)]. Some of these values were hypothetical (see below), and the other claims have not been accepted or have been refuted by authors of a number of subsequent studies (Pitt and Christian, 1968; Wynn-Williams, 1996; Beaty *et al*., 2006; Hallsworth *et al*., 2007; Kminek *et al*., 2010; Oren, 2011; Stevenson and Hallsworth, 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted).[6] The Don Juan Pond (located within the McMurdo Dry Valleys, Antarctica) is a $CaCl_2$-saturated brine pool situated in a closed basin and fed by seasonal meltwater streams and deliquescent seepages, both of which are thought to deliver $CaCl_2$ to the lake (Dickson *et al*., 2013). Its volume fluctuates but is typically ~ 3000 m^3 (slightly larger than an Olympic swimming pool), and it is among the most saline large-scale bodies of water known on Earth. This pond rarely, if ever, freezes despite winter temperatures of ≤–51°C (Siegel *et al*., 1979; Marion, 1997; Grant, 2004). While annual temperatures of the pond's water and the surrounding sediments are occasionally above 0°C, they remain below –20°C for the majority of the year (Samarkin *et al*., 2010) and, at these temperatures, microbial cell division has not been observed (for references, see Chin *et al*., 2010; Kminek *et al*., 2010).[6] Saturated solutions of divalent chloride salts, as found in the Don Juan Pond, are highly chaotropic and are therefore likely to prevent microbial growth (and may even be sterile environments; Duda *et al*., 2004; Duda *et al*., 2005; Hallsworth *et al*., 2007; Cray *et al*., 2013a; Cray *et al*., 2013b; Oren, 2013; Yakimov *et al*., 2014). Nitrous oxide emissions recorded from the surrounding sediments, frequently attributed to the biological transformation of nitrogenous compounds, are apparently the result of abiotic reactions between brine nitrates and Fe^{II}-bearing minerals (Samarkin *et al*., 2010).

The water activity of the $MgCl_2$-dominated, deep-sea hypersaline brine studied by van der Wielen and colleagues (2005) is ~ 0.382 at the *in situ* temperature of 14.5°C (Winston and Bates, 1960; Hallsworth *et al*., 2007). Culture-dependent and culture-independent studies of this and comparable brines, and investigations into the biophysics of macromolecular interactions, indicate that its potent chaotropicity prohibits life processes (even at water activity values which would otherwise be permissive for cell division) (Hallsworth *et al*., 2007; Yakimov *et al*., 2014). This finding is consistent with the behaviour and hostility of solutions of comparable salts (Winston and Bates, 1960; Hallsworth *et al*., 2003a; Duda *et al*., 2004; Kminek *et al*., 2010; Cray *et al*., 2013a,b; Oren, 2013). Speculations that microbial metabolism and cell division occur at ~ 5 M $MgCl_2$ are inconsistent with: (i) the microbiology of the Dead Sea that approaches a condition of sterility when $MgCl_2$ concentrations become elevated, but are nevertheless below 3 M (Oren, 1999; 2010; 2013), or (ii) the $CaCl_2$-dominated Don Juan Pond

[5]Habitats which have sufficiently low water-activity to exclude almost all forms of life on Earth and, therefore, have a characteristically low biodiversity (especially those of < 0.690 a_w) are fertile habitats for those extremophiles which thrive there due to minimal competition and, frequently, a lack of grazers and predators (for references, see Cray *et al*., 2013a). Such low water-activity habitats are, however, typically too biologically hostile and insufficiently biodiverse to act as open habitats for microorganisms (Cray *et al*., 2013a; Lievens *et al*., 2014; Oren and Hallsworth, 2014).

[6]See also J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished.

(Siegel *et al*., 1983; Samarkin *et al*., 2010; Oren, 2013) where concentrations of divalent chloride salts reach critical concentrations which are prohibitive for all life processes (Hallsworth *et al*., 2007; Cray *et al*., 2013b; Oren, 2013; Yakimov *et al*., 2014). Although there is a theoretical possibility that some microbes have evolved specialized substances and/or structures that insulate cells from such hostile habitats while permitting biotic activity (e.g. highly kosmotropic compatible solutes; Wyatt *et al*., 2014a), to our knowledge, no such structures have yet been reported for any microbial species in any type of extremely chaotropic (e.g. Hallsworth *et al*., 2007; Yakimov *et al*., 2014) or low water activity (≤ 0.600) environment.

Reports of germination and subsequent cell division during germ-tube formation of several Actinobacteria [i.e. *Streptomyces albidoflavus* (syn. *Streptomyces odorifer*), *Streptomyces rectiviolaceus* and a *Micromonospora* strain] at 0.500 a_w (Doroshenko *et al*., 2005; 2006; Zvyagintsev *et al*., 2009; 2012) are not consistent with data acquired by others (Stevenson and Hallsworth, 2014). Recent studies have demonstrated that none of these species was capable of growth below 0.895 a_w, and the theoretical water-activity minimum for the most xerotolerant (a strain of *Streptomyces albidoflavus*) is ~ 0.877 (Stevenson and Hallsworth, 2014). Proposed limits of 0.570 or 0.600 a_w for biotic activity of halophiles were speculative (i.e. not derived from determinations of water activity; Jaenicke and Bohm, 1998; Mormile *et al*., 2009; Cobucci-Ponzano *et al*., 2006), and likely sources of experimental error in studies of *W. sebi* germination have been discussed previously (Pitt and Christian, 1968). Furthermore, multiplication of microbes in terrestrial brine lakes which can reach values below 0.600 a_w may have actually occurred at higher water-activity values that resulted from seasonal and weather-related fluctuations in salt concentration (Oren, 1988; 1993; Cobucci-Ponzano *et al*., 2006; Mormile *et al*., 2009).

Although the established temperature minima for multiplication of the most psychrophilic microbes are in the region of −15°C to −18°C (Collins and Buick, 1989; Chin *et al*., 2010), there are numerous sources of evidence for metabolic activity considerably below this range (Fig. 1A; for references, see Kminek *et al*., 2010; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished). By contrast, there is a paucity of data to demonstrate metabolic activity below the accepted water-activity minimum for microbial cell division (i.e. 0.605; Fig. 1B; Kminek *et al*., 2010; Yakimov *et al*., 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted).[6] In relation to the water-activity limit for life, it is noteworthy that trehalose, a hygroscopic substance (Cray *et al*., 2013a) that accumulates in desiccated microbial cells (e.g. Wyatt *et al*., 2014b) and may facilitate the acquisition and retention of water, cannot efficiently absorb water from the vapour phase at equilibrium relative humidities of much less than ~ 50%, equivalent to 0.500 a_w (Fakes *et al*., 2000). Some enzymes (especially some lipases) can remain catalytic below 0.500 a_w, other enzymes can become highly inefficient as their hydration decreases, and others can lose their catalytic activity at water activities below the known limits for microbial multiplication (Dunn and Daniel, 2004; Kurkal *et al*., 2005; Lopez *et al*., 2010), though the implications of these findings for the physiological limits of cellular function at low water-activity have yet to be established. There is evidence that DNA becomes disordered, and is therefore no longer transcribable, below a water activity of 0.550 (Falk *et al*., 1963). Furthermore, strand breaks have been recorded at 0.530 a_w in bacterial cells (Asada *et al*., 1979).[7] It has, therefore, long-been considered unlikely that cellular systems could function at water activities substantially lower than 0.600 (Pitt, 1975; Brown, 1976; Brown, 1990; Sutton and Hildebrand, 1985; Kminek *et al*., 2010; Stasic *et al*., 2012; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished). However, interactions between the various factors which determine the biophysical limits for cellular integrity and biotic activity at low water-activity are complex and have yet to be fully elucidated. Macromolecular integrity and functionality can depend on the net effect of prevailing conditions such as temperature, chao-/kosmotropicity, pressure and water activity (Hallsworth 1998a; Hallsworth *et al*., 2007; Williams and Hallsworth, 2009; Bhaganna *et al*., 2010; Chin *et al*., 2010; Yakimov *et al*., 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N.

[7]Whereas intracellular water activity was not measured in this study, microbial cells in aqueous milieux are thought to be unable to maintain a water-activity gradient across the cell membrane (see Brown, 1990). This said, there is some recent evidence to the contrary (de Goffau *et al*., 2011).

Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted) and it may be possible that, in some as-yet-undiscovered environments, cells are capable of metabolism at < 0.600 a_w.

Microbial cell division via utilization of water which is not in the bulk-liquid phase

Water is more or less ubiquitous on Earth and in other parts of the Solar System (Bradley *et al*., 2014; Küppers *et al*., 2014); it may be present within the atmospheres, subsurface, rocks and regolith, polar ice sheets, glaciers, and/or subsurface oceans of planetary bodies, in vapour plumes extruded into space, and – indeed – within space itself.[8] Whereas here on Earth, we tend to be most familiar with water in its bulk-liquid phase, in both terrestrial and extraterrestrial environments, it can also be present in a variety of forms. In addition to ice and vapour, these include thin, aqueous films on/at various types of surfaces and interfaces; as molecules hydrating mineral, organic and other substances (Kminek *et al*., 2010; Toner *et al*., 2014; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished); and inside minerals in the form of fluid inclusions. Liquidity of water is determined by temperature, pressure, the presence of solutes and/or gases, and molecular interactions between other materials or substances and water molecules – as well as processes such as salt deliquescence, sublimation of ice, frost formation, condensation or dew formation on surfaces or within the gaseous phase, aerosol formation, and precipitation (Watanabe and Mizoguchi, 2002; Jepsen *et al*., 2007; Argyris *et al*., 2008; Möhlmann, 2008; 2009; 2012; Chin *et al*., 2010; Pavlov *et al*., 2010; Bing and Ma, 2011).

Thin aqueous films can exist on various surfaces including those of ice and biological and mineral structures, and the water within these films can remain in the liquid phase under a wide range of conditions (Pearson and Derbyshire, 1974; Raviv *et al*., 2001; Wolfe *et al*., 2002; Jepsen *et al*., 2007; Möhlmann, 2004; Möhlmann, 2008; Möhlmann, 2009; Möhlmann, 2011; Möhlmann, 2012; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished). The depth of thin films can range from > 1 mm to a monolayer of water molecules (~ 0.3 nm; Möhlmann, 2004; Möhlmann, 2005), and they can be stable (Möhlmann, 2012) or highly ephemeral (Burkhardt and Hunsche, 2013). At the temperatures and pressures which typically prevail in Earth's biosphere, aqueous films of ~ 1 mm are primarily made up of water which is biologically available (e.g. Qvit-Raz *et al*., 2008, Burch *et al*., 2013). Whereas, we speculate that single-monolayer films do not provide water that can be accessed by cellular systems. It has been suggested that microbes can utilize fluid films that have a mean thickness equivalent to that of three water molecules (Harris, 1981; Beaty *et al*., 2006). This hypothesis, however, may be inconsistent with the lack of solute diffusion in very thin films (Derjaguin and Churaev, 1986; Hu and Wang, 2003), which indicates that the water in films as thin as this is not in the liquid phase.[9] Despite the circumstantial evidence (see also Rivkina *et al*., 2000), there is a paucity of data that convincingly demonstrate that water is biologically available in films of less than three water molecules deep.

There are several possible sources of liquid water in otherwise desiccated and cold environments that resemble those which are characteristic of Mars, e.g.: (i) interfacial water present as a thin film (sometimes equivalent to a depth of only one or several water molecules) forming on mineral surfaces by adsorption or, on ice, as premelted ice (Dash *et al*., 2006; Möhlmann, 2011), (ii) brines forming on salt crystals via deliquescence, (iii) as the fluid inclusions of ice and salts or other minerals, and (iv) subsurface meltwater below an ice covering due to a solid-state 'greenhouse effect' (as described below) (Möhlmann, 2011). Deliquescence processes represent a particularly effective mechanism by which liquid water can be generated on Earth and, almost certainly, in extraterrestrial locations (Möhlmann, 2011). The condensing water vapour can potentially reach the dry weight of the deliquescent salt, and will exceed it if the humidity exceeds the deliquescence relative humidity. Deliquescence of NaCl, as equilibrium relative humidity increases from 65% to 80%, can be observed in Movie S1. Most

[8]See Waite and colleagues (2006); Nimmo and colleagues (2007); Tosca and colleagues (2008); Campins and colleagues (2010); Sohl and colleagues (2010); Carter and colleagues (2013); Martínez and Renno (2013); and Bradley and colleagues (2014).

[9]This inconsistency also raises the possibility that the high water-activity values reported for very thin films (Harris, 1981; Papendick and Campbell, 1981) could be a consequence of methodological error.

salts (and, indeed, many organic substances) are hygroscopic and will attract water to their surface at equilibrium relative humidities of ≤ 100%. Each salt becomes deliquescent at a specific relative humidity, thereby dissolving as the water vapour condenses. The deliquescence relative humidity for a given salt and its (usually slight) temperature dependence quantitatively correspond to both the water-activity values of, and equilibrium relative-humidity values for, saturated solutions of a given salt (Winston and Bates, 1960). If the equilibrium relative humidity is higher than a salt's deliquescence relative humidity, the water activity of the salt solution will equilibrate with the relative humidity of the atmosphere, so the salt solution will become more dilute. Mixtures of substances (e.g. mixtures of different salts or salts plus sugars) will have a deliquescence relative humidity below that of each individual component (Mauer and Taylor, 2010). In addition to the reduced water activity, salts also reduce the freezing point of water, and cryobrines may be stable far below the melting point of water, e.g. under Martian conditions (Möhlmann, 2011; Martínez and Renno, 2013). Intriguingly, one recent study indicates that deliquescence of specific salts can occur, under Martian conditions, when salts are in contact with and obtaining water from ice (Fischer *et al.*, 2014).

Within the Earth's biosphere, brine formation may play a role for diverse microbial species – especially those that are halotolerant or halophilic – which are located within bioaerosols, or on mineral or biological surfaces (e.g. leaf surfaces) and are exposed to humid air (Potts, 1994). For example, adapted species can reproduce within the phyllosphere of salt-exuding desert plants (Qvit-Raz *et al.*, 2008; Burch *et al.*, 2013) and, at subzero temperatures, in supercooled water in the atmosphere (Sattler *et al.*, 2001). *Pseudomonas syringae*, which is not halophilic, is a species widely transported within bioaerosols and its cells are highly effective as ice nuclei because they have protein coatings that cause water to freeze at relatively warm temperatures (Christner *et al.*, 2008; Morris *et al.*, 2014). Being surrounded by ice, they may benefit from water provided by the (internal) formation of thin films caused by the penetration and retention of shortwave radiation within the ice (i.e. by a solid-state 'greenhouse effect'). Pseudomonads (and other microbes) can produce substances such as hygroscopic biosurfactants and alginate that can attract and retain water within the vicinity of the cell (Chang *et al.*, 2007; Burch *et al.*, 2014).

Microbes can obtain water from the vapour phase, a process that has been observed in lichens (Pintado and Sancho, 2002; Lange *et al.*, 2006) as well as the propagules of various species (Waldham and Halvorson, 1954; Pasanen *et al.*, 1991; Reponen *et al.*, 1996; Bekker *et al.*, 2012). Other studies have demonstrated that microbial cells also generate considerable quantities of water via their metabolic activity (Oriol *et al.*, 1988; Nagel *et al.*, 2001; Marcano *et al.*, 2002; Kreuzer-Martin *et al.*, 2005; 2006; de Goffau *et al.*, 2011), up to 70% of the cell's water according to radio-labelled gas uptake experiments (Kreuzer-Martin *et al.*, 2005; 2006). Spore germination of powdery mildews, such as by the *Erysiphe* and *Uncinula* species, has been observed, at low equilibrium relative humidities (0% to 10%) without a visible extracellular source of liquid water (Brodie and Neufeld, 1942; Manners and Hossain, 1963; Carroll and Wilcox, 2003), although it is not clear whether condensation processes and/or thin films might act to shuttle water to the cell. Desiccated lichens are able to absorb water at an equilibrium relative humidity of ≥ 82% and thereby commence photosynthesis (Pintado and Sancho, 2002; Lange *et al.*, 2006). Various lines of evidence suggest that microorganisms may be capable of cell division without an extracellular supply of liquid water (see also Miller and Chibnall, 1932; Yarwood, 1950; Peterson and Cowling, 1973; Lange *et al.*, 1986; Lange *et al.*, 1994; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished). However, there is a paucity of convincing data to irrefutably affirm this hypothesis. Furthermore, systematic studies of water-activity limits for cell division of phylogenetically diverse extremotolerant and extremophilic microbes[10] suggest that cell division would be implausible at values much below 0.600 a_w (i.e. 60% equilibrium relative humidity) (Pitt and Christian, 1968; Brown, 1976; Williams and Hallsworth, 2009; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). This question is equally pertinent to life on Earth, and the aqueous milieux found elsewhere in the Solar System (not least in relation to planetary protection).

Implications for the evolution of microbial life on Earth

The most solute-tolerant Bacteria and Archaea are only able to grow at their water-activity minima

[10]As well as the evidence for failure of macromolecular systems at low water activity (see above).

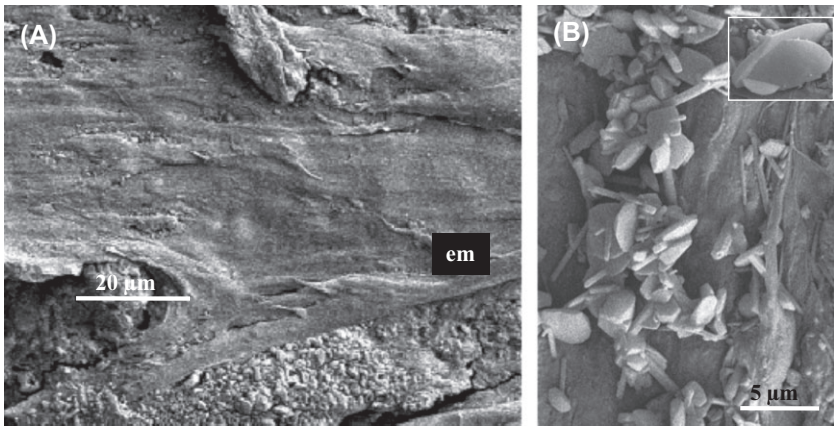

Fig. 2. Early Archaean microbes and evaporites; example from the 3.33-billion-year-old Josefsdal Chert, Barberton Greenstone Belt: (A) layer of evaporite minerals interbedded with layers of a photosynthetic microbial biofilm, (em) evaporite minerals, and (B) details of the diversity of minerals encrusted on the surface of the biofilm. They include here pseudomorphs (silica replaced) of acicular aragonite and lozenge-shaped gypsum. Reproduced from Westall and colleagues (2006) with permission from The Royal Society Press.

under hypersaline conditions (i.e. extreme, obligate halophiles).[11] Some of these organisms thrive under conditions that would have been available in saline environments on the early Earth. Extremely halophilic Archaea and Bacteria typically exhibit higher optimal growth temperatures than those of mesophilic or moderately halophilic comparators (Ratkowsky *et al*., 1983; Oren, 1992; Ramos-Cormenzana, 1992; Robinson *et al*., 2005). Indeed, the minimum and maximum NaCl concentrations at which growth of extreme halophiles can occur increase at higher temperatures (Mullakhanbhai and Larsen, 1975; Vreeland and Martin, 1980; Quesada *et al*., 1987; Rodriguez-Valera, 1992). There is some debate regarding the temperature of the early seas (those of ~3.5 billion years ago); earlier estimates of 70–80°C (Knauth and Lowe, 2003) are now considered to be too high (the $\delta^{18}O$ values on which the calculations were based may have been skewed due to inputs of hydrothermal fluids). More recent estimates based on analysis of oxygen and hydrogen isotopes (i.e. $\delta^{18}O$ and δD respectively) are about 40°C (Blake *et al*., 2010). However, the high levels of heat flow within the mantle on the early Earth drove a highly active hydrothermal circulatory system that contributed hot, salty (de Ronde *et al*., 1997), silica-rich fluids to the local environment (Westall, 2012). It has been proposed that primordial life may have first occurred within hypersaline environments on early Earth (Dundas, 1998), and recent evidence suggests that the abiotic formation of primitive versions of extant proteins can indeed occur in the presence of NaCl (Longo *et al*., 2013; Longo and Blaber, 2014).

[11]Cells are not pure-water reactors with a water activity of 1 (Trevors and Pollack, 2005), but consist of gels within which modulation of water activity, the distribution of various biomolecules, and solution chemistry that permits flexibility and stability of biomacromolecular structures are central to effective cellular function. Indeed, a metabolic ability to maintain the cellular system at his level is one of the fundamental, defining characteristics of life itself.

Understanding the way in which water-condensing chemical reactions could have led to the emergence of key biomolecules (e.g. peptides and nucleic acids) is essential to understanding the origins of life (Da Silva and Holm 2014, and references therein). Prokaryote life (anaerobic) was relatively abundant in these early environments and left behind numerous signatures of its presence (Westall, 2012). There are stratified salt deposits of various ages throughout large regions of the Earth, indicating that concentrated salt-waters/brines have existed across the planet's geologic history (Warren, 2010). Direct association of an early photosynthetic microbial community with evaporitic conditions is documented in 3.33-billion-year-old volcanic sands from the Barberton greenstone belt, South Africa (Fig. 2; Westall *et al*., 2001; 2006; 2011). The uppermost layers of a desiccated biofilm, formed on sediments deposited in shallow waters that were partially exposed to air, are interlayered with tiny evaporate crystals (microns in size and including aragonite, gypsum, halite and magnesium calcite; Fig. 2). Evaporitic precipitates have been described from other formations on the early Earth, including the 3.42-billion-year-old Buck Reef Chert in Barberton (Lowe and Fisher-Worrell, 1999) and the 3.43-billion-year-old Strelley Pool Chert of the Pilbara in Australia (Allwood *et al*., 2007). The early phototrophs were quite advanced on the evolutionary scale compared with chemotrophs. Although, to date, no direct association of chemotrophic biosignatures with the early evaporitic deposits has been identified, these more primitive organisms were nevertheless also common (Westall, 2012; Westall *et al*., 2013). Experiments simulating the entry of meteorites containing microorganisms into the Earth's atmosphere have shown that, if primitive cells did reach the early Earth through panspermia: (i) phototrophs could not have been transported to Earth by these means (Cockell *et al*., 2007), and (ii) if resilient forms of life were hidden in meteorites, they would need to be

buried at depths of at least 5 cm in cracks within the meteorite in order to withstand the heat of entry (Foucher *et al*., 2010).

Regardless of how (and where) life originated, it seems most likely that it was prokaryotes (known to have preceded eukaryotes by ~2 billion years) in hypersaline environments which were first able to multiply close to 0.605 a_w, as documented by the 3.33 Ga-old, evaporite-coated, anoxygenic photosynthetic microbial biofilm noted above (Westall *et al*., 2006; 2011). In relatively unperturbed, sediment-starved environments, these photosynthetic films built up into three dimensional, dome-shaped stromatolites (e.g. Allwood *et al*., 2007). Intriguingly, molecular analysis of modern stromatolite communities revealed that 74% of the Archaea present were closely related to the Halobacteria (Burns *et al*., 2004), which frequently dominate hypersaline environments (Oren, 2002; Cray *et al*., 2013b; Oren and Hallsworth, 2014). These prokaryotic halophiles were exposed to, and presumably inhabited, evaporitic environments containing elevated concentrations of magnesium and characterized by water activities of considerably less than 0.755 (and can, indeed, be considerably below 0.600 a_w, depending on salt concentrations; Winston and Bates, 1960; Hallsworth *et al*., 2007; Yakimov *et al*., 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). Indeed, the signatures of past life forms, including stromatolites, can be common in evaporitic deposits (Rothschild and Mancinelli, 2001).

Much later, and presumably in land-based (rather than marine) habitats, the Eukarya must have developed a similar resilience during growth at high concentrations of solutes which are produced via biogenic activity, namely sugars and polyols. Indeed, the most halophilic Eukarya are considerably less salt tolerant than their bacterial and archaeal counterparts, and it may be that the prokaryotes are yet to evolve an ability to grow at low water-activity in non-saline substrates (their current record is in the range 0.850 to 0.800; Lievens *et al*., 2014; R. Santos, A. Stevenson, C. C. C. R. de Carvalho, I. R. Grant, I. R. and J. E. Hallsworth, submitted; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted).

Extraterrestrial, aqueous milieux which resemble fertile habitats on Earth

Liquid water was, and is still, present in numerous locations in the Solar System. On Mars, for example, there is abundant geomorphological evidence for the presence of liquid water on the planet in the past (Carr, 2006) and possibly even, ephemerally, in the present (Möhlmann, 2011; McEwen *et al*., 2014; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished). Such evidence includes the formation of secondary minerals through the aqueous alteration of the basaltic rocks that cover the surface of the planet (e.g. Carter *et al*., 2013; Martínez and Renno, 2013). It has been calculated that the water activities of evaporite deposits and bodies of saline water on early Mars were as high as 0.780 to 0.860 (Tosca *et al*., 2008), which is well within the ranges for microbial species from each domain of life (Javor, 1984; Grant, 2004; Williams and Hallsworth, 2009; Stevenson and Hallsworth, 2014; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted).

There is evidence for various brines on Jupiter's moon Europa (Fig. 3A) that are composed primarily of water and salts such as $MgSO_4$, Na_2SO_4 and/or Na_2CO_3 (and, in some cases, also contain sulfuric acid; Muñoz-Iglesias *et al*., 2013). Saturated solutions of these salts have water-activity values of 0.900, 0.930 and 0.920 respectively (at 20°C, 1 atm; Winston and Bates, 1960), although it is currently unclear what the values would be under the prevailing conditions on Europa. At the lower temperatures, and the *in situ* pressures, on Europa, the solubility of ions and, conversely, the precipitation of salts can also vary leading to increases in water activity (Marion *et al*., 2003; 2005); the water activity of a saturated Na_2CO_3 solution at 10°C, for example, is 0.990 (Winston and Bates, 1960). Whereas water-activity values for individual brines will vary according to their ionic composition (and pH, which also influences solubilities of some salts), it seems likely that the *in-situ* water activities span the entire range for known life (Javor, 1984; Williams and Hallsworth, 2009; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D.

Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted).

Water has also been identified in asteroidal materials, for example the Monahans (1998) H5 chondrite which contained hypersaline fluid inclusions composed predominantly of saturated NaCl (Zolensky *et al*., 1999) having a water activity of 0.760 at 20°C and 0.750 at 2°C at 1 atm (Winston and Bates, 1960), although these values will vary with pressure. Fluid inclusions have been identified in an increasing number of asteroidal specimens including the Zag (1998) meteorite (Rubin *et al*., 2002). Furthermore, organic molecules have been detected in the fluid inclusions of some of these asteroidal bodies (e.g. Fries *et al*., 2012); thus, the composition of these fluids can be close to those of the media and substrates in which halophiles occur. For instance, halophiles in hypersaline fluid inclusions of salt crystals from evaporite deposits contain Archaea, Bacteria and algae (*Dunaliella* species).[12] Many NaCl-saturated habitats contain a remarkably high microbial biomass and are characterized by intense competition (Antón *et al*., 2002; Daffonchio *et al*., 2006; Baati *et al*., 2008; Elevi Bardavid *et al*., 2008; Khemakhem *et al*., 2010) during which some species – which are known as 'microbial weeds' (Cray *et al*., 2013a; Oren and Hallsworth, 2014) – achieve dominance of the communities including Archaea, Bacteria and Eukarya (e.g. *Haloquadratum walsbyi*, *Salinibacter ruber* and *Dunaliella salina*; for references, see Cray *et al*., 2013a; Oren and Hallsworth, 2014). The microbes that dominate and/or are most frequently isolated from the fluid inclusions of salt crystals found in evaporite deposits include a number of species known to be capable of cell division in the range 0.739 to 0.611 (or their close relations, such as *Dunaliella*, *Halocarcula*, *Halobacterium*, *Halococcus*, *Halorubrum* and *Natrinema* spp.: McGenity *et al*., 2000; Stan-Lotter *et al*., 2000; Schubert *et al*., 2009b; Lowenstein *et al*., 2011; Gramain *et al*., 2011; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). In relation to water activity, the biotic activity of microorganisms – including halophiles – is plausible for some of the aqueous milieux found in extraterrestrial environments. Indeed, some of these locations resemble highly fertile habitats for known halophiles (see also A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted).

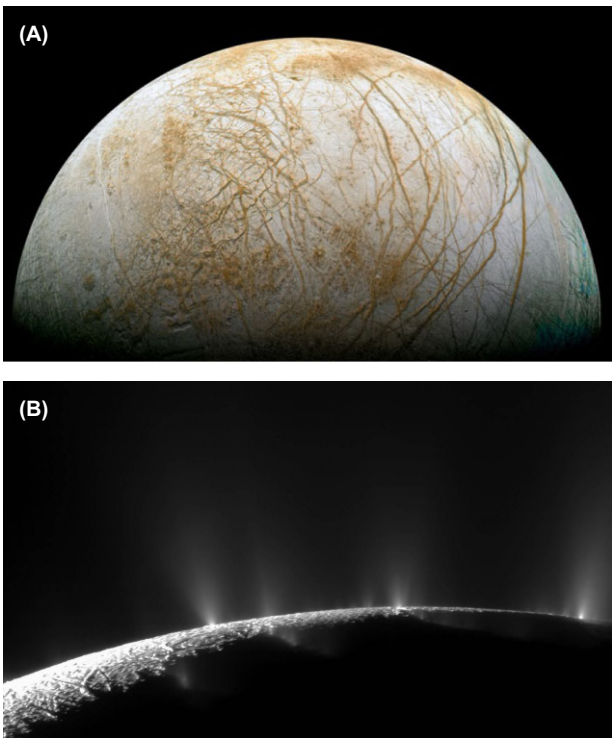

Fig. 3. Views of two planetary moons which are known to have an abundance of water, some of which may be present as subsurface oceans: (A) the icy surface of Europa, and (B) jets composed of water vapour, ice particles and organic compounds released from beneath the surface of Enceladus. Courtesy NASA/JPL-Caltech.

Planets which are neither too close to nor too far from a star and could, theoretically at least, accommodate active biological systems are said to be in the circumstellar habitable zone or Goldilocks zone of their respective solar system (Strughold, 1953). This designation is based on criteria, such as size of the planet and its absolute distance from the star it orbits, whether luminosity could permit photosynthesis, having surface temperatures which are biologically permissive for at least some of the time (variously defined as 0°C to 100°C, or –25°C to +122°C; Franck *et al*., 2007; Takai *et al*., 2008; Kminek *et al*., 2010; Harrison *et al*., 2013), and/or whether they have liquid water (Rampino and Caldeira, 1994; Von Bloh *et al*., 2011). However, these criteria (and indeed the habitable-zone concept) have limited applicability or valid-

[12]See McGenity and colleagues (2000); Schubert and colleagues (2009a); Gramain and colleagues (2011); Lowenstein and colleagues (2011); Valentine (2013). Cyanobacteria are known to be metabolically active in evaporite deposits (the *in-situ* water-activity limit for their physiological activity has yet to be determined; Rothschild *et al*., 1994).

ity for a variety of reasons. Ecosystems exist on Earth which do not depend on photosynthetic activity (Chivian *et al*., 2008; Teixeira *et al*., 2013) and, indeed, the earliest forms of life were not photosynthetic (Westall, 2012); furthermore, there is circumstantial evidence that an extracellular source of liquid water is not obligatory for microbial life (see above). What is more, biologically permissive conditions may prevail in specific environments or substrates on otherwise hostile planetary bodies (for example in relation to moons of Saturn, see Raulin, 2008; Nimmo *et al*., 2007; Parkinson *et al*., 2008). And finally, various activities of solutes can both prevent freezing of water and expand biotic windows of microbes, and may be able to do so to a greater degree than has yet been recorded (see below; Chin *et al*., 2010; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished).

Water can remain liquid at temperatures far lower than those known to permit microbial cell division (i.e. approximately −18°C; see references in Chin *et al*., 2010; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished). Liquid water (in various forms, from thin films to underground oceans) may be found in many environments on Mars as well as planetary moons (Europa, Ganymede, Enceladus, etc.). Diverse lines of evidence suggest that both photosynthetic and non-photosynthetic microbes may be capable of metabolism and cell division by hygroscopic absorption of water vapour and/or acquiring water from their substratum (as a sole extracellular source of water) both *in vitro* and in their natural habitats on Earth,[13] and utilize a variety of mechanisms for the acquisition and retention of water (e.g. production and accumulation of trehalose and other hygroscopic substances which optimize the acquisition and retention of water, morphological changes which minimize water loss, hydrotactic responses, inhabiting high humidity niches, and construction of soil features to enhance water capture and retention; Garcia-Pichel and Pringault, 2001; Garvie *et al*., 2008; de Goffau *et al*., 2011; Williams *et al*., 2012; Rajeev *et al*., 2013; Zakharova *et al*., 2013). Furthermore, as noted above, some microbial cells can generate vast quantities of water via their metabolic activities (Miller and Chibnall, 1932; Peterson and Cowling, 1973; Oriol *et al*., 1988; Nagel *et al*., 2001; Marcano *et al*., 2002; Hocking, 2003; Kreuzer-Martin *et al*., 2005; 2006). As mentioned above, bacterial cells demonstrate that up to 70% of intracellular water may be obtained in this way, and other studies suggest that bacterial cells may be able to maintain higher intracellular water-activity than that of the environment (de Goffau *et al*., 2011).

The rarefied atmosphere of Saturn's moon Enceladus can contain ≥90% water vapour (Waite *et al*., 2006) and, whereas its surface is approximately −200°C (Brown *et al*., 2006), plumes of water vapour and ice which are released into space (Fig. 3B) are thought to originate in subsurface oceans that have temperatures in the range −23°C to −3°C (Nimmo *et al*., 2007; Parkinson *et al*., 2008); i.e. temperatures which are permissive for the metabolic activity of psychrotolerant and psychrophilic microbes (Collins and Buick, 1989; Chin *et al*., 2010; Kminek *et al*., 2010; Mykytczuk *et al*., 2013). Various salts, nitrogenous compounds and organic substances have been identified in the atmosphere of Enceladus and E-ring ice grains of Saturn (which may originate from Enceladus) including NaCl, $NaHCO_3$, $NaCO_3$, N_2, ammonia, hydrogen cyanide, CO and CO_2, methane, acetylene and propane (Matson *et al*., 2007; Postberg *et al*., 2009; 2011). Under conditions prevalent on Earth, bioaerosols can be fertile habitats characterized by high levels of microbial diversity, biomass and metabolic activity (Fahlgren *et al*., 2010; Womack *et al*., 2010; 2012). In relation to the atmosphere of Enceladus and/or the watery plumes which it emits into space, it is intriguing to speculate what the water activity of liquid droplets in, or the humidity of, the gaseous phase (presumably close to 100%) might be and whether the temperatures within these plumes can ever be considerably higher than −200°C. It should be noted that, whereas definitive evidence from culture-based studies of microbial systems on Earth indicate limits for cell division of approximately +122°C or −18°C (Collins and Buick, 1989; Takai *et al*., 2008; Chin *et al*., 2010; Harrison *et al*., 2013), circumstantial evidence from other biochemical or geochemical data suggest biotic activity under more extreme conditions (down to about −40°C, and up to approximately +140°C; Kminek *et al*., 2010; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished).

[13]For example. fungi, lichens and cyanobacteria (Snow, 1949; Armolick and Dickson, 1956; Pitt and Christian, 1968; Ayerst, 1969; Bootsma *et al*., 1973; Drewello and Weissmann, 1997; Shomari and Kennedy, 1999; Lange *et al*., 2006; Wierzchos *et al*., 2011; Zakharova *et al*., 2013).

Although the Earth is located within the region allocated as the Goldilocks zone of our own solar system, it hosts many environments which do not permit life-processes and are therefore essentially sterile due to, for example, low water-activity, high chaotropicity, excessively high or low temperatures, pH of > 12, plus combinations of conditions such as high salt and low pH or high temperature and high pH (e.g. Brown, 1990; Hallsworth 1998a; Grant, 2004; Hallsworth *et al*., 2007; Harrison *et al*., 2013; Yakimov *et al*., 2014). Under all these conditions, cells also need adequate energy sources and nutrients for maintenance and growth which may require electron donors and acceptors for respiration etc. Some combinations of conditions can slightly extend extremes for growth, such as high pressure and temperatures; furthermore, survival can occur under conditions where growth cannot.[14] Conversely, planetary bodies which are basically hostile to life may nevertheless harbour small-scale, biologically permissive domains (Kminek *et al*., 2010; J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished). Solute activities represent one of the determinants for potential habitability on Earth; for example, chaotropicity can enable cellular function at low temperatures and kosmotropicity may enable cellular function in high-temperature environments or those dominated by chaotropic substances.[15] The ways in which water activity and other solute activities can interact to determine the physicochemical limits for life (e.g. Williams and Hallsworth, 2009; Chin *et al*., 2010) have yet to be fully characterized. Furthermore, there is little information on the way in which availability of nutrients and other resources can determine tolerance limits to physicochemical stress parameters (e.g. Daffonchio *et al*., 2006; J. P. Harrison, J. E. Hallsworth, and C. S. Cockell, submitted). Once the interactions between such factors are better understood, the currently accepted criteria for habitability will require revision (Beaty *et al*., 2006; Marion *et al*., 2003; Marion and Kargel, 2008; Tosca *et al*., 2008; Kminek *et al*., 2010; Harrison *et al*., 2013; J.D. Rummel *et al*., unpublished).

How sensitive are cells to minute changes in water activity? And other unanswered questions

In their environmental context, microbes are exposed to complexity at multiple levels, in relation to: (i) the dynamics of physical and chemical parameters, (ii) the antimicrobials and other substances produced by other cells in the vicinity, and (iii) varying availability of resources, and countless other factors. Water activity, in particular, can oscillate (Cray *et al*., 2013a; Lievens *et al*., 2014), and may do so across a range of timescales from a fraction of a second to days, or even longer. The majority of stress-biology studies which quantify water activity do so to either one or two decimal places. We propose here that water activity ought to be determined to an accuracy of three decimal places (Winston and Bates, 1960; Hallsworth and Magan, 1995; Williams and Hallsworth, 2009; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted) as this is more closely aligned with the sensitivity of cellular systems. All technologies used to quantify the water activity of undefined substrates are associated with some degree of error (see Winston and Bates, 1960, Greenspan, 1977, Hallsworth and Nomura, 1999, Yu *et al*., 2009). Commercially available apparatuses for water-activity determination are associated with a net variation (accounting for both accuracy and repeatability) of ± 0.010 to 0.020 water-activity units (A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). At 0.600 a_w, this is equivalent to variations of water potential between ± –2.3 and –4.5 MPa respectively. For the purposes of biological and food-related research, it has been suggested that levels of accuracy of ± 0.010 (Labuza *et al*., 1976; Roa and Tapia, 1998), ± 0.020 (Troller and Christian, 1978; Sereno *et al*., 2001), ± 0.005 (Ferro Fontán and Chirife, 1981; Hallsworth and Nomura, 1999) or ± 0.001 a_w are appropriate (Winston and Bates, 1960). More recent studies suggest that microbial cells can be sensitive to differences/changes of < 0.010 water activity (Williams

[14]The propagules/cells of many microbes are highly resilient to exposure to extremes of temperature, uv, pH, chaotropicity, desiccation and other stresses (e.g. Wyatt *et al*., 2014b; R. Santos, A. Stevenson, C. C. C. R. de Carvalho, I. R. Grant, I. R. and J. E. Hallsworth, submitted), even over long timescales, and so are capable of surviving conditions found in extraterrestrial locations (see above).

[15]See Hallsworth (1998a); (Hallsworth *et al*., 1998b; 2003a,b; 2007); Williams and Hallsworth (2009) Bhaganna and colleagues (2010); Chin and colleagues (2010); McCammick and colleagues (2010); Bell and colleagues (2013); (Cray *et al*., 2013a,b); Lievens and colleagues (2014); and Yakimov and colleagues (2014). Whereas chaotropic substances are typically less polar than water and disorder biomacromolecules, kosmotropic substances are usually more polar than water and thereby structure or rigidify macromolecular systems (see Cray *et al*., 2013a, and references therein).

and Hallsworth, 2009; A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). For example, water-activity differences of < 0.005 a_w-units have impacted growth rates for diverse strains of xerophilic fungi by between 40% and 80% (A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted) which, in turn, implies fundamental differences at every level of the cellular system, from gene expression to physiological and developmental processes. On glycerol-supplemented media at water activities of 0.799 and 0.795, growth rates for *A. penicillioides* varied between 1.13 and 0.642 mm d^{-1} for strain JH06THH and between 1.20 and 0.732 mm d^{-1} for strain JH06THJ; and on $MgCl_2$-supplemented media at water activities of 0.915 and 0.907, rates for *X. bisporus* varied between 3.96 and 1.43 mm d^{-1} for strain FRR 0025, 2.55 and 0.533 mm d^{-1} for strain FRR 2347, and 2.13 and 0.800 mm d^{-1} for strain FRR 3443 (A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). These data raise the tantalizing question of whether microbial cells are sensitive to water activity differences down to the fourth, or even fifth, decimal place.[16] It is noteworthy that, for a hypothetical microbial species that has a temperature window for cell division spanning from 5°C to 40°C (i.e. a 35°C range), a temperature change of 10°C, 1°C or 0.1°C would represent a 1/3.5, 1/35 and 1/350 fraction of this window respectively. If the water-activity window for this microbe spanned from 1 to 0.900 a_w (i.e. 0.100 a_w-units in total), 1/3.5, 1/35 and 1/350 portions of this window would correspond to 0.02857, 0.00286 and 0.00029 a_w-units respectively. This underlines the fact that water-activity determinations to one decimal place (equivalent, in this example, to ~ 29°C) can lack biological meaning, and those made to two decimal places (equivalent to an accuracy level of up to 2.9°C) are far less accurate than we would accept for biological studies of temperature or other environmental parameters. Based on our current knowledge, the water-activity and temperature windows for microbes collectively span 0.400 a_w-units and 140°C respectively (Fig. 1). In the context of stress biology, and at the scale of the biosphere, the expression of water activity to one decimal place leads to an unacceptable level of accuracy, as 0.100 a_w-units equates to a temperature difference of 35°C.[17] Even water-activity determinations to three decimal places (equivalent to an accuracy level of ~ 0.3°C) are imposed by technological limitations rather than being dictated by the sensitivity of the cell.

It remains unclear whether microorganisms are capable of subsistence without an extracellular supply of liquid water, and the biological availability of water in aqueous films of varying thickness (and at various temperatures) has also yet to be quantified. Cells may be able to acquire and retain water (de Goffau *et al.*, 2011) which can be utilized when water activity falls below biologically permissive levels (for instance, see the studies of powdery mildew cited above) but there is no definitive evidence that this does indeed occur (and, if so, what mechanisms are involved) at present (J. D. Rummel, D. W. Beaty, M. A. Jones, C. Bakermans, N. G. Barlow, P. Boston, V. Chevrier, B. Clark, J.-P. de Vera, R. V. Gough, J. E. Hallsworth, J. W. Head, V. J. Hipkin, T. L. Kieft, A. S. McEwen, M. T. Mellon, J. Mikucki, W. L. Nicholson, C. R. Omelon, R. Peterson, E. Roden, B. Sherwood Lollar, K. L. Tanaka, D. Viola and J. J. Wray, unpublished). Culture-independent studies are needed for high-solute, and other low water-activity, habitats to establish whether metabolic activity remains once water activity is below the threshold for cell division (0.605 a_w) and, if so, whether this is commonplace at different locations within the microbial biosphere. In contrast with the increasing understanding of molecular-level adaptations in many other forms of extremophile, there is a paucity of information in relation to physiological, biochemical and genetic

[16]Based on the use of Novasina technology (Novasina AG, Pfäffikon, Switzerland) and a protocol incorporating a range of precautionary measures, we achieved an accuracy of $\pm$ 0.001 a_w-units (A. Stevenson, J. A. Cray, J. P. Williams, R. Santos, R. Sahay, N. Neuenkirchen, C. D. McClure, I. R. Grant, J. D. R. Houghton, J. P. Quinn, D. J., Timson, S. V. Patil, R. S. Singhal, J. B. Antón, J. Dijksterhuis, A. D. Hocking, B. Lievens, D. E. N. Rangel, M. A. Voytek, N. Gunde-Cimerman, A. Oren, K. N. Timmis, T. J. McGenity, and J. E. Hallsworth, submitted). Whereas calculations can be carried out to enable the expression of water-activity values to the fourth decimal place, these have been based on a number of assumptions which, collectively, result in unacceptable levels of uncertainty (Greenspan, 1977; Yu *et al.*, 2009). Such a level of accuracy would be highly desirable in many spheres of biological research but empirical determinations of water activity to the fourth decimal place are currently unattainable.

[17]This further suggests a lack of parity between the safety margins used for these two parameters in relation to current planetary protection policy (Fig. 1).

mechanisms which facilitate halophile/xerophile function at < 0.690 a_w (e.g. Leong *et al.*, 2014).[18] Further work is also needed to elucidate the roles that low water-activity substrates have played, and continue to play, in the evolution of both prokaryotic and eukaryotic systems. In the context of habitability, work is also needed to elucidate the interactions between type and concentration of ions, chao-/kosmotropicity and water activity in relation to complex brines such as current those found in various locations on Earth (Siegel *et al.*, 1983; Oren, 1988; Hallsworth *et al.*, 2007; Yakimov *et al.*, 2014) and those likely to have existed on early Earth or ancient Mars (Tosca *et al.*, 2008). For ecosystems located in extremely hostile habitats, some reports hint that microbial life can be discontinuous and fragmented (Hopkins *et al.*, 2005). In some low water-activity habitats, it may be that active cells are located in otherwise biologically non-permissive zones, and pockets of sterility exist within otherwise inhabited zones. Furthermore, in some locations, microbes may be inactive for most of the time and functional for only short periods. It has yet to be determined, for example, whether slow cell divisions[19] can occur in microbial communities which may subsist in nature at water activities below the known 0.605-a_w limit. We already know much about the water-activity windows for, and stress biology of, a selection of the microbes that occur in Earth's biosphere. By contrast, we know little about the microbial limits of sensitivity to minute variations in biophysical parameters such as chaotropicity and water activity. We propose that the temporal and spatial dynamics of such parameters can constrain microbial behaviour in relation to the environment and, if this is indeed the case, will also act as determinants for microbial community composition and the evolutionary trajectories of individual microbial species.

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[18]This also acts as a barrier to the biotechnological exploitation of these extremophiles and the macromolecular systems derived from them.

[19]That is, over hundreds or thousands of years, as observed in deep-sea and subsurface sediments (Parkes *et al.*, 2000; D'Hondt *et al.*, 2002; Lomstein *et al.*, 2012).

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Supporting information

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

Movie S1. Deliquescence of NaCl crystals on the surface of a pine needle (*Pinus sylvestris*) as humidity rises from approximately 65% to 80% equilibrium relative humidity. The deliquescence point of NaCl is approximately 75% equilibrium relative humidity at 2°C. An epistomatal chamber is visible but the guard cells are located below this section and cannot, therefore, be seen. The recording was made using an environmental scanning electron microscope and equilibrium relative humidity was controlled experimentally within a chamber (see Burkhardt and Hunsche, 2013).