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Mark G. Fox-Powell[1*], John E. Hallsworth[2], Claire R. Cousins[3] & Charles S. Cockell[1]

[1]UK Centre for Astrobiology, School of Physics and Astronomy, University of Edinburgh, Mayfield Road, Edinburgh, EH9 3JZ, UK

[2]Institute for Global Food Security, School of Biological Sciences, MBC, Queen's University Belfast, Belfast, BT9 7BL, UK

[3]Department of Earth and Environmental Sciences, University of St. Andrews, Irvine Building, St. Andrews, Fife, UK, KY16 9AL, UK

*Corresponding author: Mark Fox-Powell, Room 1607, UK Centre for Astrobiology, School of Physics and Astronomy, James Clerk Maxwell Building, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JZ. Tel: +44 (0) 131 6517774; email: m.fox-powell@ed.ac.uk

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Abstract:

The thermodynamic availability of water (water activity) strictly limits microbial propagation on Earth, particularly in hypersaline environments. A considerable body of evidence indicates the existence of hypersaline surface waters throughout the history of Mars, therefore it is assumed that, as on Earth, water activity is a major limiting factor for martian habitability. However, the differing geologic histories of the Earth and Mars have driven variations in their respective aqueous geochemistry, with as-yet-unknown implications for habitability. Using a microbial community enrichment approach, we investigated microbial habitability for a suite of simulated martian brines. Whilst the habitability of some martian brines was consistent with predictions made from water activity, others were uninhabitable even when the water activity was biologically permissive. We provide evidence that high ionic strength, driven to extremes on Mars by the ubiquitous occurrence of divalent ions, renders these environments uninhabitable despite the presence of biologically available water. These findings show how the respective geological histories of Earth and Mars, which have produced differences in the planets' dominant water chemistries, have resulted in different physicochemical extremes which define the boundary space for microbial habitability.

1. Introduction:

All known life requires liquid water, thus the discovery of water on other planetary bodies is central to assessing their habitability (Hubbard *et al*., 2002). Of the planets in our solar system, Mars has received a great deal of attention regarding its potential habitability since it is known to have hosted sustained bodies of liquid water on its surface during its history (Fairen *et al*., 2003; Achille and Hynek, 2010; Carr and Head, 2010; Krasnopolsky, 2015). Furthermore, some environments are thought to have been habitable in the planet's ancient past, based on direct *in-situ* measurements (Grotzinger *et al*., 2014). It is now widely accepted that hypersaline surface waters (brines) have been pervasive on Mars, at least periodically, throughout the last 3.5 billion years, and may be present today (Vaniman *et al*., 2004; Gendrin *et al.*, 2005; Carr and Head, 2010; Martinez and Renno, 2013; Karunatillake *et al*., 2014; Ojha *et al*., 2015). Evidence for saline waters can be found in large-scale evaporite mineral sequences (Knoll *et al*., 2005) in the globally distributed martian soil (Karunatillake *et al*., 2014), putatively in Recurring Slope Lineae features (Ojha *et al.*, 2015), and in martian meteorites (Bridges and Schwenzer, 2012). Investigating the habitability of these brines is therefore crucial to understanding past and present martian habitability.

Historically, our knowledge of life in brines (where salinities exceed that found in seawater) has been derived from studies of terrestrial sodium- and chloride-rich environments which, even at saturation, are permissive for the biotic activity of some halophiles and are accordingly populated by dense microbial communities (Oren, 2008). In brine environments on Earth, microbial life is primarily limited by the thermodynamic availability of water (water activity) (Stevenson *et al*., 2015a; 2015b). The currently accepted limit to life in high salt environments is reached at a water activity of 0.611 (Stevenson *et al*., 2015b), close to the absolute limit for any cellular growth at a water activity of approximately 0.605 (Williams and Hallsworth 2009). By extrapolation, this parameter has been considered to be the major limiting factor for habitability in martian brines (Tosca *et al*., 2008). Water activity is considered by the Committee on Space Research (COSPAR) and NASA Mars Exploration Program Analysis Group (MEPAG) as a defining parameter for 'Special Regions' on Mars (those

62 regions where multiplication of known microbes could plausibly take place) (Rummel *et al*., 2014), and
63 thus plays a central role in shaping planetary protection policy and solar system exploration missions.

64 Planetary geologic evolution can, however, result in different water chemistries, with undetermined
65 implications for habitability. Investigations of terrestrial brine environments with chemistries that differ
66 significantly from the dominant brine type on Earth are relatively few, but often reveal salt-induced
67 stresses that are otherwise lacking in NaCl brines. For example, $MgCl_2$-rich brine lakes in the deep
68 Mediterranean exhibit high chaotropicity (macromolecule-disordering activity) alongside extremely
69 low water activity, exacerbating their hostility and defining the limits of colonisation in the brine-
70 seawater interface (Hallsworth *et al*., 2007; Yakimov *et al*., 2015). Chaotropicity and kosmotropicity
71 (macromolecule-ordering/-stabilizing activity) are measurable entropic phenomena exerted on
72 macromolecular systems by solutes, including salts, that can significantly, and often detrimentally,
73 affect living systems (Ball and Hallsworth, 2015). Furthermore, previous studies on salt stress have
74 highlighted adverse effects caused by salt ions that cannot be explained by osmotic stress or low water
75 activity (Lloret *et al*., 1995; Alves *et al*. 2015).

76 The surface evolution of Mars has given rise to significantly different water chemistries; notably the
77 widespread production of waters with high Mg^{2+}, $Fe^{2/3+}$ and SO_4^{2-} contents (Catling, 1999; Bullock *et*
78 *al*., 2004; Knoll *et al*., 2005; Carr and Head, 2010; Tosca *et al*., 2011). Due to high divalent : monovalent
79 ratios (Fig. 1), such waters form brines with a high charge density (ionic strength) even at relatively
80 clement water activities. Brine environments on Earth that contain elevated levels of divalent ions, such
81 as the Mg^{2+} - rich Dead Sea, and $MgCl_2$ brines in the deep Mediterranean, commonly contain Cl^- as the
82 dominant anion (Grant *et al*., 1999; Wallmann *et al*., 2002), and therefore their divalent : monovalent
83 ratios rarely exceed 1 (Fig. 1). A notable exception is the Basque Lakes, in British Columbia, which are
84 rich in magnesium sulfate salts (Eugster and Hardie, 1978). Here, the divalent content far exceeds that
85 found in the Dead Sea and other brines considered as divalent-rich, and it approaches those of some
86 martian brines (Fig. 1).

Due to a complex dependency on charge interactions in biological molecules, high ionic strength can perturb native structure and function. High charge density is capable of inducing deformations in molecules such as nucleic acids and proteins (Baldwin, 1996; Kunz *et al.*, 2004). Many adverse ion–biomolecule interactions are exacerbated in the presence of di- or multivalent ions, including water-activity reduction, chaotropicity and kosmotropicity as well as associated aggregating/ denaturing phenomena (Hofmeister effects), protein and nucleic acid destabilisation and lipid bilayer disruption (Kirkwood 1943; Green 1955; Baumann *et al.*, 1997; Dominy *et al.*, 2002; Collins, 2004; Cray *et al.* 2013; Ball and Hallsworth, 2015). We therefore hypothesized that the elevated divalent : monovalent ratios in martian waters, compared to the majority of waters on Earth (Fig. 1), causes ionic strength to play a role in defining the window for habitability, even when water activity is permissive.

As well as containing high levels of divalent ions, martian brines exert multiple physicochemical extremes, including low pH, low water activity, elevated divalent ion content and high levels of dissolved iron (depending on brine composition). The primary aim of the current study was to systematically assess the physicochemical parameters which define the habitability of typical martian brines, by seeding with natural microbial communities. In contrast to chloride-dominated brines on the Earth in which microbial propagation is primarily limited by water activity, the results presented here show that high ionic strength in martian brines constrains their habitability to a smaller window than current paradigms predict.

2. Materials and methods

2.1 Simulated martian brines

Naturally-occurring saline environments on Earth with compositions matching those modelled for martian environments have not been reported (Fig. 1). Therefore we synthesized martian brines based on computational reconstructions of evaporative brine formation on the martian surface (Tosca *et al.*, 2011). Brine compositions are known to change significantly as evaporation proceeds (Eugster and Hardie, 1978), and the computational approach employed by these authors produced two stages of concentration for each brine (Stages [a] and [b]), allowing us to probe the effects that natural evaporative

concentration can have on habitability. For information on the computational approach used to predict this evaporation and generate these two stages see Tosca *et al*. (2011).

The martian brines considered for this work were grouped into three types/classes, representative of diverse saline environments on Mars. These were: alkaline carbonate-chloride brines (Type I), which during their more dilute phase are analogous to brackish fluids that persisted at the Curiosity Rover's landing site in Gale Crater approximately 3.7 billion years ago (Léveillé *et al.*, 2014). Upon simulated concentration, Type I brines evolved a concentrated K-Na-HCO_3-Cl composition similar to fluids that interacted with the Nakhla martian meteorite (Bridges and Schwenzer, 2012). Type II brines were Mg-SO_4-Cl dominated, with comparatively low Na and K concentrations, and are characteristic of widespread large-scale Hesperian-aged salt (evaporite) deposits on Mars, such as those investigated by the Mars Exploration Rover Opportunity at Meridiani Planum (Knoll *et al.*, 2005). Type III brines were similar in composition to Type II brines, but contained higher levels of dissolved iron, resulting in brines which were extremely acidic at both stages of simulated concentration. In both Types II and III martian brines, initially high divalent : monovalent ion ratios decreased dramatically following simulated evapoconcentration due to the relative solubility of chlorides (Fig. 1). Both Type II and Type III brines were characterised by high levels of sulfates; which as well as forming the dominant salt type in many evaporite deposits on Mars, is the most abundant soluble component in the globally distributed martian dust (Vaniman *et al.*, 2004; Karunatillake 2014). Type I and II brines were each represented by one evaporation pathway, whereas two evaporation pathways were investigated for Type III brines to capture the compositional and physicochemical diversity possible in their evolution.

Brine compositions for both stages of concentration were taken from Tosca *et al*. (2011) (Table 1). Salts were dissolved in deionised water, supplemented with 4 g L^{-1} yeast extract (Oxoid), and the solutions were stirred continuously for approximately 3 hours to ensure maximum dissolution. Yeast extract was selected as a carbon source as it provides an extensive inventory of proteins, amino acids and sugars. Preliminary enrichments in Type I and Type II Stage [a] brines supplemented with peptone, casamino acids and glucose generally yielded less biomass than did yeast-extract enrichments (data not shown). Due to saturating concentrations of salts in some solutions, brines were left at 30°C for five days to

allow full equilibration of solid and liquid phases. Simulated martian brine solutions were not buffered; pH was left to vary with the salt component to simulate natural brine conditions. Solutions were then split into equal volumes for aerobic and anaerobic culture and filter-sterilised (0.22 µm diameter pores) into pre-autoclaved culture vessels; anaerobic brines were purged with N_2 to remove oxygen and sealed in sterilised 100 ml serum bottles with butyl rubber stoppers to maintain anaerobic conditions. L-cysteine-HCl was added to the anaerobic brines to a final concentration of 0.8 mM from sterile anoxic stocks. An equivalent volume (0.1% v/v) of sterile distilled water was added to aerobic brines. Finally, samples were taken for quantification of water activity, pH, chao/kosmotropic activity and ionic analyses (see below). Analysis of a pure 4 gL^{-1} yeast extract solution revealed that ionic strength was increased in all fluids in the current study by <0.004 mol $litre^{-1}$ as a consequence of yeast extract supplementation.

2.2 Environmental inoculum sources

To maximise our chances of obtaining organisms capable of colonising the brines, we sampled a range of environmental microbial habitats. All sampling was carried out using pre-sterilised sample bags and/or centrifuge tubes. Where possible, samples were obtained from ≥ 5 cm sediment depths to increase chances of sampling anaerobic organisms as well as aerobes. Samples were stored at 4° C until use. A composite inoculum, made up of two environmental samples that were each added at approximately 1 % (v/v) to prepared volumes of brine, was used to screen all brines for evidence of microbial growth. The first – local soil in Edinburgh (UK) – was selected because it has been previously shown that the physicochemical, temporal and spatial variability within top soils have selected for organisms that are tolerant of a range of extremes (Young *et al.*, 2008). Preliminary community analysis via 454 pyrosequencing of the Edinburgh soil revealed a typically high diversity of metabolically diverse taxa (Shannon's $H = 6.007 \pm 0.044$, Good's coverage = 92.65% at 97% OTU similarity). The top layers (approximately 5 cm) of this soil cycle between hydration and complete desiccation, driving extreme transitions in solute concentration(s) on a sub-millimetre scale. As such these soils represented a source of both high microbial diversity and physicochemical heterogeneity. A sample comprised of a mixture of brine and brine-saturated sediment from a 1.1 km-deep subsurface evaporite deposit (Boulby

International Subsurface Astrobiology Laboratory, Boulby Mine, Whitby, North Yorkshire, UK) formed the other half of the composite inoculum. Water pH at time of sampling was approximately 7 (Payler, unpublished). Chemical analyses showed this brine to be dominated by NaCl close to saturation, and it is known to support an active community of halophilic microorganisms (Payler, unpublished).

Where the composite inoculum failed to produce growth, additional inoculum sources were: 1) marginal mud from an acidic hydrothermal pool at Kverkjöll Volcano, Iceland (N 64° 41.205' W 16° 40.502') (Cousins *et al.*, 2013). The pool water contained high levels of dissolved iron (130 mM), sulfate (19.3 g litre^{-1}) and extremely low pH (1.75) at the time of sampling, values typical of those found in acid mine drainage sites such as Rio Tinto (Fernández-Remolar *et al.*, 2004). 2) Brine and sediments from the $MgSO_4$-brine Basque Lakes on the Cariboo Plateau, British Columbia (N 50° 35.596' W 121° 20.934'). These are some of the only known hypersaline environments on Earth where sulfate forms the dominant anion (Nesbitt, 1990), and divalent : monovalent ratios reach values much greater than 1. As such, they represent perhaps the best terrestrial analogue for divalent-rich martian brines. Lake waters are known to fluctuate in concentration dramatically depending on season (Nesbitt, 1990), and at time of sampling (February 2015), the lake water was in a relatively dilute phase, containing 252 mM Mg, 243 mM sulfate, 71 mM Na and <5 mM Cl. Lake water pH was 5.80, the sulfate : chloride ratio was 33.3, and the divalent : monovalent ratio was 5.56 (Fig. 1).

Any environmental inoculum contains a finite number of organisms. Thus for any brine that failed to support colonisation by the environmental inocula and based on the rationale that 'everything is everywhere, but the environment selects' (Baas Becking, 1934), we also placed 100 ml volumes outdoors, open to the atmosphere under a rain cover for one month to allow colonisation by airborne microbes. The rain cover was a slanted plastic ceiling placed approximately 30 cm above the vessels' openings.

Together, these samples provided a high probability of enriching for organisms that tolerate the unique combination of stresses present in martian brines. To confirm this, we designed a suite of control brines

193 (Control-1 to Control-6) that systematically validated the tolerance of these inocula to physicochemical
194 extremes of relevance to our experiments (Table 2, 3). These were prepared and inoculated with the
195 environmental samples (2 % v/v) in triplicate both aerobically and anaerobically in an identical manner
196 to the Mars-relevant brines described above, and were designed to exhibit low water activity (Control-
197 1), low pH (Control-2), combined low pH/low water activity (Control-3) and high levels of dissolved
198 iron (Control-4). Control-5 and Control-6 were designed to exhibit high ionic strength, neutral pH and
199 permissive water activity (Table 3).

200 *2.3 Incubation*

201 Coping with osmotic stress induced by high levels of salts is energetically expensive (Oren, 2011).
202 Previous analyses of growth data for 241 isolated strains revealed that aerobic organisms and anaerobic
203 organisms which use organics as a terminal electron acceptor were tolerant of a broader range of
204 extremes, including salinity, than anaerobic organisms that utilise inorganic electron acceptors
205 (Harrison *et al.*, 2015). By supplying a rich, complex source of organic carbon (4 gL^{-1} yeast extract)
206 and a temperature of 30° C, we therefore expected to increase the energetic favourability of respiratory
207 metabolisms, and thus the capabilities of microorganisms to deal with the stresses induced by our brines
208 (Oren, 2011). This ensured that apart from the extremes of the brines, the organisms had optimum
209 growth conditions with respect to temperature, energy and nutrient availability. Our experiment was
210 focused on determining whether the Martian brine chemistries alone are limiting to life.

211 All brines were inoculated in triplicate (2 % v/v) and incubated at 30° C for 60 days, then transferred
212 (1% v/v) to fresh, sterile brine media. Further transfers were carried out at appropriate time points,
213 which differed by brine and community. Brines that had been exposed to the atmosphere for one month
214 were incubated at 30° C for a further 30 days before also being transferred (1% v/v) to fresh, sterile
215 brine media. For brines that did not contain solid salt precipitate or dissolved iron, growth was
216 quantified as an increase in optical density at 600 nm. In saturated brines and those containing dissolved
217 iron, cells were enumerated by direct counts following SYBR gold or DAPI staining (see below). After
218 three transfers, when cell densities reached approximate maxima, cells were harvested by filtration onto

sterile 25 mm polycarbonate filters (Merck Millipore) for DNA extraction. Initial enrichment-stage brines that did not support growth after 60 days were incubated alongside the transfers and monitored at regular intervals for the remainder of the experiment (>300 days).

2.4 Assays for microbial growth

The ability of the martian and control brines to support microbial growth was assayed via three independent methods. Firstly, samples of brines (approx. 20 µl) were mounted on microscope slides and examined under phase contrast microscopy (Leica DM4000B). Secondly, brine samples (200 µl) were stained with 1x SYBR gold (Life Technologies) for 15 minutes in the dark, mounted on black 25 mm diameter polycarbonate filters (Merck Millipore), excited at 450-490 nm and imaged at 1000x magnification using a Leica DM4000B digital microscope and a Leica DFC 450 C microscope-mounted camera. For iron-rich brines, 1x DAPI (4',6-Diamidino-2-phenylindole) (Sigma) was found to be more reliable. For DAPI staining, samples were prepared in an identical way to SYBR-stained samples, and excited at 358 nm. Where applicable, cells were enumerated by counting 20 randomly selected fields of view and averaging over triplicate samples.

To validate microscopic approaches, we enriched communities from our composite inoculum in nutrient broth media (Oxoid), harvested aliquots by centrifugation, suspended them in samples of each brine, and subjected them to identical staining and imaging protocols as those used for the brine enrichments. Imaging of the organisms was possible in all brines (data not shown).

Thirdly, DNA was extracted from 2-10 ml of brine from the final transfer stage using a modified phenol:chloroform:isoamyl alcohol and isopropanol precipitation protocol as detailed by Urakawa *et al*. (2010). Briefly, samples were passed through 0.22 µm, 25 mm diameter polycarbonate filters. Filters were treated with proteinase K (2mg/ml) and TENS buffer (50 mM Tris-HCl; pH 8.0, 20mM EDTA, 100 mM NaCl, 1% w/v SDS) at 50°C for 1 hour. DNA, if present, was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with isopropanol. DNA extracts were quantified by spectrophotometric absorbance at 280 nm (NanoDrop Lite, BioRad), visualised in 1%

agarose gels with a SynGene G-Box UV transilluminator, and further interrogated by polymerase chain reaction (PCR) (see below).

This third approach was validated by adding communities enriched from our composite inoculum in nutrient broth media (Oxoid) to quantities of all brines, at cell densities approximately equivalent to the lowest obtained in our experiments (Type I Stage [b]), and subjecting them to identical extraction and DNA detection procedures. Positive extraction and domain-specific PCR amplification were achieved from all brines. For a brine to be labelled 'uninhabitable' in the context of the current study required concurrent negative results from both microscopic methods at all transfer stages as well as negative DNA-based detection.

2.5 Ionic strength, pH, water activity and chaotropic/kosmotropic activity quantification

Ionic strength was calculated using the following equation:

$$I = 0.5\sum c_i z_i^2$$

where c_i = the concentration of ion i (in mol litre^{-1}), and z_i = the charge of ion i. pH was measured in triplicate using an Omega PHH-37 pH meter with Omega PHE 1335 probe set-up calibrated to three points (pH 4.0, 7.0 and 10.0) with standard solutions supplied by the manufacturer. Water activity was quantified using 5 ml samples at 30°C in a Rotronic HP23-AW water activity meter, calibrated to five points (a_w = 0.325, 0.595, 0.755, 0.845, and 0.935) using saturated calibration standards ($MgCl_2$, NH_4NO_3, NaCl, KCl and KH_2PO_4, respectively) prepared as described by Winston and Bates (1960). Each brine was measured three times and results were found to be within ± 0.002 a_w (data not shown). During incubation, water activity was quantified at approximately two week (14 day) intervals and found to vary by ≤ 0.008 a_w over the course of 60 day incubation periods (data not shown).

Chaotropic/kosmotropic activities of the eight brines were quantified by measuring the increase or decrease in gelation temperature of a brine/agar solution relative to a pure agar solution as described previously (Hallsworth *et al.*, 2003; Cray *et al.*, 2013). An increase in agar-gelation temperature relative to that of pure agar was indicative of kosmotropicity, whereas a decrease in gelation temperature was

indicative of chaotropicity. Where brines caused precipitation of agar, a dilution series was made in order to construct curves that were used to derive extrapolated values (see Cray *et al.*, 2013).

2.6 Ionic composition analysis

Magnesium, potassium, sodium, chloride and sulfate ions were analysed at the University of Edinburgh, UK via ion chromatography using a Dionex DX-120 system fitted with a conductivity detector, according to manufacturer's instructions., Total iron concentrations were quantified via atomic absorption spectroscopy using a Perkin Elmer AAnalyst 200 spectrometer. Radiation was provided at 248.3 nm by an iron hollow cathode lamp (slit 1.8/1.35), and measurements were integrated over 5 seconds and performed in triplicate.

Changes in ferrous and ferric iron concentrations in Control-4 were monitored colourimetrically throughout incubation periods using the ferrozine assay as previously described (Stookey, 1970). Briefly, samples were digested in 0.5 M HCl for 1 hour and added to HEPES-buffered ferrozine solution. Absorbance was measured at 562 nm in a Helios Alpha spectrophotometer (Thermo Fischer Scientific).

Bicarbonate concentrations in Type I martian brines were quantified by titrimetric determination of alkalinity. Samples were titrated with HCl until pH 4.5 was reached, indicating all bicarbonate had been neutralised. HCO_3 concentration was then determined using the equation:

$$A = \frac{c(HCl) * v_1}{v_2} * 1000$$

where A is the total alkalinity (in mg/l), $c(HCl)$ is the concentration (mol/litre) of the HCl solution used, v_1 is the volume of HCl titrated and v_2 is the volume of sample used.

2.7 Comparison with physicochemical data from terrestrial brines

For comparisons of martian brines and terrestrial brine environments, physicochemical data was derived from sites summarised in Table 5. When not reported in the source publications, pH and water activity

of natural terrestrial brines were calculated from ionic composition using the thermodynamic model FREZCHEM version 16 (Marion and Kargel, 2008). FREZCHEM v. 16 employs Pitzer equations for calculating ion interactions at high ionic strength. Ion compositions were converted from units reported in source publications to moles kg(water)$^{-1}$ and calculations were performed at 30^{o} C, with pH controlled through equilibrium between H^{+} and CO_2 (gaseous) at approximately terrestrial atmospheric partial pressure (0.04 atm). For more information, see Marion and Kargel (2008).

2.8 PCR amplification

Community DNA was interrogated by bacterial, archaeal and eukaryotic domain-specific primers targeting ribosomal small sub-unit (SSU) RNA. For oligomer sequences used as primers in the current study, see Table 4. Each individual 25 µl PCR reaction contained 1 µl template, 0.4 µM of the relevant forward and reverse primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1x PCR buffer and 1 unit *Taq* polymerase (Invitrogen). PCR conditions were as follows: for 28F-519R, reactions were subjected to denaturation at 95^{o}C for 5 minutes, followed by 30 cycles of 94^{o}C for 30 seconds, annealing at 60^{o}C for 40 seconds and extension at 72^{o}C for 60 seconds, and finished with a final extension step at 72^{o}C for 10 minutes. For 341F-958R, reactions were subjected to denaturation at 95^{o}C for 5 minutes, followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 54^{o}C and extension at 72^{o}C, and finished with a final extension step at 72^{o}C for 10 minutes. For Euk1A-516R, reactions were subjected to denaturation at 94^{o}C for 5 minutes, followed by 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 56^{o}C for 45 seconds and extension at 72^{o}C for 60 seconds, and finished with a final extension step at 72^{o}C for 5 minutes. Positive PCR amplification was confirmed by electrophoresis in 1% agarose gels made up in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1.5 mM EDTA) and visualised using a SynGene G-Box UV transilluminator.

2.9 16S rRNA 454 pyrosequencing and bionformatic analyses

Martian brine enrichments originating from the composite inoculum that yielded positive DNA extractions and either bacteria or archaea domain-specific PCR amplification were pyrosequenced using the Roche 454 platform (Research and Testing Laboratory of the South Plains, Lubbock, Texas, USA).

A composite inoculum-derived Control-1 enrichment community was also sequenced for comparison. Initial trimming, denoising and chimera checking was carried out by Research and Testing (Edgar, 2010; Edgar, 2011; Edgar *et al*., 2013). OTU clustering and taxonomic identification was performed in the MOTHUR programme using previously described standard operating procedures (Schloss *et al*., 2009; Schloss *et al*., 2011; Quast *et al*., 2013). Pyrosequencing datasets were deposited with the Sequence Read Archive (NCBI) under the accession number SRP052574.

3. Results

3.1 Habitability of martian brines

Only three of the eight simulated martian brines supported microbial growth, despite several brines exhibiting permissive water activities and regardless of inoculum source or oxygen availability (Table 6). Amongst simulated martian brines, there were no differences in colonisation when diverse inoculum sources were used: those brines that were colonised were colonised by all environmental inocula tested, and those that remained uninhabited were consistently prohibitive across all inoculum sources (Table 6). Furthermore, initial enrichment stages of uninhabited brines did not yield any evidence of growth after incubation for more than 300 days.

Type I brines, similar to the composition of Na-K-Cl-HCO_3 hydrothermal brines that likely chemically altered the Nakhla martian meteorite (Bridges and Schwenzer, 2012), were colonised at both stages of concentration (Table 6). Type II brines, relevant to large areas of martian layered sulfate terrains including those in Valles Marineris, Margaritifer Sinus and Terra Meridiani (Gendrin *et al*., 2005), were inhabited at the initial dilute Stage [a], but evaporative Stage [b] was hostile to all sources of inoculum under all conditions (Table 6). Type III brines, which resemble an ancient Meridiani Planum and other Fe-Mg-SO_4-Cl Hesperian environments (Knoll *et al*., 2005), were not colonised at either stage of concentration. Consistently, exposure to the atmosphere for one month did not result in successful colonisation of Type II Stage [b] or Type III brines.

343 *3.2 Microbial communities in martian and control brines*

344 Amongst those brines that were colonised, biodiversity, cellular morphologies and growth dynamics
345 varied substantially between brine types and evaporitic stages (Figs. S1, S2). Furthermore, DNA-based
346 growth-detection procedures revealed domain-level differences between the inhabited brines (Table 7).
347 From all inoculum sources, each of the inhabited brines contained populations of Bacteria. However,
348 archaea were restricted to Type I Stage [a], Type II Stage [a] and the NaCl-dominated Control-1 (Table
349 7). Eukaryotes (fungi) were conspicuous members of the communities in low pH brines, Control-2 and
350 Control-3 (Table 7).

351 In brine enrichments that originated from the composite inoculum, archaeal and bacterial 16S rRNA
352 pyrosequencing revealed distinct prokaryotic communities which varied depending on the presence or
353 absence of oxygen (Fig. 2). The highest bacterial diversity was recorded in the anaerobic treatment of
354 the most dilute of all simulated martian brines: Type I Stage [a] (Shannon's H' = 3.500 ± 0.051; Good's
355 Coverage = 96.8 % at 97% OTU similarity; Fig. 2, S3). This community was dominated by members
356 of the Firmicutes; notably the genus *Anaerobranca* and an unclassified genus within
357 Peptostreptococcaceae (Fig. 2, S3). The aerobic treatment of this brine supported a lower-diversity
358 community in which the genera *Brevundimonas* and *Achromobacter*, Alpha- and Beta-proteobacteria
359 respectively, were dominant members (Shannon's H' = 1.445 ± 0.026; Good's Coverage = 99.1 % at
360 97% OTU similarity; Fig. 2, S3). Type I Stage [b], a later evaporative stage of Type I brines rich in
361 chloride salts supported a moderately diverse, mixed population of Firmicutes and Gamma-
362 proteobacteria including *Oceanobacillus* and *Halovibrio*, both genera known to exhibit halotolerance
363 (Takami *et al.*, 2002; Sorokin *et al.*, 2006) (Shannon's H' = 1.800 ± 0.081; Good's Coverage = 97.8 %
364 at 97 % OTU similarity; Fig. 2, S3).

365 Type II Stage [a], a magnesium- and sulfate-dominated brine with the highest divalent ion content of
366 any inhabited Mars-relevant brines supported a moderately diverse community of Firmicutes (including
367 *Bacillus*) and Actinobacteria (including *Arthrobacter*) under aerobic conditions (Shannon's H' = 1.731
368 ± 0.038; Good's Coverage = 98.5 % at 97 % OTU similarity; Fig. 2, S3), and a marginally more diverse

anaerobic community consisting mainly of facultatively anaerobic Firmicutes such as *Virgibacillus* (Shannon's H' = 2.507 ± 0.087; Good's Coverage = 95.9 % at 97% OTU similarity; Fig. 2, S3).

Amongst the sequenced communities found to contain archaea, the archaeal diversity was typically low. The anaerobic Type I Stage [a] (Shannon's H' = 0.841 ± 0.024; Good's Coverage = 99.3 % at 97 % OTU similarity) was dominated by methanogenic genus *Methanoculleus*, as well as an unclassified genus within the *Thermoplasmata* (Figs. 2, S3). Type II Stage [a], by contrast, was colonised by archaea only under aerobic conditions, and the community was entirely dominated by the *Nitrososphaera* genus within the Crenarchaeota (Shannon's H' = 0.614 ± 0.035; Good's Coverage = 99.4 % at 97 % OTU similiarity; Fig. 2, S3).

Control-1 exhibited a similar bacterial community to Type I brine Stage [b], including the Firmicutes *Oceanobacillus* and the Gammaproteobacteria *Halovibrio* (Shannon's H' = 1.466 ± 0.034; Good's Coverage = 98.9 % at 97 % OTU similarity). However, despite the similarities in bacterial community, the archaeal community in Control-1 (Shannon's H' = 0.959 ± 0.046; Good's Coverage = 98.8. % at 97 % OTU similarity) was markedly different from any simulated martian brine, being dominated by a single class of extremely halophilic archaea; the Halobacteria (Figs. 2, S3).

3.3 Physicochemical controls on martian brine habitability

3.3.1 Water activity

The currently accepted limit to life in high salt is reached at a_w = 0.611, and terrestrial environments that fall below this value are widely considered to be functionally sterile (Fig. 3a) (Stevenson *et al.*, 2015a; 2015b). Whilst the terrestrial brines with the lowest water activities, including the deep-sea Lakes *Discovery* and *Kryos* (located in the Mediterranean Sea) and Don Juan Pond in the McMurdo Dry Valleys, Antarctica, exhibit other biologically hostile physicochemical traits, their water activities fall below the minimum required for cellular division (Hallsworth *et al.*, 2007; Samarkin *et al.*, 2010; Yakimov *et al.*, 2015). Apart from in some localised environments, such as the brine/seawater interface in Lakes Kryos and Discovery, where chaotropicity defines microbial habitability (Hallsworth *et al.*,

2007; Yakimov *et al.*, 2015), water-activity sufficiently delineates the habitability of terrestrial saline environments (Fig. 3a).

The water activity of Type II Stage [b] (0.633 a_w) was close to the biophysical limit for proliferation of extreme halophiles (Stevenson *et al.*, 2015b), and lower than the water activity of any of the brines identified as habitable in the current study (Fig. 3a). By contrast, martian brine Type III Stage [a] exhibited permissive water activity (0.894 and 0.885) but did not allow growth of any microorganisms (Fig. 3a.). This was despite the inoculum communities' ability to tolerate lower water activities: Type I Stage [b] (0.789 a_w) and Control-1 (0.764 a_w) were successfully colonized. Control-3 (0.889 a_w), which was designed to directly simulate the water-activity of Type III Stage [a], also supported a community of organisms (Fig. 3a).

3.3.2 pH

Low pH can be ruled out as the sole inhibitory factor in Type III Stage [a] due to the colonisation by several inoculum sources of Control-2, which exhibited an equivalent pH to Type III Stage [a] (Fig. 3a; Tables 3, 6). However, combined stresses of low pH and low water-activity equivalent to those found in Type III Stage [a] restricted colonisation to just one inoculum source, under aerobic conditions only (Control-3; pH 2.5, a_w = 0.889) (Fig. 3a; Table 6). The community from Control-3 was not able to grow in Type III Stage [a].

3.3.3 Kosmotropicity

All of the simulated martian brines investigated were found to be kosmotropic (macromolecule-rigidifying) (Fig. 3b). Type III Stage [b] exhibited a kosmotropic activity approximately equivalent to a solution of 5.5 M ammonium sulfate (Fig. 3b). This is despite Type III brines possessing high concentrations of ions including Mg^{2+}, Fe^{2+} and Cl^-, the salts of which are strong chaotropes when measured as solutions made up from pure salts (Cray *et al.*, 2013). Although Type III martian brines exhibit extreme kosmotropic activities, the $MgSO_4$-rich Type II Stage [a] was densely colonized by all inoculum sources and under both aerobic and anaerobic conditions, despite imposing a kosmotropic activity higher than the uninhabited Type III Stage [a] brines (Fig. 3b).

420 *3.3.4 Iron toxicity*

421 Despite the presence of high levels of iron in Type III brines, iron-induced oxidative stress can be
422 eliminated as the sole determinant of their habitability. An aerobic community of bacteria from a single
423 inoculum source (the acidic hydrothermal pool inoculum; see Materials and Methods) became
424 established and grew successfully at pH 1.95 in the presence of approximately 600 mM dissolved iron
425 in Control-4 (Fig. S2; Tables 6, 7). This result is significant; no other inoculum source yielded
426 organisms capable of growing in Control-4. Type III Stage [a] brines, which contained 597 and 628
427 mM Fe, did not support the growth of these organisms.

428 *3.3.5 Ionic strength*

429 All uninhabited brines, including both Type III Stage [a] brines were characterized by extremely high
430 ionic strength (>10 mol litre^{-1}) (Fig. 3). Control-5 and Control-6 were designed to exhibit high ionic
431 strength but otherwise permissive physicochemical parameters. When all other stresses were
432 minimised, high ionic strength dramatically restricted habitability. Only the $MgSO_4$-rich Basque Lakes,
433 British Columbia, which possess one of the highest divalent : monovalent ratios known in terrestrial
434 brines (see Fig. 1, Materials and Methods), contained organisms capable of growth in Control-5 (ionic
435 strength = 12.141 mol litre^{-1}; 0.821 a_w; pH 7.0), and these only grew in the presence of oxygen (see
436 Figs. 3c-d, S1, S2). Domain-specific PCR revealed that the colonising population consisted solely of
437 bacteria (Table 7). Although they were tolerant of ionic strength higher than that found in Type III Stage
438 [a] brines, the bacteria that colonised Control-5 were not capable of growth in Type III Stage [a].

439 The level at which ionic strength becomes inhibitory was influenced by water activity. At moderate
440 ionic strength (5 mol litre^{-1}) and 0.764 a_w in Control-1, rapid and extensive growth was observed (Figs.
441 3d, S1). However, at a slightly higher water activity (0.801) but greatly increased ionic strength
442 (Control-6; 10.131 mol litre^{-1}), growth was inhibited under both oxygenated and anoxic conditions,
443 regardless of inoculum source (Table 6). The Control-6 brine was the only control to remain uninhabited
444 after inoculation across all inoculum sources. This was despite Control-6 exhibiting permissive water
445 activity (0.801), pH (7.1), kosmotropicity (-76.42 kJ mol^{-1}) and iron concentration (approximately 50

446 µM), levels which were directly demonstrated to be habitable by other control and martian brines (Fig.
447 3). Initial enrichments of Control-6 were also devoid of growth after incubation for a period of >300
448 days.

449

450 **4. Discussion**

451 *4.1 Microbial communities in martian brines*

452 Brines relevant to saline environments on Mars supported distinct, complex active microbial
453 communities following inoculation by a variety of environmental sources. Variations in microbial
454 community structure revealed by molecular analyses on the domain (Table 7), phylum and class (Fig.
455 2) and genus levels (Fig. S3), as well as different growth dynamics and cell densities (Fig. S1, S2)
456 demonstrated that differing ionic compositions can have an important influence in defining community
457 structure. The notable detection of methanogenic Archaea in anaerobic treatments of Type I Stage [a],
458 which was the most dilute Mars-relevant brine and most closely aligned with the Gale Crater
459 paleoenvironment (Léveillé *et al*., 2014) shows that biological methanogenesis is possible in ancient
460 Mars-relevant fluids. One plausible explanation for methanogenic growth is the production of hydrogen
461 through fermentation driven by the bacterial community in this brine.

462 One notable finding from the microbial community composition data was that in all cases, martian brine
463 microbial communities were distinct from that of Control-1, which represents the typical composition
464 of NaCl-rich terrestrial environments. The high abundance of one particular archaeal genus
465 (*Haloarcula*) in Control-1 is typical of NaCl brine lakes, which during blooms can become dominated
466 by relatively few microbial taxa (in comparison to lower salinity lakes) (Benlloch *et al*., 2002; Oren and
467 Hallsworth, 2015). Despite some martian brines supporting colonisation by known NaCl-tolerant
468 bacteria, they all lacked halophilic Archaea and other common inhabitants of NaCl-dominated brines
469 (Fig. 2, S3). Instead, they supported a diverse community of primarily non-halophilic organisms. This
470 observation provides a direct demonstration that Martian brine environments are distinct from terrestrial
471 brines and that the different geochemical histories of brines have implications for the types of

communities that they can potentially support. These data also show that the use of terrestrial brines as analogues for brines found on Mars cannot necessarily reveal the microbial habitability of the latter; instead it is important to augment field studies with the synthesis of martian brines in the laboratory to understand more empirically the factors that define microbial habitability.

4.2 Factors that influence the habitability of martian brines

We systematically investigated the factors that influence habitability in extreme martian brines. This revealed that the habitability of Type I and II brines was consistent with predictions made from water activity. These relatively dilute brines supported growth at water activities above the currently accepted limit for life (0.611), except for Type II Stage [b] which was close to this limit (0.633). There have thus far been only three halophilic bacteria or Archaea reported to grow at < 0.700 water activity, according to empirical determinations (Stevenson *et al.* 2015a; 2015b). However, Type III Fe-Mg-SO_4 brines were not habitable, even when possessing biologically permissive water activity (Fig. 3; Table 6).

The control solutions that we synthesised allowed us to identify the different physical and chemical extremes associated with the brines and to determine whether they, alone, can explain the habitability of the Type III brines. Low water activity (down to 0.764 a_w), low pH (down to 1.95) and high kosmotropic activity (up to -324.35 kJ kg^{-1}) were ruled out as sole inhibitory factors in Type III Stage [a] brines due to the colonisation of control solutions possessing these extremes (Fig. 3; Table 6). Colonisation of these control brines also rules out osmotic changes experienced by the inoculum communities during transfer from their source environment as the determinant of ability to grow in Type III Stage [a]. Organisms would have experienced equivalent or greater osmotic changes in the control solutions, and growth was not precluded.

High kosmotropocity in martian brines is notable; whilst chaotropicity can be a life-limiting parameter in diverse types of natural environments (e.g., Hallsworth *et al.*, 2007; Cray *et al.*, 2015; Yakimov *et al.*, 2015), the level of kosmotropicity encountered in Type III martian brines (Fig. 3b) is rarely, if ever, encountered on Earth (Williams and Hallsworth, 2009; Lievens *et al.*, 2015). The biophysical mechanisms which give rise to chaotropic/kosmotropic activities of solutes are extremely complex and

498 not fully understood (Ball and Hallsworth, 2015). Such a high kosmotropic activity as that found in
499 Type III martian brines, despite the presence of chaotropic salts (such as $MgCl_2$ and $FeCl_2$) highlights
500 the need for empirical determinations of these activities in studies of natural environments, as
501 kosmotropicity of complex mixtures cannot be predicted from those of pure salt values (Alves *et al.*,
502 2015; Yakimov *et al.*, 2015). Nevertheless, the establishment of microbial communities in Type II Stage
503 [a] (-270.69 kJ kg^{-1}) and Control-5 (-324.35 kJ kg^{-1}), brines with higher kosmotropicity than Type III
504 Stage [a], demonstrates that kosmotropicity at these levels alone does not limit microbial growth (Fig.
505 3b).

506 If we consider the number of environmental inocula established in each brine to be a crude proxy of its
507 habitability, the data also allow us to extract generalisations regarding the biological hostility of single
508 and combined extremes (Table 6). Combined low pH/low water activity (Control-3), iron toxicity
509 (Control-4) and high ionic strength (Control-5) all only allowed growth from one inoculum source,
510 which differed for each of these controls. This shows that although these extremes in isolation do not
511 prevent growth from all of the inocula used, they do restrict colonisation to organisms from only one
512 environment, suggesting that these extremes contribute to the limits of habitability of the most extreme
513 martian brines (Fig. 3; Table 6).

514 This finding is consistent with previous observations. Coping with co-occurring extremes of low pH
515 and low water activity demands energetically expensive homeostasis strategies, and this combination is
516 known to restrict the growth of terrestrial microorganisms (Harrison *et al.*, 2013; 2015). Iron toxicity is
517 caused primarily by the generation of oxidative hydroxide radicals through Fenton's reaction series
518 (Gutteridge and Halliwell, 1989), and the hostility of this process toward biologically-important organic
519 molecules has previously been demonstrated in simulated martian brines (Johnson and Pratt, 2010).
520 Ionic strength, a measure of charge density, is capable of inducing structural deformities and inhibition
521 of biological molecules (Baldwin, 1996; Kohn *et al.*, 1997; Kunz *et al.*, 2004; Cray *et al.*, 2013). At
522 high ionic strength, therefore, the magnitude and extent of ion-biomolecule interactions may function
523 as a stressor on microbial cells.

524 *4.3 Ionic strength is a novel factor that limits the habitability of martian aqueous environments*

525 Ionic strength was found to limit the habitability of control brines. Colonisation was restricted to only
526 one inoculum source in Control-5 (ionic strength = 12.141 mol litre^{-1}), which possessed a relatively
527 clement water activity (0.821 a_w). Furthermore, growth was inhibited entirely in Control-6 (ionic
528 strength = 10.131 mol litre^{-1}), which exhibited a lower, but still demonstrably permissive, water activity
529 (0.801 a_w) (Table 3). Given the effects that low water activity has on habitability when exerted in
530 conjunction with other extremes, such as low pH in Control-3, the difference in water activity between
531 Control-5 (0.821 a_w) and Control-6 (0.801 a_w) likely explains the capacity of the former to support some
532 restricted growth. These data indicate that in martian brines with high divalent ion content, particularly
533 the Type III brines, ionic strength can act as a barrier to habitability.

534 Ionic strength *per se* has not previously been considered as an important parameter in restricting
535 microbial growth in natural environments. This is likely due to the dearth of large-scale environments
536 on Earth with sufficient divalent ion content. Terrestrial saline waters, which typically exhibit low
537 divalent : monovalent ratios (Fig. 1) (Eugster and Hardie, 1978), only develop high ionic strength in
538 extremely concentrated brines that also impose hostile water activities (Fig. 3d). Indeed, even Mg^{2+}-
539 rich bittern brines commonly contain chloride as the dominant anion, ensuring that the divalent :
540 monovalent ratio does not exceed 1 (Fig. 1). By contrast, throughout large periods of Mars's surface
541 evolution, high divalent : monovalent ion ratios were common (Catling, 1999; Vaniman *et al.*, 2004;
542 Knoll *et al.*, 2005; Tosca *et al.*, 2011), allowing the formation of brines with high ionic strength, even
543 at moderate, biologically permissive water activities (Figs. 1, 3d).

544 It is thought that more than 99% of microorganisms on Earth resist cultivation using current techniques
545 (Amann *et al.*, 1995). Therefore, it cannot be ruled out that organisms currently resistant to cultivation
546 exist which are capable of growth under the conditions found to be uninhabitable in this study. This
547 potential bias was mitigated here by studying a wide range of inocula and using enrichment
548 communities. Cultured communities simulate the complex interdependences of organisms in the natural
549 environment and thus capture a more representative snapshot of natural microbial assemblages (Alain
550 and Querellou, 2009).

551 The data obtained in the current study demonstrate that a sampling or experimental bias does not explain
552 our results: many organisms were successfully enriched under single or combined conditions found in
553 Type III martian brines, and yet were not capable of growth in Type III Stage [a], even after incubation
554 for > 300 days. This lack of growth, observed across all inoculum sources and independent of the
555 presence or absence of oxygen, must therefore be attributable to conditions present in the Type III
556 martian brines but which are not present in the habitable martian and control brines. Based on the
557 elimination of other possible explanations, ionic strength must be one of these conditions that limits
558 habitability in martian brines.

559 *4.3 Conclusions and implications*

560 Martian brines are complex, multi-stress environments that present significant challenges to biology.
561 The results presented here support the hypothesis that high ionic strength can restrict habitability in
562 high salt environments, even if water activity is permissive. In combination with other restrictive
563 extremes such as high iron concentration and combined low pH/low water activity, high ionic strength
564 explained the lack of colonisation in Type III martian brines. Ionic strength can therefore act as a barrier
565 to martian habitability.

566 We note that our results are conservative, since when combined with other multiple stressors such as
567 low temperature, low energy availability and high radiation flux, as might be expected on Mars, the
568 brines would likely be even more hostile than under the conditions investigated hereAs brines with
569 extremely high divalent ion content have formed on Mars but do not commonly form on the Earth, these
570 findings are an example of how differing planetary-scale geochemistries, themselves dictated by
571 geologic evolution, can drive fundamental differences in habitability. On Earth, a chloride and
572 monovalent ion-rich aqueous chemistry permits the microbial colonisation of brines with exceptionally
573 low water availability; indeed close to the absolute limit for life. By contrast, on Mars a chemistry
574 dominated by divalent ions such as sulfates means that high ionic strength constrains habitability to a
575 smaller window. An enrichment of divalent ions relative to the Earth may not be limited to Martian
576 aqueous geochemistry. There is evidence that the putative subsurface ocean on Europa may contain

significant amounts of Mg^{2+} and SO_4^{2-} ions (Orlando *et al.*, 2005). Constraints placed on this composition by future missions will allow for a prediction of the habitability of this Jovian satellite.

Whereas brines are considered a reservoir of possibly habitable liquid water on present-day Mars, their prohibitively high ionic strength now casts doubt on this assumption. We question whether the definition of Mars Special Regions based on temperature and water activity alone (Rummel *et al.*, 2014) is sufficiently conservative for the purpose of planetary protection. High ionic strength may render an environment uninhabitable even if temperature and water activity (currently used to define Special Regions) are permissive. Meaningful assessments of biological permissibility for such brines is critical, both in considerations for extant or historical martian biota and in considering regions at risk from contamination with terrestrial microbes. These data also challenge the paradigm of 'Follow the Water' in Mars exploration (Hubbard *et al.*, 2002), demonstrating experimentally that aqueous environments need not be habitable. Indeed, martian brines may be some of the least promising places to search for life.

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809

Figure Legends

FIG. 1; Divalent : monovalent ratios plotted against water activity of modelled martian brines (circles) and terrestrial brine environments (squares) (Tosca *et al.*, 2011). For details of terrestrial brine calculations and sources, see Materials and Methods and Table 5.

FIG. 2; Relative abundances of bacterial phyla **(a)** and archaeal classes **(b)** in inhabited martian (Type I and II) brines and a typical terrestrial brine (Control-1), as detected by 16S pyrosequencing. Communities represented are those that originated from the composite inoculum. Legend indicates whether clades were detected in aerobic brines (A), anaerobic brines (An) or both (A/An). Shannon's H' is displayed to the right of each bar.

FIG. 3; Habitability of simulated martian brines (Type I-III, Stages [a] and [b]), control brines (C-1 to C-6) and terrestrial examples plotted as a function of water activity and pH **(a)**, water activity and chao-/kosmotropicity **(b)**, ionic strength and pH **(c)**, or water activity and ionic strength **(d)**. Categories represented are: habitable, this study (green-filled circles), restricted habitability (colonisation by only one inoculum source), this study (blue hashed triangles), uninhabitable, this study (empty circles), terrestrial, inhabited (green-filled squares) and terrestrial, uninhabited (empty squares). Red line in **(a)**, **(b)** and **(d)** indicates the currently acknowledged limit to life in high salt described by water activity at $a_w = 0.611$ (Stevenson *et al.*, 2015b). Grey dotted line in **(b)** indicates the chaotropic activity of a 2.3 M pure $MgCl_2$ solution; a level which is thought to be inhibitory to life (Hallsworth *et al.*, 2007). Orange shaded area in **(c)** and **(d)** indicates conditions at which ionic strength acts as a mediator of habitability. Arrows indicate direction of modelled evapoconcentration (Tosca *et al.*, 2011). For details of terrestrial brine calculations and sources, see Materials and Methods and Table 5.

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836

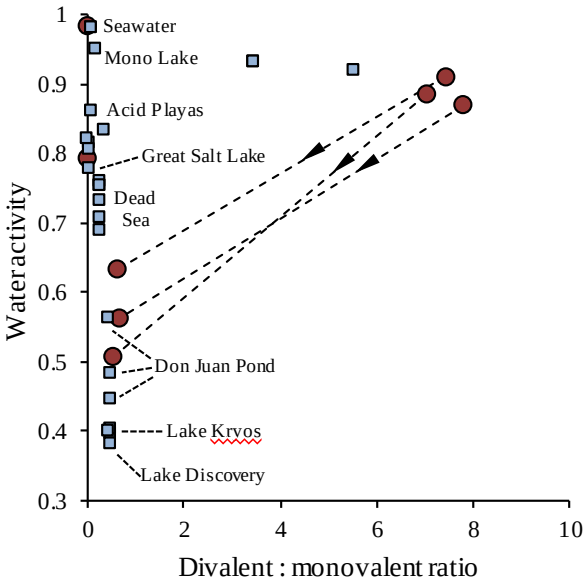

Figure 1

837

838

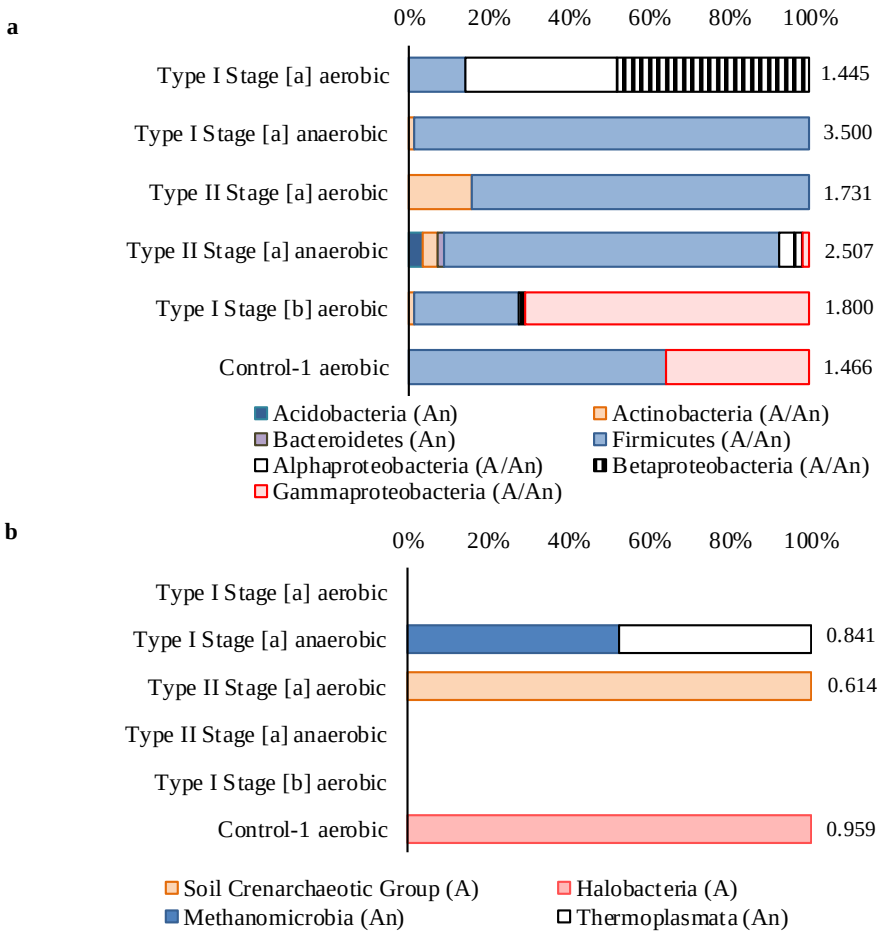
a
0%
20%
40%
60%
80%
100%
Type I Stage [a] aerobic
Type I Stage [a] anaerobic
Type II Stage [a] aerobic
Type II Stage [a] anaerobic
Type I Stage [b] aerobic
Control-1 aerobic
1.445
3.500
1.731
2.507
1.800
1.466
Acidobacteria (An)
Actinobacteria (A/An)
Bacteroidetes (An)
Firmicutes (A/An)
Alphaproteobacteria (A/An)
Betaproteobacteria (A/An)
Gammaproteobacteria (A/An)
b
0%
20%
40%
60%
80%
100%
Type I Stage [a] aerobic
Type I Stage [a] anaerobic
Type II Stage [a] aerobic
Type II Stage [a] anaerobic
Type I Stage [b] aerobic
Control-1 aerobic
0.841
0.614
0.959
Soil Crenarchaeotic Group (A)
Halobacteria (A)
Methanomicrobia (An)
Thermoplasmata (An)

Figure 2

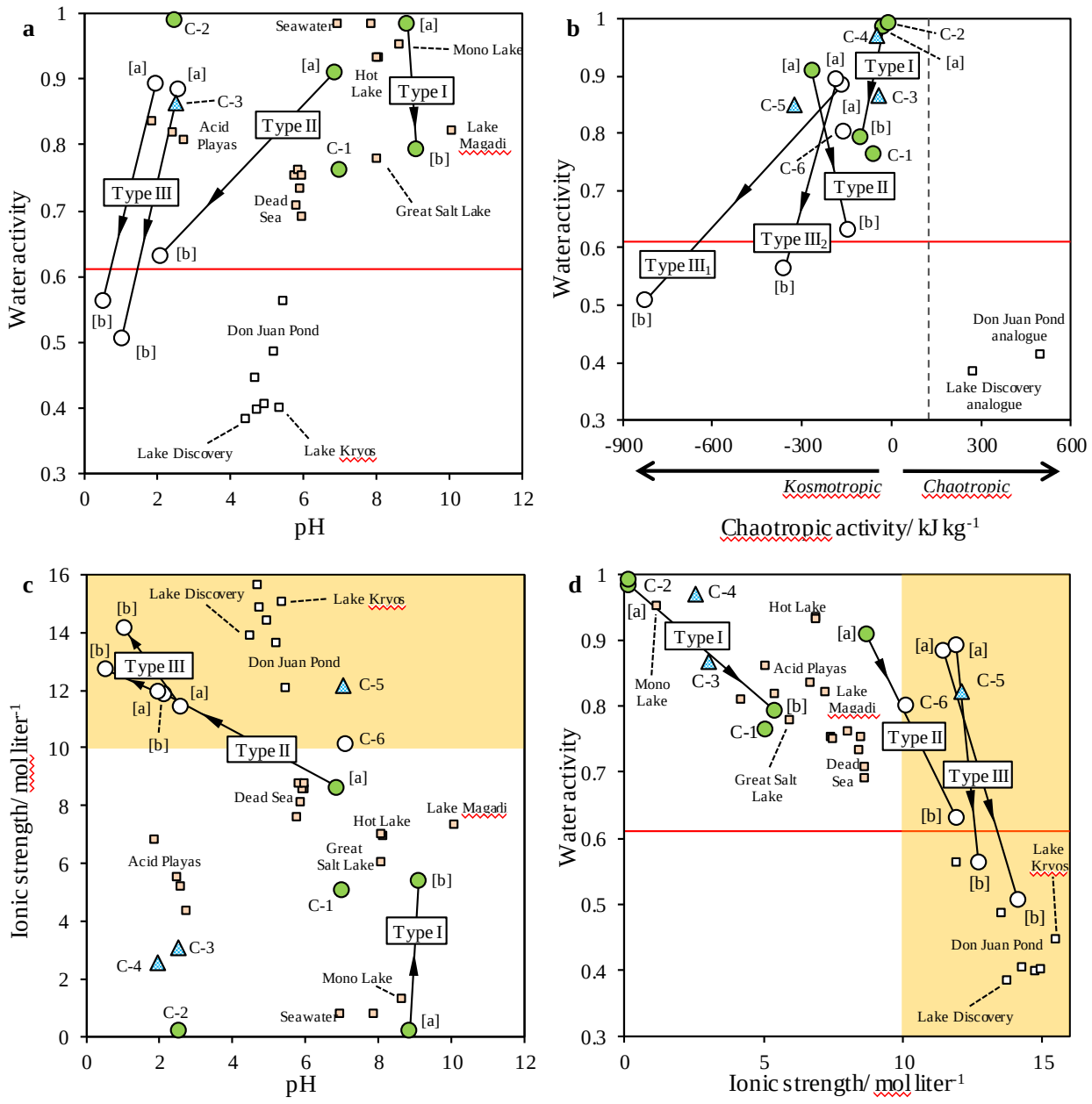
a
Water activity
pH
C-2
Seawater
[a]
Mono Lake
Hot Lake
Type I
Type II
Type III
C-3
Acid Playas
C-1
Lake Magadi
Dead Sea
Great Salt Lake
[b]
Don Juan Pond
Lake Discovery
Lake Kryos
b
Water activity
Chaotropic activity/ kJ kg-1
C-4
C-5
C-6
Type III1
Type III2
Don Juan Pond analogue
Lake Discovery analogue
Kosmotropic
Chaotropic
c
Ionic strength/ mol liter-1
pH
d
Water activity
Ionic strength/ mol liter-1

Figure 3

841
842
843 **Table 1;** Salts added during synthesis of martian brines. Concentrations are in moles litre^{-1}. All brines
844 were also supplemented with 4 g L^{-1} yeast extract. Values calculated from Table 5 in Tosca *et al.*,
845 (2011).

846

	Type I Stage [a]	Type II Stage [a]	Type III$_1$ Stage [a]	Type III$_2$ Stage [a]	Type I Stage [b]	Type II Stage [b]	Type III$_1$ Stage [b]	Type III$_2$ Stage [b]
Designation in Tosca *et al*. 2011	Brine 1, Stage 1	Brine 2, Stage 1	Brine 4, Stage 1	Brine 5, Stage 1	Brine 1, Stage 2	Brine 2, Stage 2	Brine 4, Stage 2	Brine 5, Stage 2
$NaHCO_3$	0.126	-	-	-	-	-	-	-
$KHCO_3$	0.028	0.041	-	-	2.237	-	-	-
KCl	0.022	0.020	0.075	0.086	3.776	1.033	1.142	0.583
$MgCl_2{\cdot}6H_2O$	0.001	0.056	-	-	-	1.154	3.007	1.895
NaCl	-	0.154	0.189	0.215	1.266	2.265	1.036	1.458
$MgSO_4{\cdot}7H_2O$	-	2.068	3.066	3.016	-	2.550	-	0.407
$FeSO_4{\cdot}7H_2O$	-	-	1.225	1.282	-	-	2.313	1.987
$FeCl_2{\cdot}4H_2O$	-	-	0.208	0.153	-	-	0.985	-
HCl	-	-	-	0.254	-	0.038	0.113	-
H_2SO_4	-	-	-	-	-	-	-	0.860

847

848

849 **Table 2;** Salts added during synthesis of control brines. These were designed to test the tolerance of
850 our inoculum communities to low water activity (Control-1), low pH (Control-2), combined low water
851 activity/low pH (Control-3), combined high iron concentration/low pH (Control-4) and high ionic
852 strength (Control-5 and Control-6). Concentrations are in moles litre^{-1}. All brines were also
853 supplemented with 4 g L^{-1} yeast extract.

	Control-1	Control-2	Control-3	Control-4	Control-5	Control-6
KCl	0.094	0.010	0.010	0.010	0.010	0.010
$MgCl_2 \cdot 6H_2O$	0.143	-	-	-	0.333	1.500
NaCl	4.107	0.086	2.995	-	-	-
$MgSO_4 \cdot 7H_2O$	0.142	0.002	-	-	1.75	1.75
$FeSO_4 \cdot 7H_2O$	-	-	-	0.620	-	-
$(NH_4)_2SO_4$	-	0.023	0.023	0.023	-	-
K_2HPO_4	-	0.002	0.002	0.002	-	-
Na_2SO_4	-	-	-	-	1.500	-

854

855

Table 3; Ionic composition, pH, water activity (a_w), ionic strength and kosmotropic activity of all experimental brines used in the current study. Concentrations are in mol litre^{-1}

Brine	Na	Mg	K	Fe	SO_4	Cl	HCO_3	HPO_4	NH_4	pH	a_w	Ionic strength/ mol litre^{-1}	Kosmotropicity/ kJ kg^{-1}
Type I Stage [a]	0.126	0.001	0.05	-	-	0.025	0.154	-	-	8.860	0.984	0.180	-27.05
Type II Stage [a]	0.154	2.124	0.061	-	2.068	0.307	0.041	-	-	6.860	0.929	8.667	-270.69
Type III_1 Stage [a]	0.162	2.354	0.064	0.628	2.549	0.56	-	-	-	2.580	0.885	11.456	-163.57
Type III_2 Stage [a]	0.18	2.425	0.069	0.597	2.751	0.49	-	-	-	1.96	0.894	11.916	-183.30
Type I Stage [b]	0.761	-	4.702	-	-	3.255	2.086	-	-	9.100	0.789	5.402	-101.75
Type II Stage [b]	1.631	2.974	0.664	-	1.273	4.53	-	-	-	2.090	0.633	11.906	-148.97
Type III_1 Stage [b]	0.491	2.238	0.327	2.131	0.528	7.864	-	-	-	1.020	0.507	14.133	-828.04
Type III_2 Stage [b]	1.285	1.729	0.505	1.482	1.42	5.131	-	-	-	0.5	0.563	12.722	-360.47
Control-1	4.107	0.285	0.094	-	0.142	4.201	-	-	-	7.000	0.764	5.055	-59.28
Control-2	0.086	0.002	0.006	-	0.025	0.087	-	0.002	0.045	2.500	0.991	0.166	-12.33
Control-3	2.995	-	0.012	-	0.023	3.005	-	0.002	0.046	2.500	0.889	3.077	-59.74
Control-4	0.002	--	0.015	0.618 *	0.610	0.002	-	0.002	0.046	1.950	0.969	2.558*	-45.32
Control-5	2.669	2.369	0.036	-	2.840	0.739	-	-	-	7.050	0.821	12.141	-324.35
Control-6	0.013	3.104	0.028	-	1.087	3.420	-	-	-	7.080	0.801	10.113	-160.73

*Iron concentration and resulting ionic strength taken as average measured iron concentration over incubation period. See Materials and Methods and Fig. S1.

Table 4; Primers used in this study.

Primer	Sequence (5'-3')	Specificity	Product size/bp	Reference
28F	GAGTTTGATCNTGGCTCAG	Bacteria 16S rRNA	491	La Duc *et al*., 2012
519R	GTNTTACNGCGGCKGCTG			
341F	GYGCASCAGKCGMGAAW	Archaea 16S rRNA	617	La Duc *et al*., 2012
958R	GGACTACVSGGGTATCTAAT			
Euk1A	CTGGTTGATCCTGCCAG	Eukarya 18S rRNA	560	Diez *et al*., 2001
Euk516R	ACCAGACTTGCCCTCC			

868 **Table 5;** Sources of composition and physicochemical parameters for terrestrial brine examples. a_w = water activity.

	Location	Ionic composition	a_w		pH		Ionic strength	
		Source	Value	Source	Value	Source	Value	Source
Acid Playas	Western Australia	Bowen and Benison, 2009	0.834, 0.816, 0.806, 0.860	calculated	1.90, 2.50, 2.80, 2.60	Conner and Benison, 2013	6.727, 5.488, 4.260, 5.131	calculated
Seawater	Southern Ocean, Pacific Ocean, Arctic Ocean	Bowen and Benison, 2009	0.981, 0.981	calculated	7.92, 6.99	Bowen and Benison, 2009	0.721, 0.713	calculated
Hot Lake	Washington, USA	Lindermman *et al.*, 2013	0.932	calculated	8.15	Lindermman *et al.*, 2013	6.914	calculated
Mono Lake	California, USA	Eugster and Hardie, 1978	0.950	calculated	8.70	Eugster and Hardie, 1978	1.217	calculated
Lake Magadi	Kenya	Grant *et al.*, 1999	0.819	calculated	10.13	Grant *et al.*, 1999	7.280	calculated
Great Salt Lake	Utah, USA	Eugster and Hardie, 1978	0.776	calculated	8.10	Eugster and Hardie 1978	6.000	calculated
Dead Sea	Israel	Krumgalz and Millero, 1982	0.752, 0.760, 0.751, 0.732, 0.706, 0.688	Krumgalz and Millero, 1982	5.80, 5.90, 6.00, 5.95, 5.86, 6.00	Krumgalz and Millero, 1982	7.505, 8.079, 8.536, 8.520, 8.668, 8.709	calculated
Don Juan Pond	McMurdo Dry Valleys, Antarctica	Siegel *et al.*, 1983	0.562, 0.483, 0.396, 0.445, 0.402	calculated	5.52, 5.24, 4.80, 4.72, 5.00	calculated	11.990, 13.590, 14.796, 15.579, 14.319	calculated
Lake Discovery	Deep Mediterranean	Wallman *et al.*, 2002	0.382	Hallsworth *et al.*, 2007	4.50	Wallman *et al.*, 2002	13.796	calculated
Lake Kryos	Deep Mediterranean	Yakimov *et al.*, 2015	0.399	Yakimov *et al.*, 2015	5.40	Yakimov *et al.*, 2015	15.000	calculated

869

870 **Table 6;** Habitability of simulated martian brines and control brines. Columns correspond to the
871 different inoculum sources used, and to oxygen status (whether aerobic or anaerobic conditions). The
872 + indicates successful colonisation and the – indicates lack of growth. *nd* = not determined.
873

	Composite		Kverkfjöll		Basque Lakes 874	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Type I Stage [a]	+	+	+	+	+	+
Type I Stage [b]	+	+	*nd*	*nd*	*nd*	*nd*
Type II Stage [a]	+	+	+	+	+	+
Type II Stage [b]	-	-	-	-	-	-
Type III_1 Stage [a]	-	-	-	-	-	-
Type III_2 Stage [a]	-	-	-	-	-	-
Type III_1 Stage [b]	-	-	-	-	-	-
Type III_2 Stage [b]	-	-	-	-	-	-
Control-1	+	+	+	-	*nd*	*nd*
Control-2	+	-	+	-	*nd*	*nd*
Control-3	+	-	-	-	*nd*	*nd*
Control-4	-	-	+	-	*nd*	*nd*
Control-5	-	-	-	-	+	-
Control-6	-	-	-	-	-	-

Table 7; Domain-level diversity in all inhabited brines, across all inoculum sources, as revealed by domain-specific PCR. The + and – indicate presence or absence (respectively) of domain. Oxygen status is indicated by an *A* (aerobic conditions) or *An* (anaerobic conditions).

	Type I Stage [a]						Type I Stage [b]		Type II Stage [a]				Control-1			Control-2		Control-3	Control-4	Control-5
	Composite		*Kverkfjoll*		*Basque*		*Composite*		*Composite*		*Kverkfjoll*		*Basque*		*Composite*		*Kverkfjoll*	*Composite*		*Kverkfjoll*		*Basque*
Oxygen status	*A*	*An*	*A*	*An*	*A*	*An*	*A*	*An**	*A*	*An*	*A*	*An*	*A*	*An*	*A*	*An*	*A*	*A*	*A*	*A*	*A*	*A*
Bacteria	+	+	+	+	+	+	+	-*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Archaea	-	+	-	-	-	-	-	-*	+	-	+	-	+	-	+	+	-	-	-	-	-	-
Eukarya	+	-	+	-	+	-	-	-*	+	-	+	-	-	-	-	-	+	+	+	-	-	-

*Growth demonstrated by direct cell counts only (DNA was not successfully extracted)