BACTERIAL GROWTH TOLERANCES TO HEAVY BRINES FORMED BY AN ITERATIVE MATRIX OF IONS AND THEIR SALTS.

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Life in brine: The liquid water required for life on cold arid worlds such as Mars may be salty, since high solute concentrations depress the freezing point of water, broadening the range of potentially habitable regions and periods [1]. Sulfate and chloride salts are important constituents of Mars regolith, with Ca, Fe, Mg, and Na counterions [2]. Significant concentrations of (per)chlorate salts also are present. Brines of these salts remain liquid to low eutectic temperatures, for instance –4, –27, –23, and –69 °C for $MgSO_4$, NaCl, $NaClO_3$, and $Mg(ClO_4)_2$, respectively. Dry hygroscopic salts form deliquescent brines that can support microbial life. As the brines dry, microbes become entrapped in fluid inclusions within salt crystals, which may act as the last refugia with habitable aqueous solutions on cold arid worlds.

While microbial tolerance to NaCl has been widely studied, less is known about survival and growth in dense brines of other salts. Mars regolith is enriched in sulfates relative to Terran soil and (per)chlorates are only rarely found naturally on Earth. We have previously demonstrated bacterial growth in saturated solutions of $MgSO_4$ and at its eutectic [3]. We have found surprising microbial tolerance to chlorate salts. The mechanisms by which individual ions and their salts inhibit microbial growth are not clear. It appears that no single parameter, such as water activity (Aw) or ionic strength, determines how deleterious a brine may be to microbial proliferation and survival. Effects appear to be specific to each solute, involving complex interactions, which depend on the characteristics of the organism as well. Here we explore growth tolerances to a series of ions, testing an iterative matrix of salts for their effects on a collection of salinotolerant bacteria.

Cultivation and testing of bacterial isolates from analogue sites: Our bacteria were isolated from natural environments rich in NaCl (Great Salt Plains, OK) or $MgSO_4$ (Hot Lake, WA) [3]. The collections include *Bacillus*, *Halomonas, Marinococcus, Nesterenkonia*, *Planococcus*, and *Virgibacillus* isolates that grow at ≥10% NaCl (1.7 M), ≥50% (~2.0 M) $MgSO_4$, and >20% (~2.0 M) Na chlorate [3]. For the present study, 18 salinotolerant isolates were chosen that vary in their responses to high concentrations of salts and sugars. Salt Plains (SP) medium was supplemented with salts at increasing concentrations to saturation. Microbial growth was measured in triplicate shake-tube cultures by turbidity at 600 nm.

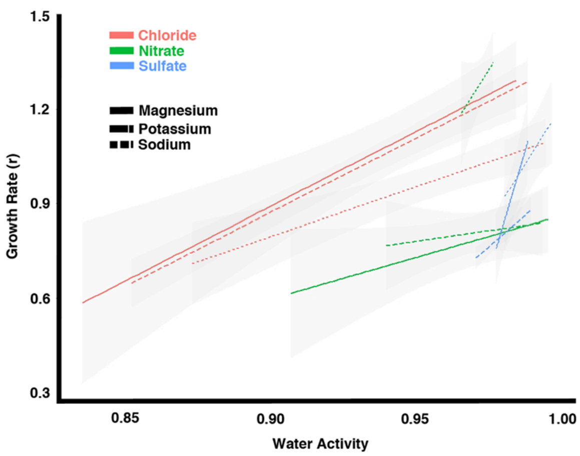
Iterative matrix of salt tolerances: We have tested the anions chlorate, chloride, nitrate, perchlorate, and sulfate coupled in every (commercially-available, water-soluble) combination with the cations Ca, Fe, Mg, K, and Na. Salts of ammonium, borate, Cs, Li, and phosphate also have been tested. Strong bacterial growth among the 18 isolates was commonly observed in salts at ≥1 M (see Table). Less tolerance was observed with (per)chlorate salts. Borate, Cs, and Fe were broadly inhibitory to growth at low concentrations. *Halomonas* sp. str. HL12 and *Marinococcus* sp. str. HL11, both from Hot Lake, were the most tolerant isolates overall.

Maximum Measured Growth Tolerances Among 18 Salinotolerant Bacteria in Dense Brines

Salt Tested	Tolerance (M)	Water Activity (Aw)
$CaCl_2$	0.5	0.984
$Ca(NO_3)_2$	0.5	0.980
$Ca(ClO_4)_2$	0.1	0.994
$MgCl_2$	2.0	0.835
$Mg(NO_3)_2$	2.0	0.907
$Mg(ClO_4)_2$	0.1	0.992
$MgSO_4$	2.0	0.978
$KClO_3$	1.0	0.980
KCl	4.0	0.890
KNO_3	2.0	0.966
$KClO_4$	1.0	0.970
K_2SO_4	0.8	0.981
$NaClO_3$	2.7	0.890
NaCl	3.0	0.883
$NaNO_3$	3.0	0.883
Na_2SO_4	1.5	0.975
$NaClO_4$	0.5	0.980

Correlations between biological effects and physical qualities of ions and salts: Over 4000 time-series were fit in MatLab (R2019a) to logistic growth curves of the 18 salinotolerant bacteria treated with various salts and salinities, to estimate the intrinsic rate of population increase (r) and maximum culture density (K). Initial analyses are presented here for an iterative matrix of 3 cations (Mg, K, Na) and 3 anions (Cl, NO_3, SO_4). We ran exploratory analyses on Aw, ionic strength, and degree of saturation to identify their interactions with the iterative salt matrix for a subset of all combinations of the ions (*N* = 1,051). To remove the influence of individual strain differences, we used mixed effects models with strain identity as a random effect (lmer in 'lmer4' package in R 3.6.1).

Initial data are presented here, showing the effects of water activity on the rate of growth of bacteria for each of the 9 ion pairs. In all cases, lower Aw led to lower growth rates (P < 2.2e-16). Further, r was affected by salt-specific responses from ion pairing (anion × cation interaction, P < 2.2e-16). Responses appear to cluster by anion (P < 9.1e-15). No 3-way interactions were significant with Aw. Note that MgSO4 does not fully dissociate in solution and does not lower Aw as dramatically as chloride salts, thus the decreases in r with Aw for sulfates are more pronounced than for chlorides.



Effect of water activity on the growth rates of 18 salinotolerant bacteria over a range of permissible concentrations for salts (to saturation) in an iterative matrix of ion pairs

Similar analyses of responses to degree of saturation and ionic strength showed significant correlations between r and the physical parameters of the ions and salts tested. However, degree of saturation had a more complex 3-way interaction with anions and cations (P <0.003), indicating that the effects of changing degree of saturation on r were salt-specific. Consistent with the Aw model, significant ion-pair interactions (P = 0.007) indicate that salt-specific effects further alter microbial growth rates. Models showed 2-way interactions of ionic strength with anions (P = 0.0008) and cations with anions (P <0.0001), but not for ionic strength with cations (P = 0.15). Three-way interactions were not significant for ionic strength with anions and cations. Our analyses found that ions and salts affect bacterial growth differentially across gradients of physical solution qualities. No single physical quality adequately accounts for the observed solute-specific effects on bacterial growth.

Microbial life in saline systems. Broad tolerances to a wide variety of salts at high concentrations were observed here for a group of salinotolerant bacteria with varied physiology and taxonomy. Natural haline and carbonate environments (e.g. Great Salt Lake, Mono Lake) harbor a wealth of microbes despite extreme salinities. Similarly, epsomic environments (e.g. Hot Lake) have rich microbial communities. Our work here suggests that potash environments rich in K salts may follow suit. Nitrate salts were compatible with our salinotolerant isolates as well. As we have demonstrated previously, chlorate salts are better tolerated than perchlorate salts.

Life detection and planetary protection: Measuring the toxicity of solutes at extremely high concentrations helps to define habitable regions on celestial bodies [1]. This informs us about the types of microbes on spacecraft that pose greater risks for forward planetary contamination. Current conditions at the near-surface of Mars may support deliquescence of perchlorate salts, while the other salts examined here would require somewhat warmer conditions [2]. Unfortunately, while perchlorate salts have the lowest eutectic temperatures, these appear to be substantially more toxic than chlorate, chloride, and sulfate salts, thus far eluding a demonstration of microbial survival at eutectic concentrations of perchlorates in the laboratory [3]. Taken together, our studies have demonstrated microbial growth under the chemical conditions that may (have) occur(ed) at certain times and discrete locations on Mars, nevertheless, the near-surface environment of Mars presents a variety of stressors that would further challenge life.

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