Deliquescence-induced formation and habitability of chloride and perchlorate brines

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von der Fakultät II - Mathematik und Naturwissenschaften der Technischen Universität Berlin zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften

– Dr. rer. nat. –

genehmigte Dissertation

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Tag der wissenschaftlichen Aussprache: 12.08.2019

Berlin 2019

Preface

This thesis was written at the Center for Astronomy and Astrophysics at the Technical University of Berlin and was embedded in the "Habitability of Martian Environments" (HOME) project (ERC grant no. 339231). Most laboratory work was carried out at the laboratory of the Astrobiology Group housed at the Institute for Chemistry, and at the Center for Electron Microscopy (ZELMI) at the TU Berlin.

This study is written in English and is presented as a cumulative doctoral thesis containing an introduction, three first-author publications, and a conclusion part as well as additional material provided in the Supplementary Information. The first publication embedded in this thesis was published in the journal "Geophysical Research Letters" with an impact factor of 4.339 (2017), while the other two publications were published in the journal "Astrobiology" with an impact factor of 3.610 (2017).

Acknowledgements

I would like to express my special thanks to my first supervisor, Prof. Dr. Dirk Schulze-Makuch, for giving me the opportunity to conduct this interesting research work in the field of astrobiology and for his invaluable guidance, continuous support and encouragement. Furthermore, I would like to specially thank Prof. PhD Sam Kounaves for additional guidance, Prof. PhD Kai Finster for evaluating my doctoral thesis, and Prof. PhD Lyle Whyte for providing input on the bacterial strain *Planococcus halocryophilus* used in this study.

This thesis could not have been achieved without the help and support of numerous excellent co-authors and members of the Astrobiology Research Group at the TU Berlin, namely Dr. Janosch Schirmack, Dr. Alessandro Airo, Deborah Maus, Annemiek Waajen, Armando Alibrandi, Laura Jentzsch, Felix Arens, Yunha Hwang, Christof Sager, and Max Riekeles. I am especially grateful to Debbie for her great help with the metabolomics experiments, to Janosch, Annemiek and Armando for their support in the lab, and to *P. halocryophilus* for surviving many torturing experiments. Finally, I want to thank my beloved family and friends for their support.

Abstract

The availability of liquid water on Mars is one of the key factors for its habitability. While there is strong morphological and geochemical evidence for the existence of large water bodies on the surface of Early Mars, at the present time, the planet is dry, cold and hostile. However, liquid water might still be found in niches like cold brines. These are especially relevant for Mars since several hygroscopic salts have been detected in the Martian regolith which cause a significant freezing point depression of water and, hence, enhance the habitability range of Mars to subzero temperatures. Formation of these brines could occur through deliquescence, i.e. water absorption by these salts followed by dissolution of the salts in the absorbed water. This process might has already been observed on Mars, namely by the formation of Recurring Slope Lineae (RSL) which are dark, flow-like features extending downslope from bedrock outcrops.

In this study, both the formation process of RSL caused by deliquescence of various perchlorate (ClO₄⁻) and chloride (Cl⁻) salts, and the survivability of the halo- and cryotolerant bacterial strain *Planococcus halocryophilus* within brines has been investigated. It was found that measuring electrical conductivity (EC) is an excellent method for following the process of deliquescence-induced RSL formation. The results of these experiments revealed that the darkening of soil typical for RSL can occur very fast, e.g. after 2.5 hours for soil containing calcium perchlorate (Ca(ClO₄)₂) under the provided experimental conditions (25°C, 70–85% RH), and requires only small amounts of intergranular water. In contrast, the formation of larger amounts of bulk water requires substantially longer, e.g. 17 days for soil containing magnesium perchlorate (Mg(ClO₄)₂) under the same experimental conditions. This suggests that RSL on Mars do actually not represent flows of briny water but a rewetting of salt-cemented soils generated by the evaporation of water tracks that flowed down the hills at a time when Mars had a warmer and wetter climate.

The brines that formed via deliquescence were too concentrated to enable growth of *P. halocryophilus* within them. However, an enhanced survivability with decreasing temperature was observed. This effect was most pronounced in calcium chloride (CaCl₂) containing samples. Additionally, we found that the presence of sodium chloride (NaCl) is

beneficial for the survival of *P. halocryophilus* during freeze/thaw cycles. To enable bacterial growth in these salty samples a dilution of the brines is necessary. Hence, the maximum salt concentration suitable for growth at 25°C and 4°C was determined for six Cl⁻ and ClO₄⁻ salts. The results showed an increased CaCl₂ tolerance of *P. halocryophilus* at 4°C compared to 25°C, while the tolerances to other salts were similar or lower at 4°C compared to 25°C. The highest ClO₄⁻ tolerance reported to date was found with 12 wt% NaClO₄ at 25°C. Growth of *P. halocryophilus* under these salty conditions yielded serval stress responses like cell clustering, formation of nanofilaments, cell encrustation, and formation of different cell colony morphologies.

Putting all together, this study provides important and coherent insights in the formation and habitability of brines as they might occur on Mars. The results of a large set of experiments give an impression on how life on Mars could have adapted to its cold and salty environmental conditions and what influence different salt species and variations in temperature and salt concentration might have.

Zusammenfassung

Flüssiges Wasser ist eine der wichtigsten Voraussetzung für die Existenz von Leben auf dem Mars. Während Oberflächenstrukturen und geologische Funde darauf hindeuten, dass auf dem Mars einst größere Gewässer existierten, präsentiert sich der Planet heute als trocken, kalt und lebensunfreundlich. Es ist jedoch wahrscheinlich, dass flüssiges Wasser nicht gänzlich von der Mars-Oberfläche verschwunden ist. So gibt es Hinweise auf flüssige Salzlösungen, sogenannte "Brines", die zumindest temporär auf der Planetenoberfläche stabil sind. Die Wahrscheinlichkeit ihrer Existenz ist durch den Nachweis hygroskopischer Salze auf dem Mars noch einmal gestiegen, denn diese können in einem "Deliqueszenz" genannten Prozess Wasser aus der dünnen Mars-Atmosphäre anziehen und sich in diesem Wasser auflösen. Es gibt Hinweise darauf, dass dieser Prozess bereits in Form von jährlich wiederkehrenden, dunklen Ablaufrinnen (Recurring Slope Lineae, RSL) beobachtet wurde.

Für die vorliegende Dissertation wurden Experimente durchgeführt und ausgewertet, die sowohl das Verständnis für die Deliqueszenz-induzierte Entstehung von RSL vergrößern, als auch die sich bildenden Brines in Bezug auf ihre mikrobiologische Habitabilität untersuchen sollen. Es hat sich gezeigt, dass die bei der Bildung der RSL beteiligte Wasserabsorption hervorragend mit der elektrischen Leitfähigkeit des Bodens korreliert. Die Ergebnisse der Experimente haben verdeutlicht, dass die für RSL typische Verdunklung des Bodens sehr schnell einsetzen kann. So treten zum Beispiel im Falle von Ca(ClO₄)₂haltigen Böden bereits 2,5 Stunden nach dem Start des Experiments (25°C, 70–85% Luftfeuchte) Verdunklungen auf. Dafür werden nur geringe Mengen intergranularen Wassers benötigt. Im Gegensatz dazu bedarf es deutlich mehr Zeit bis sich größere Flüssigkeitsmengen in Form von Tropfen herausbilden, im Fall von Mg(ClO₄)₂-haltigen Böden 17 Tage. Das lässt vermuten, dass RSL auf dem Mars keine Ströme flüssigen Wassers darstellen, sondern vielmehr nur das temporäre Feuchtwerden salz-zementierter Böden, die sich beim Verdunsten von Wasser bildeten, das einst floss, als der Mars ein wärmeres und nasseres Klima aufwies.

Die Habitabilität von Brines wurde beispielhaft am Überleben und Wachstum des salzund kältetoleranten Bakterienstammes *Planococcus halocryophilus* untersucht. Es stellte sich heraus, dass Brines, die sich durch Deligueszenz bilden, zu stark konzentriert sind, um Wachstum von P. halocryophilus zu ermöglichen. Es konnte jedoch gezeigt werden, dass dessen Überlebensrate deutlich ansteigt, wenn die Temperatur der Brines gesenkt wird. Dieser positive Effekt war am stärksten in CaCl₂-haltigen Lösungen ausgeprägt. Außerdem stellte sich heraus, dass sich die Anwesenheit von NaCl positiv auf die Überlebensfähigkeit von P. halocryophilus während des wiederholten Einfrierens und Auftauens der Brines auswirkt. Um jedoch Zellwachstum in diesen Lösungen beobachten zu können, muss die Salzkonzentration reduziert werden. Daher wurde für sechs unterschiedliche Cl- und ClO4⁻-Salze bei 4°C und 25°C die maximale Salzkonzentration bestimmt, bei der noch bakterielles Wachstum nachgewiesen werden kann. Die Ergebnisse zeigten unter anderem, dass die CaCl₂-Toleranz durch Herabsenken der Temperatur von 25°C auf 4°C merklich gesteigert werden kann, während die Toleranzen gegenüber der anderen Salze bei 4°C ähnlich oder gar geringer waren als bei 25°C. Zudem weist P. halocryophilus mit 12 Gew.% NaClO₄ bei 25°C die höchste bisher beschriebene ClO₄--Toleranz auf. Zellwachstum unter diesen salzigen Bedingungen führte zu interessanten Stressreaktionen, wie zum Beispiel zur Ausbildung von Zellclustern mit Nanofilamenten, Krustenbildung um einzelne Zellen oder die Herausbildung neuer Zellkolonie-Morphologien.

All diese Funde und experimentellen Ergebnisse liefern einen entscheidenden Einblick in die Entstehung und die potentielle Habitabilität von Brines auf dem Mars. Sie vermitteln einen Eindruck wie sich Leben an die kalten und salzigen Bedingungen angepasst haben könnte und welchen Einfluss die unterschiedlichen Salztypen und Schwankungen in Temperatur und Salzkonzentration haben können.

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Abbreviations and chemical formulas

a _w	Water activity	Na⁺	Sodium (ion)
Ca ²⁺	Calcium (ion)	NaCl	Sodium chloride
CaCl ₂	Calcium chloride	NaClO ₄	Sodium perchlorate
Ca(ClO ₄) ₂	Calcium perchlorate	РВ	Phosphate buffer
Ce	Eutectic concentration	PBS	Phosphate buffer saline
CFU	Colony forming units	RH	Relative humidity
Cl	Chlorine	RSL	Recurring Slope Lineae
Cl⁻	Chloride (ion)	SEM	Scanning electron microscopy
CIO4 ⁻	Perchlorate (ion)	ST	Sample type
DRH	Deliquescence relative	т	Temperature
	humidity	Te	Eutectic Temperature
EC	Electrical conductivity	T_{EC} , T_{V}	Time until Deliquescence could
IM	Inoculation method		be observed visually (T $_{\rm V}$) or via
М	Molar (mol/L)		electrical conductivity (T _{EC})
Mg ²⁺	Magnesium (ion)	TSB	Tryptic soy broth
MgCl ₂	Magnesium chloride	v	Voltage
Mg(ClO ₄) ₂	Magnesium perchlorate	V _x	Excitation Voltage
MSCg	maximum salt concentration	wt%	Weight percentage
	suitable for growth	wt/vol%	Weight per volume (%)
N	Normalized Voltage		

1. Introduction

1.1 Environmental conditions on Mars and the Atacama Desert, Chile, as Mars-analogue field site

Characteristic surface morphologies like fluvial valleys, large fluid-eroded channels, dendritic networks and glacial features (Masson *et al.*, 2001), as well as the occurrence of minerals like hematite (Christensen *et al.*, 2000), and sulfate deposits (Squyres *et al.*, 2004), which only form in the presence of liquid water, give evidence that 4.5 - 3.6 billion years ago Mars had a warmer and wetter climate (Ramirez, 2017). It had a denser atmosphere than today and presumably large water bodies on its surface – conditions we would call appropriate for life on Earth. It is likely that this habitable period in the Martian history lasted long enough to theoretically develop life in form of simple microorganisms since we found evidence for those on Earth in 3.5 - 3.7 billions old rocks (Schopf, 1993; Ohtomo *et al.*, 2014). However, due to the loss of its magnetic field the solar wind stripped away large parts of the Martian atmosphere which caused an irreversible climate change leading to the cold, dry and hostile planet that we know from today (Vaisberg, 2015; Jakosky *et al.*, 2017). Hence, potential Martian microorganisms would have had to adapt to a gradually decrease in water availability and to lower water activities.

Such a transition from a wet and habitable to a dry and hostile environment over geological timescales can be investigated in several environments on Earth. One of the most studied Mars-analogue field sites in this context is the Atacama Desert in Chile (Fig. 1-1A) which had a humid climate 14 - 35 million years ago but became one of the driest places on Earth due to the Andean orogeny causing a rain shadow effect at the desert's eastern border while the Humboldt Current running parallel to the desert prevents precipitation in the western coastal areas (Dunai *et al.*, 2005).



FIG. 1-1: Photo of the Yungay Valley in the Atacama Desert, Chile, taken during the field trip in 2018 **(A)** and a section from a panorama image from the top of Husband Hill on Mars taken from NASA Mars Rover Spirit (Credit: NASA/JPL-Caltech/Cornell Univ.; NASA ID: PIA17760) **(B)**.

Investigating the microbial adaptions to the increasing dryness and the survival strategies in such a Mars-analogue environment is a major goal of the Astrobiology Research Group at the Center for Astronomy and Astrophysics at the TU Berlin where this thesis was elaborated. Results achieved from several field trips gave evidence that even in the hyperarid Atacama soils microbial life can be active, at least temporally (Schulze-Makuch *et al.*, 2018).

However, the environmental conditions on today's Mars (Fig. 1-1B) are even more challenging. There, microbes would have to struggle with low temperatures (-138°C – +30°C (Jones *et al.*, 2011)), low pressures (6 – 8 mbar (Catling, D.C., Leovy C. 2006)), and high doses of galactic cosmic rays (180 – 225 μ Gy/day (Hassler *et al.*, 2014)) and UVB and UVC radiation (361 kJ/m² (Cockell, 2000)). Under these temperature and pressure conditions bulk liquid water is not stable in most locations at the Martian surface. Pure water can only sustain as water vapor in the atmosphere, in form of ice on the Martian surface, as a liquid in the deeper subsurface or as supercooled interfacial water in the Martian regolith (Martínez and Renno, 2013).

Solid water ice has been detected at the poles and in the shallow subsurface, e.g. by the sounding radar SHARAD on the Mars Reconnaissance Orbiter (Grima *et al.*, 2009) and the Gamma-Ray Spectrometer on Mars Odyssey (Boynton *et al.*, 2002), respectively. The total water vapor amount in the Martian atmosphere can reach values of 60 - 70precipitable microns (pr µm) in the northern summer (Trokhimovskiy *et al.*, 2015), while the relative humidity (RH) on Mars ranges from nearly 0% during daytime up to 100% in the morning where frost can occur (Martínez *et al.*, 2017). However, the stability range of liquid water can be expanded to subzero temperatures and lower atmospheric pressures by adding solutes.

1.2 Salts on Mars and deliquescence-induced brine formation

These solutes can be provided by various types of hygroscopic salts that occur on the Martian surface. Evidence for their occurrence exists for sulfates (Kounaves *et al.*, 2010b), carbonates (Niles *et al.*, 2013), nitrates (Stern *et al.*, 2015), bromides (Clark *et al.*, 2005), chlorates (Kounaves *et al.*, 2014a), chlorides (Hecht *et al.*, 2009), and perchlorates (Hecht *et al.*, 2009; Kounaves *et al.*, 2010a). The focus of this study is on the latter two types of salt which are widely distributed on Mars (Keller *et al.*, 2007; Kounaves *et al.*, 2014b) and cause intense freezing point depressions (Möhlmann and Thomsen, 2011).

In general, the intensity of the freezing point depression in a given salt-water system is dependent on the type of salt and its concentration and can be visualized by a water-salt phase diagram (reviewed e.g. in Hennings, 2014). The maximum freezing point depression is reached at the eutectic point composed of a specific eutectic salt concentration (c_e) and eutectic temperature (T_e). When the temperature is above the T_e of a salt-water mixture liquid salt solutions (brines) can be formed directly by water ice getting in contact with salts (Fischer *et al.*, 2014). Another brine formation mechanism is given by deliquescence, which is defined by the process where a hygroscopic salt absorbs water from the atmosphere and dissolves in the absorbed water (Davila *et al.*, 2010). To initiate this process the RH has to be above the deliquescence relative humidity (DRH) of a specific salt and the temperature above T_e . DRH values for all salts relevant for this study are summarized in Table 1-1 together with their T_e and c_e values as well as their occurrence on Mars.

On the one hand, both requirements for deliquescence, i.e. temperatures above T_e and a RH above the DRH of the salt of interest, occur only rarely at the same time on the Martian surface (Gough *et al.*, 2011; Nuding *et al.*, 2014). On the other hand, once a liquid brine has formed the recrystallization of the salt hydrate is kinetically hindered and, therefore, the brine can persist as metastable solution at temperatures below T_e (Toner *et al.*, 2014b) and at RHs below the DRH (Gough *et al.*, 2011). Investigations on the stability of Ca(ClO₄)₂ solutions showed that an eutectic brine of this salt could exist metastable on the surface of Mars for 17 hours of a Martian day (called "sol", 24.66 hours) under Martian temperature, pressure and RH conditions (Nuding *et al.*, 2014).

Although the amount of studies investigating deliquescence phenomena has been increasing recently, important questions, especially regarding the kinetics of the deliquescence process, remain unclear: Is the water uptake within the short window of RH > DRH and temperature > T_e long enough to form a brine, and which influence on the deliquescence process have parameters like temperature, pressure, hydration state of the salt, surrounding soil particles, and grain sizes?

Salt	Eutectic Point		DRH (%)*	Occurronce on Mars	
Salt	T _e (°C)	c _e (mol/l)	[at T (°C)]	Occurrence on Mars	
NaCl	-22ª	5.2ª	75 [20] ^{e,f}	Chlorine (Cl) is globally	
MgCl ₂	-33.5ª	2.79ª	33 [20] ^f	distributed ⁱ , but can be in form of	
			22 [20]e	Cl ⁻ or ClO ₄ ⁻	
CaCl ₂	-50ª	3.9ª		• ClO ₄ ⁻ : Cl ⁻ ratio of approx. 4 at	
			80 [-50]⁵	the Phoenix landing site ^j	
NaClO	-34 ^b 9.06 ^t		51 [0] ^h	• $0.4 - 0.6$ wt. % ClO ₄ ⁻ in the	
Naci04	-34	9.00	65 [-45] ^h	Martian soil ^j with probably	
	E 7¢	2 5 29	42 [0] ^h	Ca(ClO ₄) ₂ ^k or Mg(ClO ₄) ₂ ^l as parent	
	-57*	3.52°	55 [-50] ^h	salt	
	77 5d	bc ۸	13 [0] ^h	Presumably widespread on	
	-77.5*	4.2	55 [-50] ^h	Martian surface ^m	

TAB. 1-1: T_e, c_e and DRH values of various Cl⁻ and ClO₄⁻ salts as well as their occurrence on Mars.

*referring to the highest salt hydration state existing at the respective temperature. DRH values for decreased hydration states can be lower (Gough et al., 2016).

References: ^a(Möhlmann and Thomsen, 2011), ^b(Hennings et al., 2013), ^c(Stillman and Grimm, 2011), ^d(Pestova et al., 2005), ^e(Cohen et al., 1987), ^f(Greenspan, 1977), ^g(Gough et al., 2016), ^h(Nuding et al., 2014), ⁱ(Keller et al., 2007), ⁱ(Hecht et al., 2009), ^k(Kounaves et al., 2014b), ^l(Toner et al., 2015), ^m(Clark and Kounaves, 2016)

1.3 Recurring Slope Lineae (RSL) on Mars

While recently a subsurface brine lake has been discovered near the Martian south pole (Orosei *et al.*, 2018), there is no clear proof but several strong indications that deliquescence and/or temporally stable liquid brines can also occur occasionally on the surface of Mars. For example, the darkening, growth and subsequent disappearance of particles on the Phoenix lander struts were interpret as deliquescence of saline mud and down-dripping of the resulting brine (Rennó *et al.*, 2009). One of the most promising evidence for the temporal occurrence of briny surface water is the observation of RSL identified by the Mars Reconnaissance Orbiter (McEwen *et al.*, 2011a).

RSL are relatively dark flow-like features on Mars that extend downslope from bedrock outcrops and occur annually during spring and summer, especially on steep, equator-facing, southern slopes (Fig. 1-2) (Runyon and Ojha, 2014). Although the role of liquid water on the formation of RSL has been questioned (Edwards and Piqueux, 2016) and dry granular flow mechanisms have been proposed (Dundas *et al.*, 2017) there is spectral evidence for hydrated ClO₄⁻ salts being present within the RSL while being absent in the surrounding soil (Ojha *et al.*, 2015). This indicates that the formation of salt hydrates and their corresponding brines likely play an important role in the RSL formation process.

However, the exact mechanism of RSL formation is still not well understood and requires more laboratory and simulation experiments. Therefore, within the scope of this thesis lab experiments have been conducted investigating the deliquescence process as potential trigger of RSL. These experiments are described in the first publication of this thesis (chapter 2) and some follow-up experiments are shortly summarized in chapter 7.1. Furthermore, during the Atacama Desert field trip in 2018 RSL-simulating field experiments were carried out (unpublished data, chapter 7.2). The results from the field experiments showed that a saturated solution of NaCl poured down a hill forms a RSL-like darkened track that dries out slowly during several days (Fig. 7-2). Nevertheless, when the RH increased in the night and morning hours the briny RSL-like track absorbed additional water correlating with an increase in EC (Fig. 7-3). However, the water amount evaporating during daytime exceeded the water amount absorbed through deliquescence in the night and morning.

Hence, no growth but only fading of the RSL-like track could be observed. The more hygroscopic and therefore more promising ClO_4^- salts could not be used due to safety restrictions. Thus, additional field experiments also considering perchlorates should be conducted in the future.



FIG. 1-2: RSL (indicated by black arrows) occurring at Palikir Crater on Mars imaged by the HiRISE camera on NASA Mars Reconnaissance Orbiter (Credit: NASA/JPL-Caltech/Univ. of Arizona; NASA ID: PIA17933).

1.4 Microbial habitability of RSL-analogue brines

1.4.1 Water activity and other habitability-limiting factors

The habitability of salt solutions is determined by two main factors: the type of salt and its concentration. When talking about cold brines (cryobrines) also the effect of temperature has to be considered as will be discussed more detailed in the Publications II and III of this thesis (chapters 3 and 4, respectively). At high salt concentration the limiting factor for habitability at a specific temperature is usually given by the water activity (a_w) which describes the amount of free biologically available water molecules. In pure water all water molecules are accessible for microorganism and, therefore, $a_w = 1$. In brines the salt ions bind water molecules in their hydration shells and reduce therefore the a_w . On Earth, the lowest a_w supporting life is approx. 0.61 (Stevenson *et al.*, 2015).

However, additional lethal effects of the solutes can lead to a reduced halotolerance. These toxic effects include, among others, salt-induced pH changes (Yaganza *et al.*, 2009), membrane destabilization (Xie and Yang, 2016), molecular mimicry (Cianchetta *et al.*, 2010), and chaotropicity of ions causing a destabilization of macromolecules like proteins (Hallsworth *et al.*, 2003; Hallsworth *et al.*, 2007). Additionally, for brines with a high divalent:monovalent ion ratio ionic strength might be the limiting factor for life rather than water activity (Fox-Powell *et al.*, 2016). Results from recent experiments conducted in our research group indicated that one parameter, among others, effecting the habitability of brines is the strength of hydration shells around ions (especially anions) (Waajen *et al.*, 2019, in preparation). The stronger the hydration shell around an ion the lower its cell membrane permeability and, hence, its toxicity (see also Publications II and III).

1.4.2 Habitability of Cl⁻-containing brines

Halophilic microorganisms can thrive in saturated NaCl solutions (6.1 M, a_w = 0.75 at 25°C) (Oren, 2008) and, thus, inhabit entrapped salt rocks (Kunte *et al.*, 2002). Endolithic bacteria in the hyperarid core of the Atacama Desert, Chile, can utilize water condensed from the atmosphere via deliquescence of NaCl deposits (Davila *et al.*, 2008). Experiments carried out within our research group showed that halophilic methanogenic archaea can survive desiccation in Mars-analogue soil interspersed with NaCl and regain their viability (methane production) after water provision through NaCl deliquescence [Maus *et al.*, 2019].

While the habitability of NaCl brines is well investigated, studies focusing on life in non-NaCl brines are sparser although those are more relevant for simulating Mars-analogue environments (chapter 1.2). Several organisms have been isolated form the Dead Sea which contains besides Na⁺ (1.54 M) and Cl⁻ (6.48 M) large amounts of Mg²⁺ (1.98 M) and Ca²⁺ (0.47 M) (Oren, 2010). Due to halite precipitation the divalent:monovalent ion ratio in the Dead Sea is even increasing with time which decreases the habitability. Blooms of microorganism only occur after very rainy winters when salt concentrations in the Dead Sea are diluted indicating that the salt-induced limit of life is nearly reached and microbes in the Dead Sea are dying (Oren, 2010).

Two organisms isolated from the Dead Sea are *Haloferax volcanii* which can grow in the presence of 2 M Na⁺ and 1.4 M Mg²⁺ (Mullakhanbhai and Larsen, 1975) and *Halobaculum gomorrense* which can grow in the presence of 0.5 M NaCl and 1.5 to 2.0 M MgCl₂ and can even tolerate high CaCl₂ concentrations of up to 1 M (in the presence of 2.1 M NaCl and 15 mM MgCl₂) (Oren, 1983). These and other Dead Sea microorganisms require divalent cations at relatively high concentration levels (approx. 75 mM Mg²⁺) for survival (Cohen *et al.*, 1983). It has been stated that the maximum MgCl₂ concentration suitable for life is limited by its chaotropicity and is around 2.3 M in the absence of compensating kosmotropic substances (Hallsworth *et al.*, 2007). Halophilic fungi can tolerate up to 2.1 M MgCl₂ or 2.0 M CaCl₂ (Zajc *et al.*, 2014). Environments with markedly higher MgCl₂ or CaCl₂ concentrations, e.g. 5 M MgCl₂ in the Discovery Basin (Hallsworth *et al.*, 2007) or 3.7 M CaCl₂ in the Don Juan Pond, Antarctica (Marion, 1997; Samarkin *et al.*, 2010), are thought not to be inhabited by active microbial communities.

1.4.3 Habitability of ClO₄⁻-containing brines

On Earth natural occurring ClO_4^- salts can be found only in low concentrations in very dry environments like the Atacama Desert, Chile (Ericksen, 1981; Catling *et al.*, 2010), or the Dry Valleys, Antarctica (Kounaves *et al.*, 2010c). Hence, the scientific interest in the habitability of ClO_4^- brines was limited prior the detection of ClO_4^- on Mars in 2008 (Hecht *et al.*, 2009). Even today, more than 10 years later, little is known about microbial $ClO_4^$ tolerances and toxicity effects of the ClO_4^- anion to microorganisms.

There have been several studies investigating the dissimilatory reduction of CIO_4^- by bacteria especially in context of anthropogenic CIO_4^- ground water contamination (reviewed in Coates and Achenbach, 2004; Bardiya and Bae, 2011), however, in these studies CIO_4^- concentrations of only approx. 1 mM are typical which is far below the $CIO_4^$ concentrations expected to occur in Martian brines (Al Soudi *et al.*, 2017). Only a few studies focused on determining the maximum CIO_4^- concentration suitable for growth of halophilic microorganisms. The highest halotolerances to CIO_4^- described prior this study are summarized in Table 1-2.

Organism	Salt providing ClO₄⁻	Perchlorate tolerance [mol/l ClO₄ ⁻]	Reference	
	NaClO ₄	1.0	(Al Soudi at al	
Halomonas venusta	Mg(ClO ₄) ₂	0.5 (inconsistent results for 1.0 M)	2017)	
Halorubrum lacusprofundi	NaClO ₄	0.8	(Laye and	
nulorubrum lucusprojunur	Mg(ClO ₄) ₂	0.6	 DasSarma, 2018)	
Haloferax mediterranei	NaClO ₄	0.6	(Oren <i>et al.,</i> 2014)	
Hydrogenothermus		0.45	(Beblo-Vranesevic	
marinus	MaCIO4	0.45	et al., 2017)	

TAB. 1-2: A selection of the highest microbial perchlorate tolerances reported prior this thesis.

1.4.4 Microbial adaptations to salt stress

Halophilic and halotolerant microorganisms developed two approaches to cope with high salt concentrations, which are the organic-osmolyte mechanism and the salt-incytoplasm mechanism (Kunte *et al.*, 2002). The first one encompasses the production or accumulation of highly water-soluble organic solutes in the cytoplasma of the cell to counter the external osmotic pressure. These osmolytes (or osmoprotectants) can be sugars like trehalose, anions like glutamate, or zwitterionic compounds like betaine or proline (Roberts, 2005). Halophilic microorganisms using the salt-in-cytoplasm strategy enrich salts, typically potassium chloride (KCI), in their cytoplasma which requires substantial adaptation of the intracellular enzymatic machinery to the high inner-cellular salt concentration (Oren, 2008). Therefore, organisms using this strategy cannot tolerate low salt concentrations since their highly adapted proteins denature under these conditions.

For the habitability experiments described in this thesis (Publications II and III) the survival and growth of *Planococcus halocryophilus* (Fig. 1-3) in Cl⁻ and ClO₄⁻ brines was studied. This bacterial strain is adapted to thrive under the cold and salty conditions prevailing in its natural habitat, the active layer of permafrost soil in the Canadian Arctic (Mykytczuk *et al.*, 2012; 2013). Although its name indicates the opposite, *P. halocryophilus*

is not halophilic but halotolerant, i.e. it tolerates high salinities but can also grow in salt-free medium. This indicates that this organism uses the organic-osmolyte mechanism to counteract high salt concentrations, however, the occurrence of osmolytes in *P. halocryophilus* cells grown under salt stress conditions has not been investigated prior this study. Therefore, we conducted some initial metabolomics experiments (unpublished data, chapter 7.3). The results showed that cells of *P. halocryophilus* contain the osmolytes betaine and proline in large quantities fitting well to the detection of several betaine and proline transport proteins that are expressed under osmotic stress (Mykytczuk *et al.*, 2013).



FIG. 1-3: Colonies of *P. halocryophilus* grown on agar plates.

1.5 Aim of this study and overview of the publications

The major goal of this thesis was to gain a better understanding of the deliquescenceinduced formation of RSL-like brines and to investigate the habitability of those at temperatures ranging from -30°C to +25°C. For this purpose the following three first-author papers have been published in international journals:

Publication I: Deliquescence-induced wetting and RSL-like darkening of a Mars analogue soil containing various perchlorate and chloride salts.

Authors: Jacob Heinz, Dirk Schulze-Makuch, and Samuel P. Kounaves

Journal: Geophysical Research Letters (2016), 43:4880–4884, https://doi.org/10.1002/2016GL068919 (Open Access)

Aims and summary: To investigate the RSL-analogue brine formation lab experiments were conducted studying the wetting and darkening of Mars-analogue soil caused by deliquescence of Cl⁻ and ClO₄⁻ salts dispersed in the soil. The deliquescence process was followed at constant temperature (25°C) and RH (70 – 85 %) conditions through visible observations and EC measurements revealing differences in the time needed to start deliquescence and in the deliquescence rates of the different salts. Conclusions for possible RSL formation processes were drawn.

Personal and co-authors contribution: I conducted all experiments described within this study and wrote the manuscript by myself. Samuel P. Kounaves and Dirk Schulze-Makuch were involved in the data interpretation and helped to improve the manuscript.

Publication II: Enhanced Microbial Survivability in Subzero Brines.

Authors: Jacob Heinz, Janosch Schirmack, Alessandro Airo, Samuel P. Kounaves, and Dirk Schulze-Makuch

Journal: Astrobiology (2018), 18:1171–1180, https://doi.org/10.1089/ast.2017.1805 (Open Access)

Aims and summary: The goal of this study was the investigation of the habitability of Marsrelevant brines that could result from deliquescence of Martian salts (see Publication I). For this purpose the survival of *P. halocryophilus* in these salt solutions was studied. In a first set of experiments the survivability of *P. halocryophilus* in eutectic brines was investigated at temperatures ranging from -30°C to 25°C. These experimental conditions were chosen because temperatures on Mars are mostly in the subzero range and deliquescence of salts always results in saturated solutions (which corresponds to the eutectic concentration at the eutectic temperature of the respective salt) before further water absorption of the brine can dilute the solution to some extent. Since on Mars temperatures are varying and periodically drop below the freezing point of the brines, putative Martian microbes would also have to deal with freezing and thawing processes of these brines. Hence, we studied in a second set of experiments the effect of salts (in this study only NaCl) on the survival of *P. halocryophilus* during freeze/thaw cycles.

Personal and co-authors contribution: Approx. 90% of all experiments described within this study were conducted by myself. Janosch Schirmack helped in some cases with dilution and plating of the samples during the long-term growth experiments (approx. 10%). The manuscript was written by myself. All co-authors were involved in the data interpretation and helped to improve the manuscript.

Publication III: Bacterial growth in chloride and perchlorate brines: Halotolerances and salt stress responses of *Planococcus halocryophilus*.

Authors: Jacob Heinz, Annemiek C. Waajen, Alessandro Airo, Armando Alibrandi, Janosch Schirmack, Dirk Schulze-Makuch

Journal: Astrobiology (2019), in press.

Aims and summary: At the high salt concentrations investigated in Publication II no growth of *P. halocryophilus* was observed. Thus, the question arose how far the Cl⁻ and ClO₄⁻ solutions had to be diluted for bacterial growth to occur. To address this question we inoculated low-concentrated saline media with *P. halocryophilus* and adapted the cells stepwise to higher concentrations to determine the maximum salt concentration suitable for growth. These experiments were conducted at 25°C (optimum growth temperature) and at 4°C for examining the effect of a temperature decline. Additionally, the salt stress responses of *P. halocryophilus* were investigated visually on agar plates and through different microscopical techniques to better understand which adaption mechanisms putative Martian microbes could develop under the cold and briny conditions found on Mars.

Personal and co-authors contribution: I conducted all growth experiments by myself and approx. 80% of all microscopical experiments described within this study. Annemiek C. Waajen, supported by Janosch Schirmack, did the first run of the fluorescence and scanning electron microscopy experiments (15%) whereas I did run number 2 and 3. Armando Alibrandi provided some of the light microscopy images (5%). The manuscript was written by myself. All co-authors were involved in the data interpretation and helped to improve the manuscript.

Accepted manuscript of:

Heinz, J., Schulze-Makuch, D., and Kounaves, S.P. (2016) Deliquescence-induced wetting and RSL-like darkening of a Mars analogue soil containing various perchlorate and chloride salts. Geophys. Res. Lett., 43 (10) 4880-4884, https://doi.org/10.1002/2016GL068919. *Published by American Geophysical Union (AGU) under a CC BY-NC-ND 4.0 license (license terms cf. http://creativecommons.org/licenses/by-nc-nd/4.0/*)

Publication I: Deliquescence-induced wetting and RSL-like darkening of a Mars analogue soil containing various perchlorate and chloride salts

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Key Points:

- Deliquescence of salts in a Mars simulant soil sample causes a RSL-like darkening.
- Forming thicker liquid films or bulk water via deliquescence is a relatively slow process.
- The deliquescence process can be investigated by electrical conductivity measurements.

Abstract

Recurring Slope Lineae (RSL) are flow-like features on Mars characterized by a local darkening of the soil thought to be generated by the formation and flow of liquid brines. One possible mechanism responsible for forming these brines could be the deliquescence of salts present in the Martian soil. We show that the JSC Mars-1a analogue soil undergoes a darkening process when salts dispersed in the soil deliquesce, but forming continuous liquid films and larger droplets takes much longer than previously assumed. Thus, RSL may not necessarily require concurrent flowing liquid water/brine or a salt-recharge mechanism, and their association with gullies may be the result of previously flowing water and deposited salts during an earlier warmer and wetter period. In addition, our results show that electrical conductivity measurements correlate well with the deliquescence rates and provide better overall characterization than either Raman spectroscopy or estimates based on deliquescence relative humidity (DRH).

2.1 Introduction

Recurring Slope Lineae (RSL) are narrow (< 5 m) and relatively dark flow-like features on Mars that extend downslope from bedrock outcrops. The features were identified by the Mars Reconnaissance Orbiter (MRO) (McEwen *et al.*, 2011a) and occur annually during spring and summer, especially on steep, equator-facing, southern slopes (Runyon and Ojha, 2014). Several mechanisms have been proposed to explain the occurrence of RSL, ranging from dry granular flows (McEwen *et al.*, 2011b) to effects caused by rapid heating of nocturnal frost (Dundas *et al.*, 2015). The best current hypotheses for their formation involve either the melting of frozen brines, the seasonal discharge of a local aquifer, or via deliquescence of salts dispersed in the soil (Chevrier and Rivera-Valentin, 2012; McEwen *et al.*, 2015; Ojha *et al.*, 2015).

Experimental investigations into the formation of brines via deliquescence has been widely reported (Zorzano *et al.*, 2009; Gough *et al.*, 2011, 2014; Fischer *et al.*, 2014; Nuding *et al.*, 2014, 2015; Nikolakakos and Whiteway, 2015). These investigations clearly showed

that the salts present on Mars, such as magnesium and calcium perchlorates or chlorides (Kounaves *et al.*, 2010a; 2014b), are highly deliquescent and some of their solutions could be, at least temporally, stable on the surface of Mars. The relative humidity (RH) needed to start the deliquescence process of a salt, the deliquescence relative humidity (DRH), is shown in Table 2-1 for some Mars-relevant salts.

	DRH (%)	Temperature (K)	References
Ca(ClO ₄) ₂ ·4H ₂ O	13	273	(Nuding <i>et al.,</i> 2014)
CaCl ₂ ·6H ₂ O	29	298	(Lide, 2003)
MgCl ₂ ·6H ₂ O	33	298	(Lide, 2003)
Mg(ClO ₄) ₂ ·6H ₂ O	42	273	(Gough <i>et al.,</i> 2011)

TAB. 2-1: Deliquescence relative humidity (DRH) values for salts used in the experiments.

Most of the research has been conducted by investigating phase changes of the salts with the aid of Raman spectroscopy, whereby the Raman laser beam was focused on small salt particles or thin layers (Gough *et al.*, 2011, 2014; Fischer *et al.*, 2014; Nuding *et al.*, 2014, 2015; Nikolakakos and Whiteway, 2015). However, these studies do not take into consideration that the salts on Mars are probably intimately mixed with the soil.

To date, there have been no reported studies correlating the observed darkening of Mars analog soils and deliquescence as a function of the types of salts and environmental parameters such as humidity or temperature. Here we demonstrate that visual observation in parallel with electrical conductivity (EC), a technique commonly used for detection of liquid water in soils (McKay *et al.*, 2003; Davis *et al.*, 2010), can be used to monitor the deliquescence process in mixtures of soil and salt.

2.2 Materials and Methods

2.2.1 Reagents and sample treatment

The soil salt mixtures consisted of JSC Mars-1a analogue soil and either magnesium perchlorate hexahydrate $Mg(ClO_4)_2 \cdot 6H_2O$ (Sigma-Aldrich, Lot # MKBQ3075V), calcium perchlorate tetrahydrate $Ca(ClO_4)_2 \cdot 4H_2O$ (Acros Organics, Lot # A0332298), magnesium chloride hexahydrate $MgCl_2 \cdot 6H_2O$ (Fluka, Analysis # 350301/1696) or calcium chloride hexahydrate $CaCl_2 \cdot 6H_2O$ (Fluka, Analysis # 349745/163796). Salts were ground in a mortar before mixing with soil. One sample contained only pure soil. The other samples consisted of 10 g soil and 0.0123 mol of the corresponding salt hydrate (i.e. 4.075 g $Mg(ClO_4)_2 \cdot 6H_2O$; 3.825 g $Ca(ClO_4)_2 \cdot 4H_2O$; 2.5 g $MgCl_2 \cdot 6H_2O$, 2.695 g $CaCl_2 \cdot 6H_2O$). The salt contents were chosen sufficiently high to ensure a visible and measurable change during deliquescence (salt concentrations in RSL are expected to be much higher than at Martian landing sites and variable salt concentrations might affect their deliquescence). After mixing the components by shaking in a 30 mL polycarbonate container, the samples were dried for 2 days in a desiccator under vacuum over anhydrous calcium chloride.

To increase the RH in the system the desiccant in the lower part of the desiccator was replaced by water. A hot plate was used to cycle the temperature in the desiccator between 290 and 298 K to evaporate water until the relative humidity (RH) increased from 40% (which was the RH in the lab after opening the desiccator and removing the desiccant) to over 70%. This value increased over several days slowly to a RH of 85% without any treatment (Fig. 2-2). After 18 days the experiment was stopped. Afterwards the samples were dried again until there was no measurable conductivity and the samples regained their light brown color. The experiment was then repeated to ensure reproducibility of the results.

2.2.2 Measurements and data treatment

All EC measurements were made using two parallel 1 mm diameter copper wire electrodes inserted 25 mm apart into the soil samples, and connected to a CR 10 data logger (Campbell Scientific). The CR 10 is capable of applying an AC excitation voltage that prevents polarization of the electrodes. The applied excitation voltage (V_x) results in a current between the electrodes proportional to the EC of the sample. This current is converted to a measured voltage V. The observed output value of the data logger, equivalent to the conductivity, is a normalized voltage *N* given by:

$$N = \frac{V_X}{V} \cdot 1000$$

The upper detection limit of this technique is reached when *V* equals V_X . That happens when, due to increasing ion mobility in the sample, the resistance tends to zero, so that EC goes to infinity. In this case *N* tends to 1000. The *N* values can also be converted into conductivity values (in Siemens per meter) when the cell constant of the system is known (Stone *et al.*, 1993). The data logger was set to register *N* values every 5 minutes. RH and temperature were measured with a HOBO Pro v2 data logger throughout the entire experiment.

2.3 Results

The pure JSC Mars-1a analogue is a light reddish brown soil (Fig. 2-1a). Mixing it with the various salt hydrates resulted in a dark brown soil sprinkled with some remaining dry particles (Fig. 2-1b). The darkening of the sample is due to the wetting of the soil caused by some minor initial amount of liquid water adsorbed by the hygroscopic salts. After drying, the soil/salt mixtures regained the light reddish brown color of the pure JSC Mars-1a analogue soil but with the noticeable exception that near the surface larger clumps of soil are visible (Fig. 2-1c). Similar clumpy or cloddy soils were observed in the soils at the Phoenix landing site and attributed to their being wetted at some point in the past (Arvidson *et al.,* 2009).



FIG. 2-1: Soil sample photos. **a** – Pure and dry JSC Mars-1a analogue soil. **b** – Soil mixed with wet $Ca(ClO_4)_2 \cdot 4H_2O$. **c** – Dried sample of soil and $Ca(ClO_4)_2 \cdot nH_2O$.

The results of our experiments are shown in Figure 2-2, where the measured normalized voltage (*N*) and the RH in the system are plotted versus the duration of the experiment in days. Selected images of the samples taken through the glass of the desiccator at specific points of time are included to show the correlation between the increasing conductivity and the darkening of the soil due to deliquescence. As expected, at the beginning of the experiment for RH ~ 40%, the samples were dry with *N* = 0. The changes in appearance and conductivity that occurred after the RH was increased to 70% are described below and summarized in table 2-2, where T_V is the time after the start when first visible changes (wet grains on the sample surface) were observed and T_{EC} the time when EC started to increase.

About 2.5 hours after increasing the RH to 70%, some of the clumped grains on the surface of the calcium perchlorate sample started to become wet, as can be seen by the darkening of these particles compared to the brighter soil beneath (Fig. 2-2a). As the wet grains are not connected to each other, the conductivity remained at zero. About 19 hours after the experiment started a complete layer of wet soil had formed (Fig. 2-2b), coinciding

with an increase in conductivity, most likely due to a thin layer of salt water forming around the soil particles. The increase of conductivity in these first minutes of detection can better be seen in the enlarged segment of Figure 2-2.

Salt (mixed with soil)	Tv	T _{EC}
Ca(ClO ₄) ₂ ·nH ₂ O	2.5 ± 0.5 h	19 h
MgCl ₂ ·nH ₂ O	48 ± 2 h (2 d)	61 h (~2.5 d)
Mg(ClO₄)₂·nH₂O	70 ± 4 h (~3 d)	72 h (3 d)
CaCl ₂ ·nH ₂ O	108 ± 4 h (~4.5 d)	124 h (~5 d)

TAB. 2-2: Time after experimental start at which the beginning of deliquescence could be observed visually (T_v) and via electrical conductivity (T_{EC}) in hours (h) and days (d).

The other samples stayed dry and non-conductive for two days after the start of the experiment. During this time the diameter of the dark and wet layer in the calcium perchlorate sample constantly increased in size, which was also mirrored by an increase in conductivity. After two days some grains on the top of the magnesium chloride sample became dark and wet, similar to the calcium perchlorate sample. After 2.5 days, an increase in the conductivity in the magnesium chloride sample was also detected. Similar to the calcium perchlorate sold that grew with time proportional to the conductivity. Similar results were obtained for the magnesium perchlorate and calcium chloride samples after 3 and 4.5 days, respectively.

The soil sample with calcium perchlorate started to deliquesce first at a rate similar to the other salts, but continued at a lower rate after about the 8th day. The rate of increase for the calcium perchlorate sample reached its maximum after 16 days, where it remained nearly constant until the end of the experiment (Fig. 2-2). The other samples did not reach a maximum value, but they approached the detection limit of the technique.


FIG. 2-2: Changes in normalized voltage (N) and RH as a function of time, and photos of soil samples. (**a–c**) The time when sample photos were taken. Wet separated grains on surface of $Ca(CIO_4)_2 \cdot nH_2O$ sample with no measurable conductivity after 2.5 h (Fig. 2-2a). Thin wet layer of soil in the upper part of the sample causes first measurable conductivity values after 19 h (Fig. 2-2b). Wet layers and droplets of salty water in the sample of $Mg(CIO_4)_2 \cdot nH_2O$ with the highest measured conductivity after 17 days (Figure 2-2c). The enlarged segment of the graph displays the T_V values in more detail. Short data gaps in the curves are results of temporary connection problems with the CR10 data logger.

When the experiment was stopped after 18 days, all samples, except the pure soil which stayed dry, were completely wet and dark brown. In the magnesium perchlorate sample, which reached the highest conductivity, small liquid droplets and films where visible on the wall of the vessel after 17 days (Fig. 2-2c). A repetition of the experiment resulted in very similar curve progressions compared to the first run, with the exception that a slightly lower deliquescence rate for magnesium chloride was observed.

2.4 Discussion

The results clearly show that the salts in the Mars JSC-1a analogue soil deliquesce when sufficiently high RH values are reached. However, without any added salts the soil stays completely dry for 18 days at RH values up to 70 - 85%, which shows that the wetting of the other samples is not caused by adsorption or direct condensation of water onto the soil. The deliquescence process coincides with a darkening of the soil, similar to that observed for the RSL on Mars. We have shown that EC is an excellent method for monitoring soil wetting by deliquescence. Some salts (in our case calcium perchlorate) can initiate this process after only 2.5 hours at RH ~ 70%, however, other salts (here magnesium perchlorate, magnesium chloride and calcium chloride) need several days until they have absorbed enough liquid water to provide ion mobility, even if their DRH are below the starting RH of the experiment (70%) (Table 2-1). We found that it takes much more time (in our experiment 17 days) until sizable liquid droplets and films form under the experimental conditions used here. Therefore we agree with Fischer et al. (2014) who showed that bulk deliquescence (meaning the formation of greater amounts of liquids) is a slow process, but in contrast to their work, we find that some salts like calcium perchlorate can start to deliquesce much more rapidly (< 3.5 hours). This is seen as the time during which atmospheric conditions at the Phoenix landing site would meet the conditions necessary for deliquescence to occur (Möhlmann, 2011).

An interesting observation is that there seems to be no correlation between the T_V or T_{EC} values of a salt and its DRH value. The calcium perchlorate sample with the lowest DRH

(13 %) started first to deliquesce, but it was followed chronologically by the samples with magnesium chloride (DRH 33 %), magnesium perchlorate (DRH 42 %) and calcium chloride (DRH 29 %). Thus, one cannot predict the order in which different salts start to deliquesce only by taking the DRH values into consideration. There may be several reasons for this lack of correlation. One reason may be that the hydration state present at deliquescence may be different than the hydration state as received due to the desiccation of the samples at the beginning of the experiment.

Furthermore, the velocity of the deliquescence process can neither be estimated by DRH nor T_V or T_{EC} values, as can be seen in the flatter slope of the calcium perchlorate conductivity curve (Fig. 2-2). As calcium perchlorate has the lowest DRH and T_{EC} values one could expect the highest rate of deliquescence and therefore the steepest curve slope, but this is not so according to our experiments.

2.5 Conclusion

We have shown that the darkening of the soil similar to what is seen at the RSL on Mars can be reproduced by the wetting of perchlorate and chloride containing soils caused by deliquescence of these salts. However, due to the longer timescales required to produce greater amounts of liquid water in the forms of bulk or droplets, it appears likely that the RSL would not necessarily require the concurrent presence of flowing liquid water or brine. Thus, it is possible that their association with gullies may be the result of an earlier period when Mars may have had a warmer and wetter climate that allowed for the melting of subsurface ice at exposed outcrops and/or the flow of liquid water and subsequent precipitation of salts on evaporation. This process would have been most effective on the warmer equator-facing slopes of the craters and dunes, where RSLs are found today. The steep slopes of these formations may have also impeded new dry soil from covering the salt deposits. This hypothesis, consistent with the latest spectral evidence for hydrated salts in the RSL (Ojha *et al.*, 2015), does not require a salt-recharge mechanism since the liquid water films are formed in-place by the previously deposited salts.

2.6 Acknowledgments

We thank Chris McKay and Alfonso Davila for donating the CR10 data logger and helping to improve the experimental setup. Moreover, we thank Raina V. Gough for helping to improve the manuscript. This project was funded by European Research Council Advanced Grant "Habitability of Martian Environments" (HOME, # 339231). All of the numerical data for this paper are provided in the figures and are also available in tabular form from the authors upon request (dirksm@astro.physik.tu-berlin.de). Accepted manuscript of: Heinz, J., Schirmack, J., Airo, A., Kounaves, S. P., and Schulze-Makuch, D. (2018) Enhanced Microbial Survivability in Subzero Brines. Astrobiology, 18(9), 1171–1180. https:// doi.org/10.1089/ast.2017.1805 Published by Mary Ann Liebert under a CC BY 4.0 license (license terms cf. http://creativecommons.org/licenses/by/4.0)

3. Publication II: Enhanced Microbial Survivability in Subzero Brines

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Running Title: Survivability in Subzero Brines.

Search Terms: Brines; Subzero; Perchlorate; Survival; Halophile; Mars

Abstract

It is well known that dissolved salts can significantly lower the freezing point of water and thus extend habitability to subzero conditions. However, most investigations thus far have focused on sodium chloride as a solute. Here, we report on the survivability of the bacterial strain *Planococcus halocryophilus* in sodium, magnesium and calcium chloride or perchlorate solutions, respectively, at temperatures ranging from +25°C to -30°C. Additionally, we determined the survival rates of *P. halocryophilus* when subjected to multiple freeze/thaw cycles. We found that cells suspended in chloride-containing samples have markedly increased survival rates compared to those in perchlorate-containing samples. In both cases the survival rates increase with lower temperatures, however, this effect is more pronounced in chloride-containing samples. Furthermore, we found that higher salt concentrations increase survival rates when cells are subjected to freeze/thaw cycles. Our findings have important implications not only for the habitability of cold environments on Earth but also for extraterrestrial environments such as Mars, where cold brines might exist in the subsurface and perhaps even appear temporarily at the surface such as at Recurring Slope Lineae.

Keywords: Brines; Subzero; Perchlorate; Survival; Halophile; Mars

3.1 Introduction

Life as we know it requires liquid water as the principle solvent for its biochemistry, but most planetary surfaces in our Solar System never reach temperatures above the freezing point of pure water, rendering these localities as likely uninhabitable compared to the benign climate conditions on Earth. However, the presence of salts can lead to a substantial freezing point depression down to the eutectic temperature of a given salt-water mixture (e.g. -50°C for a 31 wt% CaCl₂ solution) and, thus, greatly expand the temperature range for potential habitats (Möhlmann and Thomsen, 2011). Hence, the question arises whether microorganisms can thrive or at least survive in such subzero brines.

On Earth, microbial organisms such as yeast can tolerate water activities (a_w) down to 0.61 (Rummel *et al.*, 2014). However, the lowest salt-induced water activity that halophilic microorganisms can tolerate is that of a saturated NaCl solution (a_w = 0.75) while other salts (e.g. those containing Ca²⁺ and Mg²⁺ ions) are more inhibitory to cell metabolism (Rummel *et al.*, 2014). Furthermore, it has been reported that certain cyanobacterial species embedded in hygroscopic sodium chloride (NaCl) deposits found in the hyperarid soils of the Atacama Desert are able to utilize water condensed from the atmosphere via deliquescence (Davila *et al.*, 2008; Davila and Schulze-Makuch, 2016).

Additionally, many halophilic microorganisms can also be psychrophilic or psychrotolerant (Gounot, 1986; Hoover and Pikuta, 2010). To date, the lowest reported temperature for microbial growth is -18°C for yeast on frozen surfaces (Collins and Buick, 1989). Metabolic ammonia oxidation has been detected down to -32°C (Miteva *et al.*, 2007) and, finally, there are indications for photosynthetic activity of lichens at -40°C (de Vera *et al.*, 2014).

It has been argued that low temperature and high salt tolerances are closely linked, since at subzero temperatures water ice forms which increases the solute concentration of the remaining liquid water (Bakermans, 2012). Moreover, chaotropic agents like magnesium chloride (MgCl₂), i.e. substances destroying the bulk water structure and therefore reducing hydrophobic interactions (Gerba, 1984), at moderate concentrations

can decrease the minimal temperature at which cell division can occur for certain microorganisms and increase their growth rates at low temperatures, presumably by increasing the macromolecular flexibility (Chin *et al.*, 2010). Furthermore, some microorganisms shift their salinity optimum for growth to higher salt concentrations if exposed to lower temperatures (Gilichinsky *et al.*, 2003).

Organisms have evolved several adaptations for thriving and/or surviving in cold saline environments. These include production of antifreeze or ice-binding proteins, cryoprotectants or extracellular polymeric substances (EPS) (Jia *et al.*, 1996; Gilbert *et al.*, 2005; Kuhlmann *et al.*, 2011), an increase of fatty acids branching to maintain membrane fluidity (Denich *et al.*, 2003), a higher antioxidant defense against reactive oxygen species (Chattopadhyay *et al.*, 2011), the expression of isozymes adapted to low temperatures and high salinities (Maki *et al.*, 2006), or the exclusion of inhibitory ions by accumulating intercellular compatible solutes (Csonka, 1989).

Most of the studies dealing with brines at subzero temperatures have focused on NaCl as a solute, the most common salt found in saline environments on Earth. However, certain environments on Earth are dominated by high concentrations of other salts such as calcium chloride (CaCl₂) in Don Juan Pond, Antarctica (Cameron *et al.*, 1972; Dickson *et al.*, 2013) or sodium and magnesium sulfates in Spotted Lake, Canada (Pontefract *et al.*, 2017). Furthermore, Martian soils are known to contain various chloride (Cl⁻) and perchlorate (ClO₄⁻) salts (Hecht *et al.*, 2009; Kounaves *et al.*, 2010a), emphasizing the importance of research in the field of non-NaCl briny habitats at subzero temperatures.

In this study we used the halo- and cryo-tolerant bacterial strain *Planococcus halocryophilus* Or1 (DSM 24743^T) isolated from the active-layer of permafrost soil in the Canadian High Arctic (Mykytczuk *et al.*, 2012). This organism grows at temperatures between -15°C and +37°C and under NaCl concentrations of up to 19 wt/vol.% at which metabolic activity has been detected at temperatures down to -25°C (Mykytczuk *et al.*, 2013). This bacterial strain shows many cold- and osmotic-stress responses such as the expression of cold-adapted proteins, the expression of various osmolyte transporters, a high lipid turnover rate, a high resource efficiency at cold temperatures with an

accumulation of carbohydrates as energy resource (Mykytczuk *et al.*, 2013) and complex changes in protein abundances (Raymond-Bouchard *et al.*, 2017). Furthermore, under cold growth conditions *P. halocryophilus* develops a nodular sheet-like crust around the cells (Mykytczuk *et al.*, 2016; Ronholm *et al.*, 2015).

The above described ability of *P. halocryophilus* to cope with low temperatures and high salt concentrations makes it an ideal organism for studying if and how well terrestrial life might be able to survive or even thrive in Martian environments. In particular, we have investigated how well *P. halocryophilus* can survive repeated freezing/thawing cycles and in subzero chloride and perchlorate brines, since such conditions may be temporarily present on Mars (Martínez and Renno, 2013).

3.2 Materials and Methods

3.2.1 Strain and culture conditions

We used the bacterial strain *Planococcus halocryophilus* Or1 (DSM 24743^T) obtained from the DSMZ (Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures). *P. halocryophilus* was grown in DMSZ growth medium #92 containing additional 10 wt% NaCl. Its growth curve at 25°C was determined via colony forming units (CFUs) and cell suspensions used for inoculating the experiments were either retrieved after 4 days (sample type ST 1) or 7 days (sample type ST 2) of growth (Fig. 3-1).



FIG. 3-1: Bacterial growth curve of *P. halocryophilus* in DMSZ growth media #92 + 10 wt% NaCl at 25°C. CFUs obtained as technical duplicates. Crosses mark the sampling times for inoculating of sample types ST 1 and ST 2.

3.2.2 Experiments in eutectic salt solutions

In all experiments 2 mL of the cell suspension (prepared as described in Section 2.1) was mixed with 8 mL of a salt solution resulting in 10 mL sample solution with a eutectic salt concentration. The eutectic compositions of the investigated salts are listed in Table 3-1, together with the ionic strength, the water activity at 25°C calculated from the Pitzer equation (Pitzer, 1991) with Pitzer parameters taken from *Toner et al.* (2015), and the eutectic temperature. All samples were prepared and analyzed as biological duplicates. Before mixing cell suspensions and salt solutions the suspensions were cooled to 4°C and the salt solutions to the respective incubation temperature. Additionally, for testing if ClO_4^- preconditioning of the cells has a positive effect on their survival in ClO_4^- containing

samples, cell suspensions with either up to 10 wt% NaClO₄ or 5 wt% NaClO₄ + 10 wt% NaCl were prepared and incubated for seven days at 25°C.

TAB. 3-1: Eutectic concentrations and temperatures, ionic strength and water activities at 25°C for salt solutions used in this study.

	Eut	ectic	Ionic	water	Eutectic Temperature			
	Concei	ntration	strength	activity				
	[wt%]	[mol/L]	[mol/L]	at 25°C	[°C]	[К]		
NaCl	23.3ª	5.20	5.20	0.80	-22	251ª		
MgCl ₂	21ª	2.79	8.38	0.75	-33.5	239.5ª		
CaCl ₂	30.2ª	3.90	11.70	0.65	-50	223ª		
NaClO ₄	52.6 ^b	9.06	9.06	0.68	-34	239 ^b		
Mg(ClO ₄) ₂	44 ^a	3.52	10.56	0.56	-57	216 ^c		
Ca(ClO ₄) ₂	50.1 ^d	4.20	12.60	0.52	-77,5	195.5 ^d		

^a(Möhlmann and Thomsen, 2011), ^b(Hennings et al., 2013), ^c(Stillman and Grimm, 2011), ^d(Pestova et al., 2005).

3.2.3 Cell number quantification

The concentration of viable cells in the samples were determined after specific time intervals via colony formation unit (CFU) counts, and where necessary samples were diluted in phosphate buffer saline (PBS) containing 21 wt% NaCl or MgCl₂ to avoid osmotic bursting of cells. Highest values of CFU ml⁻¹ were achieved when dilution was done with NaCl enriched PBS for samples containing NaCl, MgCl₂, NaClO₄ or Mg(ClO₄)₂, and MgCl₂ enriched PBS for samples containing CaCl₂ or Ca(ClO₄)₂. Because cell death occurred at higher temperatures during plating, especially in Ca²⁺ containing samples, plating for all experiments described in this study was carried out rapidly at cold temperatures. The NaCl/MgCl₂ enriched PBS was precooled to -15/-30°C and agar plates to 4°C.

3.2.4 Freeze/thaw cycle experiments

For investigating the effect of dissolved salts on cell survival when subjected to multiple freeze/thaw cycles, we incubated *P. halocryophilus* at 25°C for one week in six individual vials. Three of them contained 10 mL of DMSZ growth medium #92 (with no additional NaCl) while the other three samples contained additionally 10 wt% NaCl. After incubation all samples where repeatedly frozen at -50°C, stored at this temperature for between one and three days and thawed at room temperature until the unfrozen sample reached 20°C, which took approximately two hours. After taking an aliquot from each sample for CFU determination the samples were frozen again. These freeze/thaw cycles were repeated up to 70 times and the survival was tested intermittently. The results for samples with the same growth media composition were averaged and the standard deviation was calculated.

3.3 Results

3.3.1 Microbial survival rates in chloride brines

The survival rates of *P. halocryophilus* in eutectic Cl⁻ samples were significantly increased when the samples were kept at lower temperatures (Fig. 3-2). For example, if *P. halocryophilus* was left in NaCl containing samples at room temperature all cells died within two weeks, while their survival was substantially increased at 4°C, and nearly no CFU reduction occurred at -15°C. Two samples of ST 2 were investigated for the NaCl system to confirm reproducibility. Samples of ST 2 had slightly higher starting cell numbers in all cases studied. However, survival rates of ST 1 and ST 2 samples were similar, although the curve for the NaCl ST 1 sample at 4°C had a steeper slope during the first 40 days but approached the slope of the ST 2 curves afterwards.



FIG. 3-2: Survival rates of *P. halocryophilus* in eutectic CI⁻ samples. Initial cell cultures were incubated for 4 days (ST 1) or 7 days (ST 2) at 25°C in growth medium containing 10 wt% NaCl before mixing them with the salt solution. CFUs were obtained as biological duplicates. Detection limit for CaCl₂ containing samples at 103 CFU/mL results from the dilution factor of 3 that is necessary to decrease the Ca²⁺ concentration on the agar plate sufficiently for colony growth to occur.

The cell survival results for the MgCl₂ containing samples were very similar to those of NaCl, but at -15°C and -30°C there appears to be a slow reduction of surviving cells. The survival rates of *P. halocryophilus* in CaCl₂ containing samples at 25°C and 4°C were significantly lower than those containing NaCl or MgCl₂. In contrast, survival rates at subzero temperatures were comparable to the MgCl₂ system, i.e. cells were dying slower at these lower temperatures.

3.3.2 Microbial survival rates in perchlorate brines

The survival rates of *P. halocryophilus* in eutectic ClO₄⁻ samples (Fig. 3-3) were orders of magnitude lower than in Cl⁻ samples (Fig. 3-2). Although survivability at lower temperatures in NaClO₄ samples increased, the survival rate is generally so low that even at -30°C few cells survived for only one day (Fig. 3-3A and 3-3B). For Mg(ClO₄)₂ and Ca(ClO₄)₂ containing samples, survival was even lower, where CFU detection was only possible for samples stored at -30°C and none were detected for samples kept at higher temperatures.

We increased the NaClO₄ concentration in the growth media to determine if ClO₄⁻ preconditioning of *P. halocryophilus* could enhance their survival in eutectic ClO₄⁻ samples. It was found that *P. halocryophilus* can grow in the presence of up to at least 10 wt% NaClO₄ (with no additional NaCl in the growth medium) or up to 10 wt% NaCl + 5 wt% NaClO₄. However, cell growth under these conditions was markedly slower than in ClO₄⁻ free medium. Thus, for the preconditioning experiments we used cells preconditioned with 8 wt% NaClO₄ or with 10 wt% NaCl + 3 wt% NaClO₄ (Fig. 3-3B). Nevertheless, in these cases cells grew slower than in the experiments with 10 wt% NaCl in the growth media, which resulted in a lower starting cell number. Due to the slower growth rates in ClO₄⁻ containing media, the cells should still be in the exponential growth phase after 7 days of incubation. We found that changing the preconditioning salt from NaCl to NaClO₄ does not increase the survivability in ClO₄⁻ containing samples. However, increasing the total salt concentration by adding 3 wt% NaClO₄ on 10 wt% NaCl results in a slight increase in survival. Cells in these samples doubled their maximum survival time from approximately one day in samples

containing either 8 wt% NaClO₄ or 10 wt% NaCl to two days in samples containing 3 wt% NaClO₄ + 10 wt% NaCl.



FIG. 3-3: (A) Survival rates of *P. halocryophilus* in ClO₄⁻ samples. Initial cell cultures were incubated for 7 days at 25°C in growth medium containing 10 wt% NaCl before mixing them with the salt solution **(B)** Effects of different preconditioning methods at -30°C. Before mixing them with the salt solution, the initial cell cultures were incubated for 7 days at 25°C in growth medium containing salts as indicated the figure legend. CFUs were obtained in biological duplicates.

3.3.3 Arrhenius plot

For a better comparison of the temperature dependences of cell survival in different Cl⁻ and ClO₄⁻ containing samples, the data was plotted as an Arrhenius type graph, with the slopes of the survival rate-fitted lines for all Cl⁻ and NaClO₄ containing samples (values for same salt-temperature combinations were averaged) plotted logarithmically against the temperature of the sample (Fig. 3-4A). As the slope (S) of these curves is the crucial parameter for evaluating the extent to which survival is increased with lowering temperature, the slope values for each curve were plotted as well (Fig. 3-4B).

The slopes for the Cl⁻ containing samples, especially for MgCl₂ and CaCl₂, flatten below death rate constants of about 0.1 day⁻¹. However, it has to be kept in mind that the death rates are on a logarithmic scale and, therefore, the flattening might only be the result of approaching a non-lethal state, i.e. a death rate of zero. Therefore only the steeper slops of the curves towards higher temperatures were compared in Figure 3-4B.

3.3.4 Microbial survival rates during freeze/thaw cycles

P. halocryophilus survives repeated freeze/thaw cycles more readily if the growth medium contains additional NaCl. Without NaCl, the CFU reduction is 20% per freeze/thaw cycle, whereas an addition of 10 wt% NaCl lowered the death rate to 7% per freeze/thaw cycle (Fig. 3-5). Cells in the salt-free samples survived up to 70 freeze/thaw cycles, while extrapolation of the death rate curve for the samples containing 10 wt% NaCl reveals that cells in these samples could survive up to approximately 200 freeze/thaw cycles.



FIG. 3-4: (A) Arrhenius type plot for all Cl⁻ samples and NaClO₄ including slopes for linear parts of the curves and molar concentrations (c), water activities (a_w) and ionic strengths (I) for all samples.
(B) Slopes (S) of the steeper curve parts plotted as bar charts.



FIG. 3-5: Survivability of *P. halocryophilus* during freeze/thaw cycles. Cells were incubated for 7 days at 25°C in growth medium containing either no additional salt (black circles) or 10 wt% NaCl (grey triangles) before subjecting them to freeze/thaw cycles. CFUs obtained from biological triplicates.

3.4 Discussion

We have shown that survival of *P. halocryophilus* is significantly lower in eutectic CIO_4^- samples than in Cl⁻ containing samples at all investigated temperatures, although ionic strength and water activities at 25°C are similar, e.g. for $CaCl_2$ and $NaClO_4$ samples (Tab. 3-1 and Fig. 3-4A). Moreover, the water activity should not change markedly when lowering the temperature since it has been shown to remain reasonably constant at subzero temperatures for solutions containing Cl⁻ (Fontan and Chirife, 1981) and CIO_4^- (Toner and Catling, 2016). Furthermore, the oxidizing ability of CIO_4^- is negligible in solutions at these low temperatures (Brown and Gu, 2006). Thus, other ion specific properties must be

responsible for the differences in the inhibitory effects of the ClO_4^- and Cl^- containing samples.

Additionally, we demonstrated that the survival of *P. halocryophilus* cells in eutectic Cl⁻ and ClO₄⁻ samples increases systematically with decreasing temperatures. The Arrhenius plot (Fig. 3-4) indicates that this correlation is more significant in Cl⁻ containing samples. The slope for the CaCl₂ containing samples (0.225 °C⁻¹) is more than 2.5-fold steeper than for the NaClO₄ containing samples (0.079 °C⁻¹), which means that survivability in the CaCl₂ samples is increased by lowering temperature to a significantly higher extant than in the NaClO₄ samples. The slopes for MgCl₂ (0.152 °C⁻¹) and NaCl (0.135 °C⁻¹) containing samples lie between those of NaClO₄ and CaCl₂. The slow decrease of the death rate constant in the NaClO₄ containing samples with decreasing temperature is caused by the normal temperature dependence of all chemical reactions (including cell damaging reactions) described by the Arrhenius equation. The steeper slopes for the Cl⁻ samples indicate an additional effect on the decrease of death rates with lowering the temperature.

We propose the main reason for this difference in the temperature dependence of the cell survival in Cl⁻ and ClO₄⁻ containing samples is caused by the increase of size and stability of hydration spheres around the ions in the Cl⁻ brines at lower temperatures. Previous studies have shown that with decreasing temperatures the hydration number around cations such as Ca²⁺ increases (Zavitsas, 2005) and that the first hydration sphere around Na⁺ in NaCl solutions becomes more rigid (Gallo *et al.*, 2011). Furthermore, X-ray and neutron diffraction studies have shown that a decrease in temperature results in the first hydration shell of Cl⁻ ions becoming gradually more structured and a second hydration sphere forming (Yamaguchi *et al.*, 1995). Data from the method of integral equations has revealed a strengthening of the hydrogen bonding between Cl⁻ and water molecules in the first hydration shell at lower temperatures (Oparin *et al.*, 2002). These results demonstrate that lowering the temperature in Cl⁻ containing samples increases stability and size of the hydration spheres around the dissolved ions, which is known to reduce the permeability of ions through cell membranes (Degrève *et al.*, 1996; Jahnen-Dechent and Ketteler, 2012). Hence, we conclude that a reduced ion permeability caused by larger and more stable

hydration spheres minimizes the toxicity of the extracellular high ion concentration. Therefore the cell survivability in low temperature Cl⁻ brines is increased over the extent of the normal Arrhenius-like temperature dependence.

In contrast to cations and Cl⁻ ions, ClO₄⁻ ions do not tend to form stable hydration shells (Neilson *et al.*, 1985; Lindqvist-Reis *et al.*, 1998). The reason for the small size and low stability of hydration shells around ClO_4^- is its large ionic radius and its low electrical charge with an even distribution over the entire anion (Brown and Gu, 2006) resulting in weak hydrogen bonds and one of the lowest hydration energies of common inorganic anions (Moyer and Bonnesen, 1979). The low tendency of ClO_4^- ions to form stable hydration spheres at any temperature presumably correlates with a constant cell membrane permeability and is a reasonable explanation for the observed low survival rate increase in ClO_4^- containing samples with lower temperatures.

Finally, the higher membrane permeability at all investigated temperatures explains the general low survivability of cells in ClO_4^- verses Cl⁻ containing samples. However, several other structural factors may play a significant role as well, for example, the formation of chloro complexes in CaCl₂ containing samples (Phutela and Pitzer, 1983; Wang *et al.*, 2016), ion pair formations (Fleissner *et al.*, 1993; Smirnov *et al.*, 1998), molecular mimicry (Cianchetta *et al.*, 2010), or the reported formation of a crust around *P. halocryophilus* cells at low temperatures, which consist of peptidoglycan, choline and calcium carbonate (Mykytczuk *et al.*, 2016), that might provide protection against Cl⁻ but not ClO_4^- .

The freeze/thaw experiments have shown that the survivability of cells during freezing and thawing processes increases when NaCl is present. Studies have argued that the formation of large water crystals during freezing might be destructive to cell membranes and might even grow larger during thawing due to migratory recrystallization (Mazur, 1960). Greater amounts of large water crystals should only form in the salt-free samples, because in the salt-rich samples pure water crystals are formed during freezing only until the solution under the ice layer reaches the eutectic composition. After that point, eutectic freezing results in very small water ice and salt hydrate particles, which potentially could be physically less harmful to the cells. Another lethal effect during the freezing process might be the osmotic shock resulting from the increasing solute concentration in the remaining liquid solution (Harrison, 1956). It is reasonable to assume that the decreased water activity as a result of the enhanced solute concentration in the growth media during the freezing process, is less harmful to bacteria that were already preconditioned with 10 wt% NaCl during incubation. Furthermore, studies have shown that a heat or cold shock treatment of Deinococcus radiodurans cells increases their survivability against freeze/thaw cycles (Airo et al., 2004), hence an exposure to higher salt concentrations may result in a similar stress response in *P. halocryophilus* and a higher tolerance against freeze/thaw cycling. The beneficial effect of NaCl during the freeze/thaw process has been described in previous studies (e.g. Calcott and Rose, 1982). In contrast, other studies have shown the opposite trend, that the presence of NaCl decreases the percent of surviving bacteria during freeze/thaw cycles (Postgate and Hunter, 1961; Nelson and Parkinson, 1979). However, these bacteria are not known to be halotolerant and therefor might suffer more under the increased osmotic stress than P. halocryophilus does. In the future, freeze/thaw experiments with halophilic microorganisms like P. halocryophilus should also include other types of salts in the growth medium, to test their influence on the cell survivability in comparison to NaCl.

On Mars, NaCl has been detected globally, and especially at high levels by remote sensing in the Southern Highlands (Osterloo *et al.*, 2008). Perchlorates have been detected at the Phoenix lander and the Curiosity Rover sites and is also likely global in extent (Clark and Kounaves, 2016). These salts have also been suggested to be part of the brines associated with the Recurrent Slope Lineae (RSL) (Ojha *et al.*, 2015). However, more recent studies have argued that only small amounts of water might be present within RSL (Edwards and Piqueux, 2016) and that the darkening of the RSL might only be a result of a rewetting process of former flows of salty water (Heinz *et al.*, 2016). Furthermore, it has also been suggested that RSL may be the result of granular flows where water plays no or only a subordinated role (Dundas *et al.*, 2017).

However, in general, the ubiquitous presence of hygroscopic salts and of water in the form of ice on the poles or in the subsurface or as gas in the atmosphere makes the existence of cold, highly concentrated brines conceivable. Such brines could develop through deliquescence or at salt-ice contacts (Fischer *et al.*, 2014), being temporally stable at the surface of Mars and perhaps permanently stable in the subsurface as briny groundwater (Burt and Knauth, 2003; Martínez and Renno, 2013). Our data reveal that microorganism resident in such brines could survive significantly longer at subzero temperatures than previously thought and they might even thrive in slightly diluted brines as has been shown for *P. halocryophilus* in 19 wt% NaCl solution (Mykytczuk *et al.*, 2012). As temperatures on Mars are change throughout the day and the seasons it is conceivable that temperatures drop temporally below the eutectic temperature of the brine. Our freeze/thaw experiments demonstrate that the freezing and thawing of cells in eutectic brines would be less lethal than freezing and thawing in salt-free water.

3.5 Conclusion

We have shown enhanced microbial survival in subzero eutectic Cl⁻ brines compared to their warmer analogues. Based on the results, the best hypothesis is that the increase in size and stability of hydration shells around ions at lower temperatures reduces osmotic and chaotropic stress factors for microbial organisms. Although *P. halocryophilus* grew even in the presence of 10 wt% NaClO₄, higher ClO₄⁻ concentrations lower survival rates significantly even at subzero temperatures. It appears that the decreased capability of ClO₄⁻ ions to form stable hydration spheres causes the high toxicity of eutectic ClO₄⁻ solutions and the lower temperature dependence of cell survival compared to Cl⁻ brines. Furthermore, we have shown that the presence of salts like NaCl increases the survivability during freeze/thaw processes. This has broad implications for the habitability of some extreme environments on Earth and the potential habitability of Mars.

3.6 Acknowledgments

We thank Lyle Whyte for helping to improve the manuscript. This project was funded by European Research Council Advanced Grant "Habitability of Martian Environments" (HOME, # 339231). All of the numerical data for this paper are provided in the figures and are also available in tabular form from the authors upon request (j.heinz@tu-berlin.de).

Author Disclosure Statement

No competing financial interests exist.

Accepted manuscript of: Heinz, J., Waajen, A.C., Airo, A., Alibrandi, A., Schirmack, J., and Schulze-Makuch, D. (2019) Bacterial growth in chloride and perchlorate brines: Halotolerances and salt stress responses of *Planococcus halocryophilus*. Astrobiology, ahead of print, https://doi.org/10.1089/ ast.2019.2069.

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Publication III: Bacterial growth in chloride and perchlorate brines: Halotolerances and salt stress responses of *Planococcus halocryophilus*

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Running Title: Bacterial Growth in Brines.

Search Terms: Brines; Salt; Growth; Mars; Perchlorate; Halotolerance

Abstract

Extraterrestrial environments encompass physicochemical conditions and habitats that are unknown on Earth, such as perchlorate-rich brines that can be at least temporarily stable on the Martian surface. In order to better understand the potential for life in these cold briny environments, we determined the maximum salt concentrations suitable for growth (MSCg) of six different chloride and perchlorate salts at 25°C and 4°C for the extremophilic cold- and salt-adapted bacterial strain *Planococcus halocryophilus*. Growth was measured through colony forming unit (CFU) counts, while cellular and colonial phenotypic stress responses were observed through visible light, fluorescence, and electron scanning microscopy. Our data show that (1) the tolerance to high salt concentrations can be increased through a step-wise inoculation toward higher concentrations, (2) ion-specific factors are more relevant for the growth limitation of *P. halocryophilus* in saline solutions than single physicochemical parameters like ionic strength or water activity, (3) P. halocryophilus shows the highest microbial sodium perchlorate tolerance described so far, however, (4) MSCg values are higher for all chlorides compared to perchlorates, (5) the MSCg for calcium chloride was increased by lowering the temperature from 25°C to 4°C, while sodium and magnesium containing salts can be tolerated at 25°C to higher concentrations than at 4°C, (6) depending on salt type and concentration, P. halocryophilus cells show distinct phenotypic stress responses such as novel types of colony morphology on agar plates and biofilm-like cell clustering, encrustation, and development of intercellular nanofilaments. This study, taken in context with previous work on the survival of extremophiles in Mars-like environments, suggests that high-concentrated perchlorate brines on Mars might not be habitable to any present organism on Earth, but they might be able to evolve thriving in such environments.

Keywords: Brines; Salt; Growth; Mars; Perchlorate; Halotolerance

4.1 Introduction

Most subzero saline habitats on Earth are dominated by sodium chloride (NaCl) and most research has been focused on this salt (for review e.g. Gunde-Cimerman *et al.*, 2018). However, non-NaCl saline environments exist on Earth as well, such as the calcium chloriderich Don Juan Pond, Antarctica (Dickson *et al.*, 2013) or the Spotted Lake, Canada having high sulfate concentrations (Pontefract *et al.*, 2017).

Similarly, soils on Mars contain non-NaCl salts such as perchlorates (Hecht *et al.*, 2009). Accordingly, the presence of perchlorate-rich Martian groundwater has been discussed (Clifford *et al.*, 2010). Fitting well to this hypothesis, it was recently proposed that the discovered subsurface lake might contain magnesium and calcium perchlorates causing a freezing point depression of water down to its calculated temperature of -68°C (Orosei *et al.*, 2018). Furthermore, spectral investigations indicated that perchlorate salt hydrates and their brines might play a role in the formation of Recurring Slope Lineae (RSL) on Mars (Ojha *et al.*, 2015).

At the Phoenix landing site, perchlorate concentrations in the Martian soil are ranging from 0.4 to 0.6 wt% (Hecht *et al.*, 2009). However, it has to be kept in mind that the perchlorates are present as solid salts which will form highly concentrated brines whenever the temperature is above the eutectic temperature and the relative humidity is above the deliquescence relative humidity (RH) of the perchlorate salt (Davila *et al.*, 2010; Nikolakakos and Whiteway, 2015; Heinz *et al.*, 2016). For example, at -34°C the sodium perchlorate concentration of the forming eutectic brine would be 52.6 wt% (9 M) (Hennings *et al.*, 2013), which is too high for any organism we know from Earth to thrive therein. Therefore, only diluted perchlorate brines might serve as a habitat. These diluted solutions could be stable in the subsurface of Mars (Burt, 2003; Martínez and Renno, 2013).

Since there are diurnal and seasonal temperature and humidity changes on Mars, it can be assumed that salt concentrations in these brines also fluctuate due to crystallization of ice or salt hydrates at cold temperature conditions or due to water absorption from the atmosphere at high RH conditions, e.g. in the morning hours when the RH in the Martian atmosphere is highest and can reach saturation (Harri *et al.*, 2014). Thus, microorganisms would have to survive temporarily enhanced salt concentrations (including crystallization processes) at low temperatures and thrive at times of higher temperatures and brine dilution. It has already been shown that low temperatures enhance the bacterial survival in chloride (Cl⁻) and perchlorate (ClO₄⁻) brines with eutectic concentrations while, additionally, the high salt concentrations benefit the survival during freeze/thaw cycles of the brine (Heinz *et al.*, 2018). However, the question how low the salt concentration would have to be for microbial growth remained open prior to this study.

The exploration of the physicochemical limits for growth and survival of organisms thriving in cold saline environments, gives not only insight into the microbial ecosystems adapted to the most extreme habitats on Earth but also provides the necessary data for assessing the habitability of extraterrestrial environments, e.g. on Mars (Schulze-Makuch *et al.*, 2015; Schulze-Makuch *et al.*, 2017). A model organism for halo- and psychrotolerant bacteria is *Planococcus halocryophilus*, which has been isolated from the active layer of permafrost soil in the Canadian High Arctic (Mykytczuk *et al.*, 2012). It is able to grow at 19 wt/vol% NaCl (corresponding to 16 wt%) concentration and at -15°C, while showing metabolic activity down to -25°C (Mykytczuk *et al.*, 2013; Raymond-Bouchard *et al.*, 2017).

Bacterial growth of *P. halocryophilus* under these harsh conditions is enabled by the expression of various osmolyte transporters and cold-adapted proteins, a high lipid turnover rate, and a high resource efficiency at subzero temperatures with an accumulation of carbohydrates as energy resource (Mykytczuk *et al.*, 2013). Furthermore, analyses of the proteome of *P. halocryophilus* revealed intricate changes in protein expression (Raymond-Bouchard *et al.*, 2017). Under subzero growth conditions, this bacterial strain develops a nodular sheet-like crust around the cells which might provide protection against cold and osmotic stress (Ronholm *et al.*, 2015; Mykytczuk *et al.*, 2016).

The ability to tolerate these cold and salty conditions was the reason for choosing *P*. *halocryophilus* as a suitable analog microorganism to test how well microbes on Earth can adapt to the conditions prevailing on Mars and whether adaption to high-concentration perchlorate brines is possible with the available biochemical toolset of life as we know it.

P. halocryophilus is an aerobic organism and thus, at first glance, might not appear relevant to Martian habitability, given that the molecular oxygen (O_2) content in the Martian atmosphere is with 0.13 % very low. However, recent studies found that Martian brines can be enriched with dissolved O_2 up to 2 mol m⁻³, enabling aerobic microbes to metabolize (Stamenković *et al.*, 2018). In addition, there might be other suitable extraterrestrial habitats that provide both osmotic stress conditions and feasible O_2 levels.

Here, we investigated the maximum halotolerance for growth of *P. halocryophilus* at optimal growth temperature (25°C) and low temperature (4°C) for various Cl⁻ and ClO₄⁻ salts. Furthermore, we investigated the phenotypic adaptations to salt stress such as changes in cell and colony morphology and the formation of cell clusters. This study examines several major aspects important for astrobiological research especially on Mars where Cl⁻ and ClO₄⁻ brines might be the last possible refuges for life (Davila and Schulze-Makuch, 2016).

4.2 Materials and Methods

4.2.1 Organism and culture conditions

The bacterial strain *Planococcus halocryophilus* Or1 (DSM 24743^T), obtained from the DSMZ (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures) was used in all experiments described within this study. The bacteria were grown aerobically at 25°C (optimum growth temperature) or 4°C (low temperature control) in liquid DMSZ growth medium #92 (3% Tryptic soy broth (TSB), 0.3% Yeast extract) with various concentrations (1 wt% (w/w) incremental steps) of one of the following salts: sodium chloride (NaCl), magnesium chloride (MgCl₂), calcium chloride (CaCl₂), sodium perchlorate (NaClO₄), magnesium perchlorate (Mg(ClO₄)₂), or calcium perchlorate (Ca(ClO₄)₂). The media were prepared by mixing the media components, salt and water, followed by pH adjustment (pH 7.2 – 7.4), centrifugation if necessary (in calcium-containing samples yeast flocculation can occur (Stratford, 1989) which has no influence on the cells growth, because *P*.

halocryophilus readily thrives in medium containing only TSB (Mykytczuk *et al.*, 2012)), and sterile filtration.

4.2.2 Determination of the maximum salt concentration suitable for growth (MSCg)

Bacteria were monitored for growth and death using colony forming unit (CFU) counts to determine the MSCg values of the respective salts. Two identical samples were prepared and inoculated separately (biological duplicates) for each experiment. For CFU counts two aliquots of 100 µl were taken from each sample and were plated on agar plates containing DMSZ growth medium #92. CFUs for the same experimental conditions were averaged using the arithmetic mean. Where necessary, the aliquots were diluted with phosphate buffer saline (PBS) containing additional 10 wt% NaCl (for all sodium containing samples) or 10 wt% MgCl₂ (for all magnesium or calcium containing samples). The increased amount of salt in the PBS was necessary to avoid bursting of cells by osmotic pressure during the dilution of the saline growth media. Some experiments were repeated to check reproducibility.

To investigate the effect of the inoculation culture on growth and survival of *P*. *halocryophilus* in the salty samples 5 ml of the saline medium (biological duplicates) were inoculated with one of the following inoculation methods (IM):

- IM 1: The medium was inoculated with the stock culture (growth medium + 10 wt% NaCl) at 25°C.
- IM 2: The medium was inoculated with a culture grown at the respective temperature (25°C or 4°C) in medium with lower concentration of the respective salt (progressive culture adaption).
- IM 3: Medium for experiments at 25°C was inoculated with a culture grown at 4°C in medium with the same or higher concentration of the respective salt.
- IM 4: Medium for experiments at 4°C was inoculated with a culture grown at 25°C in medium with the same or higher concentration of the respective salt.

Inoculation volumes ranged from 10 μ l to 50 μ l depending on the cell density of the inoculation culture. However, due to cell clustering (section 3.2.2) the starting cell density after inoculation varied between 5·10² CFU/mL and 5·10⁴ CFU/mL.

Because progressive culture adaptions were done by a 1 wt% stepwise increase in the salt concentrations of the medium, the MSCg values had an inherent uncertainty of 1 %. Larger uncertainties (up to 2 %) occurred for some samples incubated at 4°C when cells in media with salt concentrations above the MSCg neither grew nor died within the time of the experiment.

4.2.3 Light, fluorescence and scanning electron microscopy

A set of samples was investigated under the light microscope (Primo Star, Zeiss, equipped with Axio Cam 105 color) without prior sample treatment. For fluorescence microscopy of living and dead cells samples were washed twice with PBS containing 10 wt% NaCl. Three ml of each sample were stained with 3 µl of a 1:2 mixture of component A (SYTO 9 dye, 3.34 mM) and component B (Propidium iodide, 20 mM), where component A causes green fluorescence of intact cells and component B causes red or orange fluorescence of dead cells with damaged cell walls. The stained samples were imaged with a fluorescence microscope (Polyvar 2, Reichert-Jung) equipped with a Xenon lamp (XBO 150 W/1).

Samples for scanning electron microscopy (SEM) were washed twice with PBS containing 10 wt% NaCl followed by fixation in 2.5 % glutaraldehyde solution (in 0.1 M phosphate buffer (PB), pH = 7.3). The fixed samples were washed twice with 0.1 M PB, dehydrated through a graded acetone series (50, 70, 90, 95, 100%), critical point dried in a Leica CPD300, coated with carbon, and imaged with a Hitachi S-2700 microscope.

4.3 Results

4.3.1 Growth at 25°C and 4°C

4.3.1.1 GROWTH CURVES AND MSCG VALUES: For determining the MSCg values of all Cl⁻ and ClO₄⁻ salts we used growth versus death as a distinction criterion. For example, after 6 days of incubation in CaCl₂-rich media *P. halocryophilus* shows an increase in CFU values (i.e. growth) at 25°C under all tested salt concentrations with the exception of 9 wt%; hence at this temperature the MSCg value is 8 wt% (Fig. 4-1). However, at 4°C the MSCg value is greater with 10 wt% (embedded plot of Fig. 4-1).





All growth curves generated are provided in the supplementary materials (Fig. 7-4 – 7-15) and the resulting MSCg values for all salts and temperatures, including their corresponding total ion concentrations (sum of cation and anion concentration), anion concentrations, ionic strengths and water activities are listed in Table 4-1. The MSCg (wt%), total molar ion concentrations and anion concentrations are plotted as bar charts in Fig. 4-2.

TAB. 4-1: MSCg values and corresponding total ion concentrations (sum of cations and anions), anion concentrations, ionic strengths and water activities at 25°C and 4°. Values in brackets give the deviation as described in section 2.2.

	MSCg			total ion conc.		anion conc.		ionic strength		water activity*		
	[wt.%]		[mol/l]		[mol/l]		[mol/l]		[mol/l]			
	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C
NaCl	14(1)	11(1)	2.79	2.11	5.57	4.23	2.79	2.11	2.79	2.11	0.90	0.93
MgCl ₂	11(1)	10(2)	1.30	1.17	3.89	3.50	2.60	2.33	3.89	3.50	0.92	0.93
CaCl ₂	8(1)	10(1)	0.78	1.00	2.35	3.00	1.57	2.00	2.35	3.00	0.96	0.95
NaClO ₄	12(1)	7(2)	1.11	0.61	2.23	1.23	1.11	0.61	1.11	0.61	0.96	0.98
Mg(ClO ₄) ₂	5(1)	4(1)	0.24	0.19	0.71	0.56	0.47	0.37	0.71	0.56	0.99	0.99
Ca(ClO ₄) ₂	3(1)	3(1)	0.13	0.13	0.39	0.39	0.26	0.26	0.39	0.39	0.99	0.99

* Water activity calculated for 25°C from the Pitzer equation (Pitzer, 1991) with Pitzer parameters taken from (Toner et al., 2015). The temperature dependence (25°C vs. 4°C) of the water activity is negligible for C^{Γ} (Fontan and Chirife, 1981) and ClO₄⁻ solutions (Toner and Catling, 2016) at temperatures at above 0°C.

Overall, *P. halocryophilus* shows exceptionally high halotolerances to all Cl⁻ and ClO₄⁻ salts at both 25°C and 4°C (Fig. 4-2A, B). The Cl⁻ tolerance is at least 2.5-fold higher than the tolerance to ClO_4^- in media with the same cation (Fig. 4-2C). However, with 12 wt% (1.1 M) NaClO₄ at 25°C we found the highest microbial tolerance to NaClO₄ described so far. The lowest tolerated water activity was 0.90 in 14 wt% (2.8 M) NaCl, while the highest tolerable ionic strength (3.9 mol/l) was reached in 11 wt% (1.3 M) MgCl₂ at 25°C (Tab. 4-1).





4.3.1.2 EFFECT OF THE INOCULATION METHOD (IM) ON THE MSCG: The applied IM effects the growth curves and the resulting MSCg values in the following ways:

(1) At 25°C, the MSCg values could only be reached with IM 2 (progressive culture adaptation at 25°C) but not with IM 1 (inoculation with stock culture) where cell death occurs already at lower salt concentrations. For example, the MSCg for MgCl₂ was 9 wt% at 25°C when the media was inoculated with the stock culture (IM 1), however, a stepwise (1 wt%) increase in the MgCl₂ concentration (IM 2) resulted in a MSCg of 11 wt% MgCl₂ (Fig. 7-6). It is notable that the length of the growth curve lag phase (occasionally including an initial CFU reduction) is enhanced with increasing salt concentration and decreasing temperatures (e.g. Fig. 4-1, 7-4).

(2) Appling IM 3 ($4^{\circ}C \rightarrow 25^{\circ}C$ inoculation) resulted in an increase of the MSCg values at 25°C only in the case of Ca(ClO₄)₂ samples. Here, growth in 3 wt% Ca(ClO₄)₂ was not detected after inoculation with the stock solution (IM 1) nor with a 2 wt% Ca(ClO₄)₂ culture grown at 25°C (IM 2), but only after inoculation with a 3 wt% Ca(ClO₄)₂ culture grown at 4°C (IM3) (Fig. 7-14).

(3) At 4°C, a higher MSCg value was reached by applying IM 2 (progressive culture adaptation at 4°C) than by applying IM 4 ($25^{\circ}C \rightarrow 4^{\circ}C$ inoculation). For example, at 4°C inoculation of 2 wt% Mg(ClO₄)₂ medium with a 5 wt% Mg(ClO₄)₂ culture grown at 25°C did not show growth, indicating a MSCg < 2 wt% Mg(ClO₄)₂ when IM 4 is applied. However, a 1 wt% stepwise increase in Mg(ClO₄)₂ concentration at 4°C (IM 2) resulted in an culture able to growth at 4 wt% Mg(ClO₄)₂ (Fig. 7-13). This data suggests that for growth at 4°C an adaption to the cold first has to take place before *P. halocryophilus* can adapt stepwise to higher salt concentrations at that temperature.

4.3.1.3 TEMPERATURE EFFECT ON THE MSCG: The relative shift in the MSCg that occurs by lowering the incubation temperature from 25°C to 4°C is visualized in Figure 4-3. Among all six salts investigated in this study, only cells in CaCl₂ containing media show an enhanced salt tolerance at lower temperature, where growth at 9 wt% and 10 wt% CaCl₂ did not occur at 25°C but only at 4°C (Fig. 4-1).



FIG. 4-3: Relative changes in the MSCg induced by lowering the incubation temperature from 25°C to 4°C.

Nevertheless, the observation that only IM 4 (4°C \rightarrow 25°C inoculation) resulted in growth at the MSCg for Ca(ClO₄)₂ (3 wt%) at 25°C (see point (2) of section 4.3.1.2) provides evidence that also the tolerance to Ca(ClO₄)₂ is increased at 4°C, however, to a lower extent than the 1 wt% salt concentration incremental steps used in this study (section 2.1). This suggests a general increase in the calcium (Ca²⁺) tolerance of *P. halocryophilus* at lower temperatures.

In contrast, the sodium (Na⁺) tolerance is decreased at lower temperatures for both anions, Cl⁻ and ClO₄⁻ (Fig. 4-3). The tolerances to magnesium (Mg²⁺) are only slightly reduced at 4°C (1 wt% also for both anions, Cl⁻ and ClO₄⁻). The reduction in the Na⁺ tolerance at 4°C on the one hand and the increased Ca²⁺ tolerance at 4°C on the other hand led to an equalization of the anion (Cl⁻ or ClO₄⁻) concentration at the MSCg at 4°C, while at 25°C the differences between the anion concentrations for the three different cations (Na⁺, Mg²⁺ and Ca²⁺) salts are more pronounced (Fig. 4-2).

4.3.2 Cellular and colonial phenotypic salt-stress adaptations

4.3.2.1 COLONIAL PHENOTYPIC ADAPTATIONS: *P. halocryophilus* cells grown in liquid cultures exhibit with an increase in salt concentration, particularly for perchlorate salts, the tendency to develop macroscopic cohesive biofilms that could only be disrupted by intense shaking or vortexing (Fig. 4-4A).

Furthermore, it was observed that a novel colony phenotype (type II) appears only on plates inoculated with aliquots from cells grown in ClO_4 -rich medium (especially with > 9 wt% NaClO₄ at 25°C). This type II colony is paler and duller then the usual colonies (type I) that are shiny and orange (Fig. 4-4) and does not occur in cultures grown in salt-free or Cl⁻ containing media. Occasionally both colony types occurred on plates inoculated with aliquots from cells grown in media with perchlorate concentrations of a few wt% below the MSCg (Fig. 4-4B). Sporadically such colonies underwent a transformation from the type II back to the type I after approx. 2 weeks of growth on the agar plates (Fig. 4-4C). Contamination was ruled out through 16S sequencing of both colony types (99.90% sequence similarity of type I vs type II, data not shown), suggesting that the colony type II represents a reversible multi-generational phenotypic adaptation of *P. halocryophilus* to high perchlorate salt stress. Type II colonies needed 3 to 5 times longer than type I colonies to reach comparable colony sizes.

Two additional colony phenotypes were observed on agar plates: Type III colonies are irregular jagged in shape and occurred on agar plates inoculated with magnesium-rich (Cl⁻ and ClO₄⁻) cultures (Fig. 7-17). Type IV colonies are mucoidal and shiny, merge rapidly during growth and occurred on agar plates inoculated with CaCl₂-rich cultures (Fig. 7-18).


FIG. 4-4: Macroscopically visible salt stress responses: **(A)** Biofilm-like cell clumping occurring in a 10 wt% NaClO₄ culture after 1 month of growth. **(B)** Two different colony morphologies of *P. halocryophilus* observed one week after plating a 9 wt% NaClO₄ culture at 25°C. The shiny intenseorange colonies (type I) represent the colony morphology typical for *P. halocryophilus* grown in medium with low salt concentrations. The paler and smaller colonies (type II) only occurred after plating perchlorate-rich cultures. **(C)** Transformation of a type II colony into type I after two weeks of colony growth.

4.3.2.2 CELLULAR PHENOTYPIC ADAPTATIONS: *P. halocryophilus* cells grown in liquid media containing no additional salts, seen under the light and fluorescence microscope as well as in SEM images, occur predominantly as single cells, diplococci, or small cell aggregates (Fig 5A-C) and have an overall smooth surface with nodules occurring largely along the cell division plane (Fig. 5B) as previously described (Mykytczuk *et al.*, 2016).

Cells however, grown in Cl⁻-rich media occur predominantly in larger clusters of ~100 cells (Fig. 4-5 D-F); and moreover, cells grown in perchlorate-rich media cluster into even larger aggregates of >1,000 cells (Fig. 4-5 G-I). These clusters, containing living and dead 59

cells (Fig. 4-5G), are highly cohesive and could not be disrupted by 5 minutes of ultrasonication nor by washing with 100% ethanol and killing all cells (Fig. 7-19). The cell clustering predominantly occurring in perchlorate-rich media led to higher uncertainties and irregularities in the growth curves of these samples (e.g. Fig. 7-10, 7-14). Concurrent with cell clustering under salt-stress conditions is the development of intercellular nanofilaments within a cluster (Fig. 4-5E). Additionally, cells in CaCl₂-rich media developed surface encrustation (Fig. 4-5F).



FIG. 4-5: Light microscopy **(A, D)**, fluorescence microscopy (after life/dead staining) **(G)** and SEM **(B-C, E-F, H-I)** images of *P. halocryophilus* after growth in media containing no salts (A-C), chlorides (D-F), and perchlorates (G-I) at 25°C. Cells grown in salt-free media developed smooth surfaces with some nodular features (n) and occurred as single cells, dimers, or smaller cell aggregates (A-C). Several salt stress responses were observed including formation of cell clusters (G-I) and filaments (f) (E), and encrustation (en) of some cells in CaCl₂ containing cultures (F).

4.4 Discussion

4.4.1 Salt-stress response and phenotypic adaptation

Microbial salt-stress responses and multi-generational adaptations such as microbial cell aggregation and biofilm formation have been observed for various stress conditions such as desiccation (Monier and Lindow, 2003), UV radiation (Fröls *et al.*, 2008), or NaCl exposure (Philips *et al.*, 2017). Thus far, NaCl was the only salt for which the salt-stress response of *P. halocryophilus* has been investigated, showing that the cells developed an EPS-like coating with short filament-like features if grown in 15 wt% (18 wt/vol%) NaCl media at -5°C (see Fig. 1e, f in Mykytczuk *et al.*, 2016).

For our experimental growth conditions we show a similar trend for *P. halocryophilus* at high NaCl concentrations, where the cells developed even longer nanofilaments (Fig. 4-5E). The formation of similar nanofilaments linking cells within one cluster has also been observed previously, e.g. for the halophilic archaeon *Halococcus salifodinae* (see Fig. 2 in Legat *et al.*, 2013). Furthermore, we conducted experiments with two additional Cl⁻ and three ClO₄⁻ salts showing that *P. halocryophilus* develops particularly large and highly cohesive clusters especially if grown in perchlorate-rich media (Fig. 4-5 G-I). Previous studies on *Hydrogenothermus marinus* have shown a formation of cell chains under increased perchlorate concentrations which has been speculated to result from inchoate cell division (Beblo-Vranesevic *et al.*, 2017); a physiological effect of perchlorate that could be similar for *P. halocryophilus*. Such a mechanism would be in accordance with our observation that cell clusters could neither be destroyed through ultrasonication nor through killing the cells with ethanol treatment (Fig. 7-19).

Furthermore, our results show the development of cohesive biofilms in perchloraterich media (Fig. 4-4A), and if transferred to agar plates, the occurrence of an additional colony morphology (type II, Fig. 4-4B). These are both macroscopic phenotypes consistent with the microscopic development of large cell clusters linked by numerous nanofilaments. Such stress-induced changes in colony morphology are also known for other stressor than high salinity; e.g. under nutrient starvation *Vibrio cholerae* colonies change from the normal translucent to a rugose type (Wai *et al.*, 1998), or with a pH shift *Bacillus subtilis* colonies change from the normal notched 'volcano-like' to round and front-elevated 'crater-like' shapes (Tasaki *et al.*, 2017).

The phenotypic responses described above demonstrate that organisms like *P. halocryophilus* can develop perchlorate-specific stress adaptations that are not (or only to a lower extent) used to counteract high chloride concentrations. This is an important finding for all extraterrestrial environments with natural occurring perchlorates. This might not only include Mars, but any planetary bodies with a relatively dry surface (to avoid leaching of salts) and increased UV radiation (to oxidize chlorides (Carrier and Kounaves, 2015)). For example, spectral data indicates the presence of perchlorates at the surface of the icy moon Europa (Ligier et al., 2016), which could entail delivery of perchlorates to Europa's subsurface ocean (Hand et al. 2007). Also, based on the observed phenotypic adaptations of *P. halocryophilus*, microfossils of such organisms found in perchlorate-rich environments might rather be present in form of cell clusters or biofilms rather than in form of single cells.

4.4.2 Halotolerances of P. halocryophilus at 25°C and 4°C

To our knowledge, the only MSCg data for *P. halocryophilus* has been reported for NaCl being 16 wt% (19 wt/vol%) at 25°C (Mykytczuk *et al.*, 2012) and 15 wt% (18 wt/vol%) + 7 wt/vol% glycerol at -15°C (Mykytczuk *et al.*, 2013; Mykytczuk *et al.*, 2016). In contrast, we found a lower NaCl-halotolerance of 14 wt% and 11 wt% at 25°C and 4°, respectively, which can have various causes e.g.: (1) Mutation of the lab culture and loss of part of its physiological abilities since isolation from its natural environment and first description (Mykytczuk *et al.*, 2012); (2) Different, and commonly not or only partially reported, preconditioning and adaptation procedures for cell growth such as the increment size of salt increase, growth curve phase used for transfer inoculation, and transfer or culture volume and agitation; (3) Use of a different growth media: while we used DMSZ growth media #92 containing Tryptic Soy Broth (TSB) and yeast extract, *Mykytczuk* (2013) and *Mykytczuk* (2016) used a growth media containing TSB and glycerol to maintain the medium liquid down to temperatures of -15°C. Glycerol is known to be an osmoprotectant and, thus,

might have caused the elevated NaCl tolerance at low temperatures. Whether this difference in halotolerance would have also applied to other salts remains unknown, since no other salts than NaCl have been investigated for *P. halocryophilus* prior to this study.

The determination of MSCg values for multiple salts, as presented here for the first time for *P. halocryophilus*, provides the opportunity to test if any single physicochemical aspect of saline solutions is a limiting factor for growth. Our data however, does not indicate that any individual factor (incl. total ion or anion concentration, ionic strength, water activity, see Tab. 4-1, Fig. 4-2, and Fig. 7-16) to be responsible for the growth limitation of *P. halocryophilus* in saline solutions. Otherwise, the values of one of these physicochemical factors (e.g. water activity) would be similar for all salts, which is not the case.

However, our data indicates the following ion species-dependent halotolerances for *P. halocryophilus*:

(1) The anion species, here Cl⁻ vs. ClO₄⁻, plays the most dominant role determining the MSCg values independent of physicochemical unit, showing an overall at least 2.5-fold higher tolerance to Cl⁻ than to ClO₄⁻ salts with the same cation. Nevertheless, the MSCg of NaClO₄ (12 wt%; 1.1 M) is comparatively high and exceeds earlier findings for other organisms such as *Haloferax mediterranei* (0.6 M) (Oren *et al.*, 2014), *Halomonas venusta* (1.0 M) (Al Soudi *et al.*, 2017), *Hydrogenothermus marinus* (0.44 M) (Beblo-Vranesevic *et al.*, 2017) different bacterial isolates from Big Soda Lake in Nevada, USA (0.17 M) (Matsubara *et al.*, 2017), and *Halorubrum lacusprofundi* (0.8 M) (Laye and DasSarma, 2018). It should be noted that it is not clear how *P. halocryophilus* developed such high perchlorate tolerances. Perchlorates are rare in natural terrestrial environments and often coupled to the occurrences of nitrates, e.g. in the Atacama Desert, Chile (Dasgupta et al., 2005). No nitrates have been detected in the permafrost samples *P. halocryophilus* has been isolated from (Steven *et al.*, 2007), thus it appears likely that this strain has never been exposed to environmental perchlorates and did not derive its resistance to perchlorate that way.

(2) A similar role plays the cation species for growth at 25°C, where Na⁺ is the most tolerated cation for each group of anions, i.e. NaCl among the chlorides and NaClO₄ among the perchlorates.

In summary, for growth at 25°C, Na⁺ and Cl⁻ ions are individually the most tolerated ions and NaCl is the salt to which *P. halocryophilus* has the highest halotolerance (2.8 M) at the lowest water activity (0.90). Life on Earth has generally adapted to those factors most efficiently that are the most common and/or abundant in nature; for salt this is NaCl. Hence, our data suggests, that the limitation of growth for *P. halocryophilus* is to a large degree based on evolutionary adaptations to brine veins (most likely consisting of NaCl) present in permafrost soil (Steven et al., 2007). Also, all members of the genus *Planococcus* are halotolerant and have been isolated predominantly from cold and/or saline environments like the Arctic, Antarctic, or marine habitats (Mykytczuk *et al.*, 2012).

Perchlorate ions in aqueous solutions are relatively inert and non-oxidizing due to kinetic barriers (Urbansky, 1998), but rare in Earths nature and therefore presumably exhibit an enhanced toxicity compared to chlorides. Thus, it seems plausible that putative Martian microbes could adapt to natural occurring perchlorate-rich environments to the same extent as Earth microbes such as *Planococcus sp.* did adapt to NaCl-enriched habitats. This idea is consistent with our finding that the tolerance to high salt concentrations can be increased through a step-wise inoculation toward higher concentrations. At low temperatures (4°C in this study) longer lag phases would provide even more time for adaption to higher salt concentrations to occur.

Additional to the ion species-dependent halotolerance, a temperature-dependent halotolerance of *P. halocryophilus* was observed, where at 25°C the MSCg values are different for each cation species, while at 4°C the cation species is less relevant and the MSCg values are more similar (Fig. 2). This MSCg value alignment at low temperatures appears to be largely caused by two separate trends:

(1) Na⁺ containing salts (i.e. NaCl and NaClO₄) can be tolerated by *P. halocryophilus* to higher concentrations at higher temperatures. Possibly, the elaborate biochemical

machinery evolved to cope with high Na⁺ concentrations is kinetically more effective at optimal growth temperatures enhancing the overall halotolerance.

(2) In the case of CaCl₂ we observe the opposite effect, where lowering the temperature increases the halotolerance to this salt. This observation is in accordance with previous studies showing that with decreasing temperature the survival of *P. halocryophilus* in eutectic CaCl₂ brines is enhanced to a significantly larger degree than in NaCl, MgCl₂ or NaClO₄ brines (Heinz *et al.*, 2018). A low temperature induced halotolerance enhancement has been described for example for the bacterial strain *Clostridium* sp. isolated from brine lenses in the Siberian permafrost (Gilichinsky *et al.*, 2003) and for *M. soligelidi* (Morozova and Wagner, 2007) in NaCl-rich media. However, to our knowledge, *P. halocryophilus* is the first organism described thus far that shows an increased CaCl₂ tolerance at lower temperatures and can grow at salt concentrations up to 10 wt% (1 M) CaCl₂ at 4°C in the absence of kosmotropic ions which otherwise could compensate the chaotropic stress caused by calcium ions (Oren, 2013).

However, it remains unclear what mechanism causes the enhanced CaCl₂ tolerance of *P. halocryophilus* at 4°C, perhaps a psychrophilic optimization of the relevant biochemical machinery for coping with Ca²⁺ or simply the lethal effect of calcium being decreased at lower temperatures. It has been proposed that the increased CaCl₂ tolerance at lower temperatures might be due to the formation of larger and more stable hydration shells around calcium ions with decreasing temperature (Heinz *et al.*, 2018). A possible biological explanation is a beneficial effect caused by cellular encrustation, which was only observed in CaCl₂-rich media in this study (Fig. 4-5F). Correspondingly, cellular encrustation containing 20 % calcium carbonate has been documented previously for *P. halocryophilus* cells grown at subzero temperatures in NaCl-rich media (Mykytczuk *et al.*, 2016). Similar encrustation might be triggered in the presence of high Ca²⁺ amounts and might provide an efficient calcium resistance strategy due to the microbial mediated calcium carbonate precipitation. This positive effect might be increased at lower temperatures.

Another factor that might play a role on the enhanced Ca²⁺ tolerance at lower temperatures is the chaotropicity of Ca²⁺. Chaotropic compounds increase the flexibility of

macromolecules like proteins and, thus, can limit life at high temperatures (Hallsworth et al., 2007), but they can also benefit microbial growth at low temperatures (Chin *et al.*, 2010; Rummel *et al.*, 2014). Cray *et al.* (2013) found that the chaotropic activity for CaCl₂ (92.2 kJ kg⁻¹ mol⁻¹) is significantly higher than for MgCl₂ (54.0 kJ kg⁻¹ mol⁻¹) while NaCl is a kosmotropic compound (-11.0 kJ kg⁻¹ mol⁻¹). These data suggest that the high chaotropicity of CaCl₂ (and potentially also of Ca(ClO₄)₂, but data for perchlorates are lacking) might contribute to the enhanced microbial Ca²⁺ tolerance at low temperatures.

Additional work is needed to better understand the observed general trends in the habitability of Mars-analogue brines in dependence on the type of salt, its concentration and temperature. Additional long-term studies under subzero temperatures, similar to Martian environments, should be conducted, especially since studies have shown that *P. halocryophilus* is able to grow under these conditions (Mykytczuk *et al.*, 2013; Mykytczuk *et al.*, 2016). It is possible that lowering the experimental temperature to subzero values will further increase the tolerance of *P. halocryophilus* to Ca²⁺. We also recommend that other microbial strains (including anaerobic ones) or communities should be investigated under similar experimental conditions to widen our understanding of life in cold brines.

4.5 Conclusion

For the first time, this study provides insights into the extremophilic bacterium *P*. *halocryophilus*, well-known for its tolerance to both cold temperatures and high concentration of salts, on how it survives and adapts not only to NaCl solutions, but all Mars-relevant Cl⁻ and ClO₄⁻ salts solutions at different temperatures. Although growth in highly concentrated eutectic brines is not possible (Heinz *et al.*, 2018), the tolerance to the salts investigated in this study is intriguing and can be even enhanced by a stepwise increase in the salt concentration. For example, with 12 wt% (1.1 M) NaClO₄ we found the highest bacterial tolerance to ClO₄⁻ reported to date. For CaCl₂ containing cell cultures we could show that by lowering the temperature from 25°C to 4°C the halotolerance increases from

8 wt% (0.8 M) to 10 wt% (1.0 M), respectively, while tolerances to Na^+ and Mg^{2+} are decreased for the same temperature decline.

The increased Ca²⁺ tolerance at lower temperatures corresponds well to the previously described enhanced survival in low temperature brines (Heinz *et al.*, 2018) and plays an important role for the habitability of Martian environments where Ca²⁺-rich brines might be present in the shallow subsurface (Burt, 2003). Furthermore, Ca²⁺ is thought to be the main counter ion in ClO_4^- salts (Kounaves *et al.*, 2014b) and Ca(ClO_4)₂ might be a main component of the recently discovered subsurface lake near the Martian south pole and could be responsible for its freezing point depression (Orosei *et al.*, 2018).

Additionally, we described several salt adaption mechanisms like cell clustering, the formation of nanofilaments, encrustation of cells and changes in the cell colony morphology. This data provides insight into how life could adapt to such high salt concentrations necessary for a sufficient freezing point depression allowing liquid water to be stable close to the Martian surface.

Acknowledgments

This project was funded by European Research Council Advanced Grant "Habitability of Martian Environments" (HOME, no. 339231). All of the numerical data for this paper are provided in the figures, tables and supplementary information and are also available in tabular form from the authors upon request (schulze-makuch@tu-berlin.de). We would like express our thanks to two anonymous reviewers whose comments and suggestions have helped to improve this manuscript.

Author Disclosure Statement

No competing financial interests exist.

5. Conclusions and Outlook

To better understand the potential of Martian environments in being inhabited by microorganisms one has to focus on both the environmental conditions of the putative Martian habitats, especial in regards of the occurrence of liquid water, as well as the limits of life in these environments. That is why this study investigated the formation of brines via deliquescence and their potential in being involved in the formation of RSL on the hand, and, on the other hand, the effect of these brines on growth and survival of microorganisms, exemplarily represented by *P. halocryophilus* in this study.

Publication I was focused on investigating the formation process of RSL through deliquescence of Cl⁻ and ClO₄⁻ salts interspersed in Mars-analogue soil. The main idea of this study was to demonstrate the benefits of using EC measurements to follow the deliquescence process and quantify the deliquescence rate compared to prior used techniques like Raman spectroscopy (Gough *et al.*, 2011; Gough *et al.*, 2014; Nuding *et al.*, 2014; Nikolakakos and Whiteway, 2015; Nuding *et al.*, 2015; Gough *et al.*, 2016). While in these Raman-spectroscopical experiments a laser beam was directly focused on small salt particles to investigate deliquescence-induced phase changes caused by variations in temperature and RH, the conductivity method presented here provides an experimental approach closer related to realistic environmental conditions (large-scaled experiments including Mars-analog soil). A first set of field experiments in the Atacama Desert, Chile (chapter 7.2), has already shown that the conductivity method is an excellent technique to investigate deliquescence-induced RSL formation.

In the future, similar EC measurements could also be conducted on Mars, e.g. with sensors like the Thermal and Electrical Conductivity Probe (TECP) designed for the Phoenix lander (Zent *et al.*, 2009). Furthermore, additional lab experiments should be carried out to examine which influence parameters like pressure, temperature, RH, type of soil and salts, particle sizes, salt concentration, hydration states, atmospheric composition, and air circulation have on the deliquescence rate. To increase the similarity to RSL these experiments could also be conducted at artificial slopes similar to the hills where RSL can

be observed on Mars (McEwen *et al.*, 2011a). First follow-up experiments in our lab investigating the effect of the salt concentration in the soil on the deliquescence process revealed that already 1 wt% CaCl₂ is enough to result in a measurable soil wetting caused by deliquescence (unpublished data, chapter 7.1).

After addressing the formation of brines in Publication I the next logical step from an astrobiological viewpoint was to investigate the habitability of these brines in the Publications II and III. The combined findings of those two publications revealed that halotolerant microorganisms like *P. halocryophilus* cannot survive the salt stress in eutectic brines but their survivability can be increased significantly by lowering the temperature to subzero values. To enable bacterial growth a dilution of the eutectic brines is needed, where the extent of the dilution depends on the type of salt and the temperature. Although ClO_4^- salts can only be tolerated at lower concentrations than Cl^- salts there seems to be no reason why putative microbes on Mars should have been unable to adapt to higher ClO_4^- concentrations.

It is likely that in a realistic Martian environment with diurnal and seasonal changes of environmental conditions microorganisms would be exposed temporally to both scenarios: life-inhibiting (low temperatures, concentrated brines) and life-promoting (elevated temperatures, diluted brines) conditions. The results of Publication II indicated that at cold temperatures close to the eutectic point of the brine, e.g. -77.5°C for Ca(ClO₄)₂, microorganisms might not be able to thrive but to survive until temperatures rise (up to +30°C at the Martian surface). At these higher temperatures also life-promoting lowconcentrated brines, e.g. 12 wt% NaClO₄ for growth of *P. halocryophilus* (see Publication III), could be stable. These diluted salt solutions might form through proceeding water absorption of the hygroscopic concentrated brine or when the brine gets in direct contact with water ice. The low atmospheric pressure on Mars seems not to be a limiting factor for the stability of the diluted brines since the water vapor pressure at the triple point of pure water (6.1 mbar) is below the atmospheric pressure on the lowest regions of Mars (Martínez and Renno, 2013). The water vapor pressure of brines is always below the one of pure water. Halotolerant microorganisms like *P. halocryophilus* could grow in these diluted brines readily when applying salt stress defense mechanisms like the accumulation of osmolytes, e.g. betaine or proline (chapter 7.3), or cell clustering (Publication III).

Additional to the enhanced survivability at subzero temperatures found in the experiments presented in Publication II, results from the experiments of Publication III showed an increase in the maximum CaCl₂ concentration suitable for growth of *P. halocryophilus* with decreasing temperature while tolerances to other salts are similar or lower at 4°C compared to 25°C. This complex behavior demonstrates that more research is needed to better understand general aspects of the habitability of brines, especially at cold temperatures and including non-NaCl salts. These experiments might also include other halotolerant or halophilic species or microbial communities and additional Mars-relevant salts like iron sulfates or salt mixtures like "Instant Mars" consisting of Na⁺, K⁺, Mg²⁺, Ca²⁺, Cl⁻, ClO₄⁻, and SO₄²⁻ ions (Nuding *et al.*, 2015). Since results of Publication III have shown that cells grown in ClO₄⁻ solutions form different cell colony morphologies on agar plates than cells grown in ClO₄⁻ solutions the investigation of differences in the transcriptome and proteome from both cell types would be of special interest for astrobiological research. Experiments for this purpose are already ongoing in our labs and will hopefully soon provide new and intriguing knowledge about life in brines.

6. References

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7. Supplementary Information

7.1 Effect of the salt/soil ratio on the deliquescence process

7.1.1 Materials and methods

The experimental setup for measuring deliquescence-induced soil wetting is described in Publication I, chapter 2. To investigate the effect of the salt/soil ratio on the deliquescence process the same experimental approach was used but with different concentrations of CaCl₂ in the soil (JSC-Mars-1a). To check reproducibility of the measurements three samples with 5 wt% CaCl₂ were prepared and measured separately.

7.1.2 Results and discussion

The measured normalized conductivity values (N) as function of time and salt concentration are given in Figure 7-1. The results show nicely that higher CaCl₂ concentrations yield higher deliquescence rates.



FIG. 7-1: Increasing EC caused by deliquescence-induced water absorption of the soil **(A).** Magnified segment of Fig. A showing curves for samples with concentrations of ≤ 5 wt% CaCl₂ **(B).**

Deliquescence was detectable via EC measurements down to a salt content of 1 wt% CaCl₂ which is comparable to overall salt concentrations in the Martian regolith (Toner *et al.*, 2014a). The overlapping of the curves for the 5 wt% CaCl₂ triplicates demonstrates the good reproducibility of this technique. Weighing of the samples could be used to correlate the measured EC values with the amount of absorbed water.

7.2 RSL-simulating field experiments in the Atacama Desert, Chile

7.2.1 Materials and methods

During the field trip in 2018 experiments were conducted that were dedicated to simulate Martian RSL on steep-sloped hills in the Yungay Valley of the Atacama Desert, Chile. For this purpose 10 kg NaCl (technical grade) were dissolved in water to gain a nearly saturated solution. This briny solution was gently poured down a hill. Photos were taken and HOBO and CR10 data logger (more details on the data loggers are available in Publication I, chapter 2) were used to determine the atmospheric RH and temperature as well as the EC of the wetted soil, respectively.

7.2.2 Results and discussion

The NaCl solution poured down the hill resulted in a darkened wet brine track similar to RSL on Mars (Fig. 7-2A). During daytime the high temperatures and low RH resulted in a continuous evaporation of the water correlating with a decrease in the relative EC while the increased RH in the night and morning caused a deliquescence-induced water absorption coinciding with an increase in the EC (Fig. 7-3). Since the amount of water evaporating during daytime is exceeding the water amount absorbed during the night and morning hours the net water balance is negative. Thus, no growth of the simulated RSL could be observed but only a slow fading within 4 days (Fig. 7-2B). In future field experiments the NaCl could be replaced by more hygroscopic ClO₄⁻ salts with a lower DRH. This might result in a positive net water balance and, therefore, in a down-slope growth of the simulated RSL.



FIG. 7-2: Simulated RSL directly after pouring a saturated NaCl solution down the hill **(A).** Nearly completely faded RSL after 3 days **(B).**



FIG. 7-3: Temperature, RH and relative EC data collected during the RSL-simulating field experiment in the Atacama Desert. When the RH increased during night and morning hours the NaCl in the RSLlike track absorbed water from the atmosphere correlating with an increase in the EC. The data gap in the EC measurements during the first day of the experiment resulted from connection problems with the data logger.

7.3 Metabolomics studies on P. halocryophilus

7.3.1 Materials and Methods

Cells of *P. halocryophilus* were grown at 25°C in 1.2 ml DMSZ growth media #92 (biological duplicates) under three different culture conditions:

- (1) without additional salts for 2 days
- (2) with additional 13 wt% NaCl for 13 days
- (3) with additional 11 wt% NaClO₄ for 8 days

After the noted time of growth the cells reached the late exponential growth phase. The total cell number was determined under the light microscope in a Thoma cell counting chamber for all three samples. Afterward, the cell suspensions were centrifuged at 1000 xg for 5 minutes. The cell pellets were washed first with PBS containing 10 wt% NaCl and then a second time with PBS without additional NaCl. Both washing steps were performed at 4°C. To lyse the cells the pellets were treated with a 20/80 mixture of water and acetonitrile (LC-MS grade) followed by 5 minutes of ultrasonication. After 10 minutes of centrifugation at 16,000 xg the supernatant was transferred to mass spectrometry auto sampler vials and measured in an untargeted approach with an ultra-high performance liquid chromatography linked mass spectrometry. Analytes were separated in HILIC (hydrophilic interaction liquid chromatography) mode with a gradient of acetonitrile and buffered water (5% 10mM ammonium carbonate in water to 65% after 20 minutes, flow rate 200µL per minute) on the Acquity UPLC BEH amide 1,7 µm and measured with both positive and negative electron spray ionization. The Full-Scan mode had a resolution of 75000, whilst the MS² spectra were generated with a collision energy of 50 eV and a resolution of 35000. Data analysis was performed with Compound Discoverer 2.1 by comparing acquired MS² spectra against mzCloud.

7.3.2 Results and Discussion

The following cell numbers were determined:

- (1) $1.1 \cdot 10^8$ cells per ml for the sample with no additional salt
- (2) $2.3 \cdot 10^7$ cells per ml for the sample with 13 wt% NaCl
- (3) $5.3 \cdot 10^6$ cells per ml for the sample with 11 wt% NaClO₄

In total, 4041 compounds where detected. 187 of those were annotated. Among those, 23 were defined as validated hits (high quality MS² spectra, bell shaped XIC traces, confident quantification) which are summarized in table 7-1. It is clearly recognizable that in all three samples betaine occurred at highest concentrations. It was enriched in the salty samples (2 log2-fold-change NaCl/no salt; 4 log2-fold-change NaClO₄/no salt), however, enrichment occurred for nearly all metabolites that were detected in all three samples. Most likely, the reason for that phenomenon is that the cell density is 4.8 and 20.8 times higher in the salt-free samples than in samples containing NaCl or NaClO₄, respectively. In samples with a lower cell density metabolite extraction is more efficient due to matrix effects.

Nevertheless, the large quantities found for betaine are a strong indication that this substance serves as osmolyte as it does in many other halotolerant microorganisms (Roberts, 2005). Furthermore, proline (and the related substances 4- and 5-oxoproline) were detected which might contribute to the osmoprotection machinery of *P. halocryophilus* as well. These findings fit well to the detection of several betaine and proline transport proteins that are expressed under osmotic stress (Mykytczuk *et al.*, 2013). Another interesting metabolite detected is azobenzene, an orange-red azo-dye which might contribute to the orange color of *P. halocryophilus*.
TAB. 7-1: Metabolites detected in lysed samples of P. halocryophilus grown in medium containing no additional salt, 13 wt% NaCl, or 11 wt% NaClO₄. Intensities (peak areas) are normalized to the cell number.

Metabolite	no salt (Intensity)	13% NaCl (Intensity)	NaCl log2-fold change	11% NaClO₄ (Intensity)	NaClO₄ log2-fold change
Betaine	170129729	934061711	2	2555292664	4
Ethyl myristate	31794337	176551054	2	584343423	4
Stearic acid	19792209	128830432	3	381791994	4
5-Oxoproline	10856047	2936027	-2	26519143	1
Proline	8374058	113501747	4	589835936	6
4-Oxoproline	2601005	66510510	5	90532035	5
Nonanoic acid	1825810	13855231	3	51466229	5
Decanoic acid	919838	6795369	3	24838118	5
Pipecolinic acid	753501	14421527	4	47824413	6
Lauric acid	687274	2591367	2	29161925	5
Nicotinic acid	501591	6614138	4	24916096	6
Cetrimonium	452594	2491578	2	3496061	3
Bis(2- ethylhexyl)adipate	101277	658426	3	2026420	4
2,4-Bis(2-phenyl-2- propanyl)phenol	96310	641106	3	2127732	4
2,3,5,6-Tetra- methylpyrazine	75384	545395	3	734724	3
Norharman	50771	2060568	5	6206966	7
N,N-Dimethylaniline	50355	401886	3	1289555	5
Cyclo(phenylalanyl- prolyl)	42344	2410753	6	3220598	6
Cyclo(leucylprolyl)	37765	2446710	6	4022057	7
Citrulline	36953	310218	3	34410231	10
6-Methylquinoline	24740	395596	4	749027	5
Palmitoyl ethanolamide	23551	129598	2	622668	5
Azobenzene	13741	467517	5	1315308	7

7.4 Supplementary Information on Publication III

7.4.1 Growth curves of Planococcus halocryophilus

- > All growth curves were obtained as described in chapter 2 of the main text
- Cells were grown aerobically in liquid growth medium (DMSZ #92) containing salt amounts as indicated within the figure legends
- > All curves obtained as biological duplicates (samples [A] and [B])
- > Dashed lines indicate a 2nd run of a specific experiment
- **X** indicates the detection limit (no detectable CFU within 100 μl sample)
- > Negative error bars reaching values of $y \le 0$ CFU/ml were removed.
- ▶ IM Inoculation method as described in section 2.2 of the main text



FIG. 7-4: Growth curves in NaCl containing media at 25°C. NaCl 14% (IM 2) (sample B) got contaminated.



FIG. 7-5: Growth curves in NaCl containing media at 4°C. Biological duplicates A and B were not averaged for 9%- and 10%-samples due to differences in growth. No detectable CFUs in the 12% NaCl sample after 200 days.



FIG. 7-6: Growth curves in $MgCl_2$ containing media at 25°C. $MgCl_2$ 10% (IM 2) (sample B) did not show growth.



FIG. 7-7: Growth curves in MgCl₂ containing media at 4°C. Biological duplicates A and B were not averaged for 10%-samples due to differences in growth.



FIG. 7-8: Growth curves in $CaCl_2$ containing media at 25°C. (See also Fig. 4-1 in the main text).



FIG. 7-9: Growth curves in CaCl₂ containing media at 4°C. Biological duplicates A and B were not averaged for 10%-samples due to differences in growth. See also Fig. 4-1 in the main text.



FIG. 7-10: Growth curves in NaClO₄ containing media at 25°C.



FIG. 7-11: Growth curves in NaClO₄ containing media at 4°C. NaClO₄ 2% (IM 2) was inoculated with culture grown in media with 3 wt% Ca(ClO₄)₂ at 4°C. No detectable CFUs in NaClO₄ 9% after 207 days.



FIG. 7-12: Growth curves in $Mg(CIO_4)_2$ containing media at 25°C. Biological duplicates A and B were not averaged for 4%- and 5%-samples due differences in growth.



FIG. 7-13: Growth curves in Mg(ClO₄)₂ containing media at 4°C. Mg(ClO₄)₂ 2% (IM 2) was inoculated with a culture grown in media with 3 wt% Ca(ClO4)2 at 4°C since inoculation with a culture grown in 5% Mg(ClO₄)₂ medium at 25°C (IM 4) did not result in growth.



FIG. 7-14: Growth curves in Ca(ClO₄)₂ containing media at 25°C.



FIG. 7-15: Growth curves in Ca(ClO₄)₂ containing media at 4°C.



7.4.2 Ionic strengths and water activities at the MSCg (additionally to Fig. 4-2 in the main text)



FIG. 7-16: Ionic strengths **(A)** and water activities **(B)** at the MSCg. See also Tab. 4-1 and Fig. 4-2 in the main text.

7.4.3 Cell colony morphologies of P. halocryophilus grown under salt stress conditions (additionally to Fig. 4-4 in the main text)

FIG. 7-17: Irregular jagged colonies (type III) of *P. halocryophilus* occurring after bacterial growth in medium containing 9 wt% $MgCl_2$ (A) or 4 wt% $Mg(ClO_4)_2$ (B).



FIG. 7-18: Mucoid and shiny colonies (type IV) that merge easily during colony growth occurring after bacterial growth in medium containing 6 wt% CaCl₂ (A) or 8 wt% CaCl₂ (B).



7.4.4 Fluorescence microscopy images (additionally to Fig. 4-5 in the main text)

FIG. 7-19: Fluorescence microscopy images (after life/dead staining) of cell clusters of *P. halocryophilus* in 10 wt% NaClO₄ medium before **(A)** and after **(B)** killing all cells within one cluster through ethanol treatment.

8. List of Publications

Publication I:

Title:	Deliquescence-induced wetting and RSL-like darkening of a
	Mars analogue soil containing various perchlorate and
	chloride salts.
Authors:	Jacob Heinz, Dirk Schulze-Makuch, Samuel P. Kounaves
Journal:	Geophysical Research Letters (2016), 43(10), 4880–4884,
	https://doi.org/10.1002/2016GL068919
Version:	Postprint
License:	CC BY-NC-ND 4.0 license (license terms cf.
	http://creativecommons.org/licenses/by-nc-nd/4.0/)
Embedded in Dissertation:	Chapter 2, pages 16 – 26.
Publication II:	
Title:	Enhanced Microbial Survivability in Subzero Brines.
Authors:	Jacob Heinz, Janosch Schirmack, Alessandro Airo, Samuel P.
	Kounaves, Dirk Schulze-Makuch
Journal:	Astrobiology (2018), 18(9), 1171–1180,
	https://doi.org/10.1089/ast.2017.1805
Version:	Postprint
License:	CC BY 4.0 license (license terms cf.
	https://creativecommons.org/licenses/by/4.0/)

Publication III:

Title:	Bacterial growth in chloride and perchlorate brines:
	Halotolerances and salt stress responses of Planococcus
	halocryophilus.
Authors:	Jacob Heinz, Annemiek C. Waajen, Alessandro Airo,
	Armando Alibrandi, Janosch Schirmack, Dirk Schulze-
	Makuch
Journal:	Astrobiology (2019), Epup ahead of print,
	https://doi.org/10.1089/ast.2019.2069
Version:	Postprint
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Embedded in Dissertation:	Chapter 4, pages 46 – 67.