Biofilms Confer Resistance to Simulated Extra-terrestrial Geochemical Extremes

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10 # 1 ABSTRACT

11 Biofilms improve microbes' resistance to a variety of extreme physical and chemical conditions on

12 Earth. The discovery of putative aqueous environments on other planetary bodies such as Mars

13 motivates an interest in understanding the viability of life, and the potential role of biofilms, in

14 previously unexplored geochemical extremes. We investigated the loss of viability of planktonic cells

15 and biofilms of *Sphingomonas desiccabilis* (a Gram-negative, desiccation resistant, soil crust-forming

16 organism) to simulated Martian brines. These brines were produced from geochemical modelling of

17 past aqueous environments on Mars, and their high sulfate concentrations make them different to

18 most terrestrial brines, although similar briny environments have been found in locations such as the

19 Basque Lakes in Canada or in deep subsurface groundwater systems. Biofilms grown on basaltic

20 scoria were subjected to the simulated martian brines and the viability of cells was measured over

21 time and compared to equivalent planktonic cultures. Crystal violet assay was used to measure how

22 the biomass of the biofilms changed over time in response to the brines. While certain brines were

23 highly hostile to microbial viability, we found that biofilms that were desiccated prior to being

24 treated with brines maintained viability over a longer treatment period when compared to

25 planktonic cells. Our results show that biofilms confer short-term protection to the harsh osmotic,

26 ionic, and acidic conditions of Mars-relevant brines. However, in the most extreme simulated brines,

27 even biofilms eventually lost viability. By demonstrating that biofilms confer protection to conditions

28 that are potentially analogous to current day recurrent slope lineae (thought to be produced by the

29 flow of briny fluids) on Mars, our results show that contaminant biofilm-forming microorganisms

30 may have a greater chance of surviving in so-called 'Special Regions' on Mars, with implications for

31 planetary protection in missions that aim to explore these regions.

32 ## 2 INTRODUCTION

33 Biofilms are produced by a variety of organisms and generally consist of cells attached to a surface

34 (and each other) in an exuded matrix of extracellular polymeric substance (EPS) [1]. Biofilms can be

35 formed by single species or complex microbial communities and are generally produced in response

36 to the presence of a surface or external cues and stressors. Microbial biofilms increase the resistance

37 of the inhabitants to a number of extreme conditions including immunological aggression [2],

38 antibiotics [3], bactericidal chemicals [4], rapid changes in temperature and pH [5], UV radiation [6]

39 and salinity [7,8]. Biofilm-producing microorganisms are therefore present and thrive in a range of

40 environments on Earth and may have been involved at an early stage as one of the steps in the

41 development of the first cells [9].

42 Although biofilms confer resistance to extremes found on Earth, their ability to provide protection

43 against entirely novel chemical conditions found on other planetary bodies remains unknown. The

44 search for extra-terrestrial habitable environments centres on the availability of liquid water, and

45 aqueous environments are now known to exist on a number of planetary bodies including Mars,

46 Europa, Enceladus and Titan [10-14]. Many of these aqueous environments contain combinations of

47 anions that are rare on Earth. For example, the high concentrations of sulfate and other divalent ions

48 in Martian brines, a consequence of the specific geological history of that planet, have consequences

49 for habitability, making ionic strength an important determinant of whether the brines will support

50 life [15]. Recent observations suggest that recurrent slope lineae (RSL) features on Mars are created

51 by the action of flowing water which is saturated with dissolved perchlorate salts [16]. Such brine

52 compositions have not been found on Earth. In this study, we investigated the resistance of biofilms

53 to aqueous environments analogous to environments on Mars such as RSL currently potentially

54 active [16] or in quasi-tidal areas that existed during the phase of the planet's history when it lost its

55 surface water to atmospheric escape or to retreat into subsurface ice [17,18], but also have

56   implications for the icy moons in the outer solar system, which are thought to have high levels of

57   dissolved salts in their liquid water interiors [19].

58   We hypothesise that production of biofilms might protect microorganisms and allow them to

59   maintain viability following exposure to compositionally diverse brines with high levels of dissolved

60   salts. Our results have implications for the adaptations that would be required by any putative life in

61   extra-terrestrial environments and for planetary protection, *i.e.* whether terrestrial organisms

62   accidently introduced into extra-terrestrial brines could persist.

3 RESULTS

64   3.1 VIABILITY AFTER BRINING

65   Figures 1-4 show the viability of *S. desiccabilis* biofilms over time when immersed in Mars-analogue

66   brines. Each time point was an independent culture (triplicate pieces of scoria with biofilm on them)

67   exposed to brine for a given length of time. Experimental replicates are collected on the same axes.

68   Error bars are the standard error of the biological triplicates from each experiment. Best-fit lines

69   were calculated using a linear regression of all experimental replicates.

70   In Type I brines (Fig. 1) there was only minor decreases in viability over more than 300 minutes when

71   dried or when hydrated. In some cases there appears to be growth during brine immersion (Ib). In

72   brine IIa (Fig. 2a), viability was maintained for over 300 minutes whether the biofilms had been dried

73   or not. In brine IIb (Fig 2b) the dried biofilms showed a decrease in viability, decreasing to below

74   detection levels after around 240 minutes. Hydrated biofilms showed a more rapid decrease in

75   viability, as viable cells were unrecoverable after less than 30 minutes of brine treatment. In type III

76   brines (Fig. 3), viability of cells in the hydrated biofilms reduced to below detection limits after

77   around 30 minutes, which was the shortest timepoint measured. There was more variation in the

78   maintenance of viability of dessicated biofilms treated with the type III brines. For brine IIIa there

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79   was a reduction in cell viability over the time course, but not to below detection limits, and not in a

80   linear fashion. In brine IIIb there was a rapid loss of viability to below detection levels within

81   approximately the first 60 minutes, though individual cultures in some experimental replicates

82   showed some measured viability at longer timescales. Brine IIIb has the lowest water activity, the

83   lowest pH and highest ionic strength of all the brines tested. There was a rapid and complete loss of

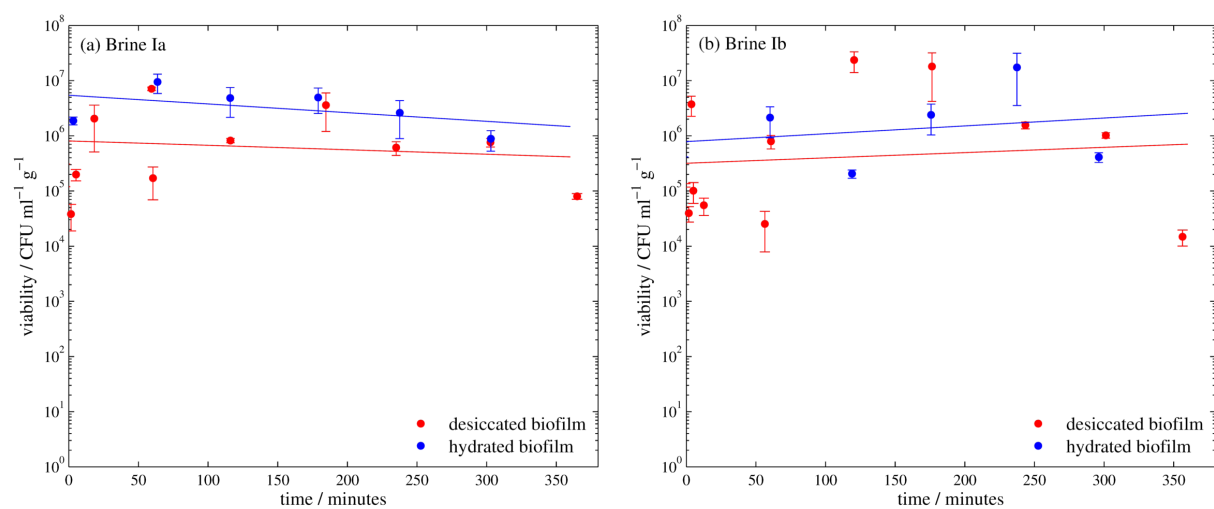84   viability in around 100 minutes with both desiccated and hydrated biofilms in type IV brine (Figure

85   4).

86

87   *Figure 1 – Viability of S.* desiccabilis *cells in desiccated and hydrated biofilms after periods of immersion in Type I brines,*

88   *measured by counting colony forming units (CFU). Each data point is a biological triplicate result, with standard error bars,*

89   *and trend lines are plotted as linear regressions of data from several systematic replicates.*

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90

91   *Figure 2 - Viability of S.* desiccabilis *cells in desiccated and hydrated biofilms after periods of immersion in Type II brines,*

92   *measured by counting colony forming units (CFU). Where no CFUs were countable, results are plotted at 10^0. Each data*

93   *point is a biological triplicate result, with standard error bars, and trend lines are plotted as linear regressions of data from*
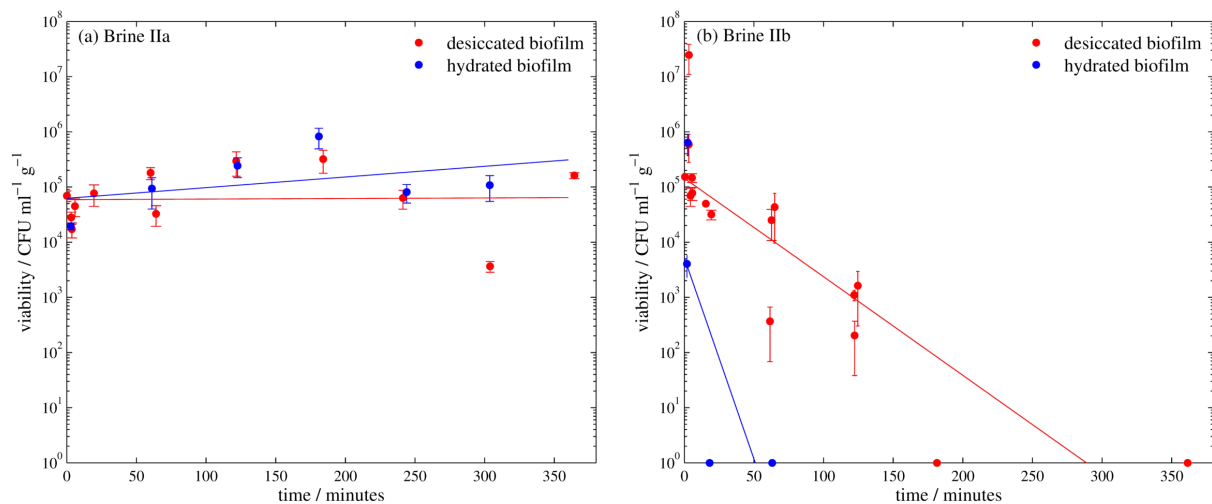
94   *several systematic replicates.*

95

96   *Figure 3 - Viability of S.* desiccabilis *cells in desiccated and hydrated biofilms after periods of immersion in Type III brines,*

97   *measured by counting colony forming units (CFU). Where no CFUs were countable, results are plotted at 10^0. Each data*

98   *point is a biological triplicate result, with standard error bars, and trend lines are plotted as linear regressions of data from*
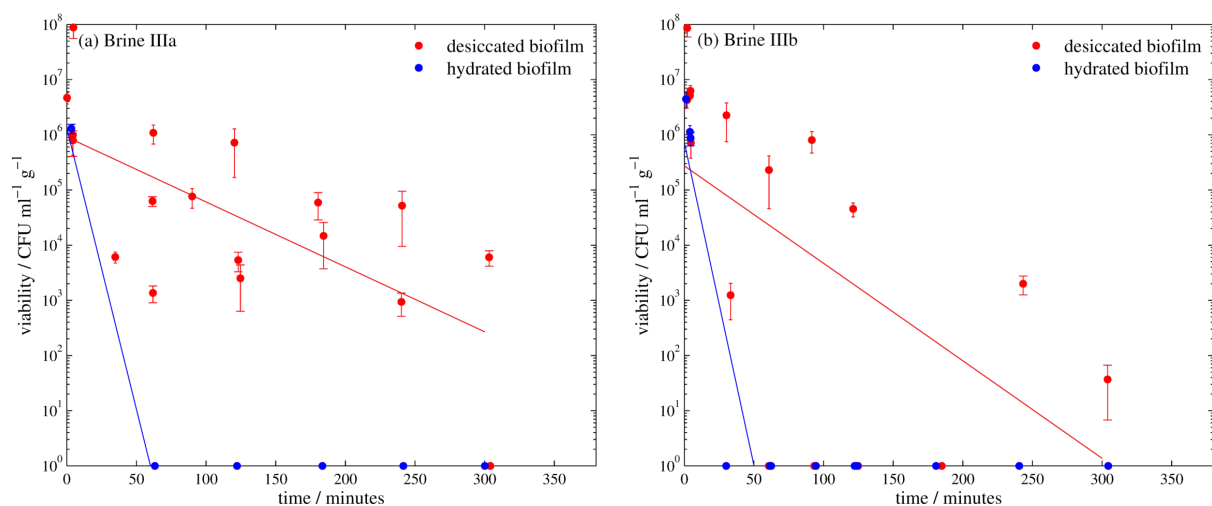
99   *several systematic replicates.*

Figure 4 - Viability of S. desiccabilis cells in desiccated and hydrated biofilms after periods of immersion in Type IV brine, measured by counting colony forming units (CFU). Where no CFUs were countable, results are plotted at 10^0. Each data point is a biological triplicate result, with standard error bars, and trend lines are plotted as linear regressions of data from several systematic replicates.
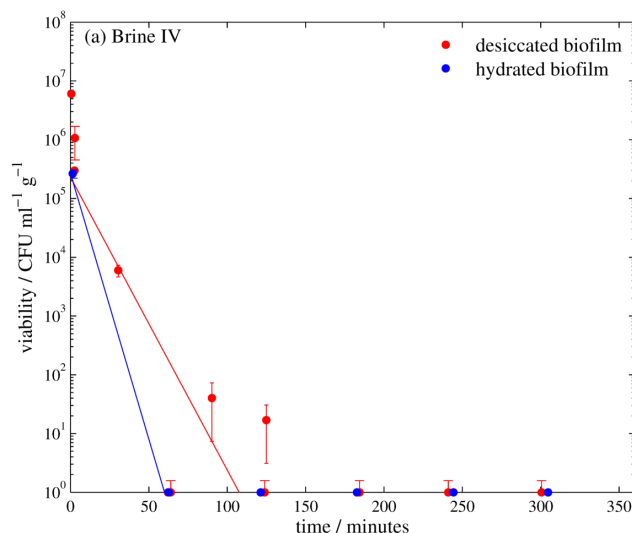
Planktonic cultures of *S. desiccabilis* showed broadly similar responses to the 'weaker' brines but far stronger responses to the 'harsher' brines compared to biofilm cultures. There was no decrease in viability over several hours of immersion in brines Ia, Ib and IIa. In brines IIb, IIIa, and IV there was a total loss in viability within less than 10 minutes (the shortest timepoint measured). In brine IIIb it was not possible to recover any planktonic cells into a pellet, suggesting they had lysed within 10 minutes.

To provide a more quantitative comparison of viability in the different brines, we calculated a linear regression for the results of each experiment and measured the gradient. Table 2 shows the gradient of these regression fits and the corresponding R^2 value to assess the quality of the fits. A larger negative gradient implies a more rapid loss of viability, and a smaller R^2 value suggests more variation in biofilm structure and growth between experimental replicates. The two instances of an R2 value of 1 are for experiments with only one measureable time point after the initial controls and so the fit is only for two points. These values are graphed in Figure 5. Brines IIb, IIIa, IIIb and IV show the most rapid loss of viability and there is a clear difference between desiccated and hydrated

119   biofilms, with the latter losing viability ~2-10 orders of magnitude more rapidly when subjected to

120   these brines.

Brine	Rate of viability loss / log(cfu l^{-1} g^{-1} min^{-1})			
	Desiccated		Hydrated	
	Fit gradient	R^2	Fit gradient	R^2
Ia	-0.0018	0.098	-0.0036	0.362
Ib	0.0022	0.095	0.0033	0.210
IIa	0.0002	0.083	0.0045	0.301
IIb	-0.0411	0.770	-0.1679	0.643
IIIa	-0.0271	0.497	-0.2346	1.000
IIIb	-0.0407	0.358	-0.2697	0.869
IV	-0.1147	0.709	-0.2081	1.000

121   *Table 1 - Rate of viability loss of S.* desiccabilis *cells in desiccated and hydrated biofilms when immersed in Brines Ia to IV,*

122   *measured from linear regressions of the curves shown in Figures 2-5.*

123

124   *Figure 5 –Rate of viability loss of S.* desiccabilis *cells in desiccated and hydrated biofilms when immersed in Brines Ia to IV,*

125   *measured from linear regressions of the curves shown in Figures 1-4.*
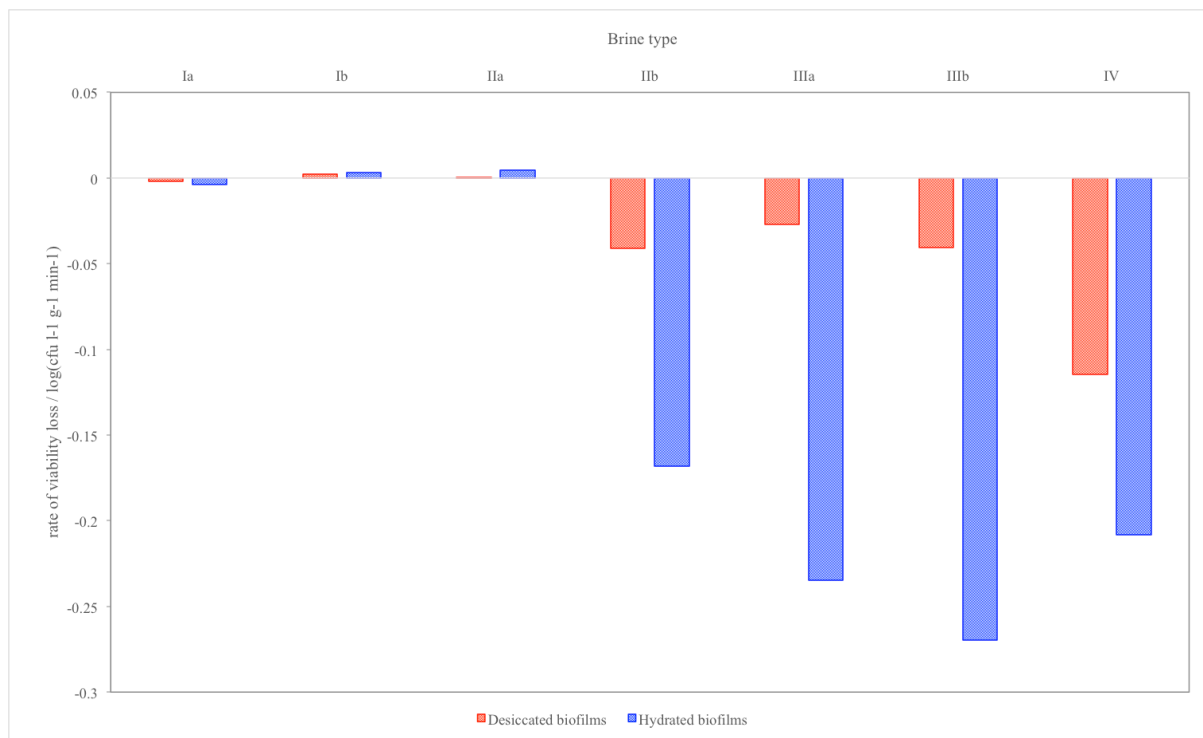
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3.2 REPEATED BRINING CYCLES

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127   We also tested the viability of biofilms on scoria after repeated cycles of brining and desiccation,

128   repeating the process used in Section 3.1. Viability was tested after two to five cycles of brine

129   immersion. CFU counts after the extraction procedure were negative for all cycles tested.

3.3 BIOMASS ASSAY

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131   As Type I brines had little effect on the viability of cells in biofilms, these were not included in CV

132   assays. Figure 6 shows the relative biomass present after a 24 hour incubation in the Type II, III and

133   IV brines for both desiccated and hydrated biofilms. All of the hydrated biofilms showed a reduction

134   in biomass after 24 hours, suggesting the biofilms were broken down by the brines. In the case of

135   the desiccated biofilms, there was also a reduction in biomass after 24 hours in brines IIa and IV, but

136   in brines IIb, IIIa and IIIb, the desiccated biofilms showed an increase in presumptive biomass over 24
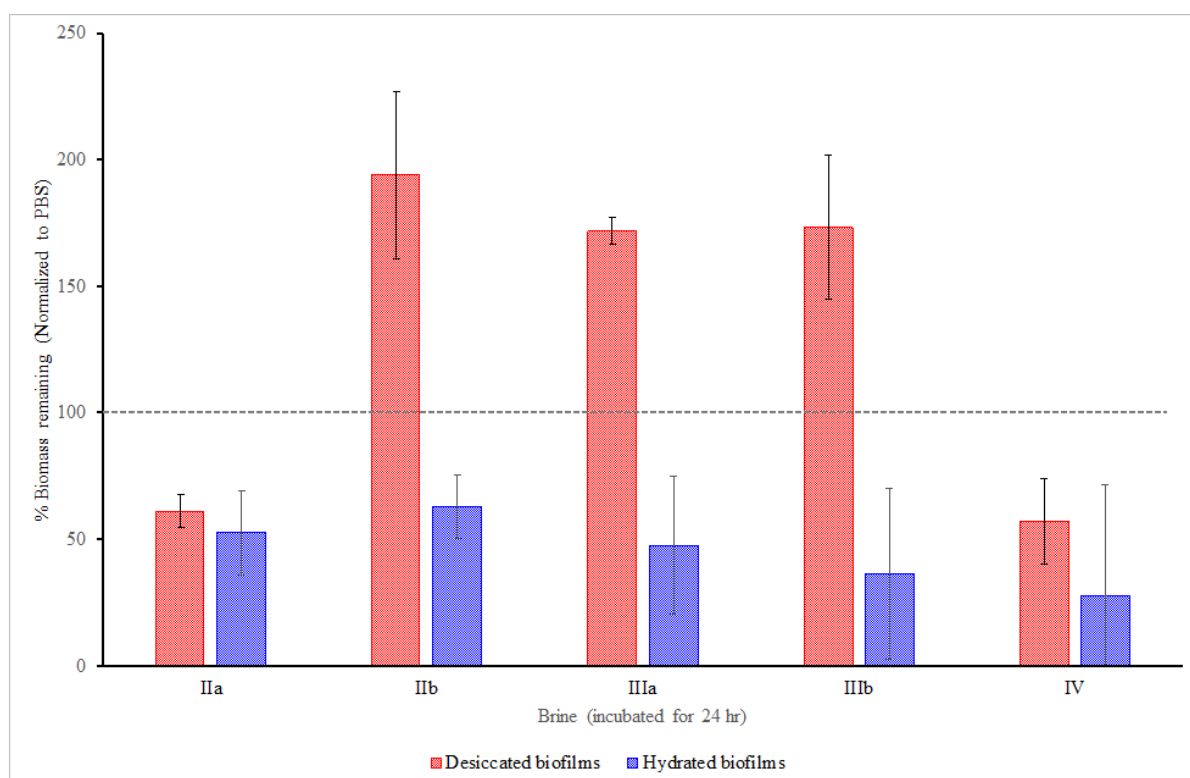
137   hours, by up to 250%.

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139 *Figure 6 – Percentage biomass remaining after immersion in brines IIa to IV for 24 hours for desiccated and hydrated*

140 *biofilms. Error bars are calculated from biological and systematic triplicates.*

141 To investigate this increase in biomass, we also performed a time series using the same

142 experimental procedure, with brine IIb over another 24 hour period (Fig. 7). During incubation in the

143 brine there was an initial reduction in biomass followed by an increase over several hours, which

144 peaked at around 6 hours after exposure to brine. After this increase, the biomass began to reduce,

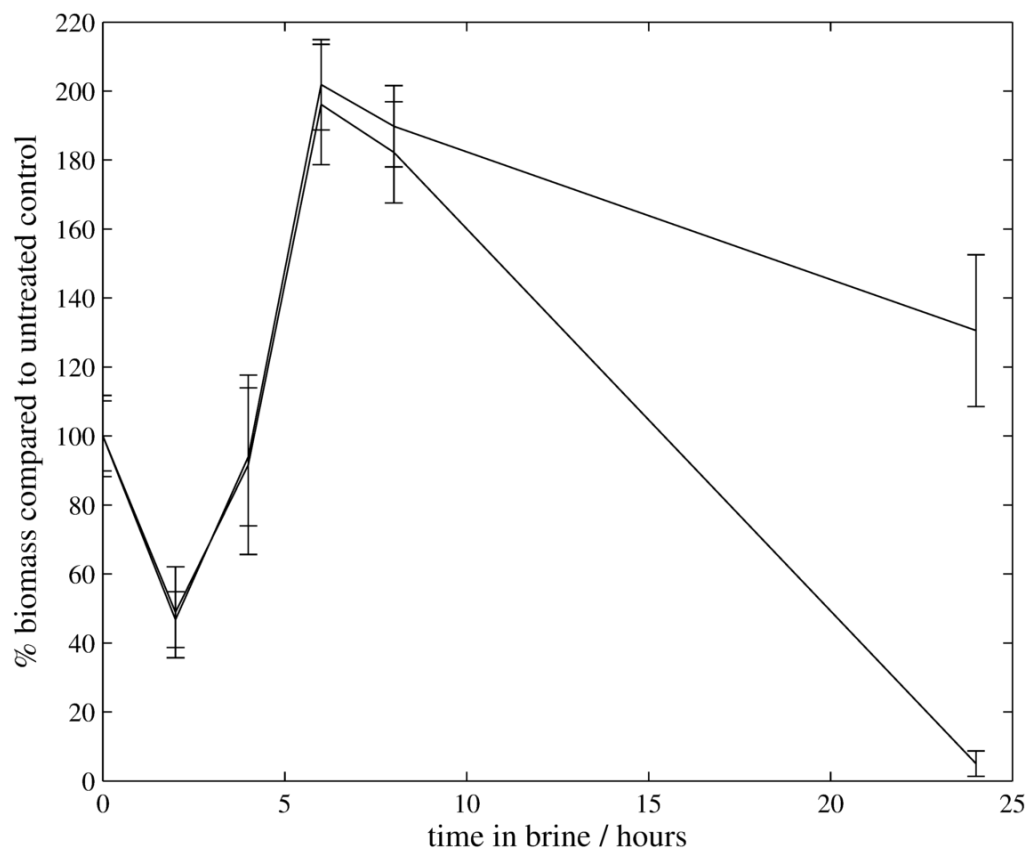145 from a peak of 200% to an average of around 100% after 24 hours.

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146

147   *Figure 7 – Biomass remaining over time for desiccated biofilms in Brine IIb. Two experiments were performed.*

4 DISCUSSION

149   Other planetary bodies in the solar system harbour aqueous environments that differ markedly in

150   geochemical composition from aqueous environments on the Earth. These differences are a

151   consequence of contrasting geological histories, which have produced the rocks and atmospheres

152   that ultimately determine water chemistry.

153   In this study we sought to address the hypothesis that as on Earth, specific adaptations of microbial

154   growth would influence the extent to which organisms could persist in extreme extraterrestrial

155   aqueous environments. In particular, biofilms are known to provide resistance to chemical extremes

156   [e.g. 4,7]. These data have implications for planetary protection that is concerned with whether

157   terrestrial microorganisms could grow in extraterrestrial aqueous environments into which they are

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158   inadvertently introduced and, more speculatively, the adaptations that would be required for

159   extraterrestrial life, if it exists, to grow in these alien geochemical extremes.

160   We found that combined physicochemical extremities of the brines had a significant effect on

161   microbial survival. Generally, those brines with high water activity, low ionic strength and moderate

162   pH show little effect on the viability of *S. desiccabilis* cells, whether grown in a planktonic state or in

163   a biofilm.

164   Brines with low pH and high ionic strength generally had a large impact on viability, making

165   planktonic cells unrecoverable in less than 10 minutes. Our data suggest that water activity is one

166   factor that influences the ability of biofilms to survive brine exposure. For example, brine IIIa has a

167   high water activity, low pH and high ionic strength and had a lesser impact on viability than brine IIb,

168   which has a similar similar pH and ionic strength, but lower water activity. This observation is

169   supported by the rapid loss of cell viability for planktonic cells and biofilms immersed in brine IIIb,

170   which has the lowest water activity of the brines tested.

171   However, factors other than water activity also appear to play a role in loss of viability in biofilms.

172   Brine IV caused a more rapid loss in viability than Brine IIb, although it has a higher water activity,

173   more moderate pH and lower ionic strength. Brine IV is the only solution with perchlorate ions,

174   which may suggest that these ions may be deleterious to cells, perhaps because of the oxidizing

175   capability of perchlorate [20].

176   The resistance to loss of viability caused by the extreme brines is likely to be caused by a

177   combination of physical protection and metabolic adaptation. Biofilms are known to provide a

178   barrier between cells and the external hostile environments [3,21], in this case providing protection

179   against the physicochemical extremes of the brine.

180   As shown by the more rapid reduction in viability measured with hydrated biofilms compared to

181   desiccated biofilms, the physical protection offered by the biofilm is not the only factor that allows

12

182   the cells to remain viable for longer in the brine. Desiccation adds yet another level of protection

183   against extreme brine exposure.

184   The crystal violet assay suggested that when desiccated, biofilms showed an increase in presumptive

185   biomass after several hours in the brine, a result not seen with hydrated biofilms and repeated

186   consistently in desiccated biofilms. One biological explanation for these observations might be that

187   desiccated biofilms are able to begin production of metabolites (such as membrane-stabilising

188   disaccharides) or additional EPS during initial exposure to osmotic stress. Production of EPS is a

189   known response to osmotic stress/desiccation [22]. Since matric (drying) water stress is equivalent

190   to osmotic water stress from high concentration solutes [22], by drying the biofilms before brining,

191   this process might act to 'prepare' the organisms by signalling the production of osmotic-resistance

192   metabolites (potentially changing the composition of the biofilm), which might continue during

193   exposure to brine prior to loss of viability. These observations merit further study.

194   Whilst our experiments show that under the most extreme brines, biofilms confer resistance to loss

195   of viability, we were unable to recover viable cells after 5 hours in the harsher brines (Brines IIb, IIIa,

196   IIIb and IV). We cannot rule out that other organisms or communities might be able to adapt to

197   these extreme conditions, but our results do not show long-term survival. Furthermore, we tested

198   the hypothesis that in the desiccated state, biofilms would enhance survival in transient aqueous

199   systems, such as those proposed to occur in Martian RSLs. Under repeated cycles of desiccation and

200   exposure to brine, which simulated the conditions that might occur in RSL, we found that viability

201   was completely lost after the first cycle in all cases.

202   We have shown that biofilms formed by microorganisms offer protection against the extreme

203   conditions of Mars-analogue brine solutions. Cells in biofilms desiccated before being immersed in

204   brines remained viable for orders of magnitude longer than planktonic cells or hydrated biofilms

205   under the same conditions. Some of these brines had low water activity, low pH and high ionic

206   content that would otherwise make them uninhabitable [15], but *S. desiccabilis* biofilms retained

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207   viability in them for short periods of time, despite them quickly destroying *S. desiccabilis* cells in the

208   planktonic state. Even though *S. desiccabilis* is not an extreme halophile, nor is it known to possess

209   exceptional tolerance to osmotic shock, its biofilm forming capability provides resistance to high

210   levels of dissolved salts. It was selected here for this ability to form biofilms and known desiccation

211   tolerance. Halophilic biofilm-forming organisms that are exposed to geochemical regimes similar to

212   those investigated here would likely possess even greater resistance.

213   These results have implications for the designation of RSL as 'special regions' for planetary

214   protection purposes [23]. Given that terrestrial spacecraft almost certainly carry a bioload, some

215   portion of which may be in biofilm form, even the transient aqueous environment suggested by RSL

216   could present an environment that hardy terrestrial microbes could exploit or leave contaminating

217   material in, especially if protected by a biofilm.

218   Finally, our results add to the expanding body of literature showing the protective properties of

219   biofilms in extreme environments. Prior studies have shown biofilms' capabilities to protect resistant

220   cells against osmotic stress, but not with brines with properties as different or as extreme as those

221   used in our study, such as concentrated sulfate brines. Although these brines are more relevant to

222   extra-terrestrial environments, they are also relevant to some terrestrial locations such as the

223   Basque lakes of British Columbia, which are sulfate-rich water bodies [24], or deep subsurface

224   groundwater systems [25].

5   MATERIALS AND METHODS

225

5.1 STRAINS AND GROWTH CONDITIONS

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227   This study examined viability and cell biomass in the organism *Sphingomonas desiccabilis* (*S.*

228   *desiccabilis*), a Gram negative, rod-shaped, chemoheterotrophic, strictly aerobic bacterium originally

229   isolated from a soil crust in the Colorado Plateau, USA [26]. Biofilms were cultured on pieces of

230   basaltic scoria (highly vessiculated Icelandic lava (*Bar-Be-Quick Barbeque Products, Burnley, UK)* see

231   Figure 8), chosen as an analogue for martian regolith and to provide a surface for the biofilm to form

232   on. Overnight cultures of *S. desiccabilis* grown in R2A medium at 20°C [27], were normalised by cell

233   density using OD_{600} measurements and inoculated onto ~5 mm pieces of scoria at a concentration of

234   50 μl in 4 ml of R2A medium and grown for one week at 20°C. Biofilm formation during this period

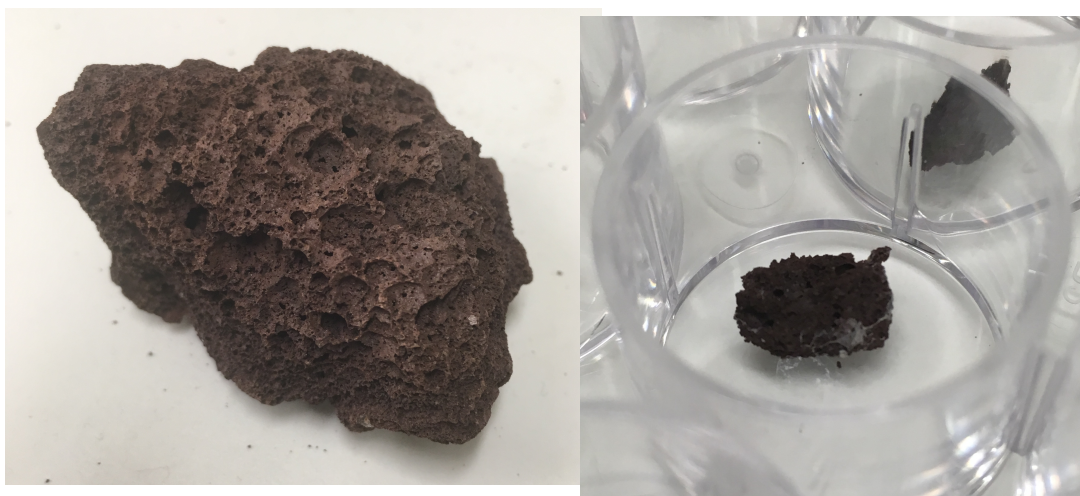235   was confirmed by visual inspection.

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237

238   *Figure 8 - Basaltic scoria used as a Mars analogue growth substrate a) larger piece and b) broken down to ~5 mm piece for*

239   *use in well plates.*

240   After a week of growth, the supernatant was removed from the scoria. The biofilm-covered scoria

241   pieces were then either a) dried in a laminar flow hood overnight or b) had their media replaced

242   with PBS in order to halt growth but prevent desiccation. This allowed us to compare the response

243   of desiccated biofilms to hydrated biofilms.

244   1-2 × 10^7 cells planktonic cells were collected from 500 μl of the same normalised overnight cultures

245   (incubated with shaking to reduce biofilm growth) for planktonic brine exposure experiments.

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5.2 EXPOSURE TO BRINES

247 The biofilm inoculated scoria was then treated with Mars-analogue brines over a time course. The

248 brines were synthesised based on computational reconstructions of putative martian brines by Tosca

249 et al. [28], as used by Fox-Powell et al. [15], and include four types at two stages in their evaporation

250 sequence (a and b). Type Ia and Ib are alkaline carbonate-chloride brines, which are analogous to the

251 conditions in Gale Crater approximately 3.7 billion years ago [29] and the fluids that interacted with

252 the Nakhla martian meteorite [30]. Type IIa and IIb are $Mg\text{-}SO_4\text{-}Cl$ dominated brines, characteristic of

253 widespread Hesperian evaporite deposits such as those at Meridiani Planum [31]. Type IIIa and IIIb

254 are similar in composition to Type II but contain higher levels of dissolved iron, and are extremely

255 acidic.

256 In addition to the six Mars analogue brines, a magnesium perchlorate solution was also included

257 (Type IV), given observations of magnesium perchlorate in the martian polar regolith by the Phoenix

258 lander [32] and more recently associated with RSL following spectral observations [16]. This brine

259 was a solution of magnesium perchlorate with a concentration to give a water activity matching that

260 of brine IIb in order to investigate any differences caused by the chemical properties of the

261 perchlorate salt. The molar recipes used to produce these brines are shown in Table 1, along with

262 measurements of their physicochemical properties.

Brine type	Ia	IIa	IIIa	Ib	IIb	IIIb	IV
$NaHCO_3$	0.126						
$KHCO_3$	0.028	0.041		2.237			
KCl	0.022	0.020	0.075	3.776	1.033	1.142	
$MgCl_2.6H_2O$	0.001	0.056			1.154	3.007	
NaCl		0.154	0.189	1.266	2.265	1.036	
$MgSO_4.7H_2O$		2.068	3.066		2.550		
$FeSO_4.7H_2O$			1.225			2.313	
$FeCl_2.4H_2O$			0.208			0.985	
HCl					0.038	0.113	
$Mg(ClO4)_2$							3.097
Water activity	0.97	0.92	0.90	0.80	0.63	0.56	0.67
pH	8.2	6.2	1.8	8.3	2	0.6	5.5
Ionic Strength	0.18	8.5	12	5	11.6	13	9.29

mol/liter								

263 *Table 2 - Salts added to mix Mars analogue brines. Concentrations are in moles litre $^{-1}$. Adapted from Fox-Powell et al. [15]*

264 *and originally calculated from Tosca et al. [28].*

265 Repeated brining was tested by removing the brines from the scoria after an hour. They were then

266 dried in a sterile flow hood until there was no visible surface moisture on the scoria, and the process

267 repeated.

5.3 VIABILITY AFTER BRINING

269 After being immersed in the brine, the scoria was washed twice in 1X PBS (Phosphate Buffered

270 Saline) and then suspended in PBS. The suspension was vortexed briefly and sonicated for two

271 minutes to extract cells from the biofilms. Planktonic cells that had been brined in the same manner

272 were centrifuged at 8 g for 10 minutes, the brine removed and the cells re-suspended in 1 ml of PBS.

273 Cell suspensions were plated onto nutrient agar plates in serial dilution and the CFUs quantified

274 after two days of growth at 20°C to give a measure of viability after immersion after a given amount

275 of time in the different brines. Results were collected for each brine for both dried and hydrated

276 biofilms in biological triplicate and systematic replicate at different time points.

5.4 DETERMINATION OF BIOMASS

278 We performed a crystal violet (CV) staining to investigate how the brines affect the overall biomass

279 of the biofilms. Biofilms were grown using the same method as described above and stained using a

280 protocol adapted from Childers et al. [33]. The biofilm-coated scoria were incubated in the different

281 brines, or PBS as a control for 24 hours, and then washed three times in PBS. Each scoria piece was

282 then stained with 0.4% crystal violet solution for 15 minutes at room temperature, washed three

283 times with PBS and destained for 15 minutes in 1 mL of 33% acetic acid solution. The optical density

284 of the destain solution was used to quantify the total biomass of biofilm left on each piece of scoria

285 after brining, with the PBS incubation forming the 'zero brine' control. The OD_{570} was measured by

286 spectrophotometer with 33% acetic acid as the blank.

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