Impact of Simulated Martian Conditions on (Facultatively) Anaerobic Bacterial Strains from Different Mars Analogue Sites

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Abstract

Five bacterial (facultatively) anaerobic strains, namely *Buttiauxella* sp. MASE-IM-9, *Clostridium* sp. MASE-IM-4, *Halanaerobium* sp. MASE-BB-1, *Trichococcus* sp. MASE-IM-5, and *Yersinia intermedia* MASE-LG-1 isolated from different extreme natural environments were subjected to Mars relevant environmental stress factors in the laboratory under controlled conditions. These stress factors encompassed low water activity, oxidizing compounds, and ionizing radiation. Stress tests were performed under permanently anoxic conditions. The survival rate after addition of sodium perchlorate (Na-perchlorate) was found to be species-specific. The inter-comparison of the five microorganisms revealed that *Clostridium* sp. MASE-IM-4 was the most sensitive strain (D_{10}-value (15 min, $NaClO_4$) = 0.6 M). The most tolerant microorganism was *Trichococcus* sp. MASE-IM-5 with a calculated D_{10}-value (15 min, $NaClO_4$) of 1.9 M. Cultivation in the presence of Na-perchlorate in Martian relevant concentrations up to 1 wt% led to the observation of chains of cells in all strains. Exposure to Na-perchlorate led to a lowering of the survival rate after desiccation. Consecutive exposure to desiccating conditions and ionizing radiation led to additive effects. Moreover, in a desiccated state, an enhanced radiation tolerance could be observed for the strains *Clostridium* sp. MASE-IM-4 and *Trichococcus* sp. MASE-IM-5. These data show that anaerobic micro-organisms from Mars analogue environments can resist a variety of Martian-simulated stresses either individually or in combination. However, responses were species-specific and some Mars-simulated extremes killed certain organisms. Thus, although Martian stresses would be expected to act differentially on microorganisms, none of the expected extremes tested here and found on Mars prevent the growth of anaerobic microorganisms.

Introduction

Mars has been a favored target in the search of extinct or extant life beyond the Earth. Various articles have been published discussing the habitability of early and present-day Mars (e.g. Tosca et al., 2008; Westall et al., 2013; Cockell et al., 2016; Eigenbrode et al., 2018). The present-day Martian surface is characterized by the absence of liquid water. If there is liquid water in the near-surface environment it likely occurs temporarily as brines (Orosei et al., 2018), *i.e*. with high concentrations of different salts, including perchlorates. In addition, the Martian surface is exposed to a high radiation flux in form of ionizing radiation and solar UV-radiation due to a thin anoxic atmosphere consisting of mainly CO_2, and by the lack of a planetary magnetic field (Horneck, 2000, Jakosky et al., 2001, Martin-Torres et al., 2015, McEwen et al., 2011, Hassler et al., 2014, Matthiä et al., 2016, Schubert et al., 2000, Gu et al., 2018).

Another potentially harmful environmental factor on the Martian surface is the ubiquitous presence of oxidizing compounds, especially perchlorates, which might have a strong impact on habitability. The Phoenix lander detected significant concentrations up to 0.6 weight percent (wt%) of

perchlorate ions at the landing site in the northern polar regions (Hecht et al., 2009). The MSL mission showed that perchlorates are present presumably on the entire surface of Mars (Archer et al., 2013). At distinct places different types of perchlorates have been detected. For example, a mixture of sodium perchlorate (Na-perchlorate) and magnesium perchlorate (Mg-perchlorate) were inferred in the Palikir and Hale crater. At Horowitz crater Na-perchlorate has been suggested and at Gale Crater calcium-perchlorate was inferred (Ojha et al., 2015; Glavin et al., 2013). The detailed formation mechanism of perchlorates is still not fully understood. Two different formation mechanisms of Martian perchlorates have been suggested. One hypothesis suggests that the perchlorates were produced on the surface whereby Martian surface minerals catalyze the photochemical oxidation of chlorides to perchlorates (Schuttlefield et al., 2011; Kim et al., 2013). It was shown that in chloride-containing Martian soil simulants, perchlorates are produced in the presence of ultraviolet light (Carrier and Kounaves, 2015). Another formation mechanism might be through the reaction of atmospheric oxidants probably on dust particles in the arid environment on Mars (Catling et al., 2010). Perchlorates, as hygroscopic substances, bind water from the atmosphere and contribute to the formation of brines with high concentrations of different dissolved salts, including chlorides, sulfates, and perchlorates. In some regions these brines are thought to remain liquid even at the low temperatures prevailing on the surface of Mars (Gough et al., 2011; Toner and Catling, 2016; Kounaves et al., 2014; Fox-Powell et al., 2016; Martín-Torres et al., 2015).

The working program of the European Community's Seventh Framework Program project MASE (Mars analogues for space exploration; grant agreement n° 607297) included sampling from terrestrial Mars analogue sites to obtain new (facultatively) anaerobic model microorganisms adapted to extreme conditions (Cockell et al., 2017). In this study, some of these microorganisms were exposed to a representative subset of environmental conditions as they occur on present-day Mars. They were perchlorates at different concentrations, absence of water, ionizing radiation, and a Martian atmosphere pressure. As these are some of the most prominent stress factors in a Martian environment, the biological effects on the microorganisms where examined when exposed to individual stress factors and in combination.

Material and Methods

Strains and culture conditions

In order to get an impression of the natural distribution of tolerances to simulated Martian conditions, only the wild type organisms were used. These strains were obtained in the context of the MASE project from various extreme environments which were considered Mars analogues sites (Cockell et al., 2017). The following microorganisms were investigated: *Buttiauxella* sp. MASE-IM-9 (DSM 105071), *Clostridium* sp. MASE-IM-4 (DSM 105631), *Halanaerobium* sp. MASE-BB-1 (DSM

Table 1. Strains, origin and cultivation conditions. [a] at the applied cultivation conditions, no spores of *Clostridium* sp. MASE-IM-4 were detectable.

Strain	Origin	Medium	Supplements in MASE medium (wt%)	Gas phase (vol%)	Temp. (°C)
Buttiauxella sp. MASE-IM-9	Islinger Mühlbach, Germany	MASE-II / TSA	0.1% Yeast extract	80% N_2, 20% CO_2	30
Clostridium sp. MASE-IM-4[a]	Islinger Mühlbach, Germany	MASE-II -$FeCl_2$ / TSA	0.01% Dimethylamine 0.001% $FeCl_2$	15% H_2, 25% CO_2, 60% N_2	30
Halanaerobium sp. MASE-BB-1	Boulby Mine, Great Britain	HACE. No growth on solid surfaces.	0.1% Yeast extract	15% H_2, 25% CO_2, 60% N_2	45
Trichococcus sp. MASE-IM-5	Islinger Mühlbach, Germany	MASE-II -$FeCl_2$ / TSA	0.01% Na_2SO_4 0.01% $C_6H_5Na_3O_7$ x 2 H_2O 0.02% KNO_3	15% H_2, 25% CO_2, 60% N_2	30
Yersinia intermedia MASE-LG-1	Lake Grænavatn, Iceland	MASE-I / TSA	0.01% KNO_3 0.01% C-Org-Mix	80% N_2, 20% CO_2	30

105537), *Trichococcus* sp. IM-5 (DSM 105632), and *Yersinia intermedia* MASE-LG-1 (DSM 102845). Medium compositions and strain-specific anoxic cultivation conditions are summarized in Table 1 and described in detail by Cockell et al. (2017). The incubation was carried out at the indicated cultivation temperature and cultures were shaken at 50 rpm.

Individual and combined stress tests

All stress tests were performed in anoxic MASE/HACE medium (Table 1). The influence of individual and combined stress factors on the selected MASE isolates were examined (Table 2).

Table 2. Overview of performed individual and combined stress tests with the MASE-strains.

Individual stress	**Conditions**
Na-perchlorate exposure	15 minutes, 4 hours, 24 hours 96 hours; ≤ 4 M
Growth in the presence of Na-perchlorate	0.5 wt% / 1 wt%, 24 hours
Combined Stress	**Conditions**
Na-perchlorate addition and desiccation	0.5 wt% / 1 wt%; 24 hours
Desiccation and X-rays	24 hours; ≤ 3000 Gy
Desiccation and Mars atmosphere	24 hours; ≤ 1 month

For the investigation of the effect of Na-perchlorate as individual stress factor the cells were exposed to different concentrations of Na-perchlorate up to 4 M including Martian relevant concentrations of 36 mM (~ 0.5 wt%) and 71 mM (~ 1.0 wt%). Na-perchlorate was added to an overnight culture grown at strain-specific optimal conditions. After anoxic incubation up to 96 hours at room temperature, the exposure was terminated by sudden dilution (1:400), followed by the determination of the survival rate by the most probable number technique (MPN) or a plating assay.

In addition to these exposure experiments, the ability of the model organisms to actively grow and multiply in the presence of perchlorates was tested. Martian relevant concentrations of Na-perchlorate (0.5 wt% or 1.0 wt%) were added before inoculation and cultivation was conducted at optimal growth conditions in the presence of Na-perchlorate.

For experiments using desiccation as an individual stress factor as well as in combination with other stresses, *i.e.* desiccation and exposure to radiation or exposure to Martian atmosphere, (see also Table 2), the cells were cultivated under optimal growth conditions until stationary growth phase was reached (~24 h). Desiccation experiments were performed as described earlier (Beblo et al., 2009). One milliliter of culture (equivalent to ~10^7 cells) was spread evenly on sterile glass slides or quartz discs and dried under anoxic conditions within an anaerobic chamber (Coy Laboratory Products Inc.; [O_2] < 5 ppm, relative humidity 13 ± 0.5 vol%). Perchlorate treated cells were desiccated in an identical way.

Exposure to ionizing radiation, in the form of X-rays, was performed as described in earlier studies with the X-ray source Gulmay RS 225A (Gulmay Medical Ltd.) at 200 kV and 15 mA (Beblo et al., 2011). The cells were irradiated at a distance of 19.5 cm below the X-ray source with 20 Gy/min ± 5 Gy/min up to 3000 Gy. The dose rate was measured with a UNIDOS dosimeter (PTW Freiburg GmbH). All irradiation experiments were performed at room temperature.

The combination of anoxic desiccation and ionizing irradiation and the combination of anoxic desiccation and exposure to a simulated Martian atmosphere (a Mars-like gas composition (2.7 vol% N_2, 1.6 vol% Ar, 0.15 vol% O_2 in CO_2) at a pressure of 10^3 Pa), were carried out using the previously described TRex-Box (Beblo-Vranesevic et al., 2017a).

If not stated otherwise all exposures and treatments were performed under anoxic conditions at room temperature.

Determination of survival rate

Growth and morphology of the unfixed native cells was observed by phase-contrast light microscopy (ZeissR AxiolmagerTM M2) with 400× or 1000× magnification.

Determination of the survival rate and enumeration of cultivable cells was achieved by the MPN-technique via dilution series with ten-fold dilution steps in anoxic MASE/HACE medium (Franson, 1985). The cells were incubated at their optimal growth temperature for up to four weeks. Alternatively, the survival rate was determined by applying a plating assay on tryptic soy agar (TSA) plates under anoxic conditions for *Buttiauxella* sp. MASE-IM-9, *Clostridium* sp. MASE.IM-4, *Trichococcus* sp. MASE-IM-5, *Yersinia intermedia* MASE-LG-1 (see also Table 1). The plates were incubated in an anaerobic chamber at room temperature up to one week. All experiments were repeated independently at least three times to represent biological replicas.

Results

Survival of MASE-strains after exposure to perchlorates as individual stress factor

To get an overview of the tolerance of the MASE-strains towards Na-perchlorate, survival curves after 15 minutes exposure and the corresponding calculation of the D_{10}-values were performed as shown in Figure 1 and Table 3. All tested strains survived the treatment with Na-perchlorate as an individual stress factor for 15 minutes at room temperature.

Table 3. Calculated D_{10}-values of MASE-strains after addition of Na-perchlorate for 15 minutes at room temperature.
* data from Beblo-Vranesevic et al. 2017b.

Strain	**D_{10}-value [M] $NaClO_4$**
Clostridium sp. MASE-IM-4	0.6
Buttiauxella sp. MASE-IM-9	1.2
Halanaerobium sp. MASE-BB-1	1.5
Yersinia intermedia MASE-LG-1	1.7
Trichococcus sp. MASE-IM-5	1.9
Escherichia coli	1.3*
Bacillus subtilis (vegetative cells)	1.9*
Deinococcus radiodurans	2.7*

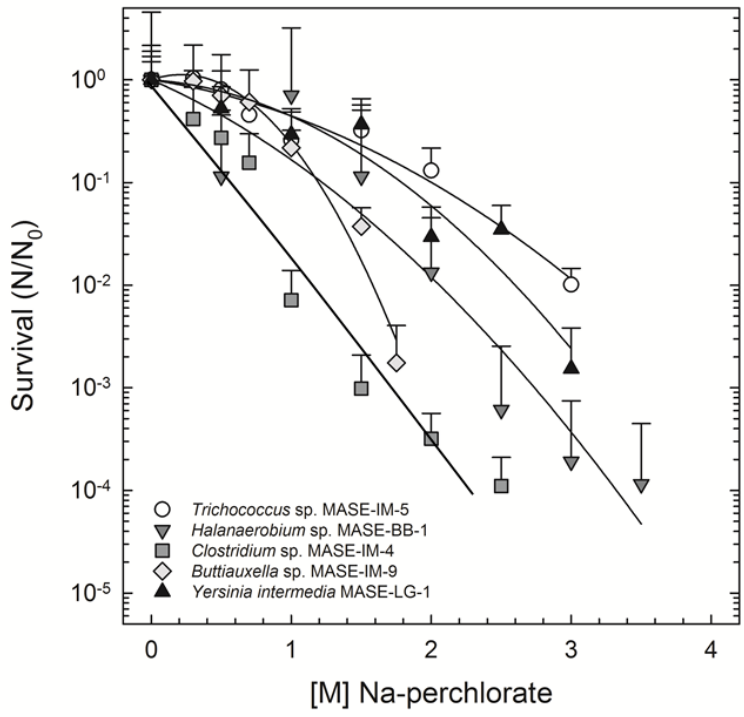

Figure 1. Survival after 15 minutes anoxic exposure to Na-perchlorate. N_0: viable non-treated cells; N: viable cells after exposure to Na-perchlorate for 15 minutes (n=3). Error bars are representing the standard deviation.

The MASE-strains were ranked in terms of their tolerance to Na-perchlorate after an exposure for 15 minutes in the following descending order from tolerant to sensitive: *Trichococcus* sp. MASE-IM-5 > *Yersinia intermedia* MASE-LG-1 > *Halanaerobium* sp. MASE-BB-1 > *Buttiauxella* sp. MASE-IM-9 > *Clostridium* sp. MASE-IM-4. This is also visible in the calculated D_{10}-values of the investigated MASE-strains ranking from 0.6 M to 1.9 M (15 min, $NaClO_4$) (Table 3).

It could be shown that exposure to lower concentrations of Na-perchlorate (36 mM = 0.5 wt% and 71 mM = 1.0 wt%), equivalent to concentrations occurring on Mars, for up to 96 hours at room temperature under anoxic conditions did not lead to an alteration in survivability of all five MASE-strains. The number of living cells remained the same or it was elevated due to further growth at room temperature for the tested time period of up to 96 hours (Figure 2).

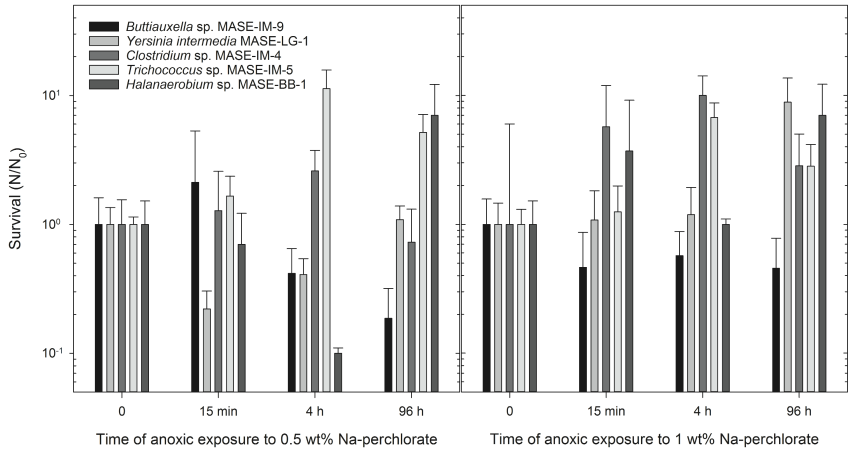

Figure 2. Response of organisms to Na-perchlorate. MASE-strain cultures (*Buttiauxella* sp. MASE-IM-9, *Yersinia intermedia* MASE-LG-1, *Clostridium* sp. MASE-IM-4, *Trichococcus* sp. MASE-IM-5, *Halanaerobium* sp. MASE-BB-1) were incubated in the presence of Na-perchlorate under anoxic conditions at room temperature. N_0: Viable cells without added perchlorate, N: Viable cells after storage in the presence of perchlorates (0.5 wt% or 1.0 wt%). Recovery was performed under standard cultivation conditions without perchlorate (n=3). Error bars are representing the standard deviation.

Growth in the presence of perchlorate

Despite the fact that all MASE-strains were growing mainly as single cells under standard cultivation conditions, microscopically observations revealed that all strains showed extensive cell agglomerations (*i.e.* cell-cell-connections) with increasing Na-perchlorate concentrations if perchlorate was present during cultivation (Figure 3). *Buttiauxella* sp. MASE-IM-9, *Clostridium* sp. IM-5, *Halanaerobium* sp. MASE-BB-1, and *Yersinia intermedia* MASE-LG-1 showed chain formation of increasing length in the presence of Na-perchlorate. The longest cell-chains were observed for *Halanaerobium* sp. MASE-BB-1 and *Buttiauxella* sp. MASE-IM-9. *Yersinia intermedia* MASE-LG-1 and *Clostridium* sp. MASE-IM-4 showed additionally an elongation of the cells itself if they were growing in chains. *Trichococcus* sp. MASE-IM-5, normally growing as diplo-coccus or three to four cells attached together, formed chains like pearls on a string.

Perchlorates and desiccation – combined stresses

The exposure to Martian concentrations of Na-perchlorate and subsequent desiccation showed additive effects of both stresses with regard to survivability. Na-perchlorate had a negative influence on the desiccation tolerance of all tested organisms (Figure 4). In the presence of Na-perchlorate, the survival rate was lowered up to five orders of magnitude compared to desiccation for 24 hours without perchlorates. The lowest

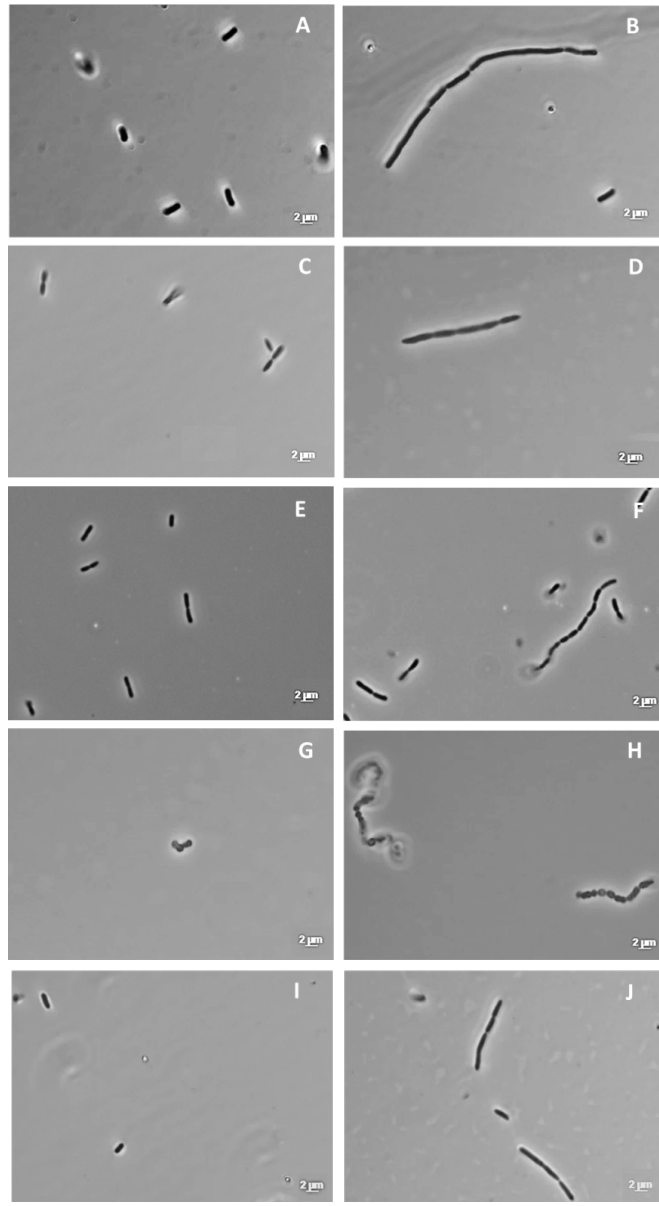

Figure 3. Light microscopy image of *Buttiauxella* sp. MASE-IM-9 (A, B), *Clostridium* sp. MASE-IM-4 (C, D), *Halanaerobium* sp. MASE-BB-1 (E, F), *Trichococcus* sp. MASE-IM-5 (G, H), *Yersinia intermedia* MASE-LG-1 (I, J) grown under standard cultivation conditions (first row, A, C, E, G, I) and cultivated in the presence of 1 %wt Na-perchlorate (second row, B, D, F, H, J).

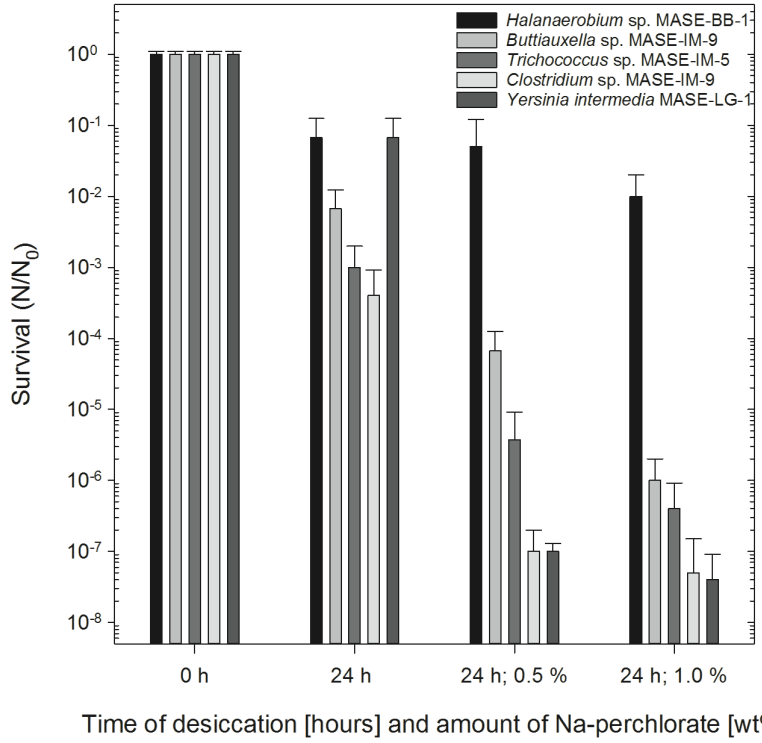

Figure 4. Influence of perchlorates on desiccation tolerance of MASE-strains (*Halanaerobium* sp. MASE-BB-1, *Buttiauxella* sp. MASE-IM-9, *Trichococcus* sp. MASE-IM-5, *Clostridium* sp. MASE-IM-4, *Yersinia intermedia* MASE-LG-1) under anoxic conditions. N_0: Viable cells without desiccation, N: Viable cells after desiccation in the absence or presence of perchlorates (0.5 wt% or 1.0 wt%). Recovery was performed under standard cultivation conditions without perchlorate (n=3). Error bars are representing the standard deviation.

effect was observed for *Halanaerobium* sp. MASE-BB-1, the highest effect was visible for *Yersinia intermedia* MASE-LG-1. In all cases, the reduction in the survival after desiccation did not reveal a substantial difference for both Mars relevant concentrations of 0.5 wt% and 1.0 wt% Na-perchlorate.

The combination of desiccation and ionizing radiation, led to additive effects of both stresses in all model organisms (Figure 5). Moreover, differential reactions to the stresses were observed: *Clostridium* sp. MASE-IM-4 and *Trichococcus* sp. MASE-IM-5 showed enhanced radiation tolerance under stress in a dried state and both strains were able to multiply after application of ionizing radiation (3 kGy) in a desiccated state. In contrast, *Buttiauxella* sp. MASE-IM-9 and *Trichococcus* sp. MASE-IM-5 only showed additive effects after irradiation in a dried state.

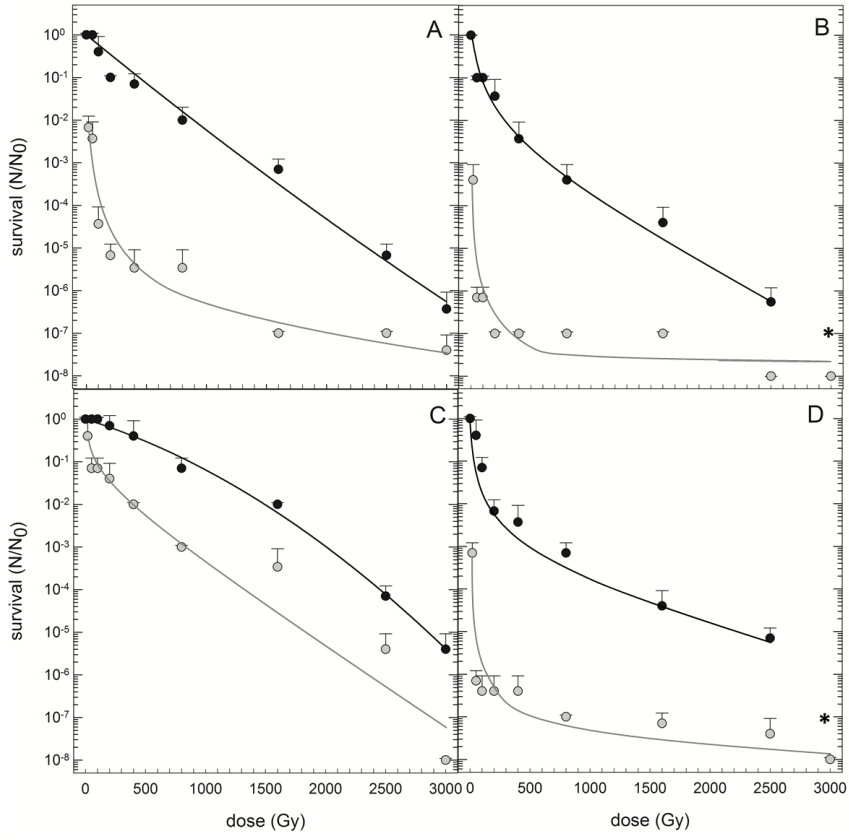

Figure 5. Survival of the MASE isolates (A: *Buttiauxella* sp. MASE-IM-9, B: *Clostridium* sp. MASE-IM-4, C: *Halanaerobium* sp. MASE-BB-1, D: *Trichococcus* sp. MASE-IM-5) after anoxic irradiation up to 3 kGy in liquid medium (black lines) and after the combination of anoxic desiccation (24 h) and subsequent exposure to ionizing radiation (up to 3 kGy) under anoxic conditions (grey lines). *: no viable cells detected. N_0: viable non-desiccated non-irradiated cells; N: viable cells after irradiation or after combined desiccation and irradiation (n=3). Error bars are representing the standard deviation.

An additional decrease of the survival of about one to two orders of magnitude was observed after the cells were exposed to desiccating conditions in combination with Martian atmosphere and pressure (2.7 vol% N_2, 1.6 vol% Ar, 0.15 vol% O_2 in CO_2 at a pressure of 10^3 Pa) (Figure 6). One exception was *Clostridium* sp. MASE-IM-4: the exposure to the Martian atmosphere enhanced the survival after desiccation after four weeks of storage (Figure 6B). In all other organisms, namely *Buttiauxella* sp. MASE-IM-9, *Halanaerobium* sp. MASE-BB-1, and *Trichococcus* sp. MASE-IM-5, additive effects of desiccation and the application of simulated Martian atmosphere were observed.

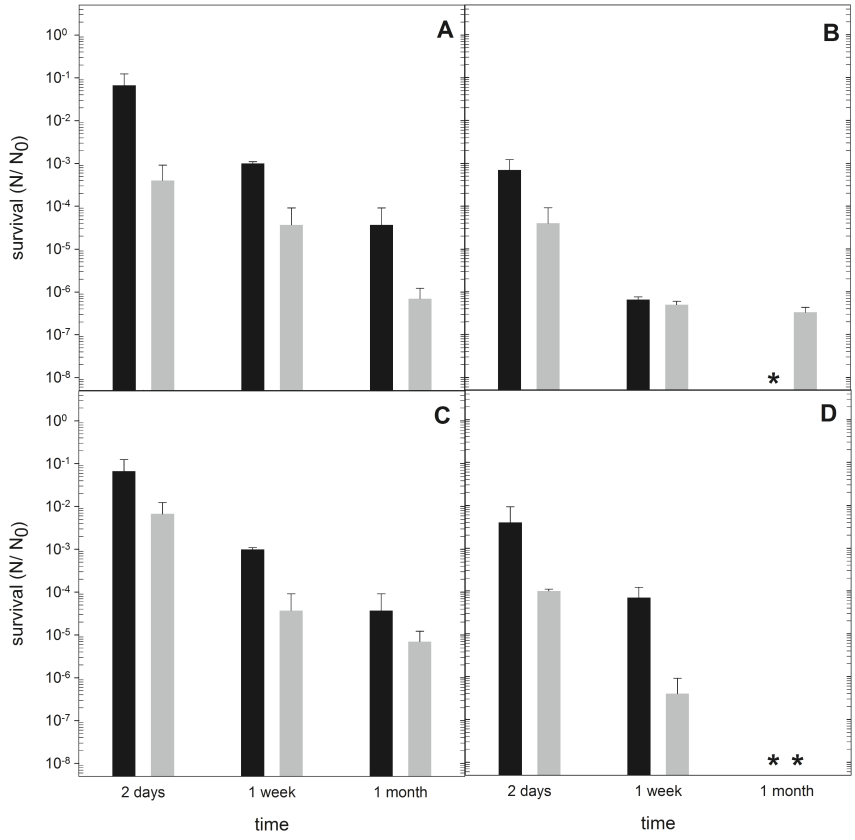

Figure 6. Survival of the MASE-strains (A: *Buttiauxella* sp. MASE-IM-9, B: *Clostridium* sp. MASE-IM-4, C: *Halanaerobium* sp. MASE-BB-1, D: *Trichococcus* sp. MASE-IM-5) after exposure to anoxic desiccation and Martian atmosphere. N_0: viable cells without desiccation and without exposure Martian atmosphere, N: viable cells after desiccation and exposure Martian atmosphere (n=3). Error bars are representing the standard deviation. *: no viable cells detected. Black: Cells were desiccated under anoxic conditions. Grey: Cells were desiccated under anoxic conditions and exposed to Martian atmosphere (Mars gas at a pressure of 10^{-3} Pa).

Discussion

In this study, we investigated the effects of individual and combined Mars-relevant stresses on (facultatively) anaerobic microorganisms. We focused on physical and chemical stress factors that are known to be prominent on the Martian surface and in the near-surface environment. They include the presence of perchlorates, desiccation, ionizing radiation and Martian atmospheric conditions. Each of the individual stress factors tested, *i.e.,* desiccation, radiation, and perchlorates led to a reduction in survival rate of the organisms. For the investigated organisms it is not known whether they

are able to metabolize perchlorates. Perchlorate concentrations similar to those studied here are not thought to exist in their natural environment. Therefore, it is not surprising that the presence of perchlorates played a major role in cell stress. Even short-term exposure of 15 minutes led to cell damaging effects. Comparison of the D_{10}-values with data from previous studies shows that the MASE-strains have a slightly lower tolerance to perchlorates than other model organisms, such as *Escherichia coli* and vegetative *Bacillus subtilis* cells (Beblo-Vranesevic et al., 2017b).

In this study the tolerance of different microorganisms to Martian relevant concentrations of perchlorates was tested (0.5 wt% and 1.0 wt% Na-perchlorate). The literature reports different tolerance levels amongst Bacteria and Archaea which do not metabolize perchlorates: one example of a sensitive organism is the acidophilic iron sulfur bacterium *Acidithiobacillus ferrooxidans,* possibly able to grow under Mars-like geochemical conditions but not able to multiply in the presence of 0.022 M (~ 0.5 wt%) Mg-perchlorate (Bauermeister, 2012; Bauermeister et al., 2014). *Halobacterium* sp. NRC-1 a model halophilic archaeon cannot grow in the presence of 0.04 M Na-perchlorate (Laye and DasSarma, 2018). Different methanogenic archaea (three *Methanobacterium* strains and two *Methanosarcina* strains) are also negatively influenced in their growth behavior at low concentrations (up to 0.01 M) of Na-perchlorate (Shcherbakova et al., 2015). It has been reported that several halotolerant strains show only slight alterations in their growth pattern in the presence of perchlorates: nearly all of the halotolerant isolates grew in the presence of 0.05 M Mg-perchlorate (Al Soudi et al., 2017). Comparable results were shown for halophilic bacteria, such as *Alkalibacillus* and *Halomonas* (Matsubara et al., 2017). Unfortunately, the absolute tolerance levels of organisms capable of metabolizing perchlorates, such as *Dechloromonas hortensis* and *Aeropyrum pernix*, are absent from the literature. These organisms were treated with perchlorate concentrations between 10 mM and 50 mM (Wolterink et al., 2005; Liebensteiner et al., 2015).

We also observed morphological changes, including filaments and cell-chain formation associated with the presence of Na-perchlorate during growth. Such modification of size and shape has been observed earlier as a response to changes in the environmental conditions or to stress factors. Filamentation is one observed shape alteration that can be influenced by several factors such as nutrient deprivation, oxidative stress, DNA damage, exposure to antibiotics and temperatures shifts (Young, 2006; Justice et al., 2008; Perfumo et al., 2014). The observation of chain formation at 0.5 wt% Na-perchlorate in *Buttiauxella* sp. MASE-IM-9, *Halanaerobium* sp. MASE-BB-1, *Clostridium* sp. MASE-IM-4, and *Yersinia intermedia* MASE-LG-1 and cluster-like structures in *Trichococcus* sp. MASE-IM-5 shows that Mars relevant concentrations of perchlorates are capable of causing microbial morphological anomalies. Morphological changes due to Na-perchlorate are also described for halophilic, thermophilic, and methanogenic

microorganisms. Grown at the highest tolerated perchlorate concentrations, *Halobacterium salinarum*, *Haloferax mediterranei*, and *Haloarcula marismortui* were unusually swollen and deformed. For instance, *Halomonas elongata* cells appeared normal up to 0.2 M Na-perchlorate, but in a medium with 0.4 M Na-perchlorate, the cells had a thin and wrinkled appearance (Oren et al., 2014). The thermophilic bacterium *Hydrogenothermus marinus* tends to grow in chains in the presence of 0.3 M Na-perchlorate (Beblo-Vranesevic et al., 2017b). The methanogenic strain, *Methanobacterium arcticum,* shows other morphological changes and builds cyst-like cells in the presence of Mg-perchlorate (Shcherbakova et al., 2015). The reason for the morphological changes within the MASE-strains is not known. It is possible that the chain formation provides a survival advantage during cell damaging conditions such as has been hypothesized for biofilms (Cvitkovitch, 2004).

For experiments with combined stress factors with desiccation and radiation, desiccation and simulated Martian atmosphere, desiccation and perchlorates mainly additive effects could be shown. The negative influence of perchlorates on the cellular metabolism is also visible in the combination of perchlorate exposure and desiccation. Even low concentrations of perchlorates led to a reduction in survivability up to four orders of magnitude compared to desiccated cells without perchlorates. Interestingly, if *Yersinia intermedia* MASE-LG-1 cells were irradiated in the presence of different perchlorates in Martian relevant concentrations no influence on the survival after exposure to ionizing radiation was detected (Beblo-Vranesevic et al., 2017a). The combination of exposure to perchlorates and UV irradiation led to additive and even synergistic bactericidal effect for vegetative *Bacillus subtilis* cells (Wadsworth and Cockell, 2017).

An enhanced radiation tolerance was visible when the *Clostridium* sp. MASE-IM-4 and *Trichococcus* sp. MASE-IM-5 were exposed to ionizing radiation in a dried form. This effect is also reported for some halophilic archaea (Leuko et al., 2015) and can be explained by the low abundance or absence of intra- and extracellular water within the desiccated samples. Consequently, radiolysis cannot occur due to exposure to ionizing radiation. A lower concentration of reactive oxygen species can be assumed. Absorption effects of the ingredients of the medium can be excluded since ionizing radiation penetrates through the (in-) organic residues of the medium. The exposure of dried cells to Martian atmosphere had no effect on the survival; i.e. once the cells survived the first desiccation step, they were stable with respect to additional desiccation in Martian atmosphere. These data are in accordance to data from literature: if *Deinococcus radiodurans* is exposed to Martian atmosphere (7 days) the survival rate was reduced less than one order of magnitude (Pogoda de la Vega et al., 2007).

Conclusion

The selected organisms obtained from extreme anoxic analogue environments on Earth were shown to possess not only a high tolerance against the environmental stresses that occur in their normal habitat, but also exhibit a substantial tolerance to individual and combined Mars relevant stress factors, *i.e.* desiccation, Martian atmosphere and pressure, ionizing radiation, and the presence of perchlorates. The observed effects of combined stress factors were found to be additive in the case of *Buttiauxella* sp. MASE-IM-9 and *Halanaerobium* sp. MASE-BB-1, and synergistic in the case of *Clostridium* sp. MASE-IM-4 and *Trichococcus* sp. MASE-IM-5. The desiccation step seems to give a relative advantage to cells to cope with other stress factors which provides constraints for the search for live on Mars. Moreover, these MASE strains were even able to grow in the presence of Martian relevant concentrations of Na-perchlorate under anoxic conditions. Our data show that survival in Martian environments, *i.e.* Martian brines, is in principle possible for some organisms. This work advances our understanding of the limits of survival in Mars-relevant conditions.

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