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Titel: Subjecting Bacteria to the Extremes of Mars

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Subjecting Bacteria to the Extremes of Mars

Master's Thesis

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Thesis in Biology
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Abstract

Throughout the time life has been present on Earth, life has evolved to grow at almost every environmental niche imaginable. One of the toughest environments are the Atacama Desert and the McMurdo valleys. Collectively these environments present extremely arid conditions, high UV radiation, subzero temperatures, and a high concentration of toxic perchlorate. All the above-mentioned factors are very similar to those found on Mars. This thesis will look at bacteria isolated from these environments and subject them to additional conditions present only on Mars. These include, but are not limited to, low pressure (0.01 bar), an atmosphere consisting purely of CO_2, low relative humidity $\geq$ 17%, and custom made soil designed to mimic the Martian soil composition.

Subjecting them to UV radiation higher than those seen on Mars, showed no decrease in CFU after 4.5 minutes of exposure. While increasing concentrations of perchlorate, 1%, 2%, and 3% decreased the growth rate of the tested isolates, it did not kill the isolates which all showed growth during the 24 hour test duration. Sequencing isolates for perchlorate reductase, PcrA, showed the potential presence in one of the isolates. Subjecting the bacteria to low pressure, 99.99% CO_2 atmosphere, low relative humidity, and Martian soil analog were all done simultaneously in the New Jens Martin Mars Chamber, the Mars chamber. The results showed a reduced CFU count by an average factor of 10^{-5} for 5 of 6 isolates. One of the isolates, did not survive the conditions presented in the Mars chamber.

In addition to testing isolates under Martian circumstances, a major part of the thesis was to prepare the Mars chamber. Step by step the Mars chamber has become increasingly closer to simulating the Martian environment.

Abbreviations

RH – Relative Humidity

Wt% - weight percentage

pcrA – Perchlorate Reductase

cld – chlorite dismutase

PRI – Perchlorate Reducing Islands

EMH – Enrichment Media Halophile

TSB – Tryptic Soy Broth

G/GS – Soil simulating Gale Crater composition/ with sulfur

J – Soil simulating Jezero Crater

- *The above abbreviations including a P, is short for perchlorate*

UV – In regards to soil samples from the McMurdo valleys, this stands for University Valley

VV – Victoria Valley

TV – Taylor Valley

F – Food

NF – No food

MGS – Mars Global Simulant

MGSF – Mars Global Simulant Food

MGSNF – Mars Global Simulant No Foo

MSC – Mars Soil Control (Isolates placed outside the Mars chamber)

MSM – Mars Soil Mars (Isolates placed inside the Mars chamber)

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1 Introduction

For a long time humanity has asked the question "*Are we alone in the universe?".* This curious question has been projected as thrilling science fiction novels, magazines and movies. Here we often see a foreign entity annihilating all living things on our planet in the search for resources, painting a very bleak outcome if we ever were to meet an extra-terrestrial entity. However, the life we are currently looking for, is not an intelligent life form, similar to ourselves, but microorganisms. As we increasingly gain more knowledge and our scientific tools improve, we are now closer than ever to answer the question "*Are we alone…?".* Trying to answer this question rovers on Mars, Deep space satellites, and powerful telescopes have been built. These tools have been deployed to look for signs of life and habitability outside our planet and solar system. Concurrent with the study and search for life in our solar system and universe, a way to look for life on other planets, is to study life on our own. By understanding where on Earth, and in which ecological and environmental niche we can find life, we can add factors to the search for habitability outside of Earth. There are many places on Earth that in the past have been deemed uninhabitable as there were no signs of multicellular life. The environment was simply too hostile for multicellular organisms to survive, which incorrectly portrayed the limits of life. However, with the introduction of affordable DNA sequencing, the current understanding of life, is that it can be found in almost every corner of the Earth. Sequencing soil, stone and water samples from extreme environments have revealed a high abundance of different microorganisms. It has taught us that the search for life elsewhere, is most likely not to be an intelligent life form but found in the form of microorganisms. Currently, the most promising place to find life is our neighbor planet Mars. One of the things that make it a promising place to find life, is its proximity, which has allowed us to send rovers to surface of Mars, and orbiting satellites. This has provided us with a lot of data of the present environment on Mars. If you are a human without a space suit, or any other Earthly organism for that matter, the present environment on Mars is not a nice place to be. With an atmospheric pressure varying seasonally between 0,007 – 0,01 bar (0,7 - 1,% of that on Earth) (Martínez et al., 2017), and consisting of 95% CO_2, 3% N_2, 1,6% argon (Franz et al., 2017). The thin atmosphere, together with an absent magnetic field (Acuña et al., 1998), means that there is not much protection from UV or cosmic radiation. The UV spectrum range lies between 200 nm (UVC) and 300nm (UVB), where UVC is the most harmful (Cockell, McKay, Warren-Rhodes, &

Horneck, 2008). The UV intensity found on Mars vary between 10-20 W/M_2 (in the Danish summer months it does not exceed more than 7 w/M2(ref: DMI)). The cosmic rays can be divided into two categories, galactic cosmic rays (GCRs) and solar energetic particles (SEPs). The SEPs and GCRs offer a radiation level measured in mGy/day. On Mars the daily amount of radiation amounts to 0.2 mGy/day (Hassler et al., 2014). This can be translated to 0.2 millisieverts and put in perspective with a standard x-ray. A standard x-ray exposes you to 1 millisievert, and a CT scan to 14 millisievert (see Appendix 1- X-ray dosage). Comparing these three figures, spending a day on Mars might not be as harmful as a standard x-ray, however for a prolonged stay, the radiation levels quickly add up. Not to mention the travel time to and from Mars, where the levels were measured to be double that of the surface of Mars (Hassler et al., 2014).

The above-mentioned factors are not the only thing that could make living on Mars a challenge. The daily temperatures can fluctuate between -113^{o}C and a more temperate level of 20^{o}C(Martínez et al., 2017). Having a mean temperature of -70^{o}C, depending on which points of temperature you include, Mars presents itself with an extremely cold environment. These low temperatures can, in part, also be found on Earth. At temperatures at -15^{o}C *Planococcus halocryophilus* has been shown to grow and divide, while keeping metabolic activity at -25^{o}C (Mykytczuk et al., 2013) and soil samples from Antarctica, incubated showed activity at -39^{o}C (Panikov, Flanagan, Oechel, Mastepanov, & Christensen, 2006). At what temperature bacterial activities ceases is difficult to say, but a means of survival for psychrophilic organisms is to reside within liquid brines trapped within the ice. Here a steady flow of nutrients can take place which organisms can utilize (De Maayer, Anderson, Cary, & Cowan, 2014). For microorganisms in these cold environments, growth rates are low, as there are temperatures that prevent the organisms from growing it is not uncommon for bacteria to enter a dormant state which allows them to survive long enough for circumstances to change to a more favorable state. Evidence shows that some bacteria can remain dormant for 500000 years and avoid DNA damage with continuous DNA repair (Johnson et al., 2007). A more recent study show a potential dormant state of up to 100 million years (Morono et al., 2020). One of the challenges with temperature below 0^{o}C is that water will be tied up in ice, making it unavailable. Together with a low atmospheric pressure which lowers the boiling point of water to 0^{o}C, the presence of water is extremely rare, it can however be present in the form of salty brines, as mentioned. The lower boiling point of water on Mars, does however contribute to

relative humidity (RH) in the atmosphere. Levels of RH have been measured to be statistically relevant at 17-23%, but levels have also been measured to reach 70% (Martínez et al., 2017).

1.1 Requirements for life on Earth

The primary requirements for life on Earth are three-fold. Organic elements, a source of energy, and water. The first two elements are not fixed to a specific carbon or energy source and can be seen as variables. Variable in the sense that the life diversity on earth, can utilize several different carbon sources to grow and undergo mitosis. The same applies for the energy source. Organisms can utilize many different elements to generate the energy needed for chemical processes. In the latter variable, most multicellular eukaryotic organisms are reliant on oxygen, with the exception of some parasites (Yahalomi et al., 2020), however in the prokaryotic kingdom a variety of elements can be used by cells to create the energy needed for sustaining life. The third requirement is water. Water is a great solvent which allows for the chemical reactions within a cell to take place. As life, as we know it, cannot exist without any of the 3 elements, the constant of water, is what makes water such an interesting and sought-after element when searching for life outside our own planet.

1.1.1 Organic elements

Organic elements are covalent bonds of nitrogen, hydrogen and oxygen to carbon. These organic elements are the building blocks for life as they are incorporated in almost every biological element, creating elements such as a cell wall, functional proteins, and information, in the form of DNA. Common as these elements are on Earth, their general composition and structure can vary according to their environment which allows for life to evolve to almost any environmental niche.

In addition to carbon, nitrogen, hydrogen and oxygen, life also needs other essential elements: calcium, magnesium, phosphorus, potassium and sodium. These elements are not present in as high concentrations in cells, as the before mentioned elements, but are essential as they provide protein structure and enzyme function.

Together with the essential elements, life also requires some trace elements: cobalt, copper, iodine, iron, manganese, molybdenum, selenium, sulfur, and zinc - that alongside the essential elements, provide structure and function for proteins and enzymes.

1.1.2 Inorganic compounds

Individually all elements from the periodic system are inorganic elements. They are arranged according to atomic number, and to some degree, abundance. It is the latter which is of interest, as the abundance of elements on Earth, have created life as we know it. The interesting part of this, is that the periodic table is not just a system referring to the abundance or presence of elements on Earth, but it applies to the entire universe.

Most of the above-mentioned elements are also inorganic compounds which one way or the other can be used for organisms in various ways. They help structure proteins and provide enzymes with specific functions or targets along with many other functions. One important inorganic compound which is not mentioned above is carbon dioxide, CO_2. The average global concentration of CO_2 is around 400ppm which corresponds to an atmospheric concentration of 0,04%. This seemingly low number is however enough to sustain all the plants on Earth, both on land and in the sea. Being autotrophic, they rely purely on CO_2, sunlight, and water, as carbon source and energy source, respectively. But it is not only plants who can use CO_2 as a carbon source, many bacteria can also grow on a CO_2 based diet, which is of high interest when looking to Mars for a potential carbon source.

1.1.3 Energy source

For a better understanding of the general production of ATP, I refer to appendix 8 - "*Uffe's appendix*"

In order to provide structure for all the essential organic elements, a cell needs energy. The energy required comes in the form of ATP, which is also described as the energy currency of the cell. ATP is created through a series of redox reactions, having an electron donor and an electron acceptor. The electron donor and acceptor is what defines the ability to make energy. For many of the organisms on Earth, especially multicellular eukaryotic life forms, oxygen serves as the sole electron acceptor where the electron donor can vary between these organisms. The redox couple

of O_2 and H_2O has a high reduction potential. As oxygen is a highly electronegative molecule and Earth's atmosphere consists of 20% oxygen, it is easy to understand that this electron acceptor has become the preferred fuel for metabolic activity for the majority of organisms. As mentioned before, there are some parasites that are able to live without oxygen, as they steal ATP from the host organism. In addition to this, some fungi live solely on fermentation (Cathrine & Raghukumar, 2009). And then there are autotrophic plants, who gain their energy from the sun and water. However, there are environments on Earth that are deprived of oxygen. At these environments, multicellular eukaryotic life forms are rare. The eukaryotic domain of life is mostly limited to oxygen, however the prokaryotes, archaea, and bacteria, which constitutes the other two of the three domains of life, are shown to thrive under anoxic conditions. The ability to live under anoxic conditions does not apply to all bacteria or archaea, but some of the species have evolved to utilize alternative electron acceptors to produce ATP. There are numerous strategies, and metabolic pathways employed by archaea and bacteria to substitute oxygen, some of which will be discussed later.

1.1.4 Water

The last of the three main factors for life, is liquid water - H_2O. Consisting of two hydrogen atoms and one oxygen atom it gives water the ability to form hydrogen bonds, as oxygen is more electronegative than hydrogen, creating a slightly charged and asymmetric molecule. The hydrogen bonds of water is what gives water a very important set of abilities. It makes water an excellent solvent for transferring charged and ionic molecules, gives it a high heat capacity which helps regulate to global temperature (the golf stream), it has a high heat vaporization, it also has cohesive and adhesive properties, with cohesiveness making it capable of forming i.e. droplets, and adhesiveness which makes water capable of sticking to surfaces and substances, and lastly the hydrogen bonds make water less dense as a solid. The importance of droplets will be discussed in the perchlorate section.

1.2 The Presence of the Building Blocks of Life on Mars

This section will briefly touch upon past signs of water on Mars, followed by a listing of known metabolites, carbon source and potential redox pairs, known to present on Mars.

1.2.1 Water on Mars, past and present

The water on Earth is what separates and shapes the continents on Earth, it runs in streams and forms lakes on land, is transferred across vast distances by evaporation, cloud formation and eventually rainfall, creating a continuous flow of water for the entire Earth. The flow of water running down mountains, hills, etc. not only shapes the rivers, but also leave specific marks on the land. Past marks can show clear indication of rivers and canyons that once were flowing with water. This is interesting as the marks made by water are very characteristic and can help geologists determine the previous presence of water. When looking to Mars, it is the same patterns scientists are looking at, and similar patterns have been found (Salese, Pondrelli, Neeseman, Schmidt, & Ori, 2019).

1.2.2 Nutrients and Potential Metabolites on Mars

Mars, has an extremely harsh environment, and signs of any form of life is yet to be found. Rovers and satellites have been sent to study the surface of Mars, and while they have discovered what is a cold, tough and dry environment, their results have also shown the presence of most nutrients required for life on Earth, to be native to Mars as well (see table 1). The presence or lack of these compounds does not prove or disproves one or the other, but merely indicates that life forms can have an Earth like appearance and/or function. In addition to the macro- and micronutrients available on Mars, metabolites in the shape of electron donors and acceptors have also been found (see table 2). The shown metabolites do not represent redox couples, but merely the presence of which of the metabolites can be used by microorganisms on Earth. The metabolites could support the presence of life regarding the redox pairs which could be used to generate ATP, and supplement with CO, CO_2 or CH_4 as a carbon source.

Table 1: A depiction of Macro- and micronutrients and their presence on Mars

Macronutrients	**Presence on Mars**
Carbon	+
Hydrogen	+
Nitrogen	+
Calcium	+
Magnesium	+
Phosphorus	+
Potassium	+
Sodium	+
Micronutrients	
Cobalt	?
Copper	?
Iodine	?
Iron	+
Manganese	+
Molybdenum	?
Selenium	?
Sulfur	+
Zinc	?

Table sources:(Foley, Economou, & Clayton, 2003; Stoker et al., 1993) (Nachon et al., 2014; Vaniman et al., 2004)

Table 2: Listed are the potential electron donors and acceptors present on Mars

Electron donors	Electron accpetors
Fe^{2+}	Fe^{3+}
H_2 – 0,004%	SO_2^{-4}
CO – 0,06%	O_2 - 0,174%
CH4 – 0,41 ppbv	CO_2 - 95,32%
	ClO_4^- (perchlorates) 0,4-0,6 %
	Manganese (Mn4+)

Knowing the redox elements on Mars, we can look for organisms on earth who can couple these elements. While the presence of both oxygen and organic carbons are low on mars, the plausibility for life on mars, require alternate ways to generate energy.

1.2.3 Carbon sources on Mars

Potential carbon sources on Mars are limited, but not absent. The carbon sources can be found in the shape of CO_2, CO and CH_4.

The most readily available source of carbon on Mars, is atmospheric CO_2 and thus provides, potential life on Mars, an abundant source for cell material. There is a wide variety of organisms on Earth which rely solely on CO_2 as cell material. Termed autotrophs and include prokaryotes and eukaryotes, like bacteria and archaea, and plants, respectively. Organisms who utilize CO_2 as their carbon source, can do so through the Calvin cycle. The Calvin cycle synthesizes fructose 6-

phospate from 6 molecules of CO_2, by employing the enzyme RubisCO, followed by reversal of the glycolysis pathway. This is an energy consuming process, which is why most autotrophic bacteria, are also chemolithotrophs obtaining their energy from inorganic compounds (ref: Brock. Biology of Microorganisms, section 20.6 P. 591-592.).

Carbon monoxide, CO, is an odorless and tasteless gas which on Earth can be seen as as result of incomplete combustion of organic materials. Prolonged exposure to CO at a concentration of 50 ppm can cause mild side effects, like headaches and nausea, and at concentrations of 1000 ppm death can occur within an hour. Human activities are a major emitter of CO, especially the motor vehicles contribute to emissions. Luckily, there is a natural sink to this toxic gas. The sink is in the form of bacteria, which are estimated to annually remove 10^8 tons of CO from the lower atmosphere (Bartholomew & Alexander, 1979). Carbon monoxide is also a part of the Martian atmosphere and is believed to be a product of CO_2 absorption of UV radiation forming CO and O (Weiss, Yung, & Nealson, 2000). With a concentration of 0,06%, or 600 ppm (Sindoni, Formisano, & Geminale, 2011) compared to the CO concentration on Earth , 100-300 ppb (Badr & Probert) concentration present a potential carbon source, and a source of electrons. An example of this, can be seen by the bacteria *Carboxydothermus hydrogenoformans* which couples CO growth to H_2O forming H_2 and CO_2 (Svetlichny et al., 1991). The pathway of CO oxidation can be through the Wood-Ljungdhal pathway (Ragsdale & Pierce, 2008).

The third potential source of carbon on Mars, is methane. The concentration of methane on Earth, is around 1,5 ppm in the troposphere, and mainly originates as a biological waste product (Ehhalt, 1967). For this reason, scientists were baffled when levels of methane were measured on Mars. Methane concentrations on Mars have been measured to be 0,41 +/- 0,16 ppbv, fluctuating seasonally reaching peaks of 0,65 ppbv (Webster et al., 2018).

1.3 Electron donors on Mars/Metabolic possibilities

1.3.1 Iron oxidation and reduction

On Earth oxidation of ferrous iron to ferric iron is not uncommon. It is a chemolithotrophic reaction which can occur anaerobically or aerobically at neutral pH or at pH level of >=2, respectively. The reason for aerobic oxidation of Fe_2^+ requires an acidic environment, is because ferrous iron

spontaneously oxidizes to ferric iron at neutral pH. A low pH of 2 is considered an extreme environment and is an environmental niche seen at i.e. coal mining facilities. At these sites bacteria like *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* can be found. These two bacteria can utilize ferrous iron as an electron donor in an aerobic environment. Normally the oxidation and reduction of compounds create a proton gradient across membranes, however in an acidic environment of a pH 2, there is an abundance of naturally occurring protons. The above-mentioned organisms utilizes these protons in the ATPases, creating ATP. As protons cross the membrane to motor the proton motive force, they pile up inside the cell, which lowers the pH of the cytoplasm. To maintain a cytoplasmic pH level the cell uses the electron from ferrous iron, and oxygen, to make H_2O, thus lowering the concentration of protons (Brock – Biology of Microorganisms).

Ferric iron can also be used separately of ferrous iron, and serve as an electron acceptor, where ferric iron is reduced to ferrous iron. Compounds that serve as dissimilatory electron donors vary from inorganic, such as hydrogen (Lovley, Phillips, & Lonergan, 1989) and organic molecules like lactate, formate, and pyruvate (Kim, 1999; Lovley et al., 1989). The mentioned electron donors can also be coupled to manganese, Mn_4^+ which serves as an electron acceptor (Lovley et al., 1989). Much like with aerobic organisms, the ferric iron and manganese serve as a terminal electron acceptor in place of oxygen, powering the steps needed for creating a proton motive force.

Ferrous iron listed as the first electron donor, is present in high abundances on Mars in the form of olivine, pyroxene minerals, and other silicates (Hecht et al., 2009). On earth, microbes have shown to be able to couple ferrous iron oxidation to NO_3^-, with the latter functioning as an electron acceptor(Straub, Benz, Schink, & Widdel, 1996), however NO_3^- is yet to be found on the surface of Mars, currently eliminating this as a redox couple. Coupling ferrous iron to perchlorate could also be an option for a redox couple, however this combination has been shown to inhibit growth of the organism (Thrash, 2009), and yet, no known organism can couple these two redox elements for ATP generation.

1.3.2 Hydrogen

As already mentioned, hydrogen oxidation can be linked to variety of electron acceptors, both organic and inorganic. Hydrogen diffuses through the membrane, where it can bind to two different

proteins, one membrane bound protein and a cytoplasmic protein. The functions of these two proteins vary, as the membrane bound (membrane-integrated hydrogenase) protein oxidizes the hydrogen and transfers the electron through an electron chain pathway, ending in the reduction of O_2, which powers the ATPases. The latter protein (Cytoplasmic hydrogenase) reduces NAD+ with hydrogen as the reducing element. The NADH then feeds into the Calvin cycle together with CO_2 to form cell material (Brock – Biology of Microorganisms).

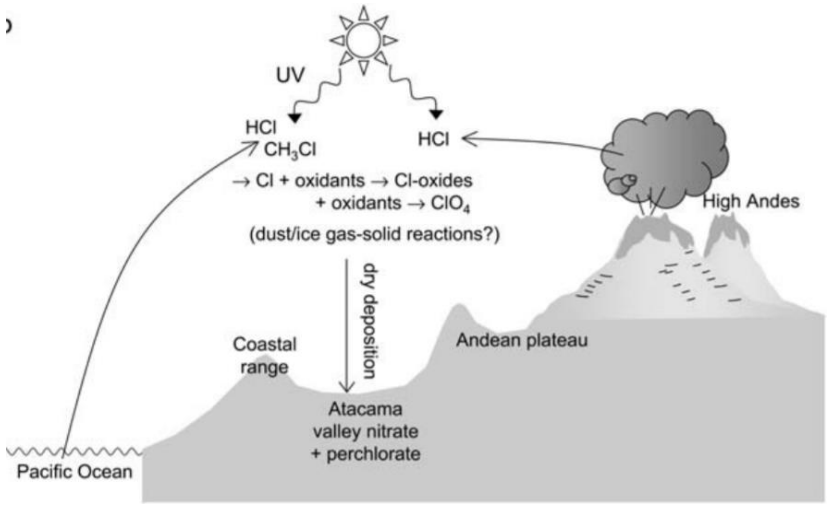
On Earth hydrogen is a common metabolite which can be found mainly in anoxic environments. It is being used by a list of bacteria and archaea as an electron donor, which are able to couple hydrogen oxidation to iron or sulfur reduction, or to i.e. acetate and other organic molecules. The source of H_2 on Mars is a photochemical process reacting with atmospheric H_2O (Liu & Donahue, 1976). Expected to mostly be suspended in the atmosphere the Curiosity rover has detected hydrogen down to 1 meters (ref: https://www.nasa.gov/jpl/msl/pia19809/curiosity-finds-hydrogen-rich-area-of-mars-subsurface). Measurements further down the surface have yet to be conducted, but are scheduled for upcoming mars missions, where drilling of the soil down to 2 meters can be done. The availability of hydrogen on the surface, or within the regolith, could provide organisms with an electron donor.

1.3.3 Perchlorate

Perchlorate is an ion with the chemical formula ClO_4^-. Perchlorate is a stable ion which tends to bind to alkali elements like, magnesium, sodium, potassium or ammonia, termed magnesium perchlorate, sodium perchlorate etc. When bound the various perchlorates are stable, but when introduced to water or humidity, the perchlorates dissolves into two ions, a positive and a negative, the latter being the perchlorate ion, or just perchlorate. Being a highly reactive ion, but stable at room temperature, it is used for making explosives, rocket fuel and gunpowder (Trumpolt et al., 2005). The perchlorates react by causing a chain reaction when heated to a high temperature, which causes the chain reaction to continuously apply heat to the system, eventually causing an explosion. Perchlorates are formed naturally and industrially. How much is manmade is an elusive number, as the military aren't fond of disclosing how much potential ammunition they might have in storage (services, 2008). Naturally the perchlorates are believed to form in the atmosphere where chlorine oxides react with ozone, O_3, forming perchloric acid which is deposited through dry deposits (Catling et al., 2010b). When deposited, the perchloric acid reacts with alkali elements

forming magnesium perchlorate or sodium perchlorate. Global distributions of perchlorate vary, with the highest concentrations of X%/w being found in the Atacama Desert, Chile (Jackson et al., 2015). In general, it is believed that the concentration of perchlorates increase with aridity, and the duration of aridity (Jackson et al., 2015). Being that the annual rainfall of the Atacama Desert is less than 4 mm/m_2 (Azua-Bustos et al., 2018), and signs of Atacama being the oldest desert on Earth, with a stable climate for 150 million years (Hartley, Chong, Houston, & Mather, 2005) it provides conditions for perchlorate to accumulate over potentially, millions of years. In addition to continuous arid environment, contributions to the accumulation of perchlorates ions is brought in by reacting chlorine oxides from the near by pacific ocean. It is also stipulated that volcanic activity from the Andes mountain range, contribute to perchlorate deposits.

Figure 1: The figure illustrates the processes of perchlorate formation in the atmosphere – image source (Catling et al., 2010b)



Perchlorate is also deliquescent meaning that it can absorb humidity from the air. A study has shown that perchlorate can contain a low liquid water content as low as ~13% RH at -30oC (Gough, Chevrier, & Tolbert, 2012). As the RH on Mars has been shown to fluctuate between day and night, RH fluctuates between <5% at day time, and can reach full saturation, 100% at night time (Zent et al., 2010). This implies that what is believed to be droplets photographed on the Phoenix lander (see figure 1) might well be droplets, rather than dust accumulations.

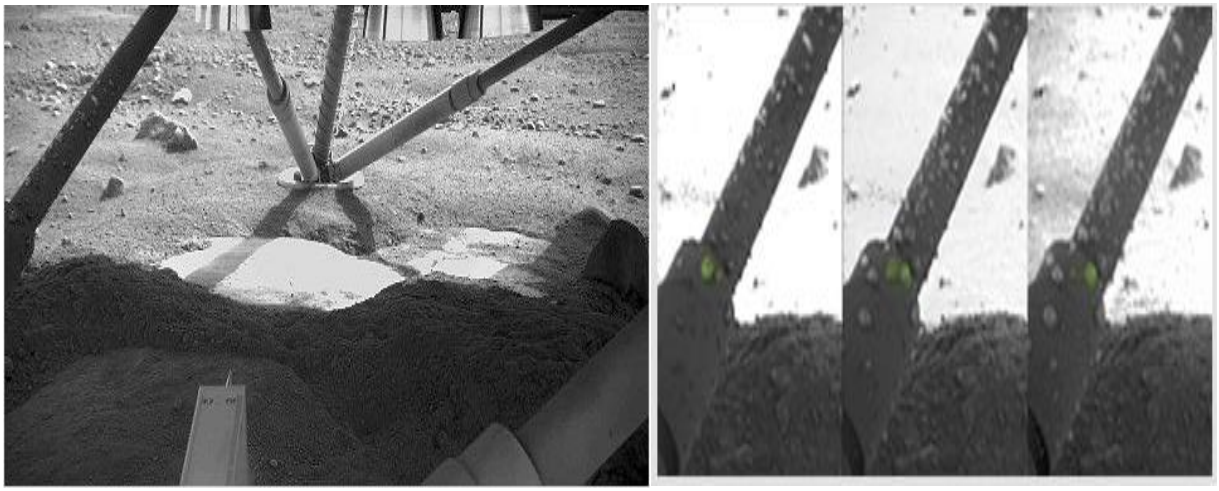


Figure 2 shows potential droplets forming on the Phoenix lander. The figure to the right shows a closeup of the droplets - image source: https://www.astrobio.net/mars/liquid-water-ice-salt-mars/

Another property which is assigned to perchlorates are their low eutectic temperatures. Mixed with water, perchlorate can lower the freezing point of water quite substantially and potentially form brines on Mars. A theoretical calculation based on 3rd party results show that sodium perchlorate and magnesium perchlorate had a eutectic temperature of -37°C and -69°C, respectively (Chevrier, Hanley, & Altheide, 2009). It is however important to mention that these eutectic points were reached at a concentration of 52 wt% for sodium perchlorate and 44 wt% for magnesium perchlorate. In the same study by Vincent el al. the loss of water measured in mm/h, showed that at higher concentrations of perchlorate and low temperatures, the perchlorates lowered the evaporation rate. In the sense of being deliquescent, it corresponds well, and the possibility of brine formation is plausible under the atmospheric and pressure conditions seen on Mars.

1.3.3.1 Perchlorate on Mars

The formation of perchlorate on Earth is partly due to the chlorine reacting with ozone, followed by dry depositions. As the source of chlorine species reacting with ozone on Earth have their primary source from the oceans, it raises questions as to where the chlorines on Mars have their origin. Another source of chlorine on Earth, are volcanos, which spew out HCl that also reacts with ozone. The contribution of perchlorates by volcanos on Earth, could explain the source of perchlorates on Mars as it has been shown that volcanic activity has been continuous through Mars'

history, and by some, stipulated that the volcanos might still be active (Greeley & Spudis, 1981; Neukum et al., 2004). The volcanic activity has also been suggested as one of the main contributors of Martian perchlorate (Catling et al., 2010a), however newer models show that volcanic activity together with atmospheric production cannot account for the perchlorate concentrations measured by the Phoenix lander, 0,4-0,6% (Smith, Claire, Catling, & Zahnle, 2014). The contribution of perchlorates to the Martian surface might still, to some degree explain the presence, but new research suggests that perchlorate could originate from NaCl. Mixing NaCl with Martian soil simulates consisting of SiO_2, Fe_2O_3, Al_2O_3, and TiO_2, subjected the soil samples to 170 hours of radiation similar to those on Mars, perchlorate and chlorate formed in sufficient volumes to account for the measured levels (Carrier & Kounaves, 2015).

Bacteria reducing perchlorate for respiration has for a long time been acknowledged. The first evidence of these organisms stems from cultivation of wastewater led out by perchlorate using/manufacturing factories. The isolated bacteria have been shown to use a wide range of organic electron donors, acetate, propionate, butyrate, lactate, succinate, fumarate, malate, and yeast extract, but also inorganic electron donors like ferrous iron, sulphide (S2-), and hydrogen ("<Bruce et al 1999 Reduction of (per)chlorate by a novel organism isolated from papermill.pdf>," ; Bruce, Achenbach, & Coates; Zhang, Bruns, & Logan, 2002). Previously thought to be a local and manmade occurrence, perchlorates have been found in pristine soils (J. D. Coates et al., 1999). Perchlorates have since then been shown to have a natural occurrence and is globally distributed and the concentrations rely heavily on the aridity the environment as perchlorate is easily dissolved in water (Jackson et al., 2015). This indicates that perchlorate reduction might be a novel thing in the environment.

Organisms that are able to reduce perchlorates do this in the absence of oxygen, as when oxygen is present it represses the transcription of the perchlorate reducing genes, *pcrA* and chlorite dismutase, *cld (De Long, Kinney, & Kirisits, 2010)*. The reduction of perchlorate is a process involving mostly electron transfer proteins, with PcrA and Cld being the two main proteins. PcrA is part of a diverse Dimethyl sulfoxide reductase protein family, which is in part characterized as having a molybdenum ion bound and contribute nitrate reductase (Melnyk et al., 2011). As mentioned before, organisms who can utilize perchlorate as terminal electron acceptor, make use

of a variety of electron donors, which feed into the perchlorate reduction pathway (figure X, source: (Youngblut, Wang, Barnum, & Coates, 2016).

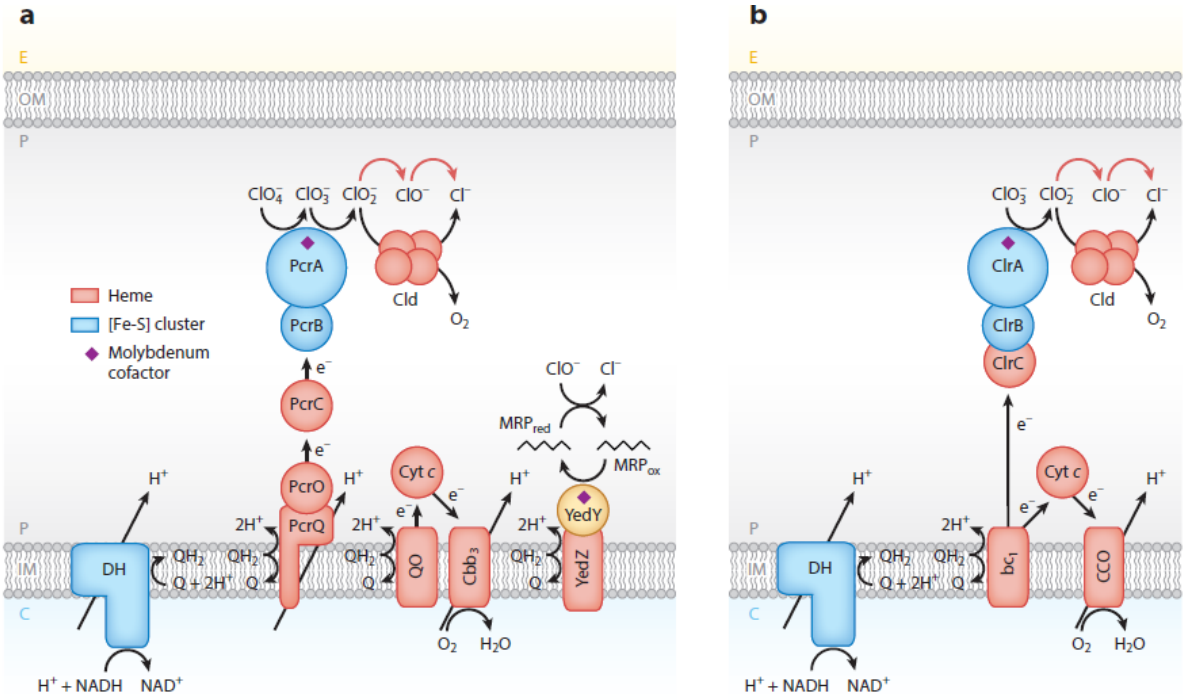


Figure 1

Biochemical depiction of (per)chlorate-reduction pathway in (*a*) dissimilatory (per)chlorate-reducing bacteria and (*b*) dissimilatory chlorate-reducing bacteria. Abbreviations: bc_1, cytochrome bc_1 complex; C, cytoplasm; Cbb_3, cytochrome oxidase; CCO, cytochrome *c* oxidase; Cld, chlorite dismutase; ClrABC, chlorate reductase; Cyt *c*, cytochrome *c*; DH, dehydrogenase; E, extracellular space; IM, inner membrane; MRP_{ox}, methionine-rich peptide in the oxidized state; MRP_{red}, methionine-rich peptide in the reduced state; OM, outer membrane; P, periplasm; PcrAB, perchlorate reductase; QO, quinol oxidase; YedY, methionine sulfoxide reductase; YedZ, putative quinol oxidase.

Figure 3(a) In this figure the perchlorate reduction pathway is illustrated. (b) The chlorate reduction pathway - image source:(Youngblut et al., 2016)

Figure 3 shows perchlorate reduction and chlorate reduction, however the latter pathway will not be addressed here. The pathway to perchlorate reduction by PcrA is a series of redox steps that transports electrons to PcrA which then reduces perchlorate. As the electron passes through the initial steps the electron carrier QH2 release two protons to the periplasmic space, which essentially can be used by an ATPases. PcrA reduces perchlorate in two steps, step 1 being perchlorate to chlorate, followed by chlorate to chlorine dioxide. Chlorine dioxide is a powerful oxidizing agent which is why Cld is crucial part of the perchlorate reduction pathway. Cld disassembles chlorine dioxide in two steps, with an intermediate of hypochlorite, ending with the final products chloride and oxygen. The chloride freely diffuses out of the cell, and the oxygen feeds into the proton motive force generation (Youngblut et al., 2016). It is **also** worth mentioning

that the dismuted oxygen not only feeds into the proton motive force, but also acts as a cosubstrate for aerobic enzymes which can oxidize hydrocarbons to CO_2 (John D. Coates, Bruce, & Haddock, 1998).

Looking at perchlorate genes across several organisms, it shows that the perchlorate reducing organisms are phylogenetically diverse within the proteobacteria. alpha, beta and gamma subclasses of proteobacteria show a monophyletic clustering of perchlorate reductase, however the *cld* gene appear to be more polyphyletic as a high sequence similarity of the *cld* gene was seen between the gamma and beta subclasses (J. D. Coates et al., 1999). This indicates horizontal gene transfer between subclasses. Looking closer at the genetics of perchlorate reduction there is evidence that some of the genes involved in perchlorate reduction are flanked by inverted repeats and make up perchlorate reducing islands, PRI. The PRIs consist of a set of core genes that appear to be universal for perchlorate reducing bacteria see figure 4.

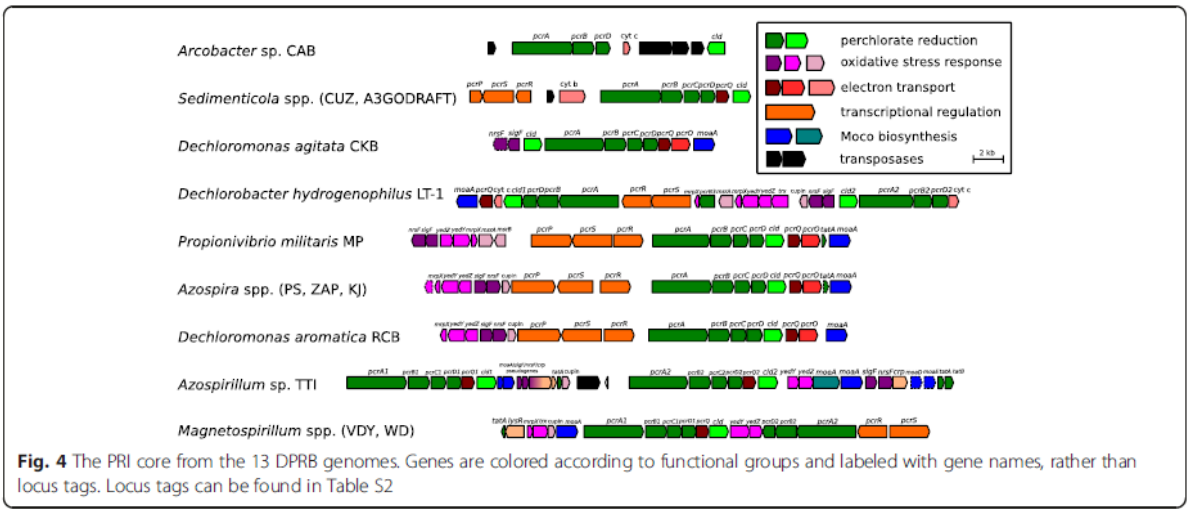


Fig. 4 The PRI core from the 13 DPRB genomes. Genes are colored according to functional groups and labeled with gene names, rather than locus tags. Locus tags can be found in Table S2

Figure 4: Depicted here are the genes consisting of the perchlorate reducing genomic islands. The figure shows color coded function of each gene and the transcriptional direction - image source:(Melnyk et al., 2011)

The core PRI contains everything needed for perchlorate reduction - perchlorate reduction genes, electron transport and oxidative stress response genes. Some of the PRI also carry transposes strongly indicating the possibility of horizontal gene transfer (Melnyk et al., 2011). Having the core genes for perchlorate reduction located on a genomic island, some with their own transposases, presents the idea that this trait can artificially be transferred to other bacteria.

There is still much to learn about how perchlorate reduction is distributed amongst bacteria. The bacteria shown and mentioned are all part of the proteobacteria phylum, which are all gram-negative bacteria. There has however also been found a gram-positive bacteria, *Morella perchloratireducens* which is able to reduce perchlorate and linking this to numerous electron donors like methanol or CO, amongst others (Balk, van Gelder, Weelink, & Stams, 2008). Another interesting bacteria, *Dechloromonas sp. Strain HZ* has also been isolated showing to able to link perchlorate reduction to hydrogen oxidation, and using CO_2 as a carbon source (Zhang et al., 2002)). The latter bacteria is of special interest, as all of the metabolites used by this bacteria can be found in high abundance on Mars. The current limit to our knowledge of perchlorate reducing bacteria comes from cultivated isolates which has taught us how perchlorate reduction can work. It is important to note that the proteins and pathways mentioned does not exclude other unknown proteins or metabolic pathways that might be available for perchlorate reduction. This also goes to show that perchlorate reduction can be a common trait for bacteria and potentially archaea aswell.

1.4 Mars on Earth and the Challenges Facing Microbes on Mars

1.4.1 Mars analogs

There are many extreme environmental niches on Earth that have shown to be habitable to microorganisms. These environments might show some resemblance to other planets and thus serve as analogs. Finding a complete analog to Mars on Earth, is however not possible. The reason for this is the low atmospheric pressure and the atmospherical composition. The closest we could get to a Mars analog in regards of atmospheric pressure would be the tallest point of Earth, Mount Everest. With a height of 8848 m the atmospheric pressure at the top lies at 0,33 bar (West, 1999). As conducting research on top of Mount Everest might be circumstantial, it might be the closest analog to Mars found on Earth in terms of pressure and UV radiation, with values of 0,33 bar and ~6 - 22 W/m^2 (ref: http://meteonews.ch/en/Weather/G1283416/Mount_Everest).

1.4.2 The Atacama Desert

An alternative analog to Mars is the Atacama Desert in Chile. With only 74,7 mm/m_2 rainfall in total over a decade (from 2008 – 2017) experienced at the Yungay area (Azua-Bustos et al., 2018), the desert is categorized as a hyper arid desert. The driest places of the Atacama Desert are also

used by Nasa to test rovers. In addition to the aridity of the place, the terrain and UV exposure is similar to that on Mars. Therefore the Atacama Desert can be seen as a close analog to Mars, which provides us with a good starting point in testing if microorganisms can survive exposure to the Martian environment.

The Atacama Desert is located in Chile between the Chilean coastal range and the Andes Mountains. The mountains create a rain shadow region, where evaporation and condensation of water is blown from the Pacific Ocean and the Andes towards the mountain ranges. As the moist air climbs the mountain sides, the temperatures decrease. As cold air cannot hold as much humidity, the water is released as rainfall drying the winds that cross the mountain tops. The cold air then descends on the opposite side of the mountain. While descending, the air heats up again, which dries the air even further. This is known as the rain shadow region (see figure 5). The low levels of precipitation also lowers the RH of the desert to roughly ~20% (see figure 6) which are close to the RH of the Martian surface, thus providing a close RH analog.

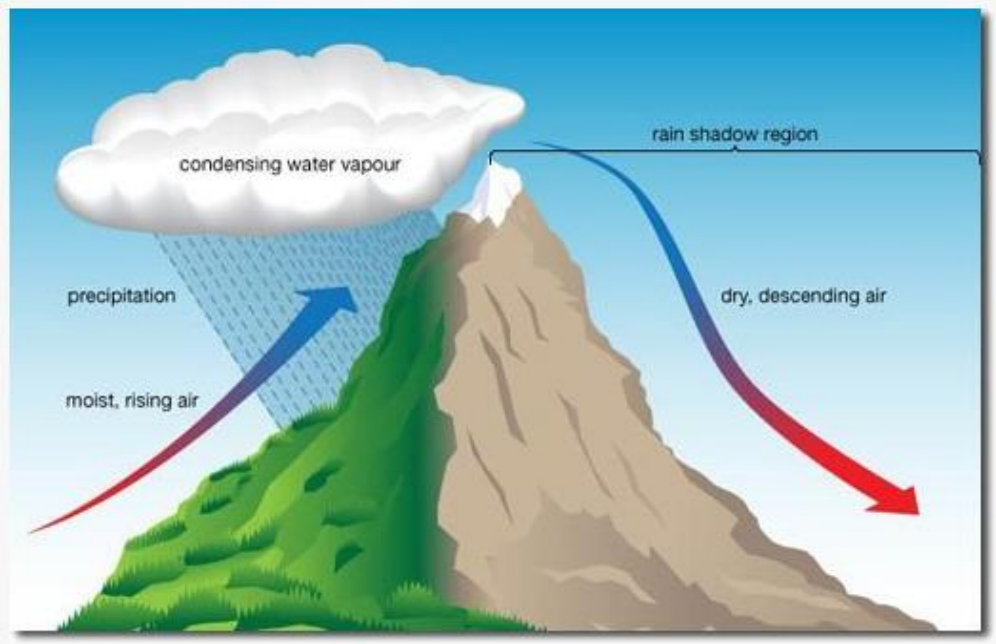


Figure 5: Illustrates the rain shadow effect aiding to the aridity of the Atacama Desert – Image source: https://www.permaculturenews.org/2013/09/04/the-atacama-desert-chile-the-driest-desert-on-earth-five-reasons-why/

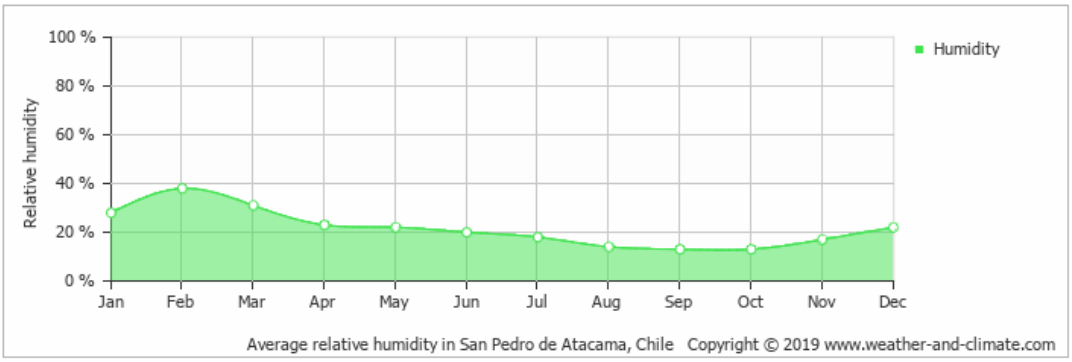


Figure 6: Shows the average humidity in the Atacam Desert – Image source: Weather-and-climate.com

An indirect effect of the rain shadow region, and low RH, is the lack cloud formation. As there are very low levels of moisture in the air and soil, cloud formation is at a minimum. The absence of clouds, high altitude (2,408m), and low ozone column of the Atacama Desert also makes the desert subject to one of the highest UV indexes on Earth . With an annual UVB exposure varying between 3500 W/m^2 to 5000W/m^2 between the coastal and the Andean Plateau, respectively, we see a daily exposure range of 9,5 and 13,7 W/m^2, but can at certain sites reach 20 W/m^2 (Cordero et al., 2018). These UV levels are similar to those found on Mars (8-20 W/m^2).

Being close to the ocean, yet trapped between mountains who cast a rain shadow from both mountain ranges, provides the Atacama Desert with dry deposits in the form of perchlorate (Perchlorates, and the formation of perchlorates, will be discussed further, later in the introduction). The location, low precipitation, low RH, and extended climate stability provide excellent circumstances for perchlorates to form, be stable, and accumulate over time. These factors have caused the Atacama Desert to have the highest natural concentration (0,3 - 0,6 wt%) of perchlorates on Earth (Catling et al., 2010b), which are similar to those measured by the Phoenix lander on Mars (0,4 - 0,6wt%) (Hecht et al., 2009). Barren and inhospitable as the Atacama Desert might seem; the desert appears to be teeming with life. Recent advances in DNA sequencing has shown that there is a microbial diverse community consisting of bacteria, archaea and fungi (Azua-Bustos, Urrejola, & Vicuna, 2012; Dirk Schulze-Makuch et al., 2018) residing in the Atacama Desert, all of which are capable of tolerating the conditions set in the desert. This goes to show that to some extent, life can survive under some Martian environmental factors.

1.4.3 The McMurdo Dry Valleys

Consisting of a group of smaller valleys, Taylor Valley, Wright Valley, and Victoria Valley, make up The McMurdo Dry Valleys located in Antarctica. The valleys, as with the Atacama Desert, provide us with a close Martian analog. The main resemblance to Mars, that we do not see in the Atacama Desert, is the temperature. With an average temperature of -14,8 to -30 ^{o}C, with the lowest temperatures measured at -65,7^{o}C at the Victoria Valley (Doran, 2002), it is the closest natural analog to the temperatures we see on Mars. Together with extremely low temperatures, The McMurdo Valleys are also a subject to extreme aridity with low annual precipitation in the form of snow, 3-50mm, and once the snow falls, the low albedo effect, which heats the uncovered soil, quickly sublimates the snowfall (Hoffman, Fountain, & Liston, 2014). Together with these factors, cold and dry katabatic winds from the Antarctic plateau ascend down the mountain slopes, surrounding the valleys, contributing to the aridity of the place (Nylen, Fountain, & Doran, 2004)

1.4.4 Upper parts of Earth's atmosphere

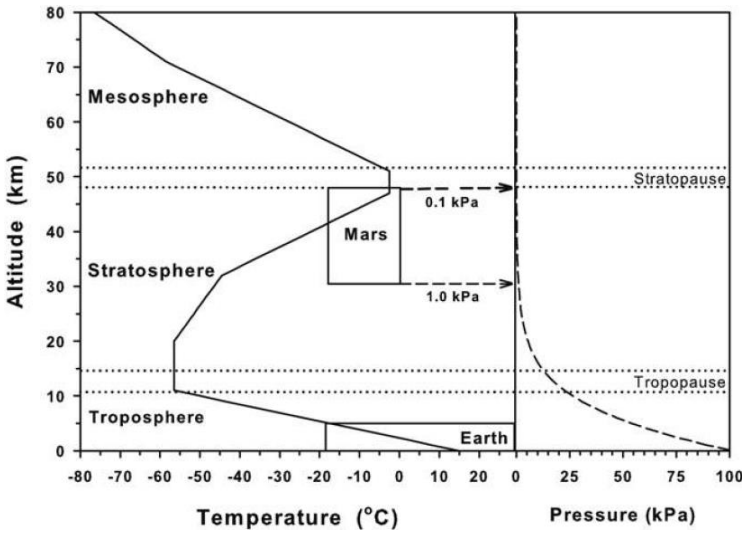


Figure 7: Illustrates the temperature and pressure in regards to altitude - image source:(Schuerger & Nicholson, 2016)

A rather alternative Martian analog could be found in Earth's atmosphere. Excluding a solid surface, Earth's atmosphere presents an analog in regard to pressure, temperature and UV radiation. This area has gained more attention as an astrobiological field, as being able to survive these conditions, is of concern, as we do not want to contaminate the surface of Mars, as we continue deploying rovers to the Martian surface.

The average atmospheric pressure of Earth at sea level is 1 bar depending on the unit of choice. Going below or above sea level will increase or lower the atmospheric pressure, respectively. Organisms residing in either end of this spectrum are termed piezophiles/barophiles for

organisms subjected to high pressure, and hypobarophiles for organisms who can grow at levels below 0.03 bar.

The study of barophiles is quite extensive and looks at bacterial growth were the atmospheric pressure exceeds 101.33 kPa. Studies of the effect of the lower end of the atmospheric pressure spectrum is however a new area of research but is equally interesting.

The spread of bacteria and other microorganisms by wind or air is a recognized means of bacterial transport. Diseases can be airborne, and wind can blow dust particles carrying potential bacteria for miles (Nunez et al., 2016; Weil et al., 2017). The latter means of transportation, might take the bacteria to high altitudes above sea level exposing the bacteria to extreme factors, such as high UV, low temperatures, low nutrient availability, low water availability, and low atmospheric pressure. These high altitudes thus present a close analog to Mars. Seeing if bacteria actively grow and divide, or merely survives, under these circumstances are of great interest, as carrying contaminants, who can survive these conditions to Mars is undesirable.

Some bacterial genus, like *Bacillus* can form endospores which can protect them from extreme environments, including hypobaria, allowing them to grow when conditions again present themselves to be favorable for growth. Not only can the endospores survive, it has been shown that *Bacillus subtilis* was able to actively grow at 5 kPa (Nicholson et al., 2010). Adaptations ensuring growth at these low levels of atmospheric pressure was seen on a genomic level with the genes *des, desK and desR* being upregulated (Fajardo-Cavazos et al., 2012). These genes encode for desaturase, a sensor kinase, and a response regulator. The desaturase is a membrane bound enzyme which can alter the structure of the fatty acid chains in the membrane, by adding or removing single or double hydrogen bonds to make the membrane more flexible to cope with a decrease in temperature. That these genes convey an increase in fitness in fitness, as it would be expected that the cell membrane would require more stiffness to cope with the decreased pressure (Verseux, 2020) Additional genes that have been shown to be involved in low pressure situations, is the stress response regulon, SigB. The SigB stress response was upregulated in *Bacillus subtilis* when incubated under 5 kPa circumstances, however, it did not increase its fitness compared to *Bacillus subtilis* with a SigB knock out (Waters, Robles-Martinez, &

Nicholson, 2014). For the *desKR* stress response, there were measured a small, but statistically relevant, increase in fitness (Fajardo-Cavazos et al., 2012).

The studies of hypobaria are still in its infancy, and the bacterial coping mechanisms in a low pressure environment are still fairly unknown. The bacteria do appear to have a response for coping in a low pressure environment, however they show none to a slight increase in fitness.

1.4.5 UV and gamma radiation

The entirety of Earth's surface is exposed to UV radiation at some point during the 24 hour rotation. The UV intensity differs on i.e. summer or winter, atmospheric density, altitude, or cloud formation. In the summer periods at either of the poles, you have a 24 hour daytime, as the tilting of the Earth makes it is that the sun does not set during this period. This increases the UV exposure, and can potentially cause a lot of stress to life during the summer months. As UV exposure can be very high 10-20 W/m^2, it is not surprise that we find life capable of tolerating such levels. An organism which has been studied extensively for its capabilities to survive high radiation, is *Deinococcus radiodurans.* As mentioned, some bacteria form endospores, which can help a bacteria survive when unfavorable conditions present themselves. *D. radiodurans*, does not form endospores, but is still highly tolerant towards radiation. The reasons for the radiation resistance of the *D. radiodurans* are numerous and be categorized as passive or active. A passive way for *D. radiodurans* to tolerate radiation, is to have several copies of it's genome, 8-10 copies (Cox & Battista, 2005). When exposed to destructive radiation, having a high genome copy number, presents several repair templates for damaged DNA. Additionally the cell has several different loci for the same gene, making the chances of one of the loci to be intact greater than at low genome copy numbers (Cox & Battista, 2005). The former passive survival mechanism go hand in hand with the active survival mechanism. Having repair proteins, which are not exclusive to *D. radidurans*, *D. radiodurans* has additional proteins that aid the repair proteins with nucleotide excision and mismatch repair (Timmins & Moe, 2016). These repair mechanisms do not only apply to UV exposure, but also apply to gamma radiation. In fact, it has been shown that *D. radiodurans* and *Rubrobacter radiotolerans* can survive 10000 and 11000 gray, respectively (Ferreira et al., 1999; Ito, Watanabe, Takehisa, & Iizuka, 2014). This corresponds to an exposure of 1000000 and 1100000 mSv. If we then look back at the measured exposure level on the surface of Mars 0,2mSv/day, we see that these two organisms would not suffer to a great extent. In regards of

tolerating high levels of radiation, it has been shown that some melanized fungi, can utilize radiation. The fungi, *Cryptococcus neoformans* and *Wangiella dermatidis* were radiated with 14 gray/min (14000 mSv) for 20 and 40 mins, the latter having the larger effect, making the fungi grow faster, and in general have a higher biomass (Dadachova et al., 2007).

2 Aim

The aim of this study will be to isolate bacteria from environments on Earth that are similar to those found on Mars. Isolates will be cultured from the Atacama Desert and the McMurdo Dry Valleys which are native to some of the factors found on Mars. They will be isolated with the belief that bacteria from these environments have naturally evolved to survive similar conditions to those found on the Mars. The isolates will subsequently be exposed to various factors present on Mars to gain a better understanding of how the environmental factors of Mars impact life on Earth.

Seeing if bacteria can survive under environmental factors present on Mars, will help us understand the limits for life on Earth. Knowing what the limits of survival for organisms inhabiting Earth, we can draw parallels to other planets. This will broaden, and at the same time, narrow down, the criteria for our search for life on other planets.

In addition, the results can contribute to answering the question, if life indeed can be present on Mars. It can perhaps also help shed light on the question if life ever was a part of the red planet in the past. If we manage to find an organism capable of surviving the harsh environment of the Martian surface, it could spark hope as to finding life on our neighboring planet. These organisms could also serve as first movers in advance of a potential human colonization, to potentially make Mars less hostile for future human excursions.

Knowing which genetic factors play a role for the survival of potential organisms could help future research in a biotechnical manner. Even though life on Earth, in a sense, has been subjected to Research and Development by natural selection for billions of years, organisms could still be genetically modified to better cope with various environmental factors.

3 Methods and Materials

This section will go through the work done in the lab. The results from all the experiments will not be discussed in the result section as there are many binary, or failed results, as a consequence of insufficient lab experience prior to starting the thesis. Having had some previous lab experience from previous courses, and the bachelor, lab work still presented quite a few challenges. Work with the Mars chamber has been a slow process as testing, and getting the environmental factors, of Mars incorporated into the chamber step by step has taken up a lot of time. During the preparation of the Mars chamber problems were encountered but identified and solved, and methods were improved to generate the results discussed in the discussion part.

3.1 Isolation of bacteria

Soil samples had been made available by Anders Priemé taken from the Atacama Desert at Yungay (S 24.0883, W 69,9946)(Dirk Schulze-Makuch et al., 2018). The soil samples were in advance finely graded, so no further handling was needed. Approximately 2 g of soil was mixed with 5 ml PBS media in a 15 ml falcon tube, vortexed vigorously, and set to rest for 1 hour. Subsequently 200 µL of inoculate was plated on Enrichment Media Halophile with 2% or 12,5% NaCl. This was done with and without including some soil to the plates. After approximately 3-4 weeks at room temperature, cultures started showing and each culture was restreaked twice to get pure isolates. The isolates were then grown in liquid culture to gain enough DNA for purification and sequencing, as performing PCR directly from the isolates on agar plates showed no results. The liquid cultures were incubated in liquid EMH 2% and incubate at 23^{o}C on a shake table set to 120 rpm.

A soil sample provided from a previous thesis work was also supplied. This soil sample also came from the Atacama Desert, but was from a salt vein 2 meters below the surface. Previous cultivation attempts had failed. The reason for the failed attempt was speculated to be due to using regular 1xPBS to mix with the soil, which could have caused osmotic bursting of the cells. A second attempt was made looking at the salt concentration and composition of the sample site, which led a “high salt PBS”. Using the “High salt PBS” we managed to cultivate a few isolates, two of which are a part of the results. The inoculum was plated on EMH 2%, EMH 12% NaCl, TSB, 0,6% perchlorate, and 1% perchlorate.

Soil samples from the McMurdo valleys were also isolated, using both 1x PBS and "High Salt PBS". The method was the same as described for the soil samples provided by Anders. The inoculum was plated on EMH 2%, EMH 12% NaCl, TSB, 0,6% perchlorate, and 1% perchlorate.

Recipes for all the media and inoculum can be found in appendices.

3.2 16s sequencing

DNA extraction of the liquid cultures were done with a DNA purification kit (DNeasy PowerSoil Pro Kit) where chemicals and spin columns were provided in addition to a description of all the steps. The clean DNA samples were then mixed with a master mix containing 1492R and 27F primers, PCRBIO reaction buffer, PCRBIO HiFi Polymerase, and sigma water, and loaded into the PCR thermal cycler with a setup of:

98^{o}C for 3 minutes, followed by 25 cycles of 95^{o}C for 15 sec, 55^{o}C for sec and 72^{o}C for 1 min, then a 4^{o}C resting temperature upon cycle completion.

The PCR products were purified using a PCR purification kit, followed by an electrophoresis to check for presence of a PCR product. The purified PCR product was sent to Macrogen for sanger sequencing using 1492R and 27F primers. The returned sequences were entered in a Blast nucleotide search in the rRNA/ITS databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Hits with the highest identity were selected, see results.

3.3 Switching to TBS media from EMH media

To ease the processes in the lab, attempts were made to grow the isolated cultures on TSB media - most isolates were able to grow on TSB media. As a result, all experiments were done with TSB media if not stated otherwise.

3.4 Perchlorate tolerance (solid media)

To test the bacteria in the presence of perchlorate, perchlorate was added to TSB media. 4 different concentrations of 0.6%, 1%, 2%, and 3% perchlorate were added to the agar media. 100 µL of undiluted culture were plated and incubate at room temperature. Binary result.

3.5 Perchlorate tolerance - growth curve (liquid media)

As solid media containing perchlorate did not seem to affect the isolates, a test with liquid media containing perchlorate was made. 100 ml of regular media and 100 ml of media containing 1% perchlorate were added to Erlenmeyer flasks. 100 µL of overnight isolates were added to each media type and placed on a shake table at 120 rpm. 1 ml of each culture was taken every 40 minutes to measure OD_{600}. 11 measurements were made.

3.6 Perchlorate and NaCl growth curves - Plate reader

Wanting to learn how the different tools of the lab could be used, I wanted to see how a plate reader could show the effect perchlorate had on growth curves, similar to the "Perchlorate tolerance (Liquid media)". Here we expanded on the types of media, including TSB, 1% NaCl, 1%, 2%, and 3% perchlorate. The plate reader was set to shake the plate for 10 seconds before reading OD_{600}. OD_{600} was measured every hour for 24 hours at 30°C temperature. The reason for 30°C and not 23°C, as the other experiments, is due to the plate reader getting hot when active, and temperatures below 30°C are highly inconsistent.

3.7 4°C temperature tolerance w/o 1% perchlorate (solid media)

Isolates were tested for growth at 4°C. This was done by diluting (10^{-1} to 10^{-9}) and plating an overnight culture each isolate, followed by 4°C incubation. The isolates were incubated until growth could be seen. After the 4°C incubation non growing isolates were reintroduced to 23°C.

The same experiment was done with plated containing 1% perchlorate.

3.8 4°C temperature and perchlorate experiment (liquid media)

In this experiment 100 µl of overnight undiluted cultures were added to Erlenmyer flask containing 100 ml TSB media or 1% perchlorate media. The flasks containing isolates were placed on a shake table and incubated in a 4°C incubator. OD and CFU were measured and counted every 24 hours for 5 days to see survival over time.

3.9 Anaerobic growth

100 µL of each isolate was plated on TSB and TSB containing 0,6% perchlorate. The plates were placed in airtight cylinders with Anaerocult (https://www.merckmillipore.com/DK/en/product/Anaerocult-A,MDA_CHEM-113829?ReferrerURL=https%3A%2F%2Fwww.google.com%2F). Anaerocult is a substance that converts O_2 to CO_2. An anaerobic indicator was also added to the cylinder - changes color from light blue to white, in the absence of oxygen. Isolates were incubated at room temperature for ~3 weeks. After 3 weeks of anaerobic conditions the isolates were reintroduced to regular atmospheric oxygen to see if non-growing isolates regrew.

3.10 UVB and UVC experiment

4 different UV experiments were done during the span of the thesis. Each experiment is explained below, and only the results from the last experiment are included. For all the experiments, overnight cultures were diluted down to an OD_{600} of 0.4. For experiment 1 and 2, the serial dilutions were 10^{-1} to 10^{-4}. For experiment 3 and 4, the serial dilutions were 10^{-1} to 10^{-9}. For all the UV experiment, the petri dish lid was taken off.

The UV lamp had been supplied by Morten Bo Madsen, and the experimental setup was made out of a moving box covered in tinfoil (see figure 8). For UV lamp specifications, see appendix.

Figure 8: Shown on this figure is the UV experimental setup

Using the formula:

$$\text{Irradiance} = \frac{\text{Power}}{4\pi r^2},$$

Irradiance was calculated to be 15.9 W/m2 with 20cm distance. As any of the exposure times with 15.9 W/m2 did not have any effect on the bacteria, compared to the controls (no exposure), we decreased the distance and increased the exposure time. The decrease in distance (from 20cm to 17.5cm) increased the irradiance to 20.7 W/m2.

First experiment was with a radiation level of 15.9 W/m^2 with an exposure time of 5 s, 10 s, 15 s, and 20 s. The second experiment was done at the same time and for the same exposure times, but with 100µl of autoclaved demineralized water with 1% perchlorate spread on top of the isolates.

The third UV experiment was done with the same radiation level as the first and second experiment, but with increased exposure times of 30s, 1min, 1.5min and 2 min.

For the fourth and final UV experiment, 6 isolates were used plus one E. coli as a UV sensitive control. Each isolate received a UV duration of 1.5 min, 2.5 min, 3.5 min, and 4.5 min, and a control (no UV exposure). For each duration a 9 times serial dilution, for each isolate, was done. The colonies were counted the next day. Some isolate colonies needed more time to grow to a visible size, so a daily check-up was done until all the plates could be counted.

3.11 The Jens Martin Mars chamber experiments (The Mars chamber)

Figure 9: The Jens Martin Mars Chamber glovebox. To the left the figure includes the control panel and the airlock. To the right is a close up of the actual Mars chamber.

When starting the project, the Mars chamber was not functional. The backplate of the chamber needed to be sealed with silicone. This does not sound like much, but due busy technicians working on higher priority projects, it took several months. Eventually the chamber got sealed and a closed environment could be established.

With a sealed chamber it was now possible to connect an inlet and an outlet. The first tests were done by properly connecting the inlet gas, N_2, which could be controlled by two pedals - a "gas" and "break". The gas pumped in the N_2, while the break stopped the inlet, and could slightly decrease the pressure inside the chamber. A steady inlet could also be established without relying on stepping on the gas pedal.

Having successfully sealed the chamber, and being able to have a constant air flow, the next addition to the Mars chamber was an electronic hygrometer. The hygrometer was added to accurately measure the relative humidity within the Mars chamber, which allowed us to perform relative humidity (RH) tests.

The Mars chamber can be divided into two parts, the glove box, which is the big box surrounding the actual Mars chamber, which is the second part. The glovebox is limited to RH and atmospheric testing, where as the Mars chamber can look at factors like pressure, temperature, UV radiation, and can measure atmospheric composition with a connected mass spectrometer (the latter three are however not operational yet).

3.12 Relative Humidity and anaerobic Experiment

For this experiment we had 6 different overnight cultures. Prepared an 0.4 OD_{600} for each culture before plating. We prepared 4x6 isolates – 6 starved isolates, and 6 isolates with media were placed inside the glovebox (17% RH). The same setup was placed outside the glovebox as a control (~43% RH). The starved cells were prepared by vortexing 1ml of liquid media cultures for 3 min at 8000 rpm, the supernatant was replaced with PBS. The first 12 isolates were incubated in the glove box, at room temperature, and the control was incubated outside the glove box which had a RH of 43%. Before incubation, the chamber was flushed with N_2 to empty the chamber atmosphere and to lower RH. This was a tedious process, as only a limited amount of N_2 could flow into the chamber while also having a limited passive outlet. After 3-4 days of a steady N_2 flow through, the RH had dropped to 17%. 3 g of triple autoclaved sand from Amager strand was added to 3 cm petri dishes and 100 µL of culture was added in the middle of the sand.

The cultures were incubated for 2 days at 1 bar atmosphere. After incubation the samples were slowly rehydrated with PBS media (see figure 10), as to minimize potential osmotic bursting of the cells.

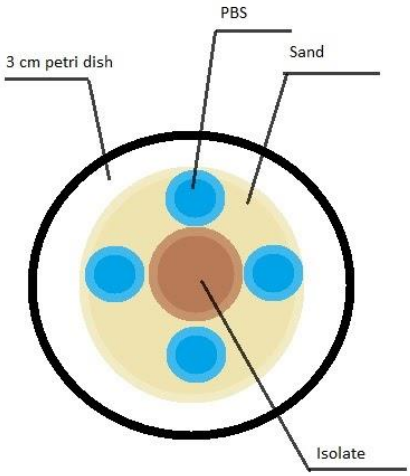


Figure 10: Illustrated here is the extraction method of the relative humidity experiment

The rehydrated cultures were then moved to falcon tubes and submerged in 10 ml PBS. Incubated for 30 mins, vortexed vigorously, incubated for an additional 30 mins, wortexed, and centrifuged for 30 s at 8k rpm. 1 ml from each culture, was added to an eppendorf tube and diluted 4 times, 10^{-4}, and each dilution, including the non-diluted sample, were plated on TSB agar plates. The

CFU could not be counted for any of the dilution steps, as there were too many to count. The results are not included but gave us an idea for what the isolates could tolerate for future experiments.

3.13 1% pressure, 17% RH, anaerobic (N_2), Martian soil analog experiment

For this experiment, we had received some Martian analogue soil (see appendix for information of the soil composition) to replace the sand from Amager Strand. We recieved 3 different types of soil, each simulating different soil compositions on Mars: Mars Global Stimulant simulating Gale Crater (G) and Gale crater with additional sulfur (GS), and the Jezero Crater(J). 100g of each soil was mixed with 1g of perchlorate to obtain a 1 wt% of perchlorate. The soils were not sterile, so they were autoclaved once as to keep the soil as close as possible to the condition in which they were received, contra 3 autoclaves as for the Amager Strand sand.

Figure 11: The silica gel used to lower humidity.

By this point we had found a faster and more efficient way to reduce the RH. This was done by filling petri dishes with silica gel (see picture 11), which had an indicator (blue - dry, and orange/pink when saturated). The amount of silica gel needed was reached by trial and error, gradually adding more silica gel in additional petri dishes, to reach the wanted RH. In addition, the vacuum pump had been connected and was fully functional. The 1% atmospheric pressure experiment thus includes several environmental factors found on Mars: 1% atmospheric pressure, a RH at 17%, Mars analogues soil, a 1% perchlorate concentration, and an anaerobic atmosphere flushed with N_2.

A fresh stock of overnight cultures was made 2 days before and diluted to an OD_{600} of 0.3. 1 day before the experiment the mars chamber was flushed with N_2 and had silica gel added, to more quickly reduce the RH. On the day of the experiment, soil was added to the wells in the microtiter plates (see picture). The soil was not weighed or evenly distributed, which later showed to be a problem. Similar to the RH experiment, we used fed and starved cells, inside the Mars chamber, and a control - outside the glove box. For each of the 3 soil types 1 wt% perchlorate was added (GP, GSP, JP). The isolates added to the 1% perchlorate well, were fed. 100 µL of the same isolate was added to G, GS, J, GP, GSP, and JP. Isolates with and without food labeled XF and XNF, food and no food, respectively. The microtiter plates were inserted in the Mars chamber, sealed of, and the pressure was reduced gradually to avoid rapid expansion of the cell causing potential bursting. The pressure was decreased with 1 hour intervals. The pressure sensor only reads changes in pressure below 0.47 bar, so until 0,47 bar was reached, a slow and steady approach was employed. Eventually the chamber seemed empty, but no change in the pressure could be seen. Suspecting a software glitch, the software was restarted, and rightly so, the pressure was now being read. The pressure showed to be 0,02 bar so we reduced it down to 0,01 bar for the last step.

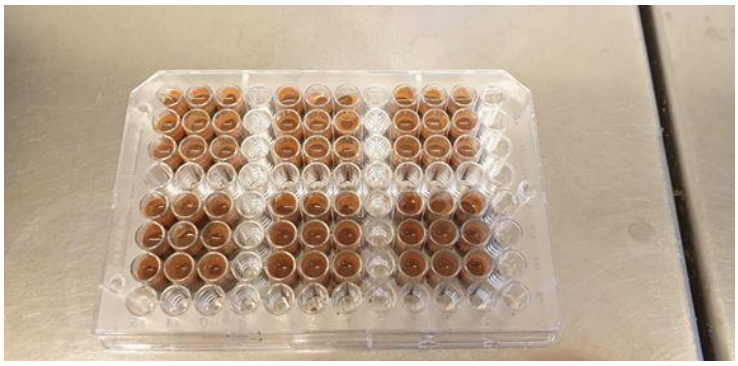


Figure 12: Microtiter plates with Mars soil analog

The reason for the sensor not registering is unknown, but for later experiments and general testing of the mars chamber, the pressure sensor has worked flawlessly. The day after the isolates had been inserted into the mars chamber, the COVID-19 lockdown was effectuated, and the isolates were left for themselves without any care for more than a month. Unattended for more than a month, I was given special permission, even though the lockdown was still in effect, to go the lab and extract the bacteria. I was supposed to limit my time at the lab as much as possible, and since there was a shortage of agar plates for CFU counting, the results were binary. The results also showed a great deal of inconsistencies, owing the size of the experiment, 14 different isolates, which meant having all the isolates close together in the microtiter plates so they could fit inside the chamber, not having a standard amount of soil in each well, made some wells who had too much soil difficult to work with, no gap (empty well) between sample wells (see figure 12). In

addition, trying to be efficient, we did several experiments at once: UV, UV and perchlorate tolerance, perchlorate tolerance, anaerobic, and of course the 1% pressure, 17% RH, and anaerobic (N2), Martian soil analog experiment. The amount of isolates and number of different experiments done at the same time, proved to be way too much, and most of the results are binary or inconclusive, and not reported in the results section.

For the UV, UV and perchlorate, and perchlorate tolerance experiments, I was given permission to take the plates home, so they could be counted. This was however a fruitless endeavor and the results are excluded.

Mistakes were made, but they were noted and improved for the next experiment.

3.14 1% pressure, 17% RH, atmospheric composition of CO_2, and Martian analog soil.

Taken into account what we had learned from the previous experiment, we limited the number of isolates to 6, we weighed the soil (~0,17g) that went into each well of the microtiter, added a gap (empty well) between each well containing soil and an isolate, had only 2 isolates (previously we had 6) per microtiter plate, and at cross-opposite corners, and we focused on the ***one*** experiment

For the final Mars experiment, we had ordered a 40L flask of CO_2 to further mimic the conditions of Mars. In addition, we also found a way to lower the RH even faster. This was done by turning on the vacuum pump connected to the airlock, while pumping in air from the recently connected CO_2 flask. The CO_2 provided had a purity of 99.9% which was more than enough to simulate the Martian atmosphere. Containing very little humidity, the RH within the chamber dropped from ~50% to 17% in less than 30 minutes. To further decrease the RH, silica gel was added, which lowered the RH down to 12. The point of the silica was at this point, to slightly lower the RH and to maintain a low RH, as a general leak, and the grease used for sealing joints could let of some humidity. Having a stable RH at 12%, the cultures were added to the chamber one day before reducing the pressure. This was to give the isolates a chance to acclimatize to the new dry environment. After approximately 18 hours, the pressure was decreased in steps. As the pressure sensor only measures pressure below 0.470 bar, the first steps were done in blind, and relying on hearing the atmosphere being sucked out of the chamber. In 45 minutes intervals pressure was let

out of the chamber. It is to be noted that after the second decrease in pressure, the pressure dropped from an unknown pressure, to 0.219 bar. This was a big step compared to what was planned. The reason for this big leap in pressure, was due to difficulties hearing the pressure decreasing. The final pressure was set to 0.00884 bar before leaving to compensate for the away time. The pressure was maintained every 24 hours, to keep the pressure at ~0,001 bar, as there was a steady incline in pressure (see list of temperature, RH, and pressure over the course of the experiment in appendix). The July 12th was a weekend, and therefore, data points could not be gathered. 8 days into the experiment the computer, which the pressure gauge was connected to, crashed. Doing some troubleshooting did not succeed in getting life back into the relic from the past. Attempting to plug in the pressure gauge to my own laptop, I was greeted with a big error message. Installing and updating drivers did not help fix the issue, so the pressure after day 8, is unknown, and was not tampered with. This meant that there was no compensation for the leak.

After 12 days of incubation all the isolates were suspended in 1x PBS and mixed/stirred while still in the microtiter plate, incubated for 30 min at room temperature, diluted to a factor of 10^{-9}, and plated on agar plates. The plates were stored at room temperature and counted daily.

4 Results

This section will mainly focus on results where isolates 5, 6, 9, 10, 201, and 202-1 had been tested, the "Primary Isolates". The "Primary Isolates" were chosen based on observations made during various experiments. The reason for focusing only on 6 cultures, rather than the total of 28 isolates was to limit experiments to a comprehensible size. Time at the lab started with a lack of experience, which meant that a structural approach also was lacking. As a consequence, some of the results are clumsy and not fit for the general results and discussion part, and will not be a part of this section.

4.1 Isolates grown from the Atacama Desert, Chile, and McMurdo valleys, Antarctica

The soil samples provided by Anders showed growth on EMH with 2% NaCl roughly 3-4 weeks after incubation. Each isolate was restreaked twice to obtain pure cultures. A total of 10 isolates were obtained. The isolates were numbered 3-12, which is also is the name of each isolate for further mentioning of the thesis. The Isolates tested for growth on TSB media all showed growth except Isolate 7, 8, and 12 and are therefore not part of the "Primary isolates".

Soil samples at a depth of 200 cm from the Yungay area were inoculated with “High salt PBS”. The soil samples were plated on agar plates containing TSB, 12,5% NaCl, 0,6% and 1% perchlorate. 9 isolates were obtained numbered 201-209 with isolate 201, 204, 205 obtained from 0,6% perchlorate, 202 and 203 from TSB, 206 from 12,5% NaCl, and 207 – 208 were isolated from 1% perchlorate. Restreaking of isolates was done, showing 202 to consisting of 2 isolates, 202-1 and 202-2, based on visual observations. Each isolate was transferred to TSB media and all were compatible.

Isolates from University Valley, Victoria Valley and Taylor (UV, VV, and TV) were also isolated, using regular PBS and “High Salt PBS”. A total of 9 isolates were obtained, however these showed slow, and very limited growth at 4^{o}C and 23^{o}C, so there was no further testing with these isolates.

Table 3: Listed are all the isolated bacteria, which inoculum was used, and which media they were isolated on.

Isolate	Inoculum	EMH 2%	TSB	12%NaCl	0,6% Perchlorate	1% Perchlorate
3	1x PBS	+	+			
4	1x PBS	+	+			
5	1x PBS	+	+			
6	1x PBS	+	+			
7	1x PBS	+				
8	1x PBS	+				
9	1x PBS	+	+			
10	1x PBS	+	+			
11	1x PBS	+	+			
12	1x PBS	+				
201	“High salt PBS”		+		+	
202-1	“High salt PBS”		+			
202-2	“High salt PBS”		+			
203	“High salt PBS”	+	+			
204	“High salt PBS”		+		+	
205	“High salt PBS”		+		+	
206	“High salt PBS”		+	+		
207	“High salt PBS”		+			+

208	"High salt PBS"		+			+
TV 301	"High salt PBS"				+	
TV 302	"High salt PBS"				+	
UV 303	"High salt PBS"				+	
VV 304	1x PBS		+			
VV 305	1x PBS		+			
TV 306	1x PBS		+			
TV 307	1x PBS		+			
TV 308	1x PBS		+			
TV 309	"High salt PBS"	+				

4.2 16s sequencing shows a primary presence of *Bacillus*

Sequencing of the isolates show most of the isolates are *Bacillus. Pseudomonas songnenensis* and *Staphylococcus capitis* were also isolated, but only one of each isolate. Due to mistakes made in the lab, slow growing bacteria, the lockdown due COVID-19, and limiting the size of the experiment, only 9 of the 28 isolated cultures were sequenced.

Table 4: Listed are the isolates that were sequenced by sanger sequencing. Shown are the genus and species, similarity and accession number

Isolate	Genus and species	Similarity %	Accession number
5	*Bacillus heynesii/paralicheniformis*	97% to both species	NR_157609.1 and NR_137421.1
6	*Bacillus sp. Strain JDMASC59*	97%	
7	*Bacillus albus FA26*	97.83%	
8	*Bacillus halosaccharovorans*	92.83%	
9	*Pseudomonas songnenensis*	97.02%	NR_148295.1
10	*Bacillus halotolerans*	96.1%	
11	*Bacillus vietnamensis*	95.2%	
201	*Staphylococcus capitis*	97.29%	NR_036775.1

202-1	*Bacillus mojavensis*	97.34%	NR_024693.1

4.3 Isolate 5, 201, and 202-1 show growth under anaerobic conditions

Under anaerobic conditions only 3 isolates (5, 201, and 202-1) show the ability to grow. They grew in the presence of 1% perchlorate and regular TSB media. When taken out of the anaerobic cylinder, all isolates showed growth. The results were binary showing growth or no growth.

4.4 Screening for p*crA* shows 2 potential candidates for perchlorate reduction

14 isolates including three negative controls, water, *E. coli* (E. c) and *P. halocryophilus* (P. h) were screened for presence of the PcrA gene. Out of the 14 isolates, four showed a presence in the vicinity of the ~300bp long PCR band, P. h, 10, 201 and 202-1. The closest of the two to the 300bp was isolate 201. The 202-1 band is slightly lower than the 300bp mark, however both bands of 201 and 202-1 were close enough to be of interest. Therefore, they were both included in the "Primary isolates" group, for further testing. Neither the water control nor *E. coli* show any band in this area, however *P. halocryophilus* shows a band at ~350 bp. The PCR result does show different bands, smeared or clear, for the different isolates, however these can be overlooked, as they do not align with the 300 bp ladder size.

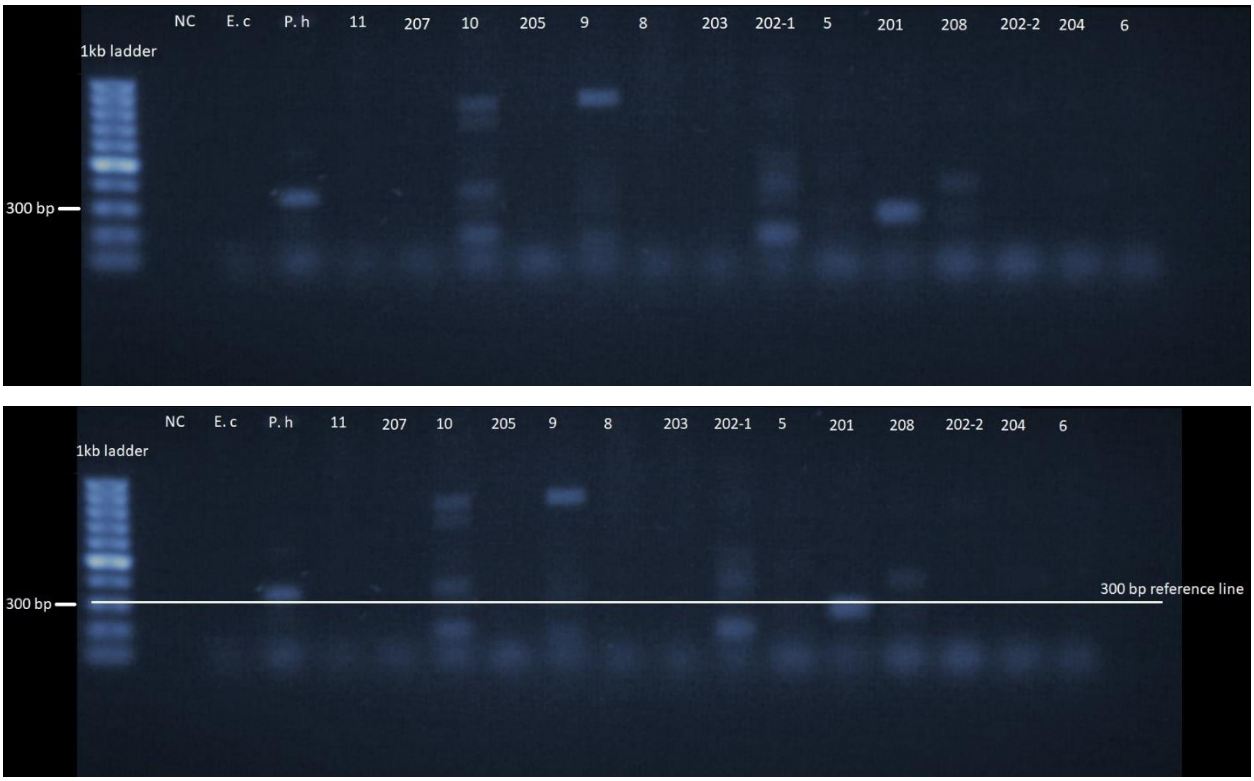


Figure 13: PCR results of the PcrA screening. The bottom figure has a 300bp reference line added

4.5 Isolates show tolerance to a 3% perchlorate concentration

Different concentrations of perchlorate on ***solid*** TSB media, with 0.6%, 1%, 2%, and 3% did not kill the bacteria, and growth of all isolates were present. However isolate 10 showed a higher CFU compared to the rest on 3% perchlorate and became part of the "Primary isolates" (CFU data not shown due to CFU inconsistencies).

4.6 Growth Curves and Growth Rate

Testing the "Primary isolates" in liquid media with 1%, 2%, 3% perchlorate, and 1% NaCl show a varying degree of tolerance for all isolates.

For this experiment, isolate 5 showed clustered growth in the liquid media, which obscured the OD_{600} values. For this reason, isolate 5 has been discarded for the results shown below. For all isolates, including isolate 5, the growth curves can be seen in appendices.

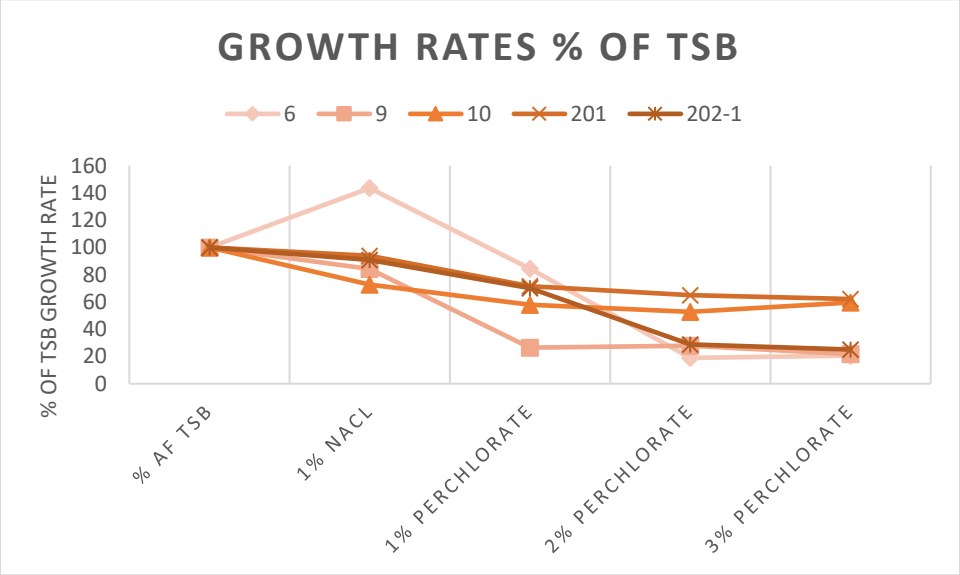


Figure 14 Values in terms of percentage are visualized as symbols with connecting lines. It is important to note that the lines are merely an aid to follow each isolate, and are not values between the points

Growth Rates	Isolate 6	Isolate 9	Isolate 10	Isolate 201	Isolate 202-1
TSB	0,195	0,336	0,235	0,193	0,319
1% NaCl	0,28	0,283	0,171	0,181	0,29
1% Perchlorate	0,165	0,089	0,136	0,138	0,224
2% Perchlorate	0,037	0,094	0,124	0,125	0,092
3% Perchlorate	0,04	0,073	0,14	0,12	0,08

Table 5: Listed are the growth rates of each isolate, excluding isolate 5, corresponding to each media tested

The values in figure 14 "Growth Rate % of TSB" are calculated by setting the growth rate on TSB for each isolate to represent 100% and then the change in percentage for the growth rate for each different media.

Example:

*Growth rate % - (Growth rate media X/Growth rate TSB)*100 Isolate 6: (0,28/0,195)*100 = 143%*

Figure 14 shows the starting point of 100%, which are the growth curves in TSB media. There is a slight growth rate drop for each isolate, except isolate 6, at 1% NaCl. Isolate 6 is shown to have a higher growth rate compared to TSB. For the 1% perchlorate media all isolates drop below 100% growth rate with isolate 9 dropping the farthest. The same implies for 2% perchlorate, where growth rates drop even further. The growth rates of 2% perchlorate and 3% perchlorate are very similar for each isolate. It is to be noted that the all the growth curves, for each isolate, grow towards and OD_{600} 0.6, but at different rates.

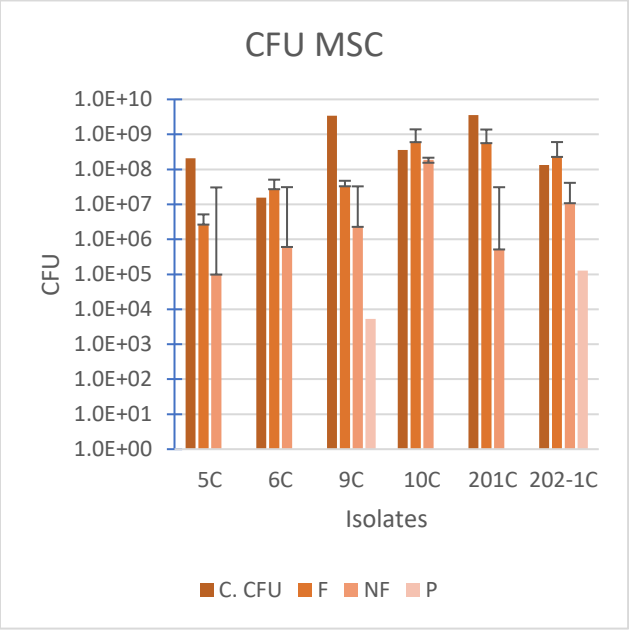
4.7 UV radiation higher than Martian levels show no effect on CFU.

The isolates, including a *E. coli* (as a UV sensitive control), were subjected to a UV intensity (20.7 W/m_2) greater than the levels seen on Mars (10-20 W/m_2) , for a duration of 1.5 min, as minimum, and 4.5 min as maximum. 4 of the isolates, including the control did not show any decrease in CFU compared to no exposure. Isolate 5 shows a decrease of CFU at 3.5 minutes and 201 show a decrease of CFU after 3.5 minutes and 4.5 minutes. (For figures see appendix 4 – Growth curves and UV CFU).

4.8 4°C incubation with and without perchlorate

This experiment looked at CFU change over time in a 4°C environment. The experiment proved not to provide any data of significance and could not be compared to anything and is therefore not part of the discussion. The only thing which can be learned from this experiment, is that the isolates 5, 6, 9, 10, 201, and 202-1 can grow at 4°C. (for figure, see appendix 6 – 4°C incubation with 1% perchlorate)

Mars experiment



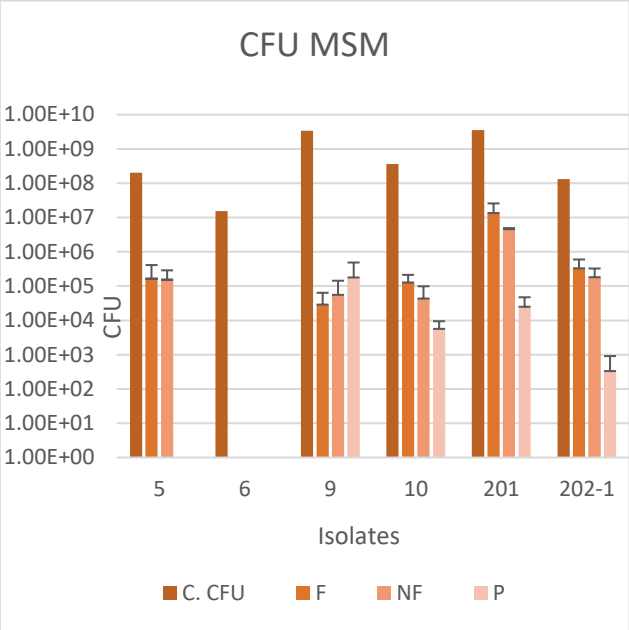


Figure 15: The left figure shows the results for the isolates outside the Mars chamber. The right figure show the results from inside the Mars chamber

The results will be described with having the three different soil samples merged to one soil sample. For a more detailed result of the CFU for the different soils, see appendix.

The first section which will describe the isolates grown placed outside the chamber, as a control – Martian Soil Control (MSC). Section 2 looks at the isolates incubated on Martian Soil inside the Mars chamber (MSM)

Looking at the CFU from isolate 6, 10, and 202-1 from outside the Mars chamber they have a higher CFU count compared to the control CFU (C. CFU) in the samples containing food (F). All the isolates show fewer CFU with no food (NF). Looking at the error bars, isolate 5, 6, and 9, show greater or equal growth to the food containing CFU. For the perchlorate containing soils (P) only isolate 9 and 202-1 show growth.

For the isolates inside the chamber, the error bars are smaller for each isolate, with and without food. All isolates have a lower CFU count compared to the C CFU and for isolates outside the Mars chamber. Isolate 6 shows no growth at all. Isolate 9, 10, 201, and 202-1 show growth on perchlorate.

5 Discussion

5.1 Isolation of bacteria from soil samples

For isolating a variety of bacteria from each soil sample, different media was used to get different bacteria. The bacteria grown on various media presented potential environmental similarities i.e. 2.5% NaCl or 0.6% perchlorate, which served to partially mimic the environment of the soil sample. The perchlorate media was not aimed at finding perchlorate reducing bacteria as all plates were, following plating, incubated in the presence of oxygen. It was to mimic the habitat of the bacteria, and showing tolerance towards perchlorate, as an early indicator. Selecting for perchlorate reducing bacteria at this stage, incubations in an anoxic environment would be required. The media used for growth should include known electron donors coupled to perchlorate reduction, where in this case, the media presented a broad range of metabolites to target a wide range of bacteria hoping to get more isolates.

Using the "High Salt PBS" showed growth where previous attempts had failed (The thesis of a former master student). This indicates that the bacteria can be very sensitive to it's environment, and a sudden change could kill the bacteria. In this case, mimicking the concentrations, and the different salts present at the natural environment allowed for 9 isolates compared to 0 on 1x PBS inoculum. It shows the importance of media for isolating bacteria at various environments, as "High Salt PBS" might not be as effective in a low salt environment i.e. freshwater environment.

5.2 16s sequencing of bacteria

Out of the 9 sequenced isolates, 8 were in the order of the *Bacillales*. 7 of the 8 were of *Bacillus* genus, with 1 being a *Staphylococcus* sp. (see table 4) These are all member of the firmicutes phylum and all gram-positive bacteria. Bacteria from the genus bacillus are known to survive harsh conditions and the Atacama desert is no exception (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000; D. Schulze-Makuch et al., 2018). It is therefore no surprise that the majority of the isolates are of the *Bacillales* order.

5.3 PcrA PCR

When searching for perchlorate reducing bacteria the search for the *pcrA* gene is apparent. Being the main protein for every known perchlorate reducing bacteria, the presence of *pcrA* would be a strong indicator as to the ability for perchlorate reduction. The electrophoresis band from a PCR

product does not prove the presence of *pcrA*, but the isolate in mention, isolate 201 also fares well in the perchlorate tolerance and growth curve experiment indicating that the PCR product could in fact be the *pcrA* gene.

In terms of perchlorate reduction, the ability is seen almost exclusively only in members of the *Proteobacteria* phylum (Youngblut et al., 2016) and is yet to be found in the *Bacillales* order. Isolate 201, *S. capitis* does however show promising results in terms of perchlorate reduction. The results do not show perchlorate reduction as such, but indications of the presence of a PCR band with a length of ~300 bp (see figure 13), corresponding to the length of *pcrA*, coupled with a high tolerance in the presence of 3% perchlorate, indicates that *S. capitis* could couple perchlorate reduction to an electron donor. Looking at the nucleotide sequence of *pcrA* from *Dechloromonas agitata* and searching for protein homologs in *S. capitis* it did not show hits for *pcrA*. Even though perchlorate reduction is almost exclusively seen in the gram negative *Proteobacteria*, an article from 2008, shows a gram positive bacteria isolated from an underground gas storage (Balk et al., 2008). The bacteria in question is *Moorella perchloratireducens,* which like *Bacillales,* is part of the *Firmucutes* phylum. These results show that perchlorate reduction is not limited to *Proteobacteria* but might be more common in the bacterial kingdom. Having this in mind, it could very well be that *S. capitis* employs other unknown proteins for perchlorate reduction. Looking at where *S. capitis* is normally found, literature suggests that it is a common skin microbe (Iwase, Seki, Shinji, Mizunoe, & Masuda, 2007; Kloos & Schleifer, 1975; Lynch et al., 2019). It indicates that it likely is a contamination which is to blame, however with a sequence identity 97.29% (see table 4) it is on the limit of accepted threshold for 16s sequence similarity (STACKEBRANDT & GOEBEL, 1994). Looking only at the 16s RNA sequence, you don't take into account foreign genetic elements which might have been passed on through horizontal gene transfer, which evidence suggests, is how perchlorate reduction can be spread to neighboring cells (Melnyk & Coates, 2015). If it is not a skin contamination, and based on 16s sequencing alone, the *pcrA* gene could have been horizontally transferred.A more definitive answer can be obtained by sequencing the pcr product, and searching for similarity in *pcrA* genes, or protein homologs in the NCBI database. If a match in the sequence was found, it could be interesting to do a full genome sequence, and discover the additional genes often attached to perchlorate reduction. It could also be interesting to see if the potential perchlorate reducing genes, were surrounded by inverted repeats, as shown by the literature to be a common thing (Melnyk et al., 2011). Being surrounded

by inverted repeats, would further support the literature, of the presence of *pcrA*, which appears to be a signature of the perchlorate reducing gene package. Sequencing of the product was however not done due to shortage of time.

The PCR results showed a couple of smeared bands, but only 3 showed a presence of bands near the 300bp mark with 201 showing to be closest. *P. halcryophilus* was a negative control but showed a band close to the 300 bp mark, but *P. halocryophilus* does not carry the *pcrA* gene, indicating that the bands are a product of a complementary primer stretch of DNA flanking a similar size piece of DNA.

5.4 UV Experiment

Isolate 5 showed lower number of CFUs after 4.5 min of exposure to UV radiation. However, if this is due UV exposure, or a plating inconsistency, is hard to tell, as similar inconsistencies can be seen for *E. coli*, isolate 9, and 202-1 (see figures in Appendix 4 – Growth curves and UV CFU). To see if this is an effect of the UV radiation or just a plating inconsistency, it would be advisable to make triplicates. In general, however, there does not seem to be any difference in CFU count for the different exposures for any of the isolates. Therefore, increasing the duration of UV exposure could be a good first step. If CFU is affected by the new exposure times, it could be followed up by making triplicates, to verify and strengthen the result.

There are several ways for bacteria to deal with UV radiation i.e. repair mechanisms, DNA copy number, pigmentation, and spore formation (Dieser, Greenwood, & Foreman, 2010; Nicholson et al., 2010; Timmins & Moe, 2016). A more thorough investigation, making use of real-time PCR and/or western blot, for each isolate in regard to genome copy number, and active repair proteins being transcribed when induced to UV radiation, could elaborate on the results and provide a more detailed discussion. These experiments were however not done, due to time constraints. It is however worth noting that pigment containing bacteria are shown to have a protective effect against UV radiation with levels of 19 W/m_2 UV-A (380-320nm)(Dieser et al., 2010) which are similar to the exposure employed in our experiments (20.7 W/m_2 – 254-366nm). To see whether the tested isolates had any pigmentation, or any absorption spectra, using a fluorescence spectral viewer could be an option. As this was not available, we had to rely on visual observations. None of the tested isolates showed any form of pigmentation, and were all pale or milky white in color. As mentioned, 8 of the 9 isolates can form spores, which protects the bacteria when circumstances

are unfavorable, including radiation (Moeller, Horneck, Facius, & Stackebrandt, 2005; Nicholson et al., 2000). Endospore formation is believed to be part of a bet-hedging strategy and as such, not a reactive trait towards environmental stress (Tan & Ramamurthi, 2014). That being the case, it is highly improbable that all of the CFU were regrown endospores. Exposing the isolates to UV radiation no later than an hour after plating, rules out the formation of biofilm as a protective barrier, which otherwise is shown to protect against UV radiation (Elasri & Miller, 1999).

Ruling out the above protectants against UV radiation, it indicates that UV intensities slightly higher than those found on Mars, are not harmful in a duration of 4.5 minutes for the tested isolates, including a supposedly UV sensitive bacteria like *E. coli.*

5.5 Martian experiment

The 3 different soil samples (GS, G, and J) were treated as one sample, MGS. The soils containing perchlorate were also grouped to show results as a single soil sample + perchlorate (GSP, GP, and JP), MGP. In terms of samples with food or no food, samples will be referred to MGSF or MGSNF. This was done to get triplicates of the experiment. If the different soil samples had any significant effect on each isolate, the effect will be ignored in this discussion.

With the Mars chamber experiment, we introduced 5 environmental factors known to Mars, RH 17%, atmosphere (CO_2, 99,99%), atmospheric pressure (0,01) bar, and Martian analog soil with and without 1 wt% perchlorate. The 5^{th} factor is an indirect factor for the low RH and low atmospheric pressure, desiccation, which also will be discussed further. As time was a constraining factor, all the factors were included in the same experiment, to see if the bacteria could survive. Previous experiments did account for separate factors like RH and anaerobic conditions but as these experiments were done as a means of testing early functionality of the chamber, as the main focus, the results were binary as they showed growth or no growth, and will not be discussed. If time was not an issue, experiments could include each factor separately. This could provide us with data showing the effect each factor had on the isolates. Comparisons could thus have been made and a more thorough analysis could be done. Another idea could be to place the MSC isolates inside the glove compartment, instead of outside. The MSC isolates, would then be subjected to a CO_2 atmosphere and a RH of 17%. This could help draw conclusions on the effect of low atmospheric pressure. It would also be interesting to include a bacteria i.e. *E. coli* that does not come from a Martian analog environment to see how well it fares compared to the tested isolates.

However, for the tested isolates, evidence points towards isolate 201 as a potential contamination, which interestingly is the one isolate that appears to be best suited for the presence of perchlorate amongst the other isolates tested here.

If we look at the soil samples containing perchlorate, the results are quite surprising (see appendix 5 – Mars experiment). For the isolates outside the mars chamber, only 2 out of 6 isolates showed subsequent growth on agar plates, and in only 2 out of 18 samples while inside the chamber 7 out 18 wells revealed subsequent growth. Comparing this to the perchlorate growth curve and growth rate experiment, we demonstrated that the concentration of perchlorate did have a negative effect on growth rates, but all isolates still showed growth even at 3% perchlorate, compared to 1% tested for in this experiment. It could appear that there has been made a mistake with an erroneous transferring of isolates to the perchlorate containing wells, but as the isolates all were allocated from the same falcon tube to all 18 samples per isolate, a personal error seems unlikely. Finding a reasonable explanation for the lack of growth on perchlorate containing soil outside the chamber compared to the slight increase of growth on perchlorate containing soil inside the chamber, did not give any results. Further discussion on this part of the results will, however, be very interesting if a common consensus for a valid reason behind these results should be reached.

Looking at survivability between MSC and MSM isolates, it clearly shows that the isolates have been stressed and survivability is decreased by a factor of up to 10^{-5}. Focusing on the difference in RH and atmospheric pressure between the MSC and MSM isolates it can be suggested that evaporation rate is most likely higher for the MSM isolates. As the pressure was decreased gradually, it would however slow down the evaporation rate. The rate of evaporation was not calculated, therefore it can only be suggested that the MSM isolates experienced a higher evaporation rate. This would imply that desiccation would affect the MSM isolates much sooner than for the MSC isolates.

In terms of desiccation, some of the nutrients in the food containing samples might also evaporate and/or become trapped as the media is mixed with the soil. In a dry environment, mobile bacteria will have a harder time swarming or swimming to places with trapped nutrients, potentially inducing starvation of the cell (Dechesne, Wang, Gülez, Or, & Smets, 2010). When presented with starvation, a bacterial cell will focus on its essential cell functions, which would prevent the cell growth and proliferation (Watson, Clements, & Foster, 1998). At a state of starvation, cells might

survive with the means of cryptic growth. Testing if the isolates in our experiment consorted to cryptic growth could be tested, by exposing a lower cell count to starvation, as a lower cell count would leave fewer lysed cells available for repurposing.

As mentioned in the introduction, perchlorate can absorb and trap moisture from the atmosphere. This could also affect the evaporation rate, by trapping some humidity in the soil, thus keeping a higher level of humidity than without perchlorate potentially easing the desiccation stress factor. An indication of this could potentially be seen for isolate 9 which showed higher a CFU count on soils containing perchlorates inside the chamber, than soils without. When grown on solid media, isolate 10 formed a thick liquid medium which quickly merged with neighboring colonies when left to grow (visual observations). This self-produced extracellular polysaccharide could be important in the early stages of exposure to low pressure and low RH. As the components of the thick liquid, is postulated to be mostly water and sugars, and therefore would require sugars and water to be produced, isolate 10 would need to grow quickly in order to produce the potential extracellular polysaccharides as water would quickly become unavailable. The extracellular polysaccharide could aid the isolate in reaching trapped nutrients, which might explain why isolate 10 fares well in all the soil samples for the MSM test, as it is the only isolate growing in 8 of 9 wells. Being suspended in a thick liquid medium, could also help influence its own environment, by holding on the water and keeping it close. A similar process, however, not including secretion of extracellular polysaccharide, has been seen by some bacteria growing on leaves, experiencing a constant desiccation, and rewetting during a diurnal cycle. Here bacteria who can hold on to water showed an increased rate of survival (Grinberg, Orevi, Steinberg, & Kashtan, 2019). Inside the Mars chamber, isolate 10 is also the only isolate which grows on all perchlorate containing soils (see figure 15).

Isolate 6 is the only isolate which completely succumbs to the factors present in the Mars chamber. Showing growth outside the chamber indicates that the soil is not the lethal factor. It could also be seen that for isolate 6, grown in MGSF that there was a higher CFU ($4{,}4*10^7$) compared to the control CFU ($1{,}5*10^7$), most likely due to the extra food. In regard to a higher CFU from the MSC samples compared to control CFU, we see the same pattern for isolate 10 and isolate 202-1 in MSC. Growing outside the chamber clearly suggests that the bactericidal factors are a result of the

factors inside the chamber. Finding out if it is desiccation, the low pressure, or potential toxic levels of CO_2, requires further testing.

For the MSM isolates the oxygen had been replaced with CO_2. CO_2 is known as food spoilage preventative (Dixon & Kell, 1989), which in part is due to replacing the O_2 in food packaging with CO_2, creating an anaerobic environment, but CO_2 can also have physical effects on bacteria. One of these effects is energy dissipation, where CO_2 freely permeates the cell membrane and reacts with water to form bicarbonate liberating protons. To avoid cytosol acidification, protons will then be actively exported causing a energy depletion (Dixon & Kell, 1989). However, in combination with low pressure, the partial pressure of CO_2 might lessen the tendency for CO_2 to diffuse through the membrane, avoiding energy depletion. For all the isolates excluding isolate 6, the low pressure, and high CO_2 concentration appears not to be bactericidal. However a study showed that incubation in 0.007 bar with a high CO_2 atmosphere, prevented the cell from recovering when reintroduced into a standard Earth pressure and atmosphere (Schwendner & Schuerger, 2018) indicating the death of isolate 6. Isolate 6 was not able to grow anaerobically at 1 bar under an CO_2 concentration of ~20%, it did however regrow, when reintroduced to a normal atmosphere. The cause for the isolate succumbing to the Mars chamber, could thus be, the low pressure combined with a high concentration of CO_2, or desiccation. Testing the factors separately could provide the results needed to answer the effect of the individual factors.

In terms of anaerobic growth, isolate 5 showed, from a previous experiment, that it was able to grow anaerobically in an atmosphere consisting of ~79% N_2 and ~20% CO_2. Looking at the results of isolate 5 from the MSM isolates, it showed that the difference between having food or no food did not affect isolate 5 in any manner. It would be expected that the food containing sample would show a higher CFU count than without. The reason might be, as mentioned before, that the onset of desiccation happened faster than for the MSC samples and could cause nutrients to co-evaporate or become inaccessible. If isolate 5 is autotrophic, the presence or absence of food would not affect the isolate in any particular manner, as the atmospheric CO_2 within in the chamber, could be utilized as carbon source. The fact that the MSM CFU count for isolate 5 in wells containing food and no food are similar, and if autotrophic, not exceeding the control CFU, could potentially be limited by the low pressure reducing the access to CO_2 for growth (Ueda et al., 2008). Looking at potential electron donors and acceptors for growth under MSM circumstances, the most likely

candidates are ferrous iron present in the Mars analogous soil as Fe-Carbonate and FeO_T (see appendix – Mars Global Simulant). Other potential electron donors on Mars include CO and H_2, none of which are present in this experiment. In terms of available electron acceptors on Mars, 4 options are present, sulfate, CO_2, Manganese, and perchlorate (see table 2). For this experiment, sulfate and perchlorate can be ruled out, as the sulfur present in the Martian soil analog is in the form of sulfur trioxide. Perchlorate can be ruled out, as no growth was seen in the perchlorate containing wells. Two possible electron pathways could therefore be present: Iron coupled manganese reduction, or iron coupled CO_2 reduction. To confirm CO_2 as a carbon source, experiments with isotopic CO_2 could be done, to see potential isotopic CO_2 assimilation. Figuring out what electron pair is utilized, removing one or the other could show which of the potential elements is the oxidizing element.

Like isolate 5, isolate 201 was also shown to grow in anaerobic conditions, confirmed *S. capitis* by the literature (Kloos & Schleifer, 1975). Looking at the at comparisons between MSM isolate 201 CFUs and C. CFUs, results showed that isolate 201 was the least affected (by a factor of 100) by the conditions in the Mars chamber, compared to the other isolates.

In this thesis we look at some of the environmental factors on Mars and see how bacteria fare under these circumstances. The tested parameters do however not include all of the factors present on Mars. Factors not included are radioactive radiation and temperatures below 0^oC. Of the two mentioned factors, adaptations to a cold environment requires major cellular adaptions, i.e. DNA, proteins, and the cell wall (De Maayer et al., 2014). In terms of radioactive radiation, it too can be decremental to cells, but does not appear to be as challenging as the cold environment. A study by directed evolution showed that *E. coli* could survive a radiation exposure of 3000 Gy (3000 sievert) (Byrne et al., 2014). It was shown that changes in the previous present DNA repair mechanisms were malleable and could be changed so that cell could survive high radiation dose.

6 Conclusion

Cultivations of isolates of soil samples from the Atacama Desert, Chile, and the McMurdo Valleys, Antarctica, showed several potential isolates for testing (see table 3). They were cultured on various media, including media containing perchlorate to increase the chances of finding different isolates (see table 3). A total of 28 isolates were obtained, 9 of those were from the McMurdo Valleys. Due to slow growth, the isolates from the McMurdo Valley were not included for further

experimentation. For the remaining 19 isolates obtained from the Atacama Desert, 6 were chosen for further testing. They were chosen on different criteria based on results of different experiments. Some of the criteria include, anaerobic growth, high growth rates, and presence of potential perchlorate reductase. The chosen bacteria were sequenced and showed mostly gram-negative bacteria (5) in the *Bacillales* order, and 1 gram positive *Pseudomanas* from the *Pseudomondales* order, which is part of *Proteobacteria* phylum. 1 of the isolates from the *Bacillales* order showed to be closely related to *S. capitis* a common skin bacterium. The chosen isolates were subsequently further tested for tolerance towards UV radiation, perchlorate tolerance compared to NaCl, low pressure, anaerobic conditions, 4^{o}C tolerance, low relative humidity, Mars soil analog, starvation, and desiccation. All the isolates showed tolerance towards 4.5 min of UV exposure with a UV intensity of 20.7 W/m_2, greater than those found on Mars, 10-20 W/m_2. Given that the duration of 4.5 minutes is only a fraction of a 24 hour diurnal cycle found on Mars, the results might not be a surprise, it does however show that short durations of UV radiation are easily tolerated, not only by the isolates from the Atacama Desert, but also a UV sensitive *E. coli*.

Searching for PcrA, was done by PCR. Primers had been designed to flank the gene of interest, to show a band sizes of ~300 bp. The primers were designed to match highly conserved regions in the *pcrA* gene. The results showed that isolate 201 might have perchlorate reductase, as a band of ~300 bp presented itself on the electrophoresis gel. Other isolates, including 202-1 and 10, also showed presence at the 300bp, but the bands appeared slightly below or above the 300bp reference line. Several additional isolates were screened for *pcrA* but did not show bands in the sought after area. As there is still much to learn about perchlorate reduction it cannot be ruled out that these isolates do not have an alternative way of reducing perchlorate.

The perchlorate tolerance showed variable results between isolates. Looking at how the growth curves and growth rates were affected, comparisons were done with media containing no perchlorate, and media with 1% NaCl, and 1%, 2%, and 3% perchlorate. The results showed that the presence of 1% NaCl had little effect on the growth curves and growth rates for any of the isolates. The presence of perchlorate did, however, show a decrease in growth rate, presenting a more horizontal growth curve, compared to exponential growth seen with TSB media (see appendix 4 – Growth curves and UV CFU). Of all the isolates, isolate 201 presented a growth curve and growth rate of all the perchlorate concentrations most similar to that of TSB. For all the

tested isolates all survived the presence of 3% perchlorate. This showed that the isolates tolerate the presence of perchlorate differently, but none of the perchlorate concentrations had a bactericidal effect.

Combining the factors, low pressure, atmospheric composition of CO_2, and a low relative humidity, was possible by making use of the Mars chamber. All the "primary isolates" were incubated under the mentioned factors. The results show that some of the isolates exceed in growth compared to the control CFU on the Martian soil analog, and under Earth atmospheric pressure and composition, indicating that the Martian soil analog is not toxic. On the contrary, isolates had a very hard time in dealing with Martian soil, containing perchlorate. For unknown reasons, the 1 wt% perchlorate mixed with Martian soil, rendered most of the bacteria unable to grow, and killed the bacteria under Earth atmospheric conditions. Regarding the perchlorate tolerance experiment this was surprising, as all isolates survived and grew at a 3% perchlorate concentration. The isolates outside of the Mars chamber all showed higher growth compared to the isolates inside the Mars chamber. This shows that the present factors in the Mars chamber had a negative effect on the isolates lowering the average CFU by 10^{-5}. Another surprising result was that the isolates inside the Mars chamber grew better in the presence of perchlorate. Isolate 10 grew on all perchlorate containing wells inside the Mars chamber, and none on the outside (see appendix – Mars experiment). The reasons for this mysterious pattern is not answered. The results showed that isolate 6 succumbed completely to the environment present within the Mars chamber. With numerous factors coming in to play, the specific factor causing cell death for isolate 6 is not answered. Besides isolate 6, all the tested isolates show survival under Mars like circumstances.

Temperature and radioactivity were not tested here, but results show that, individually, some bacteria can survive under extremely cold temperatures, and endure an enormous amount of radiation. Combining these two factors to the Mars chamber could be interesting. It would also be interesting to see how UV radiation affects isolates in a 0.01 bar CO_2 atmosphere. Both temperature and a UV exposure can be a part of the Mars chamber, however not functional yet, they will be in the near future. With factors like, subzero temperatures, low atmospheric pressure, a <95% CO_2 consisting atmosphere, low relative humidity, Martian soil analog, and UV exposure, will make the Mars chamber a close analog to the Martian environmental factors.

We have isolated bacteria from environments on Earth that share some of the same environments found on Mars. We have tested the isolates to individual factors, and also subjected them to combined factors, getting as close as possible to the conditions present on Earth. The result being that 5 out of the 6 isolates indeed survived 12 days under simulated Marian conditions. While doing so, we might even have found a perchlorate reducing bacteria in the *Bacillales* order.

7 Challenges and perspectives

"*The grass is always greener on the other side"* is a phrase that does not apply to the subject of this thesis. Looking to our neighbor, Mars, what we find is not lush green grass, but a cold and dry environment. With close to no atmosphere, the surface is exposed to high levels of radiation, including UV and radioactive γ-radiation. The environmental conditions on Mars are believed to be too hostile to harbor life. However, if we draw parallels to two of the most Mars like environments on Earth, The Atacama Desert and the McMurdo Valleys we see, what was once thought to be an environment, presenting the limits to life, to be teeming with life representing every main organisms of the tree of life. In this thesis we try to get as close to Mars as possible, without leaving the green gras. We have successfully isolated bacteria from the Atacama Desert and the McMurdo Dry valley, using different media. Even though that the used cultivation methods proved successful, alternate methods could provide even more isolates and increase the isolated bacterial diversity. The use of the "High Salt PBS" showed that by mimicking the salt composition at the sample site, could help cultivate isolates from soil previously unculturable. This could potentially be extended to the solid media used for growing potential isolates. Even though this would require more effort, it could provide us with new culturable species that could teach us a lot about surviving under stressful conditions present on Mars. We present here the "Primary Isolates", some of which showed the potential presence of perchlorate reductase. 16s sequencing of the potential organisms, showed a 97.29% similarity to *S. capitis*. Searching for perchlorate reductase through blast, showed no results, neither any homologs. To definitively see if isolate 201 carries a perchlorate reductase, sequencing of the PCR product used to define the 300 bp DNA stretch, should be done. Even though the result of this sequence could be a mismatch to known *pcrA* sequences, it could be interesting to sequence the entire genome, to look for potentially unknown genes and test their phenotype. This could reveal alternative ways bacteria are able to tolerate or reduce perchlorate. The experiment subjecting our "Primary Isolates" to various

concentrations of perchlorate showed that in a liquid state, growth is present. In this experiment it would be interesting to include a control that does not come from the Atacama Desert were perchlorate distributions are high, to see the effect of perchlorate on a bacteria that do not have perchlorate as a part of their natural environment. An example could be the same *E. coli* used for the UV experiment. The UV experiment showed that the isolates and the presumably UV sensitive *E. coli* did not react towards the UV radiation. Increasing the duration of UV exposure could potentially reveal a negative effect on the tested bacteria. It could also be interesting to make an evolutionary experiment, selecting the isolates that survive increased durations of UV and keep subjecting them to increasing UV exposure. Comparisons could then be made to see genomic and proteomic differences between isolates. It could show if bacteria evolve the same defense mechanisms independent of each other, or if they evolve sperate strategies. This could potentially provide us with a diverse toolbox of different defense mechanisms for UV protection.

For our Mars chamber experiments we managed to show survival after 12 days under Martian circumstances. This can be seen as a successful endeavor if you are a bacteria, and even though it shows proof that some of the Martian environmental factors can be tolerated by life, the results make it difficult to compare the effect of the various factors. For future experiments, it could be preferred to test each factor separately, to see the single factor's influence on the bacteria. In this experiment we had a control placed outside the Mars chamber, at ambient Earth atmospheric composition and pressure. A control is never a bad thing, the factorial gap however, from outside the chamber to factors inside the chamber was too big. Drawing conclusions based on these two observations resulted in many assumptions. Moving the control inside the glovebox, or adding samples to the glovebox as well, could reduce the gap and clearer conclusions could be have been drawn. The Mars chamber is a great tool, but currently it does not fulfill its full potential. This is however, soon to change, as a UV source has been ordered, the cooling system will be operational, adding H_2 and potentially CO (CO requires a high degree of safety measures, as it is a highly toxic gas), to the gas inlets, and properly connecting the Mass Spectrometer, will make the Mars chamber an extraordinary tool for simulating Martian conditions, and seeing how gasses released by bacteria are measured within the chamber. This will bring us closer to understanding the limits of life, and if life can tolerate the conditions present on Mars.

If we fail to find an organism capable of tackling all of the factors present on Mars, understanding which genes and proteins help under different environmental factors, could provide us with knowledge as how to genetically modify organisms, to see them better equipped to withstand the elements on Mars. As the evolution of perchlorate reduction appears to originate from mobile genetic elements, it could be valid starting point. Transforming a strictly aerobic bacteria with a plasmid containing the genes for effective perchlorate reduction and utilization, potentially making it facultative anaerobic, could be a very exciting endeavor.

Finding bacteria, archaea, or fungi that can survive all of the present factors on Mars, could be a tedious task, and maybe an impossible task. A way to potentially overcome this, besides genetic modification, is to look at bacterial communities. As bacteria mostly live in communities, forming multispecies biofilms, and have been shown to interact,(Basler, Ho, & Mekalanos, 2013; Lee et al., 2014; Xavier, Kim, & Foster, 2011) they can in many cases be seen as symbionts. It could be a valid option to put together a bacterial community that collectively tackle the forces on Mars. A potential start could be to have bacteria work together in reducing perchlorates, as already demonstrated (Clark et al., 2016), and expand the community from there.

As technology advances, so does our exploration of Mars and the universe. We have recently witnessed several missions to Mars, by several nations, showing that in a sense, Mars moves ever closer. Missions of interest is the recent successful launch of NASA's 2020 rover, Perseverance. Equipped with a variety of tools capable of measuring potential present and past life on the surface, it is also designed to take drill samples of the Martian regolith and store them in special containers. The stored samples will eventually be transported back to Earth for closer investigation. The same mission will also pave the way for human exploration on Mars with the MOXIE instrument for potentially converting the CO_2 on Mars to O_2. Another mission of interest is the ExoMars mission by ESA and the Russian Roscosmos, which similar to NASA's 2020 mission, will send a rover to Mars to search for present and past signs of life. The rover will be able to drill 2 meters down, below the radiation zone, and analyze the conditions. With these rovers we have never been closer to answer the question "*Are we alone?"*. Exciting times are ahead, and as we might discover life on Mars within 10 years, and get to analyze an extra-terrestrial organism, then perhaps the grass really is greener on the other side.

8 References

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9 Appendix

Appendix 1 – X-ray dosage

Radiation Dose to Adults From Common Imaging Examinations

Procedure		Approximate effective radiation dose	Comparable to natural background radiation for
ABDOMINAL REGION	Computed Tomography (CT) — Abdomen and Pelvis	10 mSv	3 years
	Computed Tomography (CT) — Abdomen and Pelvis, repeated with and without contrast material	20 mSv	7 years
	Computed Tomography (CT) — Colonography	6 mSv	2 years
	Intravenous Pyelogram (IVP)	3 mSv	1 year
	Barium Enema (Lower GI X-ray)	8 mSv	3 years
	Upper GI Study With Barium	6 mSv	2 years
BONE	Spine X-ray	1.5 mSv	6 months
	Extremity (hand, foot, etc.) X-ray	0.001 mSv	3 hours
CENTRAL NERVOUS SYSTEM	Computed Tomography (CT) — Head	2 mSv	8 months
	Computed Tomography (CT) — Head, repeated with and without contrast material	4 mSv	16 months
	Computed Tomography (CT) — Spine	6 mSv	2 years
CHEST	Computed Tomography (CT) — Chest	7 mSv	2 years
	Computed Tomography (CT) — Lung Cancer Screening	1.5 mSv	6 months
	Chest X-ray	0.1 mSv	10 days
DENTAL	Dental X-ray	0.005 mSv	1 day
HEART	Coronary Computed Tomography Angiography (CTA)	12 mSv	4 years
	Cardiac CT for Calcium Scoring	3 mSv	1 year
MEN'S IMAGING	Bone Densitometry (DEXA)	0.001 mSv	3 hours
NUCLEAR MEDICINE	Positron Emission Tomography — Computed Tomography (PET/CT)	25 mSv	8 years
WOMEN'S IMAGING	Bone Densitometry (DEXA)	0.001 mSv	3 hours
	Mammography	0.4 mSv	7 weeks

Note: This chart simplifies a highly complex topic for patients' informational use. The effective doses are typical values for an average-sized adult. The actual dose can vary substantially, depending on a person's size as well as on differences in imaging practices. It is also important to note that doses given to pediatric patients will vary significantly from those given to adults, since children vary in size. Patients with radiation dose questions should consult with their medical physicists and/or radiologists as part of a larger discussion on the benefits and risks of radiologic care.

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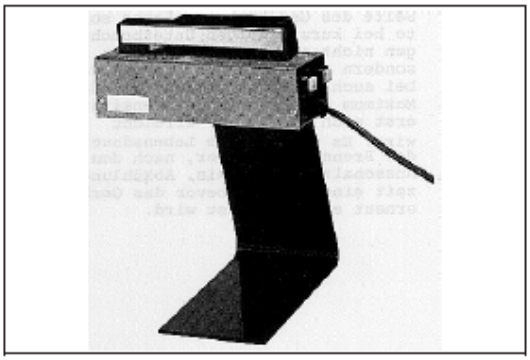
For the most current information, visit radiologyinfo.org.

Appendix 2 – UV lamp

Scientific Education	Technical Training and Education	Trade	LEYBOLD	LEYBOLD DIDACTIC GmbH

7/98-V5-



Instruction sheet 665 635

UV Analysis Lamp (665 635)

The analysis lamp for short-wave and long-wave ultraviolet radiation is used to investigate fluorescence in numerous organic and inorganic materials.

Ultraviolet radiation cannot be seen by the naked eye; only a blue-violet shimmer can be seen through the filter of a UV lamp. This is the proportion of "visible light" which the burner generates and which is absorbed by a filter with the exception of this weak residue. This lamp lets you make use of two groups of ultraviolet radiation:

Short-wave range: 254 nm
farthest from the visible spectrum. The short-wave energy is preferred for chemical analysis applications and cold sterilization.

Long-wave range: 366.0 nm
closest to visible light (also called black light). The long-wave radiation excites fluores-cence in numerous natural substances and materials made from them. It is relatively harmless to the skin and eyes.

Function

The two UV burners are switched on separately using two push-button switches on the side of the device. Do not switch off the lamp during brief interruptions, but leave it burning. Note that the maximum radiation intensity is only reached after approx. five minutes have elapsed. To ensure maximum service life, always allow a cool-down time of at least five minutes after switching off the device before switching it on again.

Technical data

Wavelength:	254 nm and 366 nm
Burner power:	2 burners, 4 W each
Dimensions:	205 x 70 x 55 mm
Weight:	1.0 kg
Power:	220 V/50 Hz

Safety notes

Never look into the beam path without suitable safety goggles! Short-wave UV radiation causes skin burns and conjunctival inflammation very rapidly and initially unnoticed!

If no safety goggles are worn, avert the beam path away from the user. This safety measure may only be omitted when UV lamps are operated behind glass, e.g. in showcases etc., as normal window glass blocks short-wave UV radiation.

LEYBOLD DIDACTICGMBH - Leyboldstrasse 1 - D-50354Hürth - Phone (02233) 604-0 - Telefax (02233) 604-222 - e-mail: info@leybold-didactic.de

Appendix 3 – Mars Global Simulant

MGS-1 Mars Global Simulant | Fact Sheet

March, 2019

Simulant Name: MGS-1 Mars Global Simulant
Simulant Type: General Purpose
Reference Material: Rocknest soil
Publication: Cannon et al. 2019
Icarus 317, 470-478
Bulk Density: 1.29 g/cm^3
Particle Size Range: 0-1 mm
Mean Particle Size: Data coming soon

Mineralogy

Weight percent, as mixed.

Plagioclase	27.1
Basaltic glass	22.9
Pyroxene	20.3
Olivine	13.7
Mg-sulfate	4.0
Ferrihydrite	3.5
Hydrated silica	3.0
Magnetite	1.9
Anhydrite	1.7
Fe-carbonate	1.4
Hematite	0.5

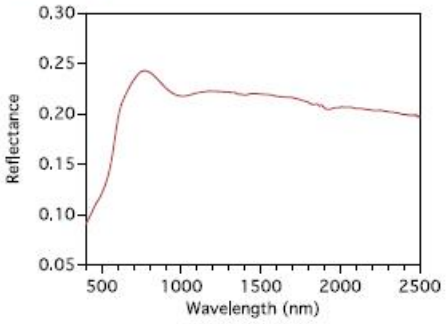
Bulk Chemistry

Weight percent, as measured by XRF.

SiO_2	45.6
TiO_2	0.3
Al_2O_3	9.4
Cr_2O_3	0.1
FeO_T	16.9
MnO	0.1
MgO	16.5
CaO	4.0
Na_2O	3.7
K_2O	0.4
P_2O_5	0.4
SO_3	2.6
Total	100.0

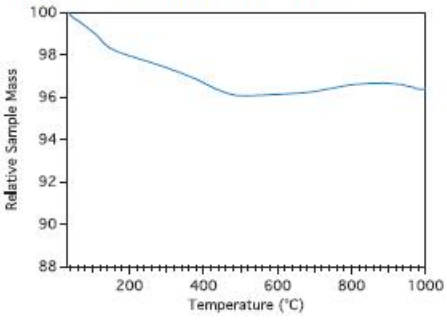
Reflectance Spectrum

As measured on an ASD Fieldspec at 30° incidence and 0° emergence angles.



Volatile Release Pattern

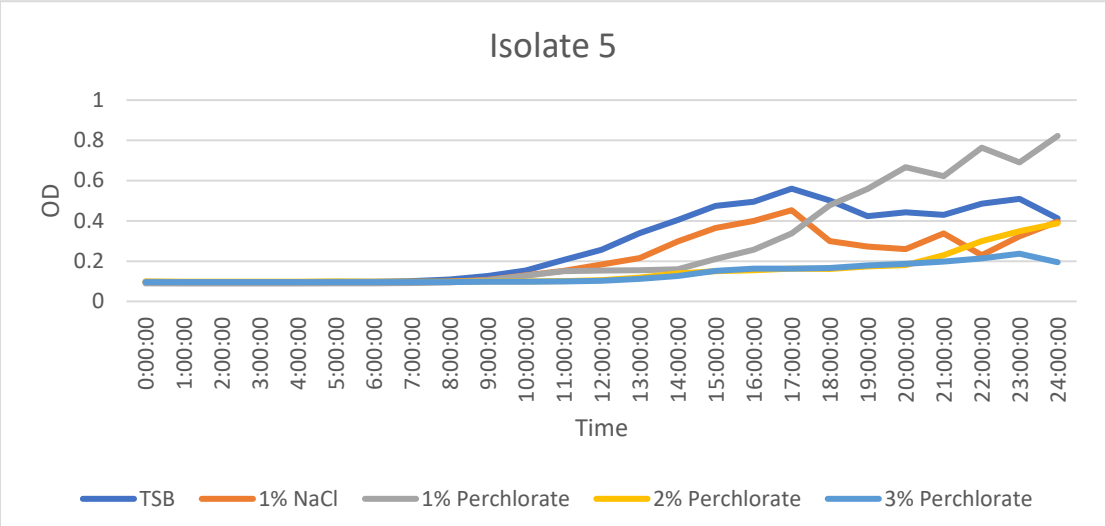
As measured on a SAM-analog TG/EGA instrument at JSC.
Total evolved water at 200° C is 2.1 wt.%.

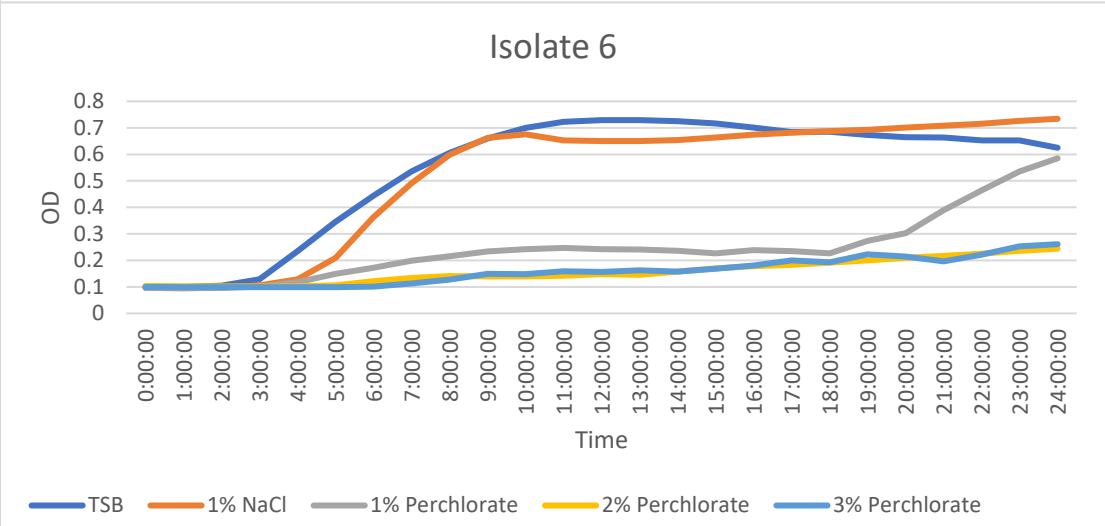


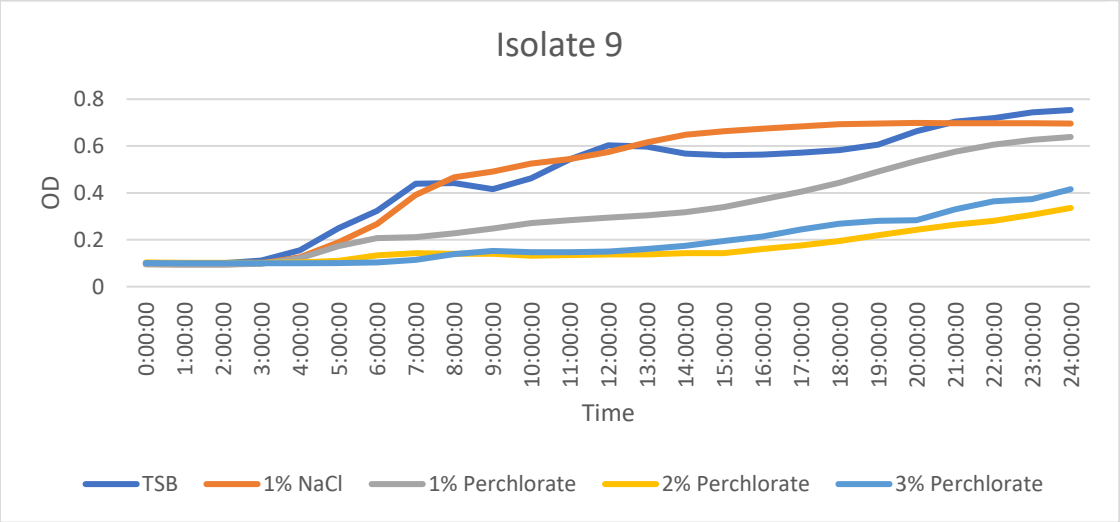
Safety Information

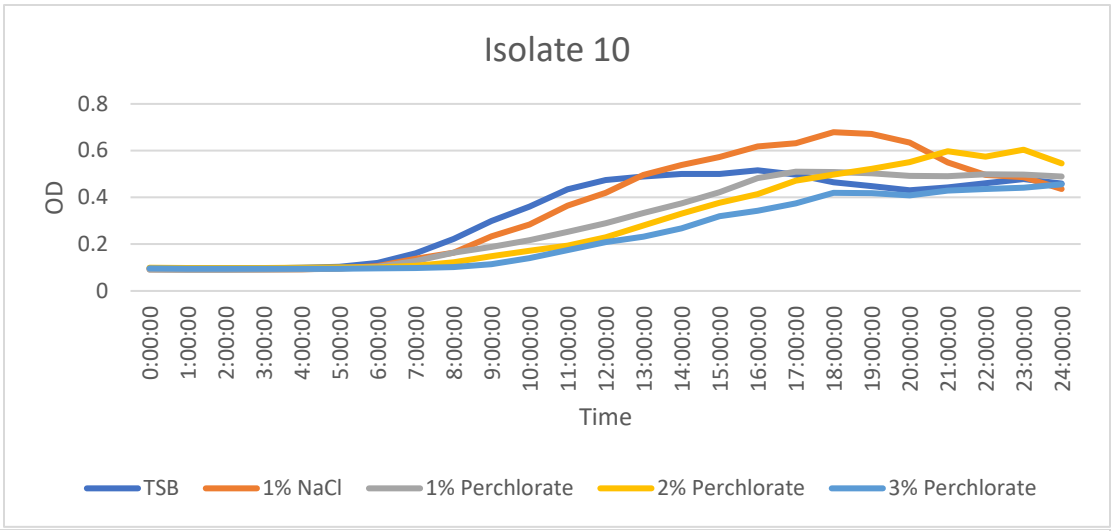
See SDS for details. Primary hazard is dust inhalation; wear a respirator in dusty conditions.

Appendix 4 – Growth curves and UV CFU





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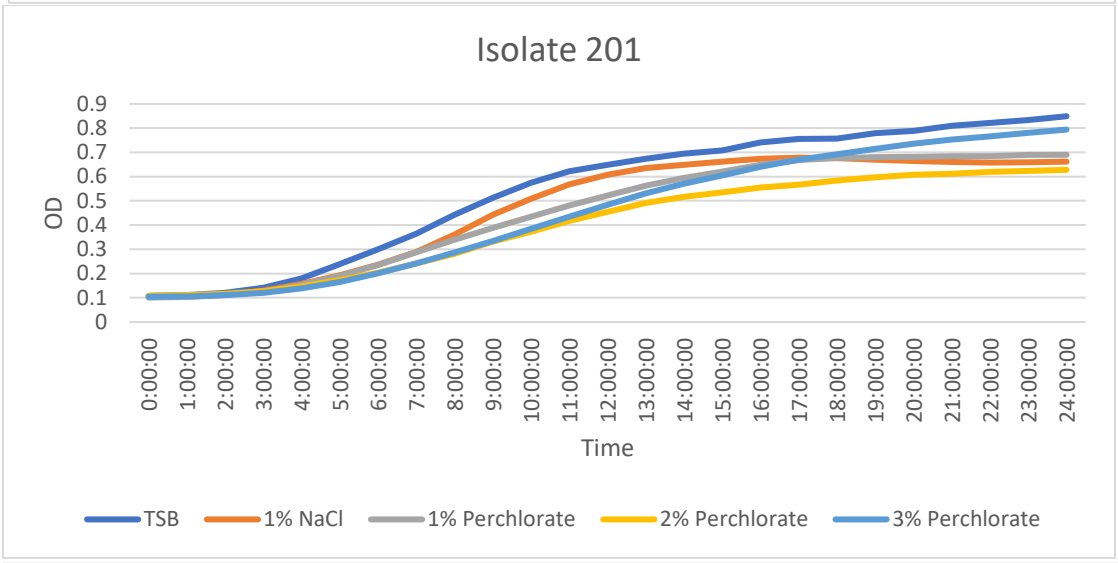
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7:00:00
8:00:00
9:00:00
10:00:00
11:00:00
12:00:00
13:00:00
14:00:00
15:00:00
16:00:00
17:00:00
18:00:00
19:00:00
20:00:00
21:00:00
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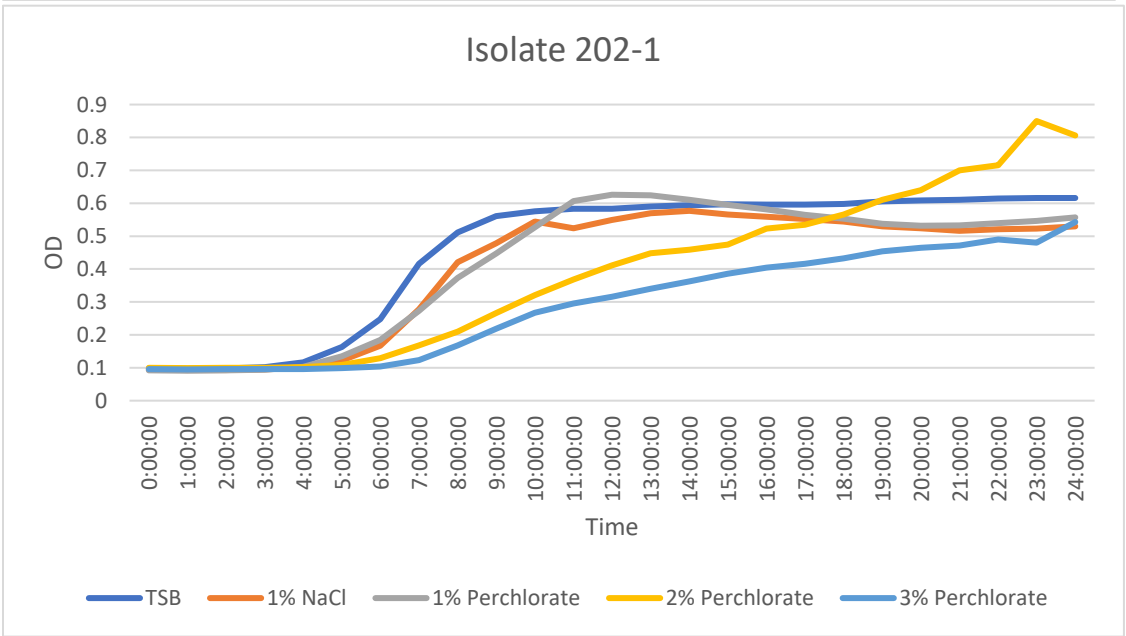
23:00:00
24:00:00
Time
TSB
1% NaCl
1% Perchlorate
2% Perchlorate
3% Perchlorate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0.7
0.6
0.5
0.4
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0.2
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0
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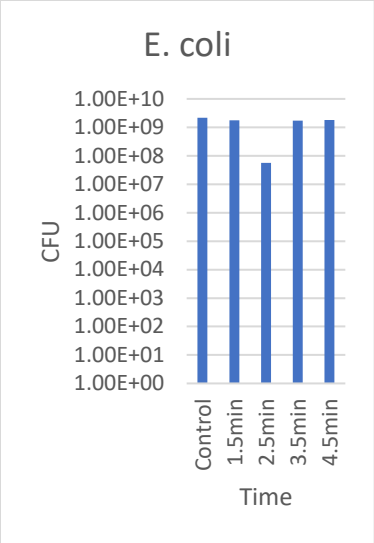
Time
TSB
1% NaCl
1% Perchlorate
2% Perchlorate
3% Perchlorate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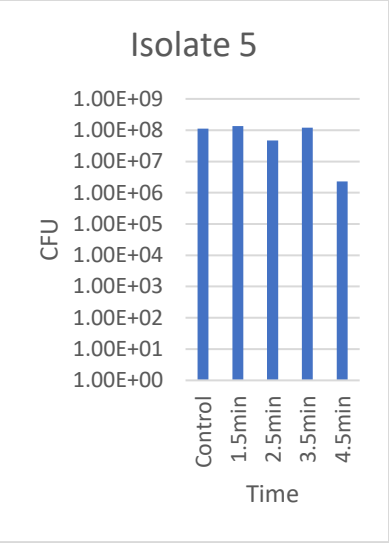
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU
Control
1.5min
2.5min
3.5min
4.5min
Time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1.00E+03
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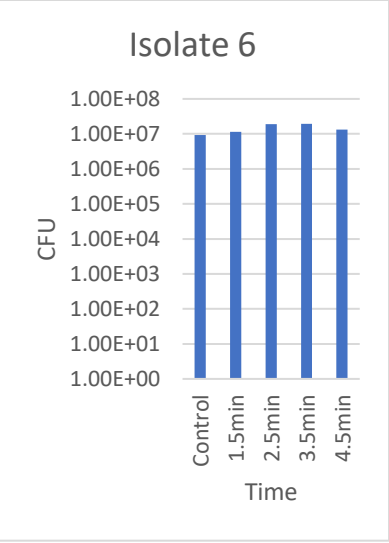
1.00E+00
CFU
Control
1.5min
2.5min
3.5min
4.5min
Time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CFU
Control
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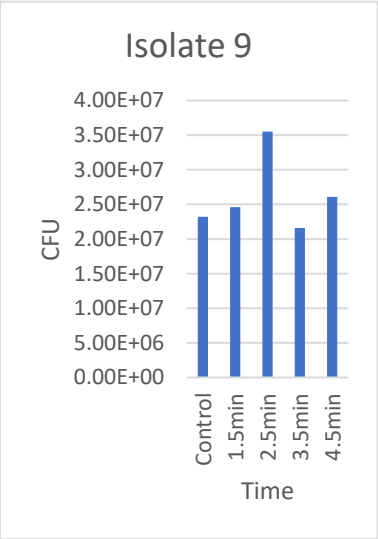
2.5min
3.5min
4.5min
Time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1.50E+07
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5.00E+06
0.00E+00
CFU
Control
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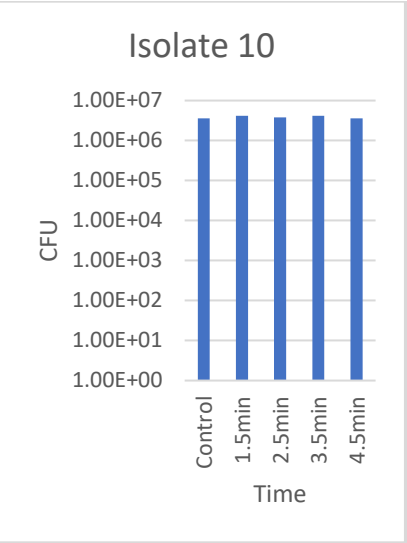
2.5min
3.5min
4.5min
Time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CFU
Control
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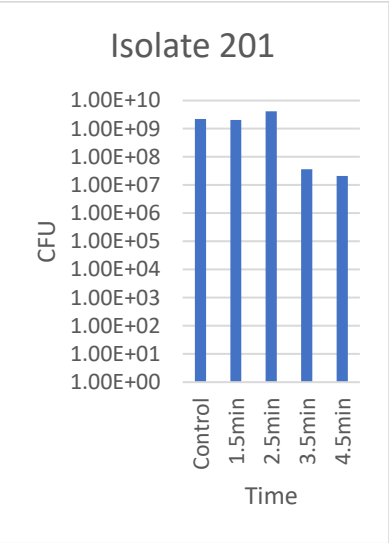
2.5min
3.5min
4.5min
Time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1.00E+03
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CFU
Control
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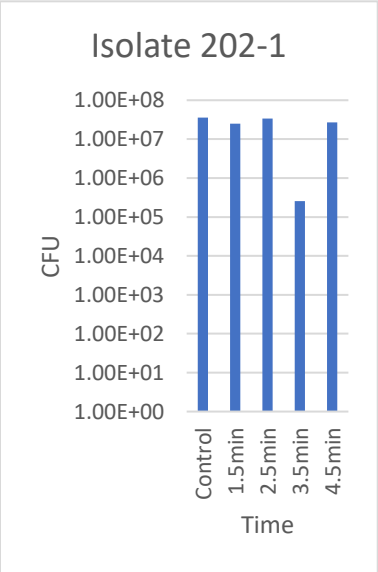
2.5min
3.5min
4.5min
Time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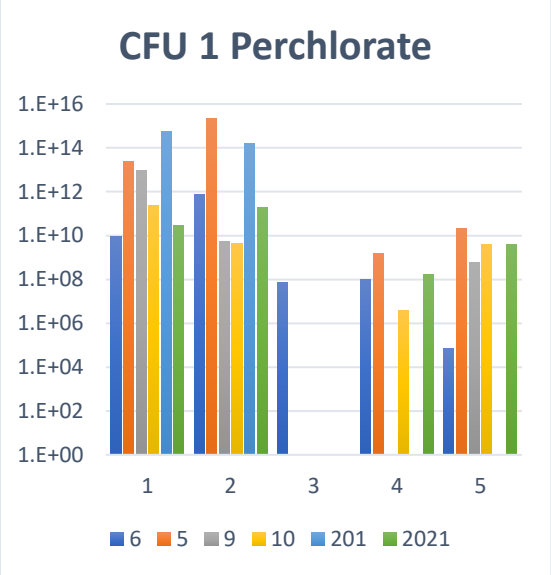
1.00E+02
1.00E+01
1.00E+00
CFU
Control
1.5min
2.5min
3.5min
4.5min
Time

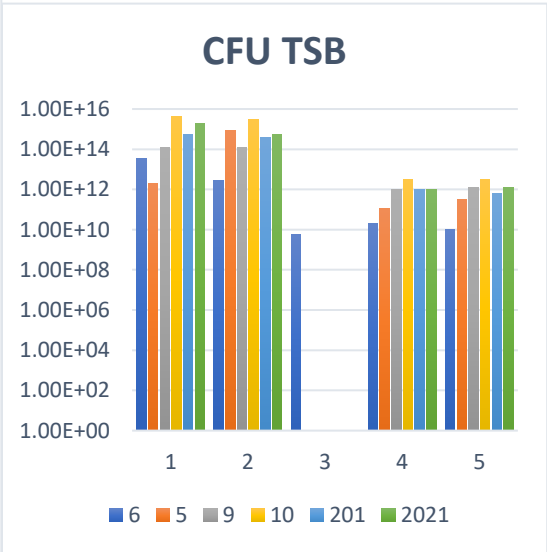
Appendix 5 - Mars experiment

MSM	5	6	9	10	201	202-1
GSF	4.50E+05	0	6.80E+04	8.10E+04	0	1.80E+05
GSNF	2.00E+05	0	1.57E+05	2.40E+04	4.30E+06	1.50E+05
GSP	0	0	5.36E+05	4.00E+03	4.30E+04	0
GF	1.80E+04	0	5.48E+02	2.26E+05	2.42E+07	6.40E+05
GNF	0	0	8.00E+03	1.06E+05	4.50E+06	5.60E+04
GP	0	0	0	3.00E+03	0	0
JF	2.30E+04	0	1.80E+04	7.50E+04	1.64E+07	1.70E+05
JNF	2.59E+05	0	2.00E+03	0	4.95E+06	3.40E+05
JP	0	0	0	1.00E+04	3.20E+04	1.00E+03

MSC	5C	6C	9C	10C	201C	202-1C
GSF	4.7.E+05	4.40E+07	4.00E+07	1.93E+08	9.50E+07	6.60E+08
GSNF	6.00E+04	2.41E+05	3.60E+06	3.50E+08	1.35E+06	9.30E+06
GSP	0	0	0	0	0	0
GF	2.02E+06	3.70E+07	1.63E+07	1.51E+09	8.40E+07	1.71E+07
GNF	1.35E+05	1.40E+06	1.92E+06	2.02E+08	0	2.08E+07
GP	0	0	0	0	0	3.80E+05
JF	5.40E+06	2.90E+04	4.20E+07	9.00E+07	1.50E+09	2.12E+06
JNF	9.90E+04	1.70E+05	1.28E+06	1.50E+06	1.89E+05	2.18E+06
JP	0	0	1.60E+04	0	0	0

Appendix 6 - 4oC incubation with 1% perchlorate





Appendix 7 - Media recipes

Enrichment Media Halophile 2% / 12.5% 1L

20g/125g Sodium Chloride

20g Magnesium Sulfate x7 H_2O

7.5g Casaaminoacids

1g Yeast extracts

5g Tryptone

0.05g Ammonium iron (II) sulfate x H_2O

0.5g DiPotassium Hydrogen Phosphate

pH ~7.2

Tryptic Soy Broth (TSB) 1L

30g Tryptic Soy Broth

3g Yeast extracts

pH ~7.2

Tryptic Soy Broth with 0.6% / 1% / 2% / 3% perchlorate 1L

30 g Tryptic Soy Broth

3g Yeast extracts

6g / 10 g / 20 g / 30 g perchlorate

- For solid media, 15 g agar can be added to any of the above recipes

"High Salt PBS" 1L

1.75g Cl^-

38.4g SO_4

3.1g NO_3

14.8g ClO_4

960 mg Mg

2.4g Ca

8.8g Na

Appendix 8 - Uffe's Appendix

Respiration

The simple way of defining respiration is by the formula:

$C_6H_{12}O_6 + 6O_2$ -Cellular respiration--> $6CO_2 + 6H_2O$ + ATP

As simple as it looks there is however, a wide variety of steps needed to generate ATP through respiration, with oxygen driving the entire process known as cellular respiration.

Besides in the intake of glucose and oxygen, cellular respiration is the first step in the process of breaking down molecules (catabolism) to be used as metabolites and the generation of energy which later is used to create new molecules (anabolism). This entire process includes the steps: Glycolysis, Krebs cycle, and electron transport/oxidative phosphorylation, which as a final result yields energy in the shape of 38 ATP molecules. Through the mentioned steps, ATP is created by ATPases and released to undergo a variety of cellular functions.

Glycolysis

The first step in the metabolism is the break down of sugar, glycolysis. Here the 6-carbon glucose ring is phosphorylated, breaking up the 6-carbon structure to two 3-carbon structures. Each of these two 3-carbon structures undergo the same process and yield the same amount of products. These 3-carbon structures (glyceraldehyde 3-phosphate) also undergo the same structural changes, which reduces NAD to give NADH - where NADH has received an electron and a hydrogen atom from the phosphorylated glucose molecule. Concurrent with the oxidation of glyceraldehyde 3-phosphate, a phosphate is added to the same molecule by an enzyme, forming 1,3 - Diphosphoglyceric acid. The recently formed 1,3 - diphosphoglyceric acid then donates its newly attached phosphate group to an ADP molecule, making 1x ATP. The last phosphate group then shifts position from 3 to 2, with the release of water which then generates an additional ATP molecule, as the rearranged phosphate group is donated to an ADP molecule finalizing the final step of the glycolysis by creating 4x ATP molecules, 2x NADH, pyruvate molecules which feed into the Kreb's cycle. As the phosphorylation of glucose requires two ATP molecules, the net value of glycolysis is 2x ATP, 2x NADH, and 2x pyruvate.

The Kreb's cycle

The Kreb's cycle also known as, tricarboxylic acid or the citric acid cycle (TCA), is where the precursors for the bulk of ATP is produced. The final product of the glycolysis, pyruvate, feeds into the Kreb's cycle which then undergoes a variety of modifications which oxidizes carbon, yielding CO_2 and NADH. The reduced NADH, carrying an electron and a Hydrogen, will subsequently feed into the electron transport and oxidative phosphorylation donating the electrons and the hydrogen atom.

Oxidative phosporylation

The last step of generating ATP is the oxidative phosphorylation, or electron transport chain. It is within this step that the oxygen functions as the molecule which drives the entire process of generating ATP. It does so by being highly electronegative. The electronegativity pulls the electrons from electron carriers NADH and FADH. The NADH and FADH are transferred to 1 of 3 large membrane bound protein complexes, NADH-Q oxoreductase, Q-cytochrome c oxidoreductase, and cytochrome c oxidase which oxidizes NADH and FADH. Besides these three transmembrane molecules are also three smaller

molecules, Succinate-Q reductase, Ubiquinone (Q) and Cytochrome c, who function by transferring the electrons between the three larger proteins. When the NAHD and FADH are oxidized they also release the hydrogen atom across the membrane creating a high concentration of hydrogen atoms on one side of the membrane. The high concentration of hydrogens on one side of the membrane creates a proton force, which will drive the hydrogen atoms to the side of the membrane where the concentrations are low. The protons however cannot simply diffuse through the membrane, but are forced to pass through a large membrane bound protein complex called ATP-synthase. The ATP-synthase uses the proton force to undergo conformational changes that link together ADP and a phosphate group forming ATP. This is the ATP which is used for cellular functions. All of the above mentioned steps, would all come to a halt, if there was no oxygen to accept electrons passed through the electron transport chain. This is the main process seen throughout the animal kingdom, where oxygen is the final electron acceptor. However, there are also organisms which are able to grow under anaerobic conditions. This means that some organisms, mostly bacteria and archaea, are able to utilize other molecules as terminal electron acceptor. One of these electron acceptors is perchlorate.

Appendix 9 – 16s sequences

16s Sequencing Results

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