Characterization of stress tolerance and metabolic capabilities of acidophilic iron-sulfur-transforming bacteria and their relevance to Mars

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Erklärung / Statement

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

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Herewith I declare that this thesis is the result of my independent work. All sources and auxiliary materials used by me in this thesis are cited completely.

Essen, 07.12.2012

Table of contents

Abbreviations

BSM	basal salt medium
cfu	colony forming units
CLSM	confocal laser scanning microscopy
ConA	Concavalin A (lectin)
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DAPI	4',6-diamidino-2-phenylindole
DPA	dipicolinic acid
DSBs	double strand breaks
EUB338	oligonucleotide probe specific for all eubacteria
FISH	fluorescence-*in situ*-hybridization
MPN	Most Probable Number
MRS	Mars regolith simulants
NA	nalidixic acid
PAC	probe active count
P-MRS	Phyllosilicatic Mars regolith simulant
qPCR	quantitative real-time PCR
RH	relative humidity
RISCs	reduced inorganic sulfur compounds
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
S-MRS	Sulfatic Mars regolith simulant
TCC	Total cell count
VBNC	viable-but-nonculturable
WGA	wheat germ agglutinin (lectin)

1 Introduction

1.1 The search for life on Mars

Is there life beyond Earth or are we alone in the universe? This question has intrigued humankind for thousands of years, but all attempts to find answers have remained largely in the realm of philosophy. Only recently, technical developments have made it possible to raise our search for life elsewhere onto a new level of scientific inquiry.

In the last decades, our neighboring planet Mars has received much attention as one of the most promising targets close to Earth on which life may have developed in the past or might even exist at present. At the turn of the 19th century, some scientists believed that Mars was inhabited by an intelligent civilization capable of building large artificial irrigation canals, and that its surface was covered with vegetation undergoing seasonal changes (Lowell 1908). Only when the Mariner mission and other Mars-orbiting probes returned the first close-up images of the surface, the existence of the canals was disproven with certainty (Sagan and Fox 1975). However, the interest in Mars as a potentially life-bearing planet has persisted until today.

1.1.1 Findings of the Viking mission

The first mission specifically designed to search for life and organic compounds on the red planet was the Viking mission, composed of two orbiters and landers and launched in 1975 (Soffen 1977). Three biological experiments were included onboard the landers to detect different types of metabolic activity including photo- or chemoautotrophic fixation of CO_2 and mineralization of organic compounds. The pyrolytic release experiment was designed to measure the incorporation of

^{14}C into biomass by adding $^{14}CO_2$ and ^{14}CO to a sample of Martian regolith[1] and incubating it in the presence and absence of light (Horowitz et al. 1977). The gas exchange experiment measured uptake and production of gases by regolith incubated with nutrients and water (Oyama and Berdahl 1977), and the labeled release experiment determined $^{14}CO_2$ generation by regolith upon addition of ^{14}C-labeled organics and water (**Table 1-1**) (Levin and Straat 1977). In addition, the landers were equipped with a gas-chromatograph mass-spectrometer (GC-MS) to analyze the the composition of the atmosphere and soil and detect organic molecules (Biemann et al. 1977).

Positive results were obtained only in the labeled release experiment, where a rapid evolution of $^{14}CO_2$ was observed, which was completely inhibited by heating of the regolith prior to incubation (160°C, 3 h) (Levin and Straat 1977). Taken together with the data from the other experiments and the failure to detect any organic compounds by GC-MS, the results of the Viking biological investigations could most consistently be explained by abiotic reactions due to the oxidative properties of the Martian regolith (Klein 1978). However, organic carbon detection by thermal volatilization, used in the protocols of both the Viking and the subsequent Phoenix lander (which descended on Mars in 2008), may not be the best method to look for trace amounts of organics, especially in the presence of strong oxidants like perchlorates. Perchlorate salts were recently found in the Martian polar region by Phoenix and were shown to oxidize organic compounds to CO_2 during pyrolysis (Hecht et al. 2009; Navarro-González et al. 2006, 2009, 2010). Thus, the question of organics on Mars remains unresolved to date, but current and planned missions such as the Curiosity rover by NASA and the ExoMars rover (ESA, scheduled for 2018) may be able to provide further data to solve this issue (Davila et al. 2010; ten Kate 2010).

[1] regolith is the loose unconsolidated rock and dust on top of the bedrock layer, will be used interchangeably with the term ‘soil’

Table 1-1: Biological investigations aboard the Viking landers (adapted from Hansen 2007; Klein 1977; ten Kate 2010).

Experiment	*Measurement*	*Results*
Pyrolytic Release	Incorporation of ^{14}C from ^{14}CO or $^{14}CO_2$ into organic matter	Small assimilation Highest with light Unaffected by heating (90°C)[a]
Gas Exchange	Production of CO_2, N_2, CH_4, H_2, and O_2 and the uptake of CO_2 by soil samples	Release of O_2, CO_2, N_2 Unaffected by heating (145°C)[b]
Labeled Release	Production of ^{14}C-labeled gas upon addition of nutritent containing ^{14}C-labeled organics	$^{14}CO_2$ production Inhibited by heating (160°C)[c]

[a]Horowitz et al. 1977
[b]Oyama and Berdahl 1977
[c]Levin and Straat 1977

The Viking mission along with an abundance of subsequent landers, rovers, and orbiters returned a wealth of data on the environmental conditions and the geochemistry of Mars, which can help to deduce the evolution of the planet and to define regions in which it is most likely to find traces of life today, if there are any. The most important factors to consider in the evaluation of Mars' habitability are the availability of liquid water as a solvent for chemical reactions[2], the availability of an energy source, which will be required by any form of life regardless of its nature, and the physical conditions on the surface or in the subsurface (Cockell 2007; Hoehler et al. 2007; Horneck 2008). The following sections will give an overview on what is known about the geological history of Mars and the present conditions, and attempt to draw conclusions regarding the potential origin and distribution of life on the red planet.

[2] Thanks to the unique physicochemical properties of water, such as its large dipole moment, hydrolytic activity, and capability to form hydrogen bonds, it not only serves as a solvent, but also as a reactant in chemical reactions, it stabilizes macromolecules, facilitates diffusion and transport processes (Brack 2001, 2007)

1.2 Mars in the past and present

1.2.1 The history of Mars and the possible origin of life

Our current knowledge about Martian history has emerged from observations of its geological features and geochemical composition, and from numerical models attempting to extrapolate past conditions. Based on impact crater density, the history of Mars has been divided into three major epochs: the Noachian (older than 3.7 Ga), the Hesperian (3.7-3.0 Ga), and the Amazonian (3.0 Ga – present) (**Fig. 1-2**, p. 7) (Carr and Head 2010; Grotzinger et al. 2011; Solomon et al. 2005). The young Mars was volcanically active, as evidenced by volcanic morphologies such as the Tharsis region and by the basaltic composition of the surface rocks (Greely and Spudis 1981; McSween et al. 2009). Remnant magnetization of parts of the crust implies that Mars once possessed plate tectonics and a global magnetic field, which both ceased to be active by the late Noachian or early Hesperian (Acuña et al. 2001; Solomon et al. 2005; Breuer and Spohn 2003). The loss of the magnetic field had strong implications for the possible origin and evolution of life on Mars; not only because of the reduced shielding from cosmic and solar ionizing radiation, but also because of an increased atmospheric escape to space due to solar wind pick-up-ion sputtering (Horneck 2008; Luhmann and Kozyra 1991). Plate tectonics are an important prerequisite for the recycling of elements between crust and atmosphere, shaping a planet's surface habitability and driving evolution by providing a constantly changing environment (Lammer et al. 2009). Water is the lubricant that helps keep plate tectonics active. Since liquid water is also considered a prerequisite for life, the existence of water on early (and present-day, section **1.2.2.2**, p. 11) Mars is a crucial question that will be discussed in the subsequent sections.

1.2.1.1 Water and environmental evolution on early Mars

Signs that liquid water might once have flowed on the surface of Mars are supplied by a diverse set of geomorphic features that are reminiscent of structures formed by the action of liquid water on Earth. These include outflow channels, valley networks, delta deposits, gullies, glacial features, and shorelines

of potential lakes or even an ocean, whose existence is, however, controversial (**Fig. 1-1**) (Carr and Head 2010; Jaumann et al. 2001; Kereszturi 2012).

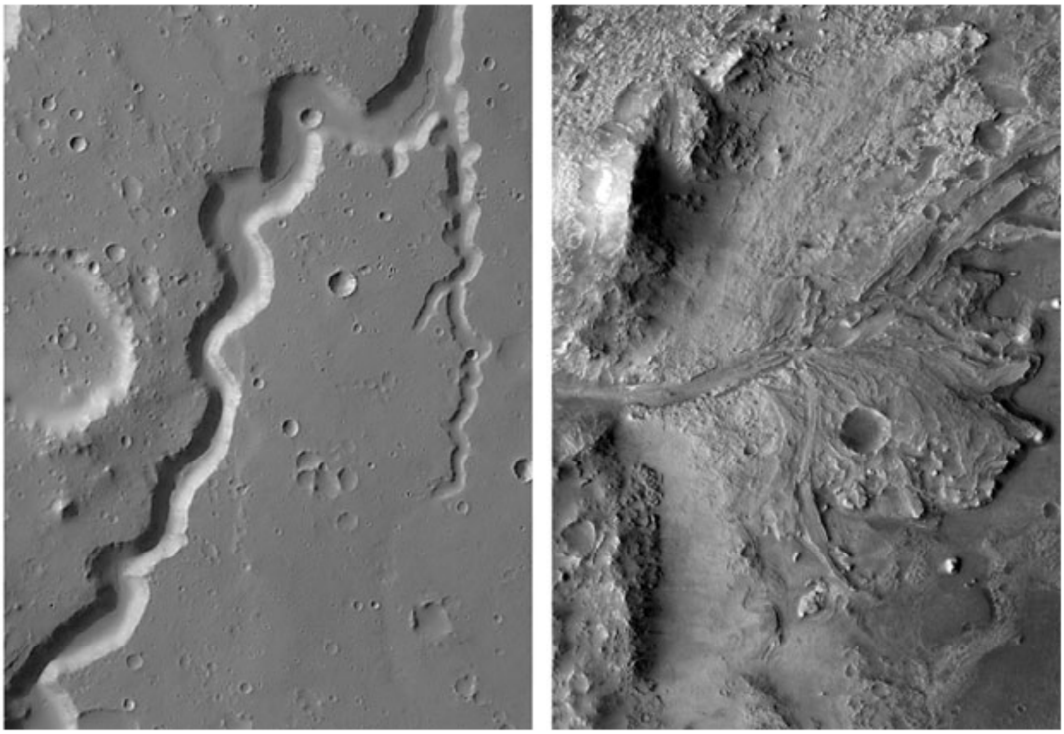

Fig. 1-1: Examples for morphological surface features on Mars that indicate the action of liquid water in the past. Left: a section of the Nanendi Vallis (H0894_000_ND3 HRSC image, 30 km), right: a delta deposit where an ancient channel entered into Jezero Crater previously filled by a lake (P04_002664_1988 CTX image, 6 km) (NASA, ESA, from Kereszturi 2012).

In addition to morphological features, the action of liquid water on early Mars is implied by chemical signatures in the form of hydrated minerals, which most likely precipitated in an aqueous environment (**Table 1-2**).

Table 1-2: Aqueous minerals identified on Mars and their possible connection to biological processes and the preservation of biosignatures.

Minerals	*Connection to biology*	*References*
Phyllosilicates	May provide the catalytic surface for biochemical evolution. Potential for long-term preservation of biosignatures.	Bibring et al. 2006; Ferris 2005; Orofino et al. 2010
Sulfates	Sulfate precipitation (e.g. jarosite) may result from bacterial sulfide oxidation. Sulfate deposits on Earth preserve intact organic molecules over geological timeframes.	Aubrey et al. 2006; Bandfield et al. 2002; Bhatti et al. 1993; Bibring et al. 2006; Martinez-Frias et al. 2006; Norlund et al. 2010
Ferric oxides	Formation is promoted by iron-oxidizing bacteria, extracellular polymers are critical in biomineralization. Magnetite crystals are produced directly by magnetotactic bacteria or indirectly by Fe^{3+}-reducers.	Bhatti et al. 1993; Bibring et al. 2006; Blakemore 1975; Chan et al. 2004; Chaudhuri et al. 2001; Le Deit et al. 2008; Lovley et al. 1987
Hydrated silica	Opaline silica are deposited in the cell wall of some organisms, e.g. diatoms.	De la Rocha et al. 1997; Milliken et al. 2008; Ruff et al 2011
Chlorides	Halophilic microorganisms affect crystallization of NaCl. Halites (NaCl) may preserve viable microbes over millions of years.	McGenity et al. 2000; Osterloo et al. 2010; Stan-Lotter et al. 2002
Carbonates	Biogenic carbonates are abundant in microbial mats (stromatolites).	Bandfield et al. 2002; Gerdes et al. 1994

These chemical surface alteration products can be used to draw inferences about the presence or absence of water and the environmental evolution of Mars (**Fig. 1-2**). Large deposits of phyllosilicates are associated with the oldest terrain on Mars of the Noachian epoch (Poulet et al. 2005). Phyllosilicates, such as smectite-type clay minerals, form in the presence of liquid water due to

weathering of primary silicates in mildly acidic to alkaline conditions (Chevrier et al. 2007; Halevy et al. 2007). On the younger, Hesperian parts of the crust, hydrated sulfate minerals, including the ferric sulfate mineral jarosite, prevail (Bibring et al. 2006). Since precipitation of sulfates requires acidic conditions, the spatial separation of Noachian phyllosilicate deposits and Hesperian sulfate sediments has been taken to suggest that a global transition occurred from the late Noachian to early Hesperian (ca. 3.7 Ga ago), presumably resulting from intense volcanic activity with increased outgassing of SO_2 (Bibring et al. 2006; Bullock and Moore 2007; Chevrier et al. 2007). However, there are some locations in which the stratigraphic order of sulfates and clay minerals appears reversed, raising the question as to whether the change from water-rich, circum-neutral conditions to water-poor and acidic conditions was on a global or on a regional scale (Grotzinger et al. 2011; Wray et al. 2010). From the late Hesperian to the present, arid conditions predominated and led to the formation of anhydrous ferric oxides distributed over large areas of Mars' surface (**Fig. 1-2**) (Bibring et al. 2006).

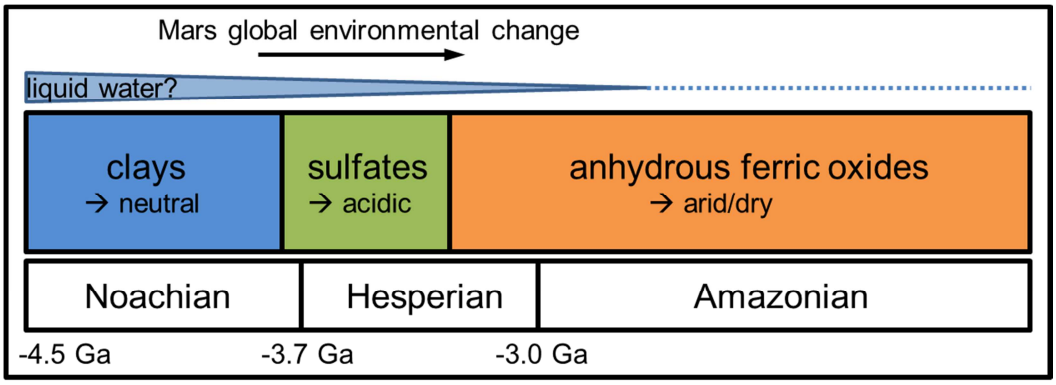

Fig. 1-2: One possible scenario for the evolution of Mars' surface environments according to Bibring et al. (2006): A period of clay mineral formation in neutral pH aqueous conditions was followed by sulfate generation in an acidic aqueous environment, accompanied by a global environmental change. The Amazonian era was dominated by the formation of anhydrous ferric oxides in a weathering regime without liquid water (modified from Grotzinger et al. 2011).

It is a matter of ongoing discussion whether the fluvial features and water-related sediments observed today on the surface of Mars have formed on a mostly dry and cold planet due to localized thermal energy sources such as large impacts (McEwen et al. 2007; Segura et al. 2002), or whether climatic conditions in the Noachian were substantially different from today, implying the existence of a thick primordial CO_2-atmosphere with sufficient greenhouse warming (provided by CH_4, NH_3, or SO_2) to sustain liquid water on the surface for transient intervals at least (Halevy et al. 2007; Jakosky and Phillips 2001; Sagan and Mullen 1972; Squyres and Kasting 1994). Alternative models have proposed that mean atmospheric temperatures on early Mars never rose above the freezing point of pure water, but that water bodies were protected against freezing by high solute concentrations, making early Mars a cold but wet planet (Fairén et al. 2009; Fairén 2010; Gaidos and Marion 2003). Recently, it was proposed that substantial phyllosilicate (clay) formation in the Noachian period might have occurred in the subsurface by interaction with hydrothermal groundwater with only transient releases of surface water, thereby indicating the subsurface as a potentially habitable environment on early Mars (Ehlmann et al. 2011).

1.2.1.2 Implication for the evolution of life

Perhaps the most basic requirement for life to originate and be sustained on Mars is the persistence of liquid water for sufficiently long periods to allow chemical evolution to take place (Davis and McKay 1996). Some of the wet environment types identified on Mars, such as geothermal and impact-induced hydrothermal systems, lakes, and valley networks, may have been in existence for periods from 10^2 up to 10^6 years (Kereszturi 2012). On the other hand, mineralogical evidence from hydrated amorphous silica, smectite clays and Fe-sulfates, which have undergone little diagenetic alteration since their formation, points to geologically brief episodes of water on the surface of early Mars, and the highly saline conditions associated with the formation of these minerals might have presented a significant challenge to the evolution of life (Tosca et al. 2008; Tosca and Knoll 2009).

But even on a cold and dry early Mars, hydrothermal environments in the subsurface, which have also been suggested as a possible location of biogenesis on Archaean Earth, could have provided reduced gases and an energy source in the form of chemical or thermal gradients thought necessary for the origin of life (Baross and Hoffman 1985; Martin et al. 2008). Clay minerals and iron sulfides, which were the precursors of jarosite precipitates on Mars (Zolotov and Shock 2005), might have played an important role in biogenesis by adsorbing relevant molecules and supplying the catalytic surface for the first metabolic reactions and polymerization of biomolecules (Cairns-Smith et al. 1992; Wächtershäuser 1988). If chemoautotrophic life indeed originated in the subsurface of the young Mars, it could have later adapted to the changing environmental conditions and might be sustained in protected niches, perhaps associated with hydrothermal activity, even up to the present (Boston et al. 1992; Jones et al. 2011).

1.2.2 Present Mars and potential microbial metabolisms

1.2.2.1 Physical and geochemical conditions on present-day Mars

The surface of present-day Mars is generally considered hostile to all known life forms. The low atmospheric pressure (on average 6 hPa) does not allow liquid water to exist over long periods of time, diurnal temperature fluctuations can be extreme (up to 100°C) with a mean surface temperature of -65°C, and biologically harmful UV-C radiation (>190 nm) as well as cosmic ionizing radiation reach the surface due to the lack of a shielding ozone layer and magnetic field (**Table 1-3**) (Clark 1998; Horneck 2008).

Table 1-3: Environmental surface conditions on present-day Mars and present-day Earth.

Parameter	*Mars*[a]	*Earth*[a,b]
Temperature range	-123 - +25°C (-65°C)[c]	-89 - +58°C (15°C)[c]
Average surface pressure	600 Pa (6 mbar)	10^5 Pa (1013 mbar)
Atmospheric composition (%)		
CO_2	95.3	0.038
N_2	2.7	78.1
Ar	1.6	0.93
O_2	0.13	20.9
CO	0.07	~1-2 x 10^{-5}
H_2O	0.02	0-4
CH_4	1.0 x 10^{-6}	1.5 x 10^{-4}
Other	0.25	2.5 x 10^{-3}
Solar UV radiation (spectrum)	≥ 190 nm	≥ 290 nm
Solar constant	589.2 W/m^2	1367.6 W/m^2
Cosmic ionizing radiation	0.1 - 0.2 Gy/a	1 - 2 x 10^{-2} Gy/a
Gravity	0.377g	1g
Length of day	24h 37min 22.7s	23h 56min 4.1s

[a]From Horneck (2000; 2008)

[b]From Lutgens and Tarbuck (2001)

[c]Mean in parenthesis

The atmosphere of Mars consists mostly of CO_2, and contains only minor amounts of O_2 (0.13%) and other gases (**Table 1-3**). A first *in situ* analysis of the Martian regolith by the two Viking landers revealed remarkably high concentrations of sulfur and iron (Clark et al. 1977; Toulmin III et al. 1976). Later missions identified massive layered deposits on the surface of Mars, consisting of such minerals as phyllosilicates, chlorides, magnesium, calcium and ferric sulfates (e.g. kieserite, gypsum, jarosite), as well as hematite (ferric oxide) (see also **Table 1-2**, p. 6) (Christensen et al. 2000; Gendrin et al. 2005; Klingelhofer et al. 2004; Le Deit et al. 2008, 2010a, 2010b; Osterloo et al. 2008; Poulet et al. 2005; Squyres et al. 2004). Recently, the Phoenix Wet Chemistry Laboratory detected the presence of high concentrations of perchlorates, probably in the

form of $Mg(ClO_4)_2$ and $Ca(ClO_4)_2$, in the northern plains (Catling et al. 2010; Hecht et al. 2009). These salts are deliquescent, i.e. capable of capturing atmospheric water, and have eutectic freezing temperatures down to roughly -70°C (Besley and Bottomley 1969; Möhlmann and Thomsen 2011).

1.2.2.2 Water on Mars today?

Liquid water cannot stably exist at the surface of present-day Mars over long periods due to the low atmospheric pressure and low temperatures. However, recent camera images by Mars Global Surveyor show new gully deposits formed in the course of one decade that might have been caused by dry landsliding or aided by a fluid with the properties of liquid water flowing transiently on the surface (**Fig. 1-3**) (Malin et al. 2006; Pelletier et al. 2008). If liquid water was responsible for these observations, it must have emerged from some subsurface reservoir, such as groundwater aquifers produced by geothermal heating (Gaidos 2001; Mellon and Phillips 2001).

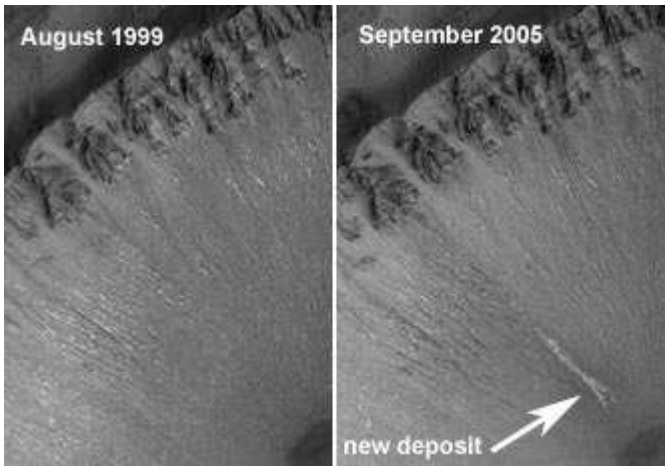

Fig. 1-3: A new gully deposit in a crater in the Centauri Montes Region on Mars that may have formed by the action of liquid water (Malin et al. 2006). Image credit: NASA/JPL/Malin Space Science Systems.

In the near subsurface regolith liquid water could occur in thin layers at the mineral-ice interface even at temperatures well below the bulk ice melting temperature (Boxe et al. 2012; Möhlmann 2010a, 2010b; Oyarzun et al. 2003). These thin films of adsorbed water could become mobile in the soil at sufficient

quantities, enabling transport processes necessary for life (Stoker et al. 2010). Furthermore, perchlorates and other soluble salts such as chlorides and sulfates lower the freezing point of water and allow the formation of cryobrines, which can extend the liquid state of water down to eutectic temperatures (Möhlmann 2010a; Möhlmann and Thomsen 2011). Undercooled liquid water in the near subsurface can also form by melting due to the solid state greenhouse effect inside ice and snow (Möhlmann 2010b). These mechanisms could provide micro-habitats with liquid water for microorganisms on Mars today even in near-surface environments.

With increasing depth, the probability of liquid water reservoirs (such as subsurface aquifers) will increase because of rising pressure and temperature. The mean geothermal gradient in the Martian crust is much lower than that of the Earth (5 K/km compared to 25 K/km; Jones et al. 2011). Based on this, estimations of the depth in which liquid water, or at least eutectic brines, may exist reach from 4 to 7 km, but the region may extend down to ~36 km (Hoffman 2001; Jones et al. 2011). Thus, it is conceivable that the deep subsurface of Mars may provide habitable conditions, especially around regions with geothermal activity (Boston et al. 1992; Dartnell et al. 2007).

1.2.2.3 Permafrost environment and methanogenesis

Water ice exists at the polar caps of Mars and in the near subsurface regolith at higher latitudes (>40°) forming permafrost soils, which has been confirmed by various measurements with instruments onboard Mars Odyssey, Mars Global Surveyor, and Mars Express (Bibring et al. 2004; Boynton et al. 2002; Mitrofanov et al. 2003, 2007; Titus 2004). The existence of permafrost soils on Mars is also indicated by polygonal ground patterns (**Fig. 1-4**). Similar environments in the polar regions on Earth such as Siberian permafrost or glacial ice harbor microorganisms, which were shown to be active at temperatures <0°C (see section **1.4.2**, p. 29 for more detail) (e.g. Christner et al. 2000; Gilichinsky et al. 2003, 2005, 2007; Mader et al. 2006; Morozova and Wagner 2007; Price 2000; Rivkina et al. 2000, 2004).

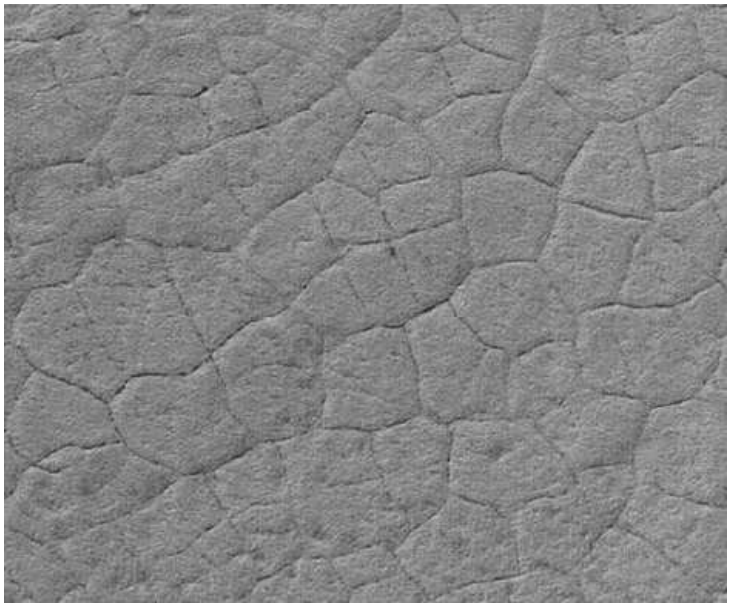

Fig. 1-4: Polygons near the south pole of Mars (86.98S, 170.68W) formed due to contraction and expansion of subsurface ice during seasonal temperature variations, image taken by Mars Global Surveyor–Mars Orbiter Camera image PIA05021. (NASA/JPL/ Malin Space Science Systems) (from Fairén et al. 2010).

Since 2003, three independent groups have reported the detection of methane (CH_4) in the Martian atmosphere in concentrations of ~10 ppb (Formisano et al. 2004; Krasnopolsky et al. 2004; Mumma et al. 2009). It was later shown that the methane originated from specific regions on Mars and that its release underwent seasonal fluctuations (Mumma et al. 2009). Because methane has a lifetime of only a few hundred years due to photochemical destruction processes (Krasnopolsky et al. 2004), the occurrence of this gas on Mars today points to a recent source of geochemical or biological origin. The most intriguing interpretation for astrobiologists is the production of methane by microorganisms in the subsurface similar perhaps to the methanogenic archaea in the permafrost soils of Earth, which generate CH_4 from H_2 and CO_2 (Gilichinsky et al. 2005; Levin and Straat 2009; Max and Clifford 2000; Wagner et al. 2001).

But even a purely abiotic methane generation could be an important finding from an astrobiological point of view because it would provide evidence for active magmatism or serpentinization processes in the Martian subsurface. Serpentinization, i.e. aqueous alteration of minerals like olivine and pyroxene, occurs already at temperatures between 40 - 90°C, which could be reached at

depths of 2 km on Mars. It can also be a source for hydrogen (Kelley et al. 2005; Lyons et al. 2005; Oze and Sharma 2005).

1.2.2.4 Potential electron donors and acceptors on Mars for chemolithotrophic metabolism

From a thermodynamical point of view, present Martian geochemistry offers various possibilities for chemolithotrophic metabolism. Chemotrophs make use of chemical disequilibria in their environment by catalyzing redox reactions, in which electrons are transferred from an electron donor to an electron acceptor via a membrane-bound electron transport chain (Seager et al. 2012). Microorganisms are extremely versatile in their ability to utilize a variety of redox couples in all conceivable combinations, if the resulting reactions are thermodynamically favorable (i.e. release free energy) (**Table 1-4**).

Availability of electron acceptors:

Sulfates, ferric iron, and perchlorates have been detected on Mars and could serve as electron acceptors in anaerobic respiration (Bibring et al. 2006; Coates et al. 1999; Hecht et al. 2009; Jorgensen 1982; Muyzer and Stams 2008; Nixon et al. 2012).

Oxygen is an important electron acceptor on Earth. It is abiotically generated in the Martian atmosphere by photochemistry, but its concentration is very low compared to Earth (0.13% at Mars surface pressure of 6 hPa) (Clark 1998). Nevertheless, aerobic respiration might be feasible as indicated by thermodynamical calculations (Jepsen et al. 2007). Aerobic metabolism could also be possible if metabolic processes producing O_2, such as perchlorate reduction, occur in close proximity (Coates and Achenbach 2004; Stoker et al. 2010). This will be further discussed in section **4.1.3**, p. 159.

Nitrate has not yet been detected on Mars, but this could have been the result of high perchlorate concentrations, which prevent its detection by the applied methods (Hecht et al. 2009). Abiotic nitrogen fixation mechanisms such as photochemical processes or impacts make its presence at least conceivable

(Clark 1998; Dasgupta et al. 2005; Jepsen et al. 2007; Manning et al. 2008; Michalski et al. 2004; Stoker et al. 2010).

Carbon dioxide as the main component of the Martian atmosphere could serve not only as an electron acceptor in methanogenesis, but also as a carbon source for autotrophic biomass formation (Jepsen et al. 2007).

Availability of electron donors:

Hydrogen is an important electron donor in many chemolithotrophic energy-generating reactions. Its concentration in the Martian atmosphere is very low, but subsurface processes like serpentinization, volcanic outgassing, and the radiolysis of subsurface water could increase the levels of Martian hydrogen considerably, at least on a local scale (Clark 1998; Kieft et al. 2005; Lin et al. 2005; Onstott et al. 2006).

If such processes take place on Mars, they could also supply other reduced gases such as CH_4 and H_2S for energy gain (Boston et al. 1992; Elwood Madden et al. 2011).

Ferrous iron is could serve as a likely electron donor because it is a major component of the basaltic rocks on Mars (Boynton et al. 2008; Brückner et al. 2008).

Table 1-4: Chemotrophic metabolic processes that could be relevant to Mars as suggested by different authors and in this work.

Microbial process	*Electron donor*	*Electron acceptor*	*Products*
	Aerobic chemotrophy		
Methane and hydrogen oxidation	CH_4, H_2	O_2	CO_2, H_2O
Reduced sulfur compound oxidation	H_2S, HS^-, S^0, $S_2O_3^{2-}$	O_2	SO_4^{2-}, S^0
Iron oxidation[a]	Fe^{2+}	O_2	Fe^{3+}, OH^- (e.g. as $Fe(OH)_3$))

continued

Table 1-4: Chemotrophic metabolic processes that could be relevant to Mars as suggested by different authors and in this work (continued).

Microbial process	*Electron donor*	*Electron acceptor*	*Products*
	Anaerobic chemotrophy		
Iron reduction[b,c]	CH_2O, H_2	Fe^{3+}, Fe-oxides (e.g. Fe_2O_3)	Fe^{2+} (e.g. FeS), or magnetite (Fe^{2+}/Fe^{3+})
Sulfur reduction[b]	CH_2O, H_2, CH_4	SO_4^{2-}, SO_3^{2-} (or SO_2), $S_2O_3^-$, S^0	SO_3^{2-} (or SO_2), $S_2O_3^-$, S^0, H_2S, H^+, CO_2
Methanogenesis/ Acetogenesis[b]	CH_2O, H_2	CO_2	CH_4, H_2O, CH_3CO_2H
Perchlorate reduction[d]	CH_2O, CH_4, Fe^{2+}, H_2S, H_2	ClO_4^-	Cl^-, CO_2, H_2O, Fe^{3+}, SO_4^{2-}
Fermentation[e]	CH_2O		Organics, H_2, CO_2, CH_4

[a]Jepsen et al. (2007)

[b]Boston et al. (1992)

[c]Nixon et al. (2012)

[d]Stoker et al. (2010)

[e]Fermentations are disproportionation reactions using endogenous electron acceptors. Organics could be provided by autotrophic primary producers

1.2.2.5 Hypothetical microbial food web in the subsurface of Mars

The metabolic processes in **Table 1-4** are interlinked and can be integrated into a potential food web that could exist in the Martian shallow or deep subsurface in regions where redox disequilibria between the oxidizing surface conditions and the more reducing subsurface environment occur (**Fig. 1-5**).

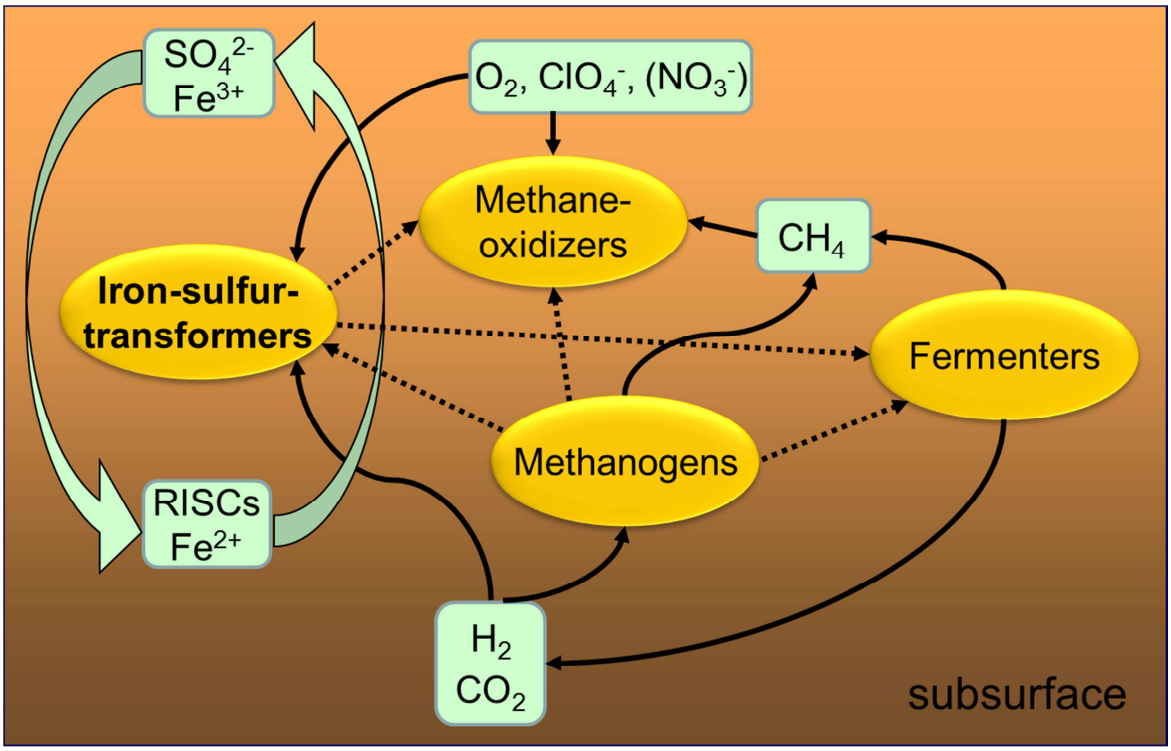

Fig. 1-5: Hypothetical food web in the shallow and deep subsurface of Mars based on electron donor and acceptor availability (see section **1.2.2.4**, p. 14). In this scenario methanogens and chemolithoautotrophic iron-sulfur transformers could be primary producers and deliver organic compounds for heterotrophic respiration or fermentation (broken arrows). Iron-sulfur transformers include autotrophic as well as heterotrophic organisms capable of ferric iron and sulfate reduction, as well as oxidation of ferrous iron and reduced inorganic sulfur compounds (RISCs). Black arrows denote uptake and production of inorganic electron donors and acceptors, green arrows show cycling of iron and sulfur compounds by a diverse group of organisms (modified from U. Szewzyk, TU Berlin).

In this hypothetical model, methanogens could play an important role as primary producers as they do in subsurface microbial communities on Earth such as permafrost soils (section **1.2.2.3**, p. 12) (Chapelle et al. 2002; Stevens and McKinley 1995; Wagner et al. 2001). This may be supported by the detection of methane in the Martian atmosphere (Formisano et al. 2004; Krasnopolsky et al. 2004; Mumma et al. 2009). Methanogenic archaea use H_2 and CO_2 (or small organic molecules like acetate) to form biomass and CH_4, which could in turn provide a substrate for methanotrophs and heterotrophic fermenters (Boone et al. 1993) (**Fig. 1-5**).

Due to the abundance of iron and sulfur minerals on Mars, bacteria involved in the transformation of these two elements are hypothesized to be another integral part of a putative Martian ecosystem (**Fig. 1-5**). The Martian mantle is more concentrated in iron than the Earth's and its dominant oxidation state is that of Fe^{2+} in the olivines and pyroxenes of volcanic rock, although Fe^{3+} is also present in the form of nanophase ferric oxides, crystalline iron oxides (hematite, goethite, magnetite), and in hydrated mineral phases like phyllosilicates and sufates (jarosite) (Boynton et al. 2008; Brückner et al. 2008; Christensen et al. 2001; 2008; Gendrin et al. 2005; Klingelhöfer et al. 2004; Milliken et al. 2008; Morris et al. 2006; Mustard et al. 2008; Poulet et al. 2005, 2007; Ruff et al. 2008). Neutrophilic iron-reducing bacteria might be plausible candidates for an analogous Martian energy-gaining metabolism, though with important limitations (Nixon et al. 2012). These bacteria usually require organics as an electron donor. Although organic compounds could be delivered to the surface by meteorites, or be produced endogenously on Mars (Abelson 1965; Chang et al. 1983; Flynn 1996; Heinrich et al. 2007), their overall abundance is probably very low. Alternatively, hydrogen can be used as an electron donor, if it is generated in the subsurface of Mars. Similar considerations would apply to sulfate reducing bacteria (Muyzer and Stams 2008; Nixon et al. 2012). While electron donor availability limits iron and sulfate reduction on Mars, metabolisms based on iron and sulfur oxidation would suffer from a scarcity of electron acceptors (Nixon et al. 2012). Yet, according to numerical modeling the most efficient lithoautotrophic metabolism under Martian surface conditions would include Fe^{2+} as an electron donor and NO_3^- or O_2 as a terminal electron acceptor despite the sparsity of oxygen and nitrogen on Mars (Jepsen et al. 2007).

Among the diversity of iron- and sulfur-oxidizing bacteria known on Earth, lithoautotrophic microorganisms that thrive in extremely acidic conditions (such as Rio Tinto) and are capable of both oxidation and reduction of iron and sulfur compounds are of particular interest with regard to possible life on Mars (Amils et al. 2007).

1.2.3 Rio Tinto as a Mars analog study site

Rio Tinto in Spain is an acidic environment driven by iron geomicrobiology, which has been proposed as a terrestrial Mars analog site (Amils et al. 2007; Clark et al. 2005; Fairén et al. 2009; Fairén 2010; Fernández-Remolar et al. 2005). The Rio Tinto system owes its existence to the oxidation of sulfidic ore deposits of the Iberian Pyritic Belt, a process, which occurs in several steps with the overall reaction:

$$4FeS_2 + 14H_2O + 15O_2 \rightarrow 4Fe(OH)_3 + 8SO_4^{2-} + 16H^+ \quad (1)$$

These oxidative reactions produce acidic water (pH <3) with high sulfate and ferric iron concentrations (**Fig. 1-6**).

Fig. 1-6: Rio Tinto waters showing the characteristic red color due to high ferric iron concentrations, and layered sediments (with kind permission of Petra Rettberg).

Despite the environmental challenges of low pH and high heavy metal concentrations, Rio Tinto water supports a diverse community of both prokaryotic and eukaryotic microorganisms, including bacteria, archaea, photosynthetic and heterotrophic protists, yeast and filamentous fungi (González-Toril 2003a, 2011; López-Archilla et al. 2001; Amaral Zettler et al. 2002). Chemolithoautotrophic iron

and sulfur-oxidizing microorganisms make a significant contribution to primary production in this habitat and play important roles in the generation and maintenance of an acidic, oxidizing environment, which drives the dissolution of iron sulfides (e.g. pyrite) and the formation of secondary minerals (Rohwerder and Sand 2007).

The iron oxides and ferric sulfates generated and preserved in sedimentary rocks deposited in terraces above the river bear close resemblance to the sulfate sediments with embedded hematite spherules detected in several regions of Mars such as Meridiani Planum or Valles Marineris (**Fig. 1-7**) (Fernández-Remolar et al. 2005; Le Deit et al. 2008; Squyres et al. 2004).

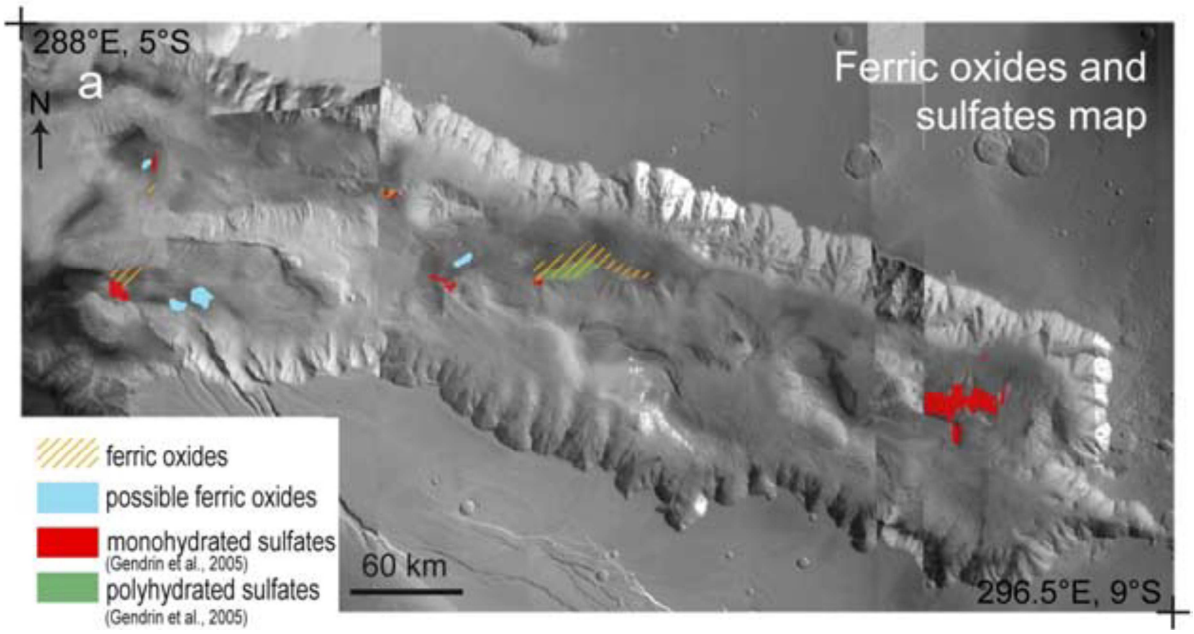

Fig. 1-7: Location map of ferric oxides and sulfates in East Candor Chasma (63.5°W-72°W, 5°S-9°S) in Valles Marineris plotted over a mosaic of HRSC (High Resolution Stereo Camera) images. Ferric oxide-rich sites are found nearby sulfate rich-areas; this suggests that both families of minerals are genetically related. Polyhydrated sulfates (in green) may be iron, magnesium or sodium-rich; monohydrated sulfates (in red) could be kieserite (from Le Deit et al. 2008).

If environmental conditions on early Mars during the deposition of these sulfate sediments were similar to those observed today in acidic environments on Earth, life could have been involved in the formation of the observed iron oxide and

sulfate minerals (Amils et al. 2007). The most important constraint for a biological origin of Martian deposits would be the availability and duration of liquid water, as discussed in section **1.2.1.2**, p. 8. The existence of jarosite, for example, does indicate a wet, acidic environment during the period of its formation on early Mars, but the fact that we can detect those deposits today suggests that the timespan of aqueous alteration was geologically short, as the preservation of jarosite requires arid or evaporative conditions (Elwood Madden et al. 2004; King and McSween 2005). However, recent laboratory experiments have shown that jarosite produced by sulfur oxidizing bacteria grown aerobically and anaerobically on pyrrhotite (an iron sulfide mineral) is more stable than jarosite produced either abiotically or by the action of iron-oxidizing bacteria and does not require arid conditions for its preservation. Thus, stable jarosite as observed on Mars, may be a biomarker for microbial sulfide oxidation (Norlund et al. 2010). Sulfides, as substrates for the proposed oxidation and mineral formation processes, might occur plausibly in the subsurface of Mars within reducing micro-niches even in otherwise oxidized sediments (Jørgensen 1977).

The chemolithoautotrophic iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, and heterotrophic iron reducers of the genus *Acidiphilium* spp. account for most of the prokaryotic biomass detected by molecular methods in Rio Tinto (González-Toril et al. 2003b). Some sulfate reducing activity was also found, associated to sediments near the origin of the river, but in rather low amount (González-Toril et al. 2011). In the subsurface sediments of the Iberian Pyritic Belt, methane and hydrogen have been detected, suggesting geothermal H_2 generation and related methanogenic activity in micro-niches with mildly acidic and reducing conditions (Amils et al. 2007; Sanz et al. 2011). This gives credit to the proposed hypothetical Martian food web which includes interactions between methanogens and iron-sulfur bacteria (**Fig. 1-5**, p. 17). To investigate the relevance of the suggested Martian food web, members of the different groups of bacteria should be selected and studied in detail with respect to their ability to grow and survive under close-to-Martian environmental conditions. This work focuses specifically on acidophilic iron-sulfur transforming bacteria.

1.3 Acidophilic iron-sulfur bacteria

Acidophilic iron-sulfur bacteria are found in acidic environments of two major origins: either associated with volcanic activities or with mining activities (Johnson 1998). In the first case, the biological oxidation of elemental sulfur from volcanic gases leads to the generation of acidity:

$$2S^0 + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+ \quad (2)$$

In the second case, sulfidic minerals like FeS_2 (pyrite) are exposed to the combined action of oxygen and water, which leads to the microbially-mediated transformation of insoluble metal sulfides into their water-soluble sulfates, a process referred to as bioleaching or biomining. The critical role of microorganisms in mineral decomposition is two-fold: i) the organisms regenerate the chemical oxidant ferric iron by enzymatic oxidation of ferrous iron (equation 3), and ii) by oxidizing intermediary sulfur compounds, such as elemental sulfur resulting from mineral dissolution (equation 2), protons are produced which aid in the hydrolysis attack of the minerals (Rawlings 2002; Rohwerder et al. 2003; Sand et al. 2001; Schippers and Sand 1999).

$$14Fe^{2+} + 3.5O_2 + 14H^+ \rightarrow 14Fe^{3+} + 7H_2O \quad (3)$$

The acidic environment offers various advantages to iron-oxidizing microorganisms. The solubility of Fe^{3+} is greatly increased at pH <2.5, and auto-oxidation of Fe^{2+} is slowed down. In addition, the energetics of Fe^{2+} oxidation are more favorable at low pH because the redox potential of the oxygen/water couple becomes more positive (Ferguson and Ingledew 2008). On the other hand, low pH poses a significant challenge as the cytoplasm of acidophiles must be maintained close to neutrality, which requires continual proton extrusion against a steep gradient (Cobley and Cox 1983).

A diversity of acidophilic iron-sulfur transforming bacteria has been characterized to date, including autotrophs, mixotrophs, and heterotrophs, as well as mesophiles and thermophiles (Johnson 1998). A more detailed description of the two species chosen in this study is given in the following subsections.

1.3.1 Acidithiobacillus ferrooxidans

Acidithiobacillus ferrooxidans (formerly *Thiobacillus ferrooxidans*) was chosen as a model iron-sulfur bacterium because it is the most widely studied meso-acidophilic chemolithotroph and displays a high metabolic versatility (Kelly and Wood 2000; Rohwerder et al. 2003; Temple and Colmer 1951). The complete genome (size ~2.9 Mbp) of *A. ferrooxidans* type strain ATCC 23270 has been sequenced and annotated (Valdés et al. 2008). The rod-shaped (0.5 x 1 µm), Gram-negative γ-proteobacterium, thriving optimally at 30°C and pH <4, is able to gain energy by the aerobic oxidation of ferrous iron, RISCs (e.g. H_2S, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$), hydrogen, and formate (Drobner et al. 1990; Lorbach et al. 1993; Pronk et al. 1991b). In addition, it has the ability to use electron acceptors other than oxygen such as ferric iron for the oxidation of sulfur and hydrogen, and sulfur for the oxidation of hydrogen (**Fig. 1-8**) (Ohmura et al. 2002; Pronk et al. 1992). *A. ferrooxidans* is tolerant against high concentrations of heavy metals such as As, Cu, and Zn, which occur in its natural habitats (Dopson et al. 2003; Valdés et al. 2008), and is even able to use some of them (Mo^{6+}, Cu^{2+}, Co^{2+}) as electron acceptors in sulfur oxidation (Sugio et al. 1988, 1990). Due to this metabolic versatility, *A. ferrooxidans* plays a crucial role in the iron and sulfur geochemical cycles in acidic environments on Earth such as acid drainage waters associated with sulfide mineral and coal mines.

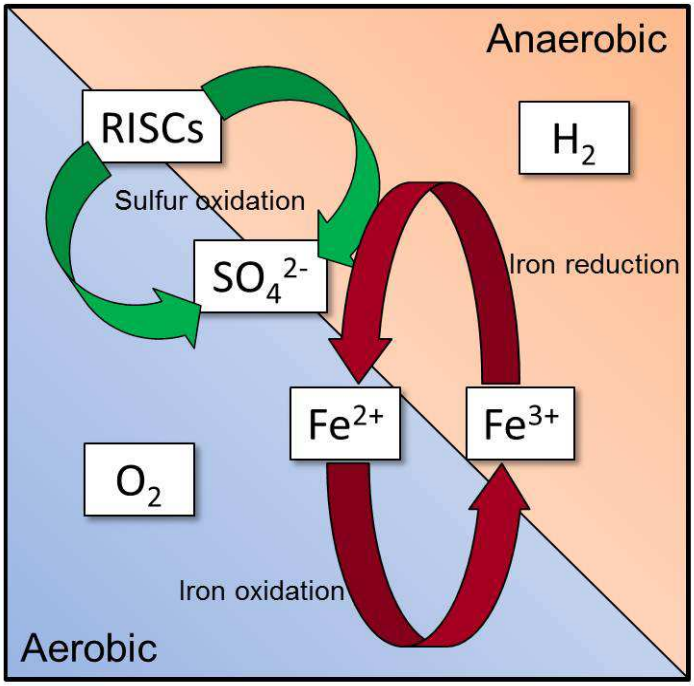

Fig. 1-8: Metabolic versatility of *A. ferrooxidans*. Oxidation of RISCs can proceed aerobically with O_2, or anaerobically with Fe^{3+} as electron acceptor. Some strains are capable of S and Fe^{3+} reduction using H_2 as an electron donor. In addition, aerobic H_2 oxidation is possible.

The obligate autotroph fixes CO_2 via the Calvin cycle (Gale et al. 1967; Heinhorst et al. 2002). Because Fe^{2+} is too weak a reductant for a direct reduction of $NAD(P)^+$, the electrons derived from the oxidation of Fe^{2+} are driven 'uphill' along a large transmembrane proton gradient by the expenditure of energy, a process known as reverse electron transport (Ferguson and Ingledew 2008). Some strains of *A. ferrooxidans* are diazotrophic, capable of fixing atmospheric nitrogen, making the organism an important primary producer (Mackintosh 1978, Norris et al. 1995).

Organic compounds, especially organic acids, are inhibitory to *A. ferrooxidans* because they dissipate the transmembrane pH gradient, which limits the cultivability of this organism on agar plates (Harrison 1984; Matin 1978; Pronk et al. 1991b). In their natural environments, association with heterotrophic acidophiles (e.g. *Acidiphilium acidophilum*) ensures the removal of potentially toxic organic compounds (Liu et al. 2011; Marchand and Silverstein 2003).

1.3.2 Sulfobacillus thermosulfidooxidans

Sulfobacilli are moderately thermophilic (T_{opt} = 50°C), Gram-positive bacteria found in acidic soils and waters of geothermal areas, mineral and ore deposits, where the exothermic biological oxidation reactions lead to an elevated temperature (Holmes and Bonnefoy 2007; Watling et al. 2008). *Sulfobacillus thermosulfidooxidans* was the first of currently four described species of this genus to be isolated (Golovacheva and Karavaiko 1978). Strains of this species are characterized by a mixotrophic metabolism with limited autotrophic and heterotrophic growth. They are able to oxidize a broad range of substrates for energy generation, including ferrous iron, RISCs, sulfidic minerals, and organic compounds such as sugars and organic acids, under aerobic conditions. Furthermore, they are able to grow in the absence of oxygen, using ferric iron as an electron acceptor and tetrathionate or glycerol as electron donors (Bridge and Johnson 1998; Norris et al. 1996). This means that they can contribute to the final stages of organic matter mineralization under aerobic and anaerobic conditions (Krasil'nikova et al. 2010).

For efficient CO_2 fixation *S. thermosulfidooxidans* requires CO_2-enriched air, trace amounts of yeast extract (0.01-0.2%), or close association with heterotrophic iron-oxidizing bacteria such as *Acidimicrobium ferrooxidans* (Clark and Norris 1996; Rawlings 2007). Low levels of intracellular ATP have been observed in *S. thermosulfidooxidans* during auto- and heterotrophic growth mode, which was suggested as a possible explanation for the cessation of growth after several transfers under these conditions (Tsaplina et al. 2007; Zhuravleva et al. 2008, 2009).

The distinguishing feature of these rod-shaped cells (1 - 4 µm in length) and the reason why they were chosen as model organisms in this study, is their ability to form endospores in response to nutrient depletion (**Fig. 1-9**) (Bogdanova et al. 2002; Norris et al. 1996). Endospores are highly specialized dormant forms with no experimentally detectable metabolic activity that remain viable over long periods of time in order to germinate into vegetative cells when conditions become more favorable. From an astrobiological point of view, endospores hold particular interest due to their high resistance against diverse kinds of

environmental stress factors such as desiccation, temperature extremes, and chemical agents (Nicholson et al. 2000; Setlow 2006).

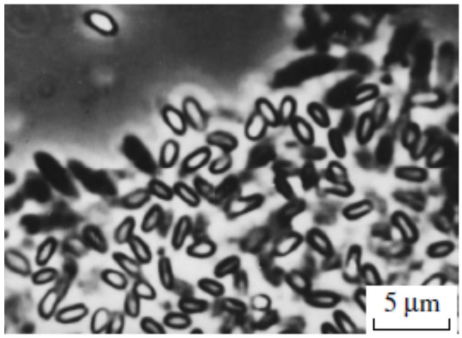

Fig. 1-9: Phase contrast microscopic image of *S. thermosulfidooxidans* endospores (oval, refractive structures) (from Bogdanova et al. 2002).

1.4 Environmental stressors and microbial resistance strategies

Many environmental factors are to be considered in assessing the ability of microorganisms to grow or survive under present Martian conditions, among them nutrient and energy availability (as discussed in **1.2.2.4**, p. 14), water availability and activity (salinity), low temperature, low pressure, and radiation environment (Beaty et al. 2006; Horneck 2000, 2008). Any organism potentially present on Mars as part of a hypothetical food web, whether existing in the permafrost soils of the present, or in an acidic ecosystems in the past, would have to cope with such physical strains at least periodically as a consequence of a constantly changing environment. The environmental stress parameters with the strongest impact on microbial life forms will be briefly summarized in the following sections.

1.4.1 Water stress

Life forms on Earth requires liquid water to maintain its structural integrity and carry out biochemical reactions. The chemical availability of liquid water can be

expressed in terms of its activity (water activity, a_w), which is related to the relative humidity (RH) of a system by:

$$a_w = RH/100 \quad (4)$$

if the water and the atmosphere of the system are in equilibrium. The a_w of pure water is 1.0, a value that decreases with increasing solute concentrations. Desiccation (matric-induced water stress) is often regarded as an extreme form of osmotic stress, though its effects on cells are quite distinct from that imposed by high salinity and lead to different adaptation mechanisms by cells (Potts 1994).

1.4.1.1 Desiccation

Removing water from cells by air-drying is fatal to the majority of organisms because of the damage inflicted on all cellular components, including lipids, proteins, and nucleic acids. The loss of the hydration shell around phospholipids during air-drying causes phase transitions of membranes, resulting in leakage upon rehydration. Proteins with reduced hydration undergo conformational changes, which lead to the loss of enzymatic activity and the accumulation of free radicals due to altered control of electron transport. In addition, the process of desiccation is inherently DNA-damaging: dried bacterial cells exhibit a substantial number of DNA single-strand breaks, double-strand breaks, oxidative base modifications, abasic sites, and cross-links (Billi and Potts 2002; Dose et al. 1992, 2001; Potts 1994).

However, a small but taxonomically diverse group of organisms, called anhydrobiotes, has acquired strategies to tolerate periods of desiccation and revive upon rehydration, either by preventing desiccation-induced damage or by repairing it after rewetting (Alpert 2005). Among the most common adaptations to desiccation is the accumulation of sugars such as trehalose and sucrose (compatible solutes) in the cytoplasm, which may replace water molecules around membrane phospholipids and proteins, preventing the transition of membranes to gel phase and protein denaturation (water replacement hypothesis) (**Fig. 4-3**, p. 176). In addition, their potential to form glasses at low water contents (vitrification), in which molecular diffusion is extremely reduced,

helps avoid uncontrolled chemical reactions (Potts 2001). Protection systems against reactive oxygen species (ROS) accumulating during dehydration are a further adaptive strategy employed by anhydrobiotes such as *Deinococcus radiodurans* (Fredrickson et al. 2008).

Desiccation-tolerance may be considered a key feature of microorganisms existing on Mars, where liquid water is scarce and probably only periodically present. In addition, the extensive salt deposits found on the surface of Mars suggest that any liquid water would take the form of highly concentrated brines, which pose an additional challenge to life (Tosca et al. 2008).

1.4.1.2 Salt stress

The lowest solute-induced water activity, for which microbial growth has been shown, is 0.62, a concentrated sucrose solution, which harbored xerophilic fungi (Harris 1981). However, the biological stresses of organic solutes differ from those imposed by inorganic salts, and for such systems an a_w of 0.75 (a saturated NaCl solution) is considered the growth limit (Grant 2004). Although microorganisms have been detected in natural environments with lower a_w, e.g. in the Dead Sea brine (a_w ~0.67), these are at present believed to be survivors from the brief periods of dilution with fresh water (Beaty et al. 2006). Calculations of the a_w at sites on Mars where salts precipitated from brines indicate that the evaporation of Meridiani groundwater led to a sustained a_w of ≤0.86, a value which is within the limits of terrestrial extremophiles (Tosca et al. 2008).

Hypersaline environments on Earth, especially in permanently cold regions, could serve as analogs for Martian brines. These include cryopegs within permafrost (Gilichinsky et al. 2005), brine veins within glacial ice (Deming 2002), or evaporation ponds in the dry regions of Antarctica (DasSarma 2006). Microorganisms thriving in these environments, must constantly balance the osmotic pressure of their cytoplasm with that of the surrounding medium in order to avoid plasmolysis. Halophilic microorganisms have developed two alternative strategies to cope with high salinity. One is based on the accumulation of molar concentrations of inorganic ions, mainly K^+ and Cl^-, in the cytoplasm ('salt-in' strategy) and is employed by halophilic archaea and some groups of bacteria (Oren 2008, 2011). These organisms have adapted their entire intracellular

enzymatic machinery to high ionic concentrations by an excess amount of acidic amino acids and a low percentage of hydrophobic amino acids. Most of their proteins even require high salt concentrations for stability and functionality (Lanyi 1974; Oren 2008).

The alternative option, which requires higher energetic costs than the 'salt-in' strategy, involves biosynthesis of organic compatible solutes (or their accumulation from the medium). Compatible solutes are small, generally uncharged, water-soluble molecules, which do not affect enzymatic activity and can therefore be amassed at high concentrations without adaptations in the proteome. Examples include polyols like glycerol, sugars such as trehalose and sucrose, and amino acid derivatives such as glycine betaine and ectoine (**Fig. 1-10**) (Galinski and Trüper 1994; Kempf and Bremer 1998). They are accumulated by many organisms, not restricted to prokaryotes and serve not only as osmo-, but also as cryo- and xeroprotectants (Kempf and Bremer 1998).

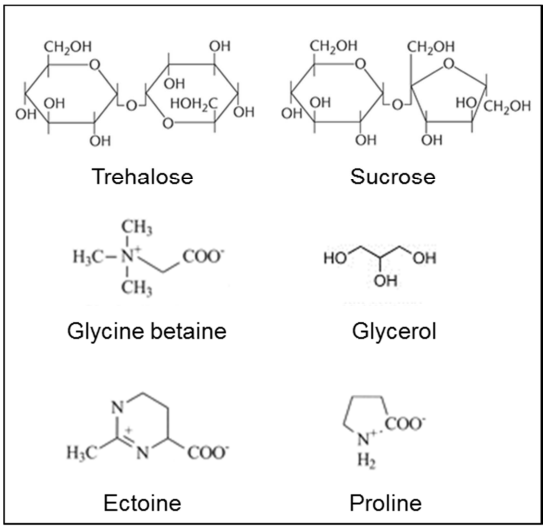

Fig. 1-10: Structure of some common compatible solutes.

1.4.2 Low temperatures

Low temperatures exert severe physicochemical constraints on living organisms including increased water viscosity, decreased molecular diffusion rates, and reduced fluidity of cellular membranes and biochemical reaction rates (Piette et al. 2011). Furthermore, at temperatures below the freezing point of water,

extracellular and intracellular ice crystal formation can physically damage cell membranes and lead to osmotic stress due to exclusion of solutes from the ice during its formation (Fuller 2004). Nevertheless, active microbial life has been found in subfreezing environments on Earth where external solute concentrations are high enough to retain liquid water, for instance in salty cryopegs in permafrost at -10°C (Gilichinsky et al. 2005), or in the brine veins of polar sea ice at -20°C (Deming 2002). Since reaction rates are slowed down with a drop in temperature, the detection of microbial activity at low temperature poses a significant challenge. Different studies have compiled evidence that bacteria can be metabolically active at subfreezing temperatures down to -33°C, albeit only at levels sufficient for maintenance and repair processes, but not for reproduction and biomass formation (Bakermans et al. 2003, 2011; Breeze et al. 2004; Carpenter et al. 2000; Christner 2002; Jakosky et al. 2003; Junge et al. 2006; Miteva et al. 2007; Murray et al. 2012; Rivkina et al. 2000, 2002). Key adaptations to cold environments include improved structural flexibility of proteins and enzymes, and changes in lipid composition to maintain membrane fluidity (Feller 2010; Russell 2007). To avoid the damaging effects of ice crystal formation, organisms can synthesize cryoprotectants such as antifreeze proteins and compatible solutes (see previous section), which serve as freezing point depressors and reduce the potential for osmotic injury during the freezing and thawing process (Cleland et al. 2004; Wang 2000).

The temperature at the surface of present Mars ranges from -123°C to +25°C depending on time of day, season, and geographical latitude, and can fluctuate diurnally over 100°C (Horneck 2000; Schofield et al. 1997). In the subsurface, these diurnal fluctuations converge to a mean temperature that depends on the depth and the thermal conductivity of the regolith. In certain latitudes, temperatures may rise >0°C at 5 m beneath the Martian surface, but at most times and in most places, Martian environments will exhibit subfreezing conditions (Clark 1998; Jones et al. 2011).

1.4.3 Radiation

Radiation damages cells in a complex manner, either through direct absorption of the energy by macromolecules, or indirectly through the production of free radicals (Nelson 2003). Damage to DNA is viewed as the major cause of the cytotoxic and mutagenic effects of radiation (Hutchinson 1966). The type of radiation determines the damage profile and the counterstrategies employed by microorganisms.

UV radiation is mainly a problem for life forms at or very close to the surface of a planetary body because of efficient shielding by most materials, while ionizing radiation can penetrate deeper into the ground and can also originate from subsurface sources (see below).

1.4.3.1 Ultraviolet (UV) radiation

UV radiation (spanning a range of the electromagnetic spectrum from 100 to 400 nm) is a strong mutagen, whose biological effects are dependent on the wavelength. DNA absorbs strongly in the UV-C part of the spectrum (190-280 nm), and the absorption of photons induces the formation of pyrimidine dimers between adjacent nucleobases and other minor photoproducts, which arrest the movement of replication forks. In addition, UV-C radiation causes protein aggregation by disrupting certain types of disulfide bonds (Chan et al. 2006). UV-A (315-400 nm) and UV-B (280-315 nm) radiation damage cells mainly indirectly through the induction of ROS, which can cause DNA strand breaks and other kinds of oxidative damage to macromolecules (Cadet et al. 2005; Wiseman and Halliwell 1996). Strategies to tolerate the physiological stress caused by UV radiation include the development of internal or external UV screens and antioxidant systems as well as cellular mechanisms to repair UV-induced damage (Friedberg 2003; Wynn-Williams and Edwards 2002).

On present-day Earth, the stratospheric ozone layer protects the surface from UV radiation wavelengths below 290 nm. However, before the oxygenation of the atmosphere, possible first life forms developing on Archaean Earth (3.8 – 2.5 Ga ago) would have been confronted with a UV radiation climate similar to that of present-day Mars, where CO_2 absorption allows radiation above 200 nm to

penetrate the atmosphere (Cockell and Horneck 2001; Kuhn and Atreya 1979). The short-wavelength UV radiation was identified as one of the most detrimental factors that leads to the inactivation of terrestrial microorganisms exposed to simulated Martian surface conditions, but it was also shown that dust layers of only 0.5 - 1 mm in thickness as well as shielding by upper cell layers in multicellular structures can provide sufficient protection against this environmental stressor (e.g. Cockell et al. 2005; Imshenetsky et al. 1967; Mancinelli and Klovstad 2000; Osman et al. 2008; Pogoda de la Vega et al. 2007; Schuerger et al. 2003, 2005, 2006; Zhukova and Kondratyev 1965).

1.4.3.2 Ionizing radiation

Ionizing radiation is high energy radiation with the ability to ionize molecules. It is divided in electromagnetic (X-ray, γ radiation) and particulate (α and β particles) radiation. Although ionizing radiation can inflict damage on macromolecules directly through the absorption of photons, damage to lipids, DNA and proteins occurs predominantly through the action of ROS produced by water radiolysis (von Sonntag 1987). A wide array of DNA damage is caused by ionizing radiation including single and double strand breaks and base modifications (Cox and Battista 2005). Among the DNA lesions most challenging to repair for most cells are DNA double strand breaks because no DNA strand can serve as an intact template in this case (Daly 2009). Some organisms are inactivated by only a few double strand breaks per haploid genome (e.g. *Escherichia coli*), while others, *D. radiodurans* being the most prominent example, are able to repair even a highly fragmented genome without error (Krasin and Hutchinson 1977; Zahradka et al. 2006). In *D. radiodurans*, the protection of proteins from oxidative damage (carbonylation) is at least equally important as the existence of highly efficient DNA repair pathways (Daly 2009).

The two major sources for ionizing radiation on a planetary body are the decay of radioactive isotopes in the crust and the flux of cosmic rays. While the surface of Earth is largely sheltered from cosmic radiation by the geomagnetic field and the atmospheric column, the unimpeded flux of cosmic rays onto the Martian surface is one of the major threats to life on the surface and shallow subsurface as the cascades of secondary particles created by the energizing radiation interacting

with regolith material can penetrate several meters underground (Dartnell 2011). Beneath this area, the remaining source of ionizing radiation is the radioactive decay, which is believed to be much lower than for terrestrial rocks, but still sufficient to inactivate dormant bacteria within an estimated time span of 40 million years (Dartnell et al. 2007; Pavlov et al. 2002).

1.4.4 Life in biofilms as a survival strategy

A universal survival strategy of organisms in their natural habitats is the formation of biofilms. Probably the dominant form of microbial life in nature, biofilms are complex communities of microorganisms, adherent to each other (aggregates) or associated to surfaces, and embedded in a matrix of extracellular polymeric substances (EPS) (Costerton et al. 1995; Flemming and Wingender 2010; Karunakaran et al. 2011; Stoodley et al. 2002). The EPS matrix, or ‘slime’, can be regarded as an emergent property of microorganisms living in a biofilm, conferring multiple advantages over the planktonic style of life (Flemming 2011). Different components of the EPS, which are excreted by the microbial cells, serve various functions. Polysaccharides provide mechanical stability and bind extracellular enzymes, which enable the EPS matrix to act as an external digestion system. A considerable proportion of the EPS is made up of extracellular DNA (eDNA) facilitating cooperative genetic exchange. Similarly, intercellular communication by quorum sensing is favored compared to the bulk liquid phase because signaling molecules are accumulated within the EPS matrix. Metabolic activity of the microorganisms may lead to the formation of strong gradients (e.g. of oxygen) creating microenvironments that can be quite distinct from the surrounding medium (Flemming et al. 2007; Flemming and Wingender 2010).

Due to the water retention capacity of the highly hydrated EPS compounds, cells in biofilms are buffered against water potential fluctuations in soil (Or et al. 2007). During dehydration, the EPS network shrinks, increasing interactions between the molecules. This may lead to skin formation, which reduces water diffusion and protects inner cell layers from further water loss (Or et al. 2007). The cyanobacterium *Nostoc commune*, for example, forms thick biofilms enabling

populations to survive desiccation and freeze-thawing (Hill et al. 1997; Tamaru et al. 2005). The EPS matrix also protects organisms from oxidizing or charged biocides, some antibiotics and metallic cations, predation, and UV radiation (Flemming and Wingender 2010). This last effect might be even enhanced in conjunction with iron precipitates formed by iron oxidizing bacteria, which are partly embedded into their EPS matrix (Braun et al. 2009; Miot et al. 2009; Schädler et al. 2009; Schieber et al. 2006).

Biofilms can play an important role in the colonization and microbial weathering of minerals by mediating the attachment of cells and forming a reaction compartment close to the surface. The biooxidation of iron sulfides by acidophilic bacteria, for instance, is considerably accelerated by the complexed Fe^{3+}-ions in their EPS matrix (Sand and Gehrke 2006).

Thus, biofilms help microorganisms create specific microenvironments, in which conditions for life are more favorable than in the surroundings. This would be a valuable adaptation for potential microbial inhabitants of the Martian subsurface, where they might be subjected to periodic desiccation and cycles of thawing and freezing. An association to soil particles in the Martian subsurface might enable metabolic activity for bacteria even at temperatures below -20 °C (Junge et al. 2004). Moreover, the spatial organization of microorganisms in biofilms, which permits the formation of microconsortia exchanging metabolic products, could be essential for a functioning food web in a low-energy environment like the Martian subsurface.

A substantial fraction of cells within a biofilm may be inactive or dormant, entering a state termed viable-but-nonculturable (see next section), in which they are also more resistant to antimicrobial treatment, and can be ‘revived’ when more favorable conditions return (Oliver 2005; Flemming 2011).

1.5 Maintenance metabolism and methods for viability determination

In most astrobiological studies to date, which have tested the ability of microorganisms to withstand Mars-like stress conditions (e.g. Diaz and Schulze-Makuch 2006; Nicholson and Schuerger 2005; Pogoda de la Vega et al. 2007;

Schuerger et al. 2003, 2006; Schuerger and Nicholson 2006; and many older studies as reviewed by Hansen 2007), survival was assessed solely by methods based on the ability of microorganisms to grow and reproduce, such as the plate count assay (growth of colonies on solid medium) and the Most Probable Number (MPN) technique (Kell et al. 1998; Koch 1994; Russek and Colwell 1983). However, periods of growth and reproduction may actually represent the exception for microorganisms in natural habitats, where they commonly exist in a mode of maintenance metabolism. Maintenance metabolism has been defined as the energy expended on all cellular functions unrelated to growth (Pirt 1965, 1982). This would include even nongrowth components (e.g. shifts in metabolic pathways or storage of polymers) that are not part of basic physiological maintenance – when maintenance is alternatively defined as the endogenous metabolism of a cell (Herbert 1958; Stouthamer et al. 1990; van Bodegom 2007). Physiological maintenance comprises osmoregulation, motility, defense and repair mechanisms and macromolecular turnover (see also section **4.2.1**, p. 169) (reviewed in van Bodegom 2007). Microorganisms with maintenance metabolism or those in a state of dormancy are obviously not dead, but would not be detected by conventional methods based on culturability, a phenomenon that has been termed viable-but-nonculturable (VBNC) (Oliver 2005).

It has been estimated that current standard cultivation techniques in the laboratory capture only ~1% of the total diversity of microorganisms from the environment, which may partly be due to a lack of knowledge of all the factors needed for their growth (Allen et al. 2004; Keller and Zengler 2004). But even strains usually cultivable in laboratories can temporarily lose their culturability in response to environmental stressors, yet remain alive and capable of renewed metabolic activity (resuscitation) (Oliver 2000). This is particularly important when pathogens, e.g. *Vibrio cholerae*, enter such VBNC states, in which they are not detectable by standard cultivation methods while retaining their virulence (Colwell et al. 1985, 1996).

To address this issue, different methods for determining the physiological state of microbial cells have been developed (**Table 1-5**) (Breeuwer and Abee 2000; Khan et al. 2010; Rochelle et al. 2011). Some of these methods are designed to detect metabolic activity such as respiration by the reduction of tetrazolium salts

(Rodriguez et al. 1992; Sherr et al. 1999; Smith and McFeters 1997), intracellular esterase activity (Breeuwer et al. 1995; Lundgren 1981), or substrate uptake (Yoshioka et al. 1996). Others are used to assess the integrity of cellular components. Membrane integrity is tested via inclusion or exclusion of fluorescent dyes as in the Live/Dead® BacLight kit (Berney et al. 2007; Boulos et al. 1999; Leuko et al. 2004). The presence of nucleic acids can be quantified by polymerase chain reaction (PCR), real-time quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR) (del Mar Lleò et al. 2000; Islam et al. 1993; Masters et al. 1994; Polz and Cavanaugh 1997; Postollec et al. 2011; Sheridan et al. 1998; Sikorsky et al. 2004; Trampuz et al. 2006), or fluorescence-*in situ*-hybridization (FISH) (Amann et al. 1995). The direct viable count (DVC), which measures cell elongation (growth in the presence of nutrients and a gyrase inhibitor to prevent cell division), can be used in combination with FISH for improved detection (Kalmbach et al. 1997; Kogure et al. 1984). Several of these methods should be used in conjunction in order to give a comprehensive picture of the effects of environmental stressors on the viability of cells (Villarino et al. 2000).

Table 1-5: Criteria and methods for the assessment of microbial viability (references given only for those methods not mentioned in the text).

Measured parameter	*Method*
Growth and reproduction	Plate count method
	Most Probable Number assay (MPN)
	Direct viable counts (DVC) (inhibition of cell division and enumeration of elongated cells)
Integrity of cellular components	Membrane: Dye exclusion methods, e.g. SYTO9 + propidium iodide (Live/Dead®), or propidium monoazide + qPCR[a];
	intact polar membrane lipids (HPLC/MS)[b]
	DNA: DAPI[c], Genomic PCR Fingerprinting, TUNEL[d], qPCR
	rRNA: Fluorescence in situ hybridization (FISH)

continued

Table 1-5: Criteria and methods for the assessment of microbial viability (references given only for those methods not mentioned in the text) (continued).

Measured parameter	*Method*
Activity (metabolism, energy generation)	Respiration: reduction of tetrazolium dyes (e.g. CTC)
	Enzyme activity: e.g. esterase activity (fluorescein diacetate), substrate uptake or product formation
	Membrane potential: potential dependent uptake of oxonol or carboxycyanine dyes[d]
	transcription (mRNA): reverse transcription-PCR
	Microcalorimetry[e] (non-specific)
	ATP content[f]

[a] Nocker et al. (2006, 2007)
[b] Sturt et al. (2004)
[c] Porter and Feig (1980)
[d] Billi (2009)
[e] Critter et al. (2004); Raubuch and Beese (1998); Schroeter and Sand (1993); Sparling (1981)
[f] Fajardo-Cavazos et al. (2008); Turner et al. (2010); Venkateswaran et al. (2003)

1.6 Open fields

One area of established astrobiological research is the investigation of the physical limits for life and the adaptability of microorganisms to extreme conditions in order to enable scientists to draw conclusions about the likelihood of life on other planets or moons in our solar system and beyond. Mars has always been of particular interest because of its closeness to Earth and its location at the edge of the habitable zone around our sun (defined as the region around a star, in which an Earth-like planet can maintain surface liquid water) (Lammer et al. 2009). On these grounds, multiple studies have been conducted since 1958 to explore the response of terrestrial prokaryotes and organic molecules to simulated Martian conditions.

The majority of these investigations have focused on the survival of prokaryotic pure-cultures of such organisms as *D. radiodurans*, *E. coli*, *Bacillus* spp., *Clostridium* spp., *Pseudomonas* spp., *Streptomyces* spp., *Vibrio* sp., halophilic archaea, cyanobacteria, lichens, and others (reviewed in Hansen 2007; de Vera

et al. 2010; Hansen et al. 2009; Johnson et al. 2011; Pogoda de la Vega et al. 2007; Smith et al. 2009a). Though some of these organisms are extremophilic, most of them are heterotrophs, relying on the availability of complex organic molecules for nutrition, which at present are not believed to be widely spread on Mars (ten Kate 2010). Studies with organisms that are, from a thermodynamical point of view, relevant to Mars, are comparatively rare and have comprised methanogenic archaea (Kral et al. 2011; Morozova et al. 2007) and only recently one study with the iron-oxidizing bacterium *A. ferrooxidans* (Gómez et al. 2010).

However, when attempting to observe active metabolism or even growth of terrestrial microbes under Mars-simulated conditions, using model organisms that could grow with potential *in situ* resources on Mars would seem more relevant than the use of heterotrophic extremophiles. Due to technical limitations, survival instead of growth under Mars conditions was the focus of most Mars simulation experiments to date with few exceptions (de Vera et al. 2010; Kral et al. 2011; Pavlov et al. 2010, Schuerger and Nicholson 2006), but only in one of these reports, thermodynamically-relevant model organisms were used (Kral et al. 2011 with methanogenic archaea).

Furthermore, as mentioned before (section **1.5**, p. 34), many of the studies focusing on survival of microorganisms, have employed solely culturability-based methods, although lack of growth in or on conventional media does not indicate death of the organisms. For a deeper understanding of the response of organisms to environmental stress parameters it is important to apply a set of different methods aimed at determining the structural and functional integrity of cells, and not to draw conclusions based solely on reproductive ability.

1.7 Objectives

By reviewing the data currently available on the surface geochemistry and physical conditions of Mars in the past and present (see section **1.2**, p. 4), different candidate organisms within a hypothetical subsurface food web were identified that might be able to use energy sources available on Mars (**Fig. 1-5**, p. 17). For this study, two species of acidophilic iron-sulfur bacteria were chosen: *A.*

ferrooxidans and *S. thermosulfidooxidans* (section **1.3**, p. 22). *A. ferrooxidans* is by far the most extensively characterized acidophilic chemolithotroph and is a suitable model organism due to its autotrophic metabolism. *S. thermosulfidooxidans* was chosen owing to its ability to form endospores.

The aim was to determine, both experimentally and theoretically, if these organisms could indeed serve as terrestrial models for putative Martian life forms by answering the following questions:

- Are the selected organisms ecologically relevant for Mars, i.e. can they grow using only the nutrients and energy sources present there?
- How resistant are they to stress conditions they are likely to encounter on Mars?
- Does biofilm formation affect stress resistance?
- Which viability indicators can be used in acidophiles to determine the effect of different stress conditions?

For the purpose of comparing the obtained data with a highly resistant model organism, the heterotrophic Gram-positive bacterium *Deinococcus geothermalis* that has been recognized for its propensity to form biofilms (Kolari et al. 2002), was also subjected to selected experiments.

2 Materials and Methods

2.1 Chemicals

Table 2-1: Chemicals used in the experiments.

Name of substance	*Chemical formula*	*Manufacturer*
Agarose		Serva[a]
Ammonium acetate	NH_4CH_3OO	Merck[b]
Ammonium sulfate	$(NH_4)_2SO_4$	Roth[c]
Bacto proteose peptone		Difco[d]
Bacto tryptone (casein hydrolysate)		Difco
Bacto yeast extract		Difco
Boric acid	H_3BO_3	Sigma[e]
Calcium chloride	$CaCl_2$ x 2 H_2O	Merck
Calcium nitrate tetrahydrate	$Ca(NO_3)_2$ x 4 H_2O	Merck
Chloroform-isoamyl alcohol (24:1)		AppliChem[f]
Citifluor AF1 (glycerol/PBS)		Science Services[g]
Cobalt chloride	$CoCl_2$	Merck
Copper(II) chloride	$CuCl_2$	Sigma
D-glucose-monohydrate	$C_6H_{12}O_6$ x H_2O	Merck
Dipotassium phosphate	K_2HPO_4	Sigma
Disodium hydrogen phosphate dihydrate	Na_2HPO_4 x 2 H_2O	Merck
DNA Ladder 1kb, 0.5 µg/µl		Fermentas[h]
DNA loading dye 6x		Fermentas
Ethanol, absolute	C_2H_6O	Merck
Ethylenediaminetetraacetic acid (EDTA)	$C_{10}H_{16}N_2O_8$	Sigma
Formaldehyde	CH_2O	Sigma
Formamide	CH_3NO	AppliChem
Glacial acetic acid	$C_2H_4O_2$	Merck
Glycerol	$C_3H_8O_3$	Sigma
Glycine betaine	$C_5H_{11}NO_2$	Fluka (Sigma)
Hydrochloric acid	HCl	Merck
Hydroxylamine hydrochloride	NH_2OH x HCl	Sigma
Iron(II) sulfate heptahydrate	$FeSO_4$ x 7 H_2O	Sigma
Iron(III) sulfate hydrate	$Fe_2(SO_4)_3$ x H_2O	Fluka (Sigma)

Table 2-1: Chemicals used in the experiments (continued).

Name of substance	*Chemical formula*	*Manufacturer*
Isopropanol	C_3H_8O	Sigma
Lysozyme		Merck
Magnesium chloride hexahydrate	$MgCl_2$ x 6 H_2O	Merck
Magnesium perchlorate	$Mg(ClO_4)_2$	Sigma
Magnesium sulfate heptahydrate	$MgSO_4$ x 7 H_2O	Merck
Manganese dichloride tetrahydrate	$MnCl_2$ x 4 H_2O	Sigma
Manganese(II) sulfate monohydrate	$MnSO_4$ x H_2O	Merck
Nalidixic acid	$C_{12}H_{12}N_2O_3$	Fluka (Sigma)
Nuclease free water		Fermentas
(1,10)-Phenanthroline hydrochloride monohydrate	$C_{12}H_9ClN_2$ x H_2O	Fluka (Sigma)
Phenol-chloroform-isoamyl alcohol (25:24:1)		AppliChem
Pipemidic acid	$C_{14}H_{17}N_5O_3$	Sigma
Potassium chloride	KCl	Sigma
Potassium dihydrogen phosphate	KH_2PO_4	Merck
R2A agar		Fluka (Sigma)
Sodium chloride	NaCl	VWR[i]
Sodium dodecyl sulfate (SDS)	$NaC_{12}H_{25}SO_4$	Sigma
Sodium molybdate dihydrate	Na_2MoO_4 x 2 H_2O	Sigma
Sodium pyruvate	$C_3H_3NaO_3$	Fluka (Sigma)
Starch, soluble		Merck
Sucrose	$C_{12}H_{22}O_{11}$	Merck
Sulfur (pure, powder)	S^0	Sigma
Sulfuric acid (95-97%)	H_2SO_4	Merck
Trehalose	$C_{12}H_{22}O_{11}$	Sigma
Tris	$C_4H_{11}NO_3$	AppliChem
Zinc chloride	$ZnCl_2$	Merck

[a]Serva Electrophoresis GmbH, Heidelberg, Germany

[b]Merck KGaA, Darmstadt, Germany

[c]Carl Roth GmbH & Co. KG, Karlsruhe, Germany

[d]Difco Laboratories, Sparks, USA

[e]Sigma-Aldrich Chemie GmbH, Steinheim, Germany

[f]AppiChem GmbH, Darmstadt, Germany

[g]Science Services, München, Germany

[h]Thermo Fisher Scientific Inc., Waltham, USA

[i]VWR International GmbH, Darmstadt, Germany

Table 2-2: (Fluorescent) stains, lectins, and PCR reagents.

Name	*Manufacturer*
ConA (Concavalin A from Canavalia ensiformis)[b], FITC-labelled	Sigma
CTC (5-cyano-2,3-ditolyl tetrazolium chloride)	Sigma
DAPI (4',6-diamidino-2-phenylindole)	Sigma
LIVE/DEAD® BacLight™ Bacterial Viability Kit L13152	Molecular probes[a]
Platinum® SYBR® Green qPCR Super Mix-UDG	Invitrogen
SYBR® Safe	Molecular probes
SYTO® 62	Molecular probes
WGA (wheat germ agglutinin from Triticum vulgaris)[b], TRITC-labelled	Sigma

[a]Eugene, USA

[b]Goldstein and Hayes (1978)

Table 2-3: Oligonucleotide probes and primers (obtained from Sigma-Aldrich).

Name	*Function*	*Sequence*
F1 Thio	Forward primer	(5'-3')-ATGCGTAGGAATCTGTCTTT
R1 Thio	Reverse primer	(5'-3')-GGACTTAACCCAACATCTCA
EUB338	Oligonucleotide probe	[Cy3]GCTGCCTCCCGTAGGAGT

2.2 Bacterial strains and cultivation

2.2.1 Bacterial strains

All bacterial strains were obtained from the DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany (**Table 2-4**).

Table 2-4: Bacterial strains used in the experiments.

Organism	*Strain*	*Origin*	*Reference*
Acidithiobacillus ferrooxidans	DSM 14882^T	acid, bituminous coal mine effluent	Kelly and Wood 2000; Temple and Colmer 1951
Sulfobacillus thermosulfidooxidans	DSM 9293^T	spontaneously heated ore deposit	Golovacheva and Karavaiko 1991
Deinococcus geothermalis	DSM 11300^T	hot springs	Ferreira et al. 1997

2.2.2 Media, buffers, and solutions

Media used for the cultivation of the bacterial strains were prepared according to specifications by the DSMZ. For *A. ferrooxidans* and *S. thermosulfidooxidans*, basal salt medium (BSM) with trace elements was complemented by the appropriate electron donors or acceptors.

2.2.2.1 Basal salt medium (BSM)

$(NH_4)_2SO_4$	132.0	mg
$MgCl_2$ x 6 H_2O	53.0	mg
KH_2PO_4	27.0	mg
$CaCl_2$ x 2 H_2O	147.0	mg
ddH_2O	950.0	ml

The medium was adjusted to pH 1.9 with 10N H_2SO_4 and autoclaved at 112°C, 30 min (Varioklav, Thermo Fisher Scientific Inc., Waltham, USA).

2.2.2.2 Trace element solution

$MnCl_2$ x 4 H_2O	76.0	mg
$ZnCl_2$	68.0	mg
$CoCl_2$	34.0	mg
H_3BO_3	31.0	mg

Na_2MoO_4 x 2 H_2O	11.7	mg
$CuCl_2$	53.0	mg
ddH_2O	1000.0	ml

The solution was adjusted to pH to 1.9 with 10N H_2SO_4 and autoclaved at 112°C, 30 min.
1 ml of trace element solution was added to 950 ml BSM after autoclaving.

S. thermosulfidooxidans was also cultivated in sporulation medium (Bogdanova et al. 2002) to obtain endospores:

2.2.2.3 Sporulation medium

$(NH_4)_2SO_4$	1.5	g
KCl	0.1	g
K_2HPO_4	0.05	g
$MgSO_4$	0.98	g
$Ca(NO_3)_2$ x 4 H_2O	0.29	g
$MnSO_4$ x H_2O	0.035	g
ddH_2O	1000.0	ml

The medium was adjusted to pH 1.9 with 10N H_2SO_4 and autoclaved at 112°C for 30 min.

2.2.2.4 Yeast extract stock solution (10%)

Bacto yeast extract	10 g
ddH_2O	ad. 100 ml

The solution was autoclaved at 121°C, 20 min.

2.2.2.5 Fe^{2+}-stock solution (200 g/l)

$FeSO_4$ x 7 H_2O	100 g
H_2SO_4 (95-97%)	3 ml
ddH_2O	ad. 500 ml

The H_2SO_4 was added to the $FeSO_4$ before the water, and everything was stirred until completely dissolved. The pH of this solution was 1.2. The solution was autoclaved at 112°C for 30 min.

2.2.2.6 Fe^{3+}-stock solution (164 g/l)

$Fe_2(SO_4)_3$ x H_2O	16.4 g
H_2SO_4 (95-97%)	1 ml
ddH_2O	ad. 100 ml

The pH of this solution was 0.8. The solution was deoxygenated as described below and autoclaved at 112°C for 30 min.

Deinococcus geothermalis was cultivated on liquid or solid R2A medium, and washed cells were resuspended in phosphate-buffered saline (PBS), both autoclaved at 121°C, 20 min.

2.2.2.7 R2A medium

Casein acid hydrolysate	0.5 g
Yeast extract	0.5 g
Proteose peptone	0.5 g
D-glucose monohydrate	0.5 g
Starch, soluble	0.3 g
Sodium pyruvate	0.3 g
Magnesium sulfate	0.024 g
ddH_2O	ad. 1000 ml
For solid medium add:	
Agar	15 g

Final pH at 7.2. The medium was autoclaved at 121°C, 20 min.

2.2.2.8 Phosphate-buffered saline (PBS)

Na_2HPO_4 x $2H_2O$	7 g
KH_2PO_4	3 g
NaCl	4 g

ddH_2O	ad. 1000 ml

The solution was adjusted to pH 7.5 and autoclaved at 121°C, 20 min.

2.2.3 Cultivation conditions

2.2.3.1 Stock cultures

Stock cultures of *A. ferrooxidans* and *S. thermosulfidooxidans* were maintained at 4°C. Every 4-6 months, fresh cultures were inoculated using cells from the original (DSMZ) stock culture as inoculum to ensure continued vability.

Stocks of *D. geothermalis* were stored at -80°C in 50% glycerol and used to inoculate solid R2A plates, which were kept at 4°C for 4 weeks (maximum).

2.2.3.2 Cultivation of D. geothermalis

Precultures were inoculated with a single colony from the solid R2A plates and grown in 50 ml liquid R2A medium in 100-ml-Erlenmeyer flasks at 45°C, 200 rpm (Multitron II incubator shaker, ATR, Laurel, USA) for 25 h (early stationary phase).

Biofilms were obtained by putting a sterilized glass slide in the flask, which was immersed halfway in the medium, and incubaed at 45°C for 25 h. Cells of *D. geothermalis* attached to the glass surface at the liquid-air-interface.

Solid R2A medium with *D. geothermalis* was incubated at 37°C for 48 h.

2.2.3.3 Cultivation of A. ferrooxidans *and* S. thermosulfidooxidans

2.2.3.3.1 Aerobic cultivation

A. ferrooxidans and *S. thermosulfidooxidans* were grown in 50 ml BSM (+ trace elements) containing 4 g/l Fe^{2+} supplied as $FeSO_4$ x 7 H_2O (added after autoclaving). For *S. thermosulfidooxidans* 0.02% (wt/v) yeast extract was added to all cultures after autoclaving. Incubation in 100-ml-Erlenmeyer flasks took place at 30°C, 200 rpm (Memmert incubator, Memmert GmbH + Co. KG, Schwabach, Germany with rotary shaker 3005, GFL, Burgwedel, Germany) for *A.*

ferrooxidans, or at 45°C (Heraeus incubator) without shaking for *S. thermosulfidooxidans*. The precultures were inoculated with 1 ml of the respective stock cultures (~1x10^6 cells/ml intial cell density). The final cell concentration under these cultivation conditions was about 8x10^7 cells/ml.

The same conditions were used for cultivation on elemental sulfur (~2% wt/v) or Mars regolith minerals (5% wt/v), but these solid substrata were added to the BSM before autoclaving and flasks were subjected to only 110°C for 1 h to avoid melting of sulfur and changes in mineral chemistry.

When *S. thermosulfidooxidans* was grown in sporulation medium, 1.5 g/l of Fe^{2+} and 0.015% (wt/v) yeast extract were added, otherwise conditions were the same.

For low-temperature cultivation of *A. ferrooxidans* on Fe^{2+} at room temperature (25.3°C ± 0.7°C), 10°C, and 4°C, inoculation and medium composition were the same as described above, but cultures were incubated without shaking.

2.2.3.3.2 Anaerobic or microaerobic cultivation

Preparation of the anaerobic or microaerobic culture media followed the protocol by Hungate (1950) and Miller et al. (1974). 0.5 or 1 l BSM (+ trace elements) was purged by N_2/CO_2 gas (80:20 v/v, purity 4.5, Praxair, Oevel, Belgium) for 30 min to drive out the dissolved oxygen. A reduction agent such as cysteine or sodium sulfite was not added because it influenced bacterial growth, and resazurin as a redox indicator did not function at pH 2. Serum flasks (120 ml, sodium silicate glass, MediPacGmbH, Rheinbreitbach, Germany) were filled with 19 ml of the purged medium under CO_2 atmosphere and closed with butyl rubber septa (Ochs, Bovenden/Lenglern, Germany) and aluminium caps (Alu-crimp caps, 10 mm with hole, WICOM, Heppenheim, Germany) (**Fig. 2-1**). All flasks were evacuated and re-filled with the respective gas mixture (either H_2/CO_2 or N_2/CO_2, 80/20 (v/v) each, purity 4.5, Praxair) by repeating this cycle three times and adjusting the final pressure to 1.5 – 2 bar. Media were autoclaved afterwards at the appropriate temperatures. For the experiment examining the switch from aerobic to anaerobic conditions, 50 ml of sterile-filtered air were added to the gas phase of N_2/CO_2 (at 1 bar).

Soluble electron donors or acceptors ($FeSO_4$ x 7 H_2O and $Fe_2(SO_4)_3$ x H_2O) were prepared separately using the same technique and were added after autoclaving with sterile needles (Terumo, Leuven, Belgium) and syringes (Ersta, Maersk, Denmark) (2 g/l Fe^{2+} for microaerobic growth experiments; 1.5 g/l Fe^{3+} for anaerobic growth). Solid additives (sulfur, Mars regolith minerals) were added before closing the serum flasks with butyl septa in the anaerobic chamber.

Fig. 2-1: Anaerobic culture flask (120 ml) with 20 ml of growth medium, and syringe used for sampling.

2.2.3.4 Cultivation of biofilms

The floating filter technique (de Bruyn *et al.,* 1990) was used for the cultivation of biofilms of *A. ferrooxidans* or *S. thermosulfidooxidans* grown on polycarbonate membrane filters. Autoclaved polycarbonate membranes (GTTB, Ø 25 mm, 0.2 µm pore size, Millipore, Merck) were inoculated by filtration of 1-5x10^6 cells from an Fe^{2+}-grown preculture (48 h) and suspended on 40 ml BSM containing 4 g/l Fe^{2+} in plastic petri dishes. The biofilms were incubated 5 days at 30°C without shaking (**Fig. 2-2**).

Fig. 2-2: Floating polycarbonate filter membrane with *A. ferrooxidans* on ferrous iron-containing liquid medium.

To compare growth and iron oxidation rate of biofilm cells on filter membranes to planktonic cells, the same amount of planktonic cells from the same preculture was inoculated into 40 ml of BSM with Fe^{2+} in a petri dish and incubated under the same conditions as the biofilms. Aliquots of 200 µl were removed daily to assess soluble iron concentration, cell number, and Most Probable Number (MPN). In the case of the biofilms, a complete filter had to be removed from the medium per time point to enumerate the total and viable cells after detachment.

2.2.3.4.1 Sequential detachment of biofilm cells of *A. ferrooxidans* from membrane filters

To test the efficiency of the removal of cells grown as a biofilm on membrane filters, 3 biofilms (after 5 days of incubation) were transferred to separate 15-ml-screw cap tubes containing 2 ml BSM each. After vigorous shaking for 1 minute (Vortex apparatus, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), cells from the suspension were counted and viable cells enumerated by MPN (see section **2.4**, p. 64). Afterwards, the filters were removed with sterile tweezers, washed by short suspension in BSM, and transferred to a new 15-ml-tube with 2 ml BSM. The procedure of shaking (1 min) and enumeration of cells was repeated (**Table 2-5**).

Table 2-5: Total cell number and culturable cell number (MPN) after two sequential detachment procedures to detach biofilm cells from membrane filters.

	cells/ml	*MPN/ml*
1st detachment	$1.6x10^9 \pm 2.9x10^8$	$9.5x10^8 \pm 4.0x10^8$
2nd detachment	$5.8x10^6 \pm 2.5x10^5$	$3.9x10^6 \pm 2.1x10^6$
% *	0.37 ± 0.08	0.45 ± 0.27

* Percentage of cells after 2nd detachment of the 1st detachment

The percentage of cells removed from the polycarbonate membrane after the second detachment procedure was 0.37 ± 0.08% of the first detachment for total cell numbers and 0.45 ± 0.27% for culturable cell numbers. It was assumed that

the detachment method was sufficiently accurate to enumerate cells of these biofilms because cell numbers after the second detachment procedure amounted to less than 10% of the first.

2.2.4 Mars regolith simulants

Two mineral mixtures of Mars regolith simulants (MRS) were provided by the Museum of Natural History, Berlin (Dr. Jörg Fritz) (**Table 2-6**). The minerals were assembled according to data on the structural and chemical composition of Martian minerals identified in meteorites (McSween 1994) and by recent orbiter and rover missions (Bibring et al. 2005; Chevrier and Mathé 2007; Morris et al. 2010; Poulet et al. 2005) to reflect current knowledge regarding environmental changes on Mars (Böttger et al. 2011).

Table 2-6: Mineralogical composition of Sulfatic Mars Regolith Simulant (S-MRS) and Phyllosilicatic Mars Regolith Simulant (P-MRS) in weight percent (wt/v%) (Boettger et al. 2011). Iron-containing minerals are marked in red.

Component	*P-MRS (wt%)*	*S-MRS (wt%)*
Pyroxene, Plagioclase, Amphibole, Ilmenite (Gabbro)	3	32
Olivine $(Mg, Fe)_2SiO_4$	2	15
Quartz SiO_2	10	3
Hematite Fe_2O_3	5	13
Montmorrilonite $[(Na,Ca)_{0.33}(Al,Mg)_2Si_4O_{10}(OH)_2 \times H_2O]$	45	–
Chamosite $[(Fe^{2+},Mg,Fe^{3+})5Al(Si_3Al)O_{10}(OH,O)_8]$	20	–
Kaolinite $Al_2Si_2O_5(OH)_4$	5	–
Siderite $Fe(CO_3)$	5	–
Hydromagnesite $Mg(CO_3)$	5	–
Goethite $FeO(OH)$	–	7
Gypsum $Ca(SO_4)x2H_2O$	–	30

Table 2-7: Mass per grain size bins of Martian Regolith simulants.

Grain size (µm)	*1000 – 560*	*560 - 315*	*315 - 160*	*160 - 63*	*63 - 0*	*Total*
P-MRS (wt/v%)	14,6	10,5	12,3	48,2	14,3	100
S-MRS (wt/v%)	19,4	22,3	22,3	24,3	11,5	100

2.2.4.1 Cultivation of A. ferrooxidans with Mars regolith simulants

For growing *A. ferrooxidans* aerobically on MRS, 50 ml BSM (+ trace elements) or 50 ml acidified ddH_2O were prepared with 5% (wt/v) S-MRS or P-MRS, and pH was lowered by the addition of 500 µl 10 N H_2SO_4 per 50 ml medium. The flasks were autoclaved at 110°C, 1 h. Afterwards, the medium was inoculated with cells from ferrous iron-grown precultures (~1x10^6 cells/ml intial cell density) after washing them twice in BSM (or three times in ddH_2O for inoculation in ddH_2O) (10000 rpm, 5 min, MiniSpin plus, eppendorf, Hamburg, Germany) (**Fig. 2-3**). For anaerobic cultivation on MRS, 20 ml BSM (+ trace elements) with 5% (wt/v) S-MRS or P-MRS (acidified with 200 µl 10 N H_2SO_4 per 20 ml medium) were prepared anaerobically as described in section **2.2.3.3.2**, p. 47 and autoclaved at 110°C, 1 h. Cultures were inoculated from precultures grown on H_2/Fe^{3+} (~1x10^6 cells/ml intial cell density) after washing them twice in BSM under CO_2 atmosphere. In parallel, sterile flasks of S-MRS and P-MRS were also prepared as abiotic controls. The cultures were incubated at 30°C, 200 rpm and culturable cell numbers and soluble iron concentrations were determined (section **2.4.3**, p. 65; section **2.5.3**, p. 68).

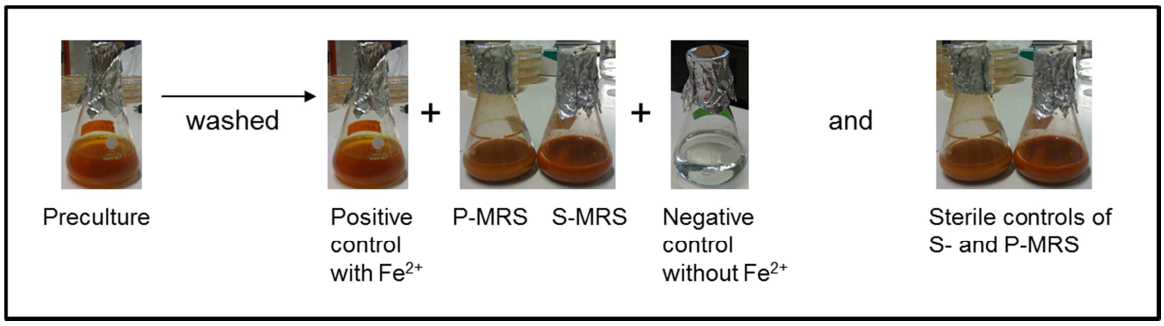

Fig. 2-3: Schematic procedure of the aerobic incubation of *A. ferrooxidans* on MRS. Negative control with bacteria was included to examine growth without additional nutrients. Sterile controls were used to check abiotic changes in iron concentrations.

2.2.4.2 Attachment of A. ferrooxidans to Mars regolith simulants.

50 ml aerobic MRS-cultures (BSM with 5% MRS) were prepared as described above for the growth experiments. A 1 l preculture of *A. ferrooxidans* was grown on ferrous iron or sulfur for 48 h, washed once, and concentrated in BSM to a volume of 4 ml (to gain a high initial cell density) using a Multifuge3 S-R (Heraeus, Thermo Fisher Scientific). 1 ml of the concentrated preculture was used as an inoculum for the MRS cultures.

The cultures were incubated at 30°C, 200 rpm, analogous to the growth experiments. At different time intervals, aliquots (400 µl) were removed after gentle stirring of the flask (to avoid detachment of bacteria). Aliquots were immediately subjected to a short spin (2000 rpm, 2 min, MiniSpin plus, eppendorf) to precipitate the mineral particles with the adherent cells, but not the planktonic cells in suspension. Cells were counted only from the supernatant liquid phase.

To observe whether the attachment was reversible by mechanical forces, the aliquots were vigorously shaken (1 min, Vortex apparatus, Heidolph Instruments) after 30 minutes of equilibration, to detach bacteria from mineral particles. After another short spin (2000 rpm, 2 min, MiniSpin plus, eppendorf), cells from the supernatant were counted again. This treatment was chosen because it has been shown to be efficient at detaching bacteria from pyrite particles (Okibe and Johnson 2004).

The pH during the test period (max. 20 h) rose from ~2 to ~2.5 or ~3.5 for S-MRS and P-MRS, respectively.

2.3 Exposure to stress conditions

A. ferrooxidans and *S. thermosulfidooxidans* were subjected to different stressors either as planktonic cells (pre-grown 48 h on ferrous iron, section **2.2.3.3.1**, p. 46) or as biofilms grown five days on polycarbonate membranes (section **2.2.3.4**, p. 48). *D. geothermalis* biofilms (grown on glass surface for 40 h) or planktonic cells (40 h preculture) (section **2.2.3.2**, p. 46) were also included in some stress resistance tests.

2.3.1 Desiccation

2.3.1.1 Strains of acidophilic iron-sulfur bacteria screened for desiccation tolerance

In addition to the type strains of *A. ferrooxidans* and *S. thermosulfidooxidans*, other strains of acidophilic iron-sulfur bacteria and one enrichment culture (of unknown species composition) were also subjected to air-drying on a glass surface for 24 h to screen for strains with a higher resistance to desiccation (**Table 2-8**). The strains were kindly provided by Dr. Mario Vera (University Duisburg/Essen, Essen, Germany).

Table 2-8: Strains of acidophilic bacteria used to screen for desiccation tolerance (source: University of Essen, Germany).

Organism	*Strain*	*Reference*
Acidithiobacillus ferrooxidans	A1 A4 R1 2Y Yellow 3 F427 WR1 PH SPIII/7	Amouric et al. 2011, Brauckmann 1985, Sand et al. 1992

continued

Table 2-8: Strains of acidophilic bacteria used to screen for desiccation tolerance (source: University of Essen, Germany) (continued).

Organism	*Strain*	*Reference*
Acidithiobacillus ferrivorans	CF27	Hallberg et al. 2010
Acidithiobacillus caldus	S2	Zhou et al. 2007
Acidiferrobacter thiooxidans	SPIII/3	Hallberg et al. 2011
Leptospirillum ferrooxidans	DSM 2705^T	Hippe 2000; Markosyan 1972
Leptospirillum ferriphilum	L101	Coram and Rawlings 2002

The strains were grown on BSM with Fe^{2+} under the same conditions as *A. ferrooxidans*^{T} (section **2.2.3.3.1**, p. 46). Aliquots were washed once with BSM and 100-200 µl were (10^7 cells) each were pipetted into the wells of a 24-well-plate with at least three parallels per strain. After 24 h of air-drying (at 30°C), the dried cells were resuspended in 2 ml BSM with Fe^{2+} and incubated at 30°C.

2.3.1.2 Sample preparation and analysis

2.3.1.2.1 Drying planktonic cells and biofilms of *A. ferrooxidans* and *S. thermosulfidooxidans*

Planktonic cells (from a 48 h preculture) of *A. ferrooxidans* and *S. thermosulfidooxidans* were either spotted on glass slides ($5x10^6$ cells in 100 µl) or, for comparison with biofilms, filtered on polycarbonate membranes (10^9 cells). Biofilms on filter membranes were prepared according to protocol (section **2.2.3.4**, p. 48) and incubated for 5 days on liquid medium. The membranes were removed from the liquid medium and exposed to drying in sterile plastic petri dishes under different conditions of relative humidity (RH) and oxygen (see section **2.3.1.2.3**, p. 56).

After drying, glass slides or membranes were returned to liquid growth medium (BSM with Fe^{2+}) and incubated at the appropriate conditions to observe regrowth and measure iron oxidation rate by taking aliquots at different times. For *A. ferrooxidans*, biofilm cells were also detached from polycarbonate membranes using the detachment procedure described in section **2.2.3.4.1**, p. 49, and

enumerated (total cell number and culturable cell number by MPN, section **2.4**, p. 64).

Positive controls were not dried, but immediately transferred to fresh medium. In addition, a sterile control was carried along to determine abiotic iron oxidation rate.

Weight loss of biofilms during drying (equilibration with air):

10 biofilms of *A. ferrooxidans* on filter membranes (grown 5 days) were weighed on an analytical scale at different time intervals during air-drying for 24 h to determine the speed of water loss and equilibration with the air. Wet, cell-free filters were used as a blank. After 10 minutes of drying, the weight of the filter membranes with and without bacteria reached a constant value, indicating that at the given relative humidity (~55%), the biofilms and filters were equilibrated with the air (**Fig. 2-4**).

The mass of the biofilms (with mass of the blank filters subtracted) after 24 h of air-drying at 55% RH was 0.89 ± 0.29 mg.

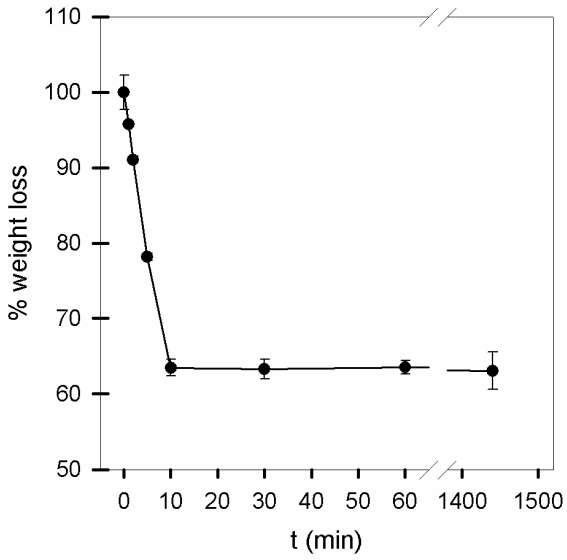

Fig. 2-4: Weight loss in % of biofilms of *A. ferrooxidans* on membrane filters during 24 h drying on air (55% RH).

2.3.1.2.2 Drying planktonic cells and biofilms of *D. geothermalis*

Planktonic cells from early stationary phase (25 h) of *D. geothermalis* were washed twice with PBS (3600 rpm, 20 min, Multifuge3 S-R, Heraeus, Thermo Fisher Scientific) and spotted on 7 mm (10^7 cells) or 11.9 mm (10^8 cells) diameter quartz discs (Heraeus Quarglas GmbH & Co.Kg, Hanau, Germany). The cells were air-dried overnight (17 h), before exposing them to different conditions. For long-term desiccation (**Fig. 3-21**, p. 113), the air-dried cells on quartz discs were transferred to desiccators (silica gel, RH <5%) and stored at 30°C for up to 1 year. In short-term experiments (**Fig. 3-28**, p. 124), the air-dried cells were stored 4 weeks under different conditions of relative humidity (RH) and oxygen or in vacuum (see section **2.3.1.2.3**, p. 56).

After drying, cells were detached from quartz discs by suspension in 1 ml PBS and vigorous shaking. Subsequently, total cell numbers and culturable cell numbers (obtained by the colony formation assay, see section **2.4**, p. 64) were determined, or Live/Dead® staining was performed (section **2.7.2**, p. 71).

Biofilms of *D. geothermalis* attached a glass surface (after 25 h incubation) were rinsed with deionized water and subjected to air-drying for 3 weeks to compare resistance to planktonic cells (on quartz discs) (**Fig. 3-24**, p. 118). Biofilms were scraped from the glass surface with a cell scraper and suspended in 1 ml PBS. The detached cells were enumerated by microscopy and the colony formation assay and stained by the Live/Dead® kit.

2.3.1.2.3 Exposure to different conditions of relative humidity (RH) and oxygen

Samples of *A. ferrooxidans*, *S. thermosulfidooxidans*, and *D. geothermalis* were subjected to drying under different conditions to examine the effects of RH and presence or absence of oxygen on survival. Relative humidity (RH) and temperature were measured by a thermo-hygrometer (LOG32, TFA Dostmann GmbH & Co. KG, Wertheim, Germany). Room temperature, at which most of the desiccation experiments took place, if not otherwise stated, was 25.3°C ± 0.7°C.

The following conditions were used (**Table 2-9**):

Table 2-9: Conditions of drying

Condition	*RH (%)*	*Oxygen?*
Ambient air	40.4 ± 6.8	yes
Desiccator (containing silica gel)	reaching 4.6 ± 2.8 after 48 h	yes/no (applied in both conditions)
Anaerobic chamber	27.7 ± 3.5	no (<10 ppb)
High vacuum	~0	no (total pressure of $5x10^{-5}$ Pa)

In the desiccator, which contained silica gel (dehydrated at 85°C prior to use), the RH reached values of 4.6% ± 2.8% after 48 h (**Fig. 2-5**) and lower after longer time periods (down to the detection limit of the hygrometer).

Anaerobic drying was done in an anaerobic box (Coy Laboratory Products Inc., Arbor, USA) under N_2/H_2 atmosphere (95:5 v/v, purity 5.0, Praxair, Oevel, Belgium) with $CaCO_3$.

High vacuum was produced in the Planetary and Space Simulation facilities (PSI, DLR Cologne, Germany) by an ion getter pumping system (rotary vane pump Duo030A + TPU510, Pfeiffer-Vacuum, Asslar, Germany) reaching a final pressure of $5x10^{-5}$ Pa.

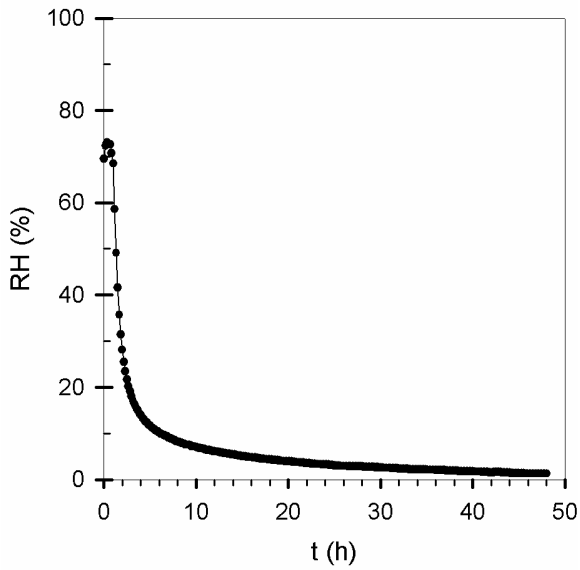

Fig. 2-5: Progress of RH in a desiccator over silica gel in the course of 48 h.

2.3.1.2.4 Vacuum-drying of *A. ferrooxidans* with compatible solutes

The vacuum-drying procedure (called 'liquid-drying' in Malik 1990) was shown to be suitable for long-term storage of *A. ferrooxidans* in the dried state, when cells were dried in a 6% (wt/v) glycine betaine solution (Cleland et al. 2004). Here, three other compatible solutes were tested with a procedure similar to the described method to preserve dried cells of *A. ferrooxidans*.

The solutions of trehalose, sucrose, glycerol, and glycine betaine (6% wt/v each, or different concentrations of sucrose) were sterilized by filtration (cellulose acetate filters, 0.2 µm, Whatman GmbH, Germany).

Cells of *A. ferrooxidans* precultures grown on Fe^{2+} were washed twice in BSM and resuspended in BSM or the respective compatible solute-solution ($8x10^7$ cells/ml) for external protection. Cells were also grown in medium supplemented with either 0.3-0.5% (wt/v) NaCl, or in 1 mM – 100 mM sucrose, trehalose, or glycine betaine to observe whether the production of compatible solutes can be induced by salt stress, and whether compatible solutes can be accumulated from the medium. These cultures were also washed twice and resuspended in BSM.

200 µl aliquots of the washed cells were transferred to into sterile glass vials (2 ml), which were loosely stoppered with sterile cotton wool. In a vacuum chamber (PSI 7, DLR Cologne) using a rotary vane pump (Duo20 + TMU261P, Pfeiffer-Vacuum), the samples were exposed to 5 hPa for 30 minutes, followed by 0.1 hPa for 2 h. Subsequently, they were transferred to a desiccator and stored there for different periods of time.

For analysis, dried samples were resuspended in 200 µl BSM and cells were enumerated by total cell counts and MPN (section **2.4**, p. 64).

2.3.2 Salt stress

Growth of *A. ferrooxidans* with different salts ($MgSO_4$, $MgClO_4$, $MgCl_2$, $CaCl_2$, or NaCl) was tested by supplementing normal growth medium containing Fe^{2+} with 0.5 or 1% (wt/v) of the respective salts. $MgSO_4$ was also tested at higher concentrations up to 20% (wt/v). The cultures were incubated under optimal growth conditions for 5 days and total cell numbers were assessed.

Survival was tested in 20% (wt/v) salt solutions at -20°C and at room temperature after 1 day and 1 week. Stock solutions of the different salts ($MgSO_4$, $MgClO_4$, $MgCl_2$, $CaCl_2$, or NaCl) were sterilized by filtration. A preculture of *A. ferrooxidans* was washed once with BSM and aliquots were resuspended in the salt solutions. Survival was assessed by MPN (section **2.4**, p. 64).

2.3.3 UV radiation

The source for monochromatic UV-C radiation was a mercury low pressure lamp (NN 8/15, Heraeus, Berlin, Germany) with a main emission line of λ = 253.65 nm. The spectral irradiance was determined before each irradiation by the use of a double monochromator (Bentham DM 150). Fluence rates were measured before each irradiation exposure with a UV-X radiometer with UV-sensor (UVX-25) for 254 nm (UVP Ultra-Violet Products, Cambridge, UK) and were ~85 µW/cm^2 at the sample site.

Polychromatic UV radiation was supplied by a metal halogenide high pressure lamp (solar simulator SOL2, Dr. Hoenle AG, Munich, Germany) emitting a spectrum of λ > 200 nm.

2.3.3.1 Irradiation of planktonic cells (A. ferrooxidans and D. geothermalis)

Washed cell suspensions (5 ml) of *A. ferrooxidans* (in BSM) or *D. geothermalis* (in PBS) containing ~1-2 x 10^7 cells/ml were irradiated in sterile petri dishes (3.5 cm diameter). The absorbance of the cell suspensions at 254 nm was measured before each irradiation in a spectrophotometer (U-3310, Hitachi) to be <0.2. To ensure homogenous exposure, irradiation took place under continuous stirring. Aliquots of 300 µl samples were taken at each fluence and stored on ice until analysis.

Viable cell numbers were determined by the colony formation assay (*D. geothermalis*) or MPN (*A. ferrooxidans*) (section **2.4**, p. 64).

A. ferrooxidans oxidizes of Fe^{2+} to Fe^{3+}, which is an efficient UV absorber (Gómez et al. 2007). For this reason, an experiment aimed to observe the effect of Fe^{3+} ions in the irradiation medium on survival of *A. ferrooxidans* during UV-C

exposure. Cultures grown on Fe^{2+}, which showed the typical red-brown coloring due to the presence of Fe^{3+}, were not washed, and only diluted with BSM to reach a cell number approximately equal to that of the washed cultures (~10^7 cells/ml). An absorption spectrum of the resulting suspension was recorded using a spectrophotometer over the range of 200-400 nm (**Fig. 2-6**) and shows a high absorption at 220 to 370 nm. Irradiation of the culture was carried out as described above.

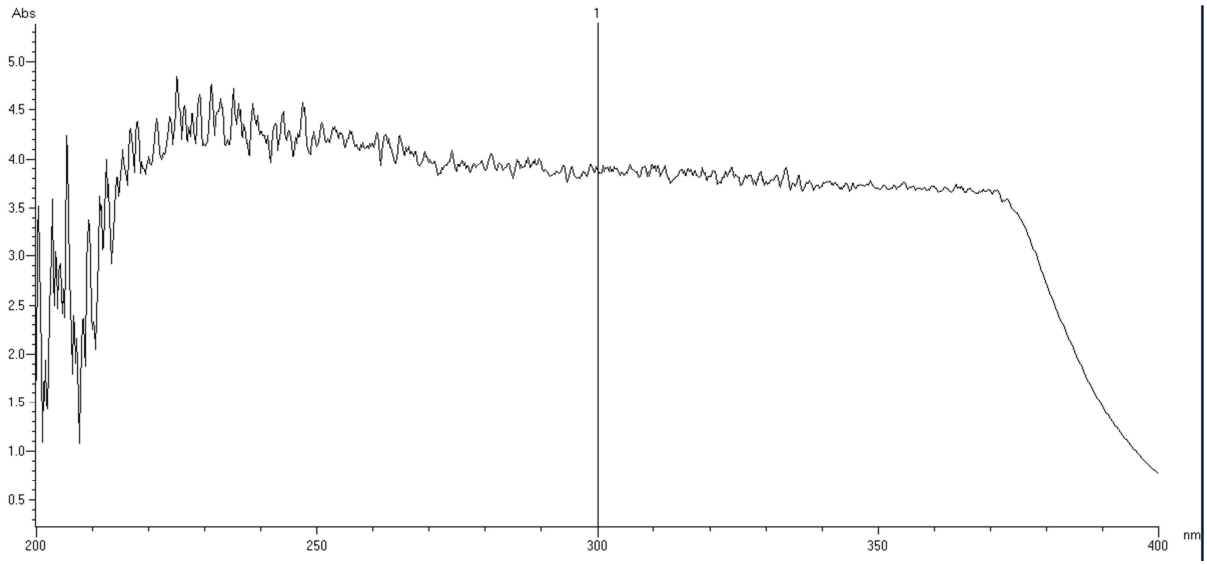

Fig. 2-6: Absorption spectrum of Fe^{3+}-containing medium with *A. ferrooxidans* over 200-400 nm.

2.3.3.2 Irradiation of biofilms of A. ferrooxidans

Biofilms of *A. ferrooxidans* on membrane filters were removed from the growth medium after five days and transferred to a petri dish (6 cm diameter) with 20 ml BSM (filters were floating on the surface). This served the purpose to irradiate biofilms under 'wet' conditions similar to the planktonic cells and avoid the additional stress of drying. Per fluence 3 biofilms were analyzed by total cell counts and Most Probable Number (section **2.4**, p. 64).

2.3.4 X-rays

Exposure to X-rays was done using an RS 225A X-ray tube (Gulmay, Chertsey, UK) at 150 kV and 20 mA with a 0.1 mm aluminum-filter. The dose rate was measured by an $UNIDOS_{webline}$ dosimeter (PTW Freiburg GmbH, Germany). Irradiation took place at a dose rate of 20 Gy/min at 20°C (for *A. ferrooxidans*) or at 46.5 Gy/min at 4°C (for *D. geothermalis*).

Washed planktonic cell suspensions (1 ml, ~10^7 cells/ml) of *A. ferrooxidans* (in BSM) or *D. geothermalis* (in PBS) were irradiated in 1.5-ml-microcentrifuge tubes. Viable cell numbers were determined by colony formation assay (*D. geothermalis*) or MPN (*A. ferrooxidans*) (section **2.4**, p. 64).

Biofilms of *A. ferrooxidans* on membranes were removed from the growth medium after five days and transferred to a petri dish (6 cm diameter) with 20 ml BSM (filters were floating on the surface). This served the purpose to irradiate biofilms under 'wet' conditions similar to the planktonic cells and avoid the additional stress of drying. Per dose 3 biofilms were analyzed total cell counts and Most Probable Number. For reasons of better comparison, planktonic cells were also filtered onto polycarbonate membranes (5x10^8 cells per membrane) and subjected to X-ray irradiation under the same conditions as the biofilms.

2.3.5 Freezing

Analogous to the desiccation experiments, protection of *A. ferrooxidans* during freezing by compatible solutes was tested. The solutions of trehalose, sucrose, glycerol, and glycine betaine (6% wt/v each) were sterilized by filtration (cellulose acetate filters, 0.2 µm, Whatman GmbH, Germany).

Cells of *A. ferrooxidans* precultures grown on Fe^{2+} were washed twice in BSM and resuspended in BSM or the respective compatible solute-solution (8x10^7 cells/ml). 200 µl aliquots (containing ~1x10^7 cells) were transferred into microcentrifuge tubes to be stored at -20°C and -80°C for up to 12 months.

For analysis, frozen samples were thawed at room temperature and cells were enumerated by total cell counts and MPN (section **2.4**, p. 64).

Biofilms of *A. ferrooxidans* and *S. thermosulfidooxidans* grown 5 days on polycarbonate membranes were removed from the growth medium and transferred to sterile petri dishes to be immediately frozen at -20°C or -80°C. Upon removal from the freezer, biofilms on polycarbonate membranes were immediately returned to liquid growth medium (BSM with Fe^{2+}) and incubated at the appropriate conditions to observe regrowth and measure iron oxidation rate by taking aliquots at different times. For *A. ferrooxidans*, biofilm cells were also detached from polycarbonate membranes using the detachment procedure (described in section **2.2.3.4.1**, p. 49) and enumerated (total cell number and culturable cell number by MPN).

Positive controls were biofilms, which were not frozen, but immediately transferred to fresh medium. In addition, a sterile control was carried along to determine abiotic iron oxidation rate.

2.3.6 Mars simulation

For Mars simulation experiments, the 'MaSimKa' (PSI 7 of the Planetary and Space Simulation Facilities, DLR Cologne, Germany) with an inner chamber volume of 0.22 m x 0.26 m was used. The chamber was evacuated by a rotary vane pump (Duo20 + TMU261P, Pfeiffer-Vacuum) and flooded with a Mars gas mixture (95.25% CO_2, 2.69% N_2, 1.64% Ar, 0.15% O_2, Praxair). For temperature adjustment a cryostat (RK 20, Lauda, Königshofen, Germany) was used and samples were exposed on a cold plate inside the chamber.

2.3.6.1 Survival of dried biofilms of A. ferrooxidans and S. thermosulifdooxidans

Biofilms of *A. ferrooxidans* and *S. thermosulfidooxidans* grown 5 days on filter membranes were removed from the growth medium, put into a sterile glass petri dish, and immediately transferred to the MaSimKa to avoid drying in ambient air. The atmosphere in the chamber was exchanged with a Mars gas mixture, and the pressure was adjusted to 7 hPa. The temperature was kept at a constant -20°C, or manually adjusted each morning to +20°C and each evening to -20°C to

simulate diurnal temperature fluctuations. The relative humidity inside the MaSimKa was ~1.5% during -20°C and 3% during +20°C.

Biofilms were exposed to these conditions for 1 week. Parallel samples of dried biofilms were stored in the anaerobic chamber for 1 week as a laboratory control.

Upon removal from the MaSimKa or the anaerobic chamber, the biofilms were immediately analyzed. For *S. thermosulfidooxidans*, they were returned to liquid growth medium (BSM with Fe^{2+}) and incubated at the appropriate conditions to observe regrowth and measure iron oxidation rate by taking aliquots at different times. For *A. ferrooxidans*, biofilm cells were also detached from polycarbonate membranes using the detachment procedure (described in section **2.2.3.4.1**, p. 49) and enumerated (total cell number and viable cell number by MPN, section **2.4**, p. 64).

2.3.6.2 Growth of A. ferrooxidans at low O_2 partial pressure

A growth experiment with *A. ferrooxidans* aimed to determine whether growth by aerobic respiration was possible at the oxygen partial pressure expected on Mars' surface.

Cultures with Mars regolith simulants (MRS) were prepared similar to the growth experiments (see section **2.2.4.1**, p. 51), because *A. ferrooxidans* was shown to grow under these conditions. 10 ml BSM with 5% (wt/v) S-MRS or P-MRS in a 15 ml screw cap tube were inoculated with washed cells of a Fe^{2+}-grown preculture (~$1x10^6$ cells/ml initial cell density). In addition, a culture (10 ml BSM with Fe^{2+}) was inoculated as a positive control. Sterile controls were also included. The tubes were covered with polycarbonate filter membranes to ensure sterility and allow gas exchange.

In one experiment, the cultures were incubated 1 week under Mars atmosphere, but at Earth-normal pressure (1013 hPa, 25°C). In a second trial, the pressure of the Mars gas mixture was lowered to Mars-relevant pressures (7 hPa, 20°C). Due to evaporation from the liquid cultures under this low pressure, the pressure rose steadily over the incubation period and had to be manually readjusted each day to remain at 15 ± 5 hPa. The high temperatures were chosen to enable fast

growth. Parallels were incubated under laboratory conditions (1013 hPa, Earth atmosphere, 25°C).

Before and after the incubation period, aliquots were removed from each culture (400 µl) after vigorous shaking to ensure homogenous distribution of cells. In the cultures that were incubated under low pressure, the evaporative loss of liquid was accounted for by restocking the volume in the tubes to the initial value before sampling.

Total and viable cells were enumerated in the samples and soluble iron concentrations determined (section **2.4**, p. 64; section **2.5.3**, p. 68).

2.4 Determination of viable (culturable) and total cell numbers

2.4.1 Total cell counts (TCC)

Total cell numbers were determined using a Neubauer-hemocytometer (depth: 0.02 mm x 0.025 mm^2, Brandt GmbH, Wertheim, Germany) with a phase-contrast microscope (Standard 16 Carl Zeiss, Göttingen, Germany) at 400-fold magnification.

Cell numbers were calculated using the following formula:

$$TCC/ml = (\text{Number of counted cells}/\text{Number of small squares counted}) * 2x10^7$$

2.4.2 Assay for colony formation ability

To determine the culturable cell number in cultures of *D. geothermalis*, the colony formation assay was applied by plating an aliquot (50 µl) on solid R2A medium after appropriate serial dilution in PBS. The agar plates were incubated 48 h at 37°C before visible colonies were enumerated and multiplied with the used dilution.

2.4.3 Most Probable Number (MPN) assay

To determine the culturable cell number in cultures of *A. ferrooxidans*, a modified Most Probable Number (MPN) assay was performed. The sample of *A. ferrooxidans* was serially diluted in BSM. From four consecutive dilutions 6 aliquots (100 µl) each were transferred to 6 wells of a 24-well-plate containing 2 ml BSM with Fe^{2+} per well (**Fig. 2-7**). The plate was incubated without shaking at 30°C under H_2O-saturated atmosphere to avoid excessive evaporation. After 10-14 days the wells in which iron oxidation had occurred were enumerated and Most Probable Number was calculated using an MPN calculator, in which quantities and dilutions could be customized:

(by Mike Curiale; http://www.i2workout.com/mcuriale/mpn/index.html, accessed 29/10/2010)

Fig. 2-7: 24-well-plate used for MPN assay with *A. ferrooxidans*. Per dilution, six wells were inoculated with 100 µl each. Wells contained 2 ml BSM with Fe^{2+}. Wells in which iron oxidation occurred were enumerated (orange color).

2.4.4 Calculation of the culturability fraction after stress exposure

Using the culturable cell numbers determined by the colony formation assay or the MPN assay, the fraction of culturable cells after stress exposure (S) was calculated using the ratio of

$$S = N/N_0,$$

with N = culturable cell number after stress exposure, and N_0 = culturable cell number before stress exposure (control).

The culturability fraction was semi-logarithmically plotted.

2.4.5 Probe Active Count (PAC)

The probe active count (PAC) is a modification of the direct viable count (Kogure et al. 1984), combining incubation of bacteria in the presence of a gyrase inhibitor with fluorescence-*in situ*-hybridization (FISH, see section **2.8.4**, p. 74) as a tool to visualize individual cell viability (Kalmbach et al. 1997). It was applied here for *A. ferrooxidans* after stress exposure. The performance of two gyrase inhibitors was evaluated by comparing total cell numbers of *A. ferrooxidans* incubated 17 h in the absence or presence of 40 – 100 mg/l nalidixic acid or pipemidic acid in BSM with Fe^{2+} (**Table 2-10**).

Table 2-10: Total cell numbers after 17 h incubation of *A. ferrooxidans* in growth medium with different concentrations of gyrase inhibitors (initial cell number was $3.7x10^6$/ml).

		cells/ml
Nalidixic acid	40 mg/l	$6.5x10^6$
	50 mg/l	$5.1x10^6$
	100 mg/l	$4.6x10^6$
Pipemidic acid	40 mg/l	$1.1x10^7$
	50 mg/l	$1.2x10^7$
	100 mg/l	$1.2x10^7$

Pipemidic acid did not inhibit cell division during 17 h of incubation at the applied concentrations, shown by the increase in total cell number by a factor of ~3. Increase of cell numbers in cultures incubated in nalidixic acid (NA) was not statistically significant. In addition, the cells incubated with nalidixic acid were elongated at concentrations >50 mg/l (**Fig. 3-19**, p. 109).

Therefore, PAC was performed by incubating *A. ferrooxidans* after stress exposure in growth medium containing Fe^{2+} with 100 mg/l nalidixic acid overnight (17 h). Subsequently, cells were immediately fixed in formaldehyde for FISH.

2.5 Chemical and analytical methods

2.5.1 pH measurement

The pH was determined using a PB-11 pH meter with a PY-P20 microelectrode (Sartorius AG, Göttingen, Germany). Before pH measurement the instrument was calibrated with commercially available pH buffer solutions (Roth) if necessary.

2.5.2 Oxygen measurement

The oxygen concentration in anaerobic culture flasks was measured with a Micro fiber optic trace oxygen transmitter (Microx TX3 trace, PreSens) with an oxygen (PSt1) or trace oxygen microsensor (TOS7) in needle-type housing (**Fig. 2-8**) and a PT1000 potentiometric temperature probe. The detection limit of the trace oxygen microsensor was 1 ppb of dissolved oxygen. Manual calibration was performed before each measurement using the supplied manufacturer's data.

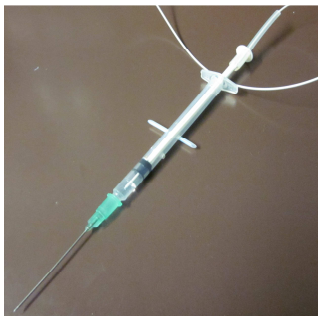

Fig. 2-8: Oxygen microsensor in needle-type housing, which could penetrate the rubber septa oft he anaerobic culture flasks to measure O_2 concentration.

2.5.3 Iron determination

Quantification of soluble iron was done by the phenanthroline colorimetric assay using ammonium acetate (16 g/l dissolved in glacial acetic acid) as a buffer and (1,10)-phenanthroline chloride monohydrate (0.2 g/l) for ferrous iron detection. Hydroxylammonium chloride (4 g/l) was used to reduce ferric iron to ferrous iron to determine the total soluble iron concentration. Samples were diluted appropriately and an iron standard curve was prepared using $FeSO_4$ x 7 H_2O. Absorption at 492 nm was measured in a spectrophotometer U-3310 (Hitachi).

2.5.4 Sulfate and thiosulfate quantification by ion exchange chromatography

Thiosulfate and sulfate concentration was determined by ion-exchange chromatography and conductivity detection using the DIONEX system DX 500. A KOH gradient was applied starting with 5 mM for 1 min followed by a linear increase over 3.5 min to 15 mM. Afterwards, the concentration declined over 1 min to 5 mM and was retained for an additional 3 minutes before the next measurement. Flow rate was 0.36 mL/ min at 170 bar. The chromatogram was processed with Chromeleon 6.70 Build 1820 software.

Before injection, samples were neutralized and ferric iron was precipitated by preparing a 10-fold dilution in 5 mM KH_2PO_4, mixing and incubating for 30 min at room temperature. After centrifuging at 8,000 rpm for 10 min (Heraeus®, Biofuge A), samples were diluted appropriately for analytical determination with double-distilled water. Standards of thiosulfate and sulfate were made of aqueous stock solutions of potassium or sodium salts.

2.5.5 Terbium dipicolinate spore detection

Dipicolinic acid (DPA) is a spore specific marker bound up as calcium dipicolinate within bacterial endospores. When it is released upon germination or cell lysis, free DPA can bind to terbium (Tb^{3+}) ions. Absorption of UV radiation by DPA leads to a characteristic emission spectrum of Tb^{3+} in the visible range due to energy transfer from the DPA. To record the spectra, a Horiba Jobin John

Fluorolog instrument was used with entrance and exit monochromators for wavelength selection of both the excitation and emission light. The excitation light source was a Xenon lamp. For the excitation spectra the exit monochromator was set to 544 nm, the peak of the Tb^{3+} emission, and the entrance monochromator was scanned from 250 – 325 nm over the DPA absorption. For the emission spectra the entrance monochromator was set at 278 nm, the peak DPA absorbance, and the exit monochromator was scanned from 450 – 650 nm over the Tb^{3+} emission.

A calibration curve was established using *Bacillus atrophaeus* spores with concentrations ranging over 10^8 – 10^4 spores/ml to translate Tb-DPA luminescence into spore number. Quantitative values should be treated with caution, as the correlation between DPA contents of *B. atrophaeus* and *S. thermosulfidooxidans* spores is not known. The 544 nm peak from the Tb^{3+} emission was integrated for quantitative comparison. Day to day instrumental variation was accounted for by normalizing to the 10 µM TbDPA reference.

Total spore concentrations in *S. thermosulfidooxidans* samples grown on different media were determined by autoclaving samples at 134ºC for 45 min and adding 100 µM Tb^{3+}. 1.5 ml aliquots were used for measurements. For comparison, aliquots of non-autoclaved spores were also brought to 100 µM Tb^{3+} concentrations, to check for background DPA. Each sample was run twice.

2.6 Fluorescence microscopy

2.6.1 Epifluorescence microscopy

Visualization of fluorescently stained samples was done with an upright epifluorescence microscope (Zeiss® AxioImager™ M2, with HPX 120C fluorescence source) using a Zeiss® plan apochromat oil-objective (100x/1,40 Ph3 M27) and the Zeiss® filter sets 38, 43, and 49. Pictures were taken by a digital microscope camera (Zeiss® AxioCam™ MRm Rev. 3 FireWire, monochromatic) and processed in the Zeiss® AxioVision™ software.

2.6.2 Confocal Laser Scanning microscopy (CLSM)

Visualization of fluorescently-stained biofilms and *D. geothermalis* samples stained with the Live/Dead® bacterial viability kit was done using a laser scanning module (LSM 510 Carl Zeiss®, Jena, Germany) coupled to an inverted Axiovert 100 M BP microscope (Zeiss®). The system was equipped with an argon laser (possible excitation wavelengths: 458 nm, 488 nm and 514 nm at 25 mW), two helium-neon lasers (possible excitation wavelengths: 543 nm at 1 mW and 633 nm at 5 mW) and one argon UV-laser (possible excitation wavelengths: 351 nm and 364 nm at 80 mW). The microscope was operated with the software LSM 510 Release 3.2 (Zeiss®).

2.7 Staining methods

2.7.1 Lectin staining

Biofilms of *D. geothermalis* on glass slides were stained with the FITC-labeled lectin WGA (wheat germ agglutinin, *Triticum vulgaris*). Biofilms were incubated in the lectin solution (50 µg/ml) for 20 min in the dark. After rinsing with particle-free deionized water, total cells in the biofilms were stained with the nucleic acid dye SYTO® 62 (20 µM), incubated 20 min in the dark, rinsed again and visualized by CLSM.

Biofilms of *A. ferrooxidans* on black polycarbonate membranes (GTBP, 25 mm, 0.2 µm, Millipore) were stained with the TRITC-labeled lectin ConA (Concavalin A, *Canavalia ensiformis*). Each filter was first stained with DAPI (4',6-diamidino-2-phenylindole) (1 µg/ml, 5 min in the dark), then washed carefully with deionized water on the filtering device. Subsequently, filter membranes were covered with ConA solution (50 µg/ml) and incubated for 1 h in humid atmosphere in the dark. After washing twice and air-drying, filters were mounted on glass slides in Citifluor AF1 (glycerol/PBS). Before visualization by CLSM samples were stored overnight in the refrigerator. The lectin solution was prepared freshly before use by dilution of 1 mg/ml frozen aliquots of stock solution with particle-free deionized

water and centrifugation for 10 min at 10,000 rpm (Hermle Z216 MK, Gosheim, Germany) to remove aggregated lectin.

2.7.2 Live/Dead® staining

For *D. geothermalis* membrane integrity staining, the Live/Dead® BacLight™ Bacterial viability kit (L13152, molecular probes, invitrogen) was used, which contained the two fluorescent nucleic acid dyes SYTO9™ and propidium iodide (PI), which differ in their ability to penetrate intact cell membranes, causing membrane-damaged cells to emit red fluorescence, while intact cells will appear green. Stock solutions of SYTO9 and PI were prepared according to instructions in deionized, sterile-filtered water and stored at -20°C.

Planktonic cell cultures of *D. geothermalis* were washed twice in PBS and resuspended in 0.14 M NaCl. Cells were stained with 3 µM SYTO9 and 30 µM PI and incubated 15 min in the dark. The protocol was adapted to *D. geothermalis* by testing different stain concentrations in fresh (live) and killed (1 h in isopropanol) cultures. Before visualization, cells were washed to remove excess staining solution and immobilized on black polycarbonate membranes (GTBP, 25 mm, 0.2 µm, Millipore). Visualization was done by CLSM with separate channels for SYTO9 (long pass filter 505) and PI (long pass filter 650) (Multitrack method).

For *A. ferrooxidans*, the Live/Dead® staining protocol was modified by using the general nucleic acid stain DAPI instead of SYTO9™ to avoid the quick fluorescence quenching of this stain. DAPI stock solutions were prepared in sterile-filtered deionized water and stored at -20°C.

Planktonic cell cultures of *A. ferrooxidans* were washed twice, first in BSM, then resuspended in 0.14 M NaCl (particle-free) to neutralize the sample. The samples were stained with 1 µg/ml DAPI and 9 µM propidium iodide for 15 min in the dark. The appropriate concentration of PI was determined by testing different concentrations in samples of fresh and killed (1 h ethanol-treated) cultures. After washing, cells were immobilized on black polycarbonate membranes and visualized by epifluorescence microscopy immersed in Citifluor AF1.

2.8 Molecular methods

2.8.1 DNA extraction

Isolation of genomic DNA from planktonic cell cultures of *A. ferrooxidans* was carried out using the chloroform-phenol extraction (Nieto et al. 2009). The samples were washed with PBS (6000 rpm, 20 min), resuspended in lysis buffer (100 mM NaCl, 50 mM Na_2EDTA, pH 7.5) with 10 mg/ml lysozyme (prepared freshly) and incubated for 30 min at 37°C in a Thermoblock (BTD, Grant Instruments, Cambridgeshire, UK), followed by 1 h of incubation at the same temperature with 1% SDS. Three cycles of freezing at -20°C and thawing at 70°C completed the cell lysis step. Samples were centrifuged (1000 rpm, 5 min, 4°C, Hermle Z216 MK) to separate the lysed cell components and the supernatant transferred to a fresh 1.5 ml-microcentrifuge tube containing an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). After mixing and centrifugation (5000 rpm, 5 min, 15°C, Hermle Z216 MK), the upper phase was recovered into a new tube and the extraction procedure was repeated once. This was followed by two similar treatments with chloroform-isoamylalcohol (24:1) to remove residual phenol. The DNA contained in the final aqueous phase was precipitated by addition of 1/10 volume of saturated ammonium acetate, layered with an equal volume of ice-cold absolute isopropanol and incubated at 4°C for 1 h. After a final centrifugation step (13000 rpm, 30 min, 4°C, Hermle Z216 MK), the isolated DNA was rinsed once with 70% ice-cold ethanol and air-dried before resuspension in TE buffer (10 mM Tris-HCl pH 8, 1 mM Na_2EDTA) or deionized water (depending on the desired use). The dissolved DNA was measured in the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific).

2.8.2 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR with genomic DNA of *A. ferrooxidans* was performed to detect DNA damage after stress treatment. A 977 bp fragment of the 16S rDNA was amplified using specific primers for *A. ferrooxidans* designed by Escobar et al. (2008) (**Table 2-3**, p. 42). Each PCR reaction mix contained 12.5

µl Platinum® SYBR® Green Super Mix (invitrogen), 9.5 µl of ultrapure deionized water, 0.5 µl of forward and reverse primer each (2 µM), and 2 µl genomic DNA in a final volume of 25 µl. The extracted DNA (in ultrapure deionized water) was diluted to reach equal concentrations in all samples and reactions were performed in triplicate assays. A negative control was included containing no DNA. The standard curve was obtained with genomic DNA of *A. ferrooxidans* (untreated culture) by preparing five serial 1:5-dilutions.

Quantitative real-time PCR reactions were carried out in a DNA Engine Opticon 2 continuous fluorescence detection system (CFD-3220, MJ Research Inc., St. Bruno, Canada) with the Opticon Monitor software (MJ Research) with the following temperature program: Initial denaturation for 10 min at 95°C; 40 cycles of 30 s denaturation at 95°C, annealing for 30 s at 58.5°C, extension for 30 s at 72°C; final extension for 2 min at 72 °C. Fluorescence was detected and a melting curve recorded from 50°C to 95°C in steps of 0.5°C. Analysis of the melting curve indicated that the amplified product was specific.

2.8.3 Agarose gel electrophoresis

Isolated DNA and PCR products were visualised by agarose gel electrophoresis. Aagarose (Serva) was melted in 1x TBE buffer (10.8 g/l Tris base, 5.5 g/l boric acid, 2 mM EDTA) at 0.8-3% (wt/v) depending on the size of the DNA. 10 µl/l of 1000x SYBRSafe (Molecular probes, Invitrogen) were added to the agarose before gelling for fluorescent-staining of nucleic acids. Samples were mixed with 6x DNA loading dye (Fermentas), loaded into the agarose gel slots and subjected to 80 V through 1x TBE buffer for 60-120 min. A standard DNA size marker (1 kb DNA Ladder, 0.5 µg/µl, Fermentas) was electrophoresed in a lane next to the DNA samples to compare with the band position (size) of the DNA fragments. After electrophoresis, nucleic acid were visualized using ImageQuant LAS 4000 digital imaging system (GE Healthcare GmbH, Freiburg, Germany).

2.8.4 Fluorescence *in situ* hybridization (FISH)

FISH with planktonic cultures of *A. ferrooxidans* was performed using the oligonucleotide probe EUB338, specific for the 16S rRNA of all eubacteria (**Table 2-3**, p. 42). Stock solutions of the probe (50 ng/µl) were stored at -20°C. Fixation of planktonic cells was carried out in 3.7% (wt/v) formaldehyde at 4°C for 3 h. Subsequently, cells were immobilized and washed by filtering samples through black polycarbonate membranes (GTBP, 25 mm, 0.2 µm, Millipore) and air-drying. Filters were stored at -20°C until hybridization.

For hybridization, filters were quartered and one piece was covered with 20 µl hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, 35% formamide, 0.01% SDS) containing 5 ng/µl of the probe. The formamide concentration was adjusted for optimal fluorescence yield. The filter pieces on glass slides were incubated under humid atmosphere in the dark at 46°C for 2 h. After incubation, the filters were quickly transferred to pre-warmed (46°C) washing buffer (80 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS) and incubated again at 46°C for 20 min to remove unbound probe. Samples were washed in deionized water and air-dried before counter-staining with 1 µg/ml DAPI (5 min in the dark). Finally, filters were washed in deionized water and 80% ethanol, air-dried completely and mounted in Citifluor AF1 for epifluorescence imaging.

All fluorescence images shown in this study were recorded at the same exposure times, if not otherwise stated. The images were not processed afterward to ensure comparability.

2.9 Numerical and statistical analyses

The data shown in this study represent mean values with standard deviations. Statistical significant differences between treatments were determined using Student's *t*-test. Differences with P values <0.05 were considered statistically significant.

D/F_{10} values (dose or fluence of radiation resulting in a 90% inactivation of a population) were determined from the linear parts of semi-logarithmic survival

curves using SigmaPlot 8.0 (Systat Software Inc., San Jose, CA, USA) by linear regression.

Growth rate and iron oxidation rate were calculated from the slope of the exponential part (total cell numbers) or linear part (iron concentration) of the growth curves using the following equations:

Growth rate (h^{-1}):

$v = \Delta lgTCC / lg2 * \Delta t$

where t is time

Generation time (h):

$g = 1 / v$

Iron oxidation rate (mg/l/h):

$R = \Delta[Fe] / \Delta t$

where [Fe] is Fe^{2+} concentration

Specific iron oxidation rate ((mg/l) / ($h*10^9$ cells)):

$\sigma = R / (\Delta TCC/10^9)$

Equations from:

http://www.uni-konstanz.de/FuF/Bio/research/Arbeitsgruppen/

Groettrup/Homepage/Skripte/Teaching/L%205%20b%20wachstum.pdf

(accessed 31/07/2012)

3 Results

Acidophilic iron-sulfur bacteria have been suggested as candidates for potential Martian life forms as they are able to utilize elements abundant on Mars (iron and sulfur compounds). In the past (>3 Ga ago), such organisms might have taken part in the deposition of the layered sediments distributed on Mars, which contain hydrated sulfates and ferric oxide minerals (Amils et al. 2007, see section **1.2.3**, p. 19). *Acidithiobacillus ferrooxidans* and *Sulfobacillus thermosulfidooxidans* were selected as model organisms to study the ability of acidophilic iron-sulfur bacteria to survive or grow under Mars-relevant environmental conditions using their diverse metabolic capacities.

The experimental strategy employed is outlined in **Fig. 3-1**. To answer the question whether the model organisms can utilize *in situ* resources present on Mars, their ability to participate in the redox cycling of iron and growth on a variety of substrates including minerals in Mars regolith was evaluated. In these growth experiments, liquid cultures were used under the assumption that liquid water, which is a pre-requisite for growth at least for terrestrial organisms, would be present in micro-habitats on Mars, e.g. as aquifers in the subsurface (possibilities for liquid water to occur on Mars are discussed in section **1.2.2.2**, p. 11). Both aerobic and anaerobic metabolism was considered because plausible sources for O_2 and H_2 may exist on Mars (see section **1.2.2.4**, p. 14). In assessing growth, total cell counts (TCC) were supplemented by Most Probable Number (MPN) to obtain information about the fraction of viable cells and observe the possible transition of culturable cells to a viable-but-nonculturable (VBNC) state.

Even though relatively protected subsurface habitats with available liquid water are considered most likely to harbor life on Mars, the Martian environment is not a static one and organisms might be exposed periodically to adverse conditions they would have to endure. Therefore, the general resistance capacities of the model bacteria to various stress factors was investigated and compared between planktonic cells and biofilms. Different methods were chosen to determine the physiological state and cellular integrity of cells after stress treatment, which is

important to include cells in a VBNC state, in which they may be unable to grow and divide.

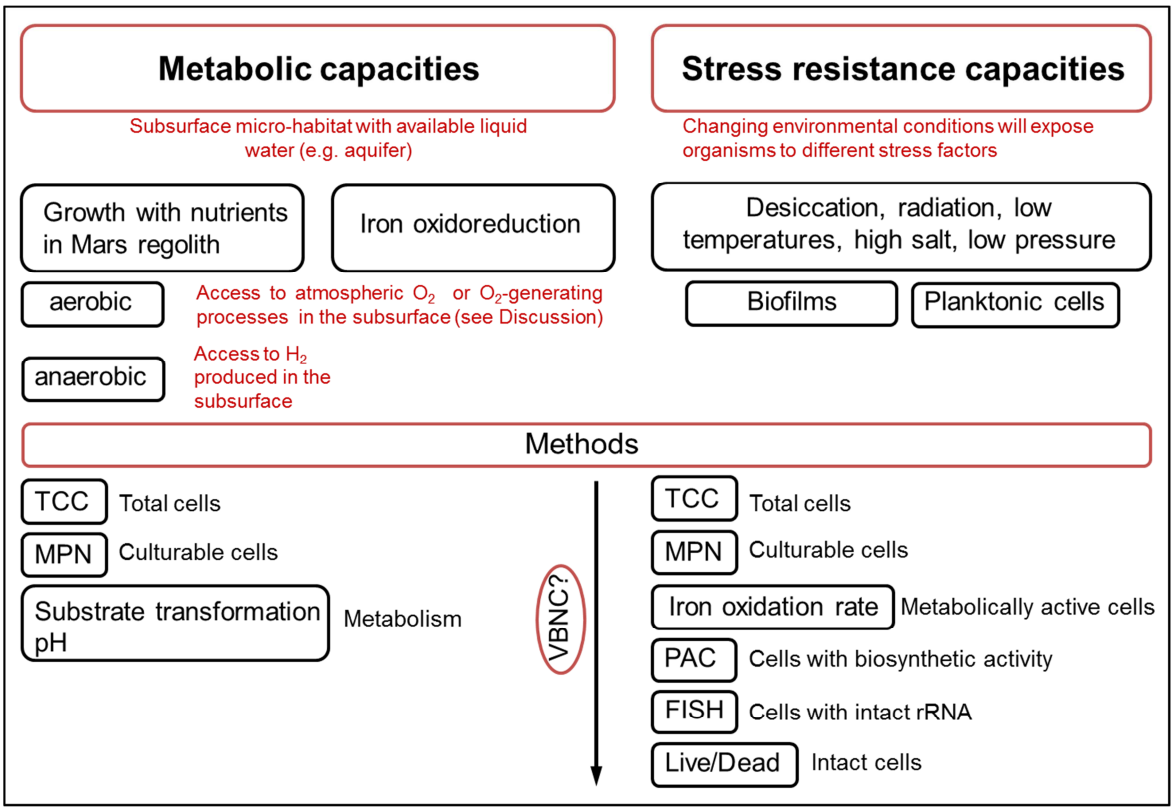

Fig. 3-1: Schematic outline of the experimental strategy designed to evaluate the capability of acidophilic iron-sulfur bacteria to survive and grow under Mars-relevant environmental conditions with *in situ* resources. Assumptions regarding potential Martian habitats are in red font.

3.1 Metabolic capacities

A. ferrooxidans and *S. thermosulfidooxidans* are characterized by a high degree of metabolic versatility expressed in the ability to use a variety of electron donors and acceptors under aerobic and anaerobic conditions. The two strains used in this study were the type strains obtained by the German Collection of Microorganisms and Cell Cultures (DSMZ). Growth experiments were performed to assess basic growth parameters of planktonic cells and biofilms grown aerobically on ferrous iron (section **3.1.1**) because these cultures served as

precultures for the stress resistance tests. Furthermore, anaerobic growth (section **3.1.2**) and iron oxidoreduction (section **3.1.3**) were studied, as well as the ability of *A. ferrooxidans* to grow on Mars regolith simulants (section **3.1.4**).

3.1.1 Aerobic growth

3.1.1.1 Planktonic cells

Precultures for stress resistance tests were generally grown aerobically on ferrous iron (Fe^{2+}) because of faster and more reproducible growth and facilitated handling compared to anaerobic cultivation. Growth of the precultures was characterized to determine the growth phase in which bacteria were exposed to stress conditions (which can have a significant impact on their resistance).

S. thermosulfidooxidans incubated mixotrophically on Fe^{2+}/O_2 with yeast extract (0.02% wt/v) grew to maximum mean cell densities of $8.6x10^7$ cells/ml, reaching stationary phase after approximately 48 h when incubated at 45°C without shaking (**Fig. 3-2**). While Fe^{2+} was being oxidized by the bacteria, the pH value initially increased from 1.8 to a peak value of 2.3 because oxidation of ferrous iron is accompanied by H^+-consumption for the generation of the proton motive force (equation 5) (Holmes and Bonnefoy 2007; Nemati et al. 1998). After ca. 90 h of incubation, the total cell number, assessed microscopically by a hemocytometer, started to decline again although iron oxidation was not complete (64% of the intial Fe^{2+} oxidized). Simultaneously with the cell number, pH values dropped, and the total soluble iron concentration decreased to approximately 82% of the initial value at the last sampling point. This resulted from the formation of ferric oxyhydroxides ($Fe(OH)_x$) and jarosite ($MFe_3(SO_4)_2(OH)_6$) by abiotic, acid-generating reactions, which remove soluble iron via precipitation (equations 6 and 7) (Nemati et al. 1998). Cells of *S. thermosulfidooxidans* were observed to attach to these ferric iron precipitates, probably accounting for the decline in cell numbers.

$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$ (5)

$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+$ (6)

$3Fe^{3+} + M^+ + 2HSO_4^- + 6H_2O \rightarrow MFe_3(SO_4)_2(OH)_6 + 8H^+$, (7)

with $M = K^+$, Na^+, NH^{4+}, or H_3O^+

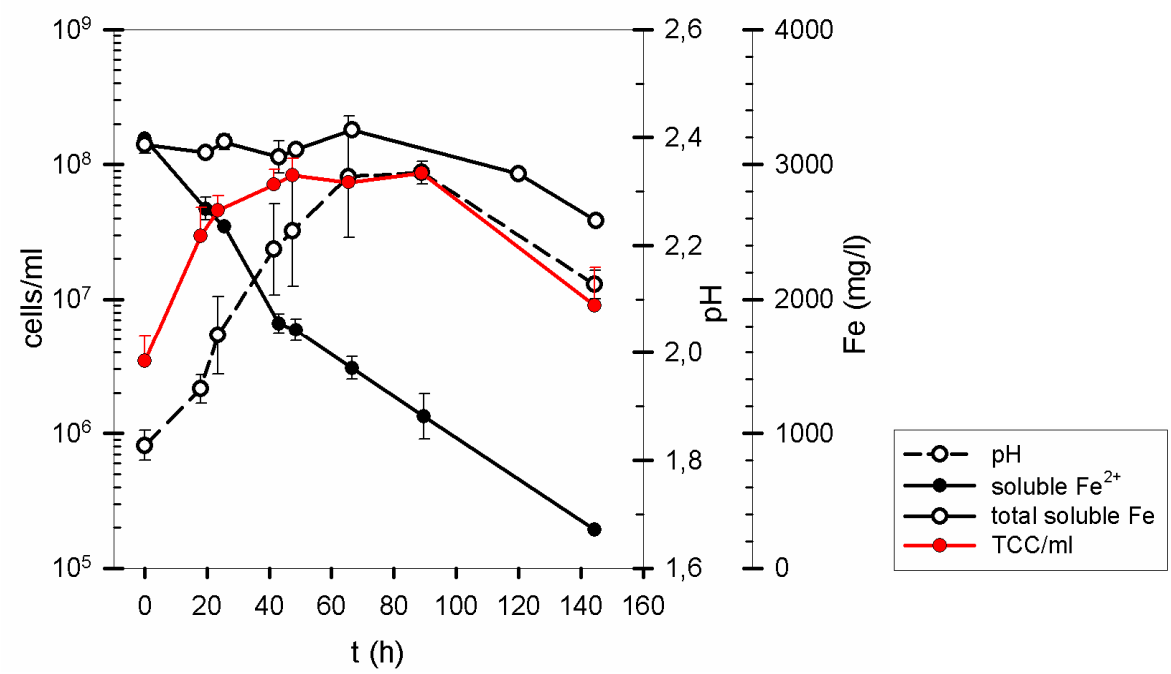

Fig. 3-2: Growth of *S. thermosulfidooxidans* on Fe^{2+}/O_2 with yeast extract (0.02%) at 45°C (without aeration), followed by total cell counts (TCC), pH, and soluble iron concentrations. Data expressed as mean (n=4) with standard deviation.

A. ferrooxidans grown autotrophically on Fe^{2+}/O_2 reached maximum cell densities of 8.9×10^7 cells/ml (**Fig. 3-3**). Microscopic total cell counts were complemented by the Most Probable Number (MPN) assay to obtain the number of culturable cells. The growth curve determined by MPN showed the same behavior as the growth curve recorded by counting total cells, and the fraction of culturable cells from total cells amounted to a mean value of 80%. Thus, in *A. ferrooxidans* MPN can be used as an estimate for growth, e.g. in cultures where direct counting is not feasible such as in Mars regolith minerals (section **3.1.4**, p. 95). For *S. thermosulfidooxidans*, the MPN assay was not practicable due to the tendency of

cells to form long chains and aggregates and the quick evaporation of medium in the higher temperature regime.

Early stationary phase in *A. ferrooxidans* incubated at 30°C with aeration began after ca. 65 h as indicated by the lack of a significant increase in cell density following this time point. At the same time, pH values also reached their peak, coinciding with the end of iron oxidation and marking the onset of energy-depleted conditions. In contrast to cultures of *S. thermosulfidooxidans*, the total soluble iron concentration in *A. ferrooxidans* cultures remained largely constant over the period of measurement with only a slight decline of ~8% toward the end of the 120 h due to ferric iron precipitation.

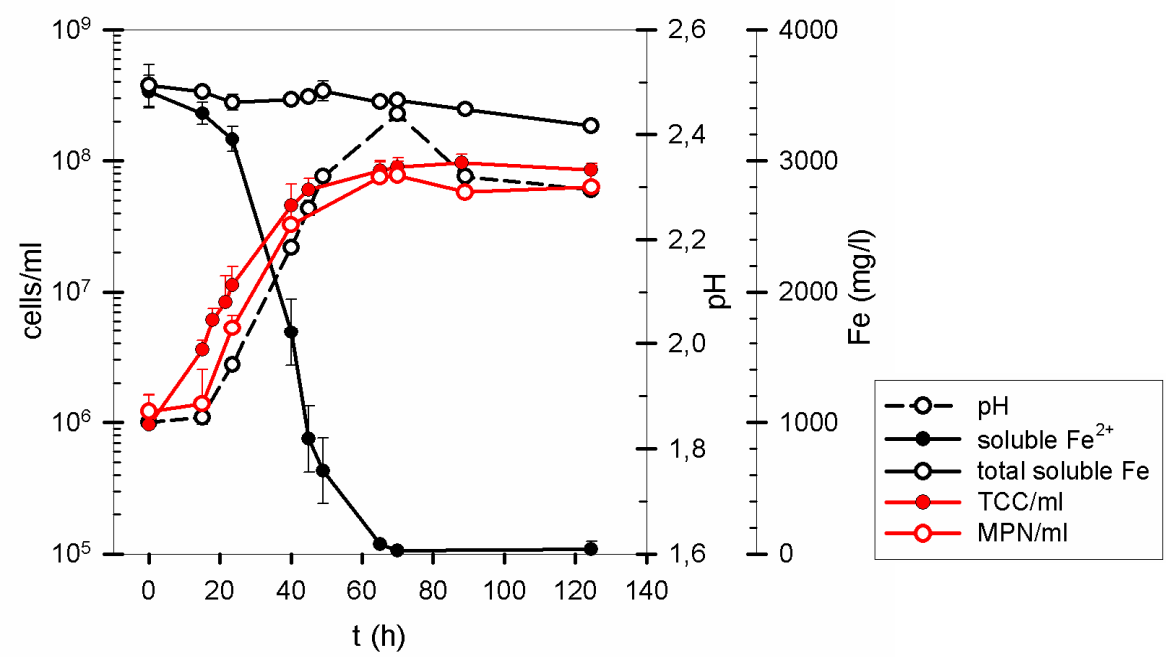

Fig. 3-3: Growth of *A. ferrooxidans* on Fe^{2+}/O_2 at 30°C (with aeration) followed by different growth parameters including total cell counts (TCC), Most Probable Number (MPN), pH, and iron concentration. Data expressed as mean (n=6) with standard deviation.

Long-term experiments showed that 20% of the ferric iron precipitated over the course of 42 days at 30°C, followed by a further decrease of pH to about 2.1 (**Fig. 3-4**). Total cell numbers remained constant over a prolonged period of time under these conditions, and despite lack of an energy source, culturable cell numbers

(MPN) exhibited the same behavior decreasing only slightly at the last sampling time point.

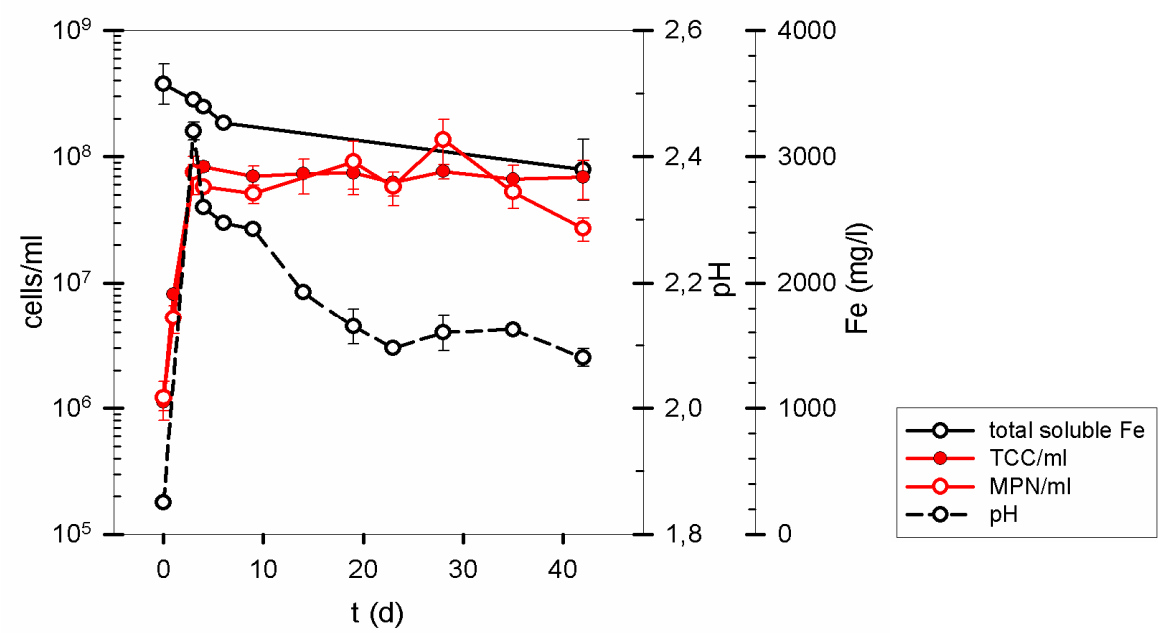

Fig. 3-4: Long-term incubation of *A. ferrooxidans* on Fe^{2+}/O_2 at 30°C (with aeration) showing progression of TCC, MPN, pH, and total soluble iron concentration. Fe^{2+}-concentration not shown for the sake of clarity. Data expressed as mean (n=2) with standard deviation.

Although the rate of cell division in exponential phase cells of *A. ferrooxidans* and *S. thermosulfidooxidans* under these growth conditions was the same, both strains exhibiting a generation time of ~6 h, the rate of substrate conversion (Fe^{2+}-oxidation) differed significantly (**Table 3-1**). *A. ferrooxidans* oxidized 65.1 mg/l of Fe^{2+} (per h and 10^9 cells) during exponential growth and Fe^{2+}-concentrations followed a sigmoid function (**Fig. 3-3**), while *S. thermosulfidooxidans* had a specific Fe^{2+}-oxidation rate of 20.7 mg/l (per h and 10^9 cells), which remained relatively independent of cell densities (**Fig. 3-2**). This may be explained by the mixotrophic incubation of *S. thermosulfidooxidans* with yeast extract, resulting in the use of organic compounds for energy gain in addition to ferrous iron. Autotrophic growth on Fe^{2+} without yeast extract was not observed with this strain.

Table 3-1: Growth rate, generation time (doubling time), and rate of Fe^{2+}-oxidation for *A. ferrooxidans* and *S. thermosulfidooxidans* grown on Fe^{2+}/O_2 (plus yeast extract in the case of *S. thermosulfidooxidans*) at their respective optimum growth temperatures. Data expressed as mean (n=4-6) ± standard deviation.

	A. ferrooxidans	*S. thermosulfidooxidans*
Growth rate (cell divisions/h)	0.17 ± 0.03	0.16 ± 0.01
Generation time (h)	6.2 ± 1.3	6.1 ± 0.5
Fe^{2+} oxidation rate (mg/l/h)	106.5 ± 16.0	30.6 ± 3.4
Specific Fe^{2+}-oxidation rate (mg/l) / (h*10^9cells)	65.1 ± 10.3	20.7 ± 3.4

Conclusion:

Aerobic growth experiments with *A. ferrooxidans* and *S. thermosulfidooxidans* were performed to characterize precultures for the stress resistance tests. These tests were carried out with late exponential to early stationary phase cells after 48 h of incubation, before depletion of the energy source. In *A. ferrooxidans*, the fraction of culturable cells remained high (~80%) up to 30 days of incubation although all ferrous iron was oxidized after 3 days. MPN can be used in this strain to assess growth under aerobic conditions, for example in cultures where total cell numbers cannot be determined.

3.1.1.1.1 Sporulation of *S. thermosulfidooxidans*

S. thermosulfidooxidans was chosen in this study due to its capacity to form endospores (Bogdanova et al. 2002; Norris et al. 1996), which is considered an important adaptation to withstand periods of adverse environmental conditions (Nicholson et al. 2000; Setlow 2006). To evaluate sporulation efficiency in this strain, *S. thermosulfidooxidans* was incubated in ‘sporulation medium’ designed to induce spore formation (Bogdanova et al. 2002). Sporulation medium contained higher amounts of calcium, magnesium, and manganese salts, and lower amounts of Fe^{2+} (1.5 g/l) and yeast extract (0.015%) compared to the usual incubation medium.

Cultures from stationary phase grown on S^0/O_2 or Fe^{2+}/O_2 were washed twice in BSM and analyzed by measuring Terbium-dipicolinate luminescence (see section **2.5.5**, p. 68), a method independent of visual examination to detect spores (done at JPL, CA, USA, in cooperation with Dr. Aaron Noell). This method is based on the release of the spore-specific substance dipicolinic acid (DPA) from spores upon germination or cell lysis (Cable et al. 2007). Spores of *Bacillus* spp. and *Clostridium* spp. contain ≥10% of their dry weight as DPA chelated with divalent cations, mainly Ca^{2+} (Murrell 1967), but it is not known whether endospores of Sulfobacilli are constructed in the same manner. DPA can be bound by terbium (Tb^{3+}) ions, yielding a Tb-DPA complex, which emits light in the visible range of the electromagnetic spectrum upon excitation by UV. Using *B. atrophaeus* spores with concentrations ranging over 10^4–10^8 spores/ml a calibration curve was established to estimate spore number from the Tb-DPA luminescence intensity. Total spore concentrations were examined by autoclaving samples at 134ºC for 45 min to release DPA.

Fig. 3-5 shows the spore calibration curve obtained with *B. atrophaeus* spores plotted with the samples of *S. thermosulfidooxidans* from different growth media at the appropriate spore concentration according to their luminescence intensity. Quantitative values should be treated with caution, as the correlation between DPA contents of *B. atrophaeus* and *S. thermosulfidooxidans* spores is not known. The estimated spore number for *S. thermosulfidooxidans* was $1.2x10^6$ spores/ml for the sample grown on S^0/O_2 in BSM and ~10^5 spores/ml for the sample from Fe^{2+}/O_2 in BSM. In comparison to the total cell counts in these samples, sporulation efficiency amounted to about 2% in S^0-medium and 0.5% in Fe^{2+}-medium. The sample of *S. thermosulfidooxidans* grown on Fe^{2+}/O_2 in sporulation medium showed no evidence of spores.

The markers with a black outline in **Fig. 3-5** are the non-autoclaved (NA) trials of each sample, included to check for background DPA, as DPA is known to leak out of spores. It is also possible that some spores form and then randomly germinate, releasing their DPA. Background DPA implies spore formation, as it is unlikely to be present in noticeable amounts for other reasons, but it makes it more difficult to obtain an accurate amount of spores present in the suspension when the signal is on the same order of magnitude as that of the autoclaved

sample. For the sample 'S^0/O_2' the amount of background (NA) signal was small relative to the autoclaved samples, but for sample 'Fe^{2+}/O_2' it was about half as strong, implying that spores had formed in the medium, but had spontaneously germinated or lysed before analysis.

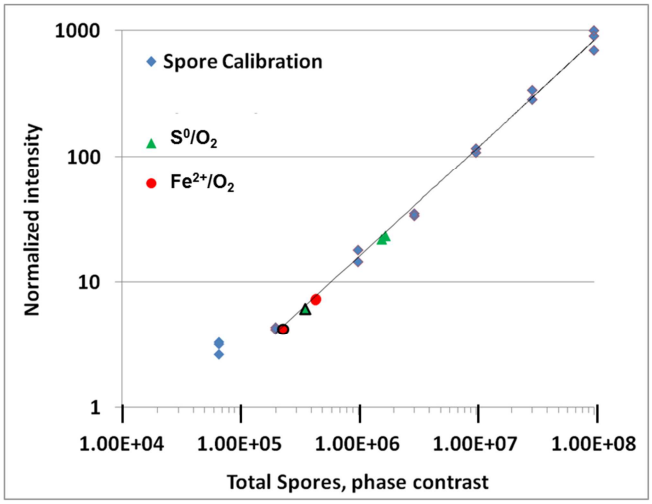

Fig. 3-5: Calibration curve for *B. atrophaeus* spores (blue diamonds) with two samples of *S. thermosulfidooxidans* (grown on S^0/O_2 or Fe^{2+}/O_2 in BSM) placed appropriately based on their normalized intensity. Markers with black outline are the non-autoclaved samples for determination of background DPA. Image courtesy by Dr. Aaron Noell (Jet Propulsion Laboratory, CA, USA).

Conclusion:

Sporulation efficiency in the type strain of *S. thermosulfidooxidans* was low under the conditions tested here, but spores could be detected by the Tb-DPA luminescence method, showing that endospores of *S. thermosulfidooxidans* share this similarity to other spore-forming bacteria such as *Bacillus* spp. The low sporulation efficiency did not allow harvesting of spores for a further characterization of their resistance properties.

3.1.1.2 Biofilms of A. ferrooxidans

To answer the question, whether the biofilm mode of growth has an influence on stress resistance in *A. ferrooxidans* or *S. thermosulfidooxidans*, a biofilm model had to be developed, for which quantitative evaluation of survival would be

feasible. The approach was to grow biofilms on membrane filters floating on liquid medium with Fe^{2+} as an energy source. After growth, these biofilms could be removed from their growth medium to be exposed to different stress conditions. Although this is a simplified model that may not be directly applicable to potential Martian biofilms, it adequately served to compare surface-attached cells to planktonic cells, which are commonly used in stress resistance tests with microorganisms.

The iron oxidation rate of growing biofilms of *A. ferrooxidans* was compared to that of a culture of planktonic cells starting with the same total number of cells (10^6) and incubated under equal conditions (without shaking at 30°C in 40 ml medium). Growth was assessed by TCC and iron oxidation rate. For enumerating biofilm cells, they were mechanically detached from the membrane surface prior to examination as described in section **2.2.3.4.1** (p. 49), necessitating the use of a separate filter at each sampling point. In the medium of the biofilm cultures, no planktonic cells were detected by microscopy during the entire incubation period, confirming that the contribution of planktonic cells to the iron oxidation rate in these cultures was minimal, if at all existent. It also indicated that, once filtered onto a membrane, few cells were released into the liquid medium, on which the filter was floating.

In the planktonic cell cultures, the final cell density reached after 120 h of incubation was 8.6×10^7/ml, amounting to a total biomass of $2.8 \times 10^9 \pm 3.1 \times 10^8$ cells. The final biomass in the biofilms was $2.9 \times 10^9 \pm 1.3 \times 10^9$ cells per membrane (no statistically significant difference to planktonic cells; $P = 0.37$). Growth and iron oxidation curves are shown in **Fig. 3-6**. Generation time (during exponential growth) of cells in a biofilm was 6.6 h, comparable to the 7.6 h calculated for planktonic cells ($P = 0.16$) (**Table 3-2**). The rate of Fe^{2+}-oxidation, on the other hand, was twice as high for planktonic cultures with 44.6 mg/l / ($h*10^9$ cells) than for biofilms with 23.8 mg/l / ($h*10^9$ cells) ($P < 0.05$). This might be an effect of reduced rate of nutrient diffusion through the filter.

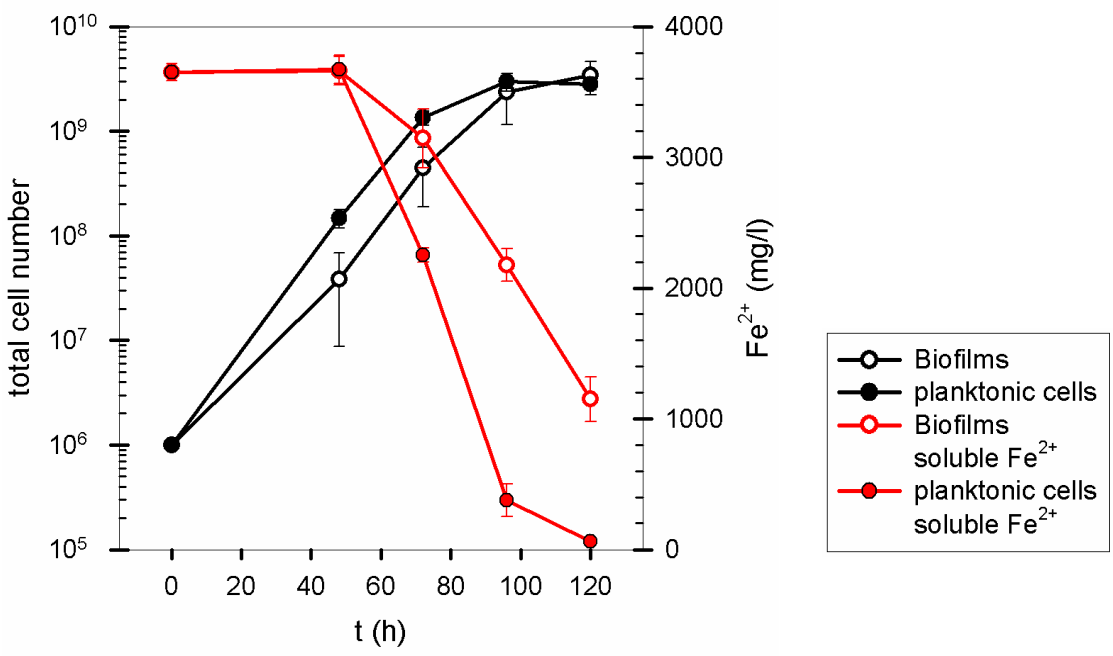

Fig. 3-6: Growth and iron oxidation of *A. ferrooxidans* biofilms on filters and planktonic cells incubated under the same conditions, expressed as total cell number (biomass) per biofilm or culture, and Fe^{2+} concentration. Data expressed as mean (n=4) with standard deviation from two independent experiments.

Table 3-2: Growth rate, generation time (doubling time), and rate of Fe^{2+}-oxidation (normalized to 10^9 cells) for *A. ferrooxidans* biofilms and planktonic cells incubated in static conditions at 30°C. Data expressed as mean (n=4) ± standard deviation.

	Biofilm	*Planktonic cells*
Growth rate (cell divisions/h)	0.15 ± 0.02	0.13 ± 0.02
Generation time (h)	6.6 ± 0.9	7.6 ± 0.9
Specific Fe^{2+}-oxidation rate (mg/l) / (h*10^9cells)	23.8 ± 6.9*	44.6 ± 4.9*

*P<0.05

To test stress resistance of these biofilms of *A. ferrooxidans*, filter membranes were removed from the liquid medium after 5 days of growth to obtain cells in the early stationary phase comparable to planktonic cell cultures, which were harvested after 48 h of growth (**Fig. 3-3**, p. 80 – the time difference results from different intial cell densities: in normal aerobic cultivation 10^6/ml was used, while this experiment – to compare with biofilms – started with 2.5x10^4/ml, i.e. 10^6 cells

in 40 ml medium). Biofilms of this growth stage consisted of about $2.9x10^9$ ± $1.3x10^9$ cells (total biomass). The corresponding MPN was consistently in the same order of magnitude as the TCC with $2.2x10^9$ ± $1.7x10^9$ culturable cells, indicating that the percentage of viable cells in these biofilms was high (~75%).

The structure of a biofilm this age was visualized by Confocal Laser Scanning Microscopy after DAPI-staining. The biofilm consisted of several layers of cells, packed more densely in some regions, while other areas were nearly devoid of cells, consistent with the typical patchy structure of biofilms. Biofilms were often covered by ferric iron precipitates formed due to the metabolism of *A. ferrooxidans* (**Fig. 3-7**). However, the surface of the biofilms did not appear 'slimy', a phenotype usually caused by extensive formation of hydrophilic polysaccharides. Staining biofilms with the fluorescently-labelled lectin Concavalin A (ConA, from *Canavalia ensiformis*, recognizes α-D-mannosyl and α-D-glucosyl groups), which has been used previously in *A. ferrooxidans* (Bellenberg et al. 2011; Gehrke et al. 1998; González et al. 2012), also revealed no significant fluorescence signal compared to planktonic cells. This does not preclude the formation of extracellular polymers, though it implies that the amount of polysaccharides containing the inhibitory sugar residues for this lectin was negligible.

Fig. 3-7: A 5-day-old biofilm on a polycarbonate membrane.

Conclusion:

Growing biofilms of *A. ferrooxidans* oxidized iron at a slower rate than planktonic cells. Cells of *A. ferrooxidans* formed patchy biofilms covered by iron precipitates, but extensive EPS production could not be detected by staining with the lectin ConA.

3.1.2 Microaerobic and anaerobic growth

The type strain of *A. ferrooxidans* has been shown to grow anaerobically on S^0/Fe^{3+} (Pronk et al. 1991a, 1992, also demonstrated in the experiment in section **3.1.3**, p. 91), and aerobically on H_2/O_2 at pH values of >2.5 (Drobner et al. 1990). Growth on H_2/Fe^{3+} was not previously reported for the type strain (Ohmura et al. 2002).

For experiments assessing growth of *A. ferrooxidans* on Mars regolith simulants (MRS) under anaerobic conditions (see section **3.1.4**, p. 95), control experiments were carried out to observe if growth occurred under the same conditions without MRS, in either N_2/CO_2 or H_2/CO_2 (80:20) atmosphere with or without Fe^{3+} added as an electron acceptor. Initial O_2 concentrations were below 1% in all cultures (i.e. microaerobic conditions).

A. ferrooxidans grew well in H_2/CO_2 atmosphere with Fe^{3+} accompanied by Fe^{3+}-reduction and decreasing pH values (**Fig. 3-8 A**), in agreement with a metabolic reaction using H_2/Fe^{3+} according to equation 8. Fe^{3+}-reduction started only after O_2 levels dropped below ~0.2%, indicating that aerobic metabolism occurred during the early stages of growth. This was followed up by a control experiment under H_2/CO_2 atmosphere without the addition of Fe^{3+} (**Fig. 3-9 A**). Here, growth also occurred in concurrence with O_2 consumption, but without a decrease in pH. This indicates usage of H_2/O_2 as an electron donor/acceptor couple by *A. ferrooxidans* (equation 9). Culturable cell numbers (MPN) initially increased with total cell numbers in both cultures in H_2/CO_2 atmosphere, but declined when the electron acceptors were depleted, in contrast to the observed long-term culturability in aerobic conditions (**Fig. 3-4**, p. 81).

$$H_2 + 2Fe^{3+} \rightarrow 2Fe^{2+} + 2H^+ \quad (8)\ \text{(Amend and Shock 2001)}$$

$$2H_2 + O_2 \rightarrow 2H_2O \quad (9)\ \text{(Amend and Shock 2001)}$$

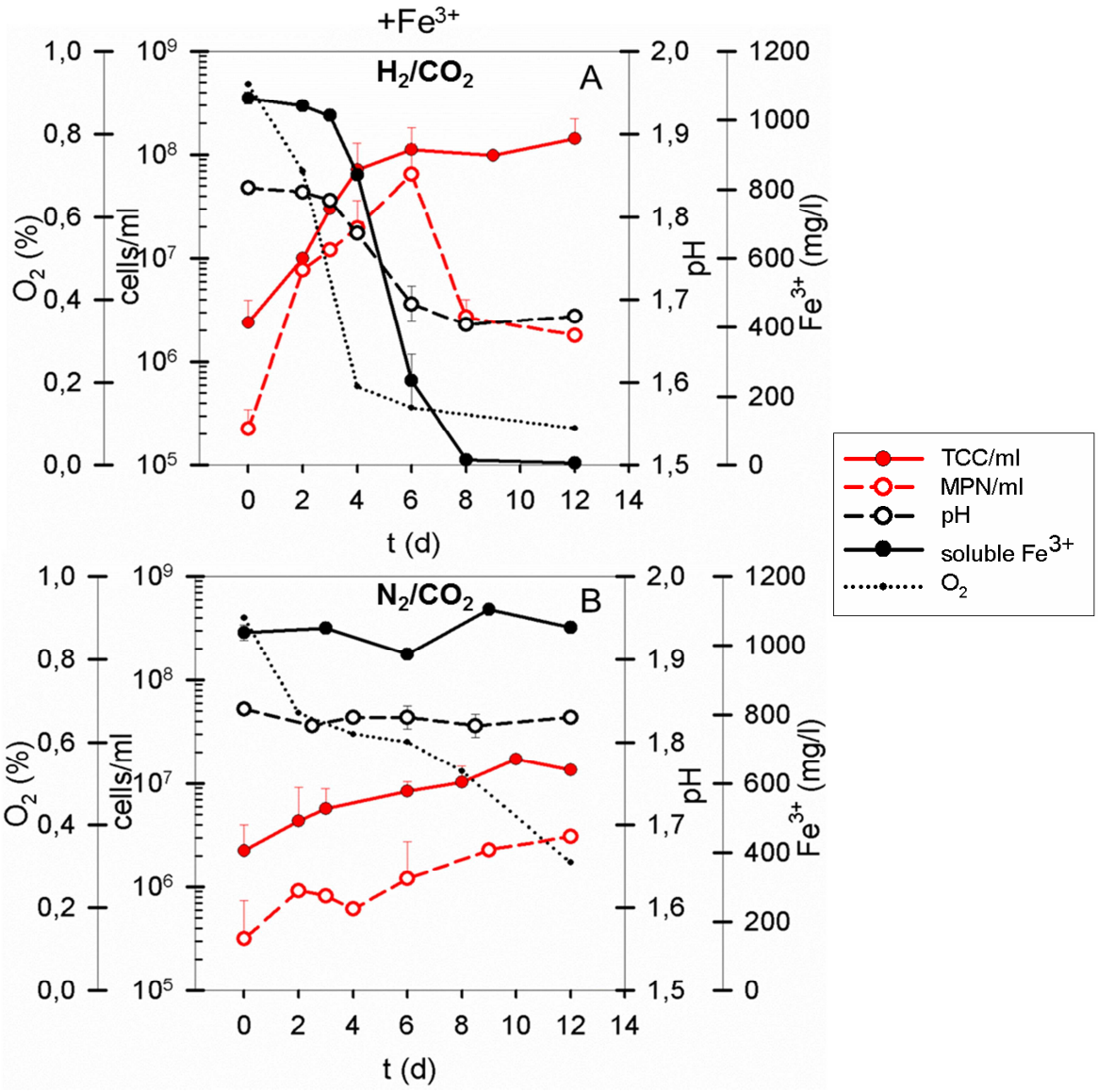

Fig. 3-8: Growth of *A. ferrooxidans* in H_2/CO_2 (80:20) atmosphere (A) or N_2/CO_2 (80:20) atmosphere (B) with Fe^{3+} added as electron acceptor, followed by total cell counts (TCC), MPN, pH, O_2 concentration, and Fe^{3+}-reduction. Data expressed as mean (n=2) with standard deviation.

Additional control experiments were performed with cultures incubated under N_2/CO_2 atmosphere to observe if growth occurred in the absence of H_2 as electron donor (**Fig. 3-8 B** and **Fig. 3-9 B**). When Fe^{3+} was present (**Fig. 3-8 B**), cell density and MPN increased, but at a slow, non-exponential rate, which may have been due to small amounts of ferrous iron and the residual oxygen in the flask. Incubation under N_2/CO_2 atmosphere without Fe^{3+} was the only control

condition in which no growth was observed (**Fig. 3-9 B**) and where O_2 concentration did not fall below 0.4%.

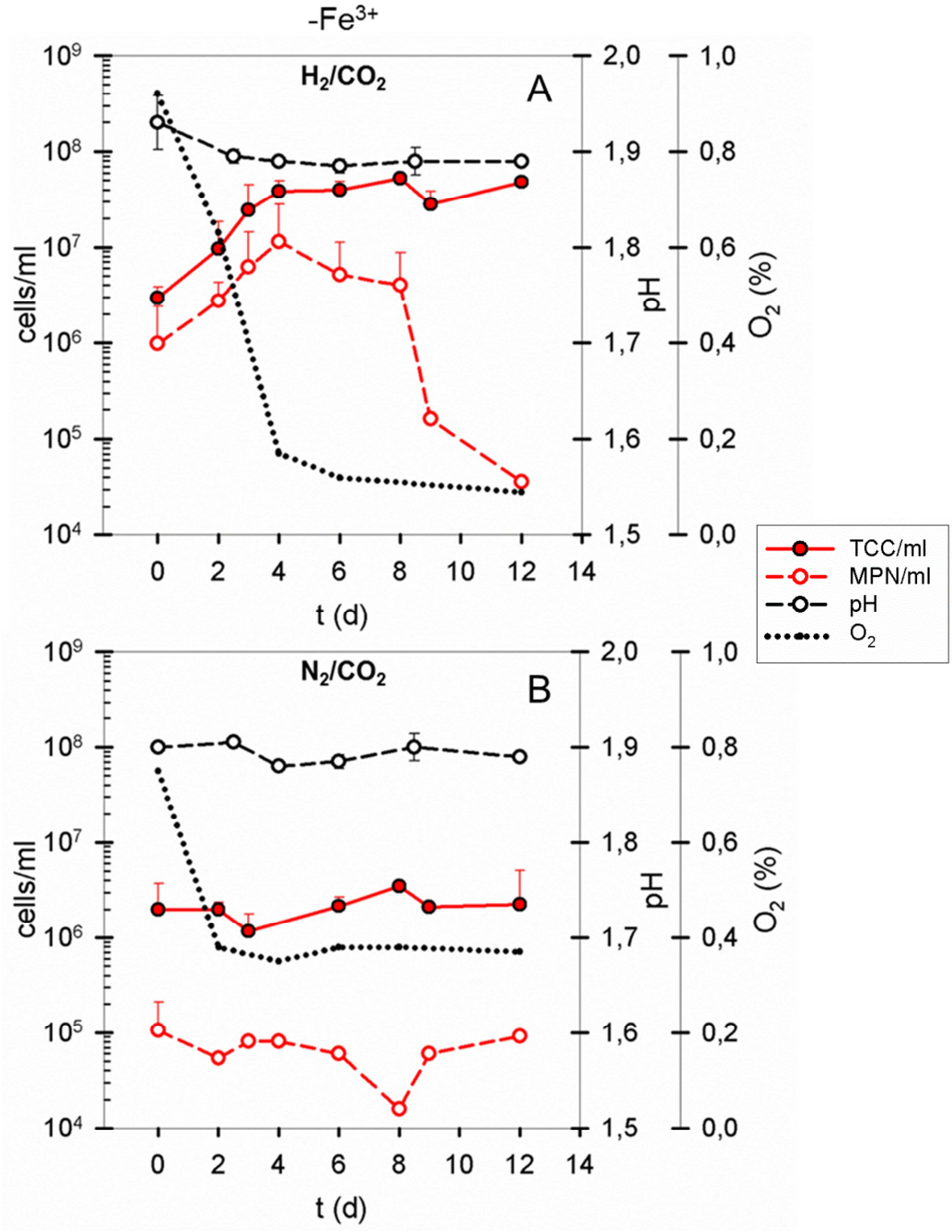

Fig. 3-9: Growth of *A. ferrooxidans* in H_2/CO_2 (80:20) atmosphere (A) or N_2/CO_2 (80:20) atmosphere (B) without addition of an electron acceptor, followed by total cell counts, MPN, pH, and O_2 concentration. Data expressed as mean (n=2) with standard deviation.

Conclusion:

Growth of *A. ferrooxidans*^{T} was demonstrated in microaerobic conditions on H_2/O_2 at pH values <2.0 and on H_2/Fe^{3+}, as opposed to an earlier study (Ohmura et al. 2002). It was observed that cell viability was related to the availability of an electron acceptor as culturable cell numbers declined after depletion of O_2 or Fe^{3+}, but not after depletion of Fe^{2+} in aerobic conditions (section **3.1.1.1,** p. 78).

3.1.3 Iron redox cycling

The ability of *A. ferrooxidans* and *S. thermosulfidooxidans* to gain energy from both ferrous iron oxidation and ferric iron reduction indicates their potential to participate in the redox cycling of iron, which could be an important metabolic feature for potential Martian organisms. Iron redox cycling was demonstrated for strains of sulfobacilli and for *A. ferrooxidans* in co-culture with acidophilic heterotrophs, but not for pure cultures of *A. ferrooxidans* (Bridge and Johnson 1998; Johnson and McGinness 1991). In a natural environment on Earth or on Mars the supply of electron donors and acceptors such as oxygen is not expected to be constant, requiring an efficient metabolic switching by the organisms to adapt to the changing environmental conditions. The following experiments show that the selected strains of *A. ferrooxidans* and *S. thermosulfidooxidans* are capable of shifting from aerobic to anaerobic metabolism and participate in iron redox cycling.

Cells were incubated in air-tight culture vessels under N_2/CO_2 atmosphere (80:20) with an initial oxygen concentration of ~15% (obtained by injecting sterile air) on ferrous iron and elemental sulfur (and yeast extract in the case of *S. thermosulfidooxidans*). In *A. ferrooxidans* cultures, a pronounced change in substrate utilization could be observed in response to the depletion in oxygen (**Fig. 3-10**).

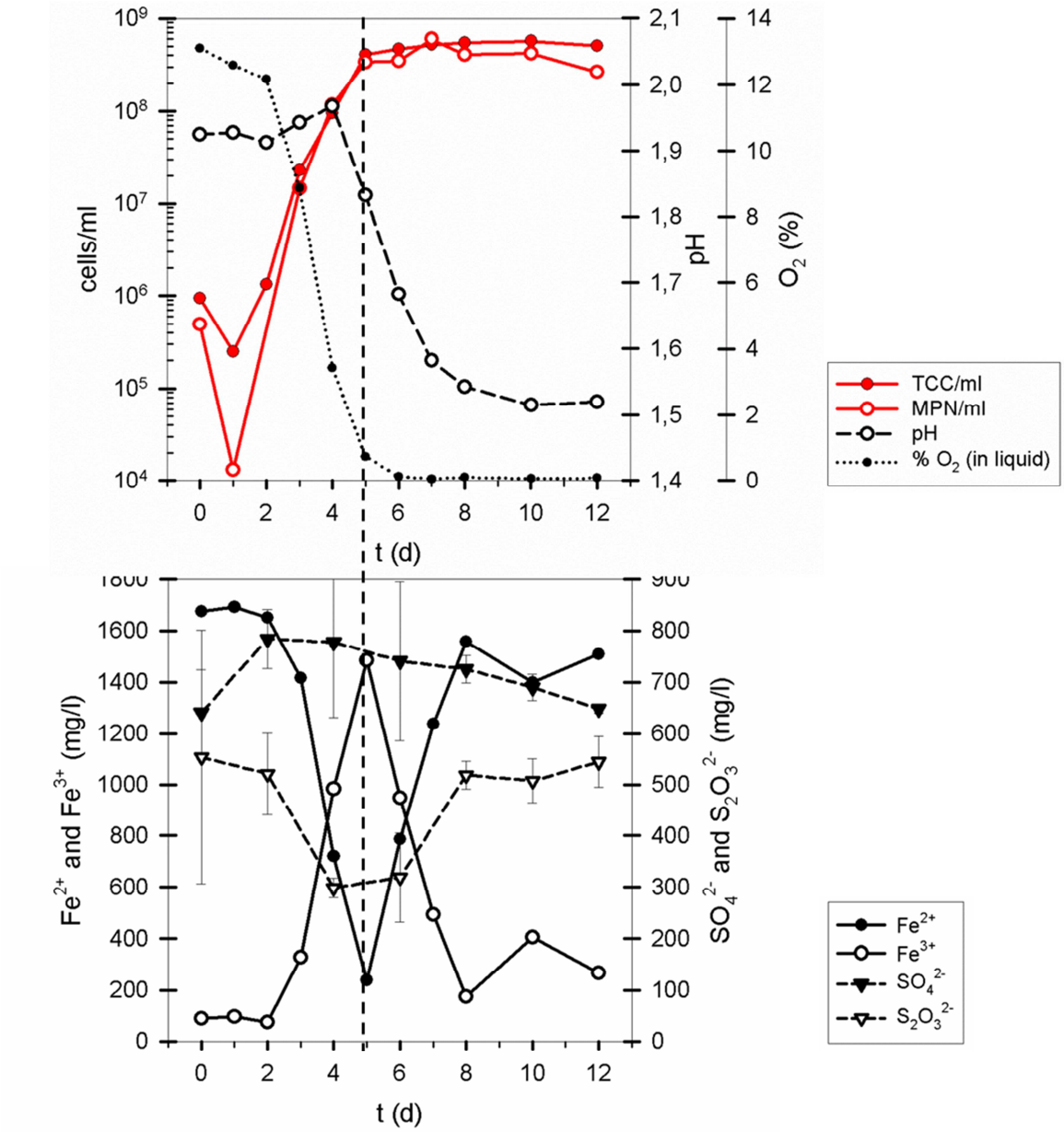

Fig. 3-10: Iron oxidoreduction by *A. ferrooxidans*. Cells were incubated in air-tight culture flasks under N_2/CO_2 atmosphere (80:20) with a reduced initial oxygen concentration, S^0 and Fe^{2+} were added. A: Total cell counts (TCC), culturable cell numbers (MPN), pH, and O_2-concentration (in the liquid phase), B: soluble ferrous and ferric iron-, sulfate- and thiosulfate-concentrations. Dashed line indicates approximate point of transition from aerobic to microaerobic (<1% O_2) conditions. Data expressed as mean (n=4) with standard deviation, error bars not included for sake of clarity if equal trends were evident in all parallels.

After a lag-phase of approximately 2 days, cell growth commenced exponentially as evidenced by the rise in total and culturable cell number (TCC and MPN) (**Fig.**

3-10 A). During aerobic growth, cells oxidized the ferrous iron completely to ferric iron in the course of 4 – 5 days, according to equation 5 (p. 79) (**Fig. 3-10 B**; although it appears as if more than 10% of the initial Fe^{2+} remained unoxidized, this was merely an effect of averaging the four different cultures, in which onset of growth and iron oxidation kinetics were not exactly congruent, which also resulted in high standard deviations, not shown in **Fig. 3-10**). The mean cell density reached when all Fe^{2+} had been consumed (day 4 - 5 of incubation in the parallel cultures) amounted to $2.8x10^8$/ml, which was 4 times higher than the value reached during aerobic growth on the same amount of Fe^{2+}, indicating that oxidation of sulfur or other inorganic sulfur species contributed significantly to the energy budget of *A. ferrooxidans*. This can also be inferred by the increase in pH during the aerobic phase, which was less pronounced than during growth on ferrous iron as sole electron donor (**Fig. 3-3**, p. 80). The oxidation of reduced inorganic sulfur compounds (RISCs) has an antagonistic effect on pH by generating protons (shown in eq. 10 and 11 for the substrates S^0 and $S_2O_3^{2-}$, exemplary for the abundance of RISCs possible).

Further indirect evidence of sulfur oxidation could be a rise in sulfate concentration as sulfate is the end product of RISC oxidation. This was, however, observed in only one out of four cultures during the aerobic growth phase (**Fig. 3-10 B**, note large error bars for initial sulfate concentration). In contrast, thiosulfate concentration decreased during the aerobic phase of incubation, indicating its oxidation by the cells (equation 11). Although thiosulfate was not added to the cultures, it was measured in the medium at initial concentrations of 550 mg/l and might have formed by chemical reactions including elemental sulfur or hydrogen sulfide (equations 12) (Oana and Ishikawa 1966; Suzuki 1999; van den Bosch et al. 2008).

$$0.25S^0 + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+ \quad (10)$$

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+ \quad (11)$$

$$S^0 + SO_3^{2-} \rightarrow S_2O_3^{2-} \text{ or } H_2S + SO_3^{2-} + 0.5O_2 \rightarrow S_2O_3^{2} + H_2O \quad (12)$$

Due to cellular respiration, the oxygen concentration decreased from 14% to a minimum value of 0.05% in the liquid phase (44.6 µM) after 7 days, by which time cell density reached its maximum value of $5.4x10^8$/ml (**Fig. 3-10 A**). However, a switch in metabolism by *A. ferrooxidans* seemed to occur already when O_2 concentration dropped below 1% after about 5 days. Here, reduction of ferric iron to ferrous iron started, suggesting that it was utilized by the bacteria as an alternative electron acceptor for the anaerobic oxidation of sulfur (equation 13), also accompanied by a decrease in pH (**Fig. 3-10 B**). A separate control experiment (not shown) demonstrated that anaerobic growth on S^0/Fe^{3+} in this strain of *A. ferrooxidans* led to an increase in cell density by about one order of magnitude. The beginning of stationary phase after 7-8 days (**Fig. 3-10 A**) was probably due to the depletion of both oxygen and ferric iron as an electron acceptor.

$$S^0 + 6Fe^{3+} + 4H_2O \rightarrow H_2SO_4 + 6Fe^{2+} + 6H^+ \qquad (13)\ (\text{Pronk et al. 1992})$$

The sulfate concentration remained relatively constant with some oscillatory fluctuations over the whole period of incubation, and even seemed to decline slightly under anaerobic conditions (**Fig. 3-10 B**). In contrast, thiosulfate concentration rose simultaneously with the ferrous iron concentration and until the majority of the ferric iron was reduced at day 8.

When *S. thermosulfidooxidans* was incubated under oxygen-limited conditions (initial O_2 concentration ~10%) with Fe^{2+}, S^0, and yeast extract (0.02%), ferric iron reduction was initiated at oxygen levels <1%, similar to *A. ferrooxidans*, but commenced at a slower rate (**Fig. 3-11**). The mean maximum cell density of $8.3x10^7$/ml was reached one day after the onset of iron reduction (day 3), followed by slowly declining cell numbers during the remainder of the anaerobic growth phase. The pH did not drop significantly below the initial values, as would be typical for sulfur oxidation, indicating that organic components might have been used as electron donor instead of sulfur. On the other hand, the hydrophilicity of the sulfur particles increased during the incubation, which is usually a result of bacterial colonization (Knickerbocker et al. 2000).

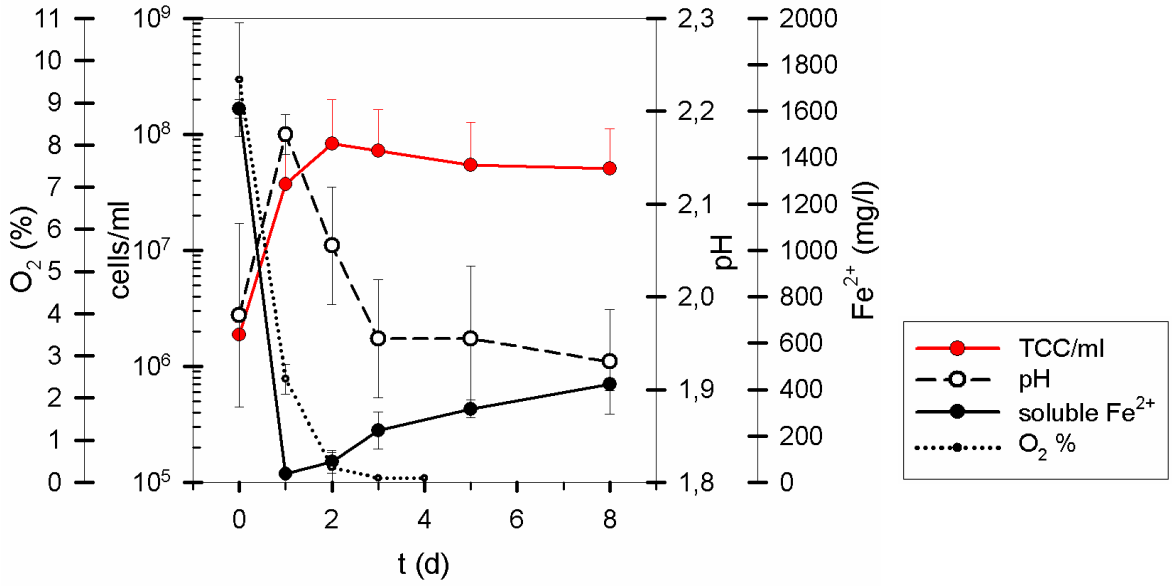

Fig. 3-11: Iron oxidoreduction by *S. thermosulfidooxidans*. Cells were incubated with shaking in air-tight culture flasks under N_2/CO_2 atmosphere (80:20) with an initial oxygen concentration of ~10%; S^0, Fe^{2+} and yeast extract (0.02%) were added. Data expressed as mean (n=2) with standard deviation.

Conclusion:

Both *A. ferrooxidans* and *S. thermosulfidooxidans* were capable of switching from aerobic to anaerobic metabolism when oxygen concentration in the liquid phase dropped <1%, carrying out iron oxidoreduction.

3.1.4 Growth with Mars Regolith Simulants

In situ analyses of Martian regolith have revealed an abundance of iron-containing minerals, which could serve as potential energy sources for chemolithotrophic bacteria. Growth experiments were conducted with *A. ferrooxidans* to determine whether autotrophic iron-sulfur bacteria can grow solely on the energy sources and nutrients provided by Martian regolith. Two different mineral mixtures were used to simulate the Martian regolith composition from different locations and periods of Mars (see section **2.2.4**, p. 50): Phyllosilicatic Mars regolith simulant (P-MRS, characterized by high clay content) and Sulfatic Mars regolith simulant (S-MRS, characterized by the gypsum

content). *A. ferrooxidans* were incubated on MRS first aerobically, but also in anaerobic atmosphere, and interaction of cells with the mineral particles was examined.

3.1.4.1 Aerobic growth on MRS

S-MRS and P-MRS were added to BSM (5% wt/v) and acidified to a pH of ~2. Bacterial cells from washed precultures were used to inoculate the cultures with MRS as well as a positive control with Fe^{2+} as energy source and a negative control without any energy source (only BSM). Cultures were first incubated aerobically.

Growth was monitored by the Most Probable Number assay because size and shape of the mineral particles compromised microscopic observations. In aerobic conditions, MPN was shown to correlate well with TCC over prolonged periods (**Fig. 3-4**, p. 81). In the negative control in BSM, culturable cell numbers did not rise indicating that carryover of ferrous iron had not occurred (**Fig. 3-12 A**). Bacteria incubated with P-MRS and S-MRS in BSM both showed a significant increase in culturable cell numbers by a factor of 11 (S-MRS) or 30 (P-MRS), compared to 87 in the positive control (**Fig. 3-12 A**).

To determine whether *A. ferrooxidans* could also grow on MRS without the nutrients (such as phosphate and nitrogen) and trace elements supplied by BSM, the experiment was repeated in the same manner with acidic deionized water (ddH_2O, pH 1.8) instead of BSM. Cells from a fresh preculture were washed three times prior to inoculation, first with BSM and twice with ddH_2O to remove salts from the medium. In the negative control without either nutrients or an energy source, culturable cell numbers declined sharply during the first 6 days, and total cell counts also fell below detection limit of the counting chamber method ($<10^5$/ml) after day 2, suggesting that cell lysis due to osmotic water influx took place (**Fig. 3-12 B**). In the positive control the $FeSO_4$ (10.9 g/l) added as an energy source probably increased osmolarity to a level that prevented cell lysis so that cell numbers did not decline. After a prolonged lag phase, culturable cell counts even started to increase as iron oxidation commenced, perhaps due to activation of endogenous reserves or nitrogen fixation. In comparison to the positive control on Fe^{2+}, growth was observed already after 2 days of incubation

in cultures containing S-MRS and P-MRS, where MPN increased by a factor of 10 (S-MRS) or 23 (P-MRS), similar to values in BSM (**Fig. 3-12 B**).

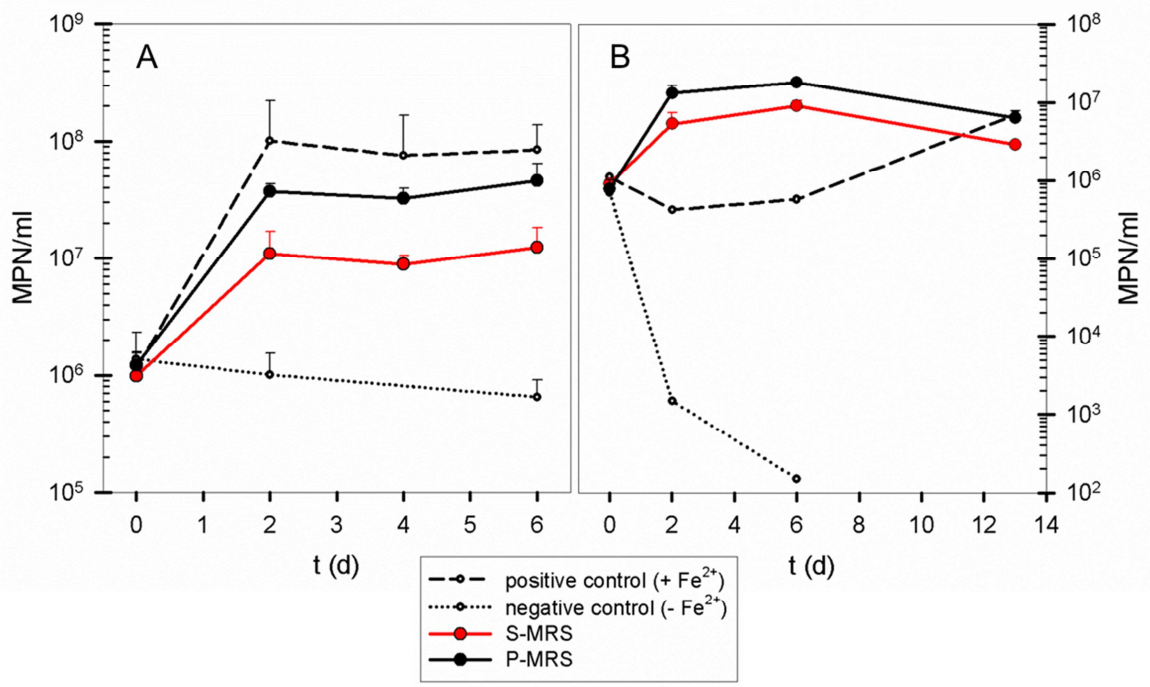

Fig. 3-12: Aerobic growth of *A. ferrooxidans* on S-MRS and P-MRS, compared to a positive control (with Fe^{2+}) and a negative control (no energy source) in BSM (A) or in ddH_2O (B), determined by Most Probable Number assay. Mean initial TCC was $1.5x10^6$/ml. Note the different scaling of the y-axis. Data expressed as mean (n=2-6) with standard deviation.

It was hypothesized that *A. ferrooxidans* grew on MRS in aerobic conditions because the mineral mixtures provided the bacteria with Fe^{2+} as an electron donor. This was confirmed by measuring the soluble ferrous iron concentrations in MRS-cultures inoculated with bacteria and in sterile controls with MRS (**Fig. 3-13**). In P-MRS cultures the soluble Fe^{2+}-concentration amounted to about 800 mg/l at the day of inoculation (day 0), while it was only ~140 mg/l in S-MRS cultures, resulting from differences in the mineral composition (**Table 2-6**, p. 50). As growth of *A. ferrooxidans* is dependent on the amount of available Fe^{2+}, the higher amount of dissolved ferrous iron in P-MRS fits well with the observed stronger growth (**Fig. 3-12 A**). After 6 days of growth, Fe^{2+} was reduced to about 2% of the initially available concentration in both the inoculated P-MRS and S-

MRS cultures (**Fig. 3-13**). This change was likely due to bacterial iron oxidation as it did not occur in the sterile MRS cultures. The values in **Fig. 3-13** are corrected for the rise in total soluble iron concentration over the incubation period. This occurred (by a factor of 2-3) in both sterile and inoculated cultures and was accompanied by a rise in pH, as protons were adsorbed by the minerals in exchange for cations such as Fe^{2+}.

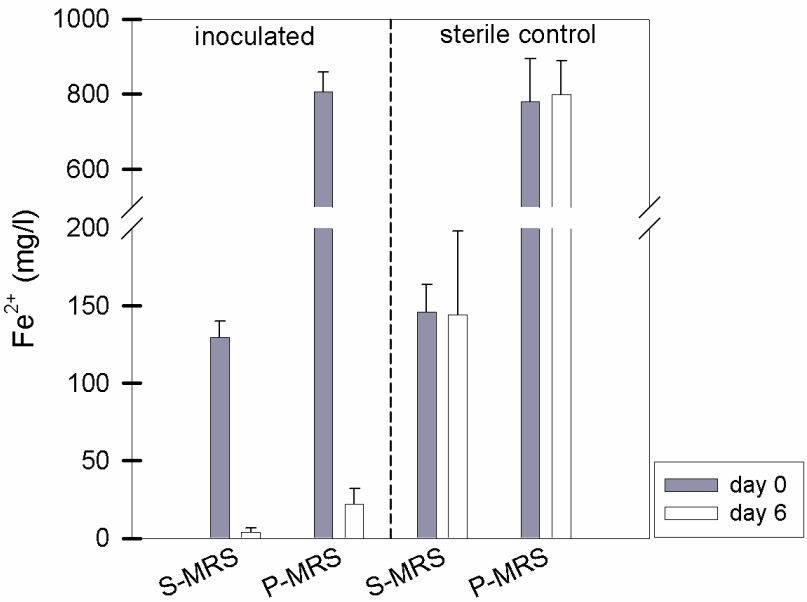

Fig. 3-13: Soluble ferrous iron concentrations in bacterial and sterile cultures of S-MRS and P-MRS at day 0 and day 6 of incubation, corrected for the increase in total soluble iron. Data expressed as mean (n=6) with standard deviation.

In a comparative test with *S. thermosulfidooxidans* incubated on MRS without an additional energy source (neither Fe^{2+} nor yeast extract), bacterial growth was not detected, probably due the lack of organic compounds. However, in some of the cultures with S- and P-MRS Fe^{2+} was consumed compared to the sterile controls indicating perhaps metabolic (iron oxidation) activity without the ability to multiply.

3.1.4.2 Anaerobic growth on MRS

Testing growth of *A. ferrooxidans* on MRS under anaerobic conditions was challenging for two reasons: i) the increase of pH occurring in all MRS cultures due to cation exchange affected the solubility of Fe^{3+} more than that of Fe^{2+}, and

ii) no reducing agent could be added to the medium as *A. ferrooxidans* was able to consume all compounds commonly used for this purpose for growth (as tested in pre-experiments). Therefore, residual amounts (<1%) of oxygen remained in the culture vessels despite taking all technical precautions possible. Results have to be interpreted in the light of this knowledge.

It was hypothesized that, just as in aerobic conditions the ferrous iron from the minerals served as an energy source for *A. ferrooxidans*, ferric iron present in the MRS mixtures might be used as an electron acceptor in the absence of oxygen. Bacteria were incubated in H_2/CO_2 atmosphere to provide H_2 as an electron donor, and in N_2/CO_2 atmosphere as a control without externally added electron donor. The control experiments of anaerobic bacterial cultures without MRS are described in section **3.1.2**, p. 88. In both H_2- and N_2-conditions, the residual oxygen was consumed within the first 2 days of incubation (**Fig. 3-14**) accompanied by an oxidation of soluble ferrous iron in both P- and S-MRS cultures that was not visible in the sterile controls (**Fig. 3-15**). Thus, growth (assessed by MPN) occurring within the first 2 days of incubation was likely due to iron oxidation under microaerobic conditions. In N_2/CO_2 atmosphere, MPN of bacteria incubated with P-MRS increased significantly in these first 2 days, but declined during the following days, indicating the depletion of an electron acceptor, while MPN in S-MRS cultures also increased, but did not exceed the initial total cell number of ~$2x10^6$/ml, probably owing to the lower amount of Fe^{2+} present (**Fig. 3-15 B**). In H_2/CO_2 atmosphere, both S-MRS and P-MRS cultures reached culturable cell densities of ~$2x10^7$/ml and retained this value over the entire incubation period, which suggests the continual supply of Fe^{3+} as an electron acceptor in contrast to the declining growth curves of bacteria cultivated without MRS in this atmosphere (**Fig. 3-14 A**, compare to **Fig. 3-8 A** and **Fig. 3-9 A**).

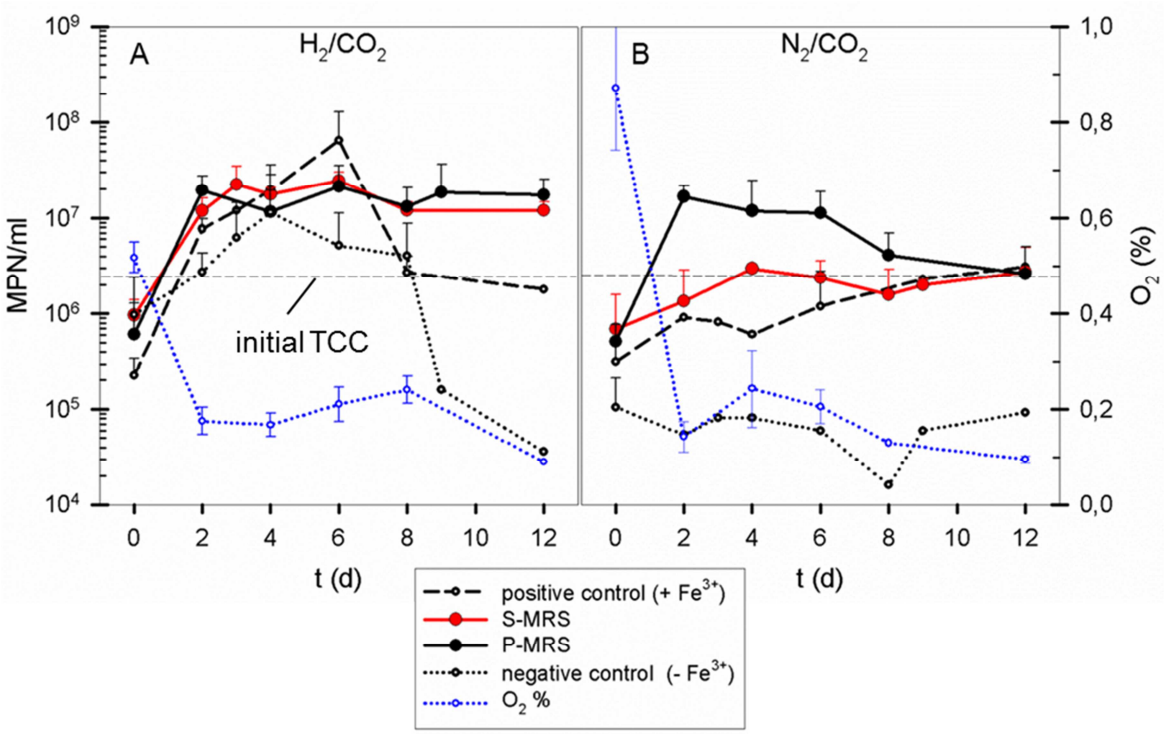

Fig. 3-14: Anaerobic growth of *A. ferrooxidans* on S-MRS and P-MRS, compared to a positive control (with Fe^{3+}) and a negative control (only BSM) in H_2/CO_2 atmosphere (A) or in N_2/CO_2 atmosphere (B), determined by Most Probable Number assay. Mean O_2 concentration in MRS cultures included. Mean initial TCC was $2.4x10^6$/ml (marked by the horizontal line). Scaling of y-axis the same for A and B. Data expressed as mean (n=4) with standard deviation.

Ferrous iron accounted for the majority of the total soluble iron measured in the MRS cultures at the start of the experiment (85% in S-MRS and 99% in P-MRS), so that the mean Fe^{3+} concentration was only 26.3 ± 2.2 mg/l in S-MRS and 11.4 ± 8.5 mg/l in P-MRS. This concentration increased due to the initial Fe^{2+}-oxidation activity in bacterial cultures (**Fig. 3-15**) to a maximum value of 167.7 ± 6.0 mg/l in S-MRS and 125.3 ± 27.2 mg/l in P-MRS, demonstrating that the residual amount of O_2 (mean value 0.7%) was sufficient for the oxidation of about 110 - 140 mg/l of Fe^{2+}. After O_2 levels had dropped to ~0.2%, cultures incubated in H_2/CO_2 atmosphere on both S-MRS and P-MRS exhibited a sharp decrease in Fe^{3+} concentrations that was significantly different from the cultures in N_2/CO_2 atmosphere (**Fig. 3-15**). It can be assumed that this resulted from the onset of Fe^{3+}-reduction with H_2 as an electron donor (as demonstrated also in **Fig. 3-8 A**). The slower decrease of Fe^{3+} concentrations, which occurred in N_2/CO_2

atmosphere, most notably in P-RMS cultures, could be attributed to the increase in pH from an initial value of 2.0 to >2.5 after 6 days (S-MRS) or after 2 days (P-MRS), where solubility of Fe^{3+} is greatly reduced. This could also explain the very low Fe^{3+} levels throughout the incubation period in the sterile control. The soluble Fe^{3+}-concentration in H_2/CO_2 atmosphere remained close to 0 mg/l after day 2 in both S-MRS and P-MRS cultures, indicating that bacteria were utilizing the Fe^{3+} as soon as it was released from the minerals. This raised the question whether release of soluble Fe^{3+} from the minerals occurred merely due to abiotic mineral dissolution at low pH, or whether bacteria actively participated in the dissolution of ferric minerals by their ferric iron reduction activity.

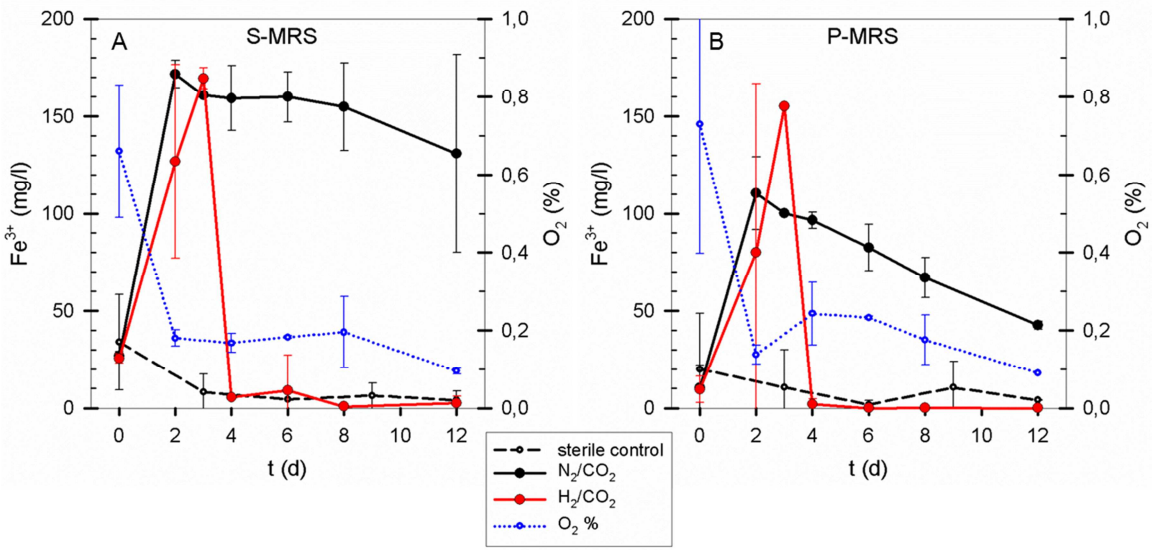

Fig. 3-15: Soluble ferric iron concentrations in bacterial and sterile cultures of S-MRS (A) and P-MRS (B) during incubation in N_2/CO_2 atmosphere or H_2/CO_2 atmosphere, corrected for the increase in total soluble iron. Mean O_2 concentration in MRS cultures included. Data expressed as mean (n=4) with standard deviation.

Ferric mineral dissolution, which can be observed by monitoring the increase in total soluble iron, has not been demonstrated for *A. ferrooxidans* before (Bridge and Johnson 1998, 2000). In this study, results indicated that *A. ferrooxidans* had indeed the ability to accelerate mineral dissolution in the presence of H_2 as an electron donor, but not in N_2/CO_2 atmosphere. This is demonstrated in **Fig. 3-16** for cells grown on S-MRS, which contained 20% (wt/v) of ferric iron minerals

(goethite and hematite). In the sterile cultures, total soluble iron increased at a constant rate (18.5 (mg/l)/d), caused by the abiotic mineral dissolution at acidic pH. This was mainly due to leaching of Fe^{2+}, probably from olivine, while the soluble Fe^{3+} concentration remained low (pH values were 2.5-3). In S-MRS cultures with *A. ferrooxidans* grown in N_2/CO_2 atmosphere (**Fig. 3-16 A**), the rate of total soluble iron increase was 10.7 (mg/l)/d. However, due to the initial iron oxidation activity by the bacteria (using up the residual oxygen in the flasks), the soluble Fe^{3+} concentration increased markedly during the first 2 days. After oxygen depletion, ferric iron concentration remained constant (at a pH of ~2.5), while total soluble iron and Fe^{2+} concentrations continued to rise, indicating that most of the soluble iron leached from the minerals after that was ferrous iron (as in the sterile controls).

The difference between the cultures incubated in N_2/CO_2 atmosphere and H_2/CO_2 atmosphere was pronounced. In H_2/CO_2 atmosphere (**Fig. 3-16 B**), initial Fe^{2+} oxidation also occurred, raising the Fe^{3+} concentration. But after 2 days, Fe^{3+} concentrations dropped again, while, at the same time, the rate of total soluble iron increase (33 (mg/l)/d) was accelerated compared to both the sterile control and the *A. ferrooxidans* culture in N_2/CO_2 atmosphere even though pH values were higher (pH >3). This indicates that *A. ferrooxidans* might have contributed to the reductive dissolution of the ferric iron minerals present in S-MRS. In P-MRS, these differences were not observable because hematite concentrations were lower (5% wt/v), and most of the Fe^{3+} that bacteria may have used for growth in H_2/CO_2 atmosphere should have come from a more accessible source (bound to the clay minerals).

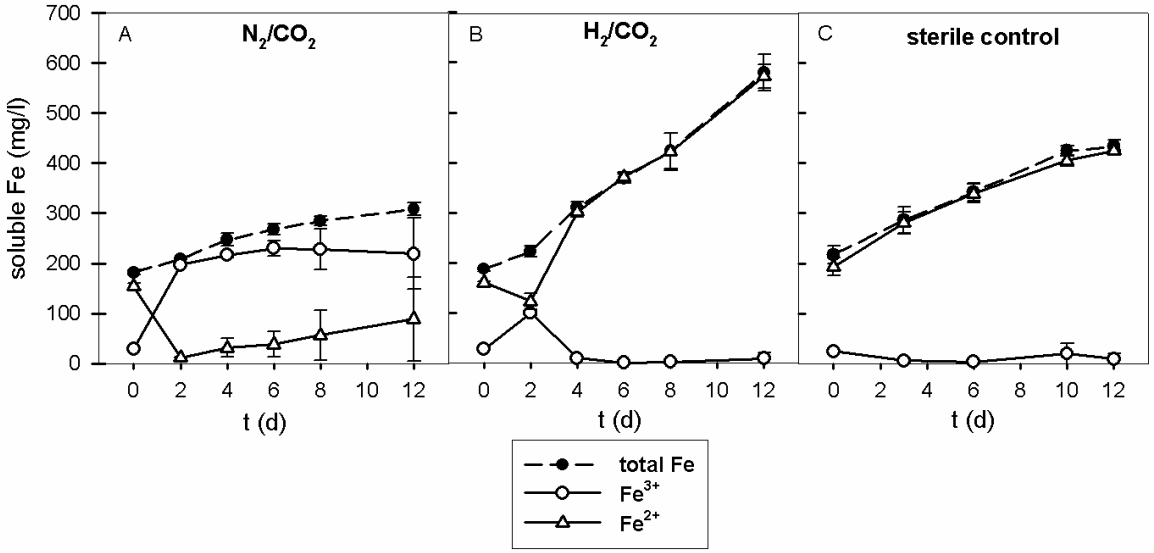

Fig. 3-16: Soluble iron concentrations in the S-MRS cultures with *A. ferrooxidans* in N_2/CO_2 atmosphere (A) or H_2/CO_2 atmosphere (B), and the sterile control (C).

3.1.4.3 Interaction of cells with mineral particles of S- and P-MRS

To further study the interactions of *A. ferrooxidans* with the mineral mixtures simulating Martian regolith, attachment behavior was examined. Attachment of bacteria to mineral surfaces is a crucial step in pyrite dissolution and biofilm formation in *A. ferrooxidans*. This process is usually followed by monitoring changes in cell number in the culture medium with the minerals (e.g. Harneit et al. 2006). In an attachment test with the MRS mixtures, cultures contained 5% (wt/v) of the minerals, analogous to the growth tests, but were inoculated with a higher number of cells ($5x10^7$/ml), pre-grown on ferrous iron. The pH was between 2 and 2.4 in all cultures during the course of the experiment.

In cultures with P-MRS, cell numbers began to decrease within minutes after inoculation and continued to do so over the first 60 min down to cell numbers close to the detection limit of the counting chamber (~$5x10^5$/ml) amounting to a loss of 98% of the initially added cells (**Fig. 3-17**). In contrast, cell numbers in the medium with S-MRS remained at a constant value over the 20 h of incubation.

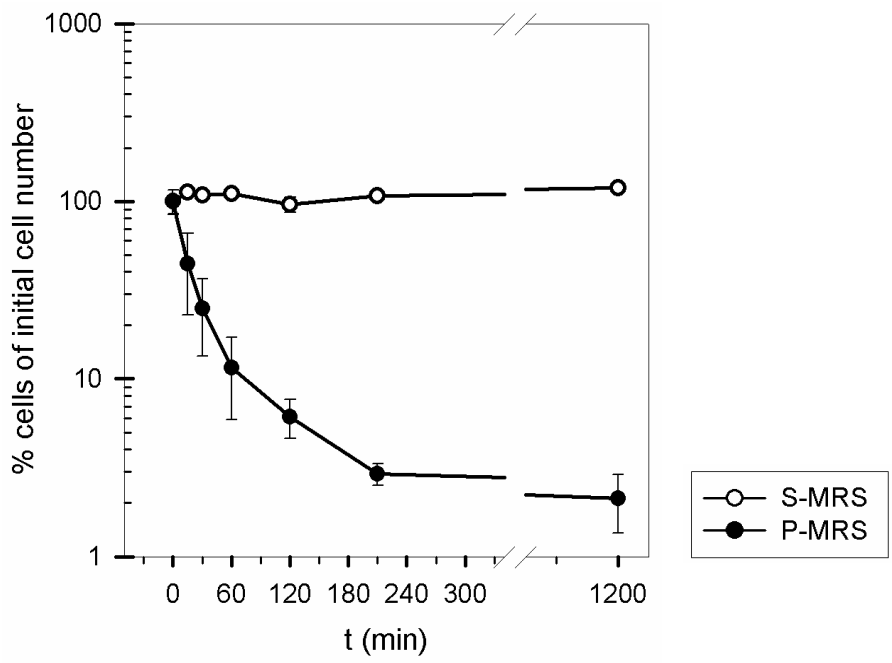

Fig. 3-17: Attachment of *A. ferrooxidans* cells pre-grown on Fe^{2+} to S-MRS and P-MRS, represented by a decrease in cell numbers in the bulk liquid, normalized to the initial cell number. Data expressed as mean (n=4) with standard deviation.

To test whether the attachment of bacteria to the P-MRS mineral particles is reversible by mechanical forces, aliquots removed from P-MRS cultures directly after inoculation (with initial cell numbers of $2x10^7$ cells/ml) were left undisturbed for 30 minutes, which was sufficient for a noticeable decrease in cell numbers (**Fig. 3-17**). After this period, they were shaken vigorously for 1 minute (Vortex apparatus, Heidolph) and centrifuged at low speed for 2 min to precipitate mineral particles (the same procedure that was used for counting all of the samples). Cell numbers counted in the supernatant of these aliquots after the 30 min were $6.3x10^5$/ml, indicating that mechanical forces of this type were not sufficient to release the cells once bound to the minerals.

Bacterial attachment to surfaces is governed by properties of both the bacterial cell wall and the surface in question (e.g. mineral particles) such as hydrophobicity and surface charge. As growth on sulfur changes the hydrophobicity of EPS (Gehrke et al. 1998; Takeuchi and Suzuki 1997), it was investigated whether this had any influence on the attachment behavior to MRS particles, possibly giving an indication as to why *A. ferrooxidans* attached to P-MRS, but not to S-MRS.

The pattern observed for the attachment of cells pre-grown on sulfur was very similar to the trials with ferrous-iron grown cells (**Fig. 3-18**). Cell numbers in S-MRS stayed relatively constant over the period of incubation, while there was a decrease in cell numbers in P-MRS cultures during the first 60 min down to cell numbers close to the detection limit of the counting chamber.

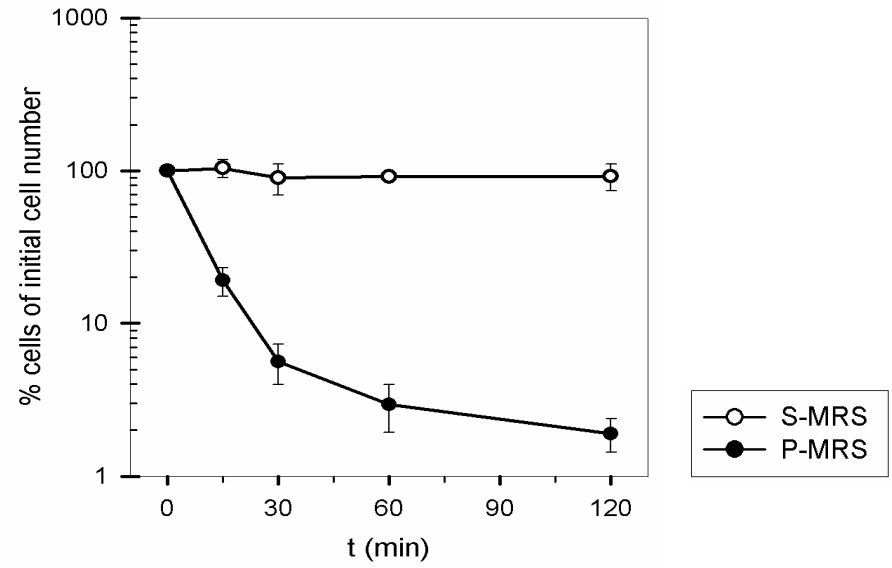

Fig. 3-18: Attachment of *A. ferrooxidans* cells pre-grown on S^0 to S-MRS, and P-MRS, represented by a decrease in cell numbers, normalized to the initial cell number. Data expressed as mean (n=2) with standard deviation.

Conclusion:

A. ferrooxidans was shown to grow on minerals in Mars regolith simulants in aerobic conditions, oxidizing ferrous iron desorbed from the minerals, and in anaerobic conditions, by ferric iron reduction if H_2 was present. It was also implicated in accelerating the reductive dissolution of ferric iron minerals in S-MRS by a mechanism that did not require direct contact of bacterial cells to the mineral particles, as *A. ferrooxidans* was not observed to attach to S-MRS. In contrast, cells disappeared from the bulk liquid phase in cultures containing P-MRS, indicating some interaction between the cells and the mineral particles that was not altered by a change in cell surface hydrophobicity after growth on sulfur.

3.2 Stress resistance capacities

Environmental conditions on Mars pose significant challenges to any form of life. In this section, different Mars-relevant stress factors, as identified in section **1.4**, p. 26, were examined with regard to their effect on the viability of iron-sulfur bacteria, focusing on *A. ferrooxidans*. Particular emphasis was put on desiccation, as one of the most prominent features of Mars is its aridity, both in the past and present.

3.2.1 Assays for viability determination

In the majority of astrobiological studies determining the ability of terrestrial prokaryotes to withstand stress conditions such as they would encounter in space or extraterrestrial environments, survival was assessed merely based on culturability (e.g. by the plate count method). However, microorganisms may enter a viable-but-nonculturable (VBNC) state of existence, in which they lose their ability to grow on conventional media (see section **1.5**, p. 34) but remain viable (e.g. intact and with maintenance metabolism) until conditions change and they may return to reproductive metabolism. This calls for the use of a combination of methods to determine the viability of microorganisms after exposure to stress conditions in order to gain a comprehensive picture of their physiological state and cellular integrity. In this study, different methods were evaluated for their suitability of use in the acidophilic model organisms and several of them were employed to determine their response to stress factors. **Table 3-3** gives an overview over the evaluated methods.

Table 3-3: Methods for viability determination tested for suitability in acidophilic iron-sulfur-bacteria (mostly *A. ferrooxidans*).

Method tested	*Measured parameter*	*Evaluation*
Most Probable Number	Growth and reproduction	Used for all stressors with good quantitation in *A. ferrooxidans* (aerobic growth), less suited for *S. thermosulfidooxidans* (chain formation)
Iron oxidation rate	Enzymatic activity, not necessarily coupled to growth	Used for non-invasive analysis of biofilm viability of *A. ferrooxidans* and *S. thermosulfidooxidans*, semi-quantitative
FISH	rRNA integrity and abundance	Used in *A. ferrooxidans* for all stressors, difficult to quantify, does not always correlate with culturability
PAC	rRNA biosynthesis and cell growth (not reproduction)	Used in *A. ferrooxidans* after incubation with nalidixic acid, better correlation with culturability than FISH, but difficult to quantify
Live/Dead®	Membrane integrity	Inconsistent results, used in *A. ferrooxidans* after desiccation and irradiation, qualitative correlation to culturability
qPCR	DNA integrity	Used in *A. ferrooxidans* after desiccation and irradiation, semi-quantitative (dependent on fragment size of the target gene)
CTC reduction (tetrazolium chloride)	Respirational activity	Unsuitable for *A. ferrooxidans*, no reduction of CTC occurred or CTC fluorescence was quenched due to acidic medium
Intracellular ATP content	Metabolic activity (ATP synthesis)	ATP may remain stable in dead cells (Fajardo-Cavazos et al. 2008), therefore unsuitable to assess metabolic activity, but useful for total biomass quantification

In *A. ferrooxidans*, culturability was assessed by determining the Most Probable Number (MPN) instead of employing the classical colony formation assay because of poor growth of chemolithoautotrophic acidophiles on agar (Tuovinen and Kelly 1973). For *A. ferrooxidans* the MPN assay yielded consistent results and was used throughout the experiments to give an estimate of cell viability after

stress exposure. For biofilms, MPN assays were complemented by measuring Fe^{2+}-oxidation rates upon resuscitation on fresh medium as an indicator for enzymatic activity.

In addition, other methods of viability determination were tested with *A. ferrooxidans* and, if suitable, applied to some of the stress factors. These included Live/Dead® staining, Fluorescence-*in situ*-hybridization (FISH) coupled to probe-active count (PAC), and quantitative Real Time-PCR (qPCR) to determine integrity of membranes, rRNA, and DNA after different stress treatments.

The Live/Dead® staining is based on the exclusion of a DNA-binding dye (propidium iodide) by an intact cell membrane, which is regarded an essential characteristic of viable cells (Davey et al. 2004). The Live/Dead® staining kit was modified for *A. ferrooxidans* by using 9 µM propidium iodide, and DAPI (1 µg/ml) as a counterstain for all cells with intact DNA (section **2.7.2**, p. 71).

Since its development, fluorescence-*in situ*-hybridization (FISH) has been widely applied to study the phylogenetic composition of microbial communities from natural environments (as reviewed e.g. by Bouvier and del Giorgio 2003) with varying success. Often, a significant fraction of the total cells (determined by DAPI staining) does not hybridize with universal oligonucleotide probes (Bouvier and del Giorgio 2003). This could be attributed, at least partly, to differences in target region availability, which may be influenced by rRNA-protein and intramolecular rRNA-rRNA interactions (Amann et al. 1995). However, the growth rate and physiological condition of microbial cells also have a profound impact on their detection using oligonucleotide probes (Christensen et al. 1999; Kerkhof and Ward 1993; Oda et al. 2000; Poulsen et al. 1993). Faster-growing or highly active cells tend to contain more ribosomes resulting in a stronger probe signal (Delong et al. 1989). Thus, the failure to detect some bacteria in environmental samples with universal phylogenetic probes could be caused by a very low rRNA content, which might indicate a state of dormancy (Mahmoud et al. 2005). If rRNA is not immediately degraded upon cell death, organisms might be detectable by FISH shortly after stress exposure even when they are dead. FISH was performed with the probe EUB338 targeting 16S rRNA, specific for all eubacteria because the use of pure cultures did not necessitate a probe specific to genus or species

level. The protocol was modified to achieve high stringency conditions with *A. ferrooxidans* (section **2.8.4**, p. 74).

The probe active count (PAC) can be used as an extension to FISH (Kalmbach et al. 1997). Based on the direct viable count, it involves the incubation of bacterial cells with a gyrase inhibitor to induce ribosome synthesis in viable cells without allowing cell division, whereas the rRNA in dead cells should be disintegrated. In this study, PAC was also applied after stress exposure of *A. ferrooxidans* to observe whether rRNA degradation is induced (as would be visible by a diminished fluorescence signal). Cell division of *A. ferrooxidans* was not inhibited by pipemidic acid, but incubation with nalidixic acid (NA) >50 mg/l for 16 h did not lead to an increase in cell number within the period of incubation and yielded slightly elongated cells: the average cell length increased from 1.2 ± 0.28 µm before incubation with NA to 1.55 ± 0.31 µm afterwards, mean of 100 cells each, statistically significant difference, $P = 7.7 \times 10^{-16}$ (**Fig. 3-19**). However, elongation was not quantified, but rather, intensity of the fluorescence signal before and after incubation with NA was compared.

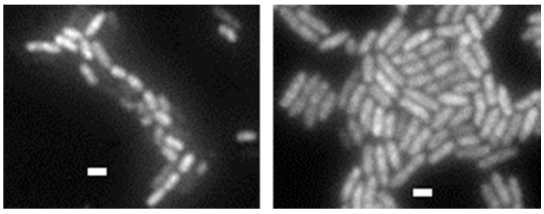

Fig. 3-19: Epifluorescence images of *A. ferrooxidans* cells hybridized with EUB338 (left), or incubated 16 h with nalidixic acid (NA) and hybridized with EUB338 (right). Scale bar denotes 1 µm. Note elongation of some of the cells in the right image.

Quantitative real-time PCR may be used for the quantification of different phylogenetic groups of microorganisms in an environmental sample (e.g. Escobar et al. 2008; Liu et al. 2006), the determination of membrane-damaged cells using propdidium monoazide (Nocker et al. 2006, 2007), and the analysis of differential gene expression (e.g. del Mar Lleò et al. 2000; Valasek and Repa 2005; Wong and Medrano 2005). In addition, qPCR has been used as a method for quantifying DNA damage after stress exposure as DNA damage results in

blocking of the polymerase and a reduction of template amplification (e.g. Sikorsky et al. 2004; Trampuz et al. 2006; Yakes et al. 1996). DNA lesions that inhibit polymerase progression can be oxidized bases, abasic sites, or strand breaks (Sikorsky et al. 2004; Trampuz et al. 2006). In these experiments, extracted DNA from stressed cells of *A. ferrooxidans* was amplified by qPCR in parallel to DNA from untreated cells to determine the relative amounts of intact and damaged DNA. The amount of input DNA from stressed and control cells was equal in the PCR reactions, which meant that any increase in threshold cycle number (C_t) should result from a decrease in amplification efficiency (Sikorsky et al. 2004). The primers targeted a 977 bp-fragment of the 16S rRNA gene (Escobar et al. 2008).

Methods tested but not included in the experiments because of unsuitability for the *A. ferrooxidans* were the assay for respirational activity by tetrazolium chloride (CTC) reduction, and measurement of the intracellular ATP content (**Table 3-3**).

3.2.2 Desiccation

Water stress is one of the most important environmental challenges any life form would encounter on Mars, but also in many habitats on Earth that are not characterized by an abundance of liquid water. Several strains of acidophilic bacteria isolated from different environments (**Table 2-8**, p. 53), including 10 strains of *A. ferrooxidans*, as well as strains of *A. ferrivorans*, *A. caldus*, *Acidiferrobacter thiooxidans*, *Leptospirillum ferrooxidans*, *L. ferriphilum*, and one environmental enrichment culture, were screened for desiccation resistance by exposure to air-drying for 24 h. None of the tested strains exhibited desiccation tolerance significantly higher than the type strains of *A. ferrooxidans* and *S. thermosulfidooxidans*. Thus, further stress resistance tests were continued with these strains.

Most experiments were performed with *A. ferrooxidans* using the strategy outlined in **Fig. 3-20**. Because initial tests with air-dried planktonic cells did not yield any reculturable survivors (section **3.2.2.1**, p. 112), conditions were investigated that might enhance desiccation tolerance of *A. ferrooxidans*. Adding

compatible solutes to planktonic cells was shown to protect them from the adverse effects of drying (section **3.2.2.2**, p. 113), and growth in biofilms improved desiccation tolerance (section **3.2.2.3**, p. 116). This was also shown for the reference organism *D. geothermalis* (section **3.2.2.3.2**, p. 117). Biofilms were dried under different conditions to investigate the influence of humidity and oxygen, and it was found that anaerobic conditions were most favorable for long-term survival of desiccation of *A. ferrooxidans* and *S. thermosulfidooxidans* (section **3.2.2.4**, p. 119).

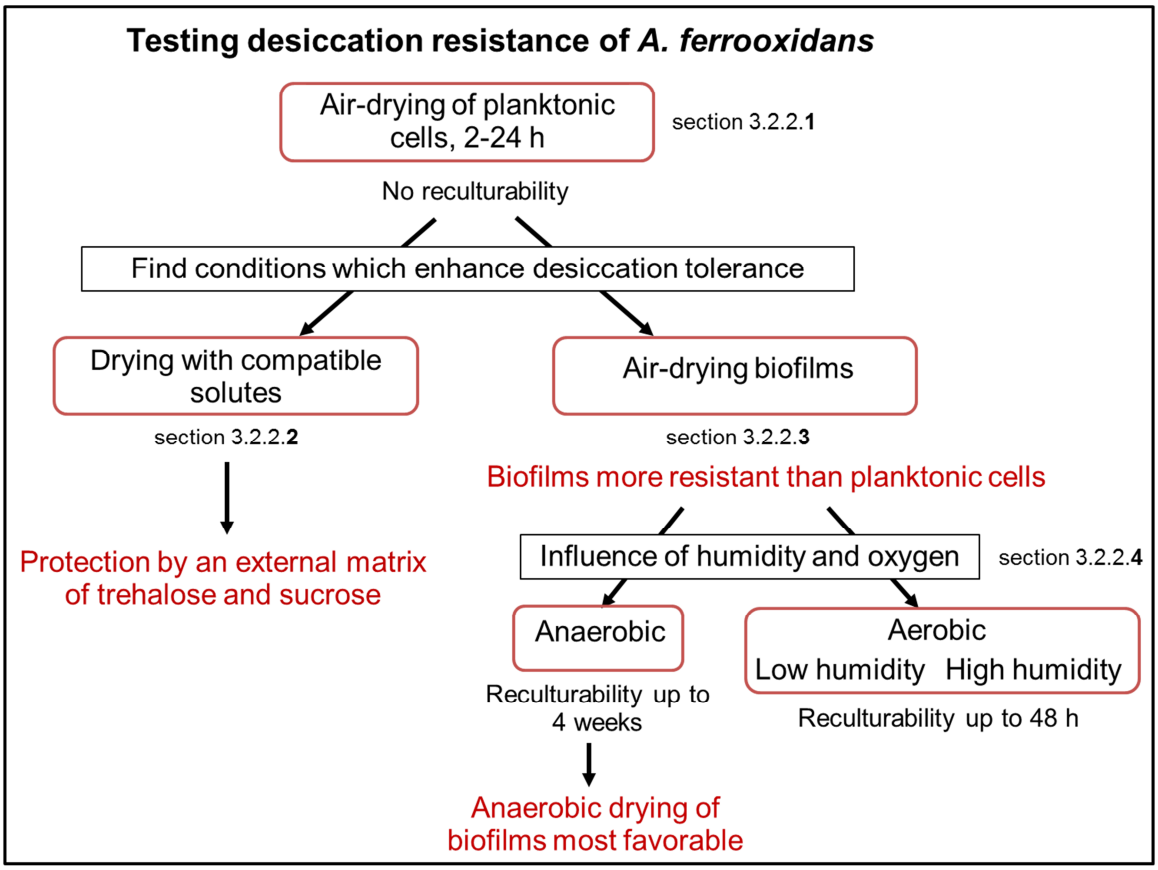

Fig. 3-20: Schematic outline of the experiments performed with *A. ferrooxidans* to evaluate factors influencing its desiccation resistance. Most important conclusions are marked in red font.

3.2.2.1 Planktonic cells of A. ferrooxidans *and* S. thermosulfidooxidans *could not be resuscitated after 2 h of air-drying on glass surface, while* D. geothermalis *survived 1 year*

In an initial experiment to assess desiccation tolerance of the model iron-sulfur bacteria *A. ferrooxidans* and *S. thermosulfidooxidans*, planktonic cells were exposed to air-drying (~40% relative humidity (RH)) on glass surfaces (10^7 cells). After 2 or 24 hours, no resuscitation in fresh medium could be achieved. This was neither influenced by the pre-cultivation conditions (different energy sources, aerobic or anaerobic growth), nor by neutralization of the medium in which cells were dried. Drying bacteria within a bentonite matrix also showed no significant protective effect. Bentonite, a natural mineral mixture consisting mostly of the smectite clay montmorillonite, was chosen on account of the high water affinity of smectites, which have been suggested to protect bacteria during periods of low water activity in soils (Marshall 1975). However, although the water retention properties of bentonite prolonged the process of drying, culturability of the bacteria was lost after the bacteria-mineral mixture had dried to equilibrium with the atmosphere (3 days in this test system).

The reference organism *D. geothermalis* was inactivated by less than 1 order of magnitude after 1 month of storing planktonic cells in ambient air on glass plates (**Fig. 3-21**). Furthermore, it could be recultured after 1 year of dried storage, showing a moderate decline in culturability rate over time. Clearly, *D. geothermalis* can be considered an anhydrobiotic, i.e. desiccation-resistant or xerophilic bacterium, while *A. ferrooxidans* and *S. thermosulfidooxidans* are not.

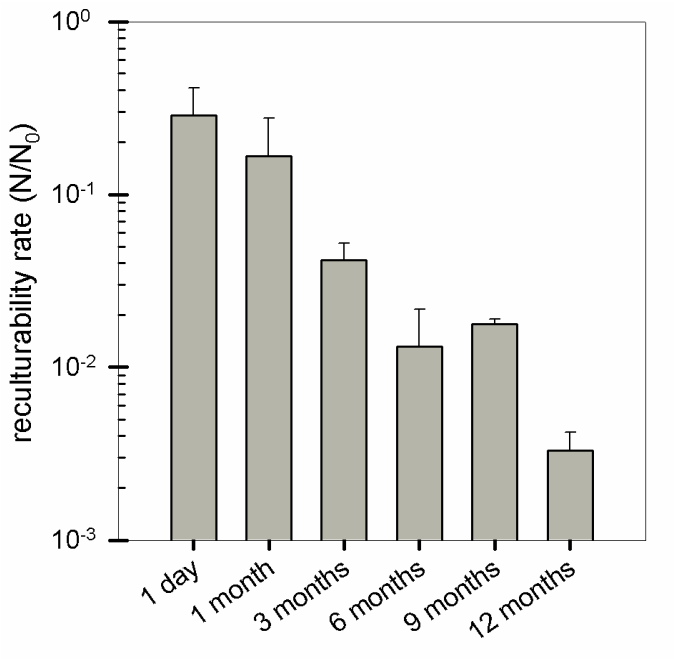

Fig. 3-21: Reculturability of dried *D. geothermalis* planktonic cells on glass plates stored in a desiccator (<5% RH, 30°C) for up to 1 year. Reculturability given as ratio of the dried sample (N) relative to the wet control (N_0) with 10^0 denoting 100% reculturability. Data expressed as mean (n=4) with standard deviation.

3.2.2.2 Influence of compatible solutes on desiccation tolerance:

Compatible solutes are used by many organisms as osmo- and cryoprotectants, and are employed commercially to ensure longer storage of microorganisms in the frozen or freeze-dried state. Compatible solutes are small, highly soluble organic molecules, which are accumulated inside the cytoplasm, but do not interfere with the normal cell metabolism. They can be synthesized *de novo* by the cells (often in response to osmotic stress), though an uptake from the environment is usually preferred because it is energetically more favorable (Brown 1976; Roeßler and Müller 2001). Glycine betaine is one of the most widespread compatible solutes. A 6% (wt/v) glycine betaine solution can protect *A. ferrooxidans* from desiccation-induced inactivation during liquid-drying (a procedure using successive periods of evacuation at different pressures and termed vacuum-drying in this work) and freeze-drying (Cleland et al. 2004). Other commonly used protective substances for freeze-drying or frozen storage of

microorganisms include glycerol, trehalose, and sucrose (Hubalek 2003; Morgan et al. 2006).

It was investigated whether: i) compatible solutes such as glycine betaine, glycerol, trehalose and sucrose can protect *A. ferrooxidans* from the adverse effects of drying, and ii) whether *A. ferrooxidans* is able to synthesize compatible solutes or iii) to accumulate them from the environment.

3.2.2.2.1 An external matrix of compatible solutes protects *A. ferrooxidans* during desiccation:

Bacteria were mixed with 6% (wt/v) solutions of compatible solutes (glycine betaine, sucrose, trehalose, glycerol) or left in BSM, and subjected to vacuum-drying under low pressure according to the procedure used by Cleland et al. (2004) (section **2.3.1.2.4**, p. 58), after which they were immediately transferred to a desiccator (RH <5%) and stored there for 5 or 15 days. At both sampling time points, only bacteria embedded in a sucrose or trehalose matrix could be reactivated in fresh medium; however, with an inactivation of four to seven orders of magnitude (**Table 3-4**).

Table 3-4: Reculturability (N/N_0) of *A. ferrooxidans* after vacuum-drying in trehalose and sucrose (6%) solutions and storage in a desiccator for 5 and 15 days (N), relative to the wet control (N_0). In all other compatible solutes tested, no recultivation could be achieved.

	5 d	*15 d*
trehalose	$3.4x10^{-4}$	$8.7x10^{-7}$
sucrose	$5.5x10^{-6}$	$6.2x10^{-5}$

To further explore the protective effect of sucrose on *A. ferrooxidans*, bacteria were suspended in sucrose solutions of different concentrations (125 mM – 1 M) and subjected to vacuum-drying to determine the effect of sugar concentration on recovery rate. Directly after vacuum-drying, cells suspended in 125 and 250 mM sucrose showed highest reculturability, while 1 M sucrose solution did not yield any reculturable cells (**Fig. 3-22 A**). When dried in a 175 mM sucrose solution

(corresponding to 6%) and stored in a desiccator for up to 30 days, a 4-log unit decline of culturability was observed immediately after the vacuum-drying procedure and this loss increased with increasing storage time (**Fig. 3-22 B**).

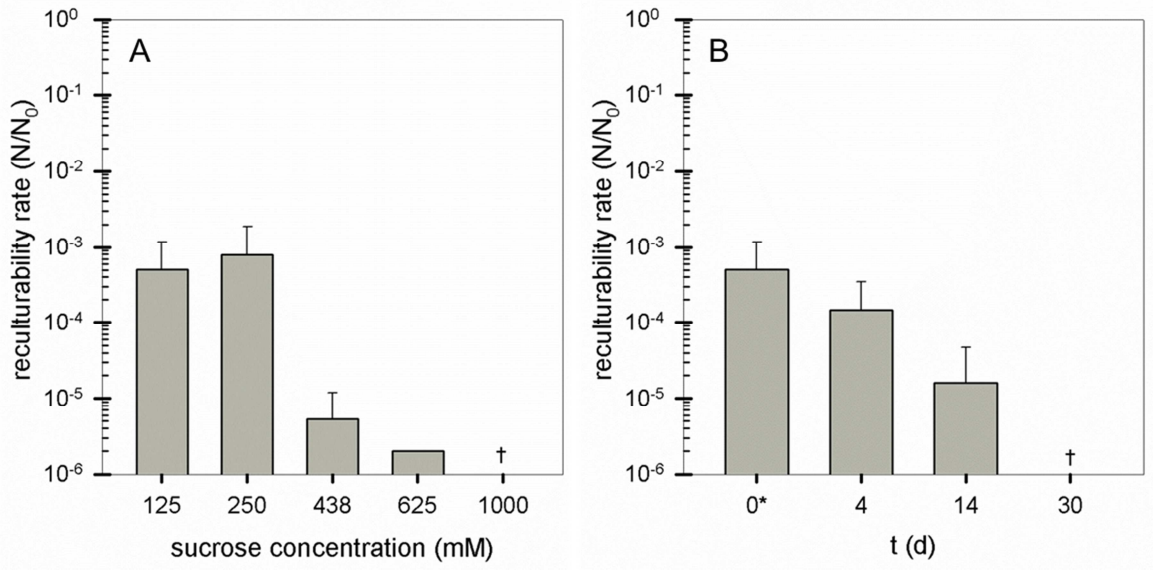

Fig. 3-22: A: Reculturability of *A. ferrooxidans* after vacuum-drying in sucrose solutions of different concentrations. B: Reculturability of *A. ferrooxidans* in a 175 mM (6%) sucrose solution directly after vacuum-drying (0*) and after storage in the desiccator for up to 30 days. 10^0 denotes 100% reculturability of the dried samples (N) compared to the wet control (N_0). Data expressed as mean (n=2) with standard deviation. † denotes no reculturability.

3.2.2.2.2 Exposure to osmotic stress to induce compatible solute synthesis did not enhance desiccation tolerance of *A. ferrooxidans*:

To determine whether salt stress can induce the synthesis of compatible solutes that may protect the bacteria during drying, *A. ferrooxidans* was grown with NaCl concentrations of 0.3%, and 0.5% (wt/v), respectively. These NaCl concentrations were selected because they resulted in significant growth retardation of *A. ferrooxidans*, but did not inhibit it completely. Washed cells of NaCl-grown cultures were exposed to vacuum-drying. Reculturability of the dried samples was lost after 24 h of storage in ambient air, indicating that compatible solutes synthesis had not been induced under these conditions.

3.2.2.2.3 Incubation of *A. ferrooxidans* with glucose, sucrose, and glycine betaine did not increase desiccation tolerance

Bacteria grown 48 h in the presence of 1 mM – 100 mM sucrose and trehalose, and glycine betaine (growth at higher concentrations was inhibited) were washed thoroughly to remove the external compatible solutes. After drying for 24 h, no reculturability was recorded. If *A. ferrooxidans* could accumulate the external compatible solutes added to the growth medium, they did not confer any increase resistance to desiccation on the cells.

3.2.2.3 Comparing desiccation resistance of biofilms with planktonic cells

3.2.2.3.1 Biofilms of *A. ferrooxidans* retain higher iron oxidation activities after desiccation than planktonic cells

The biofilm mode of life offers many advantages to microorganisms in natural environments, including the protection from environmental stress factors such as desiccation (Flemming and Wingender 2010; Tamaru et al. 2005). To test whether cells within a biofilm were more tolerant to desiccation than planktonic cells, biofilms of *A. ferrooxidans* grown 5 days on filter membranes and a similar number of planktonic cells filtered onto these membranes, were dried for 48 h under different conditions of relative humidity and in the presence or absence of oxygen. After drying, filter membranes were resuspended on fresh medium and iron oxidation was monitored to assess the resumption of enzymatic activity as an indicator of survival.

Drying in anaerobic atmosphere was most beneficial for survival (**Fig. 3-23**). Biofilms dried in the absence of oxygen had an iron oxidation rate of 72% of the wet control, while planktonic cells exhibited a prolonged lag-phase after anaerobic desiccation (not shown) before resuming iron oxidation at 51% of the respective wet control ($P < 0.05$). The iron oxidation rates of biofilms and planktonic cells dried in aerobic conditions (both at high and low RH) were significantly lower than those dried in anaerobic conditions, suggesting a reduction of viable cells or of the fitness of the cells. In all tested conditions, biofilm samples exhibited an enhanced tolerance to desiccation compared to

planktonic cells; however, due to high variability among the biological samples, especially of the biofilms, a statistically significant difference was only calculated for anaerobic conditions.

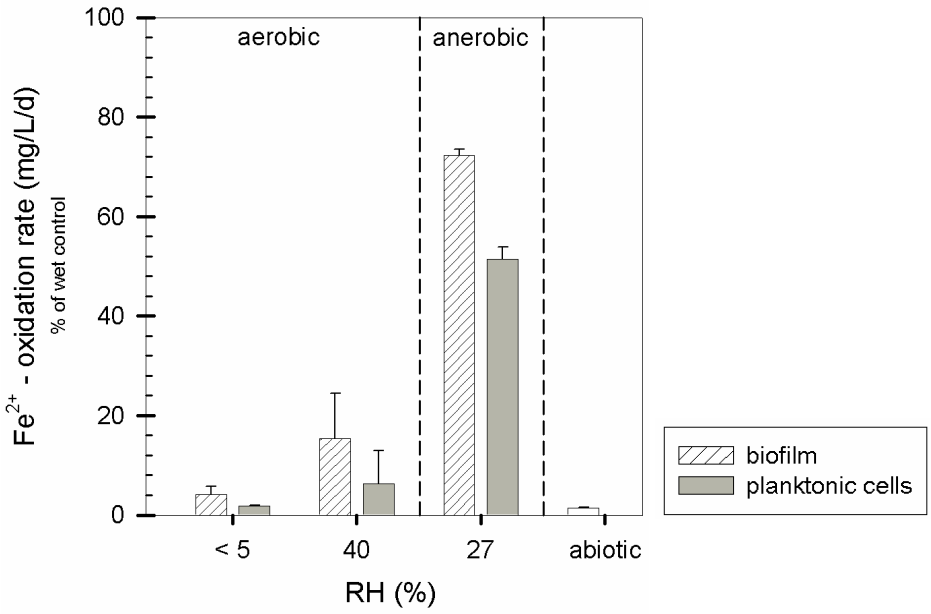

Fig. 3-23: Fe^{2+}-oxidation rates (relative to the respective wet control) of biofilms and planktonic cells of *A. ferrooxidans* on filter membranes upon resuscitation after 48 h of drying at different RH in the presence or absence of oxygen. Abiotic iron oxidation (white bar) was also measured. Data expressed as mean (n=2) with standard deviation. Asterisk denotes statistically significant difference of the biofilm-planktonic cell-pair ($P < 0.05$).

3.2.2.3.2 Viability of cells in *D. geothermalis* biofilms is less affected by drying than that of planktonic cells

To compare the results from *A. ferrooxidans* to a highly desiccation-resistant organism, biofilms of *D. geothermalis* grown on glass slides and planktonic cells on glass slides were subjected to 3 weeks of desiccation in ambient air and viability was checked by colony formation assay and membrane integrity staining (Live/Dead®, modified for *D. geothermalis*, section **2.7.2**, p. 71) after detaching cells from the glass surface. The percentage of culturable cells after drying was reduced by about 35% for planktonic cells, and by 8% for biofilm cells (**Fig. 3-24**

A), with a significant difference between the wet and dried samples only determined for planktonic cells (P=0.02). Fresh biofilms contained lower percentages of culturable cells (26%) than fresh planktonic cultures (50%).

Results obtained from Live/Dead® staining show a tendency similar to the colony formation assay. Fresh planktonic cultures were comprised by a majority (99%) of cells with intact membranes (**Fig. 3-24 B**). After 3 weeks storage in the dried state, the percentage of intact cells was significantly reduced to about 12% (P = $4.1x10^{-17}$). In biofilms, the number of cells without membrane damage remained at about 60% before and after drying.

The percentage of viable cells according to the two methods differed. In both planktonic cultures and biofilms the initial amount of membrane-intact cells was ca. 2 - 2.5 times higher than the amount of culturable cells. But with both methods, a significant reduction of viable cells after drying could only be observed for planktonic cells, thus the Live/Dead® staining corroborated results from the colony formation assay.

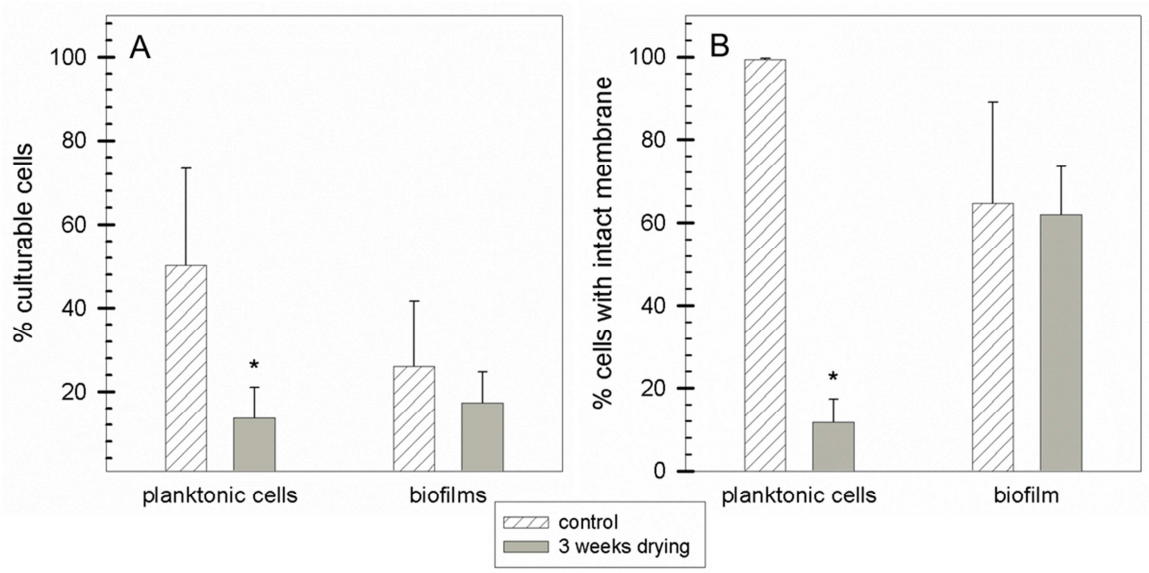

Fig. 3-24: Percentage of culturable cells (able to form colonies) (A) and of cells with intact membrane, as determined by Live/Dead staining (B), for planktonic cells and biofilms of *D. geothermalis* before (control) and after 3 weeks of desiccation. Data expressed as mean (n=4, or at least 1000 counted cells) with standard deviation. Asterisk denotes significant difference between values before and after drying (P < 0.05).

Biofilms of *D. geothermalis* formed on glass slides after 25 h of growth were also stained with the Live/Dead® BacLight™ Bacterial viability kit (3 µM SYTO9 and 30 µM PI) before detachment of cells for the quantitative analysis. The CLSM images showed a patchy structure with a heterogeneous distribution of membrane-intact and membrane-compromised cells. In aggregates consisting of many cell layers, cells closer to the glass surface were stained by propidium iodide more frequently than cells of upper layers and cells in single-layer regions, suggesting that membrane integrity was related to the time since attachment or that diffusion of nutrients and oxygen to the lower cell layers was limited. Biofilms of *D. geothermalis* also showed a fluorescence signal for the lectin WGA (Wheat Germ Agglutinin from *Triticum vulgaris*) previously used for *D. geothermalis* biofilms (Kolari 2003) indicating the presence of N-acetyl glucosamine-containing polysaccharides in the EPS matrix of *D. geothermalis* when grown on glass surface.

3.2.2.4 Influence of humidity and oxygen on desiccation tolerance

3.2.2.4.1 Anaerobic conditions are most favorable to desiccation survival of *A. ferrooxidans* and *S. thermosulfidooxidans*

Desiccation survival of microorganisms is dependent on the conditions of drying (Morgan et al. 2006) such as the relative humidity (RH) and the presence or absence of oxygen. To identify conditions most favorable for long-term desiccation of *A. ferrooxidans* and *S. thermosulfidooxidans*, biofilms were subjected to drying in

- aerobic atmosphere, either at low RH (in a desiccator over silica gel, RH <5%), or at high RH (ambient conditions ~40%),
- anaerobic (N_2/H_2) atmosphere (~27% RH due to $CaCl_2$) (section **2.3.1.2.3**, p. 56).
- In addition, *D. geothermalis* was stored under high vacuum (~$5x10^{-5}$ Pa).

Assuming equilibrium conditions in these systems, the water activity in the samples is dependent on the RH of the atmosphere and can be calculated according to equation 4 (p. 27) as a_w = RH/100. Thus, in all conditions, the a_w of the samples was below the minimal solute-induced water activity of 0.62 for which microbial growth has been demonstrated (Harris 1981) and well below the threshold of growth for desiccation (matric)-induced water stress (Beaty et al. 2006).

Biofilms of *A. ferrooxidans* and *S. thermosulfidooxidans* were dried for up to 48 h, while *D. geothermalis*, which can be stored in the dried state for several months, was dried for 4 weeks to observe an effect.

For *A. ferrooxidans*, viability after drying under different conditions was assessed by MPN (culturability) and iron oxidation rate (enzymatic activity) (**Fig. 3-25**). Both methods showed the highest viability after drying in anaerobic conditions (27% RH); however, the quantitative values differed significantly. While only 0.25% of biofilm cells were reculturable after anaerobic drying, the iron oxidation rate of these biofilms remained as high as 60% of the wet control (**Table 3-5**). Possibly, this large discrepancy could be attributed to cells in the VBNC state, which did not reproduce, but retained metabolic activity.

Drying *A. ferrooxidans* biofilms for 5 h or 24 h showed similar tendencies to the 48 h period (**Table 3-5**) with anaerobic conditions yielding higher iron oxidation rates than aerobic conditions. A significant difference between survival in high and low RH under aerobic atmosphere could not detected, although under low RH conditions generally a higher percentage of biofilm samples was resuscitated. Iron oxidation rates declined depending on the time of drying in each condition.

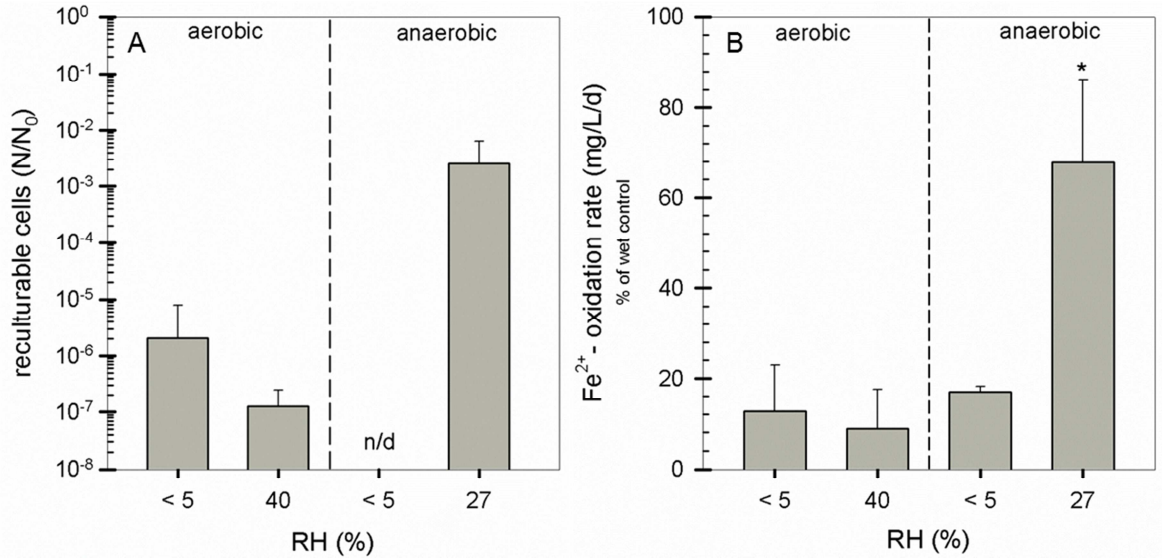

Fig. 3-25: Percentage of reculturable cells (A) and Fe^{2+}-oxidation rate (relative to the wet control) (B) of *A. ferrooxidans* biofilms after 48 h of drying at different RH in the presence or absence of oxygen. Reculturability given as ratio of the dried sample (N) relative to the wet control (N_0) with 10^0 denoting 100% reculturability. Abiotic iron oxidation rate was 1.8 ± 0.1% of the wet control. Data expressed as mean (n=6) with standard deviation. Asterisk denotes significant difference to the other treatments (P <0.05). n/d, no data could be obtained.

Table 3-5: Fe^{2+}-oxidation rates (relative to the wet control) of *A. ferrooxidans* biofilms on filter membranes upon resuscitation after 5, 24, or 48 h of drying at different RH in the presence or absence of oxygen. Abiotic iron oxidation rate was 1.8 ± 0.1%. Data expressed as mean (n=2-6) ± standard deviation.

	aerobic, <5% RH	*aerobic, 40% RH*	*anaerobic, 27% RH*
5 h	53,9 ± 11,2	37.9 ± 7.0	71,6 ± 18,5
24 h	35,9 ± 9,2	37.9 ± 22.8	63,0 ± 25,5
48 h	23,6 ± 22,8	11.1 ± 6.8	58,3 ± 14,5

Biofilms of *S. thermosulfidooxidans* were also subjected to drying for 5 h and 48 h (**Fig. 3-26**). All samples survived drying for 5 h under all conditions, with no decrease of Fe^{2+}-oxidation activity compared to the wet control. After 48 h of drying, iron oxidation rates of biofilms dried in aerobic conditions were significantly lowered to values similar to the abiotic control, indicating that iron oxidation in the cultures was not bacterially mediated and cells did not resume

enzymatic activity, while anaerobic drying resulted in a rate of 70% of the wet control (**Fig. 3-26**).

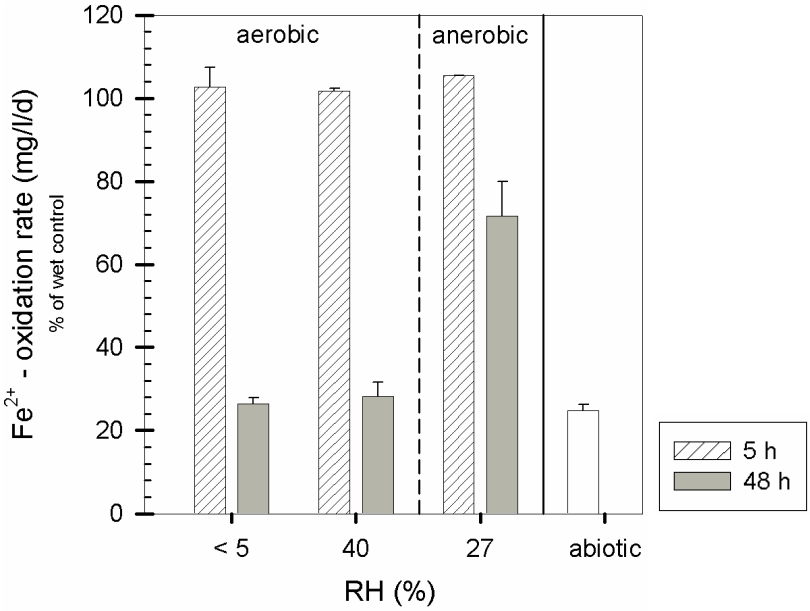

Fig. 3-26: Fe^{2+}-oxidation rates (relative to the wet control) of *S. thermosulfidooxidans* biofilms on filter membranes upon resuscitation after 5 h (shaded bars) or 48 h (grey bars) of drying at different RH in the presence or absence of oxygen. Abiotic iron oxidation rate (white bar) was 24.8 ± 1.4%. Data expressed as mean (n≥2) with standard deviation.

3.2.2.4.1.1Long-term anaerobic drying of A. ferrooxidans

The absence of oxygen was the most important determining factor for survival of desiccation in *A. ferrooxidans* and *S. thermosulfidooxidans*. Thus, the low oxygen concentration in the Martian atmosphere (0.13%) might actually favor long-term stability of desiccated organisms. This experiment aimed to determine the period of time, dried biofilms of *A. ferrooxidans* could be stored under anaerobic conditions.

The iron oxidation rate of the biofilms after resuscitation decreased continually with progressing time of drying until it reached the value of the abiotic control (1.8% of the wet control), indicating that enzymatic iron oxidation activity had ceased (**Fig. 3-27**). At this time point, no reculturable cells could be discovered

as well. After 4 weeks, biofilm iron oxidation rate was low (13 ± 6%), but still significantly higher than the abiotic iron oxidation rate. However, culturable cell numbers were inactivated by 8 orders of magnitude, again showing the discrepancy in quantification of viable cells according to different methods.

Biofilms of *S. thermosulfidooxidans* survived at least 7 days of drying in anaerobic conditions (see section **3.2.7.1**, p. 148).

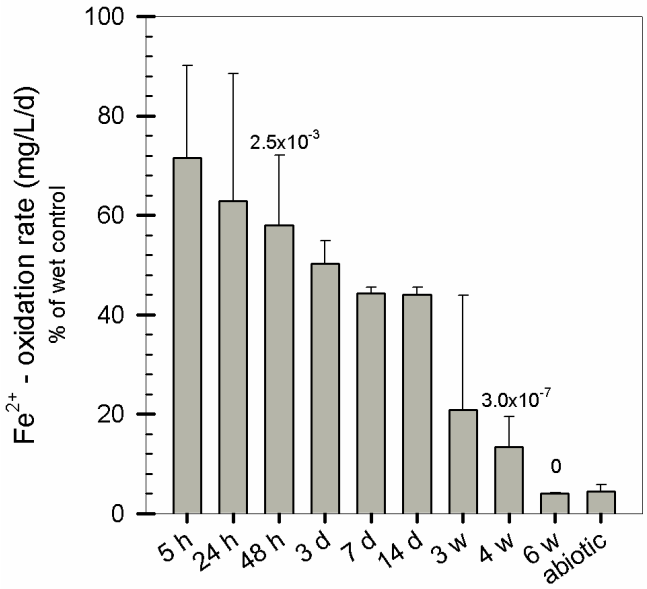

Fig. 3-27: Fe^{2+}-oxidation rates (relative to the wet control) of *A. ferrooxidans* biofilms on filter membranes upon resuscitation after drying in anaerobic conditions for 5 h – 6 weeks. Abiotic: abiotic iron oxidation. Numbers above bars for 48 h, 4 w, and 6 w denote reculturability rate at these time points given as N/N_0 (0 meaning no culturable cells could be recovered). Data expressed as mean (n≥2) with standard deviation.

3.2.2.4.2 Long-term storage of dried *D. geothermalis* is promoted in low relative humidity and anaerobic conditions

In *D. geothermalis*, reculturable cell numbers after 4 weeks of drying were determined by the colony formation assay. Highest survival rates were observed for samples stored in high vacuum (10^{-5} Pa), and lowest for those stored in ambient air (40% RH) (**Fig. 3-28**). There was no significant difference between anaerobic drying (27% RH) and aerobic drying in a desiccator (<5% RH). This was opposed to the results obtained for the iron-sulfur bacteria where drying in

aerobic atmosphere, even at low RH, always yielded lower survival than in anaerobic atmosphere.

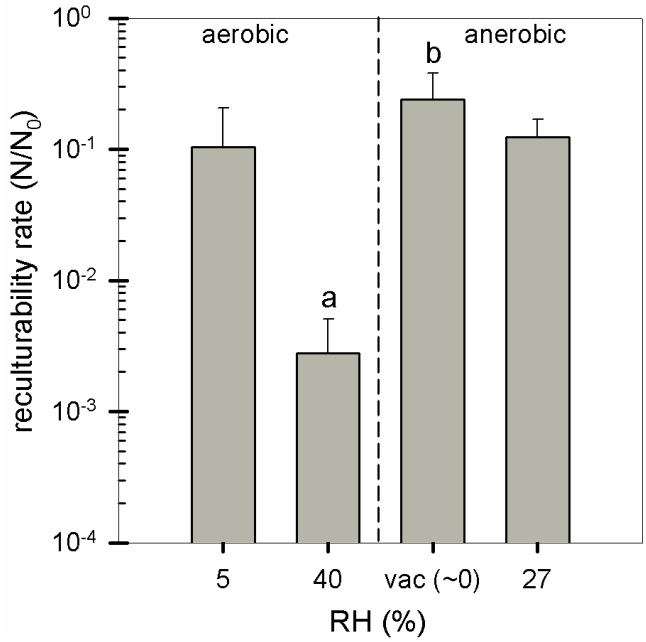

Fig. 3-28: Reculturability of *D. geothermalis* after 4 weeks of drying at different RH in the presence or absence of oxygen, or in vacuum (vac). Reculturability given as ratio of the dried sample (N) relative to the control (N_0) with 10^0 denoting 100% reculturability. Data expressed as mean (n=6) with standard deviation. a, b denote significantly different groups ($P < 0.05$).

3.2.2.5 24 h desiccation of A. ferrooxidans *leads to membrane and DNA damage and RNA degradation*

Drying planktonic cells of *A. ferrooxidans* for 24 h without any protectant led to complete loss of culturability. Using Live/Dead® staining, FISH and PAC, and qPCR, desiccation was also shown to lead to extensive damage of cell components.

The percentage of cells with intact membranes in the fresh control culture (untreated) was 53 ± 12% (**Fig. 3-29**), which corresponds approximately to the percentage of culturable cells (MPN). After drying, only 7 ± 4% of the counted cells had an intact membrane, differing significantly from the control. This seems

to indicate that most cells did not lose their culturability as a result of membrane leakage.

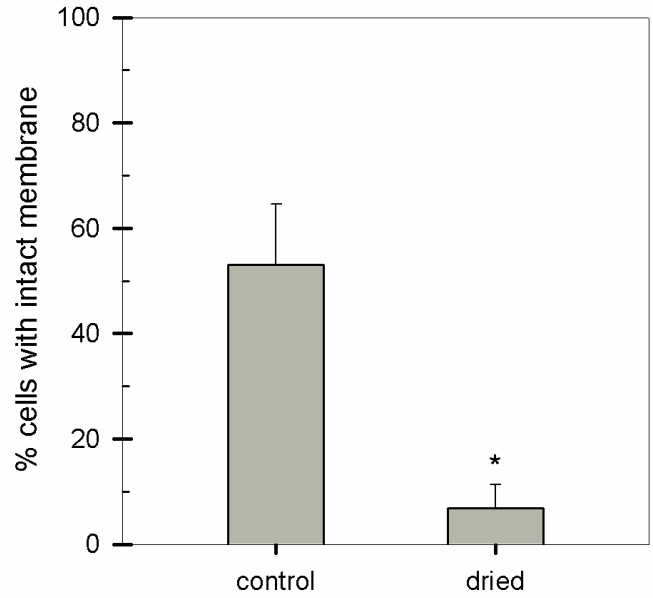

Fig. 3-29: Percentage of cells with intact membrane determined by Live/Dead staining for *A. ferrooxidans* cells after 24 h of air-drying in comparison to untreated cells serving as control. Data expressed as mean (n≥2) with standard deviation, ~700 cells counted per sample. Asterisk denotes significant difference to the control (P <0.05).

On the other hand, FISH revealed a clear degradation of rRNA inside the cells after 24 h of air-drying (**Fig. 3-30 c**). While not all cells in the untreated control exhibited an equally intense fluorescence (due to natural variations in cellular activity in a late-exponential phase culture) (**Fig. 3-30 a**), after incubation in fresh medium with NA, the fluorescence signal was uniformly high so that exposure times had to be reduced to capture the image (**Fig. 3-30 b**). In contrast, incubating dried cells overnight with a gyrase inhibitor (NA) did not lead to fluorescence recovery indicating that cells did not resume biosynthetic activity (**Fig. 3-30 d**).

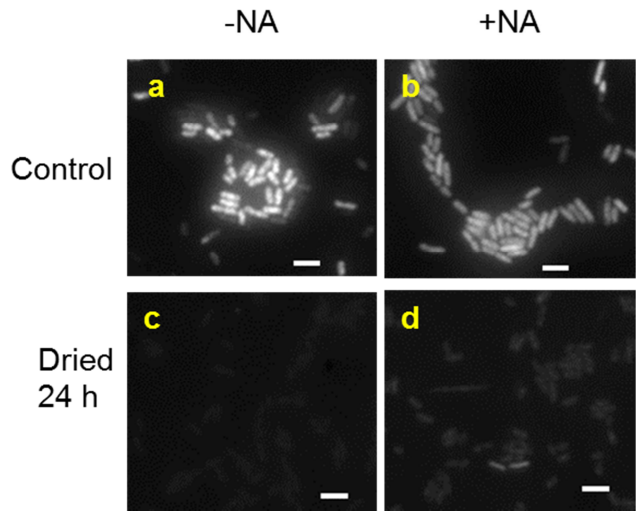

Fig. 3-30: Representative epifluorescence images of *A. ferrooxidans* cells (control or 24 h dried) hybridized with EUB338, fixed either directly after treatment (-NA) or incubated 16 h with nalidixic acid (+NA) before fixation. Exposure time: 800 ms (300 ms for b). Scale bar denotes 2 µm. n=2

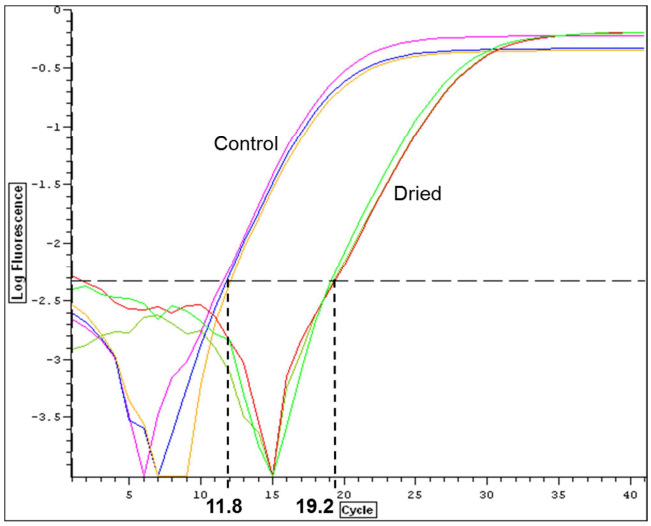

Fig. 3-31: Real-time qPCR of 16S rDNA of the dried cells and of the untreated control of *A. ferrooxidans* showing fluorescence intensity (logarithmic scale) over cycle number. DNA amount used for each PCR reaction (n=3) was 15 ng. Respective C_t values marked on the x-axis.

Extracted DNA from dried cells of *A. ferrooxidans* amplified by qPCR had a C_t value (threshold cycle, i.e. number of amplification cycle when fluorescence signal rises above threshold) higher than the untreated control although the same amount of DNA was used in the PCR reactions (**Fig. 3-31**). DNA degradation or

modification may result in a lack of amplification by PCR, thus C_t values are directly related to the amount of intact target DNA present in the sample. This suggests drying *A. ferrooxidans* led to considerable fragmentation of the genome.

3.2.3 Salt stress

High salt concentrations are one of the limiting factors for the possibility of life on Mars (Tosca et al. 2008). Some Mars-relevant salts, identified by Möhlmann and Thomsen (2011), were selected to study the ability of *A. ferrooxidans* to grow or survive in.

3.2.3.1 Growth of A. ferrooxidans *is inhibited by chlorides and perchlorates at concentrations of ≥1%*

For growth experiments, normal culture medium was supplemented with either 0.5% or 1% (wt/v) of $MgSO_4$, $MgClO_4$, $MgCl_2$, $CaCl_2$, or NaCl, and cell numbers of *A. ferrooxidans* were determined by counting. These concentrations were initially chosen arbitrarily to screen for potential salts that *A. ferrooxidans* could tolerate, which could then be tested in higher concentrations.

Maximum cell densities reached after 5 days of incubation in the salt-containing media were compared to that of the control without added salts (**Fig. 3-32**). In $MgCl_2$, $CaCl_2$, and NaCl, growth (as an increase in TCC above the initial number) could only be detected at concentrations of 0.5% and was retarded compared to the control. At 1% of these salts, cell numbers even decreased in the course of five days suggesting a toxic effect on the bacteria. Presence of $Mg(ClO_4)_2$ at both 0.5% and 1% inhibited growth entirely, but cell numbers did not drop below initial concentrations. Growth in 0.5% and 1% $MgSO_4$ was comparable to the control in terms of both maximum cell density and growth rate.

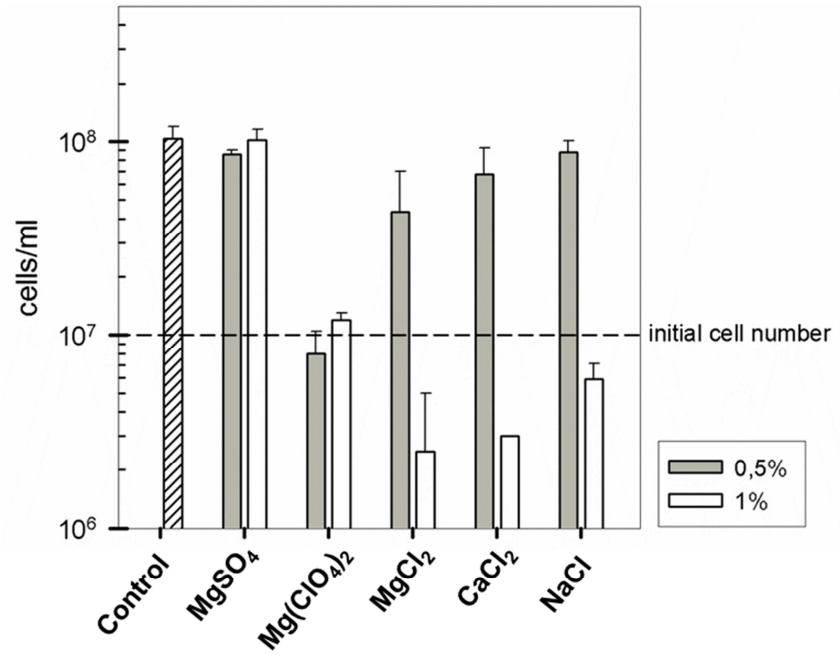

Fig. 3-32: Maximum cell number (TCC) of *A. ferrooxidans* reached after 5 days of growth in the presence of selected salts at 0.5% and 1% (wt/v). Control in BSM without additional salts. The dashed horizontal line denotes the mean initial cell number ($1x10^7$/ml). Data expressed as mean (n≥2) with standard deviation.

3.2.3.2 A. ferrooxidans *can grow in MgSO4 concentrations up to 10%*

As $MgSO_4$ concentrations of 1% (wt/v) did not inhibit growth, higher concentrations of this salt were tested. **Fig. 3-33** shows that *A. ferrooxidans* grew to the same final cell number as in the control in $MgSO_4$ concentrations of up to 10%, although growth in the 10% $MgSO_4$-solution was significantly retarded. In 20% $MgSO_4$, no cells could be detected in the counting chamber after five days of incubation, indicating cell lysis due to the high osmotic pressure.

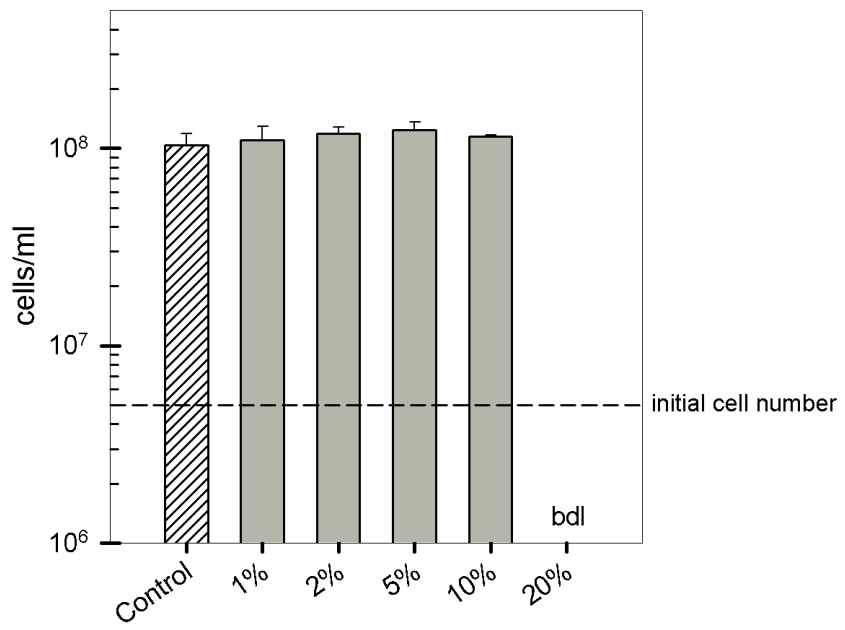

Fig. 3-33: Maximum cell number (TCC) of *A. ferrooxidans* reached after 5 days of growth in the presence of different concentrations of $MgSO_4$. Control in BSM without $MgSO_4$. The dashed horizontal line denotes the mean initial cell number ($4x10^6$/ml). bdl: below detection limit of the counting chamber. Data expressed as mean (n=2) with standard deviation.

3.2.3.3 High salt concentrations (20%) lead to a complete reduction in culturability within 24 h, while FISH signal was little affected

Survival of *A. ferrooxidans* was tested in 20% (wt/v) solutions of $MgSO_4$, $MgClO_4$, $MgCl_2$, $CaCl_2$, and NaCl by storing the cell suspensions at room temperature or at -20°C for up to 1 week. Even after only 1 min of exposure (aliquots were directly removed and diluted after mixing cells with the salt solution), no reculturability could be observed in any of the samples, except for those suspended in $MgSO_4$, where a culturable cell count of $9,2x10^5$/ml was detected (reduction of viability by two log-units). After 24 h in $MgSO_4$, culturable cell numbers were further reduced to ~$1,2x10^2$/ml (inactivation of $8.4x10^{-6}$) independent of the temperature at which they had been stored. No regrowth occurred after storage in any of the salt solutions after 1 week at room temperature or -20°C, and TCC decreased below detection limit during this time. This shows that even very short exposure to salt concentrations as high as 20% (wt/v) can inactivate or kill the majority of a population of *A. ferrooxidans*.

However, after 24 h of incubation in 20% NaCl- and $MgSO_4$-solutions, the fluorescence signal of the EUB338 probe, with which cells were hybridized, was still visible (**Fig. 3-34 c**, **e**), though slightly reduced compared to the control. intensity After incubation of the salt-stressed cells with NA there was no noticeable change in the signal intensity, but neither did cell elongation occur (**Fig. 3-34 d**, **f**).

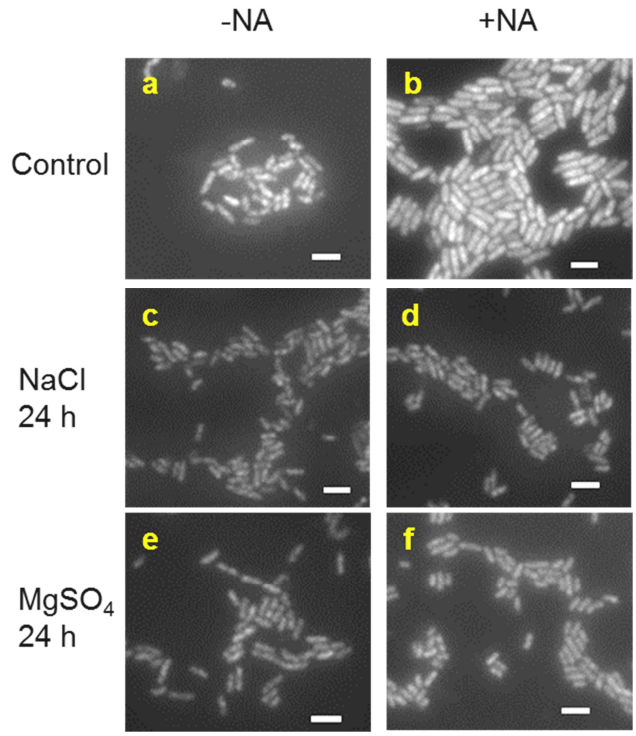

Fig. 3-34: Representative epifluorescence images of *A. ferrooxidans* cells (control or incubated 24 h in 20% NaCl- and $MgSO_4$-solutions) hybridized with EUB338, fixed either directly after treatment (-NA) or incubated 16 h with nalidixic acid (+NA) before fixation. Exposure time: 800 ms (300 ms for b). Scale bar denotes 2 µm. n=1.

3.2.4 UV radiation

UV radiation is a ubiquitous environmental stress factor on the surface of both Earth and Mars. Although UV-C radiation does not penetrate to the surface of Earth in the present, irradiation of bacteria with 254 nm is used to infer radiation resistance characteristics and compare them between different species. Monochromatic UV-C radiation of 254 nm is also often applied for sterilization purposes.

3.2.4.1 A. ferrooxidans *is sensitive to UV-C radiation, but growth in biofilms and metabolically-produced ferric iron provides efficient protection*

To investigate the resistance of microorganisms to UV radiation, bacteria are exposed while suspended in a non-absorbing medium using low cell densities (≤10^7/ml) that preclude shielding effects. In such a setup, a population of planktonic cells of *A. ferrooxidans* was inactivated by four orders of magnitude at 100 J/m^2 UV-C radiation. This was also the last of the applied fluences that yielded reculturable cells of *A. ferrooxidans* (**Fig. 3-35**). The F_{10}-value (fluence resulting in 10% survival of the initial population) amounted to ~30 J/m^2 (**Table 3-6**). This is in good agreement with the F_{10} value reported for another strain of *A. ferrooxidans* (ATCC 33020) of about 25 J/m^2 (Liu et al. 2000).

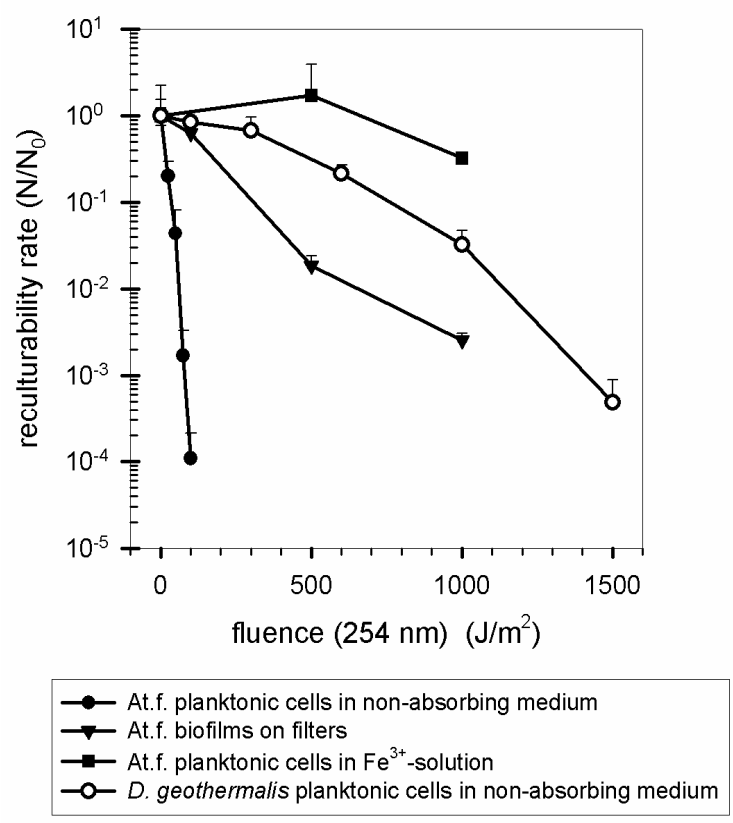

Fig. 3-35: Reculturability of *A. ferrooxidans* (At.f.) and *D. geothermalis* treated with monochromatic UV-C radiation (254 nm). Reculturability given as ratio of the irradiated sample (N) relative to the untreated control (N_0) with 10^0 denoting 100% reculturability. Data expressed as mean (n≥2) with standard deviation.

Compared with planktonic cells of *D. geothermalis*, which survived fluences of 1500 J/m^2, and had an F_{10}-value of 754 J/m^2, *A. ferrooxidans* should be considered a UV-sensitive organism. Although an earlier study (Makarova et al. 2007) showed an even higher UV-C resistance for *D. geothermalis*, this can be considered an effect of different growth conditions and of the growth phase, in which the organism was exposed to radiation, as this may have a significant impact on stress resistance (Wassmann et al. 2011). *D. geothermalis* also exhibited a distinct shoulder-shaped survival curve typical for extremely radiation-resistant organisms (**Fig. 3-35**) with no loss in viability up to a fluence of 419 J/m^2 (threshold fluence). The appearance of a shoulder in the lower fluence-parts of the semi-logarithmic diagram gives an indication of the repair capacities of the respective organism, as 100% reculturability is achieved because all DNA-damage caused by irradiation at these fluences was removed from the genome. The survival curve of *A. ferrooxidans* seems to show a steep exponential decline in **Fig. 3-35**, but at a higher resolution of the x-axis scaling, a small shoulder appears with a calculated threshold fluence of 6 J/m^2.

Table 3-6: F_{10}-values (fluences resulting in 10% survival of the initial population) for *A. ferrooxidans* and *D. geothermalis* treated with monochromatic UV-C radiation (254 nm). Data expressed as mean (n≥2) ± standard deviation.

	F_{10} (J/m^2)	*Comparison with literature*
A. ferrooxidans planktonic cells in non-absorbing medium	28.5 ± 8.3	~25 J/m^2 (other strain of *A. ferrooxidans*) – Liu et al. 2000
D. geothermalis planktonic cells in non-absorbing medium	754.2 ± 40.4	~2000 J/m^2 (different growth conditions) – Makarova et al. 2007
A. ferrooxidans biofilms on filters	313.8 ± 12.0	-
A. ferrooxidans in Fe^{3+}-solution	1969 ± 849	-

In its natural environment, *A. ferrooxidans* does not usually exist in the form of planktonic cells floating in a non-absorbing liquid. Most often, cells will be attached to surfaces such as mineral particles forming biofilms. Using the established biofilm model on filters, *A. ferrooxidans* biofilms were irradiated with UV-C radiation while floating on the surface of BSM to avoid drying as an additional stressor. Biofilms were able to withstand higher doses of UV-C radiation than planktonic cells exhibiting an F_{10}-value of about 300 J/m^2 (**Fig. 3-35**, **Table 3-6**). The survival curve of biofilms on filter membranes, after an initial small shoulder, which was followed by an exponential decline, leveled off at higher fluences. This is most likely an effect of shielding in the multilayer biofilms. After inactivation of the upper cell layers, the lower cell layers remain protected even at continued irradiation stress.

In addition, *A. ferrooxidans* planktonic cells were irradiated directly in their growth medium, which contained soluble Fe^{3+} due to bacterial iron oxidation. 100% reculturability was observed up to fluences of 500 J/m^2 (**Fig. 3-35**) due to the high absorption of Fe^{3+} in the UV-part of the electromagnetic spectrum (**Fig. 2-6**, p. 60). Thus, the environment in which these bacteria live and their own metabolic products afford them protection from UV radiation.

3.2.4.2 External UV-protection of D. geothermalis *by Mars regolith and in multilayer biofilms*

Protection from UV radiation can also be achieved by mineral particles such as the MRS mixtures used in this work. When irradiating *D. geothermalis* with the full spectrum of polychromatic UV radiation (200-400 nm) that reaches the surface of Mars, survivors could be recovered up to fluences of as much as 500 kJ/m^2 or higher, when cells were mixed with 10 mg of S-MRS, or irradiated as biofilms on filters (**Fig. 3-36**). The extrapolation of the survival curves indicates that even higher fluences will not result in a significant decrease of reculturability rates.

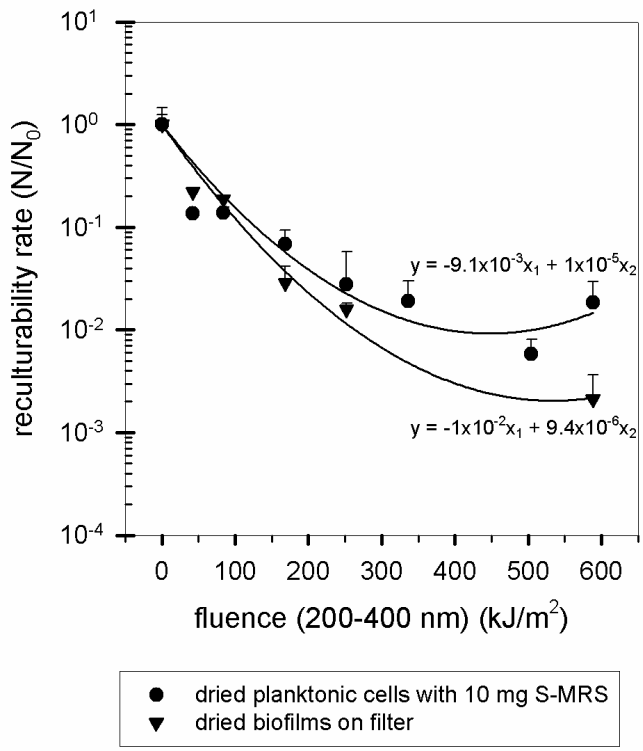

Fig. 3-36: Reculturability of *D. geothermalis* after irradiation with polychromatic UV (200-400 nm). Reculturability given as ratio of the irradiated sample (N) relative to the untreated control (N_0) with 10^0 denoting 100% reculturability. Linear regression analysis (order 2) performed with SigmaPlot 8.0 (curve equations given). Data expressed as mean (n≥3) with standard deviation.

3.2.4.3 UV-C does not directly affect RNA integrity

After irradiating planktonic cells with 100 J/m^2 UV-C radiation and fixing cells directly for FISH, the fluorescence intensity of the EUB338 probe was comparable to that of the control with some cells exhibiting a stronger signal than others (**Fig. 3-37 a**, **c**). After incubation of the irradiated cells with NA, the signal was degraded further (**Fig. 3-37 d**), in agreement with the loss of culturability at this fluence.

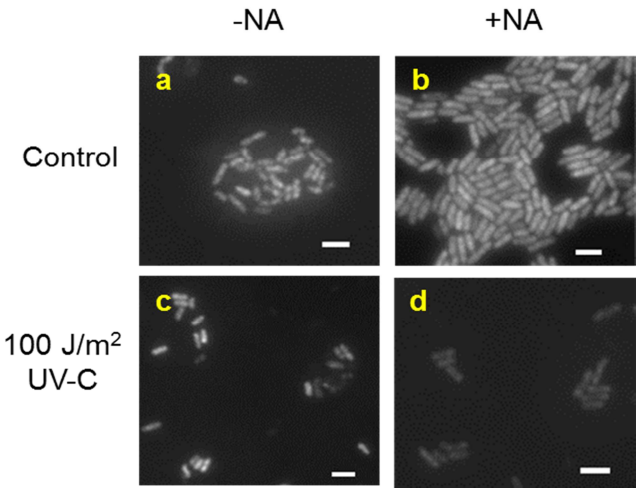

Fig. 3-37: Representative epifluorescence images of *A. ferrooxidans* cells (control and irradiated with 100J/m^2 UV-C) hybridized with EUB338, and fixed either directly after treatment (-NA) or incubated 16 h with nalidixic acid (+NA) before fixation. Exposure time: 800 ms (300 ms for b). Scale bar denotes 2 µm. n=2.

3.2.5 X-rays

While UV radiation only influences life forms directly exposed on the surface of a planet, ionizing radiation is also relevant for life in the subsurface as it is generated by the decay of radioactive elements in the planetary crust, or even when originating from space (cosmic radiation) can penetrate several meters underground (Dartnell 2011). An X-ray machine was used to subject *A. ferrooxidans* to high-energy electromagnetic radiation.

3.2.5.1 Biofilms and planktonic cells of A. ferrooxidans *are sensitive to ionizing radiation*

As ionizing radiation is not impeded by multiple cell layers or materials such as glass and plastic, washed planktonic cells of *A. ferrooxidans* and *D. geothermalis* were irradiated in microcentrifuge tubes suspended in 1 ml liquid. The difference in resistance of both strains is as pronounced as it was shown for UV-C radiation. Culturability of *A. ferrooxidans* was completely diminished by applied doses of >300 Gy and the survival curve did not exhibit a visible shoulder, while no

decrease in culturability occurred in *D. geothermalis* up to ~1.5 kGy (threshold dose) and survivors were still detected at 10 kGy of irradiation (**Fig. 3-38**). Accordingly, D_{10}-values (doses resulting in 10% survival of the initial population) differed by two orders of magnitude with ~45 Gy for *A. ferrooxidans* and 3.5 kGy for *D. geothermalis* (**Table 3-7**). The literature value for another strain of *A. ferrooxidans* (ATCC 33020) agrees with this measurement (Liu et al. 2000). However, higher ionizing radiation resistance has previously been reported for *D. geothermalis*, which could most likely be attributed to differences in growth conditions and possibly in radiation source (type of X-rays) (Makarova et al. 2007).

When biofilms of *A. ferrooxidans* grown on membrane filters were exposed to X-rays while floating on BSM to avoid desiccation, their inactivation was, surprisingly, much higher than for planktonic cells in suspension (**Fig. 3-38**) with a D_{10} of ~20 Gy. As differences in irradiation geometry could be the reason behind this discrepancy, planktonic cells were irradiated in a setup more similar to the biofilms by filtering them on membranes and suspending these membrane filters on BSM for irradiation. Here, planktonic cells revealed a slightly increased sensitivity compared to biofilms on filters which was inferred from the lack of reculturability at 300 Gy, where, in contrast, a small percentage of cells in biofilms had remained culturable. However, D_{10} values of planktonic cells and biofilms on filters did not differ significantly (**Table 3-7**).

Table 3-7: D_{10}-values (doses resulting in 10% survival of the initial population) for *A. ferrooxidans* and *D. geothermalis* treated with X-rays. Data expressed as mean (n≥2) ± standard deviation.

	D_{10} (Gy)	*Comparison with literature*
A. ferrooxidans planktonic cells in suspension	45.9 ± 12.4	~45 Gy (other strain of *A. ferrooxidans*) – Liu et al. 2000
D. geothermalis planktonic cells in suspension	3332 ± 382	~15 kGy – Makarova et al. 2007
A. ferrooxidans biofilms on filter	19.5 ± 7.1	-
A. ferrooxidans planktonic cells on filter	19.3 ± 4.0	-

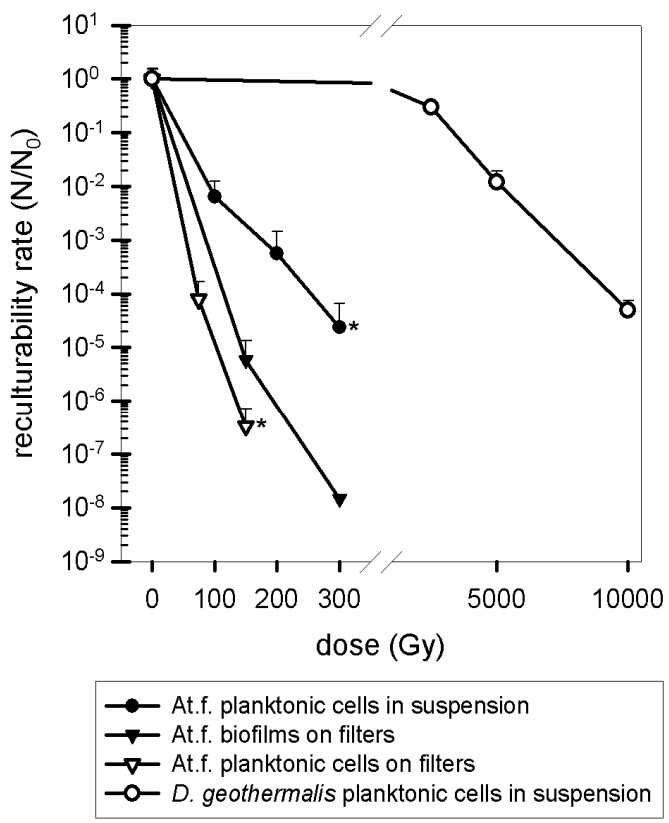

Fig. 3-38: Reculturability of *A. ferrooxidans* (At.f.) and *D. geothermalis* after irradiation with X-rays. Reculturability given as ratio of the irradiated sample (N) relative to the untreated control (N_0) with 10^0 denoting 100% reculturability. Asterisk denotes that no re-culturable cells were found at higher doses tested. Data expressed as mean (n≥2) with standard deviation.

3.2.5.2 X-rays lead to membrane damage and dose-dependent DNA damage in A. ferrooxidans*, while rRNA remains intact directly after irradiation*

A. ferrooxidans cells subjected to X-ray doses of 300 and 1000 Gy were stained by propidium iodide and DAPI (modified Live/Dead® staining).

The percentage of cells with intact membranes in the fresh, untreated culture was 53 ± 12% (**Fig. 3-39**). After irradiation with X-rays, this percentage was reduced to 20 ± 13% for both 300 Gy and 1000 Gy ($P < 0.05$). Thus, the percentage of viable (i.e. membrane intact cells) cells according to membrane integrity staining was much higher than the culturable cell number, which was decreased by at least four orders of magnitudes after exposure to 300 Gy and completely absent

after irradiation with 1000 Gy. This high percentage of cells with intact membranes that were not able to reproduce in the MPN assay, could be VBNC cells. This was also supported by FISH: cells irradiated with 300 Gy and fixed for FISH immediately, showed a probe signal intensity comparative to the control (**Fig. 3-40a**, **c**), suggesting that no degradation of RNA was directly caused by radiation. However, the strong decline of the signal after subsequent incubation in medium with NA (**Fig. 3-40d**), similar to that observed for cells irradiated with UV-C (**Fig. 3-37d**), indicates that cells were either dead or inactive and unable to resynthesize the degraded ribosomes.

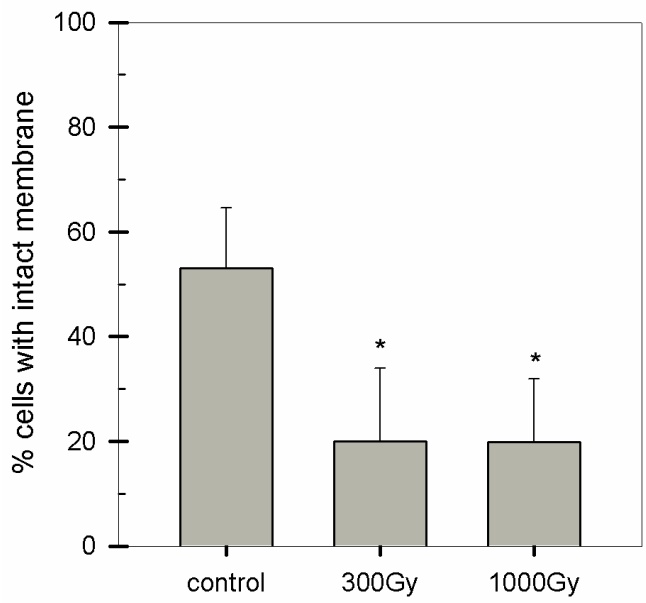

Fig. 3-39: Percentage of cells with intact membrane, as determined by Live/Dead staining for *A. ferrooxidans* cells irradiated with 300 and 1000 Gy (X-rays), in comparison to untreated cells serving as control. Data expressed as mean (n=2) with standard deviation, ~700 cells counted per sample. Asterisk denotes significant difference to the control ($P < 0.05$).

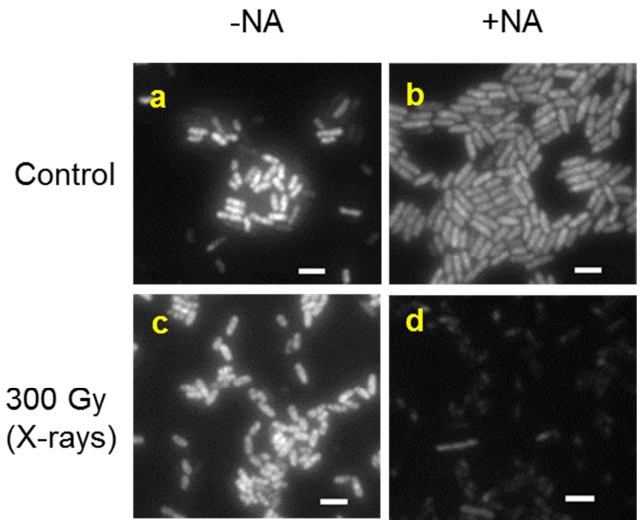

Fig. 3-40: Representative epifluorescence images of *A. ferrooxidans* cells (control or irradiated with X-rays) hybridized with EUB338, fixed either directly after treatment (-NA) or incubated 16 h with nalidixic acid (+NA) before fixation. Exposure time: 800 ms (300 ms for b). Scale bar denotes 2 µm. n=3.

DNA damage in irradiated cells was quantified by qPCR of the 16S rRNA gene. A first trial showed no differences in C_t values (threshold cycle) between the samples of *A. ferrooxidans* irradiated with 300 Gy or 1000 Gy, although both C_t values were higher than that of the untreated control. In an attempt to clarify the expected dependence of the amount of DNA damage on radiation dose, a second trial using cells irradiated with 100 – 5000 Gy of X-rays was performed. Again, C_t values for DNA from 100, 300, and 1000 Gy-treated cells were higher than that of the control (14.7 vs. 9.4), but did not differ among themselves. Only DNA from cells irradiated with 5000 Gy exhibited another increase in the C_t value (18.5) (**Fig. 3-41**). It can be concluded that this method is not suitable to quantify DNA damage at lower doses.

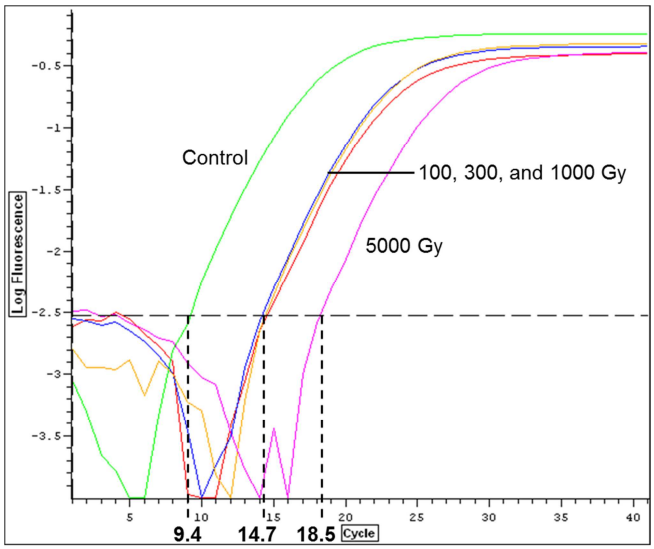

Fig. 3-41: Real-time qPCR of 16S rDNA of irradiated cells (100-5000 Gy) and of the untreated control of *A. ferrooxidans*, showing fluorescence intensity (logarithmic scale) over cycle number. DNA amount used for each PCR reaction (n=3) was 50 ng. Respective C_t values marked on the x-axis.

3.2.6 Low temperatures

The low mean temperatures on Mars might pose a significant challenge to the long-term survival of *A. ferrooxidans*, which has generally been considered sensitive to freezing and freeze-drying (Wakao et al. 1990) and is therefore primarily maintained as actively growing stock cultures in culture collections such as the DSMZ. It was tested which compatible solutes, cryoprotectants synthesized or accumulated by many microorganisms, could stabilize *A. ferrooxidans* during storage at subfreezing temperatures (section **3.2.6.1**). Furthermore, the response of *A. ferrooxidans* to low growth temperatures (10°C and 4°C) was studied to observe the transition from reproductive to maintenance metabolism (section **3.2.6.4**).

3.2.6.1 Compatible solutes protect A. ferrooxidans *during prolonged storage at subfreezing temperatures*

Subjecting *A. ferrooxidans* to -20°C and -80°C for up to 1 year not only aimed to gain relevant information on the preservation of these organisms in a Mars-like

temperature regime, but also could be helpful in optimizing storage conditions for laboratory strains. To accomplish this, different compatible solutes (glycerol, glycine betaine, sucrose, and trehalose at 6% (wt/v) which were also used in assessing desiccation tolerance, see section **3.2.2.2**, p. 113) were added as cryoprotectants to the frozen cell suspensions.

Freezing at -80°C was less deleterious to *A. ferrooxidans* than freezing at -20°C as shown in **Fig. 3-42** and **Fig. 3-43**. At -20°C, no culturable cells could be recovered after 1 week or longer if the cells were frozen without a cryoprotectant. Furthermore, glycerol, which is commonly used to preserve bacterial cultures, offered least effective protection to *A. ferrooxidans* at -20°C, resulting in a complete loss of reculturability after 1 month. Only cells stored in glycine betaine could be recovered after 9 months at -20°C with a reduction in viability by three orders of magnitude (MPN of $2x10^4$/ml) (**Fig. 3-42**).

The total cell number was determined in all samples and did not vary significantly over the period of measurement or between the different storage solutions (**Fig. 3-44**) indicating that lysis of dead cells did not occur during the storage at subfreezing temperatures.

In contrast to the slowly declining culturability of cells stored at -20°C, recovery of *A. ferrooxidans* stored at -80°C showed almost no variation over time (**Fig. 3-43**). Even without protective substances, cells in BSM could still be recovered after 12 months, although inactivation was high with 5 orders of magnitude. The presence of compatible solutes significantly enhanced long-term freezing tolerance at -80°C, glycine betaine, trehalose, and sucrose showing no significant differences, while glycerol had slightly lower protective properties. Inactivation of cells stored 12 months in these organic solutions at -80°C was <1 to 2 log units.

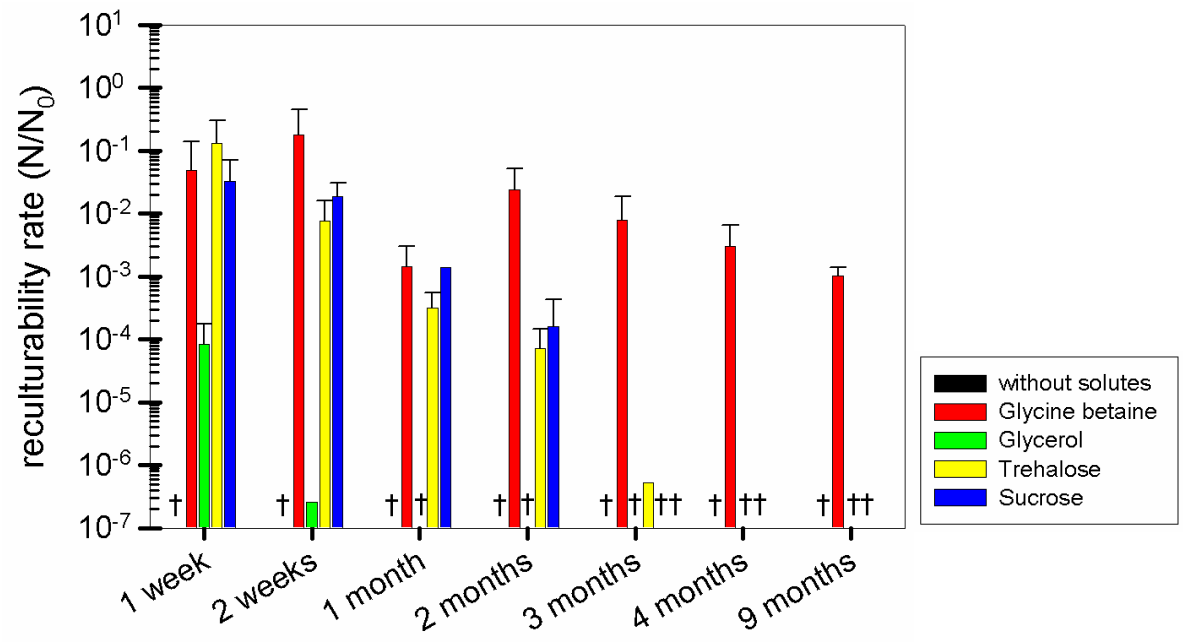

Fig. 3-42: Reculturability of *A. ferrooxidans* stored at -20°C with different compatible solutes (6% wt/v-solutions) or in BSM (without solutes) for up to 9 months. Reculturability given as ratio of the frozen sample (N) relative to the untreated control (N_0) with 10^0 denoting 100% reculturability. Data expressed as mean (n=4) with standard deviation. † denotes no reculturability.

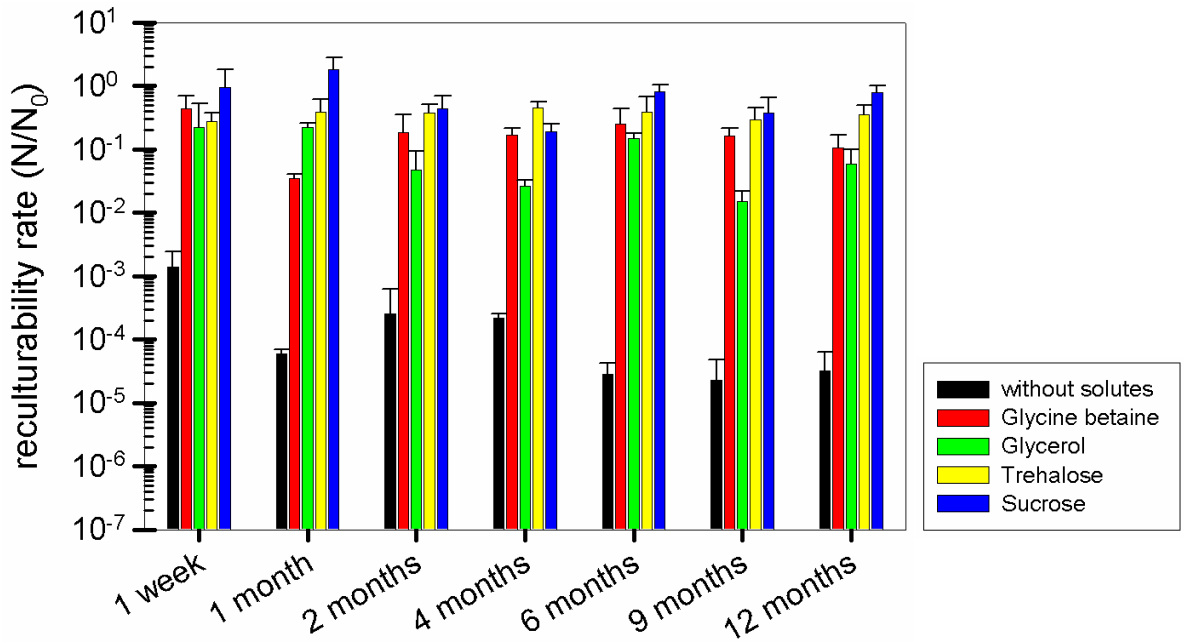

Fig. 3-43: Reculturability of *A. ferrooxidans* stored at -80°C with different compatible solutes (6% wt/v-solutions) or in BSM (without solutes) for up to 12 months. Reculturability given as ratio of the frozen sample (N) relative to the untreated control (N_0) with 10^0 denoting 100% reculturability. Data expressed as mean (n=4) with standard deviation.

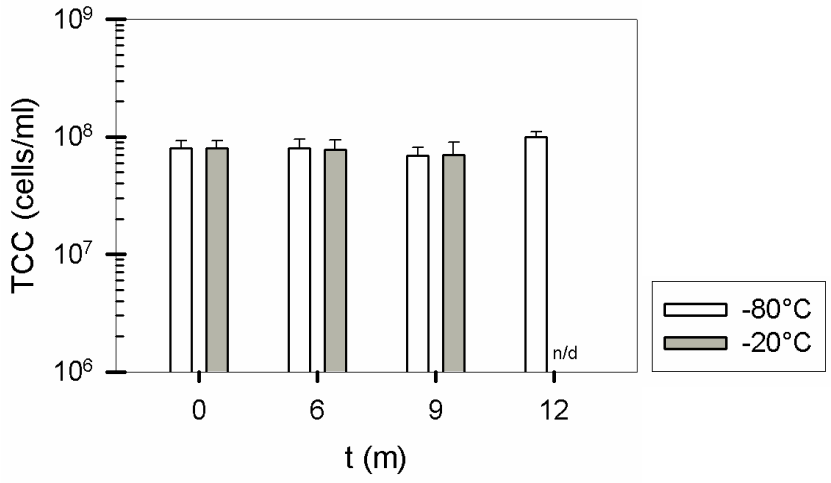

Fig. 3-44: Total cell number in *A. ferrooxidans* samples stored up to 12 months at -20°C and at -80°C. Data expressed as mean of all samples in different solutions with standard deviation; n/d: no data.

This shows that for long-term storage of *A. ferrooxidans* at -80°C, sugars like trehalose and sucrose at 6% (wt/v) are most favorable.

3.2.6.2 RNA integrity of A. ferrooxidans *is compromised after 1 week at -20°C, but not at -80°C*

After 1 week of storage at -80°C, the fluorescence intensity of the EUB338 probe was slightly reduced compared to the control, but not as much as that of cells stored at -20°C for the same amount of time, where only a low fraction of cells retained a high signal (**Fig. 3-45 c**, **e**). After incubation of the frozen cells with NA, a recovery of a uniform fluorescence signal occurred only in the cells stored at -80°C (**Fig. 3-45 f**), while the signal remained low and was even degraded further in the cells stored at -20°C (**Fig. 3-45 d**), corresponding to the different percentage of reculturable cells after these temperatures.

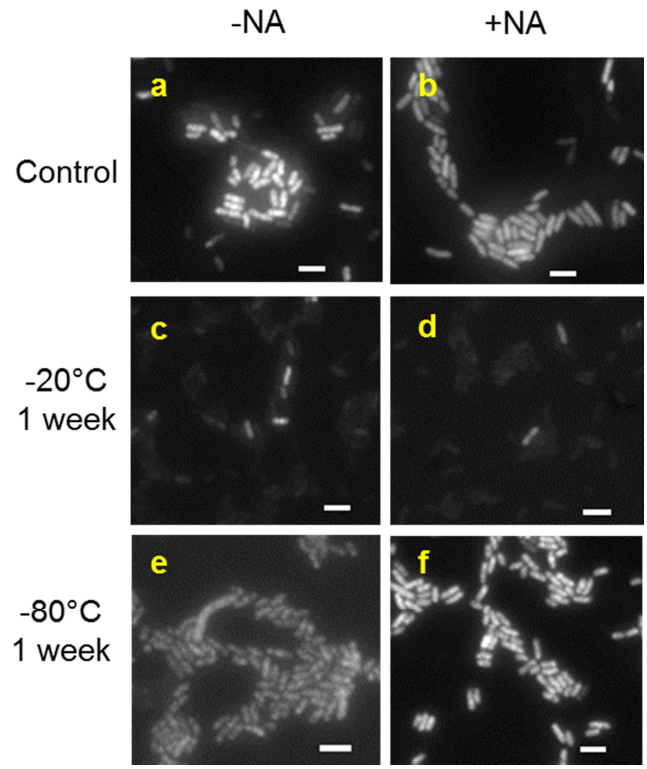

Fig. 3-45: Representative epifluorescence images of *A. ferrooxidans* cells (untreated control or frozen) hybridized with EUB338, fixed either directly after treatment (-NA) or incubated 16 h with nalidixic acid (+NA) before fixation. Exposure time: 800 ms (300 ms for b). Scale bar denotes 2 µm. n=2.

3.2.6.3 Biofilms of A. ferrooxidans *and* S. thermosulfidooxidans *exhibit a similar freezing tolerance*

Similar to desiccation experiments, tests of freezing tolerance of *A. ferrooxidans* were also conducted with biofilms on filter membranes to compare with planktonic cells stored in compatible solutes. Contrary to planktonic cells without cryoprotectants, which could not be recultivated after 1 week of freezing at -20°C, biofilm cells were recovered with an inactivation of 4 log units (**Fig. 3-46**), comparable to planktonic cells without protectant at -80°C. These biofilms also resumed iron oxidation activity at a rate of ~50% of the unfrozen control (**Fig. 3-47**). However, after 1 month at -20°C, biofilms did not show any reculturability.

At -80°C, inactivation was low (40-60%) after both 1 week and 1 month, while 2 months resulted in a decline of culturability by two orders of magnitude (**Fig. 3-46**). Iron oxidation rate after 1 week at -80°C was as high as in unfrozen

samples. For biofilms of *S. thermosulfidooxidans*, a similar response in iron oxidation rate after 1 week of freezing was observed (**Fig. 3-47**) with a rate of 60% of the positive control at -20°C and 137% at -80°C.

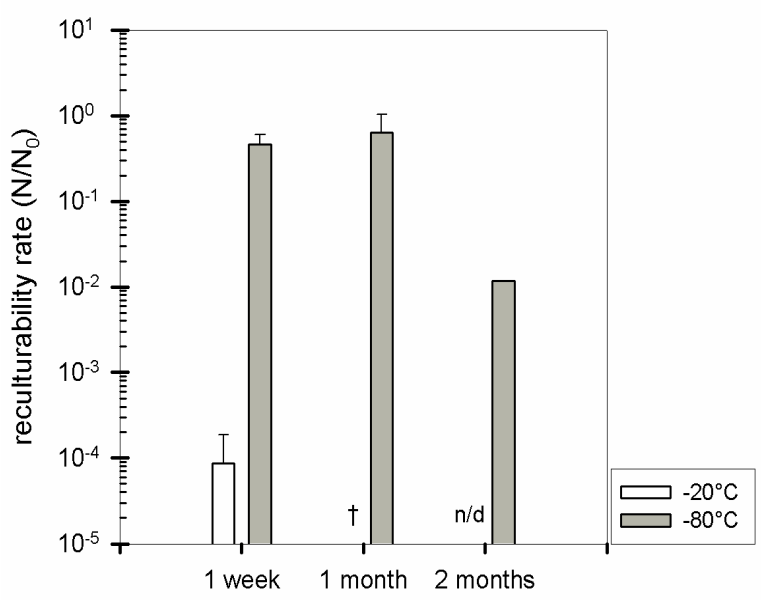

Fig. 3-46: Reculturability of *A. ferrooxidans* biofilms on filters after at 1 week - 2 months at -20°C and -80°C. Reculturability given as ratio of the frozen sample (N) relative to the untreated control (N_0) with 10^0 denoting 100% reculturability. Data expressed as mean (n=2) with standard deviation. † denotes no reculturability. n/d, not determined.

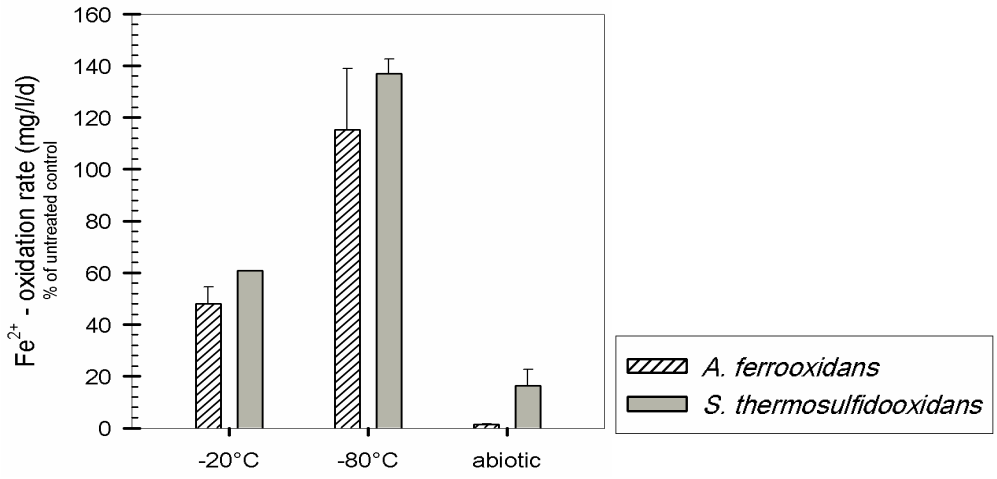

Fig. 3-47: Fe^{2+}-oxidation rates (relative to the untreated control) of *A. ferrooxidans* and *S. thermosulfidooxidans* biofilms on filter membranes upon resuscitation after freezing at -20°C and -80°C for 1 week compared to the abiotic control. Data expressed as mean (n=2) with standard deviation.

3.2.6.4 *Growth of* A. ferrooxidans *is not observable at temperatures ≤4°C, but iron oxidation takes place*

Growth at lower than optimum temperatures of *A. ferrooxidans* was assessed to predict growth kinetics at temperatures which could be applied in Mars simulation studies, in which growth instead of survival is in focus. Cultures containing Fe^{2+} were incubated without shaking at room temperature (~24°C), at 10°C, and at 4°C.

Fig. 3-48 shows the resulting growth curves for all temperatures.

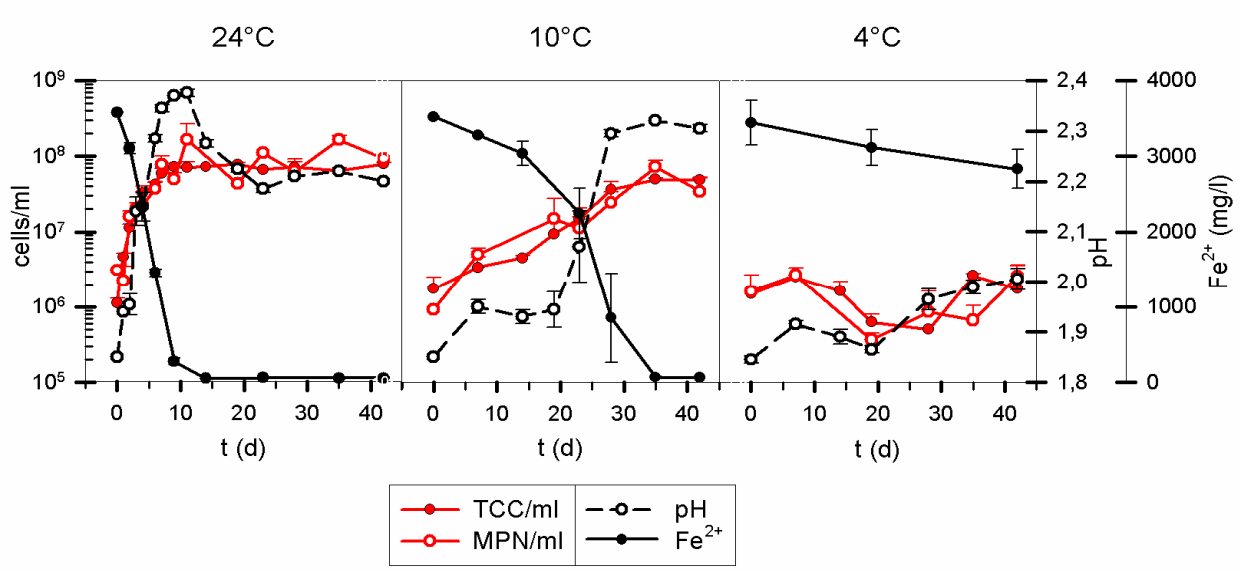

Fig. 3-48: Growth of *A. ferrooxidans* at 24°C (room temperature), 10°C, and 4°C, measured by total cell counts, Most Probable Number, pH, and Fe^{2+} concentration. Scaling of y-axes the same for all graphs. Data expressed as mean (n=2) with standard deviation.

At 24°C cells grew to a maximum density of 7.2×10^7/ml, reaching stationary phase after 9 days, which amounts to a generation time of 20 h (**Table 3-8**). At 10°C, stationary phase was reached after 35 days with 4.9×10^7 cells/ml and a generation time of 113 h. At 4°C, cell numbers did not increase significantly during the 42 days of incubation, thus, no growth rate could be calculated. However, Fe^{2+}-oxidation occurred in these cultures at a rate of ~14 (mg/l)/d, which was much lower than the 95 (mg/l)/d at 10°C and 367 (mg/l)/d at 24°C

(**Table 3-8**), but significantly higher than the abiotic iron oxidation rate at 4°C, which amounted to 1.5 (mg/l)/d. This indicates that cells were active to a small extent even at 4°C (maintenance metabolism), but the energy was not sufficient for growth and cell division. The specific Fe^{2+}-oxidation rates, normalized to the total cell number, were similar for all temperatures with about 8 mg/l / (d*10^9 cells) (**Table 3-8**), but tenfold lower than for *A. ferrooxidans* cultures grown at 30°C (**Table 3-1**), the difference probably being caused by the lack of aeration in the low-temperature cultures.

Table 3-8: Growth rate, generation time (doubling time), and rate of Fe^{2+}-oxidation for *A. ferrooxidans* grown on Fe^{2+}/O_2 at different temperatures without shaking. Data expressed as mean (n=2) ± standard deviation.

	24°C	*10°C*	*4°C*
Growth rate (cell divisions/h)	0.05 ± 0.01	$8.9x10^{-3}$ ± $8.1x10^{-4}$	-
Generation time (h)	20.0 ± 2.4	113.0 ± 10.2	-
Fe^{2+} oxidation rate (mg/l/d)	367.1 ± 4.4	95.2 ± 21.1	14.5 ± 0.9
Specific Fe^{2+} oxidation rate (mg/l) / (h*10^9cells)	8.3 ± 0.7	7.0 ± 0.6	8.1 ± 0.5

3.2.7 Mars simulation

Simulating Martian environmental conditions in the lab presents significant technical challenges, especially if the samples to be exposed contain any liquid water (such as necessary for growth tests), which will quickly evaporate at low pressures (<10 hPa). Therefore, most Mars simulation experiments up to date have been conducted with dried specimen, focusing on survival of the organisms instead of growth or metabolic activity (see section **1.6**, p. 37). After studying the survival of *A. ferrooxidans* and *S. thermosulfidooxidans* exposed to different Mars-relevant stress factors separately, the combined effect of conditions that

could be expected in a Martian shallow subsurface environment was explored, including low pressure, low oxygen concentration, and low temperature, or diurnal temperature fluctuations. In this setup, dried biofilms were used and viability of the organisms was assessed (section **3.2.7.1**).

The second experimental approach was aimed at demonstrating growth or activity of *A. ferrooxidans* in simulated Martian conditions. It has been suggested that the oxygen partial pressure in the Martian atmosphere may be sufficient to support microaerophilic metabolisms such as ferrous iron oxidation (Fisk and Giovannoni 1999; Jepsen et al. 2007; Popa et al. 2012). In an attempt to verify this hypothesis, *A. ferrooxidans* was subjected to Mars atmosphere under low pressure or under Earth-normal pressure in a liquid culture medium, simulating a potential subsurface environment with available liquid water and energy sources (section **3.2.7.2**).

3.2.7.1 Biofilms of A. ferrooxidans *and* S. thermosulfidooxidans *survive one week exposure to Mars atmosphere and pressure*

Biofilms of both *A. ferrooxidans* and *S. thermosulfidooxidans* survived one week in a Mars-like gas mixture (95.25% CO_2, 2.69% N_2, 1.64% Ar, 0.15% O_2), at low pressure (7 hPa), and low temperature (-20°C) (**Fig. 3-49** and **Fig. 3-50**) when exposed in the dried state (at RH ~1.5%).

Reculturability of *A. ferrooxidans* biofilms was reduced by 2 to 3 log units, resulting in an inactivation of 4.2×10^{-3} (**Fig. 3-49**, Mars -20°C). The lab control dried in anaerobic atmosphere showed similar values (2.1×10^{-3}) while the lab control stored at -20°C in ambient air had a reculturability ratio of 8.7×10^{-5}. Because fluctuations of temperature between subfreezing and above-freezing was thought to be more deleterious than storage at constant temperature, *A. ferrooxidans* was also subjected to Martian atmosphere and pressure while simulating a diurnal temperature fluctuation regime between +20°C (at day-time) and -20°C (at night, lower temperatures could not be achieved). Here, reculturability amounted to 9.6×10^{-4} (**Fig. 3-49**). Although biofilm samples showed a large variability, accounting for the high standard deviations, the tendency observed suggests that anaerobic (or microaerobic in the case of Mars gas)

conditions are the most determining factor for survival of *A. ferrooxidans*, even when temperature fluctuations are added as an additional stressor.

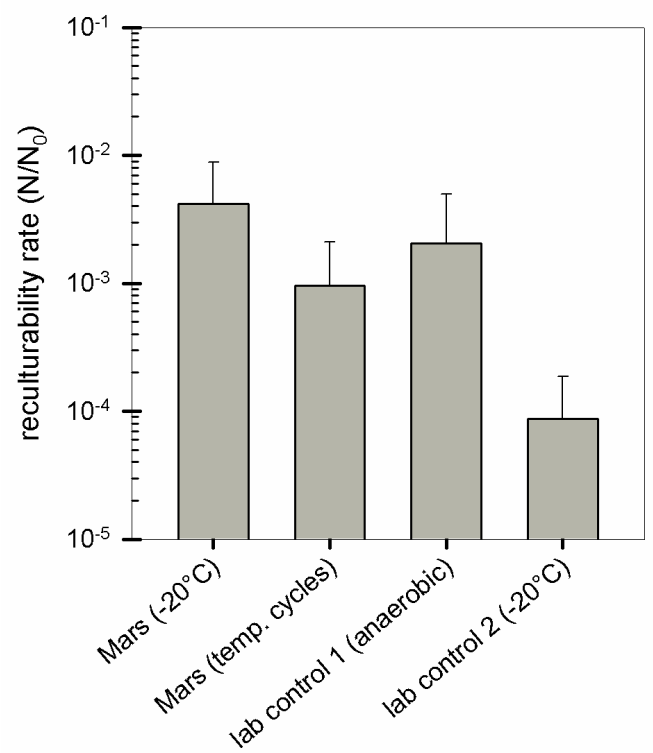

Fig. 3-49: Reculturability of *A. ferrooxidans* biofilms on filters after 1 week exposure to Mars atmosphere and pressure (7 hPa) at constant temperature (Mars -20°C) or with temperature cycles (-20°C to +20°C), compared to biofilms stored in an anaerobic atmosphere at Earth normal pressure and ambient temperature (lab control 1) or at -20°C under aerobic conditions (lab control 2). Reculturability given as ratio of the exposed sample (N) relative to the untreated control (N_0) with 10^0 denoting 100% reculturability. Data expressed as mean (n=4-8) with standard deviation.

In *S. thermosulfidooxidans* biofilms, Fe^{2+}-oxidation rates after exposure to 1 week of Mars atmosphere and pressure at -20°C were as high as that of the untreated control (**Fig. 3-50**) with 102 ± 28%. The lab controls at -20°C (in ambient air) or in anaerobic atmosphere (at ambient temperature) both exhibited reduced rates compared to the untreated samples and samples exposed to Mars-like conditions (61 ± 0.1% and 69 ± 9%, respectively). Thus, survival of *S. thermosulfidooxidans* biofilms was enhanced by a combination of anaerobic conditions and subfreezing temperatures compared to the single factors.

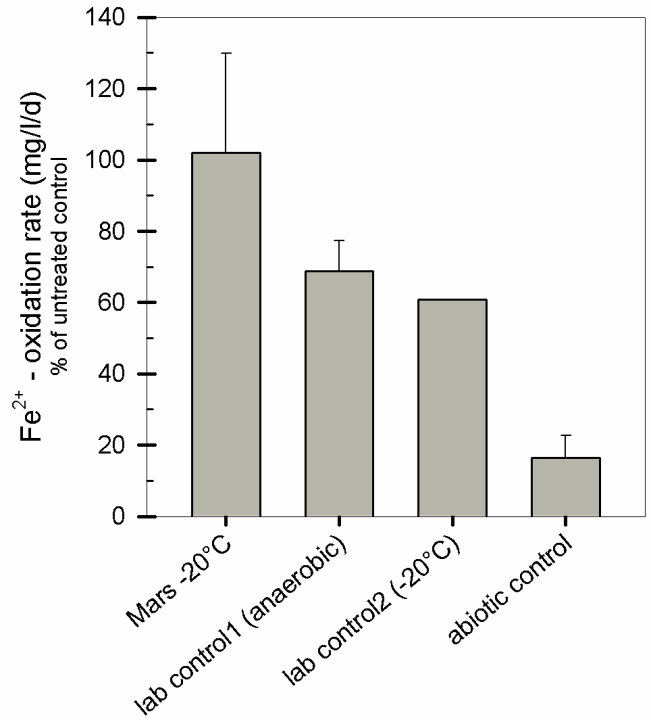

Fig. 3-50: Fe^{2+}-oxidation rates (relative to the untreated control) of *S. thermosulfidooxidans* biofilms on filters upon resuscitation after 1 week exposure to Mars atmosphere and pressure (7 hPa) at -20°C, compared to biofilms stored in an anaerobic atmosphere at Earth normal pressure and ambient temperature (lab control 1) or at -20°C under aerobic conditions (lab control 2) and to abiotic iron oxidation rate. Data expressed as mean (n=4) with standard deviation.

3.2.7.2 Growth of A. ferrooxidans *in Mars atmosphere during one week occurred at Earth-normal pressure, but not at Mars-like pressure*

These experiments aimed to test whether iron oxidation or growth of *A. ferrooxidans* could occur under the low O_2 partial pressure on Mars. This was done using liquid cultures because liquid water is required for growth and activity and may conceivably be present even in the shallow subsurface of present-day Mars (see section **1.2.2.2**, p. 11). Furthermore, temperature was retained at 20°C to accelerate metabolism of *A. ferrooxidans*. Such temperatures are not uncommon to some locations on the Mars, e.g. near the equator in the warmest time of the year (Ulrich et al. 2010).

In a preliminary experiment, *A. ferrooxidans* in Fe^{2+}-medium was observed to grow in a Mars atmospheric gas mixture (0.15% O_2) at Earth-normal pressure

(1013 hPa at 25°C). Cell numbers rose from $5x10^6$ cells/ml to $4.5x10^7$/ml after 8 days of incubation in Mars gas (compared to a cell density of $7.3x10^7$/ml reached in Earth atmosphere) with a corresponding increase in MPN from $8.2x10^5$/ml to $1.3x10^7$/ml in Mars gas and to $1.8x10^8$/ml in ambient air. In addition, 55.5% of the Fe^{2+} was oxidized in Mars gas compared to 98.6% in ambient air after 8 days.

In the follow-up experiment, the pressure was lowered to Mars-like values of 6-10 hPa. Bacteria were mixed with Mars regolith simulants (MRS) in BSM (at 5% wt/v), where they had previously been shown to grow (section **3.1.4**, p. 95). As a control, a liquid culture with Fe^{2+} as energy source was included. The cultures (~10 ml liquid with $1.5x10^6$ cells/ml) were covered with polycarbonate membranes to allow gas exchange, while ensuring sterility and were exposed to Martian atmosphere and pressure. Due to evaporation of the liquid, pressure rose constantly and was adjusted manually each day to 6 hPa. The average pressure over the course of 1 week was 15 ± 5 hPa.

MPN in each culture was assessed before and after the exposure. Parallels were also kept under ambient conditions (laboratory control). The initial culturable cell number in all samples was $6x10^5$/ml, 43% of the total cell number. An increase in MPN by 1 to 2 orders of magnitude occurred in cultures stored under Earth conditions (**Fig. 3-51**). In cultures exposed to Mars-like conditions, culturable cell numbers after 1 week of incubation did not rise significantly over the initial values in P-MRS and S-MRS, and decreased in Fe^{2+}-medium (where TCC also decreased to $8.7x10^5$/ml). The evaporation of liquid in the Mars-cultures was accounted for in the calculations. It should be noted that culturable cell numbers (MPN) in anaerobically incubated cultures might not always be good indicators of cell growth because they were observed to decrease if energy availability was low (**Fig. 3-8** and **Fig. 3-9**), but sampling in this experiment occurred at only one time point (after 7 days). Therefore, temporal changes in MPN numbers might have been missed. However, iron concentrations did not undergo changes significantly different from the sterile control cultures.

Thus, growth and iron oxidation occurred in a Mars-like gas mixture with 0.15% (v.) O_2, but not at a pressure close to the Martian surface of ~15 hPa.

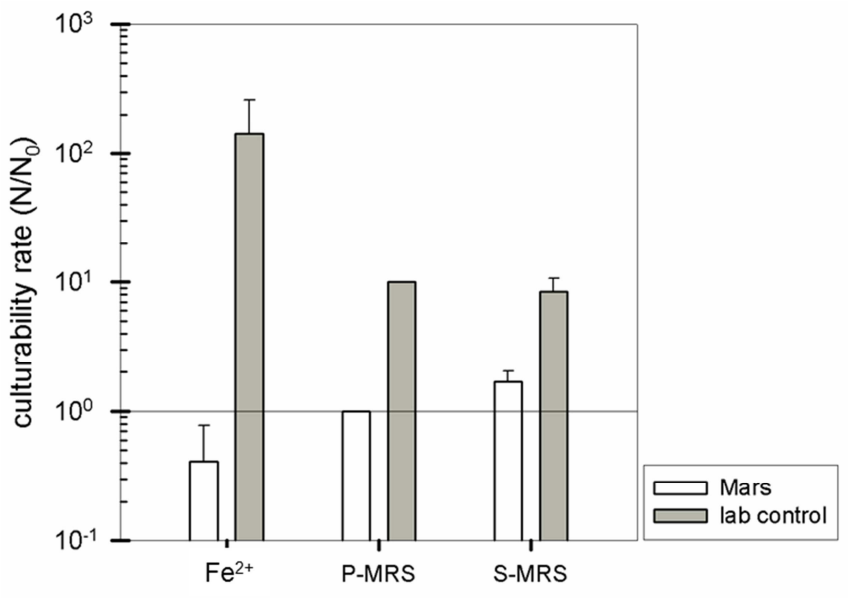

Fig. 3-51: Culturable cells of *A. ferrooxidans* in cultures with 2.5% (wt) S-MRS or P-MRS or with Fe^{2+} as a control, after 1 week exposure to Mars atmosphere and low pressure (~15 hPa) at 20°C (Mars), and 1 week under Earth conditions (Earth normal pressure, ambient temperature, aerobic) (lab control). Culturability given as ratio of the sample after exposure (N) relative to the same sample before exposure (N_0) with 10^0 denoting culturable cell counts before exposure. Data expressed as mean (n=2) with standard deviation.

4 Discussion

Growing knowledge on the geochemistry of Mars can help to identify potential metabolic reactions that could occur with the available energy sources present on the planet (section **1.2.2.4**, p. 14). Using terrestrial habitats and microorganisms as an analogy, hypothetical food webs can be constructed that could plausibly exist in the subsurface of Mars, where conditions are presently more conducive to life than at the surface. The hypothetical food web shown in **Fig. 4-1** can be divided into two distinct models, termed the ‘permafrost world’ and the ‘iron/sulfur world’. Although there may be interactions (metabolic exchanges) between the groups of organisms of these two models, they could also exist as separate entities.

The permafrost model with methanogenic primary producers at its basis is currently considered most likely due to the observation of subsurface ice deposits forming permafrost soils on Mars and the detection of methane in the atmosphere (section **1.2.2.3**, p. 12). However, the abundance of iron and sulfur minerals on Mars has led different authors to suggest iron-sulfur transforming microorganisms as likely models for Martian life forms (Amils et al. 2007; Fernández-Remolar et al. 2005; Nixon et al. 2012). This work focused on the ‘iron world’ part of the food web by studying the acidophilic iron-sulfur-oxidizing bacterial strains *Acidithiobacillus ferrooxidans* and *Sulfobacillus thermosulfidooxidans*. The hypothetical role of these bacteria in the shallow to deep subsurface of Mars is outlined in **Fig. 4-2**.

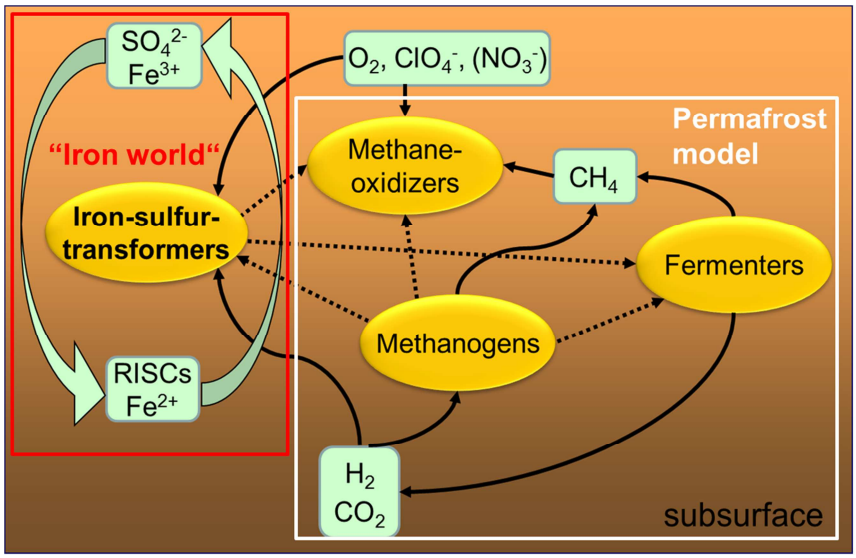

Fig. 4-1: Hypothetical food web in the shallow and deep subsurface of Mars. The white box marks the permafrost world model, in which methanogens play the central role as primary producers (section **1.2.2.3**, p. 12), possibly interacting with other organisms such as methane-consumers (methanotrophs) or heterotrophic fermenters. The red box encompasses iron-sulfur compound transforming microorganisms, which are in the focus of this work (modified from U. Szewzyk, TU Berlin).

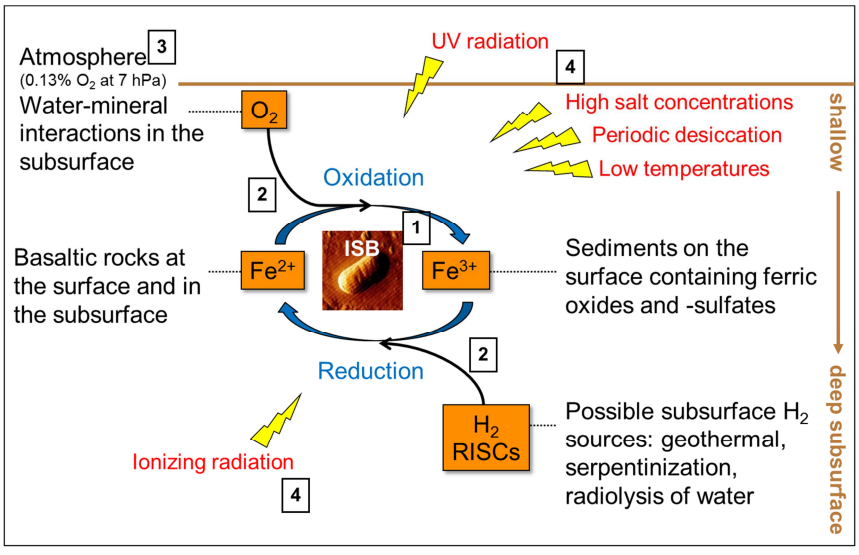

Fig. 4-2: Hypothetical role of iron-sulfur bacteria (ISB) in the cycling of iron in the shallow or deep subsurface of Mars. Sources (both validated and hypothetical) for the different electron donors and acceptors are indicated. Numbers denote the aspects that were experimentally investigated in the present study. 1 – Iron oxidoreduction; 2 – Growth on Mars regolith minerals in aerobic and anaerobic conditions, 3 – Minimal O_2 concentration for growth and iron oxidation at Mars-pressure; 4 – Effect of different stress factors on viability of ISB.

This model (**Fig. 4-2**) assumes that the iron-sulfur bacteria exist in a subsurface habitat because of the following considerations:

- Liquid water as a requirement for life is most likely to be found in the subsurface of Mars, for example as thin films on the surface of mineral particles, or in an aquifer in the deeper subsurface in regions with access to geothermal heating (section **1.2.2.2**, p. 11).
- The subsurface environment would provide protection from the high surface radiation flux (though ionizing radiation also has subsurface sources).
- Depending on the depth, the organisms would have access to different electron donors and acceptors (e.g. in the shallow subsurface O_2 from the atmosphere, or H_2 in the deeper subsurface), while the Martian regolith would provide substrates for lithotrophic growth (e.g. iron).

To study the relevance of the selected iron-sulfur bacteria for this model, several aspects were investigated (denoted by the numbers in **Fig. 4-2**):

1) The metabolic potential of the organisms, especially with respect to microaerobic and anaerobic growth and their implication in iron redox cycling (sections **4.1.1**, p. 156 and **4.1.1.1**, p. 157).
2) The ability to utilize nutrients and grow with Mars regolith minerals, which contain iron in both oxidation states (Fe^{2+} and Fe^{3+}) and the potential for ferric iron mineral dissolution (section **4.1.5**, p. 163).
3) The oxygen threshold for iron oxidation by these organisms (section **4.1.3**, p. 159). Oxygen partial pressure on Mars is very low; nevertheless, aerobic metabolism has been suggested as a thermodynamic possibility (Jepsen et al. 2007).
4) The viability of the model iron-sulfur bacteria after exposure to Mars-like stress conditions (section **4.2**, p. 169), because it can be assumed that the Martian environment is not a static one, but will exhibit fluctuations in physical conditions, e.g. diurnal and seasonal cycles and obliquity changes on geological time scales (Clark 1998; Haberle et al. 2003; Ulrich et al. 2010).

4.1 Metabolic versatility of acidophilic iron-sulfur bacteria

4.1.1 Anaerobic and microaerobic growth

Oxygen is available in the Martian atmosphere, but at a low concentration (0.13%) and at a low overall pressure (7 hPa). Therefore, growth in microaerobic or anaerobic conditions was considered an important metabolic property with respect to Mars. The selected model organism *A. ferrooxidans* is known for its high metabolic versatility, including the ability to respire anaerobically, for example using reduced inorganic sulfur compounds (RISCs) as electron donors and ferric iron as an electron acceptor (Pronk et al. 1991b, 1992). However, growth on H_2/Fe^{3+} did not previously occur in cultures of the type strain, which was used in this study, as opposed to other strains of *A. ferrooxidans* (Ohmura et al. 2002). In the experiments conducted here, cell numbers of *A. ferrooxidans*^{T} increased exponentially during incubation in cultures containing H_2 and Fe^{3+}, and this increase was linked to Fe^{3+}-reduction (**Fig. 3-8 A**, p. 89). Slow, non-exponential growth of *A. ferrooxidans* occurred also in N_2/CO_2 atmosphere with Fe^{3+}, for which no explanation can be offered at present (**Fig. 3-8** B, p. 89). Although Fe^{3+}-reduction seems to be a common feature for *A. ferrooxidans* strains, potential genes encoding for a ferric iron reduction complex could not yet be identified in the genome of *A. ferrooxidans*^{T} (Valdés et al. 2008).

Aerobic oxidation of H_2 was previously shown to occur in *A. ferrooxidans*^{T} only at pH values of 2.5 – 6 (Drobner et al. 1990), but not at pH 2 (Ohmura et al. 2002). In contrast, *A. ferrooxidans*^{T} cultivated in this study at pH <2 in microaerobic conditions (<1% O_2) under H_2/CO_2 atmosphere clearly showed an exponential increase in cell numbers (by >1 order of magnitude) that was coupled to oxygen consumption (**Fig. 3-9 A**, p. 90). This could most likely be attributed to H_2 oxidation as it also occurred after several cycles of re-inoculation and because growth did not occur in a control experiment without H_2 (**Fig. 3-9 B**, p. 90). The ability to utilize hydrogen in *A. ferrooxidans*^{T} is supported by the identification of four different types of hydrogenases encoded in its genome, among them a group 1 [Ni-Fe]-hydrogenase functioning as a respiratory enzyme within the cell

membrane (Valdés et al. 2008). Some strains of *A. ferrooxidans* have also been shown to oxidize hydrogen using sulfur as an electron acceptor (Ohmura et al. 2002).

The contrasting results to literature reported here could have been due to extended laboratory cultivation under the same conditions, which can cause a strain to lose its potential for utilizing other energy sources (Ohmura et al. 2002). To minimize cultivation-induced loss of metabolic functions during the experiments conducted here, precultures were inoculated from cultures that were as close as possible to the original strain obtained by the culture collection.

4.1.1.1 Dependence of viability on energy supply

The Most Probable Number (MPN) assay was employed in this study for *A. ferrooxidans* to obtain a quantitative estimate of 'survival' after stress exposure based on culturability, but also to monitor viability in cultures during incubation in different conditions. Culturable cell numbers of *A. ferrooxidans* remained stable over an extended period (>30 d) in aerobic cultures incubated at 30°C, even after all ferrous iron had been used up as an electron donor (**Fig. 3-3**, p. 80). In contrast, anaerobic cultivation led to a sudden drop in culturability as soon as the electron acceptor, either ferric iron (during growth on H_2/Fe^{3+}, **Fig. 3-8**, p. 89) or oxygen (during growth on H_2/O_2, **Fig. 3-9**, p. 90) had been depleted. It has been recognized early on that acidophiles need a constant input of energy in order to maintain the large proton gradient over their cytoplasmic membrane and prevent acidification of the cytoplasm (Cobley and Cox 1983). The maintenance of a high viable cell number during aerobic incubation, when O_2 availability remained high through shaking, may indicate the use of an alternative electron donor, perhaps in the form of organics. Autotrophic *A. ferrooxidans* strains were often reported to be inhibited by organics, specifically organic acids, because they easily diffuse over the cytoplasmic membrane in low pH dissociating in the neutral cell interior, which drives protons into the cell (Alexander et al. 1987; Ingledew 1982). Yet, at limiting substrate concentrations in chemostat cultures, *A. ferrooxidans* grew well on formic acid (Pronk et al. 1991b). Possibly, organic material slowly released from lysed cells can serve as an alternate electron donor for those cells still viable during stationary phase. In contrast, anaerobic cultivation did not contain a

constant source of electron acceptors due to the closed nature of the system. Thus, as ferric iron or oxygen were used up, no more energy could be generated even if organics could serve as electron donor.

4.1.2 Iron redox cycling

Iron oxidoreduction is the ability of a single strain to gain energy from both ferrous iron oxidation and ferric iron reduction, provided that suitable electron donors (such as RISCs or H_2) and electron acceptors (such as O_2) are present. Both *A. ferrooxidans* and *S. thermosulfidooxidans* have the potential for iron oxidation and reduction and can therefore play important roles in the redox cycling of this element.

In the experiment conducted here, pure cultures of *A. ferrooxidans* and *S. thermosulfidooxidans* were able to switch from aerobic iron oxidation to ferric iron reduction in the presence of only inorganic electron donors in the case of *A. ferrooxidans* (**Fig. 3-10**, p. 92) or with organic substrates in *S. thermosulfidooxidans* (**Fig. 3-11**, p. 95). Since sulfur was present in the cultures of *A. ferrooxidans*, it was likely used as an electron donor during ferric iron reduction (Pronk et al. 1992). However, a distinct increase of sulfate, which should be the end product of both aerobic and anaerobic RISC oxidation (see equations 10, 11, 13, p. 93), did not occur. It was observed before that sulfate concentrations oscillated and were not related in a linear fashion to growth and acid generation in *A. ferrooxidans* cultures incubated on elemental sulfur, thiosulfate, or tetrathionate, probably owing to chemical disproportionation reactions (Bernier and Warren 2007). This demonstrates that oxidation of inorganic sulfur compounds is governed by a complex system of chemical and enzymatic reactions, making it difficult to identify individual processes in this experiment (Suzuki 1999).

Although not added to the *A. ferrooxidans* cultures, thiosulfate was also detected in significant amounts in the culture medium, and its concentration changed with bacterial activity. Thiosulfate consumption terminated with the depletion of the majority of available O_2, and the onset of ferric iron reduction coincided with the production of thiosulfate (**Fig. 3-10 B**, p. 92). The first step during thiosulfate

oxidation in *A. ferrooxidans* is catalyzed by a periplasmic thiosulfate dehydrogenase enzyme, which condensates two molecules of thiosulfate to form tetrathionate as an intermediate. Tetrathionate is then hydrolyzed by tetrathionate hydrolase, generating thiosulfate, elemental sulfur and sulfate as final products (Ghosh and Dam 2009). Thiosulfate oxidation is oxygen-dependent because thiosulfate dehydrogenase activity should be directly coupled to oxygen reduction by a terminal oxidase (Beard et al. 2011). Thus, it can be surmised that the initial decrease of thiosulfate was caused by bacterial oxidation of this substrate, which was terminated due to lack of O_2. The following rise in thiosulfate concentration might be explained by the Fe^{3+}-reductase activity of tetrathionate hydrolase, leading to the regeneration of thiosulfate from the tetrathionate formed during aerobic thiosulfate oxidation (Beard et al. 2011, Schippers et al. 1996, Sugio et al. 2009).

Iron redox cycling by *A. ferrooxidans* was as yet only demonstrated in co-culture with acidophilic heterotrophs, which used organic compounds for ferric iron reduction. Similarly, thermophilic acidophiles like sulfobacilli have been shown to cycle iron in the presence of organic substrates (Bridge and Johnson 1998; Johnson and McGinness 1991). In all experiments, including this study, strictly anaerobic conditions were not required for ferric iron reduction; thus, the produced Fe^{2+} can later be oxidized again by the available oxygen, allowing a complete geochemical cycling (Bridge and Johnson 1998; Johnson and McGinness 1991; Warren et al. 2008).

4.1.3 Growth in Mars atmosphere at Martian surface pressure

One goal of this work was to determine whether the acidophilic model bacteria could grow with only the resources present on Mars. Growth with the minerals in Mars regolith simulants was demonstrated for *A. ferrooxidans* in aerobic and anaerobic conditions under Earth-normal pressures (see section **4.1.5**, p. 163). It has been suggested that the oxygen partial pressure in the Martian atmosphere may be sufficient to support microaerobic metabolisms such as ferrous iron oxidation (Popa et al. 2012; Fisk and Giovannoni 1999; Jepsen et al. 2007). In an attempt to verify this hypothesis, a growth test with *A. ferrooxidans* was

conducted by incubating bacterial cultures on Mars regolith simulants (P-MRS and S-MRS) as well as on ferrous iron in Mars atmosphere (0.15% O_2) at Earth-normal pressure (~1000 hPa) or Mars-like pressure (10-15 hPa). These bacterial cultures contained plenty of liquid water to reproduce the culture conditions in which growth was known to occur. For the same reason, a temperature of 20°C was applied in the Mars simulation chamber. Thus, this was not an attempt to simulate accurately the physical conditions of a putative near-surface habitat, but to investigate certain metabolic capabilities.

At Earth-like atmospheric pressure, the oxygen concentration in the Mars gas mixture of 0.15% was sufficient for the oxidation of about 1000 mg/l Fe^{2+} with an according increase in cell number by one log unit during 7 days of incubation. This is in agreement with the O_2 threshold of 0.05% (lowest oxygen concentration measured) that was observed in initial growth experiments of with *A. ferrooxidans* (section **4.1.3**, p. 159).

However, lowering the pressure to Martian surface values of 10-15 hPa, thus decreasing the oxygen partial pressure by two orders of magnitude, resulted in no measurable growth or iron oxidation after 7 days. It is possible that incubation times were too short, but extensive outgassing of water from the liquid cultures under the low pressure prevented a prolongation of the experiment.

There have not been many studies attempting to observe active metabolism or even growth of terrestrial microbes under Mars-simulated conditions. For heterotrophs like *E. coli*, *Serratia liquefaciens*, and several *Bacillus* spp., the pressure limit for growth and replication was determined to be ~25 hPa (Berry et al. 2010; Nicholson and Schuerger 2006). Methanogenesis was shown to occur at low temperatures (Gounot 1999) and at low pressures down to 50 hPa, which could be found in the near subsurface (<100 m) of Mars, based on a predicted increase of lithostatic pressure at a rate of 111 hPa/m (Kral et al. 2011). Methanogens from permafrost habitats are currently considered one of the most plausible terrestrial models for potential Martian life forms (Morozova et al. 2007; Morozova and Wagner 2007).

Pavlov et al. (2010) conducted an experiment using bacteria (*Vibrio* sp.) mixed with sand, over a layer of ice to simulate regions of Mars where shallow (<1 m

depth) ground ice is covered by dry regolith, such as at the Phoenix landing site (Smith et al. 2009b). Due to sublimation of the ice under low pressure (in that study 0.01-0.1 mbar), the water vapor could diffuse through the porous matrix of the soil, perhaps providing enough moisture (e.g. thin films of liquid water adsorbed to mineral particles; Möhlmann 2009) for growth of the organism, which was reported to occur (Pavlov et al. 2010). The experiment by Pavlov et al. might have been a good approximation to shallow subsurface environments of Mars, but it should be reproduced with organisms more relevant for Mars than *Vibrio* sp. in terms of metabolism, such as methanogens or the iron-sulfur bacteria used this study.

4.1.4 The possible relevance of aerobic metabolism for Mars

In the experiments reported in the previous sections, the minimum concentration of dissolved O_2 measured in cultures of *A. ferrooxidans* and *S. thermosulfidooxidans* after the initial O_2 consumption phase was 0.05% (0.36 mg/l or 22 µM). This fits with an earlier study, which reported that O_2 availability became the limiting factor for iron oxidation at dissolved oxygen concentrations of 0.29 - 0.7 mg/l, and that *A. ferrooxidans* did not grow (aerobically) at O_2 levels below 0.2 mg/l (Liu et al. 1988). For several other strains of the genus *Sulfobacillus*, growth coupled to ferric iron reduction occurred down to oxygen levels of 0.07% in the gas phase (Tsaplina et al. 2010).

The oxygen partial pressure, at which facultatively anaerobic bacteria switch to anaerobic metabolism is called the Pasteur Point, which is species-specific (Stolper et al. 2010). Some organisms can aerobically respire down to much lower oxygen concentrations than what was demonstrated for *A. ferrooxidans* and *S. thermosulfidooxidans*, which is probably related to the low energy yield from the oxidation of ferrous iron at acidic pH ($\Delta G° = -8.7$ kJ/mol Fe; Popa et al. 2012). A recent study determined that the threshold for aerobic respiration in *E. coli* is 3 nM O_2 (Stolper et al. 2010). Modeling data furthermore suggested that concentrations as low as 0.05 to 0.2 nM would be able to fuel an aerobic ecosystem with doubling times for single cells of 30 d (Karl and Novitsky 1988; Stolper et al. 2010). Low oxygen environments on Earth exist, for example, at

oxic-anoxic boundaries of aquatic sediments and in soils (Canfield et al. 2005). The role of aerobic metabolism in these environments is still uncertain, but anaerobic respiration may occur simultaneously to aerobic respiration (carried out by different species) even if conditions are not strictly anaerobic (Stolper et al. 2010).

The atmosphere of Mars contains oxygen at a concentration of 0.13%. At mean Martian surface pressure (~7 hPa) this corresponds to 400 nM O_2, which would be above the empirical threshold for aerobic respiration observed for *E. coli* (3 nM). Thus, Mars should not be regarded as an environment where only anaerobic metabolism can be expected. Thermodynamical calculations also indicate that metabolic reactions using O_2 (such as iron oxidation), are possible even under the low oxygen partial pressure on Mars (Jepsen et al. 2007), provided that kinetics of O_2-consumption by organisms do not exceed the rate at which O_2 is replenished. Production of O_2 on Mars can either occur by abiotic processes or, hypothetically, by metabolic reactions of other organisms.

Perchlorate reduction, where O_2 appears as an intermediary product may be one of these processes, considering the apparent abundance of perchlorates in some parts of Mars (Hecht et al. 2009; Stoker et al. 2010). After reduction of (per)chlorate (ClO_4^-/ClO_3^-) to chlorite (ClO_2^-) in terrestrial perchlorate reducers, quantitative dismutation of chlorite leads to oxygen development (eq. 14) (Coates and Achenbach 2004; Coates et al. 1999):

$$ClO_2^- \rightarrow Cl^- + O_2 \qquad (14)$$

Abiotic O_2 production in the Martian atmosphere takes place by photodissociation of CO_2:

$$CO_2 + h\nu \rightarrow CO + O \qquad (15)$$

and further reaction cascades leading to the formation of molecular oxygen (Kong and McElroy 1977).

Alternative abiotic O_2 generation can also be conceived in subsurface environments if even minor amounts of liquid water are available. Water reacting with basaltic minerals can produce H_2O_2 (Hurowitz et al. 2007), which may decompose in the presence of Fe^{3+} to yield molecular oxygen (Watts et al. 1999).

$H_2O_2 \xrightarrow{(Fe^{3+})} \frac{1}{2}O_2 + H_2O$ (16)

Radiolysis of subsurface water is also a possible source for both H_2 and O_2 and probably supports the deep chemolithotrophic biosphere in Earth's crust (Lin et al. 2005).

If subsurface aquifers exist on Mars, in which such abiotic O_2-generating processes play a role, and which also have access to a subsurface source of H_2 (e.g. by serpentinization, geothermal activity, or radiolysis of water; Clark 1998; Kieft et al. 2005; Lin et al. 2005; Onstott et al. 2006; Oze and Sharma 2005), potential organisms should have a supply of both electron donors and acceptors enabling redox cycling of elements such as iron.

4.1.5 Growth with nutrients present in Mars regolith

Growth of *A. ferrooxidans* on sulfidic minerals like pyrite and pyrrhotite has been extensively studied (e.g. Bhatti et al. 1993, Jiang et al. 2007). In this study, two artificial mixtures of minerals that were put together according to most recent data on the geochemical composition of Martian regolith, were used to examine whether these minerals could provide nutrients necessary for lithoautotrophic growth of *A. ferrooxidans* (see **Table 2-6**, p. 50). One of the Mars regolith simulants (MRS) was phyllosilicate-rich (Phyllosilicatic-MRS, P-MRS), containing a high percentage of smectite clays like montmorillonite, kaolinite, and chamosite, as well as carbonates (siderite, hydromagnesite). The other mixture (Sulfatic-MRS, S-MRS) was characterized by its high gypsum and goethite content. Both mineral mixtures consist also of pyroxene and plagioclase (gabbro), olivine, quartz, and the anhydrous ferric oxide hematite (Boettger et al. 2011). P-MRS is modeled after the phyllosilicate-rich sites on Mars, which likely formed during an aqueous weathering regime with neutral to alkaline conditions in the Noachian epoch (>3.7 Ga), either on the surface or in the subsurface at hydrothermal areas (**Fig. 1-2**, p. 7) (Bibring et al. 2006; Chevrier et al. 2007; Halevy et al. 2007; Ehlmann et al. 2011). S-MRS represents the Martian sediments with a high sulfate content (although the ferric sulfate jarosite was not included) that presumably mark the existence of acidic conditions in the Hesperian epoch (3.7-3.0 Ga) (Bibring et al. 2006; Bullock and Moore 2007; Chevrier et al. 2007).

4.1.5.1 Interaction of bacterial cells with the mineral particles of MRS

Because of the buffering capacity of the regolith minerals, the unadjusted pH of cultures with MRS (5% wt/v) suspended in deionized water was basic (pH 8-9). Growth of *A. ferrooxidans* was not observed if the culture medium was not acidified to a pH <4, in accordance with the pH optimum of the acidophilic organism. Circumneutral pH is also the limiting condition during the initial colonization of newly-exposed sulfide sites (Mielke et al. 2003). However, acidophilic bacteria are able attach to pyrite and initiate its oxidation even when the bulk liquid is at neutral pH value. This initial biooxidation is thought to be mediated by the creation of an acidic nanoenvironment between the bacteria and the mineral surface. During the leaching process bacteria are encrusted within an iron oxy-hydroxide matrix, which consolidates their association to the mineral surface. As the population increases, the pH of the external environment is also affected if the buffering capacity of the bulk liquid is sufficiently low (Mielke et al. 2003).

The most important step in this process is the attachment of bacteria to the mineral surface (Sand et al. 2001). Bacterial adhesion to surfaces is achieved by a complex combination of van der Waals and electrostatic forces, polar interactions, hydrogen bonding, as well as acid-base interactions; but hydrophobicity and surface charge of the bacterium and the surface are generally regarded as key factors (Devasia et al. 1993; Karunakaran et al. 2011; Solari et al. 1992; van Loosdrecht et al. 1987; Vilinska and Rao 2009). The EPS (extracellular polymeric substances) play an important role in the interaction of microbes with surfaces as they can confer hydrophobic or charged properties to the bacterial surface. The EPS of *A. ferrooxidans* generally consist of neutral sugars, glucuronic acid, and saturated fatty acids, but the substrate of growth influences their composition (Escobar et al. 1997; Gehrke et al. 1998; Harneit et al. 2006). In cells pre-grown on ferrous iron, Fe^{3+} ions are complexed with the glucuronic acid, mediating the attachment to the negatively charged surface of pyrite by electrochemical interactions. Sulfur-grown cells do not attach to pyrite as they do not contain these complexed iron ions, and have a higher fatty acid and lower sugar proportion, rendering the cell more hydrophobic (Gehrke et al. 1998; Harneit et al. 2006).

When examining the interaction of *A. ferrooxidans* with the minerals of the Mars regolith mixtures, it was found that the majority of cells (90%) rapidly disappeared from the bulk liquid phase during the first hour after inoculation in cultures with P-MRS, but not with S-MRS, and only 2% of the initial cell population was counted in the liquid phase after >4 h (**Fig. 3-17**, p. 104). This observation has been described in the literature for various other substrates including coal, sulfides, and elemental sulfur and is thought to indicate the process of attachment of bacterial cells to surfaces (Bagdigian and Myerson 1986; Crundwell 1996; Espejo and Romero 1987; Harneit et al. 2006; Konishi et al. 1990; Ohmura et al. 1993). No difference in the interaction of *A. ferrooxidans* with either P-MRS or S-MRS was observed, when cultures were pre-grown on sulfur, instead of Fe^{2+} (**Fig. 3-18**, p. 105). This might indicate that the observed 'attachment' of cells to the particles in P-MRS was not based on surface properties such as hydrophobicity or net charge of the bacterial cells.

It was shown that some strains of *A. ferrooxidans* can attach to quartz surface, though less efficiently than to sulfides (Harneit et al. 2006; Solari et al. 1992). Quartz was present in both mineral mixtures (3% in S-MRS and 10% in P-MRS). This alone does not appear sufficient to explain the observed large difference in attachment between the mineral mixtures. It would seem beneficial for *A. ferrooxidans* to attach preferentially to those minerals containing useable substrates such as reduced iron or sulfur compounds. The Mars regolith mixtures did not include any sulfides, but Fe^{2+} was present in many minerals, such as olivine ($(Mg, Fe)_2SiO_4$) (2% in P-MRS, 15% in S-MRS), and siderite ($Fe(CO_3)$) (5% in P-MRS), or bound to the smectite clay chamosite ($(Fe^{2+},Mg,Fe^{3+})5Al(Si_3Al)O_{10}(OH,O)_8$) (20% in P-MRS). These clay minerals made up the bulk of the P-MRS mixture and were not contained in S-MRS at all. Clay minerals are characterized by small particle size and high surface area and reactivity and play a major role in terrestrial biogeochemical cycling of metals due to their cation adsorption capacity (Sposito et al. 1999). Their net negative charge, is decreased at low pH (<3), possibly facilitating the interaction with negatively charged bacteria (Jiang et al. 2007). The high surface area of the clay particles might be the reason for the differential behavior observed here. Few studies have focused on the adsorption of bacteria to clay minerals due to

difficulties in separating cells from mineral particles of similar sizes, although some bacteria, such as *Pseudomonas putida*, were shown to associate with montmorillonite and kaolinite (Jiang et al. 2007).

4.1.5.2 Nutrients provided by Mars regolith minerals

The Mars regolith mineral mixtures seemed to provide all the essential nutrients for growth of *A. ferrooxidans*, as bacteria also grew when basal salts and trace elements were omitted and acidic, deionized water was used as medium (**Fig. 3-12B**, p. 97). In a similar study, methanogens were incubated on montmorillonite in non-buffered deionized water with only H_2/CO_2 as headspace gas. Methane production was observed under these conditions, albeit at severely reduced rates, and it was concluded that montmorillonite could supply all essential micronutrients for methanogenic metabolism (Chastain and Kral 2010).

Cations, such as K^+, Mg^{2+}, and Ca^{2+}, but also Fe^{2+}, and Fe^{3+} are important nutrients for microorganisms (Sposito et al. 1999). They are adsorbed by minerals, especially clays, and exchanged by protons from the medium ('cation exchange capacity') leading to an increase in pH, while releasing ions like Fe^{2+} for the bacteria to consume (Stotzky and Rem 1965). Since S-MRS did not contain clay minerals, the soluble Fe^{2+} concentration (probably resulting from dissolution of olivine) was about 5 times lower than that of P-MRS at the beginning of the experiments. Growth of *A. ferrooxidans* on both MRS mixtures under aerobic conditions occurred by the oxidation of the desorbed soluble ferrous iron (**Fig. 3-12**, p. 97, **Fig. 3-13**, p. 98).

Fe^{3+} was present in both MRS mixtures in such minerals as hematite (Fe_2O_3) and goethite (FeO(OH)), but also bound to clay minerals like chamosite (**Table 2-6**). In anaerobic conditions, *A. ferrooxidans* might use Fe^{3+} as an electron acceptor, for example with hydrogen as electron donor (see section **4.1.1**, p. 156). However, the initial soluble Fe^{3+} concentration was very low in both S-MRS and P-MRS cultures (initially 11 mg/l for P-MRS and 26 mg/l S-MRS at pH <2.5). During incubation of *A. ferrooxidans* on MRS under anaerobic conditions, culturable cell numbers increased more strongly in the presence of H_2 compared to the control under N_2/CO_2 atmosphere (**Fig. 3-14**, p. 100). However, the time

point, at which growth of *A. ferrooxidans* in H_2/CO_2 atmosphere was supported by iron reduction alone is not easy to define due to the initial presence of low amounts of oxygen. This oxygen was used first to oxidize available ferrous iron leached from the minerals. But the retention of high viable cell numbers (MPN) in MRS mixtures as opposed to the sharp decrease in viability in control cultures with Fe^{3+} points to the continued presence of an electron acceptor such as Fe^{3+}.

This raised the question whether *A. ferrooxidans* could actively contribute to mineral dissolution, especially of the crystalline Fe^{3+} minerals goethite and hematite, which are characterized by a high thermodynamic stability (Canfield et al. 1992; Morse and Wang 1997; Munch and Ottow 1980; Neal et al. 2003), or whether the organism merely utilized the Fe^{3+} ions released from the minerals due to the low pH. Reduction of crystalline Fe^{3+}-minerals is a common feature among neutrophilic iron-reducing bacteria such as *Shewanella* sp. and *Geobacter* sp. (e.g. Cutting et al. 2009; Roden and Zachara 1996). It has also been reported for the acidophilic species *Sulfobacillus acidophilus* and *Acidiphilium* sp. when they were grown with an organic electron donor, but not for *A. ferrooxidans* (Bridge and Johnson 1998, 2000). In these acidophilic iron reducers, dissolution of the minerals did not require direct contact to the bacteria. The mechanism of mineral dissolution was proposed to be a change in the equilibrium between the solid phase Fe^{3+} and the soluble Fe^{3+}, which was shifted toward the soluble form due to the reduction of soluble ferric iron by the bacteria (equation 17) (Bridge and Johnson 1998, 2000).

$$Fe^{3+}_{solid} \leftrightarrow Fe^{3+}_{soluble} \text{ —(bacterial iron reduction)} \rightarrow Fe^{2+} \qquad (17)$$

This mechanism could have also occurred in the *A. ferrooxidans* cultures with S-MRS, which contained a total of 20% (wt/v) of crystalline Fe^{3+} minerals (goethite and hematite). Fe^{3+} reduction by the bacteria in H_2/CO_2 atmosphere may have shifted the equilibrium (eq. 17) to favor the release of further Fe^{3+} into the solution, thereby accelerating mineral dissolution. This led to a notable difference in the rate of release of soluble iron from the minerals between cultures under H_2/CO_2 (electron donor present) and N_2/CO_2 (no electron donor present)

atmosphere (**Fig. 3-16**, p. 103). Although the pH value was similar in these cultures, or even higher in H_2/CO_2 atmosphere (sometimes exceeding values of 3.0, which may significantly reduce the amount of mineral dissolution; Bridge and Johnson 2000), the amount of total iron leached from the minerals after 12 days was twice as high in H_2/CO_2 atmosphere. Furthermore, the stronger increase of the pH value in H_2/CO_2 atmosphere indicated an accelerated desorption of iron from the minerals, which are exchanged with protons. Thus, *A. ferrooxidans* seems to have accelerated ferric iron mineral dissolution.

This effect was less pronounced for P-MRS, probably because most of the Fe^{3+} in this mixture came from a more accessible source (i.e. bound to the clay minerals). The structural Fe^{3+} in clay minerals can also serve as a substrate for neutrophilic iron-reducing bacteria (Dong et al. 2003; Jaisi et al. 2005, 2007a, 2007b; Kostka et al. 1996, 2003; Zhang et al. 2007), sulfate-reducing bacteria (Li et al. 2004) and even methanogenic archaea (Liu et al. 2011).

These experiments showed that Martian regolith consists of minerals that could provide basic nutrients for the growth of chemolithoautotrophic organisms, provided that liquid water is present. Both S-MRS and P-MRS mixtures contain ferrous iron-bearing minerals like olivine and pyroxene, which can undergo aqueous alteration processes (serpentinization) under elevated temperatures (>40°C), e.g. in the subsurface, generating H_2 and ferric iron in the form of magnetite (equation 18) (McCollom 2007). Such subsurface serpentinization processes are also considered plausible sources for the methane detected in the Martian atmosphere (Kelley et al. 2005; Lyons et al. 2005; Mumma et al. 2009; Oze and Sharma 2005).

$$2(FeO)_{rock} + H_2O \rightarrow Fe_3O_4 + H_2 \qquad (18)$$

As discussed in section **4.1.3** (p. 159), O_2 should also not be discarded as a possible electron acceptor on Mars, even in the subsurface, where it could be used for the oxidation of ferrous iron in the basaltic rocks.

4.2 Stress resistance of model iron-sulfur bacteria

The previous section (**4.1**) discussed the various metabolic capacities of iron-sulfur bacteria that would be relevant features of potential Martian organisms. It was assumed that such organisms exist in subsurface niches on Mars, in which they would have access to suitable energy sources, liquid water, and are protected from stress factors such as the high surface radiation flux or desiccation. However, Mars is a dynamic system exhibiting constantly changing environmental conditions. Liquid water, for example, may be only temporarily available, exposing life forms to periodic desiccation. Organisms in putative subsurface aquifers may also be transported to the surface by processes similar to those leading to the changes in surface morphology observable today on Mars (such as gully formation as shown in **Fig. 1-3**, p. 11), or by impact events (Malin et al. 2006). Thus, the ability to tolerate harsh environmental conditions would be an important property of potential Martian life forms.

4.2.1 Viability of organisms

Bacteria often respond to environmental stress conditions by ceasing to undergo cell division. However, if the cells remain intact and the intracellular damage is not too extensive, these organisms may still exhibit metabolic activity known as maintenance metabolism. Maintenance metabolism has been defined as the energy expended on all cellular functions unrelated to growth (Pirt 1965, 1982) or the endogenous metabolism (physiological maintenance) of a cell (Herbert 1958; Stouthamer et al. 1990; van Bodegom 2007). Physiological maintenance comprises osmoregulation, motility, defense and repair mechanisms and macromolecular turnover (reviewed in van Bodegom 2007).

This mode of metabolism appears to be common for microorganisms in cold environments (e.g. in ice down to temperatures of -33°C), where the measured respiration rates are not sufficient for reproduction, but for repair and maintenance of cellular structure as well as for adaptation to changes in the physical conditions (Amato and Christner 2009; Bakermans et al. 2011; Price and Sowers 2004; Wilhelm et al. 2012). Likewise, microorganisms in the deep

subsurface, which can be regarded as a habitat with an extremely low energy supply, are estimated to have doubling times on the order of centuries. Most of these microorganisms must use all available energy to maintain macromolecular integrity (e.g. repair of radiation-induced DNA damage) with little remaining for the production of new cell material so that maintenance or endogenous metabolism may be considered the 'normal' state of organisms in such ecosystems (Chapelle and Lovley 1990; Kieft and Phelps 1997; Phelps et al. 1994; Whitman et al. 1998).

Microorganisms in this state are sometimes referred to as viable-but-nonculturable (VBNC) if they cannot be grown on conventional media (Kell et al. 1998; Oliver 1993). Using culturability as the sole indicator of viability is the classical approach in microbiology. Many astrobiological studies (such as Mars simulation experiments with different heterotrophic organisms) have also relied almost exclusively only the plate count assay to determine survival after exposure to stress conditions (e.g. Diaz and Schulze-Makuch 2006; Nicholson and Schuerger 2005; Pogoda de la Vega et al. 2007; Schuerger et al. 2003, 2006; Schuerger and Nicholson 2006; and many older studies as reviewed by Hansen 2007). However, lack of growth in or on conventional media does not indicate death of the organisms. The border between life and death in bacteria is hard to define. It is probably more of a continuum with different 'levels' of vitality ranging from metabolically active cells that are unable to divide to cells exhibiting extensive degradation of cellular components such as DNA (Davey 2011). These different grades of viability can best be detected by employing a set of different methods aimed at determining the structural and functional integrity of cells (**Table 4-1**).

Table 4-1: Methods for viability determination employed in this study.

Method	*Measured parameter*	*Results and conclusion*
Most Probable Number (MPN)	Growth and reproduction	Culturabe cell numbers were dependent on energy supply in *A. ferrooxidans* cultures (section **4.1.1.1**, p. 157). MPN after exposure to stress often yielded lowest survival rate compared to other methods indicating cells in VBNC state/mainentance metabolism.
Iron oxidation rate	Enzymatic activity	Iron oxidation rates correlated with MPN in a qualitative, but not quantitative manner (i.e. showing the same trend but not the same numbers).
FISH (fluorescence-*in situ*-hybridization)	rRNA integrity and abundance	Certain stress factors (e.g. desiccation) led to a quick degradation of rRNA, while others (e.g. irradiation) yielded cells with a fluorescence signal similar to the control.
PAC (probe active count)	rRNA biosynthesis and cell growth (not reproduction)	Incubation with nalidixic acid followed by FISH could show if stressed cells are able to recover or if rRNA is further degraded such as in dead cells.
Live/Dead®	Membrane integrity	Percentage of membrane-intact cells was often much higher than culturability, i.e. intact, but nonculturable cells were abundant.
qPCR	DNA integrity	DNA damage could be detected after desiccation and ionizing radiation (resolution is fragment-size-dependent)

The results obtained by the different markers of viability often showed a qualitative, but not quantitative correlation as would be expected when considering their differential targets. For example, both reculturability rate and iron oxidation rate of *A. ferrooxidans* biofilms after 48 h of desiccation were higher in anaerobic than in aerobic conditions (**Fig. 3-25**, p. 121), but culturability rate decreased by two log units after anaerobic drying, while iron oxidation rate was lowered by only ~40%. Storing biofilms of *A. ferrooxidans* at -20°C for 1

week also reduced culturability by four orders of magnitude, while iron oxidation rate was still at 50% of the control (**Fig. 3-46**, **Fig. 3-47**, p. 145). This indicates that the fraction of cells in the biofilm actively participating in iron oxidation after the stress exposure might have been higher than the percentage of cells able to reproduce.

In some cases, rRNA integrity determined by FISH (performed directly after the stress exposure) was not highly affected by the stress factor, even though culturability was low (e.g. after irradiation and incubation in 20% salt solutions), while other stress factors (e.g. desiccation and storage at -20°C) led to a degradation of rRNA. In addition, membrane integrity was not affected as much as culturability by desiccation and ionizing radiation. This points out that different stressors affect cells differently, some being inherently damaging to membranes or nucleic acids while others are not (e.g. Billi and Potts 2002; Cadet et al. 2005; von Sonntag 1987). However, subsequent incubation in nalidixic acid (NA) (probe active count) could show if cells were able to recover their fluorescence signal, which often correlated with culturability.

The differing results of these methods may indicate that cells of the chemolithotrophic organism *A. ferrooxidans* can also enter a viable-but-nonculturable state in response to environmental stressors. If this is the case, then cells which were not able to reproduce in the Most Probable Number assay, might be able to recover if suitable conditions for resuscitation were identified. A more detailed discussion of the effects of different stressors on viability will be given in the following subsections.

4.2.2 Desiccation

Mars is a dynamic system. In its history, the planet has undergone a transition from conditions, under which liquid water could at least transiently exist on the surface, to a more and more arid and cold climate (see section **1.2.1.1**, p. 4) (Bibring et al. 2006; Fairén et al. 2010). Even in a putative subsurface aquifer on Mars, where liquid water could exist today due to higher pressures and temperatures, changing environmental conditions may confront organisms with the stress of desiccation. Desiccation is also one of the most significant

environmental stress factors encountered by bacteria on Earth, especially in arid and semi-arid habitats, because desiccation causes significant damage to all cellular components (Potts 1994).

The acidophilic iron-sulfur bacteria *A. ferrooxidans* and *S. thermosulfidooxidans* used in this study were sensitive to short-term desiccation when exposed as planktonically-grown cells to air-drying. This was also found with other strains of acidophiles isolated from various environments (section **3.2.2**, p. 110). Drying *A. ferrooxidans* 24 h in ambient air yielded no reculturable cells. Furthermore, binding of the oligonucleotide probe EUB338 to ribosomal RNA (FISH signal) was greatly diminished, in comparison to the control (**Fig. 3-30**, p. 126), and there was apparently extensive DNA damage that resulted in a decrease of amplification efficiency in the qPCR reaction by 3 orders of magnitude (**Fig. 3-31**, p. 126). In contrast, the amount of cells with an intact membrane as determined by Live/Dead® staining after drying in ambient air remained at about 12% (**Fig. 3-29**, p. 125), indicating a large fraction of cells which had retained their membrane integrity, but were unable to propagate, probably due to the damage to intracellular compounds such as DNA and rRNA. Incubation of air-dried cells in growth medium with nalidixic acid overnight also did not result in a recovery of a strong FISH signal or in elongation of cells, which corresponded to the low culturability rate.

However, several factors were identified that improved desiccation tolerance in *A. ferrooxidans*, including a low oxygen partial pressure, the presence of compatible solutes and growth in biofilms.

4.2.2.1 The role of oxygen and relative humidity in desiccation survival

The most determining factor for a good recovery of *A. ferrooxidans* or *S. thermosulfidooxidans* after desiccation was a low oxygen tension (**Fig. 3-25**, p. 121, **Fig. 3-26**, p. 122, **Fig. 3-27**, p.123). Drying bacteria in anaerobic conditions can alleviate oxidative stress, which is associated with almost any conceivable form of environmental stress (Aldsworth et al. 1999). Reactive oxygen species (ROS) are thought to accumulate during dehydration (Billi and Potts 2002). The accumulation of free radicals, especially in a cell that cannot actively metabolize or transport them out, can result in irreversible damage to proteins and DNA

(Fredrickson et al. 2008). In *D. radiodurans*, the high desiccation and radiation resistance have both been associated with an efficient protection of its proteome (including DNA repair proteins) from ROS by manganese complexes (Daly et al. 2007, 2010; Fredrickson et al. 2008).

Often, enzymes scavenging ROS such as peroxidases, catalases, and superoxide dismutases, are induced in response to desiccation, giving a clear indication that drying causes immense oxidative stress (Cytryn et al. 2007; França et al. 2006; Ramos et al. 2001; van de Mortel and Halverson 2004). *A. ferrooxidans* is endowed with a surprisingly low number of ROS-scavenging enzymes, considering it usually thrives in environments with high iron concentrations. Catalase-coding genes have not been detected in the genome of the type strain at all; however, non-enzymatic ROS protection systems are abundant (Valdés et al. 2008). Still, sensitivity to oxygen during drying was much more apparent for the acidophilic iron-sulfur bacteria than for *D. geothermalis*.

Viability after drying may also correlate with the residual intracellular water content, which is dependent on the extent and speed of the drying procedure and on storage conditions, especially the relative humidity (RH) (García de Castro et al. 2000; Mugnier and Jung 1985; Potts 1994). Low relative humidity was more favorable to long-term stability of dried *D. geothermalis* (**Fig. 3-28**, p. 124), which was also observed for the closely related species *D. radiodurans* (Bauermeister et al. 2011). It was hypothesized that this is related to the threshold of glass formation by intracellular solutes, which is only reached at low intracellular water contents (Bauermeister et al. 2011; Billi and Potts 2002). In *A. ferrooxidans* and *S. thermosulfidooxidans*, a clear difference between desiccation tolerance in low RH and high RH could not be observed. Although reculturability of *A. ferrooxidans* dried within a desiccator at RH <5% was greater than that of cells dried in ambient air (**Fig. 3-25**, p. 121), the differences were not statistically significant due to a high variability between the samples.

4.2.2.2 Influence of compatible solutes

Many bacteria and archaea respond to environmental stressors by the accumulation of compatible solutes including proline, glycerol, glutamine, ectoine, glycine betaine, sucrose, and trehalose (**Fig. 1-10**, p. 29), either by enhanced uptake from the medium or increased biosynthesis (Boch et al. 1996; Csonka 1989; Galinski and Trüper 1994; Imhoff and Valera 1984; Kempf and Bremer 1998; Lamosa et al. 1998; Lai et al. 2000; Madkour et al. 1990; Poolman and Glaasker 1998). Compatible solutes are defined as small, highly soluble organic molecules that do not interfere with enzymatic reactions and can therefore be amassed at high concentrations (Roeßler and Müller 2001; Brown 1976). They stabilize protein structure and increase protein solubility (**Fig. 4-3**), which is also demonstrated by the fact that many compatible solutes confer resistance to different kinds of stress conditions that involve reduced water activity such as high osmolarity, desiccation, high temperatures and freezing (Narváez-Reinaldo et al. 2010; Papageorgiou and Murata 1995; Roeßler and Müller 2001; Welsh 2000).

Accumulation of compatible solutes from the environment is energetically more favorable than *de novo* synthesis and is therefore preferred by many organisms (Roeßler and Müller 2001; Streeter 2003). Chemolithoautotrophic *A. ferrooxidans* possesses a limited set of genes involved in the uptake of organic compounds, especially of carbohydrates (Valdés et al. 2008). Although addition of glycine betaine to the growth medium apparently stimulated growth of *A. ferrooxidans*, it did not confer enhanced resistance to desiccation. The same was true for trehalose and sucrose. If uptake of these compatible solutes occurred in *A. ferrooxidans* under the tested conditions, it did not significantly affect the tolerance of planktonic cells to air-drying. Similar observations were made for *Lactobacillus plantarum* (Kets and de Bont 1994).

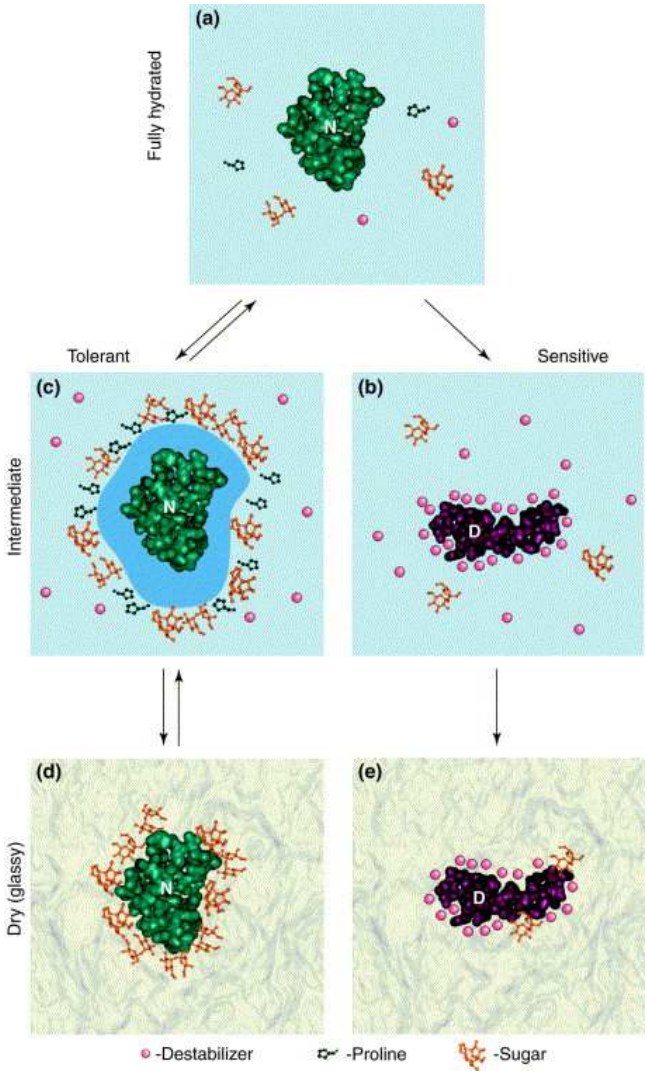

Fig. 4-3: Protein stabilization by compatible solutes (e.g. proline, sugars) by preferential exclusion during different stages of water loss: a) fully hydrated, native protein conformation (N). When the concentration of destabilizing molecules (such as some ions) increases during dehydration, the probability of interactions between destabilizers and proteins increases. In sensitive cells (b) this leads to protein denaturation (D), while in tolerant cells (c) preferential exclusion of compatible solutes causes hydration of the protein surface (blue ring). With further dehydration, when the water shell around the proteins disappears below 0.3 (g H_2O)/(g dry weight), sugar molecules (but not other compatible solutes) can replace water via hydrogen bonding, thus stabilizing the native protein structure in the glassy cytoplasm in tolerant cells (d). In sensitive cells, denaturation of the protein is not reversible (indicated by arrows) (from Hoekstra et al. 2001).

Biosynthesis of compatible solutes can be induced by osmotic stress and low temperatures (Dinnbier et al. 1988; García de Castro et al. 2000; Somero 1992; Sowers and Gunsalus 1995; Welsh and Herbert 1999; Yancey et al. 1982). When *A. ferrooxidans* was incubated with 0.5% (wt/v) NaCl, a concentration which retarded growth, but did not inhibit it completely, desiccation resistance of cells after washing and air-drying was not enhanced. *A. ferrooxidans* has a pronounced potential for biosynthesis of organic compounds including sugars and amino acids. For example, both a trehalose synthase (AFE2083) and a sucrose synthase (AFE1552) gene were identified in the genome of the type strain (Valdés et al. 2008). Apparently, conditions that induced the increased synthesis of these compounds, were not identified yet, but the bacteria should have the potential for production of these compatible solutes.

Trehalose and sucrose were also the most effective solutes, compared to glycine betaine and glycerol, when added as an external protective matrix to *A. ferrooxidans* prior to desiccation. This is probably connected to three important properties of these disaccharides:

i) the ability to form a glassy matrix (vitrification), which is a highly viscous state in which diffusion and harmful molecular reactions are slowed to a minimum,
ii) the ability to depress the phase transition temperature of membranes (which would otherwise transition to a gel phase during dehydration causing leakage or fusion of membranes during rehydration),
iii) the ability to replace water molecules around polar residues of macromolecules, which helps preserve the structure and function of proteins and membrane lipids

(Crowe et al. 1998; Hincha and Hagemann 2004; Leslie et al. 1995; Potts 1994, 2001; Ricker et al. 2003; Rudolph and Crowe 1985).

Trehalose plays an important role in many organisms that are exposed to periodic desiccation, such as soil bacteria (Cytryn et al. 2007; Strøm 1998). Trehalose or related molecules like sucrose have been used successfully to enhance desiccation tolerance of otherwise sensitive organisms such as *E. coli* and *P. putida* (Billi et al. 2000; García de Castro et al. 2000; Israeli et al. 1993;

Louis et al. 1994; Welsh and Herbert 1999). *Bradyrhizobium japonicum* could be protected from desiccation by exogenously-added trehalose, and trehalose-synthesis genes were induced during incubation of this species under low relative humidity (27% RH) (Cytryn et al. 2007; Streeter 2003).

Organic compounds have not yet been identified on the surface of Mars and are likely to be scarce if only abiotic sources are existent (Nixon et al. 2012; ten Kate 2010). However, synthesis of organic substances by autotrophic organisms in a hypothetical subsurface food web (**Fig. 4-1**, p. 154) is conceivable. Methanogens might be such primary producers, delivering organic molecules that could serve as energy source or compatible solutes for other species. Many iron-sulfur bacteria are autotrophs as well, such as *A. ferrooxidans*, and are perhaps able to produce their own compatible solutes under the right conditions.

4.2.2.3 Growth in biofilms may offer protection from desiccation

Growth in biofilms could be another adaptive mechanism employed by putative Martian organisms that may aid in their protection from the extreme physical conditions they might encounter on Mars. Biofilms are assumed to be the dominant form of microbial life in natural environments on Earth. Biofilms are bacterial communities embedded in a self-produced matrix of extracellular polymeric substances (EPS) and often attached to surfaces (Davey and O'Toole 2000; Stoodley et al. 2002). The EPS matrix is a complex network of exopolysaccharides, proteins, lipids, and extracellular DNA with a variety of functions (see section **1.4.4**, p. 33) (Flemming and Wingender 2001, 2010; Flemming 2011; Sutherland 2001). One of these functions is the protection of the embedded cells from adverse environmental conditions such as desiccation (Flemming and Wingender 2010).

Many bacteria respond to desiccation by producing EPS (Ophir and Gutnick 1994; Or et al. 2007; Roberson and Firestone 1992; Tamaru et al. 2005; van de Mortel and Halverson 2004). Their function in desiccation tolerance is probably related to their water-retention capacity. The molecules of the EPS matrix, especially polysaccharides, create a hydrated microenvironment and retard dehydration, enabling cells to respond to slowly decreasing water activity with physiological adaptations. Slow rehydration of EPS during rapid rewetting may

also help protect the cells from osmotic stress and damage to membranes caused by phase transitions of lipids (Chenu and Roberson 1996; Flemming and Wingender 2010; Halverson et al. 2000; Hill et al. 1997; Ophir and Gutnick 1994; Or et al. 2007; Shaw et al. 2003). EPS in a porous matrix such as soil provide a continuum between the bacteria and the soil particles and increase the water-retention properties of the soil (Chenu 1995; Chenu and Roberson 1996; Czarnes et al. 2000; Roberson and Firestone 1992). When subjected to desiccation, the hydrated EPS network shrinks and outermost layers may form a skin that retards desiccation of the inner layers with embedded bacteria, and reduces diffusion rates (e.g. of oxygen) (Or et al. 2007).

Model biofilms of acidophilic iron-sulfur bacteria in this study were grown on membrane filters floating on the surface of liquid medium containing soluble ferrous iron. This method was chosen, despite certain limitations, in order to obtain reproducible biofilms, for which manipulation (i.e. exposure to stress conditions) and quantitative analysis of viability would be facilitated.

When biofilms of *A. ferrooxidans* were exposed to desiccation under anaerobic conditions, they exhibited a significantly increased tolerance compared to planktonic cells dried in the same manner, although the differences were not as pronounced when desiccation took place in aerobic atmosphere (**Fig. 3-23**, p. 117). Extensive production of EPS in *A. ferrooxidans* biofilms grown under these conditions could not be observed by staining with the fluorescently-labeled lectin Concavalin A (ConA), in contrast to biofilms of *A. ferrooxidans* grown on the surface of minerals such as pyrite (Devasia et al. 1993; Gehrke et al. 1998; Harneit et al. 2006). The reason for this could be either that the amount of exopolysaccharides produced was too low, or that the EPS differed in composition, containing polysaccharides with different sugar residues that did not bind this specific lectin. It was shown that planktonic cells of *A. ferrooxidans* grown either on soluble or solid substrates produce generally less EPS than sessile cells (Devasia et al. 1993; Gehrke et al. 1998; Harneit et al. 2006). The cells in the model biofilms of *A. ferrooxidans* on filter membranes were also sessile, but their substrate of growth was soluble ferrous iron, which might explain the low amount of EPS observable by both ConA-staining and visual examination. Since extensive EPS production did not seem to be the key for

increased desiccation tolerance of biofilms of *A. ferrooxidans*, it is hypothesized that differences in growth conditions might have altered cell physiology compared to planktonic cells, which can greatly affect stress resistance (e.g. Wassmann et al. 2011).

On the other hand, biofilms of the reference organism *Deinococcus geothermalis* were shown to contain EPS by staining with the lectin WGA (Wheat Germ Agglutinin). When drying biofilms of *D. geothermalis* and enumerating culturable cells and membrane-intact cells, the viability of biofilm cells was clearly less affected than that of planktonic cells (**Fig. 3-24**, p. 118). Although untreated biofilms initially contained a lower percentage of culturable and membrane-intact cells than planktonic cultures, desiccation (3 weeks) did not significantly decrease these values in biofilms, while it did so for planktonic cells. The percentage of membrane-intact cells was 2 to 2.5 times higher than the percentage of culturable cells. The same discrepancy (also by a factor of about 2.8) was observed in *B. japonicum* after desiccation (Cytryn et al. 2007). This might indicate the presence of VBNC cells, which were not able to reproduce, but had retained membrane integrity. This data seems to corroborate the hypothesis that growth in biofilms confer a certain protection from desiccation.

4.2.2.4 Conclusion

A. ferrooxidans was sensitive to air-drying, but storage of dried cells could be prolonged if they were kept under low oxygen tension, grown as a biofilm, or embedded within an external matrix of compatible solutes such as sucrose and trehalose. Even under these conditions, however, the time in which culturable cells can be recovered was limited to a few days or weeks at maximum. Compared to *D. geothermalis*, which could be stored for >1 year in the dried state without addition of an external protective substance, *A. ferrooxidans* cannot be considered an anhydrobiotic organism. *S. thermosulfidooxidans* generally exhibited similar sensitivity to drying as *A. ferrooxidans*, although the ability to form endospores should provide this species with an adaptive advantage. It has been reported that spore-forming *Sulfobacillus* strains were the only bacteria that could be revived after 4 years of storage of dehydrated heap material (Keeling et al. 2006). However, the type strain investigated here exhibited only low

sporulation efficiency under laboratory cultivation conditions so that loss of reculturability after drying might have indicated the absence of such dormant forms in the samples. It is also possible that the resuscitation medium lacked certain essential nutrients that induce spore germination in this species (Moir 2006). The characteristics of *Sulfobacillus* endospores as well as germination conditions have not yet been extensively studied. The data obtained in this work demonstrates that spores of *S. thermosulfidooxidans* contain dipicolinic acid (DPA), which is also common in spores of other bacteria such as *Bacillus* sp. and stabilizes the water-requiring structures during dehydration of the spore core (Nicholson et al. 2000). DPA could be used as a spore-specific marker to check for *Sulfobacillus* spores in environmental samples.

The extent and duration of available liquid water on Mars are still a matter of investigation. Present Martian surface conditions are considered hyperarid due to the low atmospheric pressure. However, several possible sources for liquid water in the shallow or deep subsurface of Mars have been proposed (see section **1.2.2.2**, p. 11). For example, water in porous media such as a soil matrix or in ice may be liquid down to temperatures of -40°C, possibly due to capillary-pore effects and interfacial forces such as van der Waals interactions (Maruyama et al. 1992; Möhlmann 2010a, 2010b; Oyarzun et al. 2003). However, it is currently not known whether the thickness of the interfacial water layer would be sufficient to allow transport processes necessary for life (Möhlmann 2009). Furthermore, the presence of salts will lower both the freezing point of the liquid water as well as the triple point pressure, possibly allowing liquid water to exist at higher altitudes and lower temperatures on Mars (Landis 2001; Stoker et al. 2010; Wynn-Williams et al. 2001). This means that a potential organism living in the liquid water phase made available by such processes, must also be psychrophilic and able to tolerate high salt concentrations (see following section).

4.2.3 Salt stress

Salt deposits have been detected on the surface of Mars in many regions by the Mars rovers and landers (Viking, Spirit and Opportunity, Phoenix) and by orbital spectroscopy (e.g. OMEGA on Mars Express). The Martian soils are especially

rich in sulfur, present in the form of sulfate salts (Mg, Ca, and Fe), and contain a lower amount of chlorine compounds such as chlorides and perchlorates (Mg, Na, or Ca) (S:Cl ratio 4:1) (Clark 1993; Clark et al. 2005; Clark and van Hart 1981). An 'average Martian brine' (Jones et al. 2011) may contain magnesium, iron, and calcium sulfates at 0.4 to 8% (wt/v). Especially magnesium sulfate constitutes a substantial component of Martian soil (about 5%), and may reach up to 30% in some locations (Banin et al. 1997; Bibring et al. 2005; Clark and van Hart 1981; Gendrin et al. 2005; Jagoutz 2006; Marion et al. 2009, 2010; Tosca et al. 2008; Yen et al. 2005). If these deposits dissolve in melting ice (e.g. in permafrost regions), the resulting brines would contain salt concentrations of 25 to 40% (wt/v) (Crisler et al. 2012).

A. ferrooxidans did not exhibit halotolerance as growth was inhibited by NaCl concentrations of >0.5% (wt/v) (this was similar for the other tested chloride salts $CaCl_2$ and $MgCl_2$) (**Fig. 3-32**, p. 128). Incubation (24 h) in 20% NaCl also resulted in a complete loss of culturability. In contrast, rRNA integrity seemed to be little affected in the salt-stressed cells compared to the control (as indicated by the FISH signal). After incubation in growth medium containing nalidixic acid, the fluorescence signal of the FISH probe still remained high, but elongation did not occur (**Fig. 3-34**, p. 130).

The experiments in this study also showed that *A. ferrooxidans* may be considered an epsotolerant bacterium. Epsotolerance (from epsomite: $MgSO_4x7H_2O$) has been used as a term for organisms capable of growth in high $MgSO_4$ concentrations (Crisler et al. 2012). In contrast to halotolerance, epsotolerance has not received much attention to date because few natural ecosystems on Earth are high in magnesium sulfate (exceptions include some hypersaline lakes and ponds saturated in epsomite, e.g. Hot Lake and Basque Lake in the United States; Crisler et al. 2010). This would be different on Mars, where sulfates constitute a large fraction of salts in the regolith. *A. ferrooxidans* was able to grow in $MgSO_4$ concentrations of 10% (but not at 20%, where a rapid decrease in total cell and culturable cell number was observed) (**Fig. 3-33**, p. 129). A similar observation was reported for certain sulfate-reducing bacteria (Marnocha et al. 2011), while some aerobic heterotrophic isolates (mostly *Halomonas* and *Bacillus* spp.) could grow even at 60-70% $MgSO_4$ (Crisler et al.

2012). The frequently noticed higher potential of heterotrophs for growth at high salt concentrations might be explained by the energy expenditure required for osmoregulation, which is a particular challenge for life forms with low energy-yielding metabolisms (such as chemolithotrophs) (Crisler et al. 2012; Oren 1999).

Recently, the Phoenix lander discovered concentrations of 0.4 to 0.6% (wt/v) of perchlorates in the Martian regolith at its landing site (Hecht et al. 2009). Perchlorate is potentially toxic to many microorganisms, and *A. ferrooxidans* did not grow in the presence of perchlorate concentrations of ≥0.5% (**Fig. 3-32**, p. 128). But some species of Proteobacteria (predominantly members of *Dechloromonas* and *Azospira*) can tolerate ClO_4^- concentrations as high as those found in Martian or Antarctic soils and can even use it as an electron acceptor to oxidize a variety of organic or inorganic electron donors including hydrogen and ferrous iron (reviewed by Coates and Achenbach 2004). Fe^{2+}-oxidizing/perchlorate-reducing bacteria have been suggested as plausible candidates for potential Martian life forms based on their metabolism (Stoker et al. 2010).

The differential tolerance to chlorides, perchlorates, and sulfate salts that was observed for *A. ferrooxidans* is probably related to the chaotropic or kosmotropic properties of the salts. Chaotropic salts like $MgCl_2$, $CaCl_2$, and ClO_4^- disrupt hydrogen bonds between water molecules, reducing hydrophilic interactions, which can lead to the denaturation of macromolecules (Chaplin 2012). However, these chaotropic properties may counteract the effects of low temperatures on cellular biomolecules and membranes (Chin et al. 2010; Collins 1997; Feller and Gerday 2003; Hamaguchi and Geiduschek 1962). Thus, chaotropic salts in the Martian regolith may not only serve as freezing point depressants, but may also favour microbial growth at low temperatures (Chin et al. 2010). Kosmotropic substances, on the other hand, especially salts that consist of a mixture of a kosmotropic anion (e.g. SO_4^{2-}) with a chaotropic cation (e.g. Mg^{2+}), have a stabilizing effect on proteins and nucleic acids (Chaplin 2012; Marcus 2009). For this reason, sulfate-dominated systems exhibit higher water activities than chloride-systems (at the same concentrations) (Grant 2004; Hallsworth et al. 2003). However, the behavior of salts as chaotropes or kosmotropes may be concentration-dependent. For example, it was shown that $MgSO_4$ at

concentrations up to 1 M (12%) behaved as a kosmotropic substance, while at higher concentrations (e.g. 20%) it became increasingly chaotropic (Crisler et al. 2012). This could explain the observed lethal effect of $MgSO_4$ concentrations >10% on *A. ferrooxidans*.

In conclusion, the low water activity in highly concentrated Martian brines was identified as one of the most challenging environmental stress factors potential Martian life forms would encounter (Tosca et al. 2008). The tested strain of *A. ferrooxidans* would not be able to grow or even survive in a salt-saturated environment, although magnesium sulfate concentrations of up to 10% can be tolerated. It remains to be investigated whether other chemolithotrophic life forms, particularly those with metabolic capabilities relevant for Mars, can grow at salt concentrations approaching those in potential Martian brines.

4.2.4 Radiation

Microorganisms from a subsurface ecosystem on Mars may be transported to the surface by geological processes such as sudden releases of liquid from the underground resulting in gullies and outflow channels, or by impact cratering (Malin et al. 2006). Apart from highly desiccating conditions, they would then be exposed to a high radiation flux consisting of UV radiation and ionizing radiation from space. UV radiation is a ubiquitous environmental stress parameter in terrestrial habitats that are unshielded from solar radiation. On Mars, UV-C radiation (200-280 nm) is not blocked by an ozone layer and therefore reaches the surfaces without attenuation (Cockell and Horneck 2001; Kuhn and Atreya 1979).

A. ferrooxidans was shown to be a UV-sensitive organism compared to *D. geothermalis* (**Fig. 3-35**, p. 131). A population of the type strain of *A. ferrooxidans* was inactivated by fluences of UV-C radiation of >100 J/m^2, while *D. geothermalis* populations retained nearly 100% viability up to 400 J/m^2. Similar resistance levels were reported for another strain of *A. ferrooxidans* (ATCC 33020) and for *D. radiodurans*, respectively (Bauermeister et al. 2009; Liu et al. 2000).

The high current flux of UV radiation (589.2 W/m^2) on the Martian surface would inactivate all known terrestrial life forms within seconds of exposure, including such radiation-resistant organisms as *Deinococcus* sp. and spores of *B. subtilis*, rendering the surface of Mars more or less sterile (Pogoda de la Vega et al. 2007; Schuerger et al. 2003). However, even a shallow layer of dust is sufficient as a shielding against this type of radiation (**Fig. 3-36**, p. 134), especially if the dust particles contain ferric iron minerals as they would on Mars. Fe^{3+} absorbs strongly in the range of 220-270 nm and was proposed as an effective UV-screen for the first microbial mats on Archaean Earth (Gómez et al. 2007; Pierson et al. 1993). *A. ferrooxidans* subjected to UV-C radiation in its growth medium containing soluble Fe^{3+} (produced by bacterial metabolism) could be subjected to much higher fluences (≥1000 J/m^2) without notable inactivation, compared to cells exposed in non-absorbing medium (**Fig. 3-35**, p. 131). If the formation of Fe^{3+}-minerals like goethite and jarosite during the early history of Mars took place in an acidic aqueous environment similar to Rio Tinto on Earth, it is conceivable that soluble Fe^{3+} in the water could have been used by potential early acidophilic life forms as a natural UV-screen (Amils et al. 2007; Gómez et al. 2007).

Protection from UV radiation could also be conferred by upper cell layers in biofilms of *A. ferrooxidans* (**Fig. 3-35**, p. 131), an effect, which may be enhanced in the presence of EPS. For example, alginates in the EPS of *Pseudomonas aeruginosa* biofilms were shown to have UV-absorptive properties (Elasri and Miller 1999).

In contrast to UV radiation, which affects only the surface (and indirectly, by photochemically-produced ROS, the uppermost layers of regolith (Johnson et al. 2011), ionizing radiation is a factor that needs to be considered also for subsurface life. Ionizing radiation has two sources; it is generated by radioactive decay in the planetary crust, or it can reach the planetary surface from space (cosmic radiation and solar energetic particles) (Dartnell 2011). The dose rate from radioactive decay is lower on Mars than on Earth because of a lower abundance of potassium, thorium, and uranium, and amounts to about 130 µGy/a (Kminek and Bada 2006). On the other hand, the dose rate of galactic cosmic rays on Mars is higher than on Earth because Mars lacks a global magnetic field and the thin atmosphere provides only little shielding (Acuña et al. 1998; Drake et

al. 1988). Depending on the depth in the regolith, galactic cosmic radiation dose ranges from 200 mGy/a at the surface to 0.6 mGy/a at 3 m depth (Kminek and Bada 2006). Comparing this with the average radiation dose that could be tolerated by *A. ferrooxidans* (in which 45 Gy reduced the culturable cell number by 90%;**Table 3-7**, p. 136), a population of these bacteria could remain viable in the shallow subsurface (0-3 m) of Mars for 1000 to 100,000 years (estimation based only on damage by ionizing radiation). If bacteria were subjected to intermittent periods with liquid water availability and conditions that would allow at least maintenance metabolism, they could repair the accumulated damage caused by radiation and desiccation and extend their survival time dramatically. However, without maintenance metabolism, even the most radiation-resistant bacteria (e.g. *D. geothermalis*) or dormant forms (e.g. spores) in the near subsurface (upper meter of regolith) would be sterilized by ionizing radiation within a time span on the order of 10^4 – 10^6 years (Dartnell et al. 2007a, 2007b, 2010; Kminek et al. 2003; Moeller et al. 2010; Pavlov et al. 2002). For life in the deep subsurface, where galactic cosmic rays no longer penetrate, these periods would be extended considerably as the ionizing radiation dose caused by radioactive decay is very low (Dartnell 2011).

Ionizing radiation caused a certain amount of damage to cell membranes, which was, however, not dose-dependent in *A. ferrooxidans*, as both irradiation with 300 and 1000 Gy led to a decrease of membrane-intact cells by ~60% (**Fig. 3-39**, p. 138). It also resulted in DNA degradation (likely caused by the formation of double strand breaks, DSBs) as shown by the reduced amplification efficiencies of DNA from irradiated cells in qPCR reactions. However, the length of the amplified DNA fragment limited the capacity of the method to differentially quantify DNA damage at different doses.

A. ferrooxidans has been shown to be desiccation- and radiation-sensitive, while *D. geothermalis* is extremely resistant to both of these stress factors, suggesting a common basis in the adaptive mechanisms to desiccation and radiation. Desiccation and ionizing radiation cause similar damage profiles in cells, among them DNA DSBs and oxidative damage to macromolecules, suggesting that a combination of pathways involved in DNA repair and protection from oxidative stress may play a crucial role in the resistance to both stressors (Blasius et al.

2008; Daly 2009; Fredrickson et al. 2008; Mattimore and Battista 1996; Potts 1994). The resistance of *D. radiodurans* to high ionizing radiation doses uncommon for any habitat on Earth was suggested to be the result of an adaptation to periodic desiccation, to which this soil bacterium is subjected (Mattimore and Battista 1996), although this line of reasoning cannot be applied to all extremely resistant microorganisms. *D. geothermalis*, for example, is not a typical soil bacterium, usually isolated from geothermal springs and other aquatic habitats (Ferreira et al. 1997; Makarova et al. 2007). Thus, a high desiccation resistance would not be expected to evolve in this species. Likewise, many hyperthermophilic bacteria and archaea from deep sea hydrothermal vents, which never encounter desiccation stress, were found to be both highly radiation- and desiccation-resistant (Beblo et al. 2009, 2011).

Protection of proteins from oxidative damage has been proposed as a key to bacterial radiation- and desiccation-resistance because the retention of functional enzymes is a prerequisite for DNA repair (Daly et al. 2007; Daly 2009, Fredrickson et al. 2008). *D. radiodurans* and other radiation-resistant bacteria are characterized by a high intracellular Mn:Fe ratio compared to sensitive bacteria (Daly et al. 2004). High concentrations of iron can be detrimental in the presence of radiation- or desiccation-induced reactive oxygen species (ROS) because both Fe^{2+} and Fe^{3+} can react with these species creating a cascade of secondary ROS (Daly 2009). As mentioned above, *A. ferrooxidans* type strain contains a relatively limited set of enzymes that are involved in ROS-scavenging. Most notably, a catalase-encoding gene is apparently absent (Valdés et al. 2008). Catalase decomposes H_2O_2 (e.g. generated by radiolysis of water), which can otherwise react with ferrous iron to form the extremely reactive hydroxyl radicals, OH° (Fenton-reaction, equation 19) (Imlay 2008). OH° indiscriminately oxidizes nucleic acids, lipids, and proteins (Daly et al. 2007; von Sonntag 1987).

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH° \qquad (19)$$

Thus, high iron concentrations in its natural surroundings and lack of catalase activity may contribute to the high sensitivity of *A. ferrooxidans* type strain to

desiccation and radiation. This also explains the beneficial effect of low oxygen concentration on the desiccation survival of *A. ferrooxidans* (section **4.2.2.1**, p. 173), which would be an advantage of the Martian environment.

In conclusion, organisms exposed to the radiation flux on the surface of Mars would be inactivated within a very short time span without protection. However, in sufficient depths, organisms would be protected from radiation and could plausibly persist over prolonged periods of time (10^5 years even in the case of radiation-sensitive organisms like *A. ferrooxidans*) in a dormant state. However, intermittent phases of conditions conducive to metabolism must occur in order to repair accumulated damage. Such conditions would include the availability of liquid water and temperatures high enough for enzymatic reactions to take place at appropriate rates.

4.2.5 Low temperatures

Average temperatures on Mars are extremely low compared to Earth both at the surface (depending on latitude and season) and in the shallow subsurface (Clark 1998; Horneck 2000; Jones et al. 2011). With increasing depth in the subsurface, the extreme diurnal and annual temperature fluctuations (diurnally on the scale of ~100°C; Ulrich et al. 2010) are diminished, and mean temperatures are expected to rise continuously due to geothermal heating (e.g. from radioactive decay), which would also increase the probability of regions where liquid water could exist (deep aquifers) (Jones et al. 2011). However, temperatures do not have to exceed the freezing point of pure water for active metabolism to occur provided that liquid water is available, e.g. as brine veins or pockets within ice and permafrost soils (e.g Gilichinsky et al. 2003, 2005; Junge et al. 2004; Rivkina et al. 2000).

4.2.5.1 Influence of compatible solutes on freezing tolerance of A. ferrooxidans

If organisms in a putative subsurface environment on Mars were exposed to temperature fluctuations, they would have to endure periods of freezing without sustaining extensive damage. A common strategy among terrestrial organisms to

cope with the stress of freezing is the production of compatible solutes, which also act as osmo- and xeroprotectants (see section **4.2.2.2**, p. 175). These hydrophilic molecules retard the formation of intracellular ice crystals and reduce the potential for osmotic injury during the freezing and thawing process (Cleland et al. 2004; Hubalek 2003; Ko et al. 1994). Thus, they are also used for the preservation of frozen or freeze-dried prokaryotic stock cultures. *A. ferrooxidans* has generally been considered challenging to preserve by common freezing and freeze-drying methods, and is therefore primarily maintained as actively growing stock cultures in culture collections such as the DSMZ (German collection of microorganisms and cell cultures). An early study showed that freeze-drying of *A. ferrooxidans* inactivated initial populations by 3 log units, even when suspended in sucrose (Wakao et al. 1990). More recently, *A. ferrooxidans* was preserved by a vacuum-drying procedure with glycine betaine added as a protective substance (Malik 1990; Cleland et al. 2004).

In this work it was shown that simple freezing at -80°C with sucrose, trehalose, glycine betaine, or glycerol is effective at preserving the majority of viable cells of *A. ferrooxidans* for up to 1 year (**Fig. 3-43**, p. 142), without the requirement for high initial cell concentrations (>10^8 cells/ml). Even when no cryoprotective agent was added (only basal salt medium), culturability was reduced by 4 to 5 log units, but did not vanish completely after one year of storage.

When stored at -20°C, survival of *A. ferrooxidans* was considerably reduced and the effectiveness of the different compatible solutes varied much more than at -80°C. Glycine betaine was most effective at protecting cells at -20°C, while glycerol was least effective. The protective effect of sucrose and trehalose at this temperature was initially high, but decreased after 3 months of storage. In contrast, trehalose and sucrose had worked best during desiccation of *A. ferrooxidans* (section **4.2.2.2**, p. 175). Thus, the type of compatible solutes with the highest protective effect is dependent on the organism and the stress factor in question.

Alternatively, the EPS matrix of biofilms may serve as an exogenous protective matrix to bacterial cells during freezing, which has been demonstrated, for example, for the cyanobacteria *Chroococcidiopsis* sp. and *N. commune* (Knowles and Castenholz 2008; Tamaru et al. 2005). In this study, biofilms of *A.*

ferrooxidans also exhibited a higher survival based on culturability after freezing at -20°C and -80°C than planktonic cells frozen without a cryoprotectant (**Fig. 3-46**, p. 145). Although extensive EPS production was not detected by lectin-staining (with ConA, see section **4.2.2.3**, p. 178), this does not preclude the presence of EPS in the *A. ferrooxidans* biofilms, which may be indicated by their higher resistance to desiccation as well as freezing.

The FISH signal of cells stored for 1 week at -20°C without a cryoprotective agent was very low compared to untreated cells (**Fig. 3-45**, p. 144). Apparently, low temperatures of -20°C did not preserve rRNA very well, or did not prevent molecular motion enough to inhibit RNAse activity inside the cell. After 1 week at -80°C, the FISH signal was only slightly reduced compared to the control and gained in intensity after incubation in growth medium with NA, although culturability was reduced by 3 orders of magnitude. This indicates a good preservation of rRNA at -80°C despite an adverse effect of this temperature on reproducibility of the cells.

Viability of *A. ferrooxidans* was more affected at -20°C than at -80°C possibly because -20°C were not sufficiently low for the formation of a protective glassy matrix within and outside the cell, which, aided by compatible solutes, would stabilize frozen (or dried) biomolecules over long periods of time (Fuller 2004; Potts 1994). Another reason could be related to the rate of freezing, which determines the propagation of intracellular ice crystals that damage cell membranes, although the ideal freezing rate seems to depend on the organism (Fuller 2004; Morgan et al. 2006; Uzunova-Doneva and Donev 2000).

4.2.5.2 Reproduction versus maintenance metabolism at low temperatures

At low temperatures, reproduction of microorganisms may be put on hold, while respiration and low levels of metabolic activity continue (e.g. Amato and Christner 2009; Bakermans et al. 2011; Murray et al. 2012; Price and Sowers 2004; Wilhelm et al. 2012). To determine the threshold at which the transition from growth (i.e. increase in cell number) to maintenance metabolism occurs in *A. ferrooxidans*, cultures were incubated at different temperatures below the optimum growth temperature (which is 28-30°C).

Growth was clearly observed at 10°C by an increase in culturable and total cell number, and by iron oxidation, although at reduced rates compared to more optimal growth temperatures (20-30°C) (**Fig. 3-48**, p. 146). In contrast, in a recent paper, iron oxidation by the type strain of *A. ferrooxidans* did not take place at 10°C (Hallberg et al. 2010). This may be another example for the propensity of bacterial strains to diverge during cultivation in different laboratories (see also section **4.1.1**, p. 156).

At 4°C, growth of *A. ferrooxidans* did not occur at a measurable rate, but viable cell numbers remained as high as total cell numbers (**Fig. 3-48**, p. 146), which may be an indicator for continued energy supply and usage in *A. ferrooxidans* (see section **4.1.1.1**, p. 157). In addition, iron oxidation in bacterial cultures was greater than that observed for abiotic cultures at the same temperature. However, iron oxidation kinetics were shifted from an exponential to a linear relationship, which indicates an uncoupling of growth and iron oxidation that has often been observed under conditions of extreme stress (Kelly and Jones 1978; Leduc and Ferroni 1994; Leduc et al. 1993; Roy and Mishra 1981). The slow, linear iron oxidation rate suggests that cells were active to a small but measurable degree at 4°C, demonstrating the need for maintenance metabolism independent of cell division (see section **4.2.1**, p. 169).

Other strains of acidophilic iron oxidizers that are capable of exponential growth at low temperatures (4°C, or even 2°C) have been identified, for example psychrotolerant *Acidithiobacillus ferrivorans*, isolated from a subterranean pyrite mine, but no subzero temperatures have been tested (Hallberg et al. 2010; Kimura et al. 2011; Leduc et al. 1993).

4.2.5.3 Conclusion

The cold environment of Mars may be beneficial for the preservation of dormant life as freezing tolerance is common in microorganisms and may be aided by the production of intracellular cryoprotective substances and an extracellular polymeric matrix (Hubalek 2003; Knowles and Castenholz 2008; Tamaru et al. 2005). Metabolism at subfreezing temperatures, as it has been shown for terrestrial life forms, is also conceivable, if supercooled liquid water exists on Mars (Bakermans et al. 2003, 2011; Breeze et al. 2004; Carpenter et al. 2000;

Christner 2002; Deming 2002; Gilichinsky et al. 2005; Jakosky et al. 2003; Junge et al. 2006; Miteva et al. 2007; Möhlmann 2010a, 2010b; Rivkina et al. 2000, 2002). With sufficient depth, the rising pressure and temperature in the subsurface of Mars may create habitable conditions for life with higher temperatures and liquid water (Jones et al. 2011). In the past of Mars (Noachian epoch, >3.7 Ga ago), during the time of formation of many of the water-related sediments, microbial activity could even have been possible at the surface or in the near surface regolith. Furthermore, oscillations in planetary obliquity over the history of Mars may have allowed surface temperatures to exceed 0°C, as predicted for the Phoenix landing site 5 to 10 million years ago for up to 100 days per year (Haberle et al. 2003; Richardson and Michna 2005; Stoker et al. 2010;).

4.2.6 Simulated Martian conditions in the shallow subsurface

After assessing the resistance capabilities of *A. ferrooxidans* to single stress factors, the bacteria were exposed to a combination of low pressure (6 hPa), low temperature (-20°C) or diurnal temperature fluctuations (-20°C to +20°C) under a Martian atmospheric gas mixture (95.25% CO_2, 2.69% N_2, 1.64% Ar, 0.15% O_2). These conditions can be expected in the shallow Martian subsurface, where organisms might be transported from the deeper subsurface environments they were assumed to inhabit in the growth experiments (section **4.1**, p. 156). UV and ionizing radiation were not included in the simulation because it was already demonstrated in various studies that the high UV flux at the Martian surface would inactivate even the most resistant microorganisms within seconds. But it was also shown that it can be shielded effectively by shallow layers of regolith, while ionizing radiation doses were too low to be of relevance to a short-term experiment (section **4.2.4**, p. 184).

Since 1958 multiple Mars simulation studies have been conducted to explore the response of terrestrial prokaryotes and organic molecules to simulated Martian conditions. The majority of investigations focused on determining survival of microorganisms after exposure to Martian surface conditions similar to the ones applied here (**Table 4-2**, p. 193). In most of these studies, UV radiation and

desiccation were identified as the most detrimental factors for survival of terrestrial organisms.

Table 4-2: Some of the more recent Mars simulation studies and their results. Older studies (starting in 1958) can be found in Hansen (2007).

Organisms	*Conditions*	*Results*	*Reference*
Chroococcidiopsis sp. (endolithic cyanobacterium)	8.5 hPa, -10°C, CO_2 atmosphere, UV radiation	No survival after 30 min, but protection by 1 mm of soil or rock.	Cockell et al. 2005
A. ferrooxidans (isolate from Rio Tinto)	7 hPa, 150 K, Mars gas mixture, UV radiation	Planktonic cells spread between two layers of Mars regolith pellets survived 10 h under 2-5 mm covering.	Gómez et al. 2010
Bacterial permafrost community (freeze-dried soil cores)	8 hPa, varying temperature, Mars gas mixture, UV radiation	Synergistic effects by UV radiation, UV-generated ROS and freeze-thaw cycles on viability of organisms and stability of biomolecules. Efficient shielding by 2 cm of soil.	Hansen et al. 2009
A variety of cold- and desiccation-tolerant organisms	10-22 hPa, -40 to +24°C, Mars gas mixture, UV radiation	Stresses of desiccation and oxidation were most detrimental to viability of organisms embedded in Mars regolith, while UV radiation and temperature variations had minimal effect.	Johnson et al. 2011
Methanogenic archaea from permafrost	6 hPa, -75 to +20°C, Mars gas mixture	90% of permafrost isolates survived 22 d exposure. UV radiation was not tested.	Morozova et al. 2007

continued

Table 4-2: Some of the more recent Mars simulation studies and their results. Older studies (starting in 1958) can be found in Hansen (2007) (continued).

Organisms	*Conditions*	*Results*	*Reference*
D. radiodurans	7 hPa, diurnal temperature profile, Mars gas mixture, UV radiation	UV radiation most deleterious, but variations in temperature and humidity also severely affected survival. Mixing with hematite provided UV-protection.	Pogoda de la Vega et al. 2007
Bacillus subtilis endospores	8.5 hPa, -80°C to +23°C, CO_2 or Mars atmosphere, UV radiation	No recovery of viable spores after 15 min of UV exposure.	Schuerger et al. 2003
Psychrobacter cryohalolentis (from permafrost cryopeg)	7.1 hPa, -12.5°C, Mars gas mixture, UV-C radiation	No viable cells after 8 h of exposure. This time increased with the level of UV attenuation.	Smith et al. 2009a

The organisms exposed in the majority of older studies (see report by Hansen 2007) were heterotrophs, chosen in order to evaluate the ability of terrestrial organisms to survive or propagate on the surface of Mars, if they are transported there via space craft (‘Planetary Protection’). However, due to their organic substrate-dependent metabolism they would not be considered the most likely organisms to be encountered on Mars. Studies with organisms that exhibit metabolic capabilities relevant to Mars (such as chemolithotrophs), are still comparatively rare and have focused on methanogenic archaea as one of the most probably models for Mars (Morozova et al. 2007; Kral et al. 2011).

In this study, two strains of acidophilic chemolithotrophic iron-sulfur bacteria were selected due to their Mars-relevant metabolic capabilities. *A. ferrooxidans* and *S. thermosulfidooxidans* were exposed to Martian near-surface conditions as biofilms, which had been well characterized regarding their stress tolerance in the previous experiments and were shown to tolerate desiccation and subfreezing temperatures for several days. Biofilms have seldom been used in Mars

simulation studies, although they represent the dominant form of microbial life on Earth (Stoodley et al. 2002).

Biofilms of *A. ferrooxidans* and *S. thermosulfidooxidans* survived one week of exposure to Martian atmosphere and pressure at a constant temperature of -20°C, and in the case of *A. ferrooxidans*, also when temperature cycles (-20°C to +20°C) were applied (**Fig. 3-49**, p. 149; **Fig. 3-50**, p. 150). The percentage of cells in biofilms of *A. ferrooxidans* which could be recultured after exposure to Martian conditions (at -20°C) was similar to biofilms stored under anaerobic conditions at Earth-normal pressure (laboratory control). Temperature cycles reduced culturability by one order of magnitude, but not as much as did storage at constant -20°C under aerobic conditions. This demonstrated once more the beneficial effect of low oxygen partial pressure on survival of *A. ferrooxidans*.

Biofilms of *S. thermosulfidooxidans* did not exhibit reduced rates of iron oxidation after exposure to Mars atmosphere and pressure, relative to the unexposed wet control, while both laboratory controls (stored either in anaerobic atmosphere or at -20°C) were affected in viability (**Fig. 3-50**, p. 150). Thus, a combination of low pressure, low temperature, and absence of oxygen had a beneficial effect on survival of *S. thermosulfidooxidans*.

This study demonstrated the survival capacity of *A. ferrooxidans* and *S. thermosulfidooxidans* when exposed to Martian near-surface conditions over a period of several days. Only one other group (Gómez et al. 2010) has used these kinds of organisms in a Mars simulation experiment; however, examining only very short exposure times (up to 10 h). In contrast to this study, they also included UV radiation and could show that planktonic cells of *A. ferrooxidans* survived short-term exposure to Martian atmospheric conditions with UV radiation if protected by 2 or 5 mm of Mars regolith simulant (Gómez et al. 2010).

4.3 Conclusion

It has been shown in this study that the acidophilic iron-sulfur bacterium *A. ferrooxidans* can be considered a probable member of a potential Martian food web based on its metabolic capacities. A habitat for such iron-sulfur bacteria could plausibly exist in the shallow to deep subsurface of Mars, for example in regions where water is generated by geothermal heating of subsurface ice deposits. The interaction of water with rocks would lead to the weathering of iron-bearing minerals releasing Fe^{2+} and Fe^{3+} that can be used by the organisms. Water-mineral interactions could also generate O_2 (Watts et al. 1999) as an electron acceptor, or, in the deeper regions with higher temperatures, H_2 as an electron donor by serpentinization (Oze and Sharma 2005). Redox gradients would form in regions where reduced fluids mix with more oxidized fluids that may have access to the surface. The dynamic nature of the Martian environment would require life forms to cope with stresses like desiccation, high salt concentrations, low temperature, and radiation, which have a differential impact on viability.

After investigating different aspects of this model (marked by the numbers in **Fig. 4-2**, p. 154), the following conclusions can be drawn:

1) Acidophilic iron-sulfur bacteria may play an important role in iron redox cycling on Mars, as they can both oxidize and reduce iron using only inorganic electron donors or acceptors that are available on Mars.
2) *A. ferrooxidans* was able to grow solely on the nutrients provided by minerals in Mars regolith under aerobic and anaerobic conditions. The bacteria could also contribute to the dissolution of Fe^{3+}-containing minerals.
3) Though the oxygen partial pressure of the Martian atmosphere at the surface was not sufficient for detectable iron oxidation and growth of *A. ferrooxidans* during short-term incubation (7 d), alternative O_2-generating processes in the subsurface might yield microhabitats enriched in oxygen.

4) *A. ferrooxidans* and *S. thermosulfidooxidans* exhibited only moderate tolerance to most stress factors, but:
   a) Desiccation resistance was improved under low oxygen partial pressures and low temperatures, such as would be expected on Mars.
   b) Biofilm growth and addition of compatible solutes increased the survival of desiccation and freezing.
   c) In sufficient depths, organisms would be protected from surface radiation and could persist over prolonged periods of time (up to 10^5 years) even in a dormant state (and longer if maintenance metabolism and repair are possible).
5) Reproductive ability of *A. ferrooxidans* after exposure to different stressors was often more severely affected than other markers of cell viability such as iron oxidation activity, membrane integrity, and rRNA integrity, which might suggest that cells entered a viable-but-nonculturable state. At low temperatures (4°C), *A. ferrooxidans* did not grow, but remained metabolically active (maintenance metabolism) and culturable, while lack of an energy source led to a reduction in viability.

5 Abstract

In the last decades, our neighboring planet Mars has received much attention as one of the most promising targets close to Earth on which life may have developed in the past or might even exist at present. Recent NASA and ESA missions have provided detailed information on the physical and geochemical environment of Mars that can help to identify potential habitats and organisms that could be able to grow with the energy sources present on the planet. Due to the abundance of iron and sulfur minerals on Mars, iron-sulfur transforming microorganisms are considered likely models for putative Martian life forms (e.g. Amils et al. 2007; Fernández-Remolar et al. 2005; Nixon et al. 2012). For this study, two species of acidophilic iron-sulfur bacteria were chosen: the chemolithoautotrophic *Acidithiobacillus ferrooxidans* and the spore-forming *Sulfobacillus thermosulfidooxidans*. The goal of this thesis was to study their metabolic capacities, especially regarding the ability to grow with *in situ* resources that could be expected on Mars, and their tolerance to environmental stress conditions. A subsurface environment was assumed as the most likely habitat in these experiments, because it has a higher probability for liquid water, can provide different sources of energy for lithotrophic metabolism, and afford protection from the harsh surface physical conditions.

It was shown that acidophilic iron-sulfur bacteria such as *A. ferrooxidans* may play an important role in iron redox cycling on Mars, as they can both oxidize ferrous iron (Fe^{2+}) using O_2 as an electron acceptor, and reduce ferric iron (Fe^{3+}) using H_2, which could be generated by several processes in the subsurface of Mars. The threshold for aerobic respiration in the experiments with *A. ferrooxidans* and *S. thermosulfidooxidans* was 0.05% of dissolved O_2. Oxygen partial pressure in the Martian atmosphere was too low to yield any measurable iron oxidation activity of *A. ferrooxidans* after 1 week of incubation in Mars gas at low pressure (15 hPa). However, abiotic processes in the subsurface involving the interactions of liquid water with iron-bearing minerals could generate O_2 in sufficient quantities. Furthermore, *A. ferrooxidans* was incubated on two synthetic mixtures of Mars regolith minerals under aerobic and anaerobic conditions. Growth was observed with either O_2 as an external electron acceptor or H_2 as an external electron donor, and minerals provided not only Fe^{2+} and Fe^{3+} for energy gain, but probably also other essential nutrients for lithoautotrophic growth. The bacteria also seemed contribute to the reductive dissolution of Fe^{3+}-containing minerals like goethite and hematite, which are characterized by a high thermodynamic stability.

Because the Martian environment is not a static one, but will exhibit fluctuations in physical conditions, potential life forms would have to cope with stress factors like desiccation, high salt concentrations, low temperature, and radiation. A set of viability indicators was used to compare the effect of the stress conditions on different aspects of cellular viability such as reproduction, metabolic activity, and integrity of cellular compounds (membrane, DNA, rRNA). Reproductive ability (culturability) of *A. ferrooxidans* after exposure to different stressors was often more severely affected than other markers of cell viability such as iron oxidation activity, membrane integrity, and rRNA integrity, which might suggest that cells entered a viable-but-nonculturable state. At low temperatures (4°C), *A. ferrooxidans* did not grow, but remained metabolically active, pointing to a state of maintenance metabolism. Based on culturability, *A. ferrooxidans* and *S. thermosulfidooxidans* exhibited only moderate tolerance to most of these stress factors. However, desiccation tolerance was improved if the cells were kept under low oxygen tension, grown as a biofilm, or embedded within an external matrix of compatible solutes such as sucrose and trehalose. Freezing tolerance was also improved by the addition of compatible solutes and growth as biofilms. *A. ferrooxidans* and *S. thermosulfidooxidans* survived one week under simulated Martian surface conditions (6 hPa, -20°C, 0.13% O_2) in the form of dried biofilms. Thus, the conditions of the Martian surface (especially low temperature and low oxygen pressure) were favorable to the survival of cells. Although *A. ferrooxidans* was sensitive to UV-C radiation (F_{10} = 29 J/m^2) ample shielding is provided already by shallow layers of dust (especially containing Fe^{3+}) or by upper cell layers in a biofilm. Comparing the average ionizing radiation dose on Mars with the tolerance of *A. ferrooxidans* (D_{10} = 46 Gy), a population of these bacteria could remain viable in the shallow subsurface (0-3 m) of Mars for up to 10^5 years In sufficient depths, organisms would be protected from surface radiation and could persist even longer if intermittent maintenance metabolism and repair are possible.

The acidophilic iron-sulfur bacterium *A. ferrooxidans* can be considered a probable member of a potential Martian food web based on its metabolic capacities.

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8 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

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Publications

Bauermeister A, Bentchikou E, Moeller R, Rettberg P (2009) Roles of PprA, IrrE, and RecA in the resistance of *Deinococcus radiodurans* to germicidal and environmentally relevant UV radiation. Arch Microbiol 191:913-918

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Bauermeister A, Hahn C, Rettberg P, Reitz G, Moeller R (2012) Roles of DNA repair and membrane integrity in heat resistance of Deinococcus radiodurans. Arch Microbiol 194:959-966

Conference contributions

Talks

Bauermeister A, Moeller R, Reitz G, Billi D, Rettberg P (2010) Enhanced stress resistance of *Deinococcus radiodurans* cells in the dried state. 38th COSPAR Scientific Assembly, Bremen, Germany, July 18-25

Bauermeister A, Leon F, Sand W, Rettberg P, Reitz G, Flemming H-C (2011) Acidophilic iron-sulfur bacteria and their relevance for Mars. AbGradCon, Bozeman, MT, USA, June 5-8

Bauermeister A, Leon F, Sand W, Rettberg P, Reitz G, Flemming H-C (2011) Could acidophilic iron-sulfur bacteria be suitable model organisms for Mars? 11th European Workshop on Astrobiology, Cologne, Germany, July 11-14

Bauermeister A, Rettberg P, Reitz G, Flemming H-C (2012) Resistance of the iron-oxidizing bacterium *Acidithiobacillus ferrooxidans* to Mars-relevant stress conditions. AbSciCon, Atlanta, GA, USA, April 16-20

Posters

Bauermeister A, Moeller R, Reitz G, Rettberg P (2008) Resistance of different strains of *Deinococcus radiodurans* (wild-type and DNA repair-deficient mutants) to mono- and polychromatic UV radiation. 8th European Workshop on Astrobiology, Neuchatel, Switzerland, September 1-3

Bauermeister A, Moeller R, Reitz G, Rettberg P (2009) Survival of different *Deinococcus radiodurans* strains after exposure to mono- and polychromatic UV radiation and desiccation. VAAM, Bochum, Germany, March 8-11

Bauermeister A, Leon F, Rettberg P, Reitz G, Sand W, Flemming H-C (2009) Mars-relevant microorganisms in simulated subsurface environments under hydration/dehydration conditions. 9th European Workshop on Astrobiology, Brussels, Belgium, October 12-14

Bauermeister A, Billi D, Moeller R, Rettberg P (2011) Assessing survival and subcellular damage of *Deinococcus radiodurans* after desiccation *in situ* by molecular probes and genome stability assays. How dead is dead II, Tübingen, Germany, June 16-17