

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/327106025>

Resistance of cyanobacteria to space and Mars environments, in the frame of the EXPOSE-R2 space mission and beyond

Thesis · April 2018

DOI: 10.13140/RG.2.2.28437.88808

CITATIONS

0

READS

277

1 author:



Cyprien Verseux

ZARM

29 PUBLICATIONS 185 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



Astrobiology Graduates in Europe (AbGradE) [View project](#)



BIOMEX (Biology and Mars Experiment) [View project](#)



**UNIVERSITY OF ROME
"TOR VERGATA"**

DOCTORAL PROGRAM IN CELLULAR AND MOLECULAR BIOLOGY
XXIXth CYCLE

FACULTY OF MATHEMATICAL, PHYSICAL AND NATURAL SCIENCES
DEPARTMENT OF BIOLOGY

Resistance of cyanobacteria to space and Mars environments, in
the frame of the EXPOSE-R2 space mission and beyond

Cyprien Verseux

Y.Y. 2016/2017 (defended on 04/27/2018)

Co-Supervisor: Pr. Daniela Billi

Co-Supervisor: Dr. Lynn Rothschild

Tutor: Pr. Manuela Helmer Citterich

Program Coordinator: Pr. Francesco Cecconi

TABLE OF CONTENTS

Summary of doctoral research activities	7
PART I: RESISTANCE OF <i>CHROOCOCCIDIOPSIS</i> spp. TO SPACE AND MARS-LIKE CONDITIONS	11
Chapter 1: Introducing Part I.....	13
Cyanobacteria and the search for life beyond Earth.....	13
Exposure of cyanobacteria to space and Mars-like conditions.....	26
Aim of Part I and chapters' description.....	34
Chapter 2: Survival and biosignature detection of <i>Chroococcidiopsis</i> spp. after ground-based simulations of extraterrestrial environments.....	41
Introduction	42
Material and Methods.....	45
Cyanobacterial strains and culture conditions	45
Sample preparation.....	45
Test facilities and exposure conditions.....	48
Survival	49
Random amplification of polymorphic DNA (RAPD) assay	49
Confocal laser scanning microscopy	50
Membrane damage assessment.....	50
Results	52
Survival	52
DNA detection by PCR-based assay	53
Emission spectra of photosynthetic pigments.....	55
Membrane damage assessment.....	61
Discussion	62
Exposure conditions	62

Survival and DNA preservation	64
Preservation of pigments' autofluorescence.....	66
Potential mechanisms behind <i>Chroococcidiopsis</i> 's spp. resistance	67
Conclusion.....	70
 Chapter 3: Enhanced resistance of <i>Chroococcidiopsis</i> biofilms, compared to their planktonic counterparts, to space and simulated Martian conditions in low Earth orbit.....	73
Introduction	74
Materials and methods.....	77
Organisms and sample preparation.....	77
Exposure in low Earth orbit.....	77
Mission ground references and laboratory controls.....	79
Survival	80
qPCR assays	80
Confocal laser scanning microscopy	80
Results	83
Survival	83
DNA damage revealed by qPCR	84
Confocal laser scanning microscopy	87
Discussion	91
Differences in survival	91
Differences in DNA damage	94
Preservation of photosynthetic pigments.....	97
Acknowledgements	99
 Chapter 4: Responses of the desert cyanobacterium <i>Chroococcidiopsis</i> to simulated Martian conditions in low Earth orbit: implications for the limits of terrestrial life and the habitability of Mars	101
Introduction	102
Materials and methods.....	105

Mars regolith analogues	105
Organisms and sample preparation.....	105
Exposure in low Earth orbit.....	106
Ground references and laboratory controls	108
Colony forming ability assays	109
Random Amplification of Polymorphic DNA.....	109
Confocal laser scanning microscopy	109
Results	17411
Survival	111
DNA damage as revealed by random amplification of polymorphic DNA	111
Alteration of photosynthetic pigments as revealed by CLSM.....	113
Discussion	118
Survival	118
DNA damage as revealed by Random Amplification of Polymorphic DNA	121
Alteration of photosynthetic pigments as revealed by CLSM	121
Conclusions	124
Acknowledgements	125
PART II: CYANOBACTERIUM-BASED LIFE-SUPPORT SYSTEMS FOR THE FUTURE OF CREWED SPACE EXPLORATION.....	127
Chapter 5: Introducing Part II.....	129
Aim of Part II and chapter's description	1677
Chapter 6: Cyanobacterium biomass as a substrate for heterotrophic growth on Mars.....	169
Introduction	169
Materials and methods.....	171
Bacterial cultures	171

Lithotrophic growth of <i>Anabaena</i> sp. PCC 7120 with Mars regolith simulant	171
Cyanobacterium biomass as a nutrient source for heterotrophic bacteria	171
Characterization of the cyanobacterium medium	172
Perchlorate growth assays	172
Results	174
Lithotrophic growth of <i>Anabaena</i> sp. PCC 7120 with Mars regolith simulant	174
<i>Escherichia coli</i> and <i>Bacillus subtilis</i> can efficiently use cyanobacterium-based medium as a substrate.....	175
Characterization of the cyanobacterium-based medium.....	177
Perchlorate growth assays	178
Discussion	180
SYNTHESIS AND CONCLUSIONS	185
REFERENCES	189
LIST OF FIGURES	215
LIST OF TABLES	223
ACKNOWLEDGEMENTS	225

Summary of doctoral research activities

Those doctoral years allowed me to take part in quite a wide diversity of projects, the central theme being the assessment of cyanobacterium resistance to conditions found beyond Earth.

It started with the analysis of samples exposed to ground-based simulations of two EXPOSE-R2 projects (BOSS and BIOMEX) and of samples exposed to ionizing irradiation as part of the Starlife project. The data collected during that period of time is presented in Chapter 2. Further experimental work was performed on those samples prior to my arrival in the laboratory; the resulting data was not included in my PhD thesis (which only contains data I collected, or co-collected), but the interested reader can find them in the publications referenced as a footnote on the first page of Chapter 2.

I later prepared samples for the EXPOSE-R2 flight mission (together with Dr. Mickaël Baqué, who was still a doctoral student in Daniela Billi's laboratory at that time) and, when they landed back on Earth, worked on the analyses presented in Chapters 3 and 4. I here wish to acknowledge advice from Mickaël and the efforts from students I supervised, notably Clelia Staibano and Andrea Ianneo, who notably performed DNA extractions on a large fraction of the samples. In order to complement this data, I performed Raman spectroscopy using the facilities of the German space agency (DLR)'s center in Berlin. I was for this purpose trained by Mickaël Baqué, currently post-doctoral fellow there. Data from those analyses were not included in this thesis, but will soon be available as part of an overview article on the BIOMEX project.

In parallel, I contributed to assessing the feasibility of using cyanobacteria as a basis for life support systems, in support of future crewed missions far from Earth. Part of the data I collected (together with mass spectrometry results, for which I prepared samples and selected compounds to analyze but that I did not perform myself) is presented in Chapter 6. Efforts are ongoing to assess the

feasibility of using a similar system for feeding plants, and encouraging data was obtained, but results are too preliminary to be included in this thesis.

Aside from those main fields of investigation, I worked on various side projects. Some were completed, a few reached dead-ends, and others are ongoing at the time of writing. One example is a microbiome study, for which I collected samples (using swabs and wipes) on various surfaces (ground, furniture and crewmembers' torso skin) every two weeks for a year during the HI-SEAS IV mission (Caldwell et al., 2016), a NASA-funded experiment in an isolated and confined environment in which I took part as the Crew Biologist. Thanks to a grant from the Europlanet 2020 Research Infrastructure (funded by the EU's Horizon 2020 research and innovation program), I went to the Center for microbial life detection at Medical University Graz, Austria, where I concentrated close to 200 samples and purified DNA. DNA was then submitted to Microbial 16S rRNA gene sequencing (using Illumina MiSeq), yielding high-quality reads. Data analysis just started.

Besides performing laboratory work, a large fraction of the efforts provided for my PhD were dedicated to writing. Besides the articles mentioned and/or partially reported in the following chapters, I wrote and co-wrote grant proposals, which, although it took time away from doctoral work, led me to acquire skills of value for my future endeavors. I also contributed to peer-reviewed publications which were not included in this thesis due to being a bit outside its main focus, being quite long (and thus representing, if included, a fraction of this thesis disproportionate to their importance to it), and/or being based on results obtained prior to my PhD (during internships). A list of the papers published or submitted during my PhD is given below.

Verseux, C., and Baqué, M. (2014). Towards cyanobacterial-based life support systems on Mars. *Ital. J. Aerosp. Med.* *11*, 50–56.

Baqué, M., **Verseux, C.**, Rabbow, E., de Vera, J.-P.P., and Billi, D. (2014). Detection of macromolecules in desert cyanobacteria mixed with a lunar mineral analogue after space simulations. *Orig. Life Evol. Biosph.* *44*, 209–221.

Nieth, A., **Verseux, C.**, and Römer, W. (2014). A question of attire: dressing up bacteriophage therapy for the battle against antibiotic-resistant intracellular bacteria. Springer Sci. Rev. 3, 1–11.

Nieth, A., **Verseux, C.**, Barnert, S., Süss, R., and Römer, W. (2015). A first step toward liposome-mediated intracellular bacteriophage therapy. Expert Opin. Drug Deliv. 12, 1–14.

Samuel, T., Noack, L., **Verseux, C.**, and Serrano, P. (2015). A new network for astrobiology in Europe. Astron. Geophys. 2, 2–15.

Noack, L., **Verseux, C.**, Serrano, P., Musilova, M., Nauny, P., Samuels, T., Schwendner, P., Simoncini, E., and Stevens, A. (2015). Astrobiology from early-career scientists' perspective. Int. J. Astrobiol. 14, 533–535.

Baqué, M., **Verseux, C.**, Böttger, U., Rabbow, E., de Vera, J.-P.P., and Billi, D. (2016). Preservation of biomarkers from cyanobacteria mixed with Mars-like regolith under simulated Martian atmosphere and UV flux. Orig. Life Evol. Biosph. 46, 289–310.

Verseux, C., Baqué, M., Lehto, K., de Vera, J.-P.P., Rothschild, L.J., and Billi, D. (2016). Sustainable life support on Mars – the potential roles of cyanobacteria. Int. J. Astrobiol. 15, 65–92.

Verseux, C., Paulino-Lima, I., Baqué, M., Billi, D., and Rothschild, L. (2016). Synthetic Biology for Space Exploration: Promises and Societal Implications. In Ambivalences of Creating Life. Societal and Philosophical Dimensions of Synthetic Biology, K. Hagen, M. Engelhard, and G. Toepfer, eds. (Springer-Verlag), pp. 73–100.

Verseux, C., Acevedo-Rocha, C.G., Chizzolini, F., and Rothschild, L.J. (2016). Misconceptions of synthetic biology: Lessons from an interdisciplinary summer school. Nanoethics 10, 327.

Verseux, C., Baqué, M., Cifariello, R., Fagliacone, C., Raguse, M., Moeller, R., and Billi, D. (2017). Evaluation of the resistance of *Chroococcidiopsis* spp. to sparsely and densely ionizing irradiation. Astrobiology 17, 118–125.

Billi, D., Baqué, M., **Verseux, C.**, Rothschild, L., and Vera, J.-P. (2017). Desert cyanobacteria: Potential for space and Earth applications. In Adaption of Microbial Life to Environmental Extremes, H. Stan-Lotter, and S. Fendrihan, eds. (Springer International Publishing), pp. 133–146.

Fagliarone, C., Mosca, C., Ubaldi, I., **Verseux, C.**, Baqué, M., Wilmotte, A., Billi, D. (2017). Avoidance of protein oxidation correlates with the desiccation and radiation resistance of hot and cold desert strains of the cyanobacterium *Chroococcidiopsis*. *Extremophiles* 21, 981-991.

Heinicke, C., **Verseux, C.** (2017). Surface operations during a long-duration Mars simulation mission. 68th International Astronautical Congress 2017, Paper IAC-17, B3.5,6,x39856.

Verseux, C., Billi, D. (*in press*, 2018) Cyanobacterium-based technologies in space and on Earth.

Billi, D., **Verseux, C.**, Baqué, M., Fagliarone, C., de Vera, J.-P (*submitted*, 2018). A desert cyanobacterium under simulated Martian condition in low Earth orbit: Implications for Mars habitability.

Billi, D., **Verseux, C.**, Baqué, M., Staibano, C., Rabbow, E., Rettberg, P (*submitted*, 2018). Enhanced resistance of *Chroococcidiopsis* biofilms, compared to their planktonic counterparts, to space and simulated Martian conditions in low Earth orbit.

PART I

RESISTANCE OF *CHROOCOCCIDIOPSIS* spp. TO SPACE AND MARS-LIKE CONDITIONS

Chapter 1

Introducing Part I

Cyanobacteria and the search for life beyond Earth

Whether there is life on Mars remains an open question. It has been a key focus of astrobiology for decades, but the wealth of data gathered on the planet and on the resistance of life to extreme environments (e.g., Rothschild and Mancinelli 2001), together with the development of new spaceflight and analytical technologies promising unprecedented field investigation, has renewed interest in this question over the past few years. In the search for answers, and as detailed below, cyanobacteria are valuable model organisms.

Understanding the relevance of cyanobacteria in this context first requires knowledge of the main environmental parameters found on Mars and in interplanetary space that would most affect microorganisms, as well as the motivations behind the question of life on Mars. This first section thus starts with an overview of those topics.

Today, the surface of the red planet is likely uninhabitable to organisms as we know them: several of its environmental parameters (see Table 1.1) are extremely harsh to them. Particularly so are the low atmospheric pressure, cold temperatures and radiation, and the oxidative environment caused by the radiation regime.

Mars's atmospheric pressure varies between approximately 5 and 12 hPa, with seasonal and diurnal variations due to carbon dioxide ice sublimation as the planet warms up (see for instance Gomez-Elvira et al., 2014). Given Mars's temperatures, ranging from -150°C to +30°C with an average of -60°C, liquid water is unstable (see Figure 1.1). Some flows, called *recurring slope lineae*, have been detected (Martín-Torres et al., 2015; McEwen et al., 2011), but those are saturated in salt (which decreases water's freezing point and allows such flows under Mars's conditions), resulting in water activity too low

to support life as we know it (Martín-Torres et al., 2015). The lack of available liquid water makes metabolic reactions impossible, at the surface, for such life.

On Mars, the radiation regime includes ultraviolet (UV) and ionizing radiation. Given the limited shielding provided by the thin Martian atmosphere, the fraction of low-wavelength UV radiation (200–315 nm) reaching the surface is much greater than that reaching the surface of Earth, and the spectrum extends to shorter wavelengths (down to circa 190 nm, against circa 300 nm on Earth) (see for instance Rothschild & Cockell, 1999). The models of Cockell et al. (2000) and Schuerger et al. (2003) predict a maximum flux of around $50 \text{ W}\cdot\text{m}^{-2}$ of UV in the 200–400 nm range on the equatorial Martian surface at the mean orbital distance, and a daily fluence of circa $1.5 \times 10^3 \text{ kJ}\cdot\text{m}^{-2}$. During its first 100 sols on Mars, NASA’s Curiosity rover measured a maximum UV irradiance in the 200–380 nm range of about $20 \text{ W}\cdot\text{m}^{-2}$ at Gale Crater (4.59°S , 137.44°E) at areocentric solar longitudes 151 – 208° (Gómez-Elvira et al., 2014). Ultraviolet radiation is highly

Table 1.1. Comparison of some of the environmental parameters on Mars and Earth’s surfaces (modified from Graham [2004]) and Kanervo et al. [2005], extended with ionizing radiation data from Hassler et al. [2013] and Cockell et al. [2000]). More details, and implications for microorganisms, are given in the text.

Parameter	Mars	Earth
Surface gravity	0.38 g	1.00 g
Mean surface temperature	-60°C	+15°C
Surface temperature range	-150 to +30°C	-90 to +60°C
Mean photosynthetically radiation	$8.6 \times 10^{19} \text{ photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	$2.0 \times 10^{20} \text{ photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$
UV radiation spectral range	>190 nm	>300 nm
Ionizing radiation	$\sim 100 \text{ mGy}\cdot\text{year}^{-1}$	$\sim 1 \text{ mGy}\cdot\text{year}^{-1}$
Atmospheric pressure	5–12 hPa	1013 hPa (mean at sea level)
	N₂ 0.189 hPa, 2.7%	780 hPa, 78%
Atmospheric composition (average)	O₂ 0.009 hPa, 0.13%	210 hPa, 21%
	CO₂ 6.67 hPa, 95.3%	0.38 hPa, 0.038%
	Ar 0.112 hPa, 1.6%	10.13 hPa, 1%

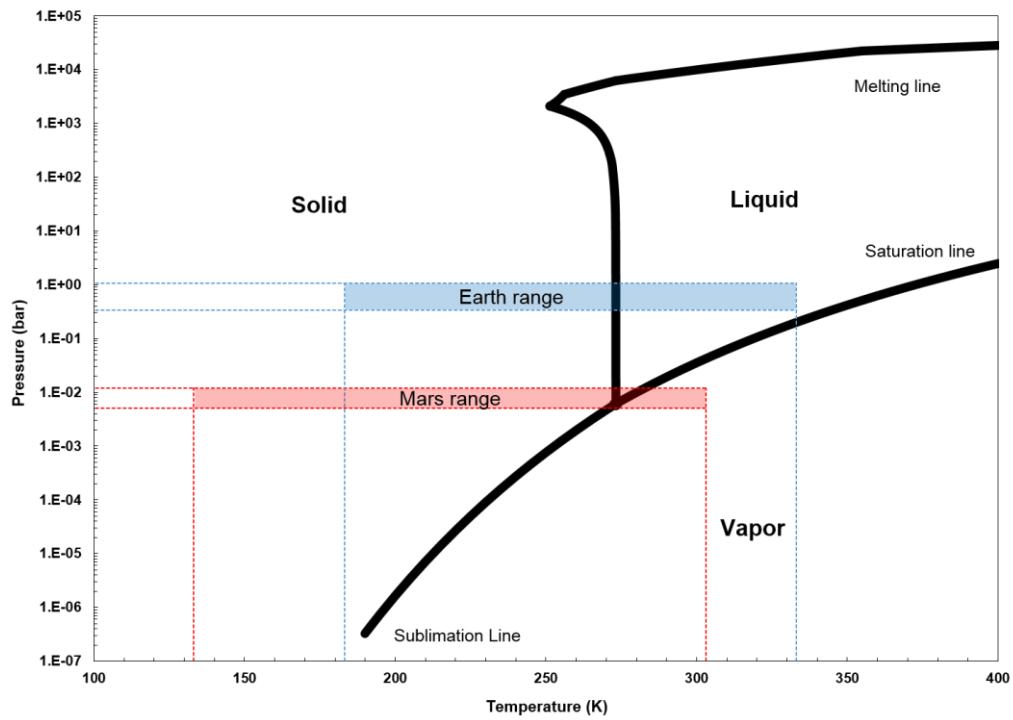


Figure 1.1. Phase diagram showing water's state as a function of pressure and temperature. The “Mars range” and “Earth range” boxes indicate the ranges of pressure and temperature found on Mars’s and Earth’s surfaces, respectively. While large amounts of water in liquid and solid states can be found on Earth’s surface, liquid water is unstable on Mars’s surface due to its low atmospheric pressure.

damaging to terrestrial microbes (e.g., Horneck et al., 2010). This is particularly true for UVC radiation (200–280 nm) given the high absorbance of DNA around 260 nm. Damage is created in large part by the direct generation of bipyrimidine lesions (e.g., Leuko et al., 2011), but also by DNA strand breaks and DNA/protein cross-linking, as well as photochemically produced reactive oxygen species (ROS) (e.g., Horneck et al., 2010).

Ionizing radiation includes solar energetic particles (SEP) and galactic cosmic rays (GCR). GCR are made of, roughly, 85-90% protons, 1-13% α particles (similar to He nuclei), 1% heavier nuclei, and 1% electrons. SEP are mostly composed of protons. On Earth, the flux is low: particles are deflected by its magnetic field and attenuated by its atmosphere. As a consequence, most

ionizing radiation on Earth comes from background radioactivity (from radioactive nuclides in the crust) and typically amounts to circa 1 mGy·year⁻¹ (Sorenson, 1986) (the highest detected levels were about 0.4 Gy·year⁻¹ [Baumstark-Khan and Facius, 2001], but this is an exceptional case). Mars, on the other hand, lost its magnetic field within its first billion years (Stevenson, 2001). As a consequence, SEP and GCR reach the Martian surface and subsurface with high energies. Pavlov et al. (2012) and Dartnell et al. (2007) assessed absorbed dose rates at the surface to be in the range of 50–150 mGy·year⁻¹, and the Curiosity rover measured an absorbed dose rate of 76 mGy·year⁻¹ at Gale Crater (Hassler et al., 2013). This kind of radiation can damage macromolecules by direct absorption, leading to ionization or radiolysis (causing, for instance, DNA strand breaks), or via highly reactive species created by interactions of radiation with water or other molecules (Baumstark-Khan and Facius, 2001).

Finally, strong oxidants have been detected on Mars's surface. Most notably, perchlorates have been found at concentrations ranging from circa 0.4 to 1.1% in weight (Sutter et al., 2016). Though they are usually quite stable at Mars's ambient temperatures, Mars's UV irradiation makes them reactive and highly detrimental to life (Wadsworth and Cockell, 2017).

To prevent overconfidence in claims related to Mars's habitability, a few facts should be considered here. First, conditions and interpretation of experiments aimed at assessing Mars's habitability are typically based on average values and/or values selected for standard locations and times (e.g., average UV flux at the equator at noon and at an average distance from the Sun [Cockell et al., 2000]), and/or on limited available datapoints (e.g., perchlorate concentrations at the Phoenix landing site [Fang et al., 2015], or radiation at the MSL landing site for the documented periods of time [Hassler et al., 2013]). On Earth, environmental conditions can vary greatly with location—even over very short distances, especially if considering microhabitats: from a microbial point of view, a few millimeters or less can separate highly supportive habitats from lethal environments. As an example: if taking average values for parameters such as dryness and irradiation, large areas of the Atacama desert are uninhabitable; and yet, in those areas, microbes can be found in spatially restricted niches (e.g., Rothschild, 1990; Wierzchos et al., 2006). It is reasonable to expect that, on Mars, large or local areas can be found with conditions (and possibly degrees of habitability) differing greatly from one another. While a few studies consider the possibility of highly spatially restricted habitats (e.g., Rothschild, 1990; Möhlmann,

2009; Westall, 2013), the diversity of Martian environments on a microbial scale remains far from being covered. Besides, accurate data on various parameters are known only at a few locations, and various factors (e.g., some chemical compounds present in the ground) remain unknown. Assertive claims on the ability of specific organisms to survive (or not) Mars conditions, as regularly found in press releases or even original papers, are consequently premature and based on a currently limited knowledge of Mars's surface (and even more limited knowledge of its subsurface). Rather than reaching definitive conclusions on Mars's capacity to harbor life, astrobiologists slowly dress a portrait of its habitability. New studies lead to incrementally better understanding of the effects of Mars's environments on living organisms and, every year, new discoveries regarding Mars's surface lead to reassessments of its potential for life.

A related observation should be mentioned once in this thesis: almost all valuable experiments and hypotheses on Mars's habitability are based on terrestrial life. It gives us a reference and frames our hypotheses, and rightfully so: while interesting as a thought experiment, assumptions on the abilities to thrive in such conditions of forms of life unlike any we know would be, at best, highly tentative. However, terrestrial life evolved—to state the obvious—on Earth, at least over most of the past three billion years. Its resistance was tuned to specific sets of environmental stressors including, for instance, Earth's highly oxidizing atmosphere. As a consequence, studies on the resistance of terrestrial organisms may lead to underestimations of the habitability of a different environment for potential organisms which would have evolved there.

While, for the sake of brevity, those facts are not systematically reminded later on, discussions in the following chapters of this thesis should be taken in such a context: they are based on the incomplete information available so far on Mars's environments, and on the only branch of life we know.

Based on currently available data, the consensus is that the surface of Mars is not, overall, a hospitable place to any known life form. However, Mars has not always been the hostile desert it is today. It is thought that the environmental conditions on Archean Earth (which hosted life) and Noachian Mars were quite similar (Carter et al., 2015; McKay, 1997; Wynn-Williams and Edwards, 2000). In particular, extensive evidence suggests that Mars's surface used to harbor large amounts of liquid water, although its abundance and persistence through time are still debated (Bibring et al., 2006; Carter et al., 2015; Ehlmann et al., 2011; Grotzinger et al., 2014a; Mustard et al., 2008;

Poulet et al., 2005). UV irradiance, as well, was presumably quite similar on both young planets: the Archean Earth supposedly lacked an ozone layer, allowing UV radiation down to 200 nm to bombard its surface (Cockell et al., 2000; Patel et al., 2004). Besides, nutrient sources suitable for autotrophic microbes were a priori available (Cockell, 2014). The surface of Early Mars might consequently have been habitable to life as we know it.

The Earth and Mars then evolved quite differently: Mars lost its intrinsic magnetic field, most of its atmosphere and its surface liquid water. However, pockets of conditions habitable to life as we know it may have persisted up to present day (Cockell, 2014; Westall et al., 2013). For instance, evidence suggests the presence of abundant liquid water below Mars's cryosphere (Ehlmann et al., 2011; Michalski et al., 2013). Even close to the surface, protected niches could have offered habitable conditions until today (Schirmack et al., 2014; de Vera et al., 2014). Besides, various energy and nutrient sources suitable for autotrophic microbial metabolisms are present on Mars (Cockell, 2014; Nixon et al., 2013; Verseux et al., 2016a). Life may thus still exist there, most likely under the surface.

This possibility has been considered for quite a long time, and the (scientific) search for life on Mars started decades ago. An important milestone was set in 1976 with the arrival on Mars of NASA's Viking landers, two robots sent to specifically search for traces of life. Unfortunately, results were inconclusive, in large part due to our lack of knowledge of the Martian surface which led to an inability to either detect life or to distinguish it from abiotic, physicochemical reactions (Schulze-Makuch et al., 2015). Since then, no Mars mission has been dedicated to a direct search for life (Levin, 2015). However, a large numbers of landers, rovers and orbiters have been sent there which greatly increased our understanding of the Martian surface (McKay et al., 2013). The direct search for life will resume in 2020, with the expected launch of two rovers: NASA's Mars 2020 and ESA-Roscosmos's ExoMars.

The ExoMars rover (Figure 1.2) will be equipped with a drill to collect material below the surface, down to depths of 2 meters, where potential biological remains may be better preserved (Vago et al., 2017). It will carry the Pasteur payload: a powerful set of instruments aimed at detecting signs of life, notably a high-resolution color camera (CLUPI) designed for close-up observations (Josset et al., 2017), a Raman laser spectrometer (RLS) which will perform Raman spectroscopy on crushed samples obtained using the drill

system (Rull et al., 2017), and a mass spectrometer (part of the MOMA instrument; Goesmann et al., 2017). The Mars 2020 rover will include, among others, a UV Raman spectrometer called SHERLOC (for Scanning Habitable Environments with Raman and Luminescence for Organics and Chemicals) at the end of a robotic arm (Fries et al., 2010; Tarcea et al., 2007). Those instruments were designed to detect biosignatures: features typical of life, which can be morphological, geochemical, or organic (Horneck et al., 2016). As examples, CLUPI could detect morphological biosignatures such as biolamination, Raman spectrometers various functional groups (noteworthy fluorescent groups from pigments found exclusively in living organisms) (Rull et al., 2017; Fries et al., 2010), and MOMA's mass spectrometer a wide range of small organics such as amino acids (Goesmann et al., 2017). While living organism could be detected by those payloads, the latter rather target traces of past life: extant microbial life on Mars, if it exists, is likely extremely scarce.

One could doubt that robotic exploration alone will give a generally agreed-upon answer to the question of life, extinct or extant, on Mars.



Figure 1.2. Rover of the ExoMars mission, a project involving the European (ESA) and Russian (Roscosmos) space agencies. Expected to be launched to Mars in 2020, its main scientific objectives will be to search for signs of life, study the evolution of Mars's geochemical environment, and track trace gases in the Martian atmosphere. Image: ESA. Available at exploration.esa.int/mars/53-910-exomars-rover (consulted on February 26th, 2018).

Negative results may only indicate that no sign of life has been found so far, while positive signals would likely be scrutinized by scientists looking for alternative explanations such as contamination or abiotic causes. Evidence for life would likely be questioned until either convincing counter-evidence is found, or until a definitive positive answer comes from astronauts on site, sample return missions followed by analysis on Earth, or rovers much beyond today's state-of-the-art. Such a willingness to refute claims of discovery of life on Mars previously occurred, for instance, after the Viking's Labeled Release experiment (Levin, 2015) or after the discovery of microfossil-like structures in the Martian meteorite ALH84001 (Schulze-Makuch et al., 2008). Even on Earth, claims on the discovery of ancient life—such as the alleged 3.7–4.3 billion year old microfossils found in the Nuvvuagittuq Supracrustal Belt (Quebec, Canada) by Dodd et al. (2016)—are often highly controversial, and the biogenic origin of those suspected remains can be highly difficult to ascertain. To paraphrase Carl Sagan, claiming that life has been found beyond Earth requires unquestionable evidence, and such evidence is unlikely to be brought by today's rovers alone. However, the detection of biosignatures by ExoMars or Mars2020 would strongly suggest that Mars has harbored life. It would likely trigger unprecedented interest and investment in astrobiology endeavors on the red planet, allowing more ambitious missions to be led in the areas of detection.

One of the challenges ahead of both foreseen rovers is that biosignatures as we know them can be affected by the harsh conditions found beyond Earth. While many are easily recognizable on Earth, the astrobiology community seeks to extensively characterize the effects of extraterrestrial conditions (such as radiation, Mars's atmosphere or space vacuum, and interaction with minerals) and develop databases of biosignatures under relevant environments (Horneck et al., 2016). Cyanobacteria are useful models in the development of such databases: besides ubiquitous molecules (such as DNA), they contain widespread pigments, including beta-carotene and characteristic photosynthetic pigments.

This approach relies on the assumption that, if biosignatures are present on Mars, they have strong similarities with known terrestrial biosignatures. An Earth-independent genesis of life on Mars has been proposed (e.g., Smith and McKay, 2005) and could lead to radically different morphologies and molecular constitutions, making the search for Earth-like biosignatures irrelevant. However, terrestrial life and a hypothetical life on Mars may be

related (see below). Furthermore, even independent geneeses may produce somehow similar organisms (Cockell, 2016).

Besides being used for testing potential biosignatures, terrestrial microorganisms are studied to assess the habitability of Mars throughout its history: astrobiologists study their survival limits as a proxy for the limits of life. This approach may be biased since, as stated above, the limits for life on Earth may be defined by evolutionary constraints found there rather than by absolute limits for life, but terrestrial life remains the only example we have.

Among interesting models for such studies are cyanobacteria. Some thrive in the most extreme habitable conditions on Earth (Billi et al., 2013, 2017), where they have an outstanding resistance to environmental factors occurring in space and on Mars's surfaces (as described in the section below). In highly irradiated deserts on Earth, endolithic cyanobacteria find refuge close to the surface of rocks, where they receive enough light for photosynthesis while being protected from lethal levels of UV irradiation—a strategy that might be valid on Mars, given the amounts of photosynthetically active radiation and UV (Cockell and Raven, 2004). Cyanobacteria are consequently an interesting model for life on the red planet. It has been suggested that, if life exists on present-day Mars, it is likely underground and photosynthesis-independent given the harsh conditions at the surface where solar light is available (see, e.g., Cockell, 2014 and Westall et al. 2013). However, even a millimeter-scale layer of regolith can reduce UV radiation down to survivable levels (Cockell et al., 2005; Johnson et al., 2010; Mancinelli and Klovstad, 2000), and may thus allow life in the close subsurface. Even photosynthetic organisms could be shielded from lethal radiation while receiving enough photosynthetically active radiation for their metabolism, as happens, for instance, with terrestrial cyanobacteria in evaporites (Rothschild, 1990) or in translucent, desert rocks (e.g., Smith et al., 2014). Besides, if environmental conditions on Mars were life-permissive long enough for life to originate there and to evolve metabolic systems as complex as oxygenic photosynthesis, and/or if life was exchanged between the two planets (according to the theory of lithopanspermia; see below), biological remains from life forms analogous to desiccation- and radiation-resistant cyanobacteria might have existed—and might even be present in a dormant form below the surface—on Mars.

If life ever existed on Mars, questions arise on its origin. Although the exact conditions for life to originate are not surely determined, elements thought to be essential (noteworthy organic molecules, liquid water, and energy sources

from the Sun and/or volcanism) are believed to have been similarly distributed on both planets at some point in their history. Therefore, an origin of life on Mars is a scenario worth considering (Cockell, 2014; McKay, 2010; Westall et al., 2013). Whether life originated there or not, one may wonder whether it could be related to life on Earth. Indeed, material transfer between planets is not uncommon at a geological scale. Rocks can be expelled by asteroids or comets, which have been hitting both Earth and Mars at a high rate, especially during the first 500 million years of our planetary system (bombardment was then two orders of magnitude more intense than today). Ejecta reaching escape velocity (e.g., $5 \text{ km}\cdot\text{s}^{-1}$ for Mars) leave the planet and begin orbiting around the Sun, until they either impact another celestial body or leave our planetary system. Consequently, large amounts of materials have been transferred from Mars to Earth and conversely. Besides, rocks can undergo this journey without being excessively heated. It has been assessed that billions of Martian ejecta landed on Earth without reaching 100°C (Mileikowsky et al., 2000) and, consistently, large parts of the famous ALH84001 meteorite never exceeded 40°C (Weiss et al., 2000).

Shock recovery experiments, performed in the assessment of lithopanspermia, showed that some microorganisms (including *Chroococcidiopsis* sp. CCMEE 029) could survive pressures that would be generated by impacts sufficient to expulse rocks from Mars (Horneck et al., 2008). The associated acceleration does not seem germicidal either (Benardini et al., 2003; Mastrapa et al., 2001). Hypothetical, viable microorganisms contained in ejecta might consequently survive expulsion from Mars.

Another critical question is whether some life forms could survive the subsequent journey in interplanetary space. Resistance of bacteria to the space environment has been extensively studied in the last decades (see for instance Cottin et al. 2017, and Olsson-Francis and Cockell 2010) and, according to Mileikowsky et al. (2000), if Mars ever harbored microbial life then its natural transfer to Earth is a highly probable process. Among the most important factors are resistance to radiation damage, DNA decay by hydrolysis, and vacuum exposure (assuming cells are inside rocks and protected from UV radiation). Vacuum is expected to be the most life-threatening factor if protected from UV but, if melting of the outer layers of the ejecta during the passage through the source planet's atmosphere creates an atmosphere-containing compartment, the most likely limiting factors are either DNA decay by hydrolysis or ionizing radiation (Mileikowsky et al., 2000). Mileikowsky et al. (2000) estimated that a fraction of ejecta not reaching sterilizing temperatures travelled for a time inferior to that needed for hydrolysis to

inactivate bacteria. It should be pointed out that, to estimate the rates of DNA decay, the authors used Arrhenius plots with constants which do not accurately describe it in an environment where pressure and temperature make liquid water unstable. However, those constants likely resulted in overestimations, not underestimations, of DNA decay by hydrolysis in such conditions. Aside from water's presence and state, the rates of DNA decay are mostly dependent on temperature (Lindahl, 1993) and could likely be predicted accurately after experimental determination of the relevant constants. On the other hand, resistance to radiation—the other suspected limiting factor for bacteria's survival in ejecta compartments—is very organism-specific and its prediction requires extensive experimentation.

Bacillus spores are often used as model organisms in the context of lithopanspermia, due to their very high resistance to space environments and their being common spacecraft contaminants. However, given that they are heterotrophs, they are unlikely to flourish in a world where organics are not abundant. While they are good models for studying survival to an interplanetary journey, they may be less relevant as models for first colonizers. Autotrophs would be more suitable for such a role. On Earth, cyanobacteria are typically the first colonizers of deserts habitats: complex ecosystems containing heterotrophs occur once cyanobacteria have produced organic compounds and fixed nitrogen, and made mineral nutrients available by leaching rocks. Various considerations may decrease the perceived likelihood of lithopanspermia involving phototrophs, such as the fact that heating of the external part of rocks during atmospheric re-entry reduces their chances of survival (compared to those of chemotrophs and heterotrophs), which are generally found close to the surface to access light (Cockell, 2008; Cockell et al., 2007; Foucher et al., 2010). However, such phenomena would act as filters rather than hermetic barriers, and cyanobacteria arguably are a model worth considering in the assessment of lithopanspermia's possibility.

To sum up this section, desert strains of cyanobacteria are relevant models for critical fields of investigation in astrobiology: the detection of biosignatures beyond Earth, the habitability of Mars, and the likelihood of lithopanspermia.

It should be pointed out, however, that no model is sufficient by itself. As an example, cyanobacteria need to access light, which restricts their potential habitats to the harsh surface or close subsurface, and one cannot exclude the possibility that radically different metabolisms exist or have existed on Mars (for well-thought examples, see Nixon et al., 2013). A common bias when one

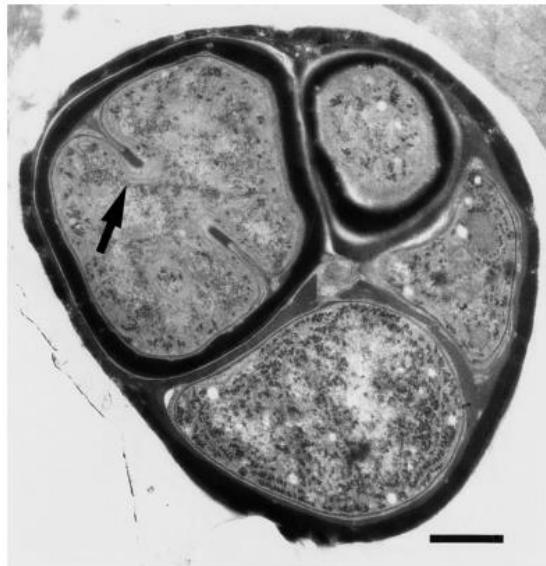


Figure 1.3. Electron micrograph of ultrathin section of a cell aggregate of *Chroococcidiopsis* sp. CCMEE 029, a cyanobacterium species used in a large fraction of the work presented here. The arrow points toward a division septum. Scale bar: 0.5 μm . Image from Billi et al. (2000).

works with only one group of organisms is to formulate hypotheses in a way that makes this group relevant, and to give limited attention to hypotheses involving other organisms. While this thesis is focused on cyanobacteria, I here want to acknowledge the relevance of other models and their complementarity to this group, at a time when we have no concrete evidence of any given kind of metabolism on Mars.

Given their legitimacy as a model for astrobiology research, cyanobacteria were exposed to conditions found beyond Earth in various experiments. An overview of those studies is given below. This overview is biased toward *Chroococcidiopsis* spp. (shown in Figure 1.3), as they are the model organisms used in the laboratory of Pr. Daniela Billi and the protagonists of most of the following chapters. Most studied species come from the Culture Collection of Microorganisms from Extreme Environments (CCMEE), established by E. Imre Friedmann and currently maintained at the University of Rome Tor Vergata. Their importance for our studies come from their extremophilic

Table 1.2. Examples of astrobiology-relevant environmental conditions survived by *Chroococcidiopsis* spp.

Factor	Parameters	References
Desiccation	Years in a dry state	Billi, 2009; Grilli Caiola et al., 1996; Fagliacone et al., 2017; Chapters 3-4
Ionizing radiation	Photons, 15 kGy	Billi et al. 2000; Verseux et al., 2017
	Heavy ions, 2 kGy	Verseux et al., 2017
UV irradiation (200-400 nm)	30 kJ/m ² (as dry monolayers)	Cockell et al., 2005
	1.5 x 10 ⁵ kJ/m ² (as dry multilayers overlain by 3 mm Antarctic sandstone)	Billi et al., 2011
	1.5 x 10 ³ kJ/m ² (as dry multilayers mixed with ~ 15 µm anorthosite)	Baqué et al., 2014; Chapter 2
UVC	13 kJ/m ² (as cell suspension)	Baqué et al., 2013a
Mars-like atmosphere	980 Pa of 95.55% CO ₂ , 2.70% N ₂ , 1.60% Ar, 0.15% O ₂ , ~370 ppm H ₂ O, for 722 days	Chapters 3-4
Vacuum	≈ 10 ⁻⁵ –10 ⁻⁶ Pa, for 672 days	Chapters 3-4
Shock pressure	5–10 GPa	Horneck et al., 2008
Extreme temperatures and temperature cycles	1 hour at -25°C + 1 hour at +60°C; 2 hours at -10°C + 2 hours at +45°C, 66 times	Baqué et al., 2013b, 2016
Low Earth orbit (various factors)	EXPOSE-E	Cockell, 2011
	EXPOSE-R	Bryce et al., 2014
	BOSS (EXPOSE-R2)	Chapter 3
	BIOMEX (EXPOSE-R2)	Chapter 4

features: species from the genus *Chroococcidiopsis* are the typical dominant microorganisms in deserts considered as Mars analogues due to cold temperatures, dryness, high UV irradiation, low levels of organic materials, and/or rock composition (Billi et al., 2013). As described below (and

summarized in table 1.2), they have also demonstrated a high resistance to conditions found beyond Earth.

Exposure of cyanobacteria to space and Mars-like conditions

Given their relevance to key astrobiology investigations, cyanobacteria were exposed to space and Mars-like conditions in a wide range of studies.

Their resistance to photon irradiation (gamma-rays and X-rays) was studied as early as the early 1950s (Bonham and Palumbo, 1951), and the high resistance of some species was described in the early 1960s (Godward, 1962; Shields et al., 1961). In the following decade, the resistance of a wide range of species from various genera was tested. Resistance appeared to be very variable among species, from sensitive ones with D_{10} (the radiation dose needed to inactivate 90% of the microbial population) below 1 kGy to highly resistant ones with D_{10} above 10 kGy (e.g., Asato, 1971; Bruce, 1964; Godward, 1962; Kraus, 1969; Kumar, 1964; Shields et al., 1961).

Results from those early studies are, however, difficult to reproduce, compare or interpret, given that one or several irradiation parameters now known to impact survival (temperature, growth phase, dose rates, oxygen levels, light intensity, etc.) were usually not controlled—or not specified. In the past 15 years, those results were deepened by more controlled studies (see Table 1.3 for an overview of recent and older studies). Some cyanobacterium species such as *Synechococcus* and *Synechocystis* spp. were shown to be sensitive to gamma rays, with D_{10} of about 0.3 kGy (Agarwal et al., 2008; Domain et al., 2004) and 0.7 kGy (Domain et al., 2004), respectively. Two *Anabaena* strains were shown to tolerate a 5 kGy gamma-ray dose without loss of survival, showed a GI_{50} (dose where growth is inhibited by 50%) of 6–11 kGy and survived doses of 15 kGy (Singh et al., 2010, 2013). *Arthrosphaera* sp. PCC 8005 cells were shown to survive exposure to doses of at least 6.4 kGy of gamma irradiation, and 1 and 2 kGy of He and Fe particle radiation

(Badri et al., 2015). *Chroococcidiopsis* spp. hydrated cells withstood several kGy of heavy ion irradiation, and 15 kGy of photon (gamma- and X-ray) irradiation (Billi et al., 2000; Verseux et al., 2017). Dried cells even withstood 24 kGy of gamma-rays (Verseux et al., 2017). For comparison, *E. coli* ATCC 10798 has been shown to have a D_{10} of 0.35 kGy (Trampuz et al., 2006). However, when comparing data from various studies and ranking bacteria according to their resistance, one should keep in mind that slight changes in sample preparation, hydration state of samples, oxygenation, light, temperature, radiation source, or growth phase, can greatly impact a species's D_{10} . While the orders of magnitude involved can be a useful estimate, a given survival fraction should be considered valid for a precise set of experimental conditions only.

A dose of a dozen kGy, survivable by *Chroococcidiopsis* spp. (Verseux et al., 2017), corresponds to roughly 100 thousand years on the surface of Mars or to 60 thousand years on the surface of a rock travelling through space.

Table 1.3. Data illustrating the resistance of cyanobacteria to ionizing radiation, in perspective with dose rates on Mars's surface. See text for more details.

	Irradiation	References
<i>Chroococcidiopsis</i> spp. survival	> 15 kGy X-rays	Billi et al., 2000; Verseux et al., 2017
	> 12 kGy γ -rays	
	> 1 kGy He	
	> 2 kGy Fe	Verseux et al., 2017
	> 1 kGy Si	
<i>Anabaena</i> spp. survival	> 15 kGy γ -rays	Singh et al., 2010, 2013
	> 6.4 kGy γ -rays	
<i>Arthospira</i> spp. survival	> 1 kGy He	Badri et al. 2015
	> 2 kGy He	
Cyanobacterial resistance (overall)	Photon irradiation: 1 kGy > D_{10} < 15 kGy	Various studies (e.g., Kraus, 1969)
Mars surface irradiation	50-150 mGy/year	Dartnell et al., 2007; Pavlov et al., 2012; Hassler et al., 2013

However, the nature of the radiation, and not only the dose, should be taken into account (more details on this are given in Chapter 2). Besides, the extent of damage created by a given radiation dose may depend on the timeframe over which this dose is applied. In order to simulate long-term effects of ionizing radiation reaching an area in space or on Mars, researchers typically use higher doses for shorter periods of time. Intuitively, such an approach seems valid for dormant organisms which will undergo damage accumulation without counterbalancing it with damage repair (e.g., Rothschild & Cockell, 1999), but it remains to be ascertained experimentally.

The resistance of *Chroococcidiopsis* spp. to doses of ionizing radiation much higher than what is found in their natural environments may be a consequence of evolutionary pressures from their dry habitats (Mattimore and Battista, 1996; Pavlopoulou et al., 2016), as desiccation and radiation can induce similar damage (e.g., oxidative stress and DNA damage). Consistently, *Chroococcidiopsis* spp. were reported to be viable after years of storage in a dry state (Billi, 2009; Grilli Caiola et al., 1996; Fagiarone et al., 2017).

Some cyanobacteria are also highly resistant to UV. Dried monolayers of *Chroococcidiopsis* sp. CCMEE 029, for instance, survived 10 minutes under a simulated Martian UV flux (amounting to 30 kJ/m^2) (Cockell et al., 2005). Their reduction in survival after 5 minutes was similar to that observed in spores of *Bacillus subtilis* after 15 seconds of a similar flux (Schuerger et al., 2003). Although the highly lethal effects of the Martian UV flux may be seen as strongly limiting for survival of photosynthetic microbes (which need to access sunlight), a 1-mm layer of rock could be enough to bring UV dose rates down to survivable levels (Cockell et al., 2005; Mancinelli and Klovstad, 2000). Consistently, multilayers of *Chroococcidiopsis* sp. CCMEE 123 overlain by 3 mm of Antarctic sandstone survived exposure to $1.5 \times 10^5\text{ kJ/m}^2$ (a dose corresponding to roughly 10^2 days on Mars's surface at the equator and at vernal equinox, based on the model of Cockell et al. [2000]) of polychromatic UV radiation at the Martian range (200-400 nm) (Billi et al., 2011), and dried multilayers of *Chroococcidiopsis* spp. CCMEE 029 and 057 survived exposure to $1.5 \times 10^3\text{ kJ/m}^2$ (roughly equivalent to one sol on Mars) of polychromatic UV when mixed with anorthosite (a dominant mineral on the Moon), for a total thickness (3–4 cell layers plus minerals) of about 15 μm (Baqué et al., 2014). A thin layer of minerals, translucent enough to let in photosynthetically active radiation, might thus allow survival without preventing photosynthesis. Besides, an ability to survive exposure to a Mars-like UV flux for a few minutes, without any protection, could favor wind-driven dissemination (Cockell et al., 2005).

Several species of cyanobacteria also demonstrated a high resistance to the most damaging part of the Martian UV spectrum: UVC radiation, from which the Earth's surface has been protected since the formation of an ozone layer (the approximate fluence rates for UVA [400–320 nm], UVB [320–280 nm], and UVC [280–200 nm] on Mars have been proposed to be around 40, 8, and 3 W/m², respectively, on the equator at midday and at the mean orbital distance from the Sun [Cockell et al., 2000; Schuerger, 2015]). *Anabaena variabilis* ATCC29413 and *Anabaena* sp. PCC7120, for instance, have an F₁₀ (fluence which inactivates 90% of the population) of approximately 750 J·m⁻² (Levine and Thiel, 1987), and *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7942 F₁₀ of about 500 J·m⁻² and 400 J·m⁻², respectively (Domain et al., 2004). *Chroococcidiopsis* sp. CCME 029 in suspension showed an F₁₀ of about 300 J·m⁻², and some cells survived 13 k J·m⁻² of UVC, presumably in large part due to their multicellular aggregates enveloped in thick envelopes, which was so that only attenuated UVC radiation reached the inner cells (Baqué et al., 2013a). Similarly, cells in dried multilayers of planktonic or biofilm samples of *Chroococcidiopsis* spp. CCME 029 and CCME 057 survived 10 kJ·m⁻² of UVC (Baqué et al., 2013b, 2014). For comparison, *E. coli* K12 has an F₁₀ of 50 J·m⁻² in similar conditions (Baqué et al., 2013a).

Chroococcidiopsis spp. cells also survived long-term exposure to vacuum or Mars-like atmosphere, extreme temperatures (1 hour at -25°C followed by 1 hour at +60°C) and extreme temperature cycles (2 hours at -10°C followed by 2 hours at +45°C, 66 times) (Baqué et al., 2013b, 2016; Chapters 3 and 4).

In the paragraphs above, resistance was described in terms of survival. However, cells' loss of viability does not mean that their biosignatures are not detectable. In some of the mentioned studies, macromolecules (notably DNA and various pigments) were still detectable (although affected by some factors, notably UV) under conditions that eradicated cell populations. For instance, DNA and photosynthetic pigments in multilayer dried cells of *Chroococcidiopsis* spp. CCME 057 and 029 were still detectable after 500 kJ·m⁻² of polychromatic UV under vacuum, and after 800 MJ·m⁻² if mixed with anorthosite as described above (Baqué et al., 2014). DNA and carotenoids were still detectable after 570 MJ·m⁻² of UV 200–400 nm irradiation under a Mars-like atmosphere, when mixed with analogues of Martian regolith for a total thickness of 10–15 µm (Baqué et al., 2016). Another element pointed out by such studies is that mineral environments (e.g., Martian minerals) should be taken into account when developing biosignature databases, due to both protective effects and possible overlaps of biotic and abiotic signatures (de Vera et al., 2012).

As briefly described above (and in more details in Chapter 2), ground-based simulations can give useful insights into the endurance of microorganisms and their biosignatures in extraterrestrial environments. However, it is currently impossible to accurately reproduce on the ground all the conditions found beyond Earth (Cottin et al., 2017). For instance, the complex fluxes of ionizing radiation reaching space and Mars, and the full solar irradiance (including low-wavelength UV), cannot be matched by ground-based facilities. Even more than individual factors, their combinations are extremely hard to reproduce on Earth. The latter point is critical, as such stressors can have synergistic effects (Harrison et al., 2013).

Fortunately, opportunities regularly occur for exposing microorganisms to space. The first experiments of that kind were reported as early as in the 1960s (Zhukov-Verezhnikov et al., 1962). Since then, various studies involved exposure of microorganisms in space (reviewed in Cottin et al., 2017), owing in large part to advances in crewed space exploration. Platforms for astrobiology included (among others) sounding rockets (Hotchin et al., 1967), Sputnik, Vostok and Gemini spacecraft (Hotchin et al., 1968; Taylor et al., 1974), the Apollo 16 mission on its way back to Earth (Taylor et al., 1974), the Soviet/Russian space stations Salyut-6, Salyut-7 and MIR (Boillot et al., 2002; Rettberg et al., 2002), various unmanned exposure facilities (e.g., Demets et al., 2005; Dose et al., 1995; Greenberg et al., 1995; Kahn and Stoffella, 1996), and the International Space Station (ISS) (Rabbow et al., 2012, 2014, 2017).

The first experiments mostly aimed at determining the effects of microgravity and did not show any effect of it on individual cells (Zhukov-Verezhnikov et al., 1962). Later on, a wide range of altered behavior and growth properties such as increased virulence, reduced lag phase, increased final cell population, increased productivity of secondary metabolites and increased conjugation rates have been reported in spaceflight and simulated microgravity (see, e.g., Benoit and Klaus 2007, Nickerson et al. 2000, and Wilson et al. 2007), in various prokaryotes including cyanobacteria (Wang et al., 2006, 2004; Xiao et al., 2010). A well-supported hypothesis suggests that these effects are motility-dependent, with non-motile cells being the most affected. This might be explained by the reduced flow of metabolites and nutrients and by the reduced exchanges between bacteria and the environment (e.g., because mass-driven convection does not occur), which result in a

modified chemical environment around cells that alters biological responses (Horneck et al. 2010).

Soon after the first (microgravity-related) microbiology experiments in space, microorganisms were exposed to space radiation on sounding rockets, suggesting that life could survive the extreme environment of space (Hotchin et al., 1967). In the half-century that followed, the effects of space radiation, vacuum, and Mars-like atmosphere on microorganisms' survival and biosignatures, were investigated. Most of the organisms tested so far are anhydrobiotes (organisms that can survive desiccation by entering a dormant state upon drying, and resume metabolism upon rehydration), due to the instability of liquid water under space or Mars conditions (although, as mentioned above, some microgravity studies involved actively growing microorganisms; those were performed under Earth-like pressure). A wide variety have been exposed to space, including bacteria (bacterial spores and non-sporulating bacteria, among which cyanobacteria), fungi, lichens, and tardigrades.

Exposure of cyanobacteria in space (see Table 1.4) began in 1994, with a halophilic strain of *Synechococcus* sp. inhabiting gypsum-halite crystals. This cyanobacterium was exposed for two weeks onboard ESA's BIOPAN-I facility, to test whether gypsum-halite (which attenuates UV) would allow a large (with respect to species tested until then) fraction to survive space vacuum and radiation, which appeared to be the case (Mancinelli et al., 1998). The same strain was later sent to space for a longer period of time (close to 2 years), on EXPOSE-R (Rabbow et al., 2014). There, samples survived with a high rate (circa 90% of laboratory controls) when kept in the dark, and to some extent under attenuated (down to 1% or 0.1%) UV, but not under unattenuated UV (Mancinelli, 2015).

During BIOPAN VI, akinetes of *Anabaena cylindrica* were dried onto limestone rocks and sent into LEO for 10 days, where they survived only if not exposed to UV. During the same space mission, endolithic and endoevaporitic communities including cyanobacteria were exposed to space for 10 days within their natural rock substrate. There as well, only some cells not exposed to UV grew after the experiment. They belonged to the genus *Anabaena* (de la Torre et al., 2010).

During the EXPOSE-E mission (Rabbow et al., 2012), a natural phototroph biofilm including the cyanobacterium *Gloeocapsa* sp., and augmented with akinetes of *Anabaena cylindrica* and vegetative cells of *Nostoc commune* and *Chroococcidiopsis* sp. CCME 029, was exposed to space for 548 days. When

shielded from UV, all mentioned cyanobacteria except for *Nostoc commune* survived. When exposed to unattenuated UV, only *Chroococcidiopsis* sp. survived (Cockell et al., 2011). *Chroococcidiopsis* sp. CCME 029 survived once again in space, for 22 month and within impact-shocked gneiss, during EXPOSE-R (Bryce et al., 2014). The resistance of *Nostoc* sp. HK-01 is currently being tested in space, as part of the Japanese Tanpopo space mission (Kimura et al., 2016).

While one could assume that all those experiments brought most of the insights the astrobiology community needs for answering its most burning questions, and that new space exposure experiments would be redundant, this is not the case. The effects of individual environmental factors can be difficult to isolate, and sometimes to differentiate from stresses induced by the logistics of spaceflight (long-term storage in non-optimal conditions, vibrations, shocks and accelerations during takeoff and landing, etc.), especially given the constraints of microbiology work in space (equipment and sample number constrained by weight, volume and safety restrictions, limited time and microbiology training of astronauts, and so on). Experiments need to accommodate harsh dimension limitations, meaning that only a restricted number of parameters can be tested at a time. Besides, new discoveries (e.g., the effects of Mars-like minerals on microbial Raman spectra) drive new questions. New missions build upon the previous ones and upon discoveries

Table 1.4. Space exposure missions including cyanobacteria and performed (or started) prior to EXPOSE-R2. See text for details.

Cyanobacterium species	Mission	References
<i>Synechococcus</i> sp.	BIOPAN-I	Mancinelli et al., 1998
<i>Synechococcus</i> sp.	EXPOSE-R	Mancinelli, 2015
<i>Anabaena cylindrica</i> (akinetes)	BIOPAN VI	de la Torre et al., 2010
Various (part of endolithic and endoevaporitic communities)	BIOPAN VI	de la Torre et al., 2010
Phototroph biofilm including <i>Gloeocapsa</i> sp., augmented with <i>Anabaena cylindrica</i> (akinetes), <i>Nostoc commune</i>, and <i>Chroococcidiopsis</i> sp.	EXPOSE-E	Cockell et al., 2011
<i>Chroococcidiopsis</i> sp.	EXPOSE-R	Bryce et al., 2014
<i>Nostoc</i> sp.	Tanpopo	Kimura et al., 2016

made since them, and benefit from technology development. Each one of them gives new insights, but we still lack a clear overview and more experiments beyond Earth are needed.

One of the most recent opportunities from exposing microorganisms to LEO was the EXPOSE-R2 mission (Rabbow et al., 2017). It took advantage of both ground-based facilities (at DLR's MUSC) and LEO facilities (the European Space Agency's EXPOSE-R2 hardware, on the URM-D external platform of the Russian Svezda module, outside the ISS) (Figure 1.4). This equipment allowed investigators to expose samples to extreme temperature cycles, vacuum or Mars-like atmosphere, and UV at the ranges found in space or on Mars. LEO facilities also included space factors not simulated on the ground such as intense (compared to Earth's surface) ionizing radiation. The mission gathered about 600 biological samples including, as part of two



Figure 1.4. Location of the EXPOSE-R2 hardware, on the external platform of the Svezda module of the International Space Station. On the right are solar panels, attached on their left side to the Station's main structure. The Earth and Sun are visible in the background. Image: NASA. Available at expose.cnes.fr (consulted on February 28th, 2018).

distinct experiments (BOSS and BIOMEX), three strains of *Chroococcidiopsis* (CCMEE 029, 057, and 064) from the laboratory of Pr. Daniela Billi. The BOSS (Biofilm Organisms Surfing Space) experiment was designed to test the hypothesis that the biofilm lifestyle is better suited to support long-term survival under space and Martian conditions than the planktonic one. The BIOlogy and Mars EXperiment (BIOMEX) project aims at investigating the resistance of selected extremophiles (mixed with Moon or Mars regolith simulants), and the stability/degradation of their macromolecules, when exposed to space and Mars-like conditions (de Vera et al., 2012).

Aim of Part I and chapters' description

The main purpose of the work presented in this part is to characterize the resistance of cyanobacteria—using, mostly, *Chroococcidiopsis* spp. as model organisms—and their biomarkers to conditions found beyond Earth, mostly in space and on Mars. It was mostly performed to help assessing the possibility to detect life—extinct or extant—on the red planet, in spite of the harsh conditions that limit habitability and the preservation of biosignatures.

To do so, we exposed cyanobacteria to space, Mars, and Moon conditions, simulated or not, using both ground-based facilities and an exposure platform in low Earth orbit. We then assessed, most notably, cell survival and the preservation of some macromolecules. The methods used for those analyses, as well as the rationale behind them, are summarized below.

The main reasons for assessing survival were refining our knowledge and understanding of i) the habitability of Mars, so as to improve our assessment of the likelihood of Martian life, ii) the timeframes of survival on Mars's surface, so as to better estimate the risk represented by microbial contaminants to search-for-life endeavors, and iii) the resistance to conditions found in interplanetary space, so as to give new elements to the debates around lithopanspermia. More generally, we aimed at better defining the limits for life in our solar system.

Defining whether a bacterial cell is dead or not is not as straightforward as it may seem at first. Membrane integrity is often seen as a defining criterion, but situations occur where cells have intact membranes and no metabolism, or vice-versa (e.g., Shi et al., 2007). Thus, although membrane integrity can give

a quick estimate of cell viability percentage, it does not firmly indicate whether populations will be able to grow and divide. We rather decided to rely on growth assays (colony forming ability assays, and in some cases growth in liquid medium), as a proxy for cells' preservation in a state where proliferation is possible: for our purposes, proliferation is the most critical consequence of survival. An ability to replicate in standard laboratory conditions may not be a perfect indicator, as stressed cells sometimes can resume growth only when in some specific conditions, which may differ significantly from optimal conditions for healthy cells (Alper & Gillies, 1958; Harris, 1963; Oliver, 2005; Panitz, Wingender, & Flemming, 2017; Stapleton, Billen, & Hollaender, 1953). Besides, given the low amounts of samples available for most tests, only a limited number of cells could be plated; a lack of colonies thus do not allow for claims of total eradication, but only indicate that cell populations were reduced by several orders of magnitude. However, replication-based assays was deemed most relevant overall given our questions and available amounts of samples.

Aside from survival, we assessed the preservation of some macromolecules, mostly DNA and pigments.

DNA preservation was investigated for three main reasons. First, it is often a limiting factor for microbial survival in extraterrestrial environments: desiccation, UV, ionizing radiation, and other factors can cause lethal levels of DNA damage (e.g., Horneck et al., 2010). Second, it is considered an important biomarker (e.g., Carr et al., 2013) given its universality among life on Earth, its highly unlikely formation by non-biological means, and its instability after cell death (Pääbo et al., 2004) which would make it a sign of extant or recently extinct life (Parnell et al., 2007). Third, DNA amplification-based methods are the most widely used for microbial life detection in extreme terrestrial environments, and various instruments are being developed for DNA detection on Mars (Carr et al., 2013b; Isenbarger et al., 2008). Assessing its preservation under extraterrestrial conditions can thus help estimate both the chances of detecting DNA from potential life on Mars and the risk represented by microbial contaminants.

Our choice method for assessing DNA damage was based on quantitative PCR (qPCR). The principle is that a lesion in the targeted DNA sequence will block the progression of the polymerase on the template, preventing its amplification (Ponti et al., 1991; Ayala-Torres et al., 2000). Using a known amount of starting DNA, a standard curve for relating amplification data to initial template numbers, and standard qPCR calculations, we can thus estimate the number of amplifiable (undamaged) templates per mass unit of

DNA, and thus obtain semi-quantitative assessments of DNA damage resulting from exposure to test conditions. While other methods aimed at characterizing DNA damage may have been insightful, for instance to characterize base modifications, the high sensitivity of qPCR was needed for our experiments: we often could not obtain more than a few tens of ng, or even a few ng, of DNA per sample.

Unfortunately, qPCR did not give reliable results with samples mixed with Moon or Mars regolith analogues. A likely explanation is the adsorption of DNA to minerals (especially clay) after extraction (see for instance Direito et al., 2012), which interfered with qPCR. For those samples (as well as for a few others, for instance to confirm qPCR results), we used random amplified polymorphic DNA (RAPD) assays. While less quantitative than qPCR, those tests can be used to avoid its pitfalls in samples containing PCR inhibitors (e.g., Atienzar et al., 2002).

Pigment preservation was assessed due to pigments' relevance as biomarkers. Indeed, although a wide variety of highly sensitive and discriminatory tests have been suggested for the detection of biosignatures, including for instance mass spectrometry (Li et al., 2015; McKay et al., 2013), optical spectroscopy (Edwards et al., 2014; Preston et al., 2014), PCR and/or sequencing (Carr et al., 2013a; Isenbarger et al., 2008), and immunoassays (Parro et al., 2011; Sims et al., 2005), such methods can be hard to implement quickly and over extended geographical areas (Weinstein et al., 2008). As a consequence, excitation and detection of biomolecule autofluorescence has been proposed as a way of quickly surveying target locations and sorting samples, before using more discriminatory instruments which require sample preparation, take more time and require higher amounts of consumables (Dartnell and Patel, 2014). Fluorescence-based systems have been widely tested on Earth for the detection of microorganisms and biomolecules, including in harsh natural environments (Groemer et al., 2014; Storrie-Lombardi and Sattler, 2009; Weinstein et al., 2008). Assessing pigments' stability under different extraterrestrial conditions is of high interest for validating their use as biosignatures.

As mentioned above, *Chroococcidiopsis* spp. contain pigments which are highly relevant in this context. First, photosynthetic pigments: phycobiliproteins and chlorophyll *a*. Those are interesting targets due to their high fluorescence (Parnell et al., 2007; Dartnell and Patel, 2014; Keränen et al., 1999) and wide distribution: phycobiliproteins are found in cyanobacteria and some algae, primarily rhodophytes and cryptophytes, while chlorophyll *a*

is present in most photosynthetic organisms. Second, beta-carotene, which is widespread among Earth's biological organisms.

In order to assess the preservation of photosynthetic pigments' autofluorescence, we relied on confocal laser scanning microscopy. This allowed us to obtain 3-dimensional information on fluorescence preservation, which enabled us to observe fluorescence on specific, spatially restricted zones. It was particularly relevant to multilayered samples and samples mixed with regolith, as we could thus determine the evolution of fluorescence with depth and around mineral grains.

For assessing the preservation of beta-carotene, we instead relied on Raman spectroscopy. This technology is highly relevant to the search for life as i) it is nondestructive, ii) no sample preparation is needed, iii) it can be used at either close or long distances (micro- and macro-modes), iv) it is very fast (down to a fraction of a second per spectrum), and v) it allows for unambiguous identification of some materials (Rull et al., 2017). Beta-carotene is a particularly relevant target due to its strong and unique Raman spectrum (Böttger et al., 2012; Vítek et al., 2009a). Since, as mentioned above, both ExoMars and Mars 2020 rovers will carry Raman spectrometers, the BIOMEX consortium is assessing the stability and detectability of beta-carotene's Raman spectra under Mars-like conditions (de Vera et al., 2012). Our Raman results were not included in this thesis, but the interested reader is referred to a joint paper, in preparation at the time of writing, gathering results from the whole consortium.

We had the opportunity—as discussed above—to include samples in two experiments of the EXPOSE-R2 space mission: BOSS and BIOMEX.

In the BOSS project, we used biofilm and planktonic samples of two strains of *Chroococcidiopsis*: *Chroococcidiopsis* sp. CCME 029 (N6904), isolated from cryptoendolithic growth in sandstone, Negev Desert, Israel, and CCME 057 (S6e), isolated from chasmoendolithic growth in granite, Sinai Desert, Egypt. Biofilm samples of *Chroococcidiopsis* CCME 064 (S8c), isolated from hypolithic growth under stones of desert pavement in the Sinai Desert, were also included, but not their planktonic counterparts due to payload limitations. In the BOSS project, we used planktonic samples of *Chroococcidiopsis* spp. CCME 029, mixed with minerals.

Each space mission is a unique opportunity, so various tests (including simulations of the space mission) were performed on the ground in the preceding years to test handling procedures, analysis protocols, hardware integration and performances, and samples' preparation and resistance. In addition to validating our intended process, those so-called experiment



Figure 1.5. EXPOSE-R2 outside the International Space Station, during extravehicular activities. A cosmonaut and the Earth are visible in the background. Image credits: NASA.

Available at eea.spaceflight.esa.int/attachments/spacestations/545a48ba741bf.jpg (consulted on February 27th, 2018).

verification tests (EVTs) and science verification tests (SVTs) yielded new insights into the impact of space and Mars environments on *Chroococcidiopsis* spp. Some of the results from those ground-based simulations were gathered in Chapter 2. For more details, the interested reader is referred to Baqué et al. (2013b, 2013c, 2014, 2016).

One of the limitations of space missions (such as EXPOSE-R2) is that they do not allow for exposure to doses of ionizing radiation corresponding to space or Mars exposure over timeframes longer than the mission itself. This is critical given that the effects of such radiation on cyanobacteria become detectable only at very high doses (that would need exposure times longer than the cumulative duration of all astrobiology experiments performed in space so far) and that ionizing radiation can penetrate the Martian ground much deeper than UV does, thereby potentially affecting microorganisms shielded from UV. There has been quite a few studies on the effects of ionizing radiation on cyanobacteria in the past decades, mostly using photon (gamma and x-ray) irradiation (see above); however, a given dose of ionizing radiation can have different effects on microorganisms if it comes from different kinds of particles. One key parameter of ionizing radiation is its linear energy transfer (LET): the amount of energy transferred to a material when traveling a given distance through it. Besides, how damaged a microbial population will be by a given radiation flux depends on its physiological state, notably whether it is metabolically active or dormant. As part of a project called STARLIFE, involving a large consortium of laboratories, we exposed *Chroococcidiopsis* cells, hydrated or dried, to various kinds of radiation ranging from gamma rays (LET: 0.2 keV/ μ m) to Fe nuclei (LET: 198 keV/ μ m). Some results from those experiments are described in Chapter 2; for more information, the reader is referred to Verseux et al. (2017).

Following the successful EVT and SVT, the EXPOSE-R2 space mission started. Samples took off to the International Space Station on July 23rd, 2014 from Baikonur (Kazakhstan) onboard the Progress cargo spacecraft 56P. Later, it was installed on the outside platform of the Russian Zvezda module. Samples exposed in LEO (Figure 1.5) stayed for close to 18 months outside the International Space Station, including more than 15 months when samples in upper carriers were also exposed to UV. Samples in “Space” conditions were exposed to vacuum for 22 months, and those in “Mars” conditions were exposed to a Mars-like atmosphere for close to 2 years (times of exposure to vacuum and Mars-like atmosphere differ from exposure time in LEO because the Mars-like atmosphere was filled on the ground, and because samples were

exposed again to Earth atmosphere only some time after landing). Ground reference samples were exposed to conditions mimicking exposure in LEO, based on available information and as far as technically possible. Additional samples were stored in the dark, under ambient laboratory conditions. Due to time periods between sample preparation and mission start, and between mission completion and sample analysis, samples remained in a dry state for 2.5 years before analysis. Results from the BOSS experiment are described in Chapter 3, and those from the BIOMEX experiment in Chapter 4.

The most important results and implications from those studies, together with those from the work presented in Part II of this thesis, are summarized in section “Synthesis and conclusions”.

Chapter 2

Survival and biosignature detection of *Chroococcidiopsis* spp. after ground-based simulations of extraterrestrial environments¹

This chapter presents some of the results obtained after exposing *Chroococcidiopsis* spp. to ground-based simulations of space, Mars and Moon environments. It gathers selected data from verification tests performed in preparation for the BIOMEX space mission, as well as from the “Starlife” series of experiments with ionizing radiation. For more data from those studies, the reader is referred to Baqué et al. (2014, 2016) and Verseux et al. (2017).

In preparation for BIOMEX, *Chroococcidiopsis* cells, mixed or not with analogues of Mars or Moon regolith, were exposed to fractionated fluencies of polychromatic UV, UVC (254 nm), space vacuum, and/or a simulated Martian atmosphere. Cells and biomarkers showed an overall high resistance, particularly so when mixed with minerals for a total thickness of circa 15 µm or less. In particular, DNA and pigments were still detectable after exposure to conditions expected during the EXPOSE-R2 experiment.

As part of the Starlife project, *Chroococcidiopsis* cells were exposed in both hydrated and dried states to ionizing radiation fluxes with different linear energy transfer values (0.2 to 200 keV/µm). Irradiation with up to 1 kGy of He or Si ions, 2 kGy of Fe ions, 5 kGy of X-rays, or 11.59 kGy of gamma rays

¹This chapter is based on selected data from three articles:

- Baqué, M., Verseux, C., Rabbow, E., de Vera, J.-P.P., and Billi, D. (2014). Detection of macromolecules in desert cyanobacteria mixed with a lunar mineral analogue after space simulations. *Orig. Life Evol. Biosph.* *44*, 209–221.
- Baqué, M., Verseux, C., Böttger, U., Rabbow, E., de Vera, J.-P.P., and Billi, D. (2016). Preservation of biomarkers from cyanobacteria mixed with Mars-like regolith under simulated Martian atmosphere and UV flux. *Orig. Life Evol. Biosph.* *46*, 289–310.
- Verseux, C., Baqué, M., Cifariello, R., Fagliarone, C., Raguse, M., Moeller, R., and Billi, D. (2017). Evaluation of the resistance of *Chroococcidiopsis* spp. to sparsely and densely ionizing irradiation. *Astrobiology* *17*, 118–125.

(^{60}Co) did not eradicate *Chroococcidiopsis* populations; neither did it induce detectable damage to DNA or plasma membranes.

Data gathered during those ground-based simulations will help in the interpretation of results from space experiments (such as those presented in Chapters 3 and 4) and are relevant to the search for life on Mars and other planetary bodies of interest.

Introduction

On July 24th, 2014, the Progress 56 cargo spacecraft left Earth. It carried the EXPOSE-R2 platform, itself containing *Chroococcidiopsis* cells. Results from the successful space mission that ensued are presented in Chapters 3 and 4.

Given that each space mission is unique and the amounts of material that can be sent upward are extremely limited, various tests were performed on the ground in the preceding years to test handling procedures, analysis protocols, hardware integration and performances, and samples' preparation and resistance. Besides their preparatory purposes, those tests were an opportunity to yield insights on the resistance of *Chroococcidiopsis* cells, as well as their potential biomarkers, under conditions found beyond Earth. The rationale for such investigations is described in Chapter 1.

Some of those tests aimed at reproducing the conditions expected in the Mars simulations planned in low Earth orbit, as part of the BIOMEX experiment. For those assays, dried cells of *Chroococcidiopsis* sp. CCME 029 were mixed with two Martian mineral analogues, Phyllosilicate Mars Regolith Simulant (P-MRS) and Sulfatic Mars Regolith Simulant (S-MRS), which are simulants of minerals formed during the Noachian (Early Mars) and Hesperian/Amazonian (Late Mars) periods, respectively (Bibring et al., 2006; Böttger et al., 2012; Ehlmann and Edwards, 2014; Mustard et al., 2008; de Vera et al., 2012). Cells mixed with minerals were then exposed to a Mars-like atmosphere in combination or not with 570 MJ/m² of polychromatic UV radiation (200-400 nm), a dose simulating that expected during a one-year exposure mission in low Earth orbit (without the neutral density filters used during EXPOSE-R2), or 380 sols on the Martian surface (Cockell et al., 2000; Dachev et al., 2014; Rabbow et al., 2012, 2014). Cells without minerals were exposed to similar conditions but with UV attenuated down to 0.1% by neutral

density filters, as was finally selected for the experiment in low Earth orbit (see Chapters 3 and 4). We then assessed survival, detectability of biosignatures from photosynthetic pigments (chlorophyll *a* and phycobiliproteins) and DNA, and the protective effect of Mars mineral analogues. Our main motivation (besides preparing for the EXPOSE-R2 mission) was to contribute to the search for traces of life forms naturally present on Mars, but these biosignatures might also be useful for the detection of microbial contamination of Mars caused by human activity. Such contamination might come from spacecraft contaminated on the ground (COSPAR, 2011) or, in a more distant future, leak from a biological life support system (see Verseux et al. 2016a). As a secondary objective, we also assessed the survival of *Chroococcidiopsis* spp. under space- and Mars-like stress factors, which brings elements to the debate around the lithopanspermia theory (for a review, see Nicholson 2009) and Mars's habitability.

Other tests were performed under space- and Moon-like conditions, again in preparation for BIOMEX. Dried cells of two desert strains of *Chroococcidiopsis*, namely CCMEE 029 and CCMEE 057, were mixed with a lunar regolith analogue (anorthosite) and exposed to either monochromatic UVC radiation, polychromatic UV radiation, or a combination of vacuum and UV radiation. While our closest celestial neighbor is not often associated with astrobiology studies, it could be a valuable testing ground for the search for life on Mars. Indeed, simulating the red planet's surface is still highly challenging: these include low atmospheric pressure and temperatures (resulting in liquid water instability), lower gravity than Earth's, and a lack of both a magnetic field and an ozone layer allowing harmful—in a biological sense—UV and ionizing radiation to reach the surface (Cockell et al., 2000; Patel et al., 2004). Mars analogues on Earth regarding extreme temperatures and prolonged absence of liquid water have been identified in hot and cold deserts such as the Dry Valleys in Antarctica and the Atacama desert in Chile, where life takes refuge within or under rocks (Bahl et al., 2011; Friedmann, 1980), but no terrestrial analogue exists with an atmospheric pressure, gravity and radiation comparable to that of Mars. In this context, the Moon could prove useful: lacking an atmosphere and being outside the influence of the Earth's magnetosphere, its surface is subject to radiation similar to that on Mars and could therefore represent a test platform for instrumentation and life resistance studies (Carpenter et al., 2012; de Vera et al., 2012). More generally, the Moon is considered a valuable site to address a wide range of life science and astrobiology questions dealing with i) the habitability of the Earth through time, ii) the possibility of life elsewhere in the universe, and iii)

advances in human exploration and settlement of space (Crawford et al., 2012). In addition, the potential existence of preserved organic molecules in lunar ice, which could yield valuable clues about the origins of life (Schulze-Makuch, 2013), will be the focus of future analyses and require the development of life detection technologies in the Lunar environment. Finally, the search for biogenic material transferred from the Early Earth to the Moon (Armstrong et al., 2002), due to asteroid impacts, could also be worth conducting on the Lunar surface.

In the experiments performed in preparation for EXPOSE-R2, ionizing radiation was not reproduced since i) it is technologically challenging, and ii) the dose received during a mission of about two years is quite low when compared to cyanobacteria's resistance to them. However, it is interesting to test the effects of ionizing radiation over longer timeframes, as it can affect the survival of dormant organisms and degrade biomarkers. There has been quite a few studies on the effects of ionizing radiation on cyanobacteria in the past decades, mostly using photon (gamma and X-ray) irradiation (see Chapter 1); however, particles in the radiation flux have different masses, energies and charges, so damaging organisms in different ways (Dartnell, 2011; Horneck et al., 2010). An indicator of a particle's effects on organisms is its linear energy transfer (LET), the amount of energy transferred to a material when travelling a given distance through it (Moeller et al., 2010). We consequently assessed the resistance of dried and liquid samples of *Chroococcidiopsis* spp. to photon and ion radiation, with various LET values. This study was part of the "Starlife" series of experiments (Moeller et al., 2017).

Taken as a whole, results from those different studies point out the resistance of *Chroococcidiopsis* spp. to conditions found beyond our atmosphere, and confirm the relevance of the materials and methods foreseen for the EXPOSE-R2 mission.

Material and Methods

Cyanobacterial strains and culture conditions

Chroococcidiopsis spp. CCME 029 and CCME 057 were isolated by R. Ocampo-Friedmann from, respectively, cryptoendolithic growth in sandstone in the Negev Desert (Israel) and chasmoendolithic growth in granite in the Sinai Desert (Egypt). *Synechocystis* sp. PCC6803 (hereafter referred to as *Synechocystis*) was purchased from the Pasteur Culture Collection of Cyanobacteria. All strains were grown under routine conditions at 25°C, in BG11 medium, under a photon flux density of 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by fluorescent cool-white bulbs with a 16h/8h light/dark cycle.

Sample preparation

For experiments performed in preparation for BIOMEX, multilayered planktonic samples were obtained by plating, on top of BG-11 agarized medium, pellets from 2-month-old liquid cultures (approximately 2×10^9 cells) mixed (or not) with circa 0.2 g of mineral analogues of either Mars regolith (for samples belonging to the series of experiments hereafter referred

Table 2.1. Mineralogical composition of the S-MRS and P-MRS analogues of Martian regolith. The importance of each component is given, for both P-MRS and S-MRS, in weight percent of the analogue (modified from Böttger et al. 2012).

Component	P-MRS (wt %)	S-MRS (wt %)
Gabbro (pyroxene, plagioclase, amphibole, ilmenite)	3	32
Olivine - $(\text{Mg},\text{Fe})_2\text{SiO}_4$	2	15
Quartz - SiO_2	10	3
Hematite - Fe_2O_3	5	13
Montmorillonite - $(\text{Na},\text{Ca})_{0.33}(\text{Al},\text{Mg})_2\text{Si}_4\text{O}_{10}(\text{OH})_2 \cdot \text{H}_2\text{O}$	45	-
Chamosite - $(\text{Fe}^{2+},\text{Mg},\text{Fe}^{3+})_5\text{Al}(\text{Si}_3\text{Al})\text{O}_{10}(\text{OH},\text{O})_8$	20	-
Kaolinite - $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$	5	-
Siderite - $\text{Fe}(\text{CO}_3)$	5	-
Hydromagnesite - $\text{Mg}_5(\text{CO}_3)_4(\text{OH})_2 \cdot 4 \text{ H}_2\text{O}$	5	-
Goethite - $\text{FeO}(\text{OH})$	-	7
Gypsum - $\text{CaSO}_4 \cdot 2 \text{ H}_2\text{O}$	-	30

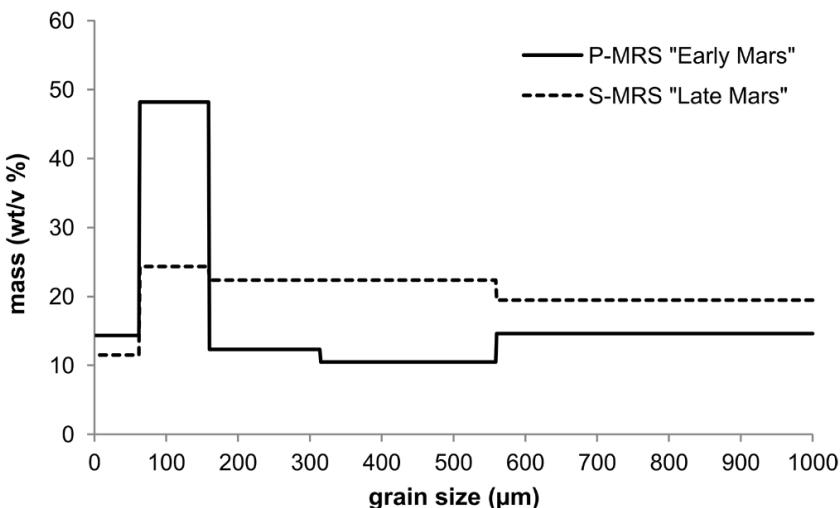


Figure 2.1. Size distribution of the mineral mixtures determined by sieving. P-MRS has a smaller grain size, with a large fraction below 200 μm while S-MRS grains are more evenly distributed in the 0–1 mm range.

to as “Mars series”, and corresponding to work published in Baqué et al., 2016) or Moon regolith (“Moon series”, corresponding to work published in Baqué et al., 2014). The mineral mixtures used as Mars regolith simulants were the Phylosilicate Mars Regolith Simulant (P-MRS) and Sulfatic Mars Regolith Simulant (S-MRS). Their composition is given in Table 2.1. They were prepared by the Naturkunde-Museum (Berlin) in the framework of the Helmholtz-Alliance “Planetary Evolution and Life”. Size distribution of the two mineral mixtures is reported in Figure 2.1. The Moon regolith simulant was anorthosite from the Ukrainian shield (Korosten Pluton, Zhytomyr region; Mytrokhyn et al., 2003). Samples were allowed to dry before cutting disks to the size of the exposure carrier cells ($\sim 110 \text{ mm}^2$) under sterile conditions.

For irradiation assays performed in the framework of Starlife, approximately 1.5×10^8 cells were collected from cultures in the early stationary phase. Dried samples were prepared by filtering cells on Millipore filters and air-drying them overnight in a sterile hood, while liquid samples were prepared by resuspending cells in 50 μL of BG11.

Table 2.2. Parameters obtained using the Planetary and Space Simulation facilities at DLR (Cologne, Germany) for ground-based simulations preformed in preparation for BIOMEX, and reported in this chapter (modified from Baqué et al., 2014).

ATMOSPHERE	MINERALS	UV IRRADIATION
“Mars” series		
Mars-like atmosphere* for 89 days.	P-MRS, S-MRS, or none	None.
Mars-like atmosphere* for 89 days.	None.	Polychromatic UV (200-400 nm) attenuated with 0.1% neutral density filter. Dose: 570 kJ/m².
Mars-like atmosphere* for 89 days.	P-MRS or S-MRS.	Polychromatic UV (200-400 nm), non-attenuated. Dose: 570 MJ/m².
“Moon” series		
Ambient	Anorthosite, or none.	UVC (254 nm). Doses: 10 J/m², 100 J/m², 1000 J/m², 10000 J/m².
Ambient	Anorthosite, or none.	Polychromatic UV (200-400 nm). Doses: 1.5 x 10³ kJ/m², 1.5 x 10⁴ kJ/m², 1.5 x 10⁵ kJ/m², 5.0 x 10⁵ kJ/m², 8.0 x 10⁵ kJ/m².
Vacuum (2 x 10⁻⁴ Pa) for 89 days	Anorthosite, or none.	None.
Vacuum (2 x 10⁻⁴ Pa) for 89 days	None.	Polychromatic UV (200-400 nm) attenuated with 0.1% neutral density filter. Dose: 5 x 10² kJ/m².

*Composition: 95.55 % CO₂, 2.70 % N₂, 1.60 % Ar, 0.15 % O₂, ~ 370 ppm H₂O. Pressure: 780 Pa.

Test facilities and exposure conditions

Preparatory experiments for BIOMEX were performed using facilities of the Planetary and Space Simulation (PSI) at the DLR-Institute of Aerospace Medicine in Cologne (Germany). An overview of exposure conditions is given in Table 2.2. Laboratory controls were kept at DLR in the dark, at room temperature.

Samples from the “Mars” series of experiments were exposed, as part of the Scientific Verification Tests (SVTs), to a simulated Martian atmosphere (95.55 % CO₂, 2.70 % N₂, 1.60 % Ar, 0.15 % O₂, ~ 370 ppm H₂O, Praxair Deutschland GmbH) to a final pressure of 780 Pa for 89 days, in combination or not with polychromatic UV (200-400 nm) radiation produced by the solar simulator SOL2000. The dose of 570 MJ·m⁻² was reached by running the SOL2000 for 125 hours at 1,271 W·m⁻². Neutral density filters attenuating 99.9 % of the radiation spectrum (0.1% ND filters) were used for samples exposed without minerals. Irradiation was performed at intervals during working hours to allow constant monitoring of the temperature during irradiation, to avoid the risk of sample heating. Temperature varied from -25°C to +30°C throughout the experiment but remained below 10°C during irradiation times. Laboratory controls were kept at DLR in the dark, at room temperature.

Samples from the “Moon” series of experiments were exposed, as part of the SVTs, to vacuum (2×10^{-4} Pa) for 89 days, in combination or not with

Table 2.3. Parameters of the four irradiation series reported in this study. All irradiation sessions were performed as part of the “Starlife” project, except for irradiation with Si ions. C: *Chroococcidiopsis*; S: *Synechocystis*. LET: linear energy transfer.

Series	Strain			State		Irradiation (highest dose, in kGy) [LET, in keV/ μ m]*					
				Liquid	Dried	Heavy ions			Photons		
	C 029	C 57	S 6803			He [2.24]	Fe (2) [198]	Si (1) [50]	X-rays (5) [0.3-3.0]**	γ -rays (113.25) [0.2]**	
1	x	x	x		x	x	x		x		
2	x	x			x	x	x		x		
3	x	x		x		x		x	x		
4	x	x	x	x	C only						x

* Values from Moeller et al. (2010; 2017).

** From secondary electrons

polychromatic UV irradiation. The dose of 500 kJ/m² was reached by running the SOL2000 for 99 hours at 1,370 W/m², with 0.1% ND filters. Within the first part of the Experiment Verification Tests (EVT 1), samples were exposed to doses of polychromatic UV irradiation ranging from 1.5 to 800 MJ·m⁻² using the SOL2000 at 1,370 W·m⁻². Within the second part (EVT 2), samples were exposed to doses of UVC (254 nm) ranging from 10 to 10,000 W·m⁻², using an Hg low-pressure lamp at 80 mW·cm⁻².

EVTs involved *Chroococcidiopsis* spp. CCME 029 and 057 and were performed in triplicates. However, SVTs involved only *Chroococcidiopsis* sp. CCME 029 and, since the accommodation plan was as scheduled for the EXPOSE-R2 platform, allowed for only one replicate per sample (de Vera et al., 2012).

Ionizing irradiation experiments were performed as part of the “Starlife” series of experiments (Moeller et al., 2017), except for irradiation with Si ions. They were conducted over 4 series, whose parameters are summarized in Table 2.3. X-ray irradiation was performed using a Gulmay RS225 irradiation cabinet (Gulmay Medical Ltd.) at the German Aerospace Center (DLR) in Cologne, Germany, with a dose rate of 30 Gy/min, at room temperature. Heavy ion irradiation was performed using the Heavy Ion Medical Accelerator in Chiba (HIMAC), at the National Institute for Radiological Sciences in Chiba, Japan, with dose rates of 4.2 Gy/min (He), 4-5 Gy/min (Si) or 12.1 Gy/min (Fe) at 23±1°C. Gamma irradiation was generated using a C-188 ⁶⁰Co source provided by Beta-Gamma-Service in Wiehl, Germany, with a dose rate of 100 Gy/min, at room temperature.

Survival

Samples were serially diluted and spread on BG11-agar plates with up to 10⁸ cells per plate, without disrupting the cell clusters naturally formed by *Chroococcidiopsis* spp., and any single cell or cellular aggregate was considered one colony forming unit.

Plates were incubated under routine conditions, either until colonies appeared or for five months (whichever came first). Due to aggregates formed by dried samples, only colonies from samples irradiated in the liquid state were counted.

Random amplification of polymorphic DNA (RAPD) assay

Subsamples were resuspended in 50 µL of sterile MilliQ water (after being pelleted and having the supernatant discarded, for liquid samples), washed twice by centrifuging 10 min at 10,000 rpm, and resuspended in 20 µL sterile MilliQ water. They were then subjected to three cycles of freeze-thawing (-80°C for 10 min and 60°C for 1min) and boiled for 10 min. After centrifugation, 5 µL of lysed cell suspensions were used for PCR amplification with the HIP1-CA primer (5'-GCGATCGCCA-3'). PCR conditions were as follows: 94°C for 3 min; 30 cycles at 94°C for 30 s, 37 °C for 30 s, and 72°C for 1 min; and 72 °C for 7 min. Genomic DNA from 1-month-old cultures of *Chroococcidiopsis* was used as control. PCR products were loaded on a 1.5 % agarose gel and electrophoresis was run at 90 V in TAE buffer. RAPD patterns were revealed under UV lamp with ethidium bromide staining.

Confocal laser scanning microscopy

Microscopy analyses on samples from the “Mars” series of experiments were performed using a confocal laser scanning microscope coupled with spectral analysis (CLSM-λscan). Small fragments (about 2 mm²) were put onto slides and examined using a CLSM (Olympus Fluoview 1000 Confocal Laser Scanning System). Photosynthetic pigment (chlorophyll *a* and phycobiliproteins) autofluorescence was investigated by successively exciting the samples with 543-nm and 635-nm lasers, and collecting the emitted fluorescence in the 555–609 nm and 655–755 nm channels, respectively. Mineral reflectance was recorded after excitation at 488 nm by collecting the reflective signal between 490 and 510 nm. Three-dimensional images were captured every 0.5 µm and processed with Imaris v. 6.1.0 software (Bitplane AG Zürich, Switzerland) to obtain maximum intensity projections. The spectral analysis of regions of interest (ROI) was performed using the 543-nm laser at 0.54 mW and collecting the emission from 543 to 800 nm. Mean fluorescence intensity (MFI) was measured on 15 cells or more from 3 different areas. Spectral analyses on minerals were performed using the 488 and 543-nm lasers at 1 mW and collecting the emission of ROIs from 488 to 800 nm and from 543 to 800 nm, respectively. Curve plotting and normalization were performed using the GraphPad Prism program (GraphPad Software, San Diego, CA).

Membrane damage assessment

SYTOX-Green assays were performed, in the framework of Starlife, on *Chroococcidiopsis* 029 and *Synechocystis*, non-irradiated or exposed to the highest tested doses of heavy ions and X-rays (dose values are given in Table 2.3), as well as on *Chroococcidiopsis* 029 exposed to 23.92 kGy of gamma radiation. Assays were performed by staining cells with 50 µM SYTOX-Green (Molecular Probes, S-7020) for 5 min in the dark, and acquiring images at constant exposure time, gain and offset using a Nikon TE200 inverted microscope.

Results

Survival

Dried cells of *Chroococcidiopsis* sp. CCMEE 029 survived exposure to a simulated Martian atmosphere or vacuum for 89 days, regardless of the presence of a mineral matrix, but failed to yield colonies under any of the other conditions of the “Mars” series of experiments, or after exposure without minerals to $1.5 \text{ MJ}\cdot\text{m}^{-2}$ of polychromatic UV or $5 \times 10^2 \text{ kJ}\cdot\text{m}^{-2}$ UV in combination with space vacuum. However, cells mixed with the lunar mineral analogue formed colonies after exposure to (but not after a higher dose than)

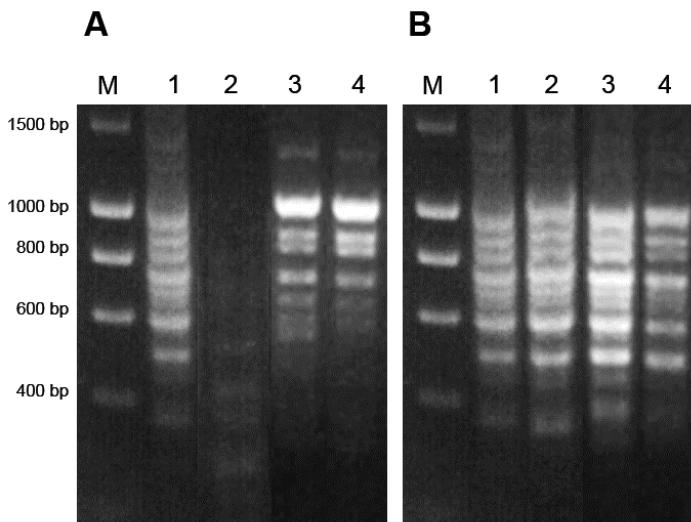


Figure 2.2. PCR-based evaluation of DNA persistence in dried *Chroococcidiopsis* under polychromatic UV and Mars-like atmosphere, after the “Mars” series of experiments, by random amplification of polymorphic DNA (RAPD) assay. A: PCR fingerprint from liquid culture (lane 1), altered fingerprint from dried cells without minerals exposed to $570 \text{ kJ}/\text{m}^2$ of UV and simulated Martian atmosphere (lane 2), and altered fingerprints from dried cells exposed to $570 \text{ MJ}/\text{m}^2$ of UV and simulated Martian atmosphere, and mixed with S-MRS (lane 3) or P-MRS (lane 4). B: PCR fingerprint from liquid culture (lane 1), and unaltered PCR fingerprint from dried cells exposed to simulated Martian atmosphere without minerals (lane 2) or in the presence of S-MRS (lane 3) or P-MRS (lane 4). Lanes M: DNA marker.

$1.5 \text{ MJ}\cdot\text{m}^{-2}$ polychromatic UV, and all samples survived exposure to all tested doses of UVC radiation (up to $10 \text{ kJ}\cdot\text{m}^{-2}$).

After irradiation with up to 1 kGy of He or Si ions, 2 kGy of Fe ions, or 5 kGy of X-rays, all *Chroococcidiopsis* samples showed colony forming abilities with a decrease in survival below one order of magnitude, even at the highest tested doses of heavy ions and X-rays (1 kGy He, 2 kGy Fe, 1 kGy Si and 5 kGy X-rays). *Synechocystis* samples failed to yield colonies even for non-irradiated controls, as expected given the desiccation sensitivity of this strain. Liquid samples of *Chroococcidiopsis* spp. CCME 029 and 057 (but not liquid samples of *Synechocystis*) formed colonies after 11.59 kGy of gamma irradiation, with a reduction in survival of approximately one order of magnitude, but not after 23.92 kGy. Dried samples of *Chroococcidiopsis* sp. CCME 057 formed colonies after exposure to 11.59 kGy of gamma irradiation but not after 23.92 kGy, and dried samples of *Chroococcidiopsis* sp. CCME 029 formed colonies after exposure to 23.92 kGy but not after 46.88 kGy.

DNA detection by PCR-based assay

RAPD profiles obtained from *Chroococcidiopsis* sp. CCME 029 exposed to the “Mars” series of experiments are shown in Figure 2.2. Positive amplification of DNA from dried cells mixed with the two Mars mineral analogues was verified for unexposed controls (not shown), even though the composition of the mineral matrix (especially clay-containing minerals such as montmorillonite in P-MRS; see Table 2.1) is known to be problematic for PCR-based amplification (Alvarez et al., 1998; Direito et al., 2012).

When dried cells without minerals were exposed to $570 \text{ kJ}\cdot\text{m}^{-2}$ of polychromatic UV radiation ($570 \text{ MJ}\cdot\text{m}^{-2}$ with 0.1% ND filter) combined with a simulated Martian atmosphere, RAPD profiles were highly modified, with only low-abundance and small-size amplicons (Figure 2.2A, lane 2). When cells mixed to S-MRS and P-MRS were exposed to $570 \text{ MJ}\cdot\text{m}^{-2}$ of polychromatic UV radiation, marked PCR fingerprints were obtained, although with altered profiles compared to the liquid control (lanes 3 and 4). On the other hand, PCR profiles virtually identical to the control were obtained from dried cells exposed to simulated Martian atmosphere for 89 days, with or without minerals (Figure 2.2B).

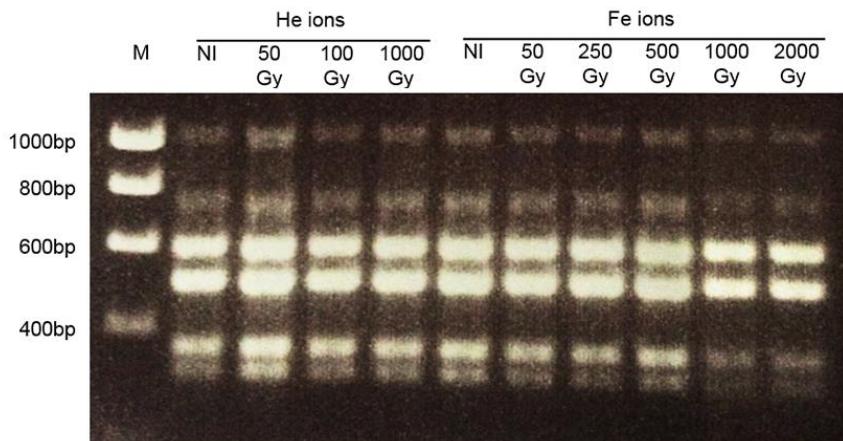


Figure 2.3. PCR-based evaluation of DNA persistence in dried *Chroococcidiopsis* sp. CCME 057, non-irradiated (NI), or exposed to doses of He ions or Fe ions up to 1 and 2 kGy (respectively), in the framework of the “Starlife” project, by random amplification of polymorphic DNA (RAPD) assay. Similarly unaltered band profiles were obtained for *Chroococcidiopsis* spp. CCME 057 and 029 exposed to all tested heavy ion and X-ray treatments (not shown). M: DNA ladder.

After ionizing irradiation series 1 to 3 of the Starlife project (see Table 2.3 for details on irradiation parameters), no DNA damage was detected in liquid or dried *Chroococcidiopsis* samples after irradiation with up to 1 kGy of He or Si ions, 2 kGy of Fe ions, or 5 kGy of X-rays, as revealed by DNA fingerprints qualitatively identical to non-irradiated controls (Figure 2.3). No DNA damage was visible either after 11.59 kGy of gamma-rays either, but some was detectable after 46.88 kGy or more, as indicated by reduced relative intensities of bands corresponding to large amplicons (Figure 2.4). As for the radiation-sensitive *Synechocystis*, DNA was well-preserved in non-irradiated, desiccated samples but highly damaged by one kGy or more of any of the tested heavy ions. The number of amplifiable targets after exposure to 11.59 kGy was reduced below detection (not shown here; see Verseux et al. 2017 for details).

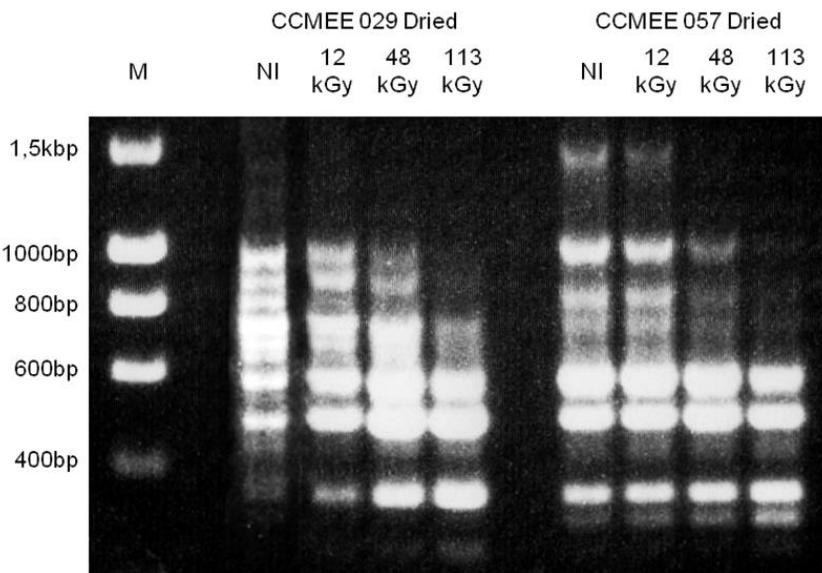


Figure 2.4. PCR-based evaluation of DNA persistence in dried *Chroococcidiopsis* spp. CCME 029 and 057, non-irradiated (NI) or exposed to γ irradiation, in the framework of the “Starlife” project, by random amplification of polymorphic DNA (RAPD) assay. Doses indicated above the lanes are those targeted prior to performing irradiation; the doses actually reaching samples were 11.59, 46.88, or 113.25 kGy. Similarly altered patterns were obtained from irradiated liquid samples (not shown). M: DNA ladder.

Emission spectra of photosynthetic pigments

CLSM imaging of dried samples of *Chroococcidiopsis* sp. CCME 029 mixed with Mars analogue minerals revealed that cells were non-homogeneously associated with S-MRS (Figure 2.5A, B) or P-MRS (Figure 2.5C, D). The experimental set-up yielded dried samples of about 10 μm , corresponding to 2-3 cell layers when mixed with S-MRS, but were significantly thicker (about 15 μm , including 4–5 cell layers) when mixed with P-MRS. This is probably due to the different grain size distribution in the two prepared analogues, P-MRS presenting thinner grains than S-MRS (Figure 2.1). After exposure to a simulated Martian atmosphere, no morphological alteration occurred in dried cells mixed with S-MRS (Figure 2.5B) or P-MRS (Figure 2.5D) compared to controls (not shown). On the other hand, exposure

to a simulated Martian atmosphere in combination with 570 MJ/m² of polychromatic UV irradiation resulted in bleached photosynthetic pigments and altered cell morphology in cells mixed with S-MRS (Figure 2.5A) or P-MRS (Figure 2.5C, upper left corner). However, cells with unaltered morphology and unbleached photosynthetic pigments were scored in samples mixed with P-MRS (Figure 2.5C, middle).

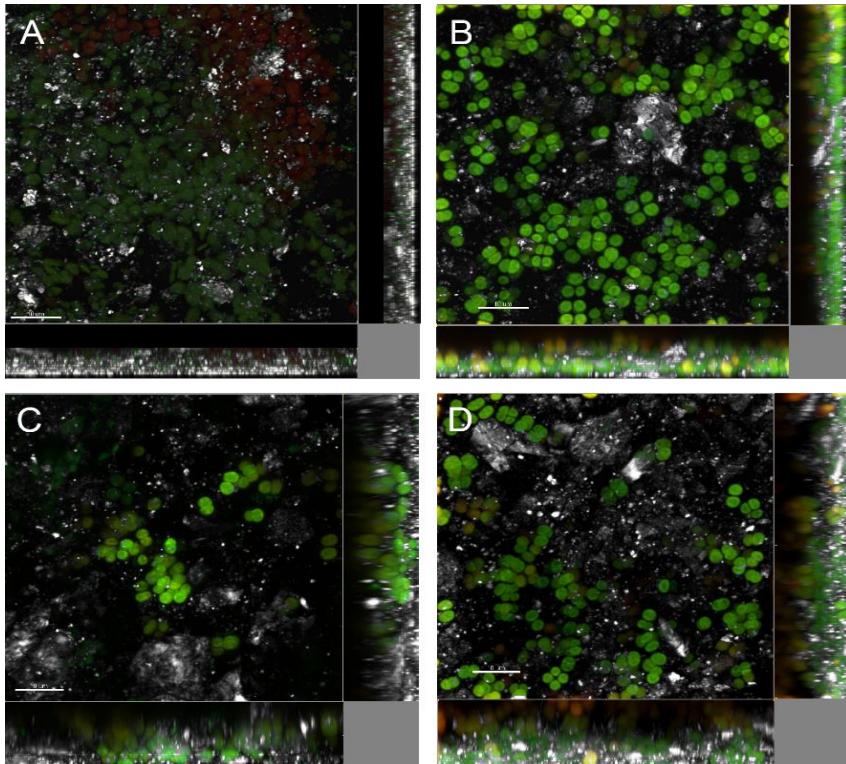


Figure 2.5. Confocal scanning laser microscope (CLSM) images with lateral projections showing mineral reflectance (white color; emission in the 490–510 nm range after excitation at 488 nm) and autofluorescence of chlorophyll *a* (emission in the 555–609 nm range after excitation at 543 nm) and phycobiliproteins (emission in the 655–755 nm range after excitation at 635 nm) of dried *Chroococcidiopsis* sp. CCMEE 029 cells mixed with S-MRS (A, B) or P-MRS (C, D), after exposure to simulated Martian atmosphere alone (B, D) or in combination with 570 MJ/m² of polychromatic UV (A, C), as part of the “Mars” series of experiments. Scale bar: 10 µm.

CLSM- λ scans with a 543-nm laser were used to reveal the effect of simulated Martian atmosphere and polychromatic UV radiation on the emission spectrum of the photosynthetic pigments of dried cells of *Chroococcidiopsis* sp. CCMEE 029 mixed with either P-MRS (Figure 2.6A) or S-MRS (Figure 2.6B). Cells from liquid cultures showed an emission spectrum with a peak at 650–660 nm due to the overlapping emission of phycobiliproteins (phycocyanin and allophycocyanin) and chlorophyll *a* (Roldán et al., 2004). The emission spectrum of dried cells without minerals was identical to that of liquid controls (not shown), whereas the emission spectrum of unexposed dried cells mixed with P-MRS or S-MRS presented an altered shape, shifted towards lower wavelengths, starting from 580 nm instead of 600 nm (Figure 2.5A, B). However, the maximum emission intensity at 653 nm was not significantly different from that of controls, with $104.1 \pm 4.1\%$ for P-MRS and $104.6 \pm 5.3\%$ for S-MRS (Table 3.2). In addition, the emission spectrum of dried cells mixed with P-MRS or S-MRS showed a peak at 563 nm that was significantly higher than that of controls, $34.0 \pm 7.5\%$ for P-MRS and $31.0 \pm 4.1\%$ for S-MRS compared to $4.6 \pm 0.4\%$ for cells in liquid culture (Table 2.4). These differences might be due to minerals interfering with fluorescence excitation and emission; indeed, the projection images showed a high reflectance of the minerals (Figure 2.5). Furthermore, when mineral particles spotted on a glass slide without cells were excited with the 543-nm laser, an emission peak at 543 nm, extending to ~ 580 nm, occurred and can be attributed to reflectance (Figure 2.7).

The maximum emission intensity of the emission spectrum of the photosynthetic pigments of dried cells mixed with P-MRS or S-MRS was not affected by the exposure to a simulated Martian atmosphere (representing $95.8 \pm 5.6\%$ and $104.0 \pm 1.7\%$ of the maximum emission intensity of the spectrum of cells from liquid culture, respectively). However, the lower wavelength shift observed in unexposed dried cells mixed with minerals was less marked in S-MRS than in P-MRS samples. This was probably due to a reduced mineral interference in the regions selected for the analysis (not shown), as supported by the lower emission at 563 nm of S-MRS ($3.5 \pm 0.5\%$) than P-MRS ($16.7 \pm 4.6\%$).

Exposure to a simulated Martian atmosphere combined with 570 MJ/m^2 of polychromatic UV resulted in an overall bleaching of the photosynthetic pigments in cells mixed with minerals, as pointed out by a reduction in the emission peak at 653 nm (compared to the liquid control) for S-MRS and P-MRS down to, respectively, $1.6 \pm 0.1\%$ and $4.4 \pm 0.1\%$ (Figure 2.6). However, the non-homogeneous cell-mineral association and the thin layering of the

dried samples (Figure 2.5) impaired an accurate discrimination between top and bottom layers, as previously reported for thick, dried biofilms (Baqué et al., 2013c). CLSM-λscan analysis showed that cells in P-MRS samples had an emission peak at 653 nm reduced to either $57.9 \pm 4.3\%$ or $1.6 \pm 0.1\%$, thus reflecting the presence of differently shielded cells as shown in Figure 2.4C. Results are summarized in Table 2.4.

Moreover, some fluorescent minerals were spotted on both Martian analogues with two small emission peaks at 620 nm and 660 nm, as shown on the samples mixed with the P-MRS analogue and exposed to the full Mars simulation (Figure 2.6A). Mineral fluorescence from both preparations (without cells) was therefore further investigated with both the 488 and 543 nm lasers. The emission spectra of both mineral analogues produced fluorescent peaks, most notably around 620 and 660 nm, after 543-nm excitation. In addition, high reflectance peaks, extending from 490 to 520 nm after 488-nm excitation and from 550 to 580 nm after 543-nm excitation, were observed (Figure 2.7).

Table 2.4. Fluorescence emission at 563 nm and 653 nm of photosynthetic pigments in *Chroococcidiopsis* sp. CCMEE 029 cells from liquid culture and dried cells mixed with P-MRS or S-MRS, non-exposed (controls) or exposed to Mars simulations. Data are expressed as percent of the emission intensity at 653 nm of liquid culture (\pm standard error for $n \geq 15$ cells).

		Liquid culture	Dried sample Control	Dried sample	
				570 MJ/m ² + Martian atmosphere	Martian atmosphere
563 nm	4.6 ± 0.4		P-MRS	34.0 ± 7.5	16.5 ± 1.8 42.5 ± 8.8
			S-MRS	31.0 ± 4.1	23.0 ± 2.0
653 nm	100.7 ± 3.2		P-MRS	104.1 ± 4.1	57.9 ± 4.3 1.6 ± 0.1
			S-MRS	104.6 ± 5.3	4.4 ± 0.1
					95.8 ± 5.6
					101.0 ± 1.7

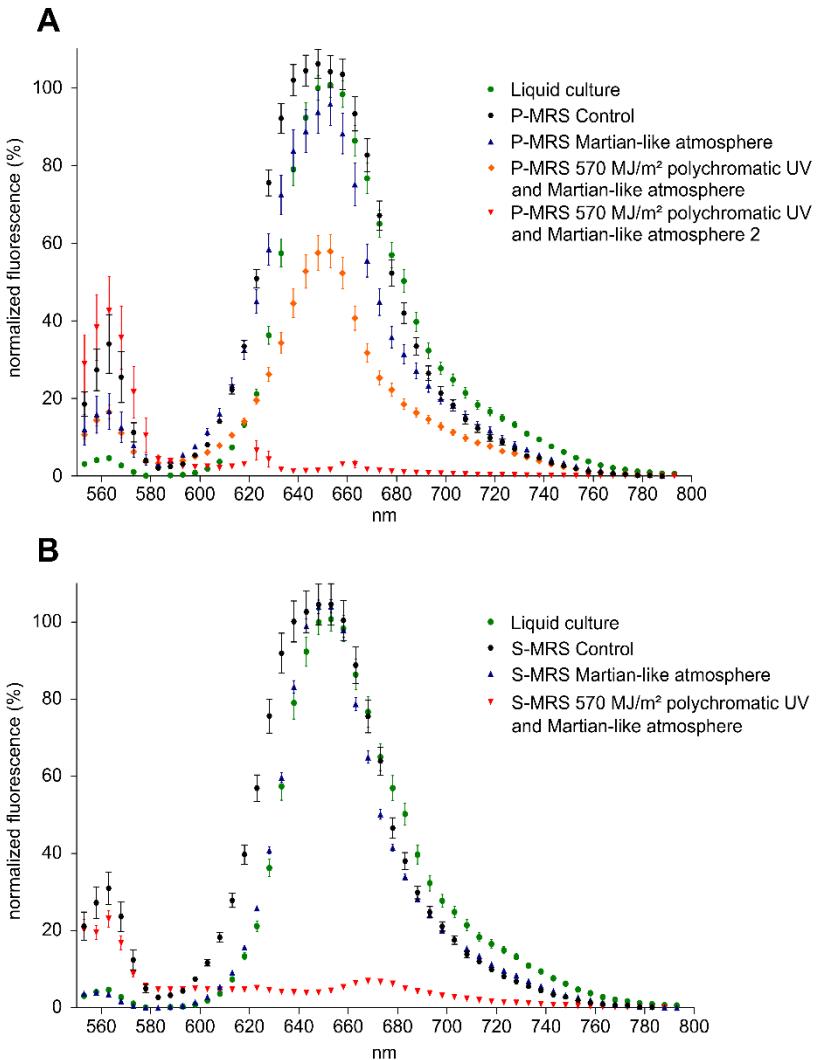


Figure 2.6. Photosynthetic pigment autofluorescence in *Chroococcidiopsis* sp. CCMEE 029 from the “Mars” series of experiments, as revealed by CLSM- λ scan performed by exciting samples with a 543-nm laser at 0.54 mW and collecting emission from 543 to 800 nm. Analyzed *Chroococcidiopsis* cells were either from liquid culture controls or dried cells mixed with P-MRS (A) or S-MRS (B) mineral analogues, unexposed (control) or exposed to all simulated Martian conditions (570 MJ/m² polychromatic UV and simulated Martian atmosphere) or simulated Martian atmosphere alone. Data points represent normalized fluorescence intensity at 653 nm \pm standard error, for $n \geq 15$ cells, as a function of emission wavelength.

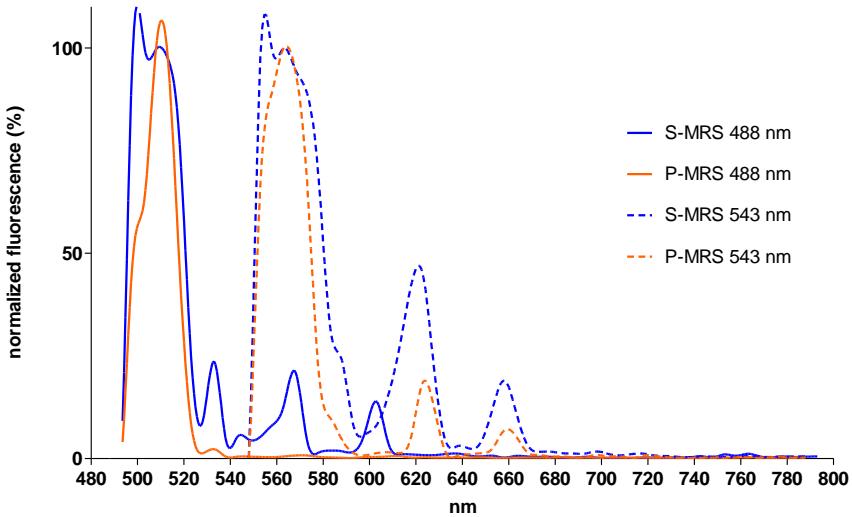


Figure 2.7. Reflectance of the P-MRS (orange) and S-MRS (blue) mineral analogues, as revealed by CLSM- λ scan performed by hitting minerals on a glass slide with 488-nm (plain lines) and 543-nm (dashed lines) lasers at 1 mW, and collecting emission of regions of interest from 488 to 800 nm and from 543 to 800 nm, respectively.

Membrane damage assessment

Images acquired after SYTOX-green staining are shown in Figure 2.8. A positive control was obtained by boiling *Chroococcidiopsis* sp. CCME 029 cells from a liquid culture for 15 minutes—a treatment known to inactivate them—which resulted in the staining of all cells (Figure 2.8A), while only a low fraction of cells were stained in a control (non-boiled) sample (Figure 2.8D) or in dried, non-irradiated *Chroococcidiopsis* cells (Figure 2.8B). Exposure of dried *Chroococcidiopsis* sp. CCME 029 to 5 kGy of X-rays (Figure 2.8B), 1 kGy of He ions, 2 kGy of Fe ions, or 23.92 kGy of gamma irradiation (not shown), did not lead to a significant increase in detectable damage to plasma membranes. On the other hand, desiccation itself caused extensive membrane damage to *Synechocystis* cells (Figure 2.8E), leading all cells to be stained even in non-irradiated samples.

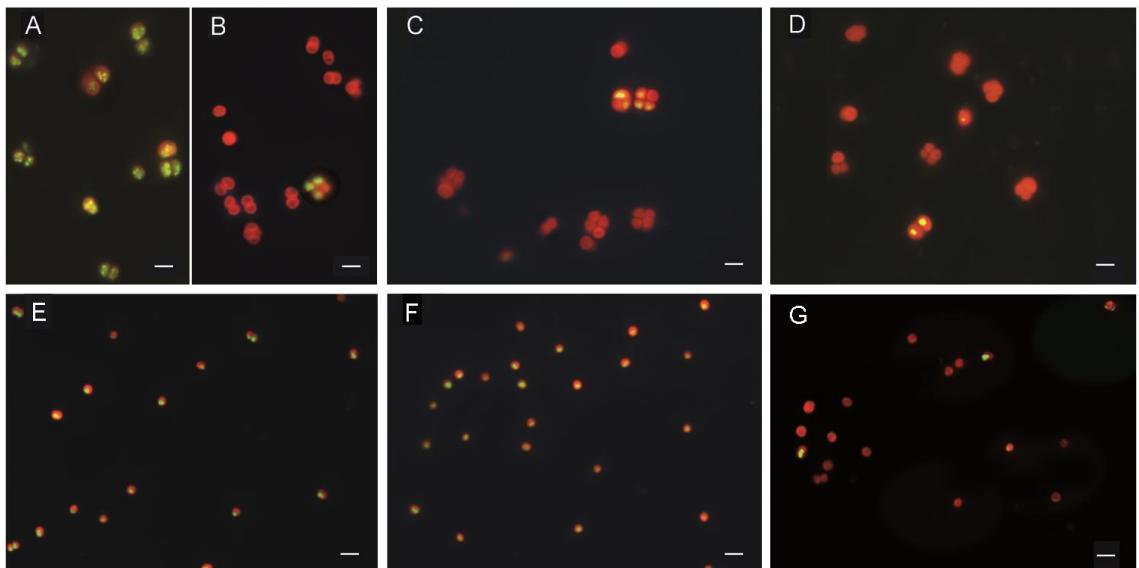


Figure 2.8. Damage to plasma membranes after 5 kGy of X-ray irradiation, as revealed by SYTOX Green. First row: *Chroococcidiopsis* sp. CCME 029 cells boiled for 15 min (A), non-irradiated, dried cells (B), dried, irradiated cells (C), and non-irradiated liquid samples (D). Second row: *Synechocystis* sp. PCC 6803 dried cells (E), dried, irradiated cells (F), and non-irradiated liquid samples (G). Photosynthetic pigments appear red, and cells with damaged membranes show green fluorescent nucleoids. Scale bar: 10 μ m.

Discussion

Chroococcidiopsis spp. were exposed to various ground-based simulations of space, Mars and Moon environments, within preparatory experiments for BIOMEX and as part of the Starlife project. We then studied both cell survival and the preservation of some biomarkers (photosynthetic pigments and DNA). Overall, our results demonstrate the high resistance of desert *Chroococcidiopsis* cells to various factors found within hundred millions of kilometers of their natural environments. They also allowed for the validation of exposure conditions, as well as methods for sample analysis, of the EXPOSE-R2 flight mission.

Exposure conditions

Given that the results presented in this chapter come from three different studies, each with different objectives in addition to the common goal of assessing the resistance of *Chroococcidiopsis* spp. and their macromolecules to extraterrestrial environments, quite a wide range of experimental conditions are reported here. The following paragraphs aim at clarifying the rationale behind the choice of various parameters.

In preparation for BIOMEX, we exposed dried *Chroococcidiopsis* cells to various doses of polychromatic UV irradiation at the range reaching Mars's surface ($\sim 200\text{-}400$ nm). The lowest tested fluency ($1.5 \times 10^3 \text{ kJ}\cdot\text{m}^{-2}$) corresponds approximately to the dose reaching Mars' surface at the equator, at the mean orbital distance from the Sun, in one day (Cockell et al., 2000). The highest ($800 \text{ MJ}\cdot\text{m}^{-2}$) corresponds roughly to what would have been expected from exposure during an 18-month EXPOSE-R2 mission (the exact duration not being known prior to flight; see Chapter 3 for actual flight data) with an average flux of $16\text{W}\cdot\text{m}^{-2}$ (Rabbow et al., 2012)—although, given that this dose would not allow for any organism to survive, neutral density filters were used during the EXPOSE-R2 space mission and reduced the flux reaching samples down to 0.1% (see Chapters 3 and 4).

Some of those irradiations were performed in the presence of a Mars-like atmosphere, in combination or not with analogues of Martian regolith, P-MRS and S-MRS (see Table 2.2, “Mars” series). Those minerals were used because it is suspected that Mars's regolith could have a critical impact on biosignature preservation and identification (more details in Chapter 4), which is among the investigated aspects of the BIOMEX experiment. For this series in Mars-like conditions, two polychromatic UV doses were used. The highest, $570 \text{ MJ}\cdot\text{m}^{-2}$, corresponds to circa 380 sols on the Martian surface (Cockell et al.,

2000; Schuerger et al., 2003). It is also, roughly, the dose which was expected to reach the EXPOSE-R2 platform during its stay in LEO (Rabbow et al., 2012, 2014). The lowest ($570 \text{ kJ}\cdot\text{m}^{-2}$) corresponds to the same scenario but where UV radiation is attenuated with neutral density filters, as would later be the case during the EXPOSE-R2 mission. Cells without minerals were exposed to the lower dose, as damage would otherwise have been expected to be too extensive for meaningful results to be obtained, while cells mixed with minerals were exposed to the higher dose.

Some polychromatic UV irradiation experiments aimed at simulating, rather than Mars's surface, space and Moon environments (see Table 2.2, "Moon" series). Consequently, they were performed in combination with vacuum (which characterizes both space and the Moon) or with anorthosite, an analogue of Moon regolith. In the same series were performed irradiation with UVC alone (254 nm), around the most damaging wavelength of the UV spectrum in space and on Mars, in the presence or not of anorthosite.

Ionizing irradiation was left out of the exposure experiments performed in preparation for BIOMEX, but its effects on *Chroococcidiopsis* cells were investigated as part of the Starlife project. We used various kinds of both photon and heavy ion irradiation, covering a wide range of LET values, and used cells in both dried and liquid states. The applied doses would be received, in space, on the Moon or on Mars, over timeframes much longer than the EXPOSE-R2 mission: while we applied kGy, or even tenth of kGy, an ionizing radiation dose of $675.4 \mu\text{Gy}\cdot\text{day}^{-1}$ (resulting from GCR, energetic protons of the inner radiation belt and electrons from the outer radiation belt [Dachev et al., 2014]) was recorded during the EXPOSE-R mission, and the total dose received by the EXPOSE-R2 platform amounted to approximately 0.5 Gy (Dachev et al., 2017). Absorbed dose rates on the surface of Mars (where the thin atmosphere and the lack of intrinsic magnetic field provide limited shielding) were assessed to be in the range of $50\text{--}150 \text{ mGy}\cdot\text{year}^{-1}$ at the surface (Dartnell et al., 2007; Pavlov et al., 2012), and an absorbed dose rate of $76 \text{ mGy}\cdot\text{year}^{-1}$ was measured by the Curiosity rover at Gale Crater's surface (Hassler et al., 2013). Dose rates can be slightly higher below the surface: secondary particles form when primary radiation penetrates substrates, and dose rates increase with shielding until reaching a peak, after which particle energy is below the threshold for new particle production and dose rates decrease (see, e.g., Dartnell et al. 2007). As an example, from $76 \text{ mGy}\cdot\text{year}^{-1}$ at the surface, dose rates can reach $96 \text{ mGy}\cdot\text{year}^{-1}$ under 10 cm of Martian rock with a density of $2.8 \text{ g}\cdot\text{cm}^{-3}$ (Hassler et al. 2013). However, the doses used as part of the Starlife study remain far in excess. As for the absorbed dose rate

reaching the surface of a rock travelling through our solar system, it was estimated to be about 200 mGy·year⁻¹ (Mileikowsky et al., 2000).

Survival and DNA preservation

In space, on Mars or the Moon, the limiting factor for survival is often DNA preservation (Horneck et al., 2010): DNA is sensitive to both radiation (by direct absorption and production of ROS) and vacuum-induced desiccation. Survival and DNA damage are consequently discussed together in this subsection. Mechanisms which may contribute to the high resistance demonstrated here by *Chroococcidiopsis* spp. are discussed in a separate subsection.

Chroococcidiopsis cells mixed with no mineral were eradicated by exposure to 500 kJ·m⁻², 570 kJ·m⁻², or 1.5 MJ·m⁻² of polychromatic UV under (respectively) vacuum, a Mars-like atmosphere, or ambient air. Similarly, cells mixed with P-MRS or S-MRS were eradicated by a dose of 570 MJ·m⁻² under a Mars-like atmosphere, and cells mixed with anorthosite by 15 MJ·m⁻² under ambient air. On the other hand, cells mixed with anorthosite survived a dose of 1.5 MJ·m⁻², demonstrating the positive effect of this mineral environment on survival. Cells also survived the highest tested dose (10 kJ·m⁻²) of UVC, and a 3-month exposure to either simulated Martian atmosphere or vacuum, regardless of the mineral matrix.

In cells mixed with no minerals, DNA detection was strongly affected by exposure to 570 kJ·m⁻² of polychromatic UV under a Mars-like atmosphere (although exposure to 3 months of a Mars-like atmosphere did not, by itself, result in detectable DNA damage). However, things were quite different for cells mixed with minerals: even at the highest fluencies (up to 570 MJ·m⁻² for cells mixed with Mars analogues, or 800 MJ·m⁻² for cells mixed with the Moon analogue), DNA remained detectable by RAPD. The thin layers of regolith thus provided significant protection in spite of their thinness (the total thickness of cells plus minerals was in the order of a few micrometers, or tens of micrometers, depending on minerals and location). This protective role of minerals is discussed in Chapter 4. A quantitative evaluation of the accumulated DNA damage by using real-time quantitative polymerase chain reaction (qPCR), as previously reported for *Chroococcidiopsis* (Baqué et al., 2013a, 2013b), was not performed due to the low purity of the yielded DNA. Indeed, DNA extraction from samples mixed with some mineral substrates is impaired by DNA's adsorption to clay (Direito et al., 2012).

Irradiation with several kGy of heavy ions, or up to 11.59 kGy of gamma rays, did not eradicate populations of *Chroococcidiopsis* spp. and did not induce detectable damage to DNA or plasma membranes. Above 11.59 kGy (or above 23.92 kGy, for dried samples of *Chroococcidiopsis* 029), gamma irradiation was lethal to both liquid and dried populations of *Chroococcidiopsis* 057 and 029. Accordingly, DNA damage (but no membrane damage) was detectable in all *Chroococcidiopsis* samples exposed to more than 11.59 kGy of gamma rays.

Based on the dose estimates provided in the subsection above, the survivable dose of 11.59 kGy corresponds to approximately 100 thousand years or 60 thousand years on the surface of, respectively, Mars or an ejectum travelling through space. However, even though considering the dose by itself is a convenient simplification, the radiation flux in space is not composed exclusively of gamma rays. Gamma rays have low LET values and space radiation includes a large proportion of protons and α -particles, as well as some heavier nuclei. But our results point out the orders of magnitude involved. Besides, *Chroococcidiopsis* spp. survived 1 kGy of He nuclei (similar to α particles), 1 kGy of Si nuclei and 2 kGy of Fe nuclei with no detectable DNA damage; even if a given dose of the Martian radiation flux was creating as much damage as a dose of the radiation flux used here several orders of magnitude higher, the replication time and repair dynamics of even slow-growing *Chroococcidiopsis* strains would be way below the time needed to receive a sterilizing dose. That being said, extant but dormant organisms under the Martian surface or undergoing lithopanspermia would accumulate DNA damage over extended periods of time. To put our results in context of the possible survival of dormant microbial forms on Mars or of a space travel through lithopanspermia, the reader is referred to Dartnell et al. (2007), Mileikowsky et al. (2000), and Paulino-Lima et al. (2011).

Here, with a D_{10} around 12 kGy, *Chroococcidiopsis* spp. showed a high radioresistance by microbial standards (*Escherichia coli* ATCC 10798, for instance, has been shown to have a D_{10} of 0.35 kGy [Trampuz et al., 2006]), and a moderate radioresistance (at least to gamma-rays) by cyanobacterial standards, following Kraus (1969) classification. For comparison, when irradiated at room temperature, the cyanobacterium *Anabaena* sp. PCC 7120 showed DNA fragmentation after exposure to 3 kGy of gamma radiation (Singh et al., 2013), and *Deinococcus radiodurans* showed D_{10} values of approximately 5.5 kGy (for liquid samples) and 12.5 kGy (for dried cells) (Bauermeister et al., 2011). However, when comparing results from different experiments, one should keep in mind that differences in growth phases,

irradiation conditions and sample preparation can greatly influence survival and subcellular integrities. It has for example been shown that cells of *D. radiodurans* were more sensitive to ionizing radiation when in late stationary phase than when in exponential or early stationary phase (Sukhi et al., 2009), although bacteria are generally most resistant to external stresses during the stationary phase (e.g., Wassmann et al. 2011). Hydration state of the irradiated samples, temperature during irradiation, dose rates and other factors have also been shown to influence D_{10} values (Badri et al., 2015; Bauermeister et al., 2011) and lead to differential DNA fragmentation (Billi et al., 2000; Singh et al., 2013). Consistently, DNA fragmentation was reported in liquid cultures of *Chroococcidiopsis* spp. irradiated with 5 kGy of X-ray with a dose rate of approximately 5 kGy·h⁻¹ in presence of bubbling air (Billi et al., 2000). It is unclear whether those differences can be explained by the lower dose rate used in the present study: it could also be a result of the different irradiation conditions. Indeed, aeration of the culture maintains sensitivity to radiation regardless of cell concentration: it prevents cells from forming clusters, inside which oxygen concentrations would be reduced, favoring radiation resistance (Slade and Radman, 2011).

Preservation of pigments' autofluorescence

Polychromatic UV radiation caused a strong decrease in the intensity of the emission spectrum of phycobiliproteins and chlorophyll *a*. Exposure to 570 MJ·m⁻² of polychromatic UV irradiation led to a strong reduction (down to 1–4% of control values) of the emission spectrum of photosynthetic pigments in *Chroococcidiopsis* sp. CCME 029 mixed with either P-MRS or S-MRS. This pointed out that neither the 4–5 cell layers mixed with P-MRS nor the 2–3 cell layers mixed with S-MRS did provide efficient shielding against a dose of UV radiation corresponding to more than a terrestrial year on Mars. Consistently, when dried monolayers of *Chroococcidiopsis* sp. CCME 029 were exposed to 72 kJ·m⁻² of a Martian-like UV flux, the pigment autofluorescence dropped down to less than 5% of controls (Cockell et al., 2005). That being said, cells mixed with P-MRS were found whose pigment autofluorescence was only reduced to 60 % of control values. This might be a consequence of the thinner grain-size of P-MRS that impaired the preparation of homogeneous samples, thus leading to thicker and protected areas in the samples, as well as of the high phyllosilicate contents of this mineral preparation (see Chapter 4 for more details). Hence, although photosynthetic pigments are expected to bleach when exposed on the Martian surface for a period as long as 380 sols, the right

mineral environment might allow them to retain their fluorescence for months within a few tens of micrometers of the surface.

Photolytic destruction of photosynthetic pigments was reported in *Synechocystis* sp. PCC 6803 exposed to UV and ionizing radiation, yielding fluorescent breakdown products with an emission peak at ~ 450 nm (Dartnell and Patel, 2014; Dartnell et al., 2011). Here, an emission peak at 563 nm, probably due to chlorophyll photolysis, occurred in unshielded *Chroococcidiopsis* sp. CCME 029 exposed to 800 MJ·m⁻² of polychromatic UV irradiation. It was however absent from cells mixed with anorthosite, as shown by the lambda scan spectra obtained on mineral-free areas (Baqué et al., 2014; MB, personal communication). As for cells mixed with S-MRS or P-MRS, the presence or absence of degradation products was not determined due to both interfering mineral reflectance and our failure to obtain cell pigment spectra in mineral-free areas.

While P-MRS and S-MRS have autofluorescence peaks at 620 nm and 660 nm, the typical emission peaks of phycocyanin, allophycocyanin and chlorophyll *a* could be detected in UV-exposed *Chroococcidiopsis*. However, fluorescent signals were also reported for minerals and salts found on Mars when excited in the UV (266 and 355 nm) and visible (532 nm) regions. In particular, calcium perchlorate has a strong fluorescent peak at around 600 nm after 532 nm excitation (Smith et al., 2014). Hence, a more thorough analysis is needed to insure that Martian soil does not interfere with the identification of selected autofluorescent molecules. This might be relevant when using the foreseen instrumentation on-board the ESA/Roscosmos mission, for example the UV/Vis camera filters or the Ma_Miss VIS-NIR spectrometer (De Angelis et al., 2014).

After exposure to 2 kGy of heavy ions and 113.25 kGy of photon radiation, pigments remained detectable in *Chroococcidiopsis* spp. Those results are consistent with the previously observed resistance of cyanobacterial pigments to ionizing radiation (Dartnell et al., 2011, 2012).

Potential mechanisms behind *Chroococcidiopsis* spp.'s resistance

Overall, our results point out a high resistance of desert *Chroococcidiopsis* cells to conditions found beyond Earth. It is likely ascribable to a wide variety of protection and repair mechanisms, each having a relatively weak impact, rather than to a single factor or a few factors. This is well-known for the highly-studied extremophile *Deinococcus radiodurans*: a very wide range of features have been identified in this species, including efficient DNA repair mechanisms, anti-oxidation defenses and specific morphological

characteristics (e.g., Slade and Radman, 2011; Munteanu et al., 2015). While work is still ongoing to determine the reasons behind *Chroococcidiopsis*'s resilience, several assumptions can be formulated, albeit to be taken with caution.

In the absence of minerals, lethal doses of UV resulted in extensive DNA damage, as revealed by strongly modified RAPD patterns. One may assume that at survivable doses, *Chroococcidiopsis*'s resistance is due to efficient DNA repair and/or protection mechanisms. Accordingly, the lack of detectable DNA damage after 11.59 kGy of gamma rays suggests the presence of strong DNA protection mechanisms. Several desert strains of *Chroococcidiopsis* were also shown to avoid genome fragmentation (as well as covalent modifications) during dehydration (e.g., Billi, 2009; Billi et al., 2011; Fagliarone et al., 2017)—at least for a fraction of cells within desiccated populations. Efficient DNA repair mechanisms in *Chroococcidiopsis* were observed as well, following its survival to 5 kGy of X-rays in presence of bubbling air: extensive DNA damage occurred but was repaired within 24 hours (Billi et al., 2000).

Part of *Chroococcidiopsis* spp.'s UV resistance may also be related to their structure. Cells are relatively large, form clusters and are surrounded by a thick, multilayered cell envelope (Grilli-Caiola et al., 1993). Those features may have a significant impact by shielding DNA from UV, especially the low-penetrating UVC. Beta-carotene and photosynthetic pigments may also absorb a non-negligible fraction of UV: Lao and Glazer (1996) calculated that the light-harvesting proteins (phycobiliproteins and chlorophyll proteins) account for more than 99% of the UV-B absorption in cyanobacteria, while DNA absorbs up to 50% of UVB flux in non-photosynthetic organisms. Consistently, the fact that DNA damage was significantly higher in *Synechocystis* than in *Chroococcidiopsis* spp. after irradiation in a dry state with 1 kGy of He ions or 2 kGy of Fe ions suggests efficient protection mechanisms in the absence of metabolic activity (e.g., protective structural features) in dried *Chroococcidiopsis*.

Dried *Chroococcidiopsis* cells are also more resistant than hydrated cells of the same strains (e.g., dried *Chroococcidiopsis* 029 survived 23.92 kGy of photon irradiation, against 11.59 kGy for hydrated cells). An increased radioresistance of cells when in a dry state was previously observed in other bacteria, for instance *Deinococcus radiodurans* (Bauermeister et al., 2011) and *Halobacterium salinarum* (Leuko et al., 2015). This could be explained by various factors including structural advantages, reduced generation of

reactive oxygen species, and lower rates of potentially harmful chemical reactions, when cells are in a dry state (see Bauermeister et al., 2011).

While all those factors may play a role, the central coping strategy in desert *Chroococcidiopsis* spp. may lie in their antioxidant defenses. Indeed, damage caused by desiccation, ionizing radiation and UV is thought to be largely mediated by ROS (e.g., Fredrickson et al. 2008; Daly et al. 2007; Slade and Radman, 2011). ROS can form via a wide range of mechanisms, not all of which are fully elucidated. Ionizing radiation, for instance, creates hydroxyl radicals (HO^\bullet)—some of which will react with each other and form hydrogen peroxide (H_2O_2)—by radiolysis of water, as well as superoxide (O_2^\bullet) from dissolved dioxygen (O_2) (Daly, 2009). During desiccation, ROS can be the consequence of reduced hydration-induced conformational changes in proteins, which leads to dysfunction in enzymes and/or electron transport chains (Potts, 1994). This is especially true in photosynthetic organisms, where light initiates reactions that the disrupted photosystems and photosynthetic electron transport chain cannot complete (Potts, 1999). ROS can be highly damaging to cells: HO^\bullet will react with a wide range of organic molecules, while H_2O_2 and O_2^\bullet will more specifically damage some proteins (Daly, 2009).

Consistently, a recent study from our group showed that desert strains of *Chroococcidiopsis* spp. can prevent protein oxidation under ROS-inducing conditions. Neither irradiation with up to 25 kGy of gamma rays, nor desiccation followed by one year of storage in a dry state, nor a 30-minute incubation in 0.5 M H_2O_2 (H_2O_2 is a ROS itself, which can diffuse inside cells and be converted into other ROS) did induce any protein carbonylation (an irreversible, oxidative protein modification) in *Chroococcidiopsis* 029 (Fagliarone et al., 2017). By contrast, high levels of protein carbonylation were observed in *Synechocystis* sp. after 10 kGy of gamma rays, 1 year of desiccation, or an identical H_2O_2 treatment.

This ability to prevent protein oxidation may be critical under the experimental conditions tested here: *Chroococcidiopsis* would have protected proteins then available for repairing damage to macromolecules, notably DNA (see Daly, 2012). This may partly explain the efficient DNA repair observed in *Chroococcidiopsis* spp. after exposure to X-rays (Billi et al., 2000) or a Mars-like UV flux (Cockell et al. 2005).

If strong antioxidant systems are key to *Chroococcidiopsis*'s resistance, those systems remain to be fully elucidated. Interestingly, an iron superoxide dismutase (which catalyzes the dismutation of O_2^\bullet into the less damaging H_2O_2 and O_2) was shown to accumulate in the peripheral cytoplasm of

Chroococcidiopsis sp. after one year in a dry state (Caiola et al., 1996), while ROS were found restricted to the inner cytoplasm 4 years after desiccation (Billi, 2009). This suggests an activity of superoxide dismutase (and possibly other protective enzymes) in dried cells. This phenomenon may be favored by *Chroococcidiopsis* spp.'s thick, EPS-rich cell envelopes (Grilli Caiola et al., 1996): such sheaths are known to stabilize enzymes and other molecules during desiccation (Baqué et al., 2013a; Helm and Potts, 2012). EPS may also have a more direct role in preventing ROS accumulation (Chen et al., 2009) and inhibiting their reactions with organic molecules (Gao and Garcia-Pichel, 2011). *Chroococcidiopsis*'s carotenoids may also play a significant role in this battle. A wide range of other antioxidant defenses have been discovered in other bacteria (e.g., Slade and Radman, 2011; Paulino-Lima et al., 2017), and work is ongoing to determine which ones exist in desert *Chroococcidiopsis* spp.

Finally, one may wonder how desert *Chroococcidiopsis* spp. developed such resistance mechanisms against environmental conditions not found on Earth. A likely explanation is that they evolved under the pressure of other, terrestrial stressors. It was for instance suggested that the resistance of some non-spore forming bacteria to high doses of radiation is a side effect of desiccation (Cox & Battista 2005), which like radiation induces damages to DNA and other cell components via the production of ROS. Desert *Chroococcidiopsis* cells undergo dehydration/rehydration cycles in their natural environment, which led to an exceptional resistance to desiccation. Those may have also have reinforced—as a side effect—*Chroococcidiopsis*'s ability to cope with other factors inducing oxidative stress and more direct forms of molecular damage (Billi 2009), including condition found in space, on the Moon, and on Mars.

Conclusion

Insights into the endurance of cyanobacterium cells and biosignatures were obtained from *Chroococcidiopsis* spp. exposed to space, Mars and Moon environments on the ground, in preparation for the BIOMEX space mission and as part of the Starlife series of experiments. Overall, desert *Chroococcidiopsis* spp. demonstrated a high resistance to those extreme conditions. While UV in space, on Mars or on the Moon would be quickly lethal to unprotected *Chroococcidiopsis* cells (or to any known microorganism) and destroy biosignatures, even a micrometer-scale layer of minerals and/or a few cell layers could provide enough protection for long-term survival and biosignature preservation. Such a protection is far from

being sufficient for blocking ionizing radiation, but *Chroococcidiopsis* spp. survived doses that they would receive in thousands of years on Mars or in space. Long-term exposure to vacuum or Mars-like atmosphere were not lethal either, suggesting that *Chroococcidiopsis* could survive extended periods of time in various extraterrestrial environments if protected from UV. However, while ground-based simulation provide valuable insights, some conditions found beyond Earth cannot be accurately simulated (Cottin et al., 2017), notably the complex fluxes of ionizing radiation reaching space and Mars, or the full solar irradiance (including low-wavelength UV). Since the ground-based tests described above validated the foreseen process, we took part as planned in the EXPOSE-R2 mission and obtained complementary results from exposure in low Earth orbit. Those are described in the following two chapters.

Chapter 3

Enhanced resistance of *Chroococcidiopsis* biofilms, compared to their planktonic counterparts, to space and simulated Martian conditions in low Earth orbit²

The main goal of the Biofilm Organisms Surfing Space (BOSS) experiment, part of the EXPOSE-R2 space mission, is to test whether biofilms are more resistant to long-term exposure to space and Martian conditions than planktonic cells. Among samples exposed in BOSS were three desert strains of *Chroococcidiopsis*: CCME 029, CCME 057, and CCME 064. Dried biofilms and dried, multi-layered planktonic samples (except for *Chroococcidiopsis* sp. CCME 064, for which only biofilms were included) were exposed for over a year to space and simulated Martian conditions in low Earth orbit within the ESA facility EXPOSE-R2, outside the International Space Station. Exposure parameters included extreme temperature cycles, ionizing radiation, space vacuum or Mars-like atmosphere, and differentially attenuated low Earth orbit (LEO) UV irradiation at the wavelength ranges found in space or on Mars. In parallel to exposure in LEO, replicates of the experiment were performed on the ground. Some were kept in the dark under ambient conditions, while others were exposed to stressors (extreme temperature cycles, Mars-like atmosphere or vacuum, and UV flux) mimicking those undergone by the EXPOSE-R2 samples, based on data recorded in-flight. The effects of those environments on cyanobacterial samples were investigated by using confocal laser scanning microscopy (to visualize the biofilm architecture and quantify photosynthetic pigments' autofluorescence), PCR-based assays (to assess DNA damage) and growth assays (to test cell viability). Results from the flight mission are consistent with previous ground-based simulations and, while even planktonic

²This chapter is in preparation for publication as: Daniela Billi, Cyprien Verseux, Mickaël Baqué, Clelia Staibano, Elke Rabbow, and Petra Rettberg. Enhanced resistance of *Chroococcidiopsis* biofilms, compared to their planktonic counterparts, to space and simulated Martian conditions in low Earth orbit.

Chroococcidiopsis cells showed a high resistance to space and Mars-like environments, biofilms were less affected by the mission's harsh conditions.

Introduction

How resistant microorganisms are to conditions found in space and on Mars has deep implications (e.g., Cottin et al., 2015; Horneck et al., 2016). Some stem from the fact that interplanetary life transfer could happen in an accidental but artificial way during future missions to Mars. This would be critical in view of the desire to preserve the pristine nature of celestial bodies, as reflected by leading policies on planetary protection (COSPAR, 2011). Then, life could theoretically have been carried from one planet to another within rocks expelled by asteroid or comet impacts, most probably during the first half billion years of our planetary system. Various studies suggest that some microorganisms could survive expulsion from a planet such as Mars, as well as atmospheric reentry and landing on Earth, if deep enough inside a rock (for a review, see Nicholson, 2009). Another critical step is the journey through interplanetary space and, to assess its outcome, life's boundaries are critical parameters. However, our knowledge of those boundaries remains limited (Cottin et al., 2015; Horneck et al., 2016).

In this context, studying the survival of biofilms beyond Earth is of high interest: microorganisms in nature usually do not live as individual cells in homogenous suspension but rather accumulate at interfaces and form biofilms. Biofilms are even one of the most widely distributed forms of life on Earth (Stoodley et al., 2002). In those highly organized structures, cells often have properties (morphology, metabolism, interactions, ...) differing neatly from that of planktonic cells (e.g., Corning, 2002) and are usually encased in a self-produced matrix of extracellular polymeric substances (EPS). EPS are made of a wide range of macromolecules (polysaccharides, proteins, nucleic acids, lipids...) which can remain attached to the cell surface or be released into the surrounding environment. EPS's composition, structure, function and abundance can vary greatly based on species and external conditions (Flemming & Wingender, 2010; Flemming et al., 2016). This matrix, as well as outer layers of cells and metabolic modifications, can increase microbial resistance to a wide variety of chemical and physical stressors such as, for instance, UV and gamma irradiation (Niemira & Solomon, 2005), disinfectants (Bridier, Briandet, Thomas, & Dubois-Brissonnet, 2011),

antibiotics (Hoiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010), heat (Frank & Koffi, 1990), and high salinity (Dufour et al., 2012; Zhang et al., 2015).

One may consequently wonder whether biofilms can be more resistant than planktonic cells to conditions found beyond Earth, in particular in space and on Mars. The BOSS (Biofilm Organisms Surfing Space) experiment, part of the EXPOSE-R2 space mission outside the International Space Station (ISS), was designed to test this hypothesis. Within this experiment, biofilms and planktonic samples of various microorganisms (*Deinococcus geothermalis*, *Bacillus horneekiae*, *Chroococcidiopsis* spp., *Halococcus morrhuae*, *Halomonas salina*, and natural biofilms within volcanic rocks) were exposed to space and simulated Mars conditions in low Earth orbit (LEO).

Members of the genus *Chroococcidiopsis* are often dominant phototrophs in hot and cold deserts (Billi et al., 2013, 2017) considered the closest environmental analogues of Mars on Earth due to dryness, low organic contents, similar mineralogy, and/or cold temperatures (Martins et al., 2017). Due to those natural living conditions, they are relevant model organisms for studies on long-term survival and adaptation to Mars-like conditions (Billi et al., 2017; Martins et al., 2017). In extreme environments, *Chroococcidiopsis* spp. colonize porous rocks, forming endolithic communities, or the soil-rock interface, forming hypolithic communities (Friedmann and Ocampo, 1976; Friedmann et al., 1967). Desert strains of *Chroococcidiopsis* are well known for being highly resistant to desiccation and—possibly as a side effect of desiccation resistance mechanisms (Mattimore and Battista, 1996; Pavlopoulou et al., 2016)—radiation (reviewed in Billi et al., 2017). For instance, survival after 4 years of air-drying was reported (Billi, 2009; Grilli Caiola et al., 1996), as well as after exposure to $13 \text{ kJ}\cdot\text{m}^{-2}$ of UVC irradiation (Baqué et al., 2013a), 15 kGy of X-rays (Billi et al. 2000), or 12 kGy of gamma radiation (Verseux et al., 2017). Multilayers of *Chroococcidiopsis* sp. CCMEE 123 overlain by 3 mm of Antarctic sandstone survived $1.5 \times 10^5 \text{ kJ}\cdot\text{m}^{-2}$ of polychromatic UV radiation at the Martian range (200-400 nm) (Billi et al., 2011). *Chroococcidiopsis* sp. CCMEE 029 survived $13 \text{ kJ}\cdot\text{m}^{-2}$ of UVC (Baqué et al., 2013a). Besides, when irradiated in the air-dried state, desert strains of *Chroococcidiopsis* showed an enhanced survival potential, as reported for dried monolayers that survived $30 \text{ kJ}\cdot\text{m}^{-2}$ of a simulated Martian UV flux (Cockell et al., 2005) or for dried multilayers after 24 kGy of gamma radiation (Verseux et al., 2017). Moreover, strain CCMEE 029 withstood long-term exposure in LEO, both during the EXPOSE-E mission for which it was added to an epilithic community (Cockell et al., 2011) and within impact-shocked gneiss during the EXPOSE-R mission (Bryce et al., 2014).

In this work, we investigated the resistance of biofilm and planktonic samples of two endolithic, desert strains of *Chroococcidiopsis*: CCMEE 029, isolated from the Negev Desert (Israel), and CCMEE 057, isolated from the Sinai desert (Egypt). Biofilm samples of the hypolithic strain CCMEE 064 (from the Sinai desert) were also included, but not their planktonic counterparts due to payload limitations. These strains had been selected on the basis of ground-based simulations, called Experiment Verification Tests (EVTs) and Science Verification Tests (SVTs), performed to test various parameters such as doses of monochromatic UV (254 nm) up to $10 \text{ kJ}\cdot\text{m}^{-2}$, and a simulation of one year in LEO including polychromatic UV (200-400 nm) up to $5 \times 10^5 \text{ kJ}\cdot\text{m}^{-2}$, attenuated with 0.1 % neutral density (ND) filters, combined or not with space vacuum (10^{-4} Pa) or Mars-like atmosphere (Baqué et al., 2013b, 2013c).

Although ground-based simulations can give highly valuable insights, no terrestrial facility can substitute space experiments when it comes to simulating complex extraterrestrial environments (Cottin et al., 2017; Martins et al., 2017). Here, we report the effects of exposure to space and simulated Mars conditions, performed in LEO as part of the BOSS experiment, on biofilm and planktonic samples of 3 desert strains of *Chroococcidiopsis*. We assessed survival as well as sub-cellular damage, investigating the integrity of genomic DNA and photosynthetic pigments (phycobiliproteins and chlorophyll *a*).

Results point out the high resistance of desert strains of *Chroococcidiopsis* to space and Mars environments, and suggest an overall increased resistance of biofilms when compared to their planktonic counterparts.

Materials and methods

Organisms and sample preparation

Chroococcidiopsis spp. CCME 057 (S6e), CCME 029 (N6904) and CCME 064 (S8c), hereafter referred to as CCME 057, CCME 029 and CCME 064, were isolated by Roseli Ocampo-Friedmann from chamoendolithic growth in granite in the Sinai Desert (Egypt), cryptoendolithic growth in sandstone in the Negev Desert (Israel), and hypolithic growth under stones of desert pavement in the Sinai Desert, respectively. Those isolates are part of Culture Collection of Microorganisms from Extreme Environments (CCME), established by E. Imre Friedmann and currently maintained at the University of Rome Tor Vergata. They were grown under routine conditions at 25°C, in BG11 medium, under a photon flux density of $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by fluorescent cool-white bulbs with a 16/8h light/dark cycle.

Biofilms were obtained by growing cyanobacteria on top of BG-11 agarized medium, in Petri dishes sealed with Parafilm, for about 2 months. They were then air-dried by removing the Parafilm and leaving them for about 15 days under routine conditions. Dried biofilms were then stored in the dark at room temperature. Multilayered planktonic samples were obtained by pelleting samples from a culture in exponential phase, resuspending cells to a density of $5 \times 10^9 \text{ cells}\cdot\text{ml}^{-1}$ in PBS, and plating 400 µl of cell suspension per 100 mm diameter Petri dish on agarized BG11-medium. They were immediately air-dried for two days under a laminar flow hood, in the dark. 12-mm-in-diameter disks were cut out of biofilm and planktonic dried samples and stored in the dark at room temperature.

Exposure in low Earth orbit

The core facility of the EXPOSE-R2 mission was previously used for the EXPOSE-R mission in 2008-2011 (Rabbow et al., 2014). Only a new set of trays, carriers and samples were therefore uploaded to the ISS. The trays and sample carriers were identical to those of EXPOSE-R (except for the PSS experiment, for which new specific sample holders were developed) but a new exposure procedure was put in place to tackle window contamination issues that occurred during EXPOSE-R (Demets et al., 2015).

Chroococcidiopsis flight samples were accommodated in exposure Trays 1 and 2 of the EXPOSE-R2 hardware, in two-layer stacked sample carriers (Figure 3.1). Tray 1 was covered with MgF₂ windows (for exposure in-flight to UV radiation from $\lambda \approx 110$ nm), while Tray 2 was covered with quartz windows and a long-pass cutoff filter of approximately 50% transmission at 216 nm (for exposure to a Mars-like UV spectrum, $\lambda > 200$ nm). Samples were further covered with 0.1% neutral density (ND) filters, attenuating solar UV radiation by approximately 3 orders of magnitude, as previously used during the EXPOSE-R mission (Demets et al., 2015; Rabbow et al., 2014). Samples in the bottom layers of sample carriers were fully protected from solar UV radiation. Both trays were closed under inert nitrogen atmosphere at ambient pressure. After closure, nitrogen in Tray 2 was exchanged with a Mars-like atmosphere (980 Pa of a gas mixture composed of 95.55% CO₂, 2.70% N₂, 1.60% Ar, 0.15% O₂, and circa 370 ppm H₂O). From sample integration to arrival at the launch site (Baikonur, Kazakhstan), temperatures remained within 4 to 25°C, except for one hour when temperatures dropped down to 4-2°C.

The EXPOSE-R2 hardware containing the samples was launched to the ISS on July 23rd 2014, on-board the Progress 56 cargo spacecraft. After 26 days on-board the ISS, it was installed (covered, so not exposed to UV) on the Universal Workplace D (URM-D) of the Russian Svezda module.

Two days later, Tray 1's valve was open, causing nitrogen to leave and be replaced with space vacuum. The hardware remained covered for 62 days for hardware and samples to outgas in the dark, before the cover was removed and samples were exposed to UV for 469 days.

Samples were then brought back inside the ISS, where they remained for 136 days before being sent back to Earth on-board the Soyuz 45S capsule. The sample racks were opened 5 days after landing, leading to a repressurization of the sample compartments. *Chroococcidiopsis* samples were then sent back to the University of Rome Tor Vergata for analysis.

More information on flight hardware and flight parameters can be found in Table 3.1 and in Rabbow et al. (2017).

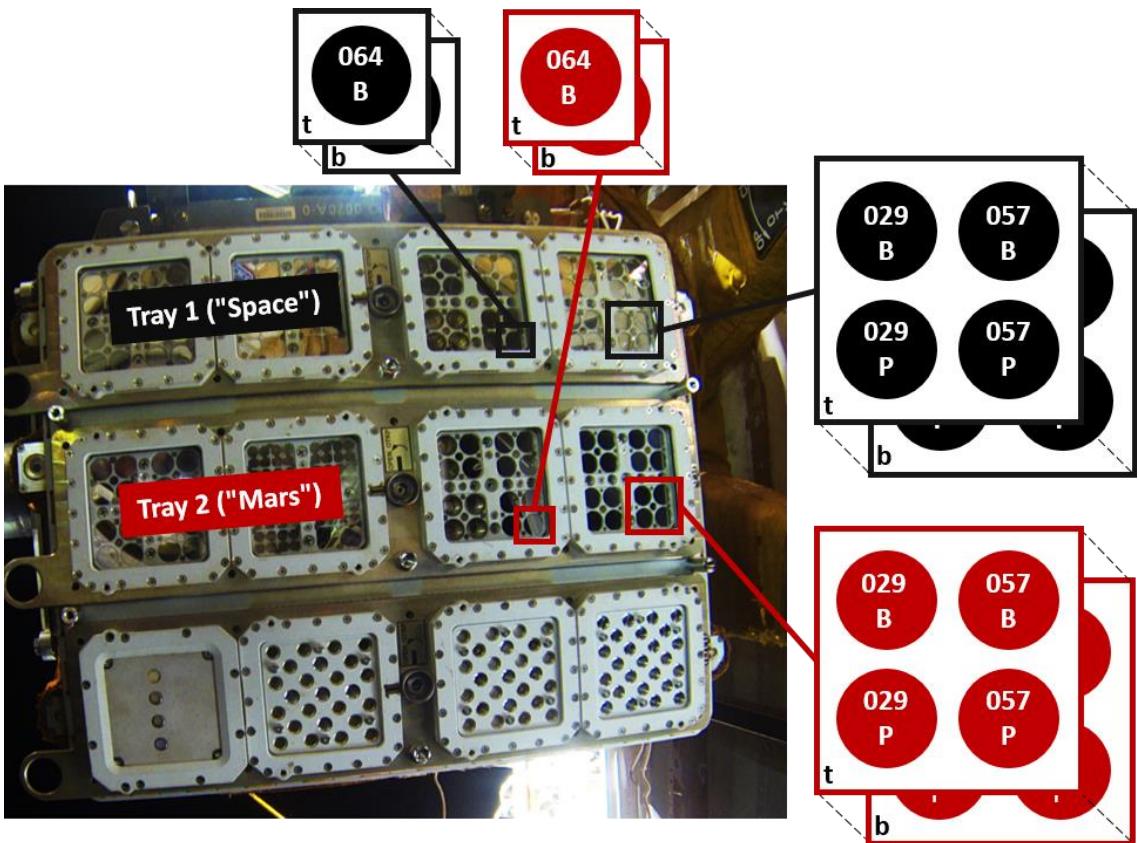


Figure 3.1. Location of *Chroococcidiopsis* samples part of the BOSS experiment in the EXPOSE-R2 facility. “Space” samples were located in Tray 1, compartments 3 and 4, and “Mars” samples in Tray 2, compartments 3 and 4, according to the EXPOSE-R2 terminology. B: biofilm; P: planktonic; 029, 057, 064: *Chroococcidiopsis* strains CCMEE 029, CCMEE 057 and CCMEE 064 (respectively). Each sample location had stacked sample carriers; samples in top carriers (t) were exposed to UV radiation, while samples in bottom carriers (b) were shielded from it. Compartment 4 of both trays also had a middle sample carrier, not shown here.

Mission ground references and laboratory controls

A simulation of the flight mission, the Mission Ground Reference, was performed at DLR’s Microgravity User Support Center (MUSC) in Cologne (Rabbow et al., 2016). Duplicates of the flight samples were kept in flight-similar hardware. This hardware was exposed to environmental conditions (temperatures, atmospheric conditions and UV irradiation) mimicking the

flight mission environment, based on available data and within the limits of the simulation facilities.

No temperature data were available for the dark outgassing period (from opening Tray 1's valve to removing the cover), as data acquisition systems were turned off to avoid heating up of the samples under the cover. During the simulation of this period and until data was available, MGR trays were kept at 15°C. Afterwards, MGR conditions lagged approximately 2 months behind the flight mission conditions. More information on MGR parameters can be found in Table 3.1 and in Rabbow et al. (2017). Laboratory controls were stored in the air-dried state, at about 45% relative humidity, in the dark and at room temperature.

Survival

Approximately 10^6 cells per sample were resuspended in BG11, spread on agarized BG11 and incubated under routine growth conditions, either until colonies appeared or for 6 months (whichever came first). From samples that did not yield colonies, circa 2×10^7 cells were placed in 2 ml of BG11 medium and incubated for 3 months in routine conditions.

qPCR assays

DNA was extracted as described by Billi et al. (1998) but omitting the lysozyme and DNaseI treatment steps. Quantitative polymerase chain reactions (qPCRs) were performed as described by Baqué et al. (2013), carrying amplification with the SensiFAST SYBR Green Supermix (Bioline, Italy) and the following program: 95°C for 3 min; 40 cycles at 95°C for 5 s and 60°C for 10 s; and 72°C for 20 s. Melting point determinations were programmed at the end of all qPCR assays. All assays were performed at least three times, each including three replicates per sample.

Confocal laser scanning microscopy

Small fragments (about 2 mm²) of dried samples were immersed in water, placed between two cover glasses and observed with a confocal laser scanning microscope (CLSM; Olympus Fluoview 1000 Confocal Laser Scanning System). Images were acquired using a 60X objective and sequences were obtained by scanning optical sections throughout samples, moving along the

z axis with a step size of 0.52 μm . Images were processed with the Imaris v. 6.1.0 software (Bitplane AG Zürich, Switzerland).

To characterize the EPS composition, cells were stained for 30 minutes in the dark with the lectin concanavalin A (which specifically binds α -mannopyranosyl and α -glucopyranosyl residues) conjugated with the fluorophore Alexa Fluor 488 (Molecular Probes C-11252) at a final concentration of 100 $\mu\text{g}\cdot\text{ml}^{-1}$. Lipidic compounds were stained for 15 minutes in the dark with BODIPY FL C12 (Molecular Probes D-3822) at a final concentration of 100 nM. Both concanavalin A- and BODIPY-stained samples were then washed twice in PBS and immersed in water before analysis. Images were taken by exciting samples with a 488-nm laser and collecting the emission between 510 and 530 nm.

Sample thickness was assessed at six locations or more for each biofilm and planktonic sample of CCMEE 029 and 057. For each location, thickness was approximated as the step size multiplied by the number of steps needed to go through the sample along the *z* axis.

3D representations of the autofluorescence of photosynthetic pigments (chlorophyll *a* and phycobiliproteins) were obtained from CCMEE 057 after exciting cells with a 543-nm laser and collecting the emitted light between 555 and 609 nm, or after exciting at 635 nm and collecting between 655 and 755 nm. ROIs were selected across 211.5 x 211.5- μm areas, and the average intensity was determined in those ROIs for all optical sections throughout the sample. Peak intensity was determined as the average intensity within the optical section where intensity was highest. For each sample type, at least nine areas were averaged.

Table 3.1. Overview of exposure conditions. Flight: low Earth orbit (LEO), in the EXPOSE-R2 facility; MGR: ground-based facilities (mission ground reference); Lab: closet in the laboratory; Space: space (in LEO) or space-like conditions (in MGR); Mars: Mars-like conditions; top: upper sample carrier of a two-layer stack; bottom: lower sample carrier of a two-layer stack.

Parameter	Flight				MGR				Lab	
	Space		Mars		Space		Mars			
	top	bottom	top	bottom	top	bottom	top	bottom		
Microgravity	695 days				None					
UV irradiation	> 110 nm; ≈ 445-505 kJ/m ² at 200-400 nm.	None	> 200 nm; ≈ 446-505 kJ/m ²	None	> 200 nm; ≈ 445-505 kJ/m ²	None	> 200 nm; ≈ 446-505 kJ/m ²	None	None	
Ionizing irradiation	≈ 0.5 Gy				≈ 1 mGy (background radiation)					
Temperatures	-15.0°C to 48.5°C		-15.2°C to 48.3°C		Within 2°C of “Flight” conditions				Room temperature	
Experimental atmospheric conditions	Vacuum (≈ 10 ⁻³ –10 ⁻⁴ Pa), 672 days		Mars-like atmosphere (see text), 722 days		Vacuum (≈ 10 ⁻⁵ –10 ⁻⁶ Pa), 672 days	Mars-like atmosphere (see text), 722 days		None (Earth- ambient only)		
Desiccation	> 900 days									

Results

Samples exposed in LEO stayed for close to 18 months outside the International Space Station, including more than 15 months when samples in top-layer carriers were also exposed to UV (Rabbow et al., 2017). Samples in bottom-layer carriers remained in the dark throughout the mission. Samples in Tray 1 (“Space”) were exposed to vacuum for 22 months, and those in Tray 2 (“Mars”) to a Mars-like atmosphere for close to 2 years (exposure times to vacuum and Mars-like atmosphere differ from LEO exposure times due to filling in the Mars-like atmosphere pre-flight and equilibrating carriers with ambient atmosphere post-flight). Ground reference samples were exposed to conditions mimicking exposure in LEO, based on available information and as far as technically possible, although ionizing radiation—to which EXPOSE-R2 was exposed to an absorbed dose of approximately 0.5 Gy (Dachev et al., 2017)—were not reproduced. Additional samples were stored in the dark, under ambient laboratory conditions. Due to time periods between sample preparation and mission start, and between mission completion and sample analysis, samples remained in a dry state for 2.5 years before analysis.

Survival

Survival results from desert strains of *Chroococcidiopsis* after exposure in LEO to space and Mars simulated conditions, ground-based simulations or storage in the laboratory, are given in Table 3.2.

After plating about 10^6 cells per sample, biofilm samples of CCMEE 057 formed colonies following all exposure conditions, both in LEO and under ground-based simulations, with and without exposure to UV. Their planktonic counterparts formed colonies only after exposure without UV irradiation, both in LEO and on the ground. Biofilms samples of CCMEE 029, on the other hand, formed colonies only after exposure to Mars simulations, in LEO and on the ground, in the dark. Planktonic samples of that strain formed colonies after exposure to Mars simulations in LEO, in the dark. Biofilms samples of CCMEE 064 formed colonies after all exposure conditions, both in LEO and on the ground, except under LEO exposure to UV radiation and Mars-like atmosphere.

After inoculating liquid medium with larger amounts of cells (about $2 \cdot 10^7$ cells per sample), growth was observed in all samples except for planktonic CCMEE 029 exposed to Mars simulations on the ground, with or without UV.

DNA damage revealed by qPCR

Genomic DNA from CCME 057 samples exposed in LEO and ground-based simulations was generally better preserved in biofilms than in their planktonic counterparts, as shown by a higher number of amplifiable copies of 16S gene per mass unit of DNA (Figure 3.2c). The only exceptions are samples exposed to space conditions (in LEO, or simulated on the ground) in the dark, for which there was no significant difference between planktonic and biofilm samples. In particular, for samples exposed to space or space-like conditions in the presence of UV, the number of amplifiable copies of the target gene was reduced by two orders of magnitude (compared to laboratory controls) in planktonic samples, but less than one order of magnitude in biofilms.

Genomic DNA from CCME 057 exposed to Mars-like conditions was more damaged than that from samples exposed to space or space-like conditions. For example, the number of amplifiable copies of the target gene in samples exposed to simulated Mars conditions in LEO, including UV, were one order of magnitude (for planktonic samples) or two orders of magnitude (for biofilms) lower than in the corresponding samples in space conditions. This occurred even for samples in the dark: while DNA from samples exposed

Table 3.2. Survival of desert strains of *Chroococcidiopsis* spp. following exposure in low Earth orbit (LEO) or ground-based simulations, or laboratory storage. Flight: exposed in LEO; GR: exposed to ground-based simulations; Lab: stored in the dried state in the laboratory during the mission; Space: exposed to space or space-like conditions; Mars: exposed to Mars-like conditions; top: exposed in the upper sample carrier of a two-layer stack (exposed to UV); bottom: exposed in the lower sample carrier of a two-layer stack (kept in the dark); ++ (green): colonies appeared after plating approximately 10^6 cells; + (orange): growth was observed after inoculating growth medium with circa 2×10^7 cells; - (red): no growth was observed.

Strain	Lifestyle	Flight				GR				Lab	
		Space		Mars		Space		Mars			
		top	bottom	top	bottom	top	bottom	top	bottom		
CCMEE 057	Planktonic	+	++	+	++	+	++	+	++	++	
	Biofilm	++	++	++	++	++	++	++	++	++	
CCMEE 064	Biofilm	++	++	+	++	++	++	++	++	++	
CCME 029	Planktonic	+	+	+	++	+	+	-	-	++	
	Biofilm	+	+	+	++	+	+	+	++	+	

to space conditions (in LEO, or simulated on the ground) in the dark showed no marked difference with DNA from laboratory controls, the number of amplifiable copies of the target gene in samples exposed to simulated Mars conditions in LEO, in the dark, was about three orders of magnitude (for planktonic samples) or two orders of magnitude (for biofilms) lower than the respective laboratory controls.

In CCME 029 (Figure 3.2b), genomic DNA was generally better preserved in planktonic samples exposed to space, space-like conditions or Mars-like conditions than in their biofilm counterparts. DNA from samples exposed to space, space-like conditions or Mars-like conditions in the dark, in LEO or on the ground, was either similarly or better preserved than DNA from the respective (planktonic or biofilm) laboratory controls.

Genomic DNA from CCME 029 and 64 samples exposed to simulated Mars conditions, including UV, was better preserved than DNA from equivalent samples exposed to space or space-like conditions (Figure 3.2a, b). On the other hand, DNA from CCME 029 planktonic samples in the dark was generally better preserved in space or space-like than Mars-like conditions.

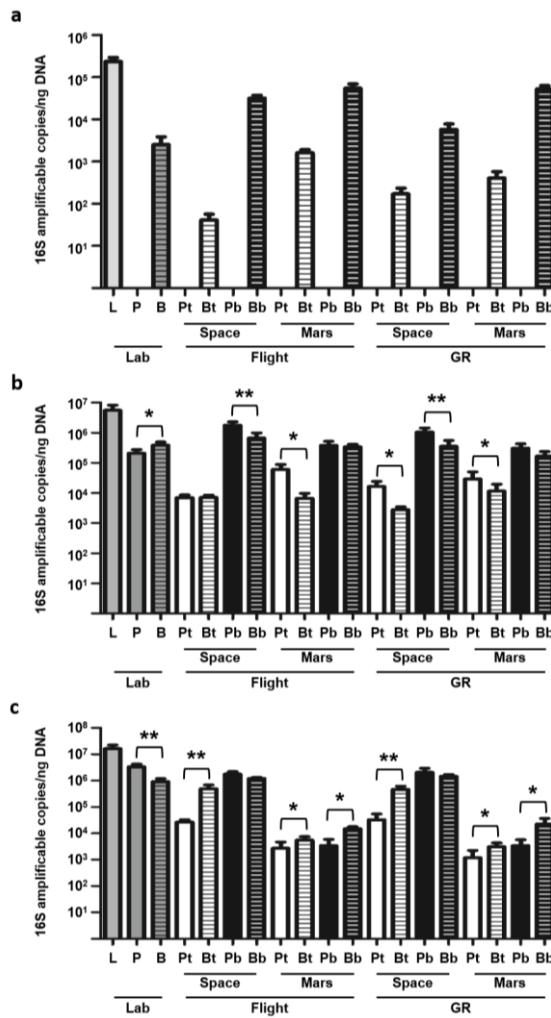


Figure 3.2. DNA damage in planktonic (P) and biofilm (B) samples of *Chroococcidiopsis* sp. CCME 064 (a), 029 (b), and 057 (c), as revealed by qPCR. For *Chroococcidiopsis* sp. CCME 064, only planktonic samples were included in the experiment. Bars show the number of amplifiable copies of the intact (amplifiable) target gene (encoding the 16S rRNA), normalized to the total mass of extracted DNA, for each sample. Data is shown as averages \pm standard deviations. Lab: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; GR: exposed to ground-based simulations; Space: exposed to space or space-like conditions; Mars: exposed to Mars-like conditions; L: liquid culture; P: planktonic, dried sample; B: biofilm, dried sample; t: exposed in the upper sample carrier of a two-layer stack; b: exposed in the lower sample carrier of a two-layer stack. Single asterisks indicate significant differences ($p < 0.05$) between planktonic and biofilm samples, based on planned Student t-tests uncorrected for multiple comparisons. Pairs of asterisks indicate significant differences ($p < 0.05$) after correcting for multiple comparisons using the Bonferroni method.

Confocal laser scanning microscopy

Sample thickness

Measurements of sample thickness revealed that biofilm samples were significantly thicker ($p < 0.05$ based on Student's t-tests) than their planktonic counterparts, for both strains: $52 \pm 5.7 \mu\text{m}$ (biofilm samples) vs $43 \pm 5.1 \mu\text{m}$ (planktonic samples) for CCME 057, and $65 \pm 1.5 \mu\text{m}$ (biofilm samples) vs $46 \pm 3.8 \mu\text{m}$ (planktonic samples) for CCME 029.

Presence of EPS in biofilm samples

The presence of EPS in 057 and 029 biofilm and planktonic samples was verified by CLSM imaging combined with two fluorescent dyes. In both strains, the lipophilic green fluorescent BODIPY revealed a much higher abundance of extracellular lipidic compounds in biofilms when compared to planktonic cells (Figure 3.3a, b). Staining with the lectin concanavalin A revealed no differences in the abundance of α -mannopyranosyl and α -glucopyranosyl residues in the EPS of biofilms and planktonic cells (Figure 3.3b, c).

Maintenance of photosynthetic pigment autofluorescence in biofilm bottom layers

The autofluorescence of photosynthetic pigments of strain CCME 057 (phycobiliproteins and chlorophyll *a*) in samples exposed to LEO, ground-based simulations or laboratory storage are given in Figures 3.4 and 3.5.

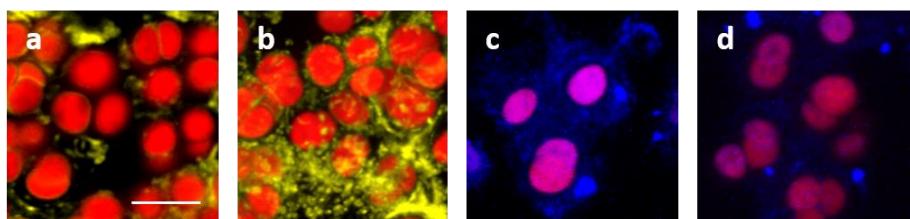


Figure 3.3. Confocal laser scanning microscope images of stained EPS in biofilm (b, c) and planktonic (c, d) samples of *Chroococcidiopsis* sp. CCME 057, confirming the higher abundance of lipids (but not polysaccharides) in biofilms than in planktonic samples. Lipids were stained using BODIPY FL C12 (a, b) and polysaccharides (α -mannopyranosyl and α -glucopyranosyl residues) using labelled concanavalin A (c, d). Scale bar: 7 μm .

CLSM images of laboratory biofilms, showing photosynthetic pigment autofluorescence, are presented in Figure 3.4. Imaging of biofilms revealed that for both laboratory (control) samples and samples exposed to Mars-like conditions in LEO, the first cell layer was completely bleached, the following two or three layers were partially bleached, and the layers below were unbleached (Figure 3.4, upper row). On the other hand, laboratory planktonic cells showed a low level of bleaching in the upper cell layer. Pigment autofluorescence in biofilms exposed to Mars-like conditions in LEO was only slightly reduced compared to laboratory controls. By contrast, the first 2-3 layers of planktonic samples exposed to the same conditions (Figure 3.4, lower

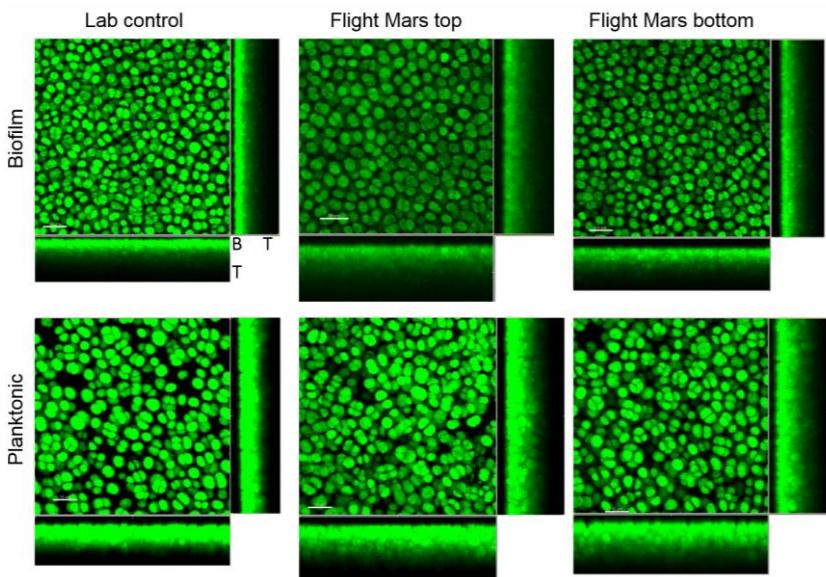


Figure 3.4. Confocal laser scanning microscopy imaging of phycobiliprotein autofluorescence in biofilms (top row) and planktonic samples (bottom row) of *Chroococcidiopsis* sp. CCME 057, with lateral projections showing fluorescence along a plane perpendicular to the main images. Samples were excited at 635 nm, and emission was collected between 655 and 755 nm. Lab control: stored in the dried state in the laboratory during the mission; Flight Mars top: exposed to Mars-like conditions in the upper sample carrier of a two-layer stack; Flight Mars bottom: exposed to Mars-like conditions in the lower sample carrier of a two-layer stack; T: top part of the sample; B: bottom part of the sample. Scale bar: 10 μ m.

row) were bleached, with a higher level of bleaching after UV exposure. Pigments were bleached slightly less in samples in space conditions than in the corresponding samples in Mars-like conditions, except for the planktonic sample in space conditions including UV, which was more bleached than the equivalent sample in Mars-like conditions (not shown).

The CLSM optical section with maximum photosynthetic pigment autofluorescence was determined in various areas for each sample, after determining the average intensity of all optical sections throughout those

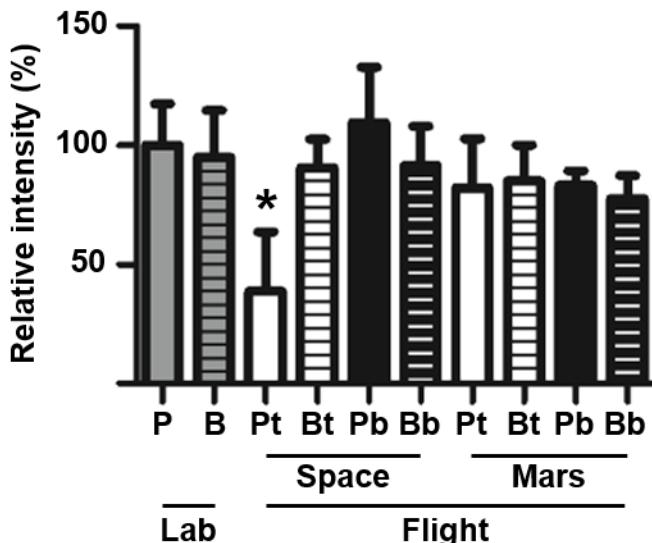


Figure 3.5. Peak intensity (average fluorescence emission intensity at the CLSM optical section where it is maximum) of phycobiliprotein autofluorescence, as percentage of planktonic laboratory samples, in *Chroococcidiopsis* sp. CCME 057. Lab: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; Space: exposed to space or space-like conditions; Mars: exposed to Mars-like conditions; P: planktonic, dried sample; B: biofilm, dried sample; t: exposed in the upper sample carrier of a two-layer stack; b: exposed in the lower sample carrier of a two-layer stack. Similar results were obtained for chlorophyll a autofluorescence (not shown). Data is shown as averages \pm standard deviation. Asterisks indicate significant differences ($p < 0.05$) with the respective (P or B) Lab control, based on Dunnett multiple comparison tests.

areas. For each sample, the average intensity of the optical sections where intensity is maximum (“peak intensity”) is shown in Figure 3.5. Data revealed that in biofilms, the peak intensity did not vary significantly between samples; pigment autofluorescence was preserved at the level of control cells, at a certain depth, in each sample (strongly suggesting that fluorescence in bottom cells was fully preserved). In planktonic samples exposed to space conditions including UV, the peak intensity was significantly reduced (down to below 40% of controls), showing some level of bleaching throughout the sample.

Discussion

If microbial life ever existed on Mars, it likely formed communities: planktonic ones in fluids, biofilms in sediments, or both. While organic material from both lifestyles may then have been preserved in sediments at concentrations high enough for detection with state-of-the-art instruments, most Mars-related biosignature and resistance studies involving microbial communities have so far relied on planktonic cells. Biofilms may however have been a more efficient lifestyle for microbial communities on Mars, and may have been better preserved, considering their generally higher resistance to environmental stressors on Earth.

The BOSS project mostly aims at comparing the resistance of prokaryotic biofilms, in space and Mars-like conditions, to that of planktonic cultures. It took advantage of both ground-based facilities (at DLR's MUSC) and LEO facilities (the European Space Agency's EXPOSE-R2 facilities, on the URM-D external platform of the Russian Svezda module). Those facilities allowed for the exposure of microbial biofilms and planktonic samples to extreme temperature cycles, vacuum or Mars-like atmosphere, and UV at the ranges found in space or on Mars. LEO facilities also included space factors non-simulated on the ground such as intense (compared to Earth's surface) ionizing radiation.

In this work, we assessed the survival of biofilms and planktonic samples of *Chroococcidiopsis* spp., the integrity of their genomic DNA, and the fluorescence of their photosynthetic pigments, after exposure to ground-based and LEO facilities, as part of the BOSS experiment. We notably investigated the role of upper cell layers within the biofilm organization, and compared the resistance of biofilms to their planktonic counterparts.

It should be noted that some properties of biofilms formed in microgravity, such as thickness and structure, may differ significantly from those of biofilms formed on Earth (e.g., Kim et al., 2013). However, given that we are here focusing on biofilms formed in non-microgravity environments (e.g., theoretical biofilms grown on Mars, or biofilms formed on spacecraft surfaces pre-flight), and not on, for instance, potentially pathogenic or technophilic biofilms forming in inhabited spacecraft (e.g., Mora, 2016), this should not affect the relevance of our conclusions.

Differences in survival

Overall, survival results demonstrate once again the high resistance of *Chroococcidiopsis* spp. to space and Mars-like conditions. All samples, except for planktonic samples of CCME 029 exposed to Mars-like simulations on the ground, survived exposure to even the harshest experimental conditions of the BOSS project. This was the first time that *Chroococcidiopsis* cells survived exposure to space and simulated Mars conditions in LEO, including full-range (though attenuated) UV, without the protective environment of a rock substrate. During the EXPOSE-R mission, *Chroococcidiopsis* 029 survived exposure to comparable conditions when within porous rocks (impact-shocked gneiss), but neither laboratory controls nor flight samples on glass discs survived (Bryce et al., 2014).

Those results also revealed the higher resistance of *Chroococcidiopsis* biofilms to space and Mars conditions compared their planktonic counterparts. Indeed, colonies were obtained (after plating circa 10^6 cells) from all CCME 057 biofilm samples but not from planktonic samples exposed to UV radiation (although the latter showed growth after inoculating liquid medium with larger amounts of cells). Similarly, all CCME 029 and CCME 064 biofilm samples showed growth, while planktonic samples of CCME 029 exposed to Mars-like conditions on the ground did not (no planktonic sample of CCME 064 was included). Part of this resistance can be explained by the multilayer structure of our samples, where upper cells protected lower cells (this hypothesis is supported by qPCR and microscopy results discussed below). Such a shielding effect was previously observed in *Chroococcidiopsis* 029: the survival fraction after exposure to the Atacama UV flux was roughly equivalent to the fraction of cells that clumped as four or five cell aggregates, in an otherwise monolayer sample (Cockell et al., 2008).

As a side note, the lack of growth in CCME 029 samples exposed to Mars-like conditions on the ground do not necessarily indicate that populations in those samples were eradicated: only limited amounts of material were available, and the fraction of surviving cells may be too low to make probable the occurrence of living cells in the number used for growth assays. It is also possible, as mentioned in Chapter 1, that a number of cells in those samples were viable but would have required different culture conditions to resume growth. It has for instance been shown in *Deinococcus geothermalis* (also part of the BOSS experiment) that UV radiation can induce a viable but non-culturable state in which cells are no longer able to grow on their usual medium but enter a dormancy stage until more favorable environmental conditions are provided (Oliver, 2005; Li et al., 2014; Frösler et al. 2017).

After plating about 10^6 cells of CCMEE 029, only flight samples in the dark and in Mars-like atmosphere (except for the ground reference planktonic sample), and the planktonic laboratory controls, gave colonies (although most samples resumed growth when rehydrating larger amounts of cells). This can be explained by the fact that desiccation and long-term storage reduced survival by several orders of magnitude, as evidenced by the lack of colonies from biofilm laboratory controls and the very low number of colonies overall. This issue is similar to what occurred during EXPOSE-R, where laboratory controls of CCMEE 029 on glass discs failed to yield colonies (Bryce et al., 2014). On the other hand, both EXPOSE-E and EXPOSE-R controls grew on rock substrates (Bryce et al., 2014; Cockell et al., 2011). This suggests a critical role of the substrate on which cells are dried. Viable CCMEE 029 cells, dried on Petri dishes, were recovered after 4 years of storage in the desiccated state (Billi, 2009), and colony forming abilities of CCMEE 029 desiccated over polycarbonate filters were only reduced by about one order of magnitude after 4 years (Fagliarone et al., 2017). However, the long-term survival of CCMEE 029 dried on agar had not been tested.

CCMEE 064 biofilms formed colonies in all conditions except for UV-including Mars simulation in LEO, suggesting a resistance intermediate between CCMEE 029 and 057. It should be noted, however, that the UV fluence received by CCMEE 064 was less than that received by the other two strains ($445\text{--}446 \text{ kJ}\cdot\text{m}^{-2}$ in the $200\text{--}400 \text{ nm}$ range, against $505 \text{ kJ}\cdot\text{m}^{-2}$), due to light-brown discoloration of the windows (Rabbow et al., 2017).

The fact that colonies were obtained from planktonic cells only in samples kept in the dark, both in LEO and ground-based simulations, is consistent with the consensus that UV is the most limiting factor to microbial survival in space (e.g., Horneck, Klaus, & Mancinelli, 2010). It is also consistent with EVTs and SVTs results conducted in preparation for the mission (Baqué et al., 2013c).

During EVTs and SVTs, CCMEE 057 showed, as in the present study, higher survival than CCMEE 029: its biofilms formed colonies (from circa 10^8 cells) after both space simulations (vacuum and $5 \times 10^5 \text{ kJ}\cdot\text{m}^{-2}$ of polychromatic UV radiation attenuated with 0.1% ND filter) and Mars simulations (780 Pa of Mars-like atmosphere and $5 \times 10^5 \text{ kJ}\cdot\text{m}^{-2}$ of polychromatic UV radiation attenuated with 0.1 % ND filter), while neither biofilms nor planktonic samples of CCMEE 029 survived UV exposure (Baqué, Scalzi, et al., 2013; Baqué, unpublished). Biofilms of CCMEE 064 had shown a survival potential similar to that of biofilms of CCMEE 029 and CCMEE 057 during EVTs (colonies formed after up to $1,500 \text{ kJ/m}^2$ of

polychromatic UV irradiation) and survived better than 029 under Mars simulated conditions (including UV) during the SVTs (Baqué et al., unpublished).

Although survival to the worst conditions here does not allow for conclusions on survival over timeframes relevant to lithopanspermia, it raises concerns on planetary protection. It is extremely difficult to sterilize spacecraft carrying modern instrumentation, and microbial contaminants could jeopardize search-for-life missions or even contaminate indigenous ecosystems if present (Moissl-Eichinger et al., 2016). As a consequence, the prevention of Mars contamination by terrestrial organisms is the main focus of planetary protection regulations. All samples survived exposure to space, when shielded from UV, for durations higher than Earth-to-Mars transit of probes or foreseen crewed Mars missions (transit time for the Mars Science Laboratory, for instance, was less than 9 months). This supports the idea that organisms able to survive a journey through space after contaminating a probe may belong to a wide range of genera. Besides, our results suggest that contaminants might survive months on Mars if shielded from UV, for instance by Martian dust (a few millimeters of it might be enough to confer full UV protection; see for instance Cockell & Raven 2004 or Mancinelli & Klovstad 2000). Under an average UV flux of $1.5 \times 10^3 \text{ kJ} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ (as predicted by Cockell et al., 2000 at the Martian equator for vernal equinox), biofilms of CCMEE 057 could survive about 8 hours. This could favor dissemination of viable organisms (Cockell et al., 2005): if carried, for instance, by winds at $5 \text{ m} \cdot \text{s}^{-1}$ (see Gomez-Elvira et al., 2014) and with the average flux mentioned above, they could travel more than 100 km without losing viability (this number is an example and would greatly vary based on latitude, solar longitude, atmospheric opacity, wind speed, and time of day). On the other hand, other factors found on Mars (e.g., oxidative species) may reduce the planetary protection risk: perchlorates, for instance, have been shown to be highly damaging to life, even at Mars-like temperatures, when activated by UV (Wadsworth and Cockell, 2017).

Differences in DNA damage

Microorganism inactivation in space is thought to be driven primarily by DNA damage caused by UV, due to the high absorption of UVB and UVC by DNA (Horneck et al., 2010). In the present work, DNA damage was assessed by qPCR.

Results for CCMEE 057 show that DNA in planktonic cells was strongly damaged by UV. Biofilms generally underwent lower levels of DNA damage, in agreement with ground-based simulations performed prior to the mission (Baqué et al., 2013c). This is consistent with the survival results described above, where no colonies appeared from planktonic cells (circa 10^6 per sample) exposed to UV. However, damage to biofilm samples exposed to Mars-like conditions (which yielded colonies) resulted higher than damage to planktonic samples exposed to UV under vacuum (which did not). Similarly, values for DNA damage resulted higher in CCMEE 029 biofilms than in the planktonic samples of the same strain, in spite of the better-preserved viability of the former. A likely explanation for this discrepancy between survival and DNA integrity is an unequal repartition of DNA damage: survivors in biofilms may be barely-damaged lower cells, protected by upper cells which are highly damaged (and thus increase the average number of lesions per mass unit of DNA). EPS may play a role in this shielding effect. The exact composition of EPS samples is hard to determine due to their wide range of components, each of which requiring different extraction methods (Flemming et al., 2007), and only a rough composition assessment was performed here. However, EPS generally contain various UV-absorbing macromolecules such as, for instance, nucleic acids and proteins. Some cyanobacterial EPS also contain UV-screening pigments (Böhm et al., 1995), but the presence of such compounds among *Chroococcidiopsis* spp.'s EPS was not investigated in the present study. Here, the abundance of lipids but not of polysaccharides in biofilms of this strain, as well as in strain CCMEE 029, is consistent with observations by Baqué, Scalzi et al. (2013). Another factor at play may be the presence of a fraction of highly resilient cells in the biofilms: even mono-species biofilm communities often comprise phenotypically distinct subpopulations with varying degrees of stress tolerance (e.g., Boles et al., 2004; Dufour et al., 2012).

Space vacuum by itself did not decrease DNA integrity (compared to laboratory controls) further than Earth's atmosphere in *Chroococcidiopsis* spp., consistently with previous work by Baqué et al. (2013). It could be due to the fact that the ambient atmosphere to which laboratory controls were exposed favors oxidative stress. Consistently, it has been reported that a high fraction (15 to 35%) of dried cells of *Deinococcus radiodurans* survived 17 months of storage in dry argon, but that a much lower fraction (< 0.01%) survived in the same conditions if relative humidity was above 20% (Dose et al., 1995).

DNA in samples of CCMEE 057 exposed to Mars-like atmosphere was much more degraded than that of laboratory samples. A similar effect was observed after the pre-mission SVTs (Baqué et al., 2013c), although to a lower extent (likely because of the shorter exposure time: 99 hours there against 722 days here). A likely explanation is spontaneous DNA decay by hydrolysis, which, according to Mileikowsky et al. (2000), would be one of the two possible limiting factors for survival in atmosphere-containing compartments of ejecta traveling through space (the other possible factor being ionizing radiation, depending on the microorganism's resistance to it). Mileikowsky et al. (2000) estimated that DNA decay by hydrolysis would occur much more slowly than DNA degradation by vacuum, which may at first seem inconsistent with our results. However, this phenomenon (as well as other chemical alterations such as oxidation) is highly dependent on temperature (Lindahl, 1993). As an example: based on the Arrhenius equation and the constants found by Lindahl & Nyberg (1972), the reaction rate for depurination of DNA in solution is approximately 9 orders of magnitude lower at 20°C than at Mars's average surface temperature (-63°C). Assessing hydrolysis rates in our case would be tentative: the mentioned constants were determined for DNA in solution. The Arrhenius equation may not describe hydrolysis when liquid water is unstable, and rates may be reduced by the scarcity of water. On the other hand, air-dried DNA remains partially hydrated and is still subject to hydrolysis. More drastic drying changes the conformation of DNA (as water is needed for structure), making it more sensitive to damage (Lindahl, 1993). Besides, fully dry DNA becomes highly hygroscopic; DNA may thus have been partly hydrated when temperatures allowed for some liquid water at 980 Pa. Data is consequently lacking to accurately assess hydrolysis rates but, if this is the reason for the relatively low preservation of *Chroococcidiopsis* 057 DNA in Mars-like atmosphere, this phenomenon would be expected to be several orders of magnitude slower at Mars's average temperature or temperatures expected during lithopanspermia.

This damaging effect of a Mars-like atmosphere was not observed in CCMEE 029 or 064. A difference in sensitivity was previously documented: in ground-based simulations where biofilm and planktonic samples from both strains were exposed to vacuum or Mars-like atmosphere, in the presence or not of UV, CCMEE 057 DNA was more damaged in samples exposed to Mars-like atmosphere than in samples exposed to space vacuum (Baqué et al., 2013c) while the reverse was true for CCMEE 029 (Baqué, unpublished data). One explanation could be the presence of defense mechanisms in *Chroococcidiopsis* 029 against DNA hydrolysis: such mechanisms have been

observed in spores and can be mediated, for instance, by DNA-binding proteins (Setlow, 1992).

It could be interesting to further study DNA degradation in Mars-like atmosphere in different temperature regimen, as a negative effect of the Martian atmosphere may reduce the likelihood of lithopanspermia transfer in atmosphere-filled cavities as described by Mileikowsky et al. (2000).

Preservation of photosynthetic pigments

Results from photosynthetic pigment (phycobiliproteins and chlorophyll *a*) autofluorescence assessment by confocal microscopy clearly show that in CCME 057, the upper layers of cells provide to the layers below a protection against vacuum, Mars-like atmosphere and ionizing radiation, consistently with previous ground-based simulations (Baqué et al., 2013c). In all samples except for planktonic samples exposed to space vacuum and UV, there is an optical section where the average intensity is equal to that of laboratory samples at the depth where intensity is maximum, suggesting that bottom cells were fully protected from pigment photobleaching.

Those results are particularly interesting in the context of the search for life on Mars: as described in Chapter 1, the photosynthetic pigments phycobiliproteins and chlorophyll *a* are targets for fluorescence-based search for signs of life. However, they are readily photobleached (Dartnell & Patel, 2014; Sinha et al., 1995; Sinha et al., 2002) and, on Mars's surface, may be quickly destroyed by UV and the highly oxidizing surface conditions they create (Yen et al., 2000). Assuming they do and only shielded samples have a chance to be preserved, the question has been raised as to whether the fluorescence from samples exposed to the surface for fluorescence-based detection of biosignatures could be preserved long enough to be detected after being exposed to UV radiation during probe operations (Dartnell and Patel, 2014). Previous studies showed that the autofluorescence (emission above 590 nm after excitation at 568 nm) of dried samples of *Chroococcidiopsis* 029 was reduced to 14% of controls after a 1-hour exposure to the Martian UV flux, and 5% after 4 h (Cockell et al., 2005c). In multilayer samples, autofluorescence of chlorophyll *a* and phycobiliproteins was reduced to below 20% of controls after one day of simulated Mars UV flux (Baqué et al., 2014). Here, the dose received by samples exposed to Mars-like conditions corresponds to approximately 8 hours under the average UV flux of Mars on the equatorial Martian surface, at the vernal equinox, based on Cockell et al. (2000)'s model. Although the first layers were bleached, the intensity is

preserved in the layer of maximum intensity. Such results are comforting regarding the likelihood of pigments to remain detectable after a short-term exposure to UV during probe operations.

Conclusion

Chroococcidiopsis spp. once again demonstrated their high resistance to space and Mars conditions: cells survived exposure to all space and Mars-like experimental conditions of the BOSS experiment in low Earth orbit. They also survived the ground-based simulations performed in parallel, except for planktonic cells of CCMEE 029 exposed to Mars-like conditions on the ground.

Our results also demonstrate a higher resistance of biofilms than planktonic cells, consistently with ground-based simulations performed prior to the mission (Baqué et al., 2013b, 2013c). In particular, all biofilm samples of CCMEE 057 yielded colonies after plating a million cells, while no colony was obtained from planktonic samples exposed to UV radiation at the space or Mars range. Similarly, DNA and photosynthetic pigments were better preserved in biofilms of this strain. All biofilm samples of CCMEE 029 showed growth after exposure, while their planktonic counterparts did not after exposure to Mars-like conditions (including UV) on the ground. All samples of CCMEE 064 biofilms also resumed growth, although no comparison is possible with their planktonic counterparts.

A large part of the high resistance of *Chroococcidiopsis* samples seems to be related to the protective effect conferred by upper cell layers. This passive mechanism would favor biofilms, as they naturally occur as multilayer aggregates. The presence of (lipid-rich) EPS likely contributes significantly to radiation shielding. Other biofilm-specific mechanisms, such as metabolic adaptations, are probably at play. As an example, cells at the bottom of biofilms are often physiologically similar to planktonic cells in a stationary phase (Stewart & Franklin, 2016), a stage at which they are often most resistant to external stresses (Wassmann et al., 2011). Biofilm cells may also express stress-responsive genes, leading to more resistant phenotypes (Foley et al., 1999; Layton & Foster, 2003), and some bacteria (including cyanobacteria) have been shown to release UV-screening pigments and oxidation-scavenging compounds among their EPS (Böhm et al., 1995; Shirkey et al., 2000). Some structural roles suspected to be played by EPS during dehydration, namely replacing the water shell around bacteria's

macromolecules with hydrogen bonds and stabilizing cell membranes, may have limited damage created by vacuum. EPS may also have helped preventing ROS accumulation and/or reactivity (Chen et al., 2009; Gao & Garcia-Pichel, 2011), and/or stabilized repair enzymes (Helm & Potts, 2012).

While extensive work would be worth performing to elucidate the exact mechanisms at play, our results add to the evidence that focusing on planktonic cells when assessing life's limits may lead to strong underestimations of its resilience. We suggest that more emphasis should be given to what is, after all, microorganisms' most common organizational state: biofilms.

As for our desert strains of *Chroococcidiopsis* spp.: planktonic samples could survive exposure to space and simulated Mars conditions for a significant amount of time, but biofilms would eventually be more likely to survive an interplanetary journey, be it as spacecraft contaminants or as inhabitants of a rock naturally expelled from its planet of origin.

Acknowledgements

This research was supported by the Italian Space Agency (ASI 053-R.0 to DB) and the German Helmholtz Association through the Helmholtz-Alliance “Planetary Evolution and Life”. We acknowledge the European Space Agency (ESA) for the selection of BOSS. We thank Dr. Elke Rabbow (DLR Cologne) for conducting the ground-based simulations, assembling and de-integrating the samples, Dr. René Demets (ESA) for supporting EXPOSE experiments, and all ISS staff for taking care of EXPOSE-R2. The authors thank Dr. Elena Romano, Centre of Advanced Microscopy “P.B. Albertano”, University of Rome “Tor Vergata”, for her skillful assistance in using the CLSM. MB acknowledges support from the Alexander von Humboldt Foundation.

Chapter 4

Responses of the desert cyanobacterium *Chroococcidiopsis* to simulated Martian conditions in low Earth orbit: implications for the limits of terrestrial life and the habitability of Mars³

The aim of the project BIOlogy and Mars EXperiment (BIOMEX), part of the EXPOSE-R2 space mission, is to assess the endurance of extremophiles and the stability of their macromolecules under space, Moon, and Mars conditions. Results will be fed to a biosignature database, as a reference for future life-detection missions beyond Earth such as the European Space Agency's ExoMars. Among the selected extremophiles for the BIOMEX experiment is a desert cyanobacterium, *Chroococcidiopsis* sp. CCME 029. Cyanobacterial cells mixed with analogues of Mars minerals were exposed to space and simulated Martian conditions in low Earth orbit, within the ESA facility EXPOSE-R2, outside the International Space Station. Survival and biosignature detectability were then investigated. Survival was assessed by growth-dependent assays, spectral features of photosynthetic pigments (chlorophyll *a* and phycobiliproteins) were evaluated by confocal laser scanning microscopy, and DNA preservation was assessed by performing PCR-based assays. Results are consistent with previous ground-based simulations of the mission and demonstrate a very high tolerance of *Chroococcidiopsis* to space and Mars-like conditions—with dried multilayers surviving long-term exposure to a Mars-like atmosphere in low Earth Orbit, even when exposed to 0.1% of the incoming UV flux at a Mars-like range—and a strong protective role played by Mars mineral analogues.

³This chapter is in preparation for publication as: Daniela Billi, Cyprien Verseux, Mickaël Baqué and Jean-Pierre de Vera. Responses of the desert cyanobacterium *Chroococcidiopsis* to simulated Martian conditions in low Earth orbit: implications for the limits of terrestrial life and the habitability of Mars

Introduction

The question of whether Mars ever harbored life remains open.

The Archean Earth and Noachian Mars are thought to have borne similar climatic and environmental conditions (Carter et al., 2015; Wynn-Williams and Edwards, 2000). In particular, extensive evidence suggests that Mars's surface used to harbor large amounts of liquid water, although its abundance and persistence through time are still debated (Bibring et al., 2006; Carter et al., 2015; Vago et al., 2017). The UV irradiation environment is also thought to have been similar: the Archean Earth supposedly lacked an ozone layer and UV radiation down to 200 nm likely reached both planets' surfaces (Cockell et al., 2000; Patel et al., 2004). Although the exact conditions for life to originate are not surely determined, elements thought to be essential (noteworthy organic molecules, liquid water, and energy from the Sun and/or volcanism) are believed to have been present on both planets at some point in their history (McKay, 2010). Therefore, an origin of life on Mars is a scenario worth considering (Cockell, 2014; McKay, 2010; Westall et al., 2013). Besides, wherever life originated, early lifeforms may have been exchanged between both planets: according to the lithopanspermia theory, viable microbes could be carried by rocks expelled by impacts (Horneck et al., 2008; Nicholson, 2009).

The Earth and Mars then evolved quite differently: Mars lost its intrinsic magnetic field, most of its atmosphere and its surface liquid water. However, pockets of conditions habitable to life as we know it may have persisted up to present day (Cockell, 2014; Westall et al., 2013). For instance, evidence suggests the presence of abundant liquid water below Mars's cryosphere (Ehlmann et al., 2011; Michalski et al., 2013). Even close to the surface, protected niches could have offered habitable conditions until today (Cottin et al., 2015; Schirmack et al., 2014; de Vera et al., 2014). Besides, various energy and nutrient sources suitable for autotrophic microbial metabolisms are present on Mars (Cockell, 2014; Nixon et al., 2013; Verseux et al., 2016a). As a consequence, there is strong interest in searching for extant or extinct life on the red planet (Vago et al., 2017).

In 1976, NASA landed the Viking probes on Mars to search for life there. Results were inconclusive, in large part due to our lack of knowledge of the Martian surface, which led to an inability to either detect life or distinguish it from physico-chemical reactions (Schulze-Makuch et al., 2015). Since then, our understanding of the Martian surface has considerably increased. Our

knowledge of the limits for life on Earth also were extended. In many terrestrial environments believed before the 1960s and 1970s to be devoid of life, organisms were discovered with unusual protection and adaptation mechanisms allowing them to thrive there (Rothschild and Mancinelli, 2001). Some of those environments gained a lot of attention in recent years due to the similarities they bear with presumed Martian environments. As examples, the Dry Valleys in Antarctica, the hyperarid core of the Atacama Desert, and the Siberian permafrost are considered Martian analogues due to their aridity, temperatures, and/or geological features (Martins et al., 2017). Organisms thriving in those environments are therefore relevant models to assess the habitability of Mars.

The resistance of such organisms to conditions found beyond Earth, notably on Mars and in space, has been tested in ground-based simulations and in low Earth orbit (LEO). Organisms from the three domains of life have been shown to survive exposure to space (Horneck et al., 2010; Jönsson et al., 2008; Leuko et al., 2014) and/or simulated extraterrestrial conditions (Cottin et al., 2017). In addition to organisms' survival, the preservation of biomarker molecules (whose detection on Mars may indicate the presence of life, extinct or extant) in such conditions is subject to numerous investigations (Hays et al., 2017; Westall et al., 2015).

The BIOLOGY and Mars EXperiment (BIOMEX) project aims at further investigating the resistance of selected extremophiles (mixed with Moon or Mars regolith simulants), and the stability/degradation of their macromolecules, when exposed to space and Mars-like conditions in ground-based facilities and in LEO (de Vera et al., 2012). The LEO part of the experiment was carried within the EXPOSE-R2 mission. Organisms were exposed on the Russian Svezda module, outside the International Space Station (ISS), from August 2014 to February 2016.

Among the extremophiles selected for BIOMEX were desert cyanobacteria of the genus *Chroococcidiopsis*, chosen for their resistance to extreme conditions found on Earth and beyond (Billi et al., 2017). *Chroococcidiopsis* spp. thrive in extreme deserts on Earth, including the Atacama desert in Chile and the Dry Valleys in Antarctica (Friedmann, 1980; Stivalletta et al., 2012). They are highly resistant to desiccation [at least to 4 years of air-dried storage (Billi, 2009)], ionizing radiation [up to at least 24 kGy of photon irradiation, 2 kGy of heavy ions, and 24 kGy of γ -rays (Billi et al., 2000; Verseux et al., 2017)], UV irradiation [up to 13 $\text{kJ}\cdot\text{m}^{-2}$ UVC (Baqué et al., 2013a), and 30 $\text{kJ}\cdot\text{m}^{-2}$ of a simulated Martian UV flux (Cockell et al., 2005)]. Furthermore, they showed an outstanding resistance after 18 months in space as part of an

epilithic microbial community onboard EXPOSE-E (Cockell et al., 2011) and after 22 months on impact-shocked gneiss onboard EXPOSE-R (Bryce et al., 2014).

In the frame of the BIOMEX experiment, *Chroococcidiopsis* cells were mixed with analogues of Mars regolith: the Phyllosilicate Mars Regolith Simulant (P-MRS) and the Sulfatic Mars Regolith Simulant (S-MRS) (Böttger et al., 2012; de Vera et al., 2012). Ground-based simulations were performed prior to the mission to test sample preparation and handling, and optimize protocols, which provided insights into *Chroococcidiopsis*'s resistance to exposure conditions expected during this mission (Baqué et al., 2013b, 2014, 2016). Results showed that minerals have an overall protective effect on survivability and preservation of cellular components, notably DNA and pigments (Baqué et al., 2014, 2016). In the present work, dried cells of *Chroococcidiopsis* mixed with P-MRS or S-MRS were exposed to Mars-like conditions, both in LEO and on the ground, including a simulated Martian atmosphere and UV radiation at the range found on Mars's surface ($\lambda > 200$ nm). Ground-based simulations mimicked the flight mission environment, based on available data and within the limits of the simulation facilities (Rabbow et al., 2016), but not ionizing radiation from solar energetic particles (SEP), galactic cosmic rays (GCR) and inner and outer radiation belts. Additional air-dried controls were stored in the dark, under ambient laboratory conditions.

After exposure, we assessed the protective effects of P-MRS and S-MRS on survivability and preservation of molecular biomarkers (Parnell et al., 2007), namely genomic DNA and photosynthetic pigments (phycobiliproteins and chlorophyll *a*).

Materials and methods

Mars regolith analogues

The Phyllosilicate Mars Regolith Simulant (P-MRS) and Sulfatic Mars Regolith Simulant (S-MRS) are simulants of Martian regolith during the Noachian (“Early Mars”) and Hesperian/Amazonian (“Late Mars”) periods, respectively. They were prepared as described in Böttger et al. (2012), in the framework of the Helmholtz-Alliance “Planetary Evolution and Life”. Their composition is reported in Table 4.1.

Organisms and sample preparation

Chroococcidiopsis sp. CCME 029 was isolated by Roseli Ocampo-Friedmann from cryptoendolithic growth in sandstone in the Negev Desert (Israel). It is part of the Culture Collection of Microorganisms from Extreme Environments (CCMEE), established by E. Imre Friedmann and currently maintained at the University of Rome Tor Vergata. Before preparing dried

Table 4.1. Mineralogical composition of the Phyllosilicate Mars Regolith Simulant (P-MRS) and the Sulfatic Mars Regolith Simulant (S-MRS). Modified from Böttger et al. (2012).

Component	P-MRS (wt %)	S-MRS (wt %)
Gabbro (pyroxene, plagioclase, amphibole, illmenite)	3	32
Olivine	2	15
Quartz	10	3
Hematite	5	13
Montmorillonite	45	-
Chamosite	20	-
Kaolinite	5	-
Siderite	5	-
Hydromagnesite	5	-
Goethite	-	7
Gypsum	-	30

samples, *Chroococcidiopsis* sp. CCMEE 029 was grown under routine conditions at 25°C, in BG11 medium, under a photon flux density of 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by fluorescent cool-white bulbs with a 16/8 h light/dark cycle.

Dried samples were obtained by pelleting samples from a culture in exponential phase, resuspending cells to a density of $5 \times 10^9 \text{ cells}\cdot\text{ml}^{-1}$ in PBS and plating, on 100 mm diameter Petri dishes, 400 μl of cell suspension mixed with 0.2 g of either P-MRS or S-MRS. Samples were then air-dried for two days under a laminar flow hood, in the dark, before cutting out 12-mm-in-diameter disks. Disks were stored in the dark at room temperature until exposure (ground reference and flight samples) or throughout the mission (laboratory controls). Samples used as no-mineral controls were prepared in a similar way (though cells were not mixed with minerals before being spread on agarized BG-11 medium) and belong to the BOSS_Cyano experiment (Billi et al., in preparation), part of the BOSS project of EXPOSE-R2.

Exposure in low Earth orbit

The core facility of the EXPOSE-R2 mission had been used previously for the EXPOSE-R mission in 2008-2011 (Rabbow et al., 2014). Only a new set of trays, carriers and samples were therefore uploaded to the ISS. The trays and sample carriers were identical to those of EXPOSE-R (except for the PSS experiment, for which new specific sample holders were developed) but a new exposure procedure was followed to tackle window contamination issues that occurred during EXPOSE-R (Demets et al., 2015).

Chroococcidiopsis 029 flight samples were accommodated in Tray 2 of the EXPOSE-R2 flight hardware, in two-layer stacked sample carriers (Figure 4.1). Tray 2 was then covered with quartz windows and a long-pass cutoff

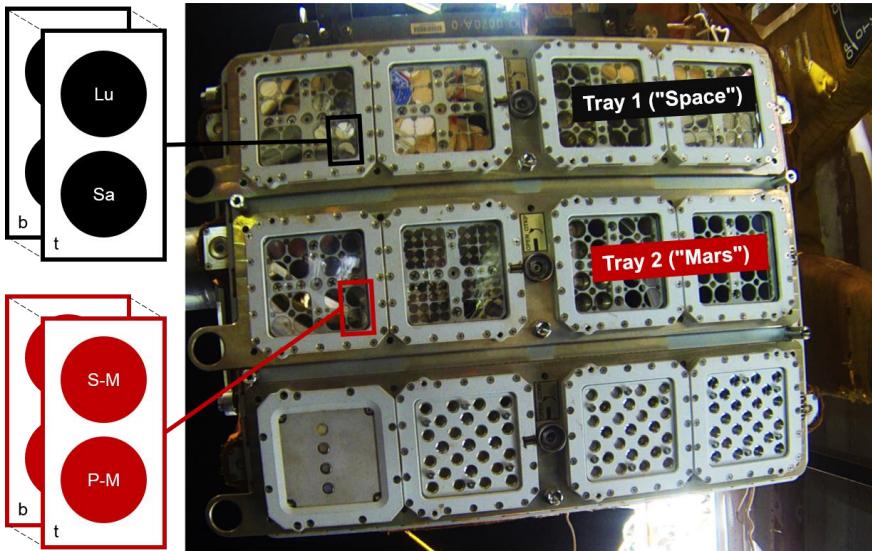


Figure 4.1. Locations of the *Chroococcidiopsis* sp. CCMEE 029 samples loaded to the EXPOSE-R2 facility for the BIOMEX experiment. Cells were dried and mixed with a Moon regolith analogue (Lu), sandstone (Sa), or one of both Mars regolith analogues: S-MRS (S-M) or P-MRS (P-M). Results reported in this work pertain to samples in the “Mars” tray. Those were exposed to LEO ionizing radiation, extreme temperature cycles, a Mars-like atmosphere and, for samples in upper carriers (t), UV at the Martian range ($\lambda > 200$ nm). Samples in bottom carriers (b) were shielded from UV. Samples without minerals used as controls belong to another experiment (Billi et al., in preparation) and were located in the rightmost compartment of Tray 2.

filter of approximately 50% transmission at 216 nm (for exposure to a Mars-like UV spectrum, $\lambda > 200$ nm). Samples were further covered with 0.1% neutral density (ND) filters, attenuating solar UV radiation by approximately 3 orders of magnitude, as previously used during the EXPOSE-R mission (Demets et al., 2015; Rabbow et al., 2014). Samples in the bottom layers of sample carriers were fully protected from solar UV radiation.

Trays were closed under inert nitrogen atmosphere at ambient pressure. After closure, nitrogen in Tray 2 was exchanged with a Mars-like atmosphere (980 Pa of a gas mixture composed of 95.55% CO₂, 2.70% N₂, 1.60% Ar, 0.15% O₂, and circa 370 ppm H₂O).

The EXPOSE-R2 hardware containing the samples was launched to the ISS on July 23rd 2014, on-board the Progress 56 cargo spacecraft. After 26

days on-board the ISS, it was installed (covered, so not exposed to UV) on the Universal Workplace D (URM-D) of the Russian Svezda module. The hardware remained in the dark for 9 weeks, before the cover was removed and samples were exposed to UV for 469 days.

UV radiation reaching top samples was quantified using two polyphenylene oxide (PPO) passive UV dosimeter stacks, covered with density filters and accommodated in the filter frames. Radiation (including ionizing radiation) was further measured by the Radiation Risk Radiometer-Dosimeter (R3D-R2) located in Tray 3, Compartment 1. Temperature was recorded in 9 locations of the platform and a heating system prevented it from falling below 21°C.

Samples were then brought back inside the ISS, where they remained for 136 days before being sent back to Earth on-board the Soyuz 45S capsule. The sample racks were opened 5 days after landing, leading to a repressurization of the sample compartments. *Chroococcidiopsis* samples were then sent back to the University of Rome Tor Vergata for analysis.

More information on flight hardware and flight parameters can be found in Rabbow et al. (2017).

Ground references and laboratory controls

A simulation of the flight mission, the Mission Ground Reference (MGR), was performed at DLR's Microgravity User Support Center (MUSC) in Cologne (Rabbow et al., 2016). Duplicates of the flight samples were kept in flight-similar hardware. This hardware was exposed to environmental conditions mimicking the flight mission environment, based on available data and within the limits of the simulation facilities. Mimicked factors included temperatures (from transportation to the launch site to return to DLR), atmospheric conditions, and UV irradiation (calculated by RedShift, and as far as technically feasible).

No temperature data was available for the dark outgassing period, as data acquisition systems were turned off to avoid heating up of the samples under the cover. During the simulation of this period and until data was available, MGR trays were kept at 15°C. Afterwards, MGR conditions lagged approximately 2 months behind the flight mission conditions. More information on MGR parameters can be found in Rabbow et al. (2017).

Laboratory controls were stored in the air-dried state, at about 45% relative humidity, in the dark and at room temperature.

Colony forming ability assays

Approximately 10^6 cells per sample were resuspended in BG11, spread on agarized BG11 and incubated under routine growth conditions, either until colonies appeared or for 6 months (whichever came first). From samples that did not yield colonies, circa 2×10^7 cells were placed in 2 ml of BG11 medium and incubated for 3 months under routine conditions.

Random Amplification of Polymorphic DNA

Cells from fragments (circa 2 mm^2) of each sample were resuspended in cold, bidistilled water, and DNA was extracted as described by Billi et al. (1998) but omitting the lysozyme and DNaseI treatment steps. DNA concentrations were determined using the Qubit dsDNA HS Assay Kit and a Qubit 2.0 Fluorometer (Invitrogen). 100 ng of DNA per sample were used as a template for amplification with the HIP1-CA primer (5'-GCGATCGCCA-3') and the following program: 94°C for 3 minutes; 30 cycles at 94°C for 30 seconds, 37 °C for 30 seconds and 72°C for 1 minute; and 72 °C for 7 minutes. Genomic DNA from a culture of *Chroococcidiopsis* 029 in exponential phase was used as a control. PCR products were loaded on a 1.5 % agarose gel and an electrophoresis was run at 90 V in TAE buffer. RAPD patterns were revealed using a UV transilluminator.

Confocal laser scanning microscopy

Small fragments (about 2 mm^2) of dried samples were immersed in water, placed between two cover glasses and observed with a confocal laser scanning microscope (CLSM; Olympus Fluoview 1000 Confocal Laser Scanning System). Images were acquired using a 60X objective and processed with the Imaris v. 6.1.0 software (Bitplane AG Zürich, Switzerland). The autofluorescence of photosynthetic pigments (chlorophyll *a* and phycobiliproteins) was revealed by successively exciting the samples with 543-nm and 635-nm lasers, and collecting the emitted fluorescence in the 555–609 and 655–755 nm ranges, respectively. The reflective signal of minerals was recorded between 490 and 510 nm after excitation at 488 nm. Three-dimensional images were obtained after scanning optical sections throughout samples, moving along the z axis with a step size of 0.52 μm . Spectral analysis of at least 15 cells per sample was performed in the optical sections where

autofluorescence was most intense, using the 543-nm laser at 0.54 mW and collecting the emission from 543 to 800 nm.

Results

Survival

The presence or absence of colonies was determined after plating approximately 10^6 cells per sample and incubating plates under routine conditions (Table 4.2). All flight samples exposed to Mars-like atmosphere in the dark (i.e., in bottom-layer carriers) yielded colonies, but those exposed to UV (i.e., in top-layer carriers) did not. No ground reference sample yielded colonies. Lab controls without minerals or with P-MRS yielded colonies, but those with S-MRS did not.

From samples that did not yield colonies, about $2 \cdot 10^7$ cells per sample were used to inoculate BG11 medium. Growth was observed in all samples except for those exposed to Mars simulations on the ground, either without minerals (with or without UV) or with S-MRS under UV.

DNA damage as revealed by random amplification of polymorphic DNA

Table 4.2. Colony forming abilities of *Chroococcidiopsis* sp. CCME 029 samples following exposure to Mars-like conditions in low Earth orbit (Flight samples) and ground-based simulation (GR samples), or after storage in laboratory conditions (Lab). top: exposed in the top-layer carrier of a two-layer stack; bottom: exposed in the bottom-layer carriers of a two-layer stack; P-MRS: mixed with the P-MRS regolith simulant; S-MRS: mixed with the S-MRS regolith simulant; No mineral: mixed with no mineral; ++ (green): colonies appeared after plating approximately 10^6 cells; + (orange): growth was observed after inoculating growth medium with circa 2×10^7 cells; - (red): no growth was observed.

Mineral	Lab	Flight (Mars-like)		GR (Mars-like)	
		top	bottom	top	bottom
No mineral	++	+	++	-	-
P-MRS	++	+	++	+	+
S-MRS	+	+	++	-	+

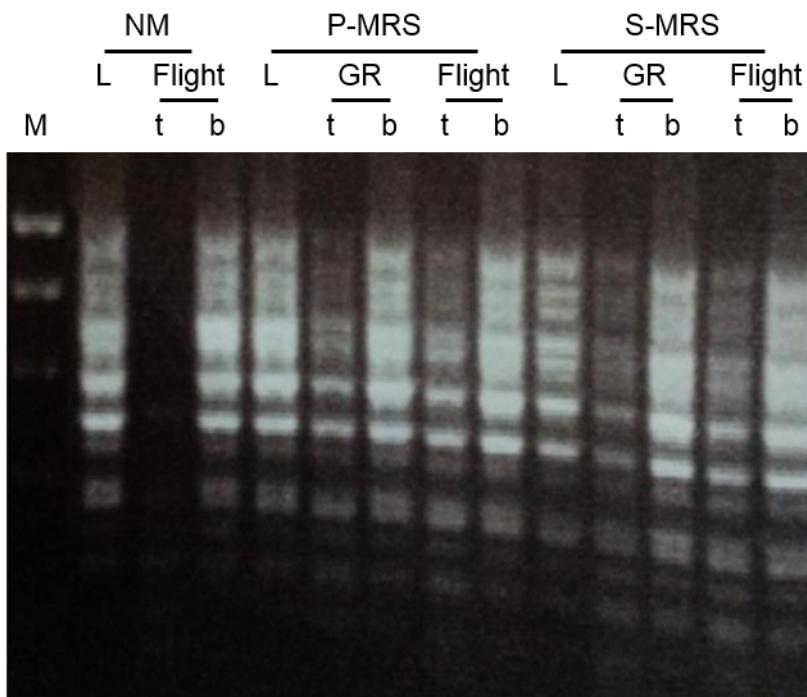


Figure 4.2. DNA fingerprints obtained after random amplification of polymorphic DNA (RAPD) assay, from *Chroococcidiopsis* sp. CCME 029 cells without mineral (NM) or with mineral mixtures (P-MRS or S-MRS). The gel is distorted; there is no steady reduction in fragments' size from left to right. M: DNA ladder; L: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; GR: exposed to ground-based simulations; t: exposed in the top-layer carrier of a two-layer stack; b: exposed in the bottom-layer carrier of a two-layer stack; P-MRS: mixed with the P-MRS regolith simulant; S-MRS: mixed with the S-MRS regolith simulant; NM: mixed with no mineral.

DNA damage was assessed by RAPD assays. The resulting fingerprints (Figure 4.2) showed a great reduction reduced in band numbers and intensity for samples exposed without minerals to UV and Mars-like atmosphere (no mineral top-layer carrier), indicating extensive DNA damage. DNA damage was attenuated (although still detectable, as shown by altered fingerprint profiles compare to laboratory controls) by the presence of P-MRS or S-MRS minerals.

Fingerprints from samples exposed to a Mars-like atmosphere in the dark are comparable to those from laboratory controls.

Alteration of photosynthetic pigments as revealed by CLSM

CLSM imaging revealed a heterogeneous association of dried *Chroococcidiopsis* cells with minerals: at a scale of tenths of μm , some areas are mineral-rich and cell-poor while others are cell-rich and mineral-poor (Figure 4.3). Sample thickness was mostly comprised between 5 and 15 μm , corresponding to approximately 2 to 6 cells, and never exceeded 30 μm (not shown).

The effects of exposure conditions on the autofluorescence of dried cells' photosynthetic pigments were assessed by performing CLSM λ -scans (Figure 4.4) and imaging (Figure 4.5). Cells were excited at 543 nm and emission was recorded over a 553–793 wavelength range (Figure 4.4). Averages show a peak at 648 nm for most of the samples (Figure 4.4). However, peaks from ground reference samples exposed without minerals, P-MRS laboratory samples, P-MRS flight samples exposed to UV and ground reference S-MRS samples exposed to UV were slightly shifted toward higher wavelengths (maximum at 653 nm), likely due to slight changes in phycobiliprotein-to-chlorophyll *a* fluorescence ratios.

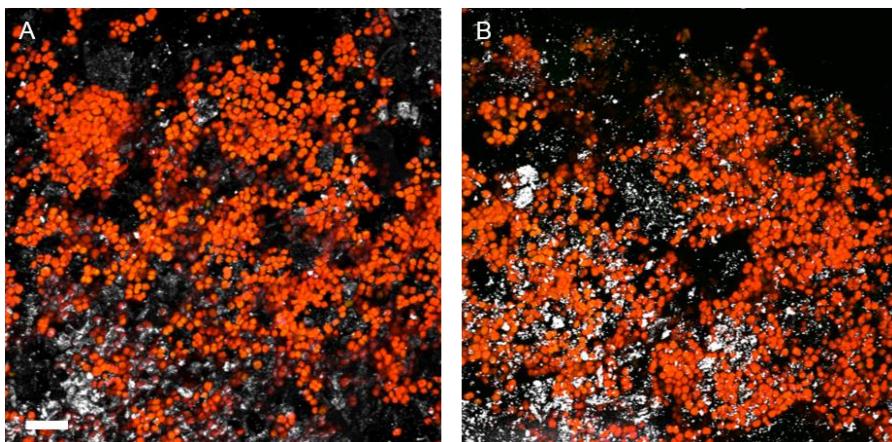


Figure 4.3. Heterogeneous association of cells with Mars regolith analogues, as revealed by confocal scanning laser microscope imaging. Minerals are visible through their reflectance (emission between 490 and 510 nm after excitation at 488 nm; shown in white) and dried *Chroococcidiopsis* sp. CCME 029 cells through the autofluorescence of phycobiliproteins and chlorophyll *a* (after successively exciting pigments with 543-nm and 635-nm lasers and collecting the emitted fluorescence in the 555–609 and 655–755 nm ranges, respectively; shown in orange) mixed with

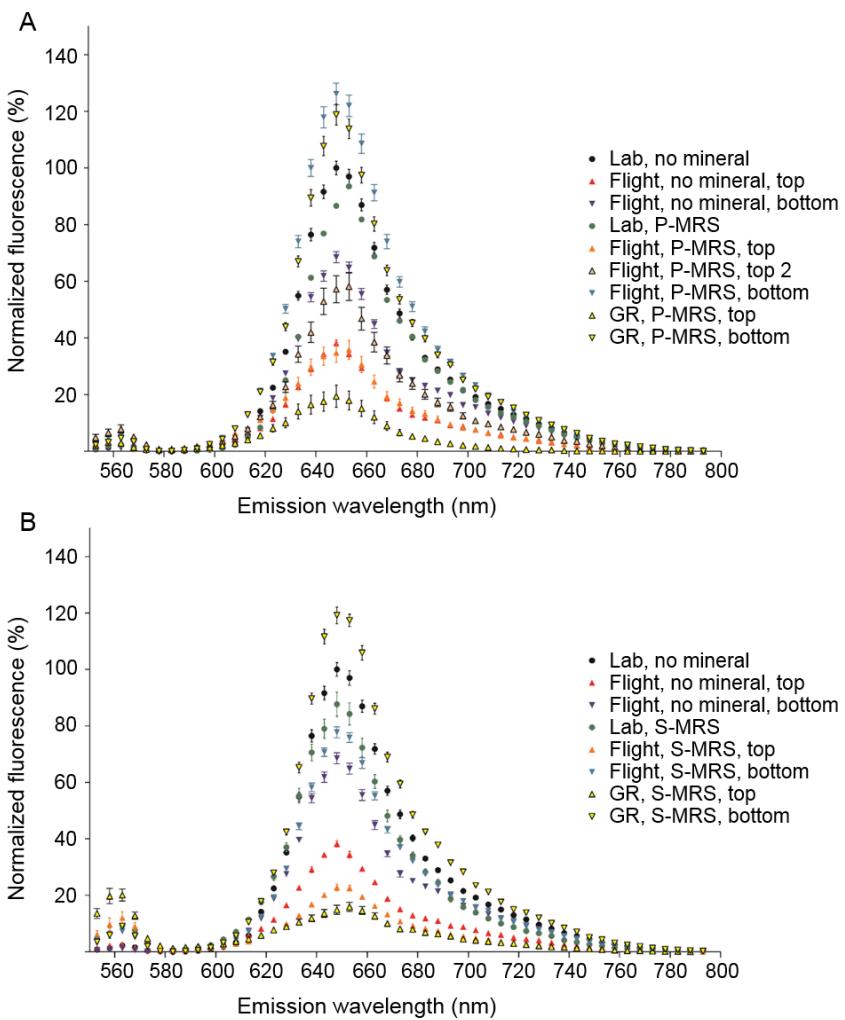


Figure 4.4. Autofluorescence of photosynthetic pigments in dried *Chroococcidiopsis* sp. CCMEE 029, mixed or not with the P-MRS (A) or S-MRS (B) mineral mixtures, after excitation with a 543-nm laser at 0.54 mW. Samples were exposed (Flight and GR samples) or not (Lab samples) to Mars-like atmosphere, in combination (top samples) or not to UV. Lab: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; GR: exposed to ground-based simulations; top: exposed in top-layer carrier of a two-layer stack; bottom: exposed in the bottom-layer carrier of a two-layer stack; P-MRS: mixed with the P-MRS regolith simulant; S-MRS: mixed with the S-MRS regolith simulant; No mineral: mixed with no mineral. Data points represent fluorescence intensity normalized to the laboratory control without minerals (Lab, no mineral) at 653 nm. Values are average \pm standard error for $n \geq 15$ cells.

In the absence of minerals, exposure to UV in addition to Mars-like atmosphere (no mineral top) sharply decreased autofluorescence (Figure 4.4), with the peak of intensity (at 653 nm) dropping down to below 40% of its initial value (Figure 4.6). Besides, autofluorescence signals were lowest in the absence of minerals under ground-based simulations (Figure 4.4), reaching a peak in intensity about 20-fold lower than controls (Figure 4.6).

Exposure of dried cells to Mars-like atmosphere on the ground did not result in any noticeable morphological alteration (Figure 4.5, “Flight bottom”). However, it did decrease autofluorescence in the absence of minerals (down to 80% of laboratory controls; Figure 4.6). This reduction was seemingly counteracted by the presence of minerals: cells mixed with either P-MRS or S-MRS and exposed to Mars-like atmosphere on the ground, in the dark, showed higher autofluorescence levels than their no-mineral counterparts and Lab controls ($\approx 119\%$ of no-mineral laboratory controls, Figure 4.6). Exposure

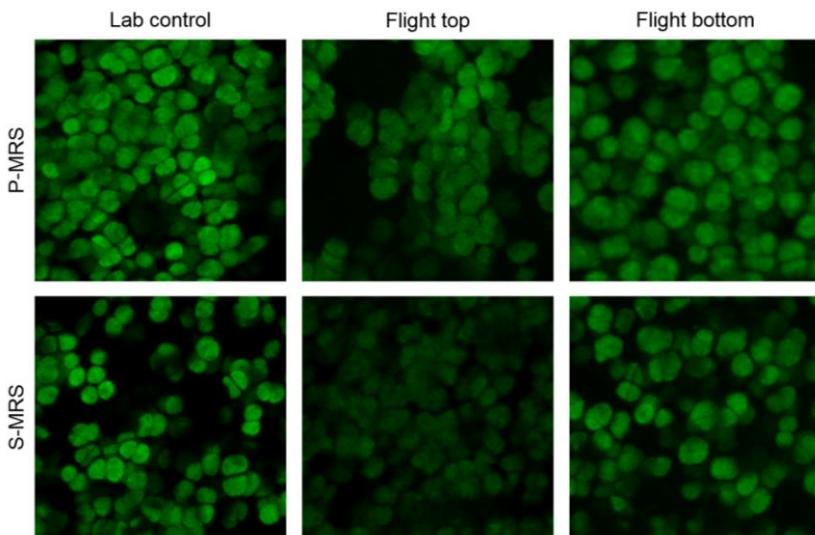


Figure 4.5. Phycobiliprotein autofluorescence in *Chroococcidiopsis* sp. CCME 029 mixed with P-MRS or S-MRS mineral mixtures, as revealed by CLSM imaging. Lab control: stored in the laboratory during the mission; Flight top: exposed to space conditions, including UV (top-layer carrier), in LEO; Flight bottom: exposed to space conditions, without UV (bottom-layer carrier), in LEO.

to Mars-like atmosphere in LEO, in the dark had similar effects for P-MRS samples (126% of no-mineral controls) but not for S-MRS ones (78%).

For samples exposed to UV in LEO, the protection provided by minerals was area-dependent: even though the average pigment autofluorescence was reduced or equal compared to no-mineral controls (for S-MRS and P-MRS respectively), cells were found whose morphology was unchanged and whose autofluorescence was better preserved when protected under mineral grains (such cells are visible in Figure 4.5, upper row, “Flight top”). In particular, an area was found in a flight P-MRS sample exposed to UV where some cells retained more than 60% of no-mineral laboratory controls’ autofluorescence on average (Figure 4.6) and up to 86% in some individual cells (not shown).

The combination of UV irradiation and Mars-like atmosphere exposure on the ground seemed more destructive than equivalent conditions in LEO. Almost no pigment autofluorescence (4%) was retained by cells exposed without minerals (Figure 4.6). The presence of the P-MRS or S-MRS analogues had a protective role, with maximum values around 19% and 16% of no-mineral laboratory controls, respectively. These values remain lower than equivalent Flight samples (38-64% and 22% respectively; Figure 4.6).

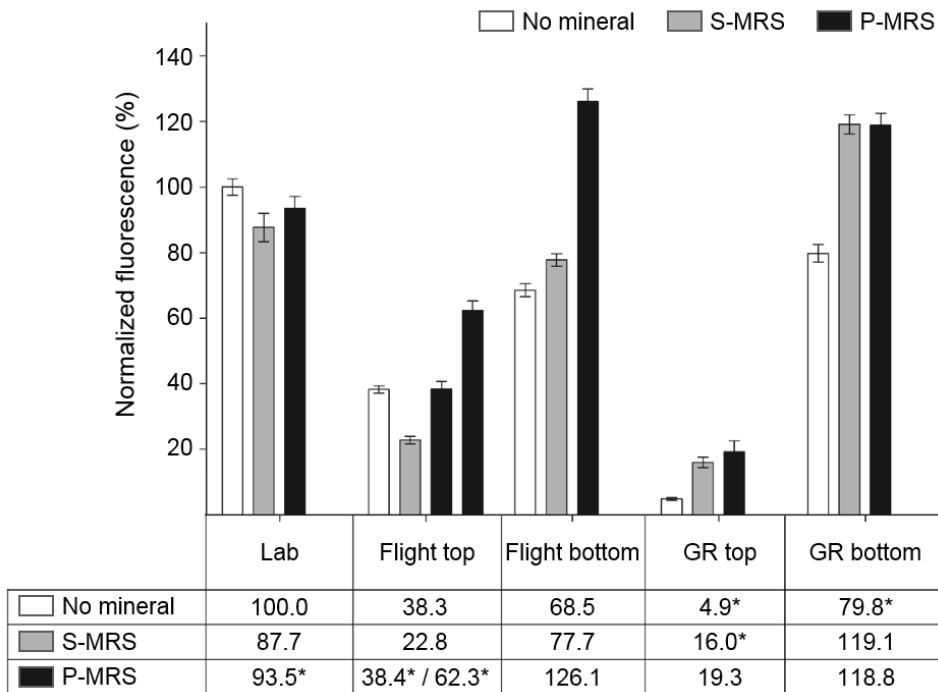


Figure 4.6. Fluorescence emission maxima at 648 nm (or 653 nm if indicated by an asterisk in the table) of photosynthetic pigments in *Chroococcidiopsis* sp. CCME 029, after excitation with a 543-nm laser at 0.54 mW. Those values correspond to peak values of curves shown in Figure 4.4. Lab: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; GR: exposed to ground-based simulations; top: exposed in the top-layer carrier of a two-layer stack; bottom: exposed in the bottom-layer carrier of a two-layer stack; P-MRS: mixed with the P-MRS regolith simulant; S-MRS: mixed with the S-MRS regolith simulant; No mineral: mixed with no mineral. Two bars are indicated for P-MRS Flight top samples and correspond to two areas with differently preserved autofluorescence. Data are expressed as percentage of the emission intensity maximum of the Lab no mineral sample (\pm standard error for $n \geq 15$ cells).

Discussion

One way of refining both the assessment of Mars's habitability and our ability to find signs of Martian life, extinct or extant, is to expose terrestrial extremophiles to simulated Mars conditions. In this context, the aim of the BIOMEX experiment was to investigate the survival of a selection of extremophiles, as well as the preservation of potential biomarkers, when exposed to space- and Mars-like conditions.

Dried cells of *Chroococcidiopsis* sp. CCME 029, mixed with analogues of Martian regolith (P-MRS or S-MRS), were exposed to simulated Mars conditions both in the EXPOSE-R2 platform in LEO (Rabbow et al., 2017) and in ground-based facilities (Rabbow et al., 2016). LEO samples remained outside the ISS for close to 18 months, including more than 15 months when samples in top-layer carriers were also exposed to UV (though attenuated down to 0.1% of the incoming flux). Survival and preservation of cellular components of *Chroococcidiopsis* cells were investigated upon completion of the flight mission.

Overall, results from the BIOMEX experiment demonstrate a very high tolerance of *Chroococcidiopsis* to space and Mars-like conditions—with dried multilayers surviving LEO, even when exposed to UV—and a protective role played by Mars mineral analogues. They are consistent with ground-based simulations, called Experiment Verification Tests (EVTs) and Science Verification Tests (SVTs), performed prior to the space mission (Baqué et al., 2016). UV irradiation, of which samples received approximately 445–505 kJ/m² (see Table 3.1 in the preceding chapter) was confirmed to be the major damaging factor, but a thin layer of cells mixed with mineral analogues conferred bottom cells with a strong protection. Cells seemed less affected (in terms of inactivation and damage to cellular components) in LEO than in the ground-based experiments performed simultaneously. This observation supports the consensus that no ground-based simulation yet can fully substitute exposure to space (Horneck et al., 2016).

Survival

All LEO samples exposed in the dark to a Mars-like atmosphere formed colonies after plating circa 10⁶ cells. No colony was scored from samples exposed to 445–505 kJ/m² of UV, consistently with Baqué et al. (2015). This highly lethal effect of UV irradiation is consistent with previous studies; for

instance, dried monolayers of *Chroococcidiopsis* 029 survived 30 kJ/m² (administered over 10 minutes) of a UV flux simulating Mars's (~50 W/m² at 200-400 nm) (Cockell et al., 2005c). In a similar setup, the survival of a monolayer of *Bacillus subtilis* spores was reduced by 99.9% after 30 seconds (1.4 kJ/m²) and no survivors were scored after 15 minutes (41.7 kJ/m²) (Schuerger et al. 2003). Moreover, during the ground-based experiments performed in preparation for BIOMEX, multilayered dried cells of *Chroococcidiopsis* 029 mixed with anorthosite, an analogue of Moon regolith, were able to survive up to 1.5 MJ/m² of polychromatic UV radiation (200-400 nm) but not a tenfold higher dose (Baqué et al., 2014).

It should be noted, however, that ground-based simulations performed prior to the mission had already indicated that this number of cells (chosen in this first attempt to spare sample material) led to false-negatives (Baqué et al., 2014, 2016). After inoculating liquid medium with larger amounts of cells (about 2×10^7) per sample, growth was here observed from all samples exposed to Mars-like conditions (including UV) in LEO, with or without minerals. It is worth noting that *Chroococcidiopsis* sp. CCME 029 cells had survived both previous EXPOSE missions (Bryce et al., 2014; Cockell et al., 2011), with UV fluencies similar to those received during EXPOSE-R2 (Rabbow et al., 2012, 2014), but in both cases cells were associated with rocks that provided shielding to some of the cells. The ND filters used here reduced UV irradiation down to 0.1% of the incoming flux, a reduction equivalent to that which would be conferred by a layer of basalt dust of about 300 µm (Caro et al., 2006) or 2 mm of gypsum (Stromberg et al., 2014). The survival of cells exposed to UV in LEO supports Cockell et al. (2005)'s estimate that a 1-mm layer of rock could bring UV dose rates on Mars's surface down to survivable levels.

Besides space (or space-like) and Mars-like conditions, part of the reduction in colony forming abilities observed here can be attributed to desiccation and storage: due to time periods between sample preparation and mission start, and between mission completion and sample analysis, samples remained in a dry state for 2.5 years before analysis. Although *Chroococcidiopsis* sp. CCME 029 was reported to withstand 4 years of air drying (Billi, 2009; Billi et al., 2013), survival is dependent on conditions during desiccation and storage, and the protocol performed here had previously been shown to greatly reduce colony forming abilities (Baqué et al., 2016).

Results from the ground reference simulation, where cells without minerals did not resume growth after exposure (with or without UV), are consistent with previous ground-based experiments: dried cells lost their colony-forming abilities when exposed to a polychromatic UV dose slightly higher than here (570 kJ/m^2) in combination with a simulated Martian atmosphere (Baqué et al., 2016). However, the fact that ground exposure was harsher to the cells than LEO exposure (also evidenced by microscopy results, as described below) was unexpected and emphasizes the need to perform exposure experiments in space: while Earth-bound tests are cheaper and allow for factor isolation and more parameter control, flight experiments can help avoid erroneous conclusions induced by simulation artifacts.

Samples in ground-based experiments were mostly meant as no-ionizing radiation controls of the LEO samples. Although platforms in LEO are partially protected by Earth's magnetic field, monitoring systems have pointed out the presence of ionizing radiation composed of solar protons (dominantly) and heavier, more penetrating particles (Dachev et al., 2012, 2014, 2016; Häder et al., 2009). The lack of increased mortality in LEO (a fortiori, the higher mortality on the ground) suggests that ionizing radiation did not significantly affect cell viability (neither did it affect molecular components' integrity, as described below). This result, on the other hand, comes as no surprise given the high resistance of *Chroococcidiopsis* sp. CCMEE 029 to ionizing radiation, as previously demonstrated on the ground (Billi et al., 2000; Verseux et al., 2017).

The mission's absorbed ionizing radiation dose from SEP, GCR, and radiation belts reached approximately 0.5 Gy (Dachev et al., 2017). Based on the absorbed dose rate of $76 \text{ mGy}\cdot\text{year}^{-1}$ measured by the Curiosity rover at Gale Crater's surface (Hassler et al., 2013), and although yearly doses vary with, for instance, solar activity, the absorbed ionizing radiation dose to which samples were exposed within EXPOSE-R2 corresponds to more than half a decade on Mars. It is, however, critical to document the effects of such radiation over much longer periods of time under Mars-like conditions, so as to better assess the preservation of hypothetical biomarkers from long-extinct life on Mars. For this, there is no alternative to ground-based facilities where doses rates can be adjusted to simulate exposure over geological timeframes.

Minerals (especially P-MRS) had a positive impact on cells exposed to ground-based simulations, in spite of the samples' thickness (including both cells and minerals) not exceeding $30 \mu\text{m}$. Cells mixed with P-MRS survived ground-based simulations with and without UV, and cells mixed with S-MRS survived them if kept in the dark. Assumptions regarding the mechanism of

protection by minerals, as well as on the difference in protection levels conferred by both minerals, are given below in the context of fluorescence results.

DNA damage as revealed by Random Amplification of Polymorphic DNA

Microorganism inactivation in space is thought to be driven primarily by DNA damage caused by UV, due to the high absorption of UV-B and UV-C by DNA (Horneck et al., 2010). Consistently, DNA in UV-exposed samples was highly damaged (as visible in RAPD fingerprints), in agreement with the lack of colonies after plating 10^6 cells. Minerals had a strong protective role, as in the ground-based preparatory studies (Baqué et al., 2014, 2016).

DNA is considered an important biomarker (e.g., Carr et al., 2013) given its universality among life on Earth, its highly unlikely formation by non-biological means, and its instability after cell death (Pääbo et al., 2004) which would make it a sign of extant or recently extinct life (Parnell et al., 2007). Its high detectability after a year in Mars-like atmospheric conditions and shallow subsurface UV conditions supports its relevance as such an indicator. Its persistence in space and Mars-like conditions also emphasizes the risk represented by contaminants on spacecraft carrying DNA-targeting, life-detection instruments.

Alteration of photosynthetic pigments as revealed by CLSM

Photosynthetic pigments were still detectable in all samples by CLSM. Carotenoids were as well, by Raman spectroscopy (Verseux et al., unpublished data) Those results are relevant to the search for life on Mars: although a wide variety of highly sensitive and discriminatory tests have been suggested for the detection of biosignatures, fluorescence-based methods can be hard to implement quickly and over extended geographical areas (Smith, 2016; Storrie-Lombardi and Sattler, 2009). Phycobiliproteins and chlorophyll *a* pigments are considered prime targets for fluorescence detection due to their high fluorescence and wide distribution (Dartnell and Patel, 2014; Parnell et al., 2007). However, because of their sensitiveness to UV and oxidants (e.g., Dartnell & Patel, 2014), only sub-surface samples could be expected to bear detectable fluorescent molecules on Mars. A risk would be to lose fluorescence signals when exposing pigments to the surface during probe

operations (Dartnell and Patel, 2014). In this regard, our results are rather comforting.

Previous studies showed that the autofluorescence (emission above 590 nm after excitation at 568 nm) of dried monolayers of *Chroococcidiopsis* sp. CCME 029 was reduced to 14% of controls after a 1-hour exposure to a Mars-like UV flux (Cockell et al., 2005). In multilayer samples mixed with anorthosite, a lunar mineral analogue, autofluorescence of chlorophyll *a* and phycobiliproteins was much better preserved: it was brought down to about 20% of controls after one day of a simulated Mars UV flux (Baqué et al., 2014). During preparatory experiments for BIOMEX, exposure of samples similar to those used here to both 570 MJ·m⁻² of polychromatic UV radiation (200-400 nm) and Mars-like atmosphere led to a complete bleaching of photosynthetic pigments' autofluorescence in most areas (Baqué et al., 2016). However, protected zones were found in samples mixed with P-MRS where fluorescence remained above 50% of liquid culture controls (Baqué et al., 2016), as was observed here.

In the present study, laboratory controls showed a 50% reduction in pigment autofluorescence compared to cells in liquid culture (not shown), likely due to oxidative damage during long-term storage under ambient atmosphere. Fluorescence attenuation in cells with no minerals was more extensive under a Mars-like atmosphere. Interestingly, this effect was counteracted by the Mars mineral analogues: in P-MRS and S-MRS samples, fluorescence remained higher than laboratory controls (except for the S-MRS sample in LEO). Those results reinforce the assumption that spectral biosignature preservation is highly dependent on the mineral environment (e.g., Stromberg et al., 2014), a topic which is currently under intense scrutiny: besides being able to detect biomarkers if present in a given sample, astrobiologists leading the search for life on Mars should have the capacity to identify areas where potential biomarkers have the highest odds of being preserved.

Some parameters of the mineral environment matter for biosignature preservation over the long term, for instance minerals' stability (e.g., Cady et al., 2003), but could not be observed in the exposure's timeframe. On the other hand, some protective effects were observable even over the short duration of the experiment. Most of the minerals' protective effect under UV has an intuitive explanation: direct shielding and energy absorption. Gypsum, for instance, which represents 30% in weight of S-MRS and has strong UV-shielding properties, has been shown to preserve chlorophyll spectral features under Mars-like conditions (Stromberg et al., 2014). But the protection

minerals confer in the dark is more intriguing and may lie in their sorption properties. P-MRS, in particular, contains a large fraction of clay minerals, notably montmorillonite which accounts for 45% of its dry weight. Clays are good matrices for organic matter (and therefore biosignature) preservation, supposedly due to adsorbing macromolecules—or even structures as large as bacteriophages (Moll & Vestal, 1992)—to their charged surfaces (e.g., Farmer & Des Marais, 1999). More generally, phyllosilicates are prone to fixing organic molecules to their surfaces and interlayer regions due to their large, negatively charged walls (Summons et al., 2011) (even anionic molecules can bind to those surfaces, via cation bridges). This binding is thought to increase macromolecules' stability (Keil et al., 1994; Hedges & Keil, 1995), although the exact mechanisms are poorly understood.

P-MRS's high phyllosilicate contents may thus explain its having a better preservation potential than S-MRS in spite of its smaller grain size. This is made relevant by the fact that phyllosilicates from the Noachian are still found at various surface locations of present-day Mars (e.g., Mustard et al., 2008). Since they likely formed in aqueous environments (e.g., Poulet et al., 2005), may have catalyzed prebiotic reactions (e.g., Bishop et al., 2013), can favor survival under extreme conditions (e.g., Foster et al., 1978; Moll & Vestal, 1992), and favor fossilization and organic matter preservation through various mechanisms (e.g., Summons et al., 2011; Wacey et al., 2013), phyllosilicate-rich areas of Mars's surface are considered choice targets for astrobiological endeavors. The presence of phyllosilicates was even a key criterion when choosing a landing site for the Mars Science Laboratory (Michalski et al., 2010; Golombek et al., 2012). Our results emphasize the relevance of those minerals: even layers in the order of 10 μm had a strong protective effect on biosignatures under Mars-like atmospheric conditions, superior to that conferred by a thicker layer of Mars-like minerals poorer in phyllosilicates.

During ground-based preparatory experiments, and contrary to present results, no significant difference was noted in average photosynthetic pigment autofluorescence between cells mixed with P-MRS, and those mixed with S-MRS, under Mars-like atmosphere in the dark (Baqué et al., 2016). This may, however, be due to the much shorter exposure period in preparatory experiments (89 days, against 722 days in the present study).

Conclusions

Recent data from NASA's Mars Science Laboratory Curiosity rover confirmed and consolidated models on Mars' past habitability. It revealed, for instance, a lacustrine environment (Grotzinger et al., 2014a) and sedimentary rocks recently exposed (78 ± 30 million years ago; Farley et al., 2014). If life emerged independently on Mars and on Earth, and/or if early life forms were exchanged between the young Earth and Mars (McKay, 2010; Westall et al., 2013), life may have persisted as Mars became less habitable, possibly until today (Cockell, 2014b). There are consequently strong incentives to search for life, extinct or extant, on this planet (Schulze-Makuch et al., 2008). One way of focusing the search is to assess the survival limits of terrestrial extremophiles, and the preservation of potential biomarkers, in Martian environments. Since all stress factors found beyond Earth's envelope (let alone their combinations) cannot be accurately reproduced in the laboratory, space experiments such as BIOMEX bring valuable insights.

UV radiation is known to be the factor most harmful to microorganisms in space and on Mars (Horneck et al., 2010), and it sharply decreased survival in exposed samples. On the other hand, DNA and photosynthetic pigments were still detectable at high levels in areas protected by the minerals mixtures, even when those were only a few μm thick. The received UV dose corresponds to approximately 8 hours under the average UV flux of Mars on the equatorial Martian surface, at the vernal equinox, based on Cockell et al. (2000)'s model. Obviously, this duration can vary greatly with time of day, latitude and season, atmospheric opacity, and distance from the Sun. For instance, although Cockell et al. (2000)'s and Schuerger et al (2003)'s models predict a maximum flux of approximately $50 \text{ W}\cdot\text{m}^{-2}$ at the equatorial Martian surface at average orbital distance, the rover Curiosity showed a UV (200–380 nm) irradiance of about $20 \text{ W}\cdot\text{m}^{-2}$ at midday at Gale Crater (4.59°S , 137.44°E) at areocentric solar longitudes 151° to 208° (Gómez-Elvira et al., 2014). However, the order of magnitude indicates that biosignatures mixed with regolith could withstand some exposure to UV radiation during probe operations. This claim should be moderated by the presence of oxidant molecules, including perchlorates, which are present in Mars's regolith (e.g., Fang et al., 2015) but were absent from the mineral simulants. While perchlorates are quite stable at Mars's temperatures, they are made reactive by UV irradiation (Wadsworth and Cockell, 2017) and may thus strongly limit biosignature preservation time close to the surface.

While UV radiation would rapidly degrade biomolecules directly on the surface, it can be blocked by a layer of regolith in the order of a millimeter.

Underneath, exposure to Mars's atmosphere could degrade macromolecules over the course of months. This effect, however, seems to be counteracted by Mars-like regolith, especially that rich in phyllosilicates. More generally, the differences in physicochemical properties between both analogues led to differential preservation of cellular components. This observation may help selecting areas where to search for remnants of life on Mars.

When the Viking rovers landed in the 1970s, we were not ready to unambiguously identify life on Mars (Schulze-Makuch et al., 2015). Since then, we accumulated knowledge on the Martian environment and its interactions with biological systems thanks to various landers, rovers, orbiters, and space exposure missions such as EXPOSE-R2. When the ExoMars and Mars 2020 rovers land on the red planet, expectedly in 2020, the odds will be much more in their favor.

Acknowledgements

This research was supported by the Italian Space Agency (ASI grant 051-R.0 to DB) and the German Helmholtz Association through the Helmholtz-Alliance "Planetary Evolution and Life". We acknowledge the European Space Agency (ESA) for the selection of BIOMEX. We thank Dr. Elke Rabbow (DLR Cologne) for conducting the ground-based simulations, assembling and de-integrating the samples; Dr. René Demets (ESA) for supporting EXPOSE experiments; and all the ISS staff for taking care of EXPOSE-R2. The authors thank Dr. Elena Romano, Centre of Advanced Microscopy "P. B. Albertano", University of Rome "Tor Vergata", for her skillful assistance in using the CLSM. MB acknowledges support from the Alexander von Humboldt Foundation.

PART II

CYANOBACTERIUM-BASED LIFE-SUPPORT SYSTEMS FOR THE FUTURE OF CREWED SPACE EXPLORATION

Chapter 5

Introducing Part II⁴

Cyanobacterium-based life-support systems on Mars

As described in the preceding chapters, the peculiar metabolic properties of cyanobacteria, together with their high resistance to extreme conditions found beyond Earth, make them relevant models for basic questions in astrobiology. But they also make them promising organisms for the development of biotechnologies beyond Earth in support of long-term, crewed space exploration. In particular, they could provide a strong basis for production processes on Mars.

Although the idea may sound far-fetched at first, sending humans to Mars within a few decades is now a realistic objective (e.g., Horneck et al., 2006). It is notably one of NASA's stated goals (NASA, 2017), and that of private spacecraft companies such as the Space Exploration Technologies Corporation (Musk, 2017). These might fail to meet their announced deadlines, or at all; postponing and cancelling is common in space missions. But other Mars colonization projects and private spaceflight companies are emerging, who can benefit from the advances of their predecessors.

⁴This chapter is based on the following two articles:

- Verseux, C., Baqué, M., Lehto, K., de Vera, J.-P.P., Rothschild, L.J., and Billi, D. (2016). Sustainable life support on Mars – the potential roles of cyanobacteria. *Int. J. Astrobiol.* *15*, 65–92.
- Verseux, C., Paulino-Lima, I., Baqué, M., Billi, D., and Rothschild, L. (2016). Synthetic Biology for Space Exploration: Promises and Societal Implications. In *Ambivalences of Creating Life. Societal and Philosophical Dimensions of Synthetic Biology*, K. Hagen, M. Engelhard, and G. Toepfer, eds. (Springer-Verlag), pp. 73–100.

However, even though leaving a footprint and planting a flag may possibly be achieved with not much more than the current state-of-the-art of engineering, a definite pay-back is still in doubt. On the other hand, if a Mars mission can allow extensive human scientific activity and yield meaningful scientific data, the effort is justified. In such a case scientists will need to spend a considerable period on site, and multiple short-term missions are not a viable option: given the time, costs and challenges associated with the journey, long-term human bases will likely be needed.

While the vision of a long-term human presence on Mars is compelling, providing consumables to sustain crews is a challenge. Even though launch costs vary depending on mission scenarios and might be reduced in the coming decades, they have been estimated to be in the order of \$300,000 per kilogram (Massa et al., 2007). Sending from Earth all the needed resources does not seem financially sustainable. Should Mars colonization be consequently deemed too expensive to be realistic? Maybe not. There are alternatives.

One is recycling – using regenerative systems. Such systems should have biology-based components: various life support functions can be provided by physicochemical processes, but some valuable products such as high-protein food cannot currently be produced by the latter (Drysdale et al., 2003; Montague et al., 2012). In addition, many components of physicochemical life support systems are heavy, highly energy-consuming and/or require high temperatures. Even in the case where these are the backbone of life support systems, biological modules could both complement them and provide safe redundancies. Consequently, various bioregenerative life support systems (BLSS) projects are or have been under development for recycling food, water and gases both in space (e.g., Godia et al. 2002; Lobascio et al. 2007; Giacomelli et al. 2012; Nelson et al. 2010; Gitelson et al. 2003; Drysdale et al. 2004) and within lunar and Martian outposts (e.g., Tikhomirov et al. 2007; Gitelson 1992; Blüm et al. 1994; Nelson et al. 2010). This may sound promising: instead of sending resources in amounts almost proportional to the mission length, only a few weeks worth of consumables would be sent and recycled. The issue is: 100 % recycling efficiency cannot be reached and losses are unavoidable. For quantitative information regarding the theoretical recycling efficiencies of the European Space Agency's Micro-Ecological Life Support System Alternative (MELiSSA), for example, see Poughon et al. (2009).

A regenerative system's running time without re-supply is consequently limited. It also cannot be expanded, as the mass of cycling components is, at a given time, at most equal to their initial mass. In addition to this, most current BLSS projects represent a large initial volume and mass, as well as a high power consumption. Re-supply is thus needed and the most advanced BLSS projects heavily depend on materials imported from Earth—although a theoretical physicochemical/biological resource production system relying on Martian resources has been patented (Cao et al. 2014). These projects are consequently not suitable for autonomous, long-term human bases on Mars: the mass problem is reduced, but not solved.

There is however a promising solution: producing resources from local materials. Like all human settlers of previous generations, the Martian pioneers must “live off the land”. A critical question this fact raises is whether it is possible to feed biological systems with local resources. On one hand water (H_2O), solar energy, carbon (C), nitrogen (N) and many other life-supporting nutrients are found on Mars (Meyer & McKay 1989; Olsson-Francis & Cockell 2010; Cockell 2014). But on the other hand, they cannot be directly exploited in the form in which they are found there by key organisms of current BLSS projects. This limitation may lead one to think that BLSS based on local resources is irrelevant for Mars exploration. What if, however,

Table 5.1. Main sources of nutrients for cyanobacterium-based biological processes on Mars.
Reproduced from Verseux et al. (2016b).

Source	Elements
Atmosphere*	CO_2 , N_2
Soil, rocks*	P, S, Mg, Fe, Ca, Na, K, and metal micronutrients
Ice caps, subsurface ice, atmosphere, hydrated minerals*	H_2O
Solar radiation*	Energy for photosynthesis, heat
Human waste	Fixed N, organic material, CO_2 , H_2O
Side effects of other artificial processes (fuel combustion, manufacturing...)	CO_2 , H_2O
Cyanobacteria (fed with the above)	O_2 , fixed N, organic material, metal nutrients

* Naturally present, independently of human activity

Martian resources could be exploited and processed into suitable forms by an additional biological module? What if there was a biological link between on-site resources and BLSS systems? This link might be created by cyanobacteria. All the inputs needed to sustain a diazotrophic (N-fixing), bioleaching cyanobacterium's metabolism could in theory be obtained from Mars's mineral resources, water, atmospheric gases and incident solar energy. First, some (e.g., *Anabaena* spp. and various desert species) have the ability to extract and biologically fix nutrients from analogues of Martian rocks (Brown and Sarkisova, 2008; Olsson-Francis and Cockell, 2010b). Due to these abilities and to their useful products, cyanobacteria have been suggested as a basis for creating planetary BLSS relying on local resources (Brown, 2008a; Brown et al., 2008; Verseux et al., 2016a; Rothschild, 2016). They are also able to fix C from carbon dioxide (CO_2) and some species can fix N from dinitrogen (N_2), both of which are found in the Martian atmosphere, leading to organic matter and dioxygen (O_2) production without dependency on

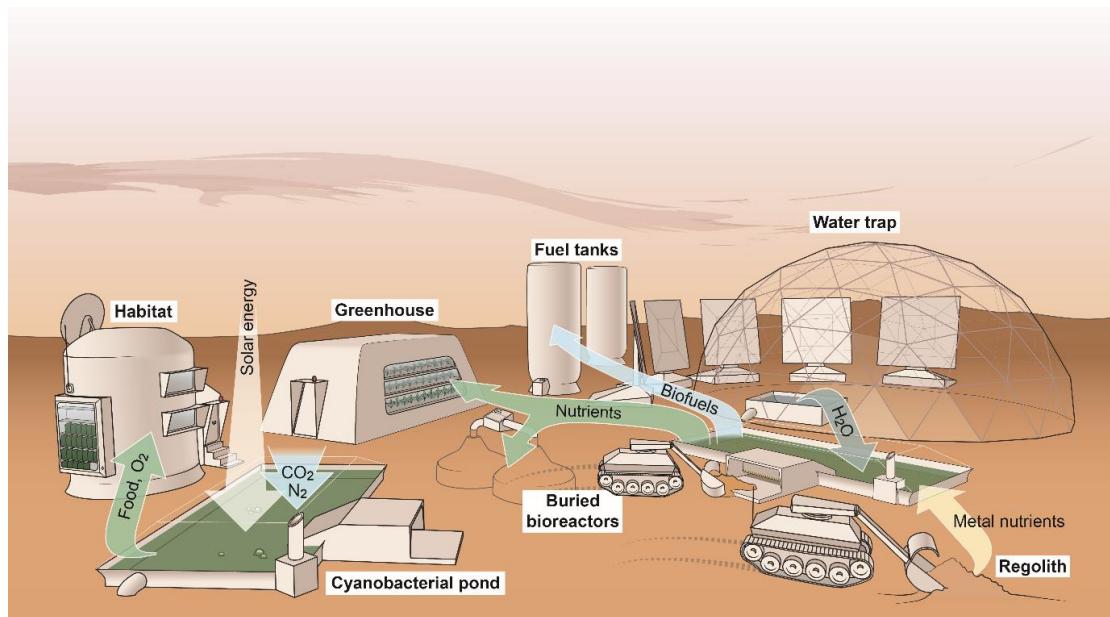


Figure 5.1. Artist's rendering of a cyanobacterium-based biological life-support system on Mars. Artistic work by Sean McMahon. Reproduced from Verseux et al. (2016a).

carbohydrate feedstock. Some species are highly tolerant to extreme environments (e.g., Rothschild & Mancinelli 2001); consequently their culture is less demanding in terms of hardware than that of more environmentally sensitive microbial species. Cyanobacteria could be used directly to produce resources such as food and O₂, but products from their cultures could also be used to feed other living organisms, so opening the way to a wide range of life-support biological processes.

Cyanobacteria could thus provide the key link between BLSS and Martian resources, making the former sustainable and expandable in human bases on Mars. An artist's rendering of such a cyanobacterium-based BLSS ("CyBLiSS") is given in Figure 5.1.

Potential roles of cyanobacteria in Mars-specific BLSS

Feeding other microorganisms

Heterotrophic microorganisms have been used throughout human history and would be highly useful on Mars. Potential applications include the production of drugs, food, biomaterials and various industrially useful chemicals, metal leaching and food processing for taste improvement (Cumbers and Rothschild 2010; Langhoff et al. 2011; Montague et al. 2012; Menezes et al. 2014; Verseux et al. 2016b). As most of these applications require relatively small culture volumes and no solar light, cultures could be performed under Earth-like conditions with reasonable costs. However, heterotrophic microorganisms rely on organic materials whose availability on Mars remain very poorly known and are not expected to be abundant there (Ming et al., 2014). Could the local resources be processed into suitable substrates by cyanobacteria? Related phenomena naturally occur on Earth, where cyanobacteria are known to support different heterotrophic communities. They can for instance be the earliest colonizers of desert habitats and allow the development of local ecosystems (including heterotrophic bacteria) through the production of organic compounds, N fixation and rock leaching (e.g., Eldridge & Greene 1994; Danin et al. 1998; Herrera et al. 2009). In aquatic ecosystems as well, cross-feeding and metabolite exchange occur between cyanobacteria and heterotrophic microorganisms (see for instance Stevenson & Waterbury 2006 and Beliaev et al. 2014).

The question of water is addressed in section "Water" below. Heterotrophic organisms also need, first of all, organic compounds as a source of C and

energy. Lysed cyanobacterial biomass could be used as such, (and as a source other critical macronutrients such as hydrogen [H], oxygen [O], phosphorus [P] sulfur [S]; for N, see following paragraph). Consistently, a filtrate of ground *Anabaena* sp. PCC7120 was used as the only source of organic compounds and fixed N to grow different strains of heterotrophic bacteria (see Chapter 6). Note that these results are preliminary and that no optimization step (e.g., choice of a strain that can metabolize sucrose, alteration of culture conditions to modify cyanobacterial biomass's composition and/or more efficient extraction method) has yet been performed. Lysed cyanobacterial biomass has also been shown to be a suitable substrate for ethanol production in yeasts (Aikawa et al., 2013; Möllers et al., 2014). In lysogeny broth (LB), the most common growth medium for heterotrophic bacteria in laboratories, the concentration of fermentable sugars and sugar equivalents (sugar phosphates, oligosaccharides, nucleotides, etc.) utilizable by *E. coli* is very low (< 100 µM), constraining bacteria to use amino acids as C sources (McFall et al. 1996; Sezonov et al. 2007). However, non-destructive ways of harvesting nutrients could lead to more efficient processes. Substrates could for instance be secreted. This solution has been investigated in Lynn Rothschild's laboratory (at NASA Ames Research Center, Moffett Field, CA) since the 2011 Stanford-Brown iGEM team engineered *Anabaena* sp. PCC7120 to secrete sucrose (Stanford-Brown 2011 iGEM team 2011), which was used as a C source to grow *Bacillus subtilis* (Ryan Kent et al., unpublished data). Prior to this, *Synechococcus elongatus* PCC7942 was engineered to produce and secrete either glucose and fructose, or lactate, then used as a substrate for *E. coli* (Niederholtmeyer et al., 2010). In this kind of system a major limitation arises from low sugar yields, which are due to relatively low synthesis rates and to the consumption of sugars by the cyanobacteria themselves. Production rates could be increased by further genetic engineering to either increase synthesis or decrease processing of these products by the producer strains. External conditions could also be modified; for instance, osmotic stress induces sucrose accumulation in many cyanobacteria, especially when synthesis of other osmoprotectants is impaired (Miao et al., 2003).

Then, fixed N is needed. Heterotrophic bacteria can obtain N from various organic and inorganic sources such as single amino acids (e.g., Crawford et al. 1974) and amino acid chains (e.g., Hollibaugh & Azam 1983; Coffin 1989), nucleic acids (Paul et al. 1989) and ammonium (NH_4^+). Diazotrophic cyanobacteria can produce all these compounds after N fixation and, here again, a simple way of making this N available to heterotrophic bacteria is to

lyse cyanobacteria. But NH_4^+ can be naturally released by some diazotrophic cyanobacteria, without cell lysis; for instance, extracellular NH_4^+ can reach several mM in cultures of *Anabaena* spp. (mutants or wild-type, depending on species) relying on atmospheric N_2 as a sole N source (Spiller et al. 1986; Subramanian & Shanmugasundaram 1986). NH_4^+ is the preferred N source for most microorganisms and becomes limiting only at extremely low concentrations (e.g., below a few μM for *Escherichia coli*; see Kim et al. 2012), several orders of magnitudes below the above mentioned concentrations. Released NH_4^+ could be used for feeding not only heterotrophic microorganism, but also some phototrophic species of interest which cannot fix N. Within the MELiSSA loop (e.g., Godia et al. 2002), NH_4^+ (there resulting from human and plant waste processing by thermophilic anaerobic bacteria) is converted into nitrates by nitrifying bacteria before being transferred to *Arthrospira* sp. cultures. Even though nitrate is considered the preferred N source for this species (as for most non-N fixing filamentous cyanobacteria), using NH_4^+ instead of nitrates has been shown not to reduce growth rates (Filali et al., 1997). A drawback is that NH_4^+ becomes growth-limiting from relatively low concentrations (a few mM) and should consequently, if limiting below these concentrations, be regularly (or continuously) added to the culture medium. Semi-separated cultures, where cyanobacteria's and heterotrophic microorganisms' culture vessels communicate through membranes that allow medium but not cell exchange, can also be considered. In such a case, extensive system characterization is needed to predict outputs, and processes could be optimized by improving the culture setup and by performing evolutionary selection of the co-culture to improve metabolic interactions. In any case, the rates of NH_4^+ release by strains of interest under Mars-like constraints, as well as the use of this NH_4^+ as an N source for non-N fixers, should be further investigated.

Many heterotrophic microorganisms also need O_2 . *Escherichia coli*, for example, roughly needs 1 g of O_2 ·gram of dry weight (gdw)⁻¹ (Shiloach & Fass 2005). O_2 consumption and cell mass vary according to strain and cultures conditions, but this figure corresponds to approximately 1 g of $\text{O}_2\cdot\text{l}^{-1}$ to reach a culture of 2×10^9 cells· ml^{-1} . Cyanobacteria produce O_2 through photosynthesis; details are given in section “Producing oxygen”.

Finally, metals must be provided. Some (macronutrients) are needed in relatively large amounts; these are mostly potassium (K), magnesium (Mg) and iron (Fe) and, for some species, sodium (Na) and calcium (Ca). Others

(micronutrients) are needed in trace amounts by some microorganisms and include, for instance, chromium (Cr), manganese (Mn), nickel (Ni) and zinc (Zn) (Madigan et al. 2000). As an example, *Escherichia coli* needs about 10^8 atoms·cell $^{-1}$ of K and Mg, 10^5 atoms·cell $^{-1}$ of Ca, Zn, and Fe, and 10^4 atoms·cell $^{-1}$ of Mn, molybdenum (Mo) and selenium (Se) (Finney & O'Halloran 2009). All the needed elements seem to be present in Martian regolith (Cockell 2014), but some of them may be poorly available to most organisms with no leaching abilities. However, rock-dwelling cyanobacteria can extract metal nutrients from a wide range of rocks (see Olsson-Francis et al. 2012). Besides mobilizing them in their own cells, they help release them in the aqueous phase, increasing their concentration there (Olsson-Francis & Cockell 2010; Olsson-Francis et al. 2012) and availability for non-leaching organisms. *Anabaena cylindrica* has for instance been shown to release elements including K, Mg, Na, Ca, Fe, Mn, Ni, and Zn from a Mars basalt analogue (Olsson-Francis and Cockell, 2010b). The metals needed in highest amounts by *E. coli*, namely K and Mg, reached concentrations of 125 and 55 μM , respectively, in large excess compared to *E. coli*'s needs (roughly, 0.3 μM to reach 2×10^9 cells·ml $^{-1}$). Even though lysing cyanobacterial biomass is the most straightforward option for transferring nutrients from cyanobacteria to heterotrophic microorganisms, it may thus not be necessary. What solution minimizes the cost-to-productivity ratio is still to be determined.

Supporting plant growth

The majority of BLSS projects include plants for air and water regeneration and food production. Interestingly, all of the nutrients needed to grow plants (C, H, N, O, P, S, K, Mg, Fe, Na, Ca, and micronutrients) seem to be present on Mars. Banin (1989) proposed using Martian regolith to grow plants and this approach is still under investigation (e.g., Silverstone et al. 2003; 2005; Nelson et al. 2008; Maggi & Pallud 2010; Wamelink et al. 2014). However, even though Martian regolith is mostly basaltic and weathered basalt can yield extremely productive soils on Earth (Dahlgren et al., 1993), regolith will require physicochemical and/or biological treatment before it can be used as a growth substrate for plants. Besides excess salts, oxides and toxins, the main limiting factors are its low nutrient bioavailability and poor water-holding potential caused by low organic C contents (Cockell, 2011; Maggi and Pallud, 2010). Enrichment of N, in particular, is critical: most plants cannot fix

atmospheric N (a few, mainly legumes, benefit from symbiotic N fixation by specific bacteria harbored in their root tissues).

Some elements could be obtained by recycling human waste and inedible biomass from previous crops. However, relying solely on this would strongly limit sustainability and scalability of the system: without adding new components to the loop, the pool of supplies can only decrease over time. Again, inputs for plant cultivation should rather be provided from local resources. An interesting approach has been proposed in the context of lunar exploration. Following this approach, plant species which are tolerant to harsh growth conditions would be grown in local regolith, after inoculating seeds with a carefully chosen consortium of bioleaching bacteria to deliver nutrients to plant roots and to protect them against excessive accumulation of toxic elements. Once these “first-generation plants” would be grown, their biomass would be converted by microorganisms into a fertile protosoil used to support the growth of more demanding plants (Kozyrovska et al., 2006; Zaets et al., 2011). An alternative strategy could rely on cyanobacteria to process elements from rocks and fix N. Even though no plant cultures are currently produced using cyanobacteria as exclusive nutrient sources, the latter are used in agriculture to improve soil fertility, balance mineral nutrition and release biologically active substances that promote plant growth and increase plant resistance (Singh, 2014). In the context of space exploration, cyanobacterial cultures have already been proposed as a way of releasing chemical elements from rocks to hydroponic solutions (Brown and Sarkisova, 2008). N fixed by cyanobacteria could also be used in hydroponic and/or soil-based systems. It could come from two main sources: from processed biomass containing for instance amino acid chains, and from released NH_4^+ .

Within the MELiSSA loop, NH_4^+ is converted into nitrates before being transferred to plants, but plants can also efficiently uptake NH_4^+ (see, e.g., Howitt et al. 2000). In theory, plant growth rates can even be higher with NH_4^+ than with nitrates due, first, to the energy needed to reduce nitrates to NH_4^+ before its incorporation into organic compounds (Bloom et al. 1992) and, second, to a reduced need for photons, water and catalytic metal use per unit of C fixed when relying on NH_4^+ (see Raven et al. 1992). However, experimental data often do not support this hypothesis (e.g., Raven et al. 1992). NH_4^+ induces toxicity at lower concentrations than nitrate, with a threshold which varies widely among species (Britto & Kronzucker 2002). As a consequence, preference for NH_4^+ over nitrate is species-dependent (Barker

& Mills 1980; Zhou et al. 2011). Even though NH_4^+ toxicity is not fully understood, it can presumably be explained in large part by the effect of the absorbed N's form on the uptake of other ions: NH_4^+ leads to higher anion uptake and lower cation uptake. It also affects pH: nitrate and NH_4^+ absorption induce, respectively, a release of hydroxyl ions (HO^-) and a release of protons (H^+), and for some plants NH_4^+ 's negative effects can be reversed by buffering the medium (Britto & Kronzucker 2002). Harmful effects could consequently be mitigated by controlling pH and adjusting the supply of other nutrients (Muhlestein et al., 1999), and by keeping NH_4^+ concentrations below toxic levels. Further studies could ascertain to what extent the growth-limiting effects of high NH_4^+ concentrations can be mitigated; more generally, work is needed to determine whether the benefits brought by nitrifying bacteria would justify the complexity and costs associated with their culture. Besides NH_4^+ , organic N from cyanobacterium biomass can be considered as an N source: plants can uptake amino acid chains (e.g., Lipson & Näsholm 2001; Näsholm et al. 2009). Uptake of single amino acids and short peptides, and possibly di- and tri-peptides, occurs via membrane transporters in root cells (Rentsch 2007). Some plants can also uptake proteins after degradation by fungal symbionts (e.g., Bajwa et al. 1985) and at least some can do it without prior degradation by other organisms, either directly (presumably by endocytosis) or via the secretion of proteolytic enzymes (Godlewski & Adamczyk 2007; Paungfoo-Lonhienne et al. 2008).

Symbioses between plants and diazotrophic cyanobacteria naturally occur. The best-known instances involve aquatic ferns from the genus *Azolla* and their symbionts from the genus *Anabaena* (Peters and Meeks, 1989), and angiosperms from the genus *Gunnera* and their symbionts from the genus *Nostoc* (Bergman et al., 1992), but interactions naturally occur between cyanobacteria and representatives of most plant groups. Artificial symbioses between diazotrophic cyanobacteria and plants which normally do not harbor cyanobacterial symbionts, where the former provides fixed N to the latter, have also been successfully created with various plants including cereals (Gusev et al., 2002). In a hydroponic system, an *Anabaena variabilis* strain has even been shown to provide fixed N to wheat grown in an otherwise N-free medium, yielding plant growth comparable to that of control plants grown in nitrate-containing medium (Spiller and Gunasekaran, 1990). Assessments of the cyanobacterium-to-plant biomass conversion efficiency, when all substrates besides CO_2 and water come from cyanobacteria, require further experiments.

Even though experimental research is needed to quantify the nutrient composition of culture supernatants (with and without cell lysis), to determine the most efficient way of transferring nutrients from cyanobacterial cultures to other organisms and to get quantitative estimates of the system's requirements, cyanobacteria could thus be considered as a useful tool for processing local inorganic resources into a form which is available to other organisms. But cyanobacteria could also be used directly for various applications. The most critical are outlined below.

Producing food

If all food came from Earth and assuming the easiest option of providing shelf-stable, prepackaged food similar to International Space Station's provisions, the amount to be sent would be about 1.8 kg per day and per crewmember (Allen et al., 2003). Adding the needed vehicle and fuel weight to carry it and assuming a 10:1 vehicle-to-payload ratio (Hoffman and Kaplan, 1997), one-year food supply for a crew of 6 would add more than 29 metric tons to the initial mass of the transit vehicle. Worse, the load needed for a healthy diet would be much higher: even though convenient due to a reduced need for storage facilities and contamination risks, a diet composed exclusively of this type of food would be nutritionally incomplete and thus not adequate in the long term. Frozen food could be seen as an interesting complement but would be extremely demanding in terms of storage facilities, could not be kept at commercial temperatures for more than about a year without losing palatability and would be unreliable due to potential freezing failure. Thus, even though current space food systems are relevant for short-term space missions and missions close to Earth (in low Earth orbit and possibly on the Moon), they must be developed further to meet the requirements of a manned mission to Mars (Perchonok et al., 2012). Establishing long-term human settlement on Mars seems unrealistic without local food production systems.

Growing plants might appear as an obvious option, even though creating an adequate environment on Mars would be particularly challenging at the first steps of colonization (details are given in the "Plants or cyanobacteria?" section below). However, cyanobacteria could be an interesting alternative.

Some species are edible, can be grown at a large scale and are expected (based on comparisons of metabolic pathways and human nutritional requirements) to be a nearly complete nutritional source, lacking only vitamin C and possibly essential oils (Way et al., 2011). Some species, notably from the genus *Arthrospira*, have consequently been studied as a potential food source in life support systems (Hendrickx et al., 2006; Lehto et al., 2006). *A. platensis* is already used on Earth as a food supplement, and its nutritional value and lack of toxicity are well-characterized. Its dried biomass has been categorized “Generally Recognized As Safe” (GRAS) for human consumption by the Food and Drug Administration (FDA) of the United States (FDA GRAS Notice No. GRN 000127). It has high protein contents (roughly, 50-70 % of the dry weight) and a high productivity: cultures in outdoor ponds under non-optimized conditions typically yield from 1 to 3×10^7 gdw·ha⁻¹·year⁻¹ (Jiménez et al., 2003) and produce 20 times more proteins per hectare than soya's (Hendrickx et al., 2006; Henrikson, 2009). Yields can be much enhanced in more controlled conditions. Cultures in photobioreactors can produce around 1 gdw·l⁻¹·day⁻¹ and, with such yields and assuming energetic contents of 3.75 kcal·gdw⁻¹ (Tokusoglu & Unal 2003), 2000 kcal a day can be produced with about 0.53 m³. That being said, this figure is a very rough approximation of the actual needs: even though it covers mean caloric needs for a 30-50 year old adult, it doesn't represent the amounts needed to cover nutritional needs. On the other hand, production rates can be further increased; for instance, a productivity of 16.8 gdw·m⁻²·h⁻¹ (there corresponding to 1.2 gdw·l⁻¹·h⁻¹) has been attained for a short period of time using high *A. platensis* cell densities, a very short light path (14 mm), a very high photon flux (8000 µmol·m⁻²·s⁻¹; about 4 times the solar flux on Earth when the Sun is directly overhead) and highly turbulent mixing (Qiang et al., 1998). *Arthrospira* spp. have health-promoting properties, including antiviral and antimutagenic functions which are especially relevant in Mars's highly irradiated environment (Lehto et al., 2007). They are also much more digestible than eukaryotic microalgae from the genus *Chlorella*, their main competitor as a photosynthetic microorganism-based food source, which have poorly digestible cellulose-containing cell walls.

However, *Arthrospira* spp. biomass currently cannot be used as a staple food: in addition to a taste that very few people would qualify as appealing, its carbohydrate-to-protein ratio is low, it contains very high levels of vitamin A, and it lacks vitamin C and possibly essential oils. Due to this nutritional imbalance, it is generally not recommended to consume more than 10 g a day.

Cyanobacteria could be mixed with other microalgae and plants to optimize nutrient and fiber contents, as well as to diversify taste and texture, but limitations could also be addressed using synthetic biology (Way et al., 2011). Modifying the sugar, protein and lipid proportions, as well as introducing molecules that humans require (e.g., vitamin C) could be achieved using metabolic engineering and other genetic manipulations (see section “Potential inputs from synthetic biology” below). It should also be taken into account that, depending on its adjusted pressure, Martian atmosphere can strongly affect the composition of cyanobacterial cultures: *Anabaena cylindrica* cells grown under low pressure and high CO₂ concentrations showed decreased protein contents and increased sugar contents compared to cells grown under ambient conditions (Lehto et al., unpublished data), getting the protein/carbohydrate ratio closer to humans’ needs.

Research has been extensively focused on *Arthrospira* spp., but other cyanobacterial species such as *Nostoc commune*, *N. flagelliforme* and *Anabaena* spp. in symbiosis with *Azolla* spp. are traditionally consumed and more are edible. *Arthrospira* cultures require extensive nutrient supply (including fixed N), a high temperature with an optimal around 35°C and an alkaline pH. Other species which are less demanding in terms of culture conditions might thus be more suitable on Mars and, even within the genus *Arthrospira*, species could be screened for the highest productivity under on-site constraints.

Finally, high-protein food could be suggested to come from animals. Granted, it is unrealistic to envision growing large species on Mars during its earliest colonization steps, due for instance to the need for an Earth-like environment, to low protein yields-to-allocated resources (area, water, vegetal-originated proteins, working time, etc.) ratios and to the risk of pathogen transmission to humans. However, aquaculture of densely growing fish species (e.g., *Tilapia* spp.), crustaceans and shellfish may be considered (McKay et al., 1993) and cyanobacteria could be used for feeding them. Cyanobacteria are already used on Earth as a main food source for larvae of many species of fish, zooplankton (itself used for feeding fish larvae), crustaceans and shellfish (Pulz and Gross, 2004).

Producing oxygen

O₂ will be one of the most critical resources in human bases on Mars, the most obvious reasons being human respiration and fuel oxidation. But it

represents only 0.13 % of Mars's atmosphere, against 21 % of Earth's. Given the total pressures of both atmospheres, its partial pressure on Mars is more than 20 thousand times lower than on Earth. O₂ thus needs to be either brought to Mars or produced there and, as for other resources, the second option is likely the most viable in the long term. On-site O₂ production could be performed using physicochemical methods: by processing regolith, water and/or atmospheric CO₂ (e.g., by combining a CO₂ scrubber, a Sabatier reactor and an electrolyzer). However, cyanobacterium-based O₂ production from H₂O and CO₂ could provide a safe redundancy (Sychev et al., 2003) and complement physicochemical methods. It could also be less energy demanding, and faster to set up and expand to face unexpected needs.

How much O₂ is needed? Each crewmember consumes about 1 kg of O₂ per day for respiration, assuming 2 hours a day of intensive physical exercise (Horneck et al., 2003). If produced photosynthetically, this requires the fixation of more than 1.3 kg of CO₂ a day, part of which could be provided, if useful, by recycling CO₂ produced by the crew's metabolic activity (about 1 kg a day·crewmember⁻¹) and manufacturing activity in addition to using atmospheric CO₂.

Cyanobacteria are very efficient O₂ producers: whereas trees release about 2.5 to 11 tons of O₂·ha⁻¹·year⁻¹, industrial cultivation in open ponds of *Arthrospira* species in Southeastern California release about 16.8 tons of O₂·ha⁻¹·year⁻¹. Cultures have been stated to be about 2.5 times more productive in the tropics (Henrikson, 2009); under these conditions, about 80 m² of culture per crewmember would be needed to cover human respiration needs. However, O₂ production rates can be dramatically increased by photobioreactor-like culture systems which optimize for instance temperature, nutrient flow rates, cell density and illumination. Photosynthetic microorganisms from a eukaryotic microalgal genus, *Chlorella*, have been well-studied in the context of O₂ production for life support systems. For instance, an experimental system ("BIOS-I") was designed and tested in the 1960s where a man living in a sealed volume had its atmosphere and water regenerated by a *Chlorella vulgaris* culture. This work was preceded by experiments aimed at investigating the potential use of *Chlorella* spp. for air regeneration, performed by Y. Y. Shepelev and G. I. Meleshko at the Institute of Aerospace Medicine, Moscow, in 1960-1961 (Salisbury et al., 1997). In BIOS-I, CO₂ excreted by the man and biogenous elements from his urine (N, P, S and K) were fed into the algal culture, which in turn produced O₂ and

purified water. Investigators showed that producing 500 g of dry algal biomass per person and per day was enough for water and air generation, and could be achieved in 20 l only (with a 0.5 cm thick cultivation compartment between 8 m² plane parallel walls to maximize light absorption), using one-side illumination with photosynthetically active radiation at 250-300 W·m⁻² (Gitelson, 1992; Kirensky et al., 1968). The system was further developed, plants were added and various manned closure experiments were performed within “BIOS-2” and “BIOS-3”, where *Chlorella* sp. was used to recycle part of the air (even though the *Chlorella* compartment was later replaced with additional plant compartments, mainly for food-related issues; see Salisbury et al. 1997).

Productivity will depend on conditions which will be provided on site. It should also be noted that the needed resources do not need to be dedicated to O₂ production, as O₂ will anyway be a side product of other processes relying on cyanobacteria and could be coupled with, for instance, food production.

Producing biofuels

If sending it from Earth, fuel would represent most of the load’s mass to be sent from Earth and (for the journey back, assuming a return mission) from Mars. If it could be produced on-site, costs and technical challenges would be much reduced.

The next question concerns fuel-type. Dihydrogen (H₂) can be used for reducing local CO₂ to hydrocarbons, and it has been proposed to bring some to Mars to produce methane (CH₄) and H₂O (the latter can then be hydrolyzed into O₂ and H₂). H₂ could theoretically be directly used as a fuel: a mixture of liquid H₂ and O₂ have suitable propulsion performances. It is however much less attractive than CH₄, among other reasons because of its very low density which induces a need for specialized combustion apparatus. In the Mars Direct plan (Baker & Zubrin 1990; Zubrin et al. 1991; Zubrin & Wagner 2011), 6 tons of H₂ would be used to generate enough CH₄ and O₂ to bring the return vehicle back to Earth and to power ground vehicles on the Martian surface. Mass could be further reduced if H₂ was also obtained on Mars. Many cyanobacteria can produce it; among them, N-fixing heterocystous species such as *Nostoc* and *Anabaena* spp. are the most promising candidates. Their H₂ production activity comes from their nitrogenases. These enzymes mainly

catalyze the reduction of N₂ to NH₄⁺ but also, in the absence of N, reduce protons (H⁺) to H₂. On Earth, though, H₂ photoproduction is currently too low for it to have practical applications. As an example, *A. cylindrica* grown in a standard medium for cyanobacteria (BG11), at ambient air composition and pressure, yields only about 0.2 μmol H₂·mg Chl a⁻¹·h⁻¹ (Murugesan et al. 2015). One of its main limitations is the inhibition by atmospheric O₂ and N₂: nitrogenases are irreversibly inactivated by O₂, while N₂ strongly inhibits proton reduction. The high CO₂, low O₂ and N₂ composition of the Martian atmosphere could thus be an advantage here. Consistently, yields above 20 ml·l⁻¹·h⁻¹ (around 0.8 mmol·l⁻¹·h⁻¹) were obtained with concentrated cultures of about 50 mg Chl a·l⁻¹ of *Anabaena variabilis* (so, roughly, 16 μmol H₂·mg Chl a⁻¹·h⁻¹) under a 99 % argon (Ar), 1 % CO₂ atmosphere (Liu et al., 2006). Recently, levels of around 25 μmol·mg Chl a⁻¹·h⁻¹ were also reached with *A. cylindrica* cultures under high CO₂/low N conditions (Murugesan et al. 2015). With a concentration of 50 mg Chl a·ml⁻¹, these rates could lead to 1.5 mmol·l⁻¹·h⁻¹ (around 35 ml·l⁻¹·h⁻¹ at room temperature, with 30 μmol H₂ produced per mg Chl a·h⁻¹) of H₂. Producing 6 tons of H₂ would then require about 228 m³·year. It corresponds, for example, to 22 days in a culture system the size of 3-meter-deep Olympic swimming pool, or to 2 years and 4 months in 100 m³, assuming yields can be maintained at this scale. But these figures are very rough approximations: rates that would effectively be obtained on Mars depend on the mission scenario and technology choices for culture systems. Finally, current productivity is still far from its maximum, and increases are regularly obtained by culture conditions' optimization and metabolic engineering.

Leaving H₂ aside, cyanobacteria can generate various biofuel precursors and components including alkanes, ethylene, hydrogen peroxide (H₂O₂; which can be used as a monopropellant) and lipids, without relying on organic precursors (see for instance Quintana et al. 2011). They could also provide organic substrates for production of biofuel precursors by other organisms, particularly relevant on Mars where importing substrates from Earth would be impractical. For instance, as mentioned above, lysed cyanobacterial biomass has been used as a fermentation substrate for ethanol production in yeasts (Aikawa et al., 2013; Möllers et al., 2014). However, genetic engineering could remove the need for other organisms even for production of biofuel precursors which are not naturally produced by cyanobacteria in adequately large amounts—if at all. For instance, some cyanobacteria have been engineered to produce ethanol. Whereas yeasts rely on a sugar-based pathway

and thus on the availability of agricultural substrates, engineered cyanobacteria produced it directly from CO₂ and solar energy, following a much simpler process where pyruvate is first converted to acetaldehyde by a pyruvate decarboxylase and then to alcohol by an alcohol dehydrogenase (Dalton and Roberto, 2008; Deng and Coleman, 1999; Dexter and Fu, 2009). Generally speaking, a lot of work has been done to engineer new biofuel (or biofuel precursor) production pathways—or to improve existing ones—in cyanobacteria (see, *e.g.*, Ducat et al. 2011). And there is still much room for improvement, especially under Mars-like conditions.

Thus, even though extensive development and optimization is needed, cyanobacterium-based biotechnologies could represent original contributions to the production of rocket fuel for the return flight, for powering surface vehicles and, more generally, for powering equipment that can be operated by combustion engines.

Other applications

Various other applications involving cyanobacteria have been proposed for human outposts on Mars. One, for instance, is biomining. Microorganisms are used on Earth to extract metals of industrial interest (*e.g.*, copper and gold) from rocks, and their use on Mars to mine basalt and potential ores has been suggested (Cockell, 2010, 2011; Navarrete et al., 2012). Cyanobacteria are known to leach a wide range of rock types, including terrestrial volcanic rocks with compositions similar to Mars's regolith (Brown and Sarkisova, 2008; Olsson-Francis and Cockell, 2010b; Olsson-Francis et al., 2012). The use of cyanobacteria, possibly engineered to increase their ability to dissolve rocks and harvest specific elements (Cockell, 2011), can thus be considered for simple bioleaching processes that would not rely on imported C sources (needed for heterotrophic microorganisms) or toxic elements such as cyanide (used in non-biological leaching processes). Extracted elements could be used within a wide range of chemical and manufacturing processes such as, for instance, CO₂ cracking, electroplating, production of alloys and manufacturing of solar cells (Cockell, 2011; Dalton and Roberto, 2008).

Cyanobacteria have also been suggested for controlling Mars's surface dust, through the production of biological crusts, in enclosed structures such as greenhouses and habitats (Liu et al., 2008). Indeed, some can grow and form

biofilms within the interstices of desert minerals and produce extracellular polysaccharides that bind the particles together, preventing wind-induced dust release. Such crusts could also be used as an air filter to remove dust from the atmosphere (Cockell, 2010). Then, the use of dehydrated *Chroococcidiopsis* cells as a gene repository for on-site molecular biology has been suggested (Billi et al. 2013), as they can preserve plasmids during long-term desiccation (Billi 2012) and presumably repair them when damaged. Cyanobacteria could also be used to process human waste products and recycle their organic C, water, nitrates and mineral nutrients. Cultures could be used directly (Fiflali et al. 1997; Godia et al. 2002; Lehto et al. 2006; Yang et al. 2008) but also indirectly; for instance, H₂O₂ generated from cyanobacterium-produced O₂ and H₂O could be used to oxidize human wastes following a physicochemical process developed by researchers of the Institute of Biophysics of the Siberian Branch of the Russian Academy of Sciences (Kudenko et al., 2000); nutrients could then be recycled in cyanobacterial cultures (Tikhomirov et al., 2007). Cyanobacteria have also been suggested for the production, beyond Earth, of various chemicals including nutritional molecules, drugs, bioplastics and cellulosic building materials (Menezes et al., 2014; Way et al., 2011).

Finally, the ability of cyanobacteria to produce organic material from Martian resources, coupled to our increasing abilities in metabolic engineering, make it possible to consider many other applications ranging from performing basic life support functions to generating comfort products.

Plants or cyanobacteria?

Assuming that both plants and cyanobacteria can be grown on Mars, some functions such as food and O₂ production could be performed by either or both of them. For these functions, plants are the most commonly proposed photosynthetic organisms. This choice, however, is mostly due to our historical reliance on – and experience with – them: they have been a highly available food source throughout most of human history. Matters need to be reconsidered where environmental conditions, resources and other constraints are different.

Photosynthetic microorganisms are more efficient, on a volume basis, at capturing solar energy than plants. Their culture in photobioreactors could yield much more biomass (especially proteins) and O₂ for a given volume and

light intensity than greenhouse-type cultures of staple edible plants (Dismukes et al. 2008; Way et al. 2011; Wang et al. 2012). This is critical where resources are scarce and cultivation areas are highly controlled. Moreover, a considerable part of plant biomass (*e.g.*, roots or stems, depending on species) is inedible and hard to recycle: plant cell walls are among the least degradable polymers in BLSS (Hendrickx and Mergeay, 2007).

Plants are also much more demanding in terms of environmental conditions. For instance, they are harmed by anoxia (roughly, a partial pressure of O₂ of at least 50 hPa is needed for proper development, mainly for non-photosynthetic tissues; see Thomas et al. 2005) and high concentrations of CO₂ (negative effects have been observed in some plants above 4 hPa [Wheeler 2004], while the partial pressure of CO₂ [pCO₂] on Mars is above 6 hPa). Photosynthetic cultures on Mars, be they vegetal or bacterial, will have to be protected from biocidal environmental conditions. The needed level of protection – and the associated costs – will depend on the ability of cultured organisms to withstand these conditions, and many cyanobacteria are much more resistant than staple plants to the Martian surface’s environmental stressors. They thrive in the most extreme habitable conditions on Earth, such as in dry deserts and ice lakes of Antarctica, and within ices of high Arctic seas (Scalzi et al., 2012; Wierzchos et al., 2006). Some have an outstanding resistance to environmental factors occurring on Mars’s surfaces, including ultraviolet (UV) and ionizing radiation (see for instance Billi et al. 2013 and Thomas et al. 2006) and can survive in space when protected from UV radiation (Olsson-Francis et al., 2010).

In habitable compartments, gas consumed and produced by crewmembers must be balanced with gas consumed and produced by an atmosphere regenerating system. Since CO₂ will be available in the atmosphere and since both CO₂ and O₂ will be generated as byproducts of other processes, this system could be more flexible on Mars than in places where simple closed systems would be used: losses could be compensated and excess vented out. However, extensive control of the atmospheric balance would both increase safety and reduce resource consumption. By adjusting culture conditions, controlling cultures’ assimilation quotient (CO₂ consumed/O₂ produced) to match humans’ respiratory quotient (CO₂ produced/O₂ consumed) is much easier with cyanobacteria than with plants, making the atmospheric O₂/CO₂ balance much more manageable with the former (Averner et al., 1984; Horneck et al., 2003).

As crewmembers should be available for research and colony settlement and maintenance, time spent on culture management will have to be kept to a minimum. Culturing plants requires significant manpower; within Biosphere 2, for instance, agricultural and food-related tasks took about 45 % of crew time (Silverstone and Nelson, 1996). In this framework, automation should be extensive. Cyanobacterial cultures are much more suitable for automation than plants' due to their culture homogeneity, growth in liquid medium and lack of inedible parts that should be sorted out. They are also much more manageable; culture parameters could be more easily adjusted to cover human needs, with a safety margin but without excess. Outputs could thus be much more controllable and predictable.

The time needed to establish cultures also matters. Even though shelf-stable food can be sent to sustain crews before cultures are first set up, it is important to be able to re-establish cultures in case of accidental loss—likely enough, and something with catastrophic consequences if not rapidly fixed. Being able to quickly extend cultures to cover unexpected needs (*e.g.*, to compensate an O₂ loss) can also be critical. Even though some plants can be grown faster, staple crops can take three to four months to mature even under favorable conditions (Drysdale et al., 2003). On the other hand, microbial cultures can be quickly expanded and re-deployed from very small amounts.

Finally, cyanobacteria are much easier to engineer than plants due to their rapid division times, compatibility to transformation, unicellularity and relatively simple genetic background (Koksharova & Wolk 2002; Way et al. 2011; Berla et al. 2013). They could therefore be much more easily modified for new functions and adaptation to Martian conditions (see section “Potential inputs from synthetic biology” below).

It should however be noted that plants have some advantages over cyanobacteria: they could provide tasty and carbohydrate-rich comfort food, and have beneficial psychological impact on crewmembers (Allen, 1991). Establishing small-scale cultures is not obviously unrealistic, especially if nutrients are provided from local resources as described above.

Besides plants, one may wonder why the present paper focuses on cyanobacteria rather than eukaryotic microalgae, which could also perform some of the functions described above. Reasons include cyanobacteria's overall better abilities to use Martian resources (*e.g.*, by N fixation and regolith

leaching) and to withstand Martian conditions, the higher digestibility of their edible species and their higher growth and photosynthetic rates. They are also more suitable for genetic engineering, in part due to current transformation systems which are much simpler and well-developed for cyanobacteria than for eukaryotic microalgae (Wang et al. 2012; Wijffels et al. 2013).

What about non-photosynthetic (chemotrophic) microorganisms? Heterotrophs may be useful on Mars, but not as primary producers (see section “Feeding other microorganisms”). Resources needed to feed the metabolism of some chemoautotrophs, on the other hand, may be found on Mars; for example, reduced iron could be used as an energy source by iron-oxidizing bacteria (Nixon et al. 2013). Chemoautotrophs could thus be considered for some applications, such as the extraction of industrially useful minerals (Cockell 2010; 2011). However, none has the versatility of cyanobacteria to be the basis for BLSS: none combines, for instance, high N fixation rates, high growth rates, ability to rely exclusively on Martian resources, O₂ production, H₂ production, amenability to genetic manipulation and edible biomass.

Growing cyanobacteria on Mars

All organisms we currently know have evolved on Earth and none of them would be able to grow efficiently on the Martian surface. Cyanobacteria must be provided with shielding and an environment suitable for metabolism and growth. Elaborated hardware systems providing Earth-like conditions have been proposed but they rely on complex technology and require accurate control of all the process parameters (e.g., gases, temperatures and pressures in each compartment), are very demanding in terms of construction materials and energy consumption, need to be constructed on Earth, are very massive and expensive to carry to Mars, and can consequently be applied to small-scale cultures only. In order to be cost-effective and reliable, a culture hardware for large-scale, long-term, sustainable BLSS on Mars should be much simpler.

Fortunately, reproducing Earth-like conditions is not needed: cyanobacteria can grow under conditions which are much closer to Mars’s. In

addition, most inputs – if not all – needed for growing cyanobacteria can be found on-site. An adequate culture system could thus provide a set of parameters (radiation shielding, atmospheric composition and pressure, gravity, nutrient supply etc.) resulting from a compromise between (i) efficient support to growth and metabolism, and (ii) system feasibility and substrate availability on Mars’s surface.

This culture system should be able to resist an inside/outside pressure difference, fine dust, large temperature gradients and strong radiation fluxes. Whatever its final design, efforts should be made to keep its weight, cost and energy consumption as low as reasonably possible given the other requirements. Ideally, the design should allow manufacturing from on-site compounds (e.g., regolith-based materials for radiation shielding, glass manufactured using silicon dioxide from Martian soil, and metallic parts derived from metal oxides mined in the regolith), assuming that equipment needed for processing is available on site. The potential of ultimately creating many of these facilities with local resources is currently being explored in Lynn Rothschild’s laboratory.

Nutrient sources

Most elements needed for feeding plants and microorganisms can be found in Martian regolith.

Data on the composition of Martian soils and rocks have been obtained from analyses of the SNC (Shergottites, Nakhlites, Chassignites) group of meteorites (McSween, 1994), NASA’s Viking (Clark et al., 1982), Pathfinder (Rieder, 1997) and Phoenix (Hecht et al., 2009) landers, NASA’s Spirit rover (Morris et al., 2004), instruments operated from orbiters—noteworthy the Thermal Emission Spectrometer (TES) on NASA’s Mars Global Surveyor (see Christensen et al. 2001), the Gamma Ray Spectrometer (GRS) on NASA’s Mars Odyssey (Boynton et al. 2007) and the Compact Reconnaissance Imaging Spectrometer for Mars (CRISM) on the NASA’s Mars Reconnaissance Orbiter (Mustard et al., 2008)—and, recently, using X-ray spectrometers aboard NASA’s Opportunity and Curiosity rovers. In particular, the latter two have allowed detailed mineral compositions to be deduced at multiple sites in the Endeavour Crater (Arvidson et al., 2014) and Gale Crater (Grotzinger et al., 2014b; McLennan et al., 2014; Ming et al.,

2014; Vaniman et al., 2014). On Earth, basalt is the dominant rock type on the surface. It harbors much of the biosphere and, as an abundant source of redox couples and macronutrients, provides an efficient support to microbial life (for a review in the context of the search for life on Mars, see McMahon et al. 2013). Martian regolith is also in large part composed of basaltic minerals; more generally, Mars's surface seems to be mostly basaltic (McSween et al., 2009; Taylor and McLennan, 2009). All basic elements needed for cyanobacteria and other organisms (C, H, O, N, P, S, K, Mg, Na, Ca, and Fe), as well as other elements needed in smaller amounts (Mn, Cr, Ni, Mo, Cu, Zn...), have been detected there.

The most convenient sources of C and N will probably be atmospheric CO₂ and N₂ (see "Atmospheric pressure and composition" section below). Additional C can be found in the CO₂ ice caps, in the surface and subsurface regolith due to exchange with the atmosphere, and possibly in reservoirs formed when the atmosphere was thicker (Kurahashi-Nakamura and Tajika, 2006). It has also been suggested that fixed N, derived from Mars's atmospheric N₂, may be buried in the regolith (Mancinelli & Banin 2003; Boxe et al. 2012). Consistent with this, N-bearing compounds have been detected there (Ming et al., 2014). However, the exact nature and bioavailability of these compounds has not yet been determined.

Thus, all elements needed to support life seem to be present in Mars's rocks (Cockell 2014) and atmosphere. These nutrients can be directly made available to cyanobacteria, as multiple species thrive in a lithotrophic lifestyle, extracting all their needed mineral nutrients from rocky substrates (including basalt) and obtaining their whole N and C supply via photosynthesis and biological N fixation. Accordingly, some strains (e.g., *Anabaena cylindrica*) have been grown in distilled water containing only powdered Mars basalt analogues (experiments were performed under terrestrial atmosphere; considerations on what atmospheric conditions are suitable are given in the "Atmospheric pressure and composition" section below). Non-N fixing cyanobacteria could also grow when NaNO₂ was added. On Mars, fixed N could come from N fixers (see above sections), while the possibility that nitrate beds are present cannot be ruled out. Supplementing the media with a sulfate source, (NH₄)₂SO₄, had a positive impact on some of the tested species. The authors suggested that, on Mars, gypsum (NaSO₄·2H₂O) could be used as such a supplement (Olsson-Francis and Cockell, 2010b). Gypsum dunes have indeed been found in the northern polar region of Mars by the OMEGA

instrument on ESA's Mars Express orbiter (Langevin et al., 2005) and the CRISM and High Resolution Imaging Science Experiment (HiRISE) instruments on NASA's Mars Reconnaissance Orbiter (Roach et al., 2007), and later confirmed by the rover Opportunity (Squyres et al., 2012). Other studies showed that the growth of several siderophilic cyanobacterial species isolated from iron-depositing hot springs in Yellowstone National Park was stimulated by the presence of Martian soil analogues in culture media (Brown and Sarkisova, 2008) and that *Nostoc* sp. HK-01 could grow on a Mars regolith simulant for at least 140 days, without other nutrient source besides atmospheric gas (Arai et al., 2008). Other cyanobacteria have been grown using other Martian soil analogues as substrates, in distilled water or spread on gellified water plates. DLR's P-MRS and S-MRS simulants (Böttger et al., 2012), and NASA JSC's Mars-1A simulant (Allen et al., 1997), for instance, supported the growth of *Matteia* sp. and *Anabaena* sp. PCC7120, respectively (Chapter 6; Verseux, Baqué et al., unpublished data). The need for providing regolith might be an issue for automation. However, using drilling photobioreactors that extract raw materials from the surface and directly bring them to cultures has been suggested (Cumbers and Rothschild, 2010) and technologies have been designed to excavate large amounts of regolith with minimal weight and time (see, e.g., Mueller & Van Susante 2011).

Thus, even though it might be relevant to adequately mix rock types to have all nutrients in appropriate proportions and suitable pH, and even though some salts, oxides and toxins might need to be removed, all nutrients and micronutrients needed to support cyanobacterial metabolism seem to be present on Mars. Additional nutrients could come from human waste. If some micronutrients (e.g., some cofactors) could not be mined or produced on site, bringing them from Earth would add a negligible mass to the initial payload as they are needed in trace amounts only. Methods for physicochemical preprocessing of Martian regolith and atmosphere (to generate, for instance, a broth of pre-leached regolith and nitric acid in which CO₂ is bubbled; see Cao et al. 2014) could be considered, if the increased productivity outweighed the increased running cost and complexity.

Atmospheric pressure and composition

Use of the minimal suitable pressure would greatly lower construction weight and cost of cyanobacterial culture systems on Mars, and would

minimize the risk of organic matter leakage (Lehto et al., 2006). Relying on a gas composition which is as close as possible to Mars's would make the establishment of such systems even simpler and cost-effective.

What is this minimal suitable pressure? No clear answer has been given to this question, as little work has been focused on microbial growth at low pressure. The lowest pressure at which biological niches are naturally present on Earth is about 330 hPa (at the top of the mount Everest), way above Martian's surface pressure of about 5-11 hPa (Fajardo-Cavazos et al., 2012). Although viable bacteria have been sampled a few times from stratospheric air above 30 km (e.g., Wainwright et al. 2003), where atmospheric pressure goes down to Mars's surface pressure, no microbial growth there was indisputably evidenced. Some methanogens can keep metabolic activity (shown by detectable CH₄ production) at 50 hPa of pressure under simulated Martian environmental conditions (Kral et al., 2011; Schirmack et al., 2014), and a few bacteria such as *Serratia liquefaciens* (Schuerger et al., 2013) and isolates from Siberian permafrost samples (Nicholson et al., 2013) have been grown under 7 hPa of CO₂-enriched anoxic atmospheres, but a wide range of microorganisms have been shown to be unable to grow on semisolid agar medium at pressures below 25 hPa of ambient air (Nicholson et al., 2010).

It might be possible to decrease the lowest suitable pressure. As no biological niche is naturally present on Earth at pressures close to Mars's, selective pressure is virtually nonexistent for current terrestrial microorganisms and the full potential for growth at low pressures is probably far from being reached. There might thus be much room for improvement by artificially evolving cyanobacteria to grow faster under low (and to grow at lower) pressures, including low pressures of Mars-like gas compositions. Consistently, an isolate of *Bacillus subtilis* showed increased fitness at 50 hPa after a 1000 generation-culture at this pressure (Nicholson et al., 2010). The minimal suitable pressure can also be dependent on atmospheric composition, as described below, and on medium type (liquid or solid). It should however be noted that a physical limitation derives from the need to maintain a liquid phase at growth-permissive temperatures.

In recent experiments, a decreased atmospheric pressure (50 kPa instead of the ambient 100 kPa) negatively affected the growth of cyanobacteria from several genera (Quin et al. 2014). But this was performed under ambient gas composition and, like atmospheric pressure, atmospheric composition matters.

CO_2 and N_2 are present in Mars's atmosphere but their partial pressures differ from Earth's (see Table 1). Mars's higher-than-Earth pCO_2 (6.67 vs 0.38 hPa) might actually be beneficial: elevated levels of CO_2 can have a fertilizing effect on cyanobacterial cultures (Murugesan et al. 2015). Below one atmosphere of pressure, CO_2 seems to become the limiting factor and cyanobacteria benefit from much higher-than-normal CO_2 concentrations; *Synechocystis* sp. PCC 6803 was shown to grow more than 3 times faster under 100 hPa with 5 % CO_2 (as well as under 1 bar with 5 % CO_2) than under 1 bar of ambient air (0.04 % CO_2), and to grow under 33 hPa of 100 % CO_2 with growth rates close to those obtained under 1 bar of ambient air (Lehto et al. 2007). Later results showed that an increase in CO_2 concentration (at least up to 20 %, even though rates started to decrease after 10 %) under either 1 bar or 100 hPa leads to higher growth rates than ambient air composition at the corresponding pressure (Murugesan et al. 2015; unpublished data). Thus, this strain seems to benefit from higher-than-usual pCO_2 , with a saturation around 4 hPa, at which level an about 3.5 fold increase in growth rates is observed for cells previously grown in ambient terrestrial atmosphere. When CO_2 is not limiting, its growth rates are not negatively affected by a 10-fold reduction in atmospheric pressure. Similarly, cultures of *Arthrospira platensis* and *Anabaena cylindrica* were shown to benefit from higher-than-ambient CO_2 concentrations (Murugesan et al. 2015). Finally, at least some cyanobacteria are able to survive (*Synechococcus* PCC7942, *Anabaena* sp.) and even to grow (*Plectonema boryanum*) in liquid culture under one bar (1000 hPa) of pure CO_2 , at least in the short term, when pCO_2 is gradually increased by 150 hPa a day (Thomas et al., 2005). If needed, bacterial resistance to high CO_2 levels could probably be further increased by preventing pH decrease in the medium, which happens due to carbonic acid (H_2CO_3) formation when CO_2 dissolves in water.

The most striking shortage of substrates on Mars derives from the low partial pressure of N_2 (pN_2) in the atmosphere and the presumed low availability of N in the regolith. Some work performed with *Azotobacter vinelandii* and *Azomonas agilis* showed that microbial N fixation is possible at a pN_2 of 5 hPa, even though below 400 hPa growth rates decreased with decreasing pN_2 (Klingler et al., 1989). The lower limit for N fixation might vary among species and is still to be defined, but it has been proposed to be within the range of 1-10 hPa (McKay and Marinova, 2001). Crossing this limit using Mars's air composition would require at least 5 to 50 times the local ambient pressure (reaching a total pressure of approximately 40 hPa to 40

kPa). However, pN₂ would still be limiting and higher values are needed for efficient processes. Rather than only increasing the total pressure to reach adequate pN₂ values, N₂ could be concentrated by separating CO₂ from the other atmospheric gases (mainly N₂ and Ar) and mixing them in different proportions to reach an appropriate pN₂ value in an otherwise optimized total pressure. Gas separation techniques are well-developed (see for instance Meyer & McKay 1996 and Zubrin & Wagner 2011) and could be based on processes routinely used by industry on Earth. Assuming similar pN₂ needs for cyanobacteria as for *Azotobacter vinelandii* and *Azomonas agilis* (see Klingler et al. 1989), a pN₂ of 95 hPa would only slightly limit growth,(experiments are planned to define the lowest pN₂ allowing efficient growth of diazotrophic cyanobacteria). For wild-type, diazotrophic cyanobacteria, a 100-hPa atmosphere with 95 % N₂ and non-limiting (5 %) CO₂ could thus lead to higher growth rates than an Earth-like atmosphere. Cultures could also be supplemented with N recycled from human and biomass waste, even though this should not be the only N source as sustainability and expandability would be compromised.

Depending on its adjusted pressure, a Martian-like atmosphere can strongly affect other aspects of the lithotrophic growth of cyanobacteria through indirect effects (e.g., a lowered pH due to H₂CO₃ formation can influence both cell viability and nutrient release from substrates). As for all processes suggested here, extensive and faithful simulations of culture conditions expected to be provided on-site are needed.

Atmosphere of selected gas composition and pressure could be provided within inflatable, tunnel-like containments (Lehto et al., 2006). Tight sealing should allow the desired pressure to be maintained, and adjusted gas supply systems could allow CO₂ and some N₂ to be provided from Martian atmosphere – possibly after N enrichment. An onion-like structure, with a pressure gradient throughout layers, has also been proposed. This would offer the additional advantages of a better filtering of the Martian dust and a better thermal insulation (Lehto et al., 2006).

Water

One of the most critical resources to provide to cyanobacterial cultures will be water. As water is needed within human outposts, regardless of the use of

cyanobacteria, various hardware systems for its extraction and processing on Mars have been suggested elsewhere. There follow a few examples of water sources and mining techniques under consideration. This list is not exhaustive.

Water could be generated on Mars by importing H₂ from Earth and combining it to O from local CO₂ (Zubrin et al., 1991) but, even though H represents only 11 % by weight of pure H₂O, relying on imported H₂ would strongly limit the autonomy and sustainability of human outposts. However, water can be found in various forms throughout Mars (reviewed in Tokano 2005, Rapp 2007 and Cockell 2014). It is present in large amounts as ice at the north polar ice cap and under the south carbon ice cap, and throughout the planet as near-surface deposits of water ice. The presence of liquid water has to date not been unquestionably established, but several lines of evidence strongly suggest transient liquid brines (Zisk & Mouginis-Mark 1980; McEwen et al. 2011; Martín-Torres et al. 2015). It might also exist below the cryosphere, in areas where temperatures and pressures are high enough (e.g., Clifford et al. 2010), possibly within drilling range. The abundance, location and nature of near-surface water deposits is still to be accurately determined, but mining these to generate usable water may be practical (e.g., Rapp 2013). If it appears that mining liquid water or large ice deposits cannot be done, water could be extracted from the soil's hydrated minerals. Energy requirements for heating soil at 500°C in an oven, before collecting steam, have been assessed to be about 5.2 kWh·kg of water⁻¹ in a 2 %-water soil, plus a small amount (below 0.1 kWh·kg of water⁻¹) for excavating and conveying the soil (Stoker et al. 1993). Other processes where soil is heated for water extraction are under consideration, including the use of focused light and of microwaves, the latter being one of the most promising regarding energy requirements, mass and reliability (Ethridge & Kaulker 2012; Wiens et al. 2001). A team of the Colorado School of Mines designed a system, referred to as the Microwave Pizza Oven, aimed at extracting water from the soil of Mars using microwaves (generated using about 12 kWh·kg of water⁻¹, provided by silicon solar cells) in conjunction with a conveyor belt mechanism to process soil and extract bound water. Expected yields are in the order of 1 kg of water per 2.5 hours in a 2 %-water soil, for a system's mass below 20 kg (Wiens et al. 2001). The use of microwave heating could also reduce dramatically excavation needs, as microwaves could be inserted down bore holes to heat at desired depths (Ethridge & Kaulker 2012). But energy and excavation needs for water extraction from the soil could be further reduced: according to Zubrin & Wagner (1996), a 0.1-mm-thick polyethylene dome

could be used to farm selected soil sites by increasing temperature and collecting volatilized water in a cold trap device. Using this approach, a 25-meter, 100-kg dome ringed by reflectors could allow about 150 kg of water to be farmed in an 8-hour day from a soil with 2 % water (Zubrin and Wagner 1996).

There is also water vapor in the atmosphere. Relying on it rather than on soil water would remove the need for processing regolith and/or to move to new locations once a site has been dried up. Concentrations vary through time and location and are very low, but total atmospheric water has been assessed to amount to approximately 1.3 km^3 ($1.3 \times 10^9 \text{ l}$), in very large excess compared to human needs for a research base, and extracted water would be renewed naturally by exchange with regolith and polar caps (McKay et al. 1993). In spite of the large volumes of atmosphere that needs to be processed, water extraction from the Martian atmosphere could be done with an energy consumption below $100 \text{ kWh}\cdot\text{kg}^{-1}$ using a process of atmosphere cooling/compression and water condensation designed by Meyer and McKay (1984) and optimized by Clapp (1985). Note that energy used in this process would not be dedicated to water extraction, as atmospheric gas could be isolated using the same setup. Energy needs for water extraction from the atmosphere could be brought further down, as shown by a system referred to as the Water Vapor Adsorption Reactor (WAVAR). In WAVAR, atmosphere is filtered and drawn through a regenerative adsorbent bed of zeolite molecular sieve, from which water is later desorbed using microwave radiation (Coons et al. 1997; Schneider & Bruckner 2003; Williams et al. 1995). Energy consumption has been assessed as ranging from approximately 3 to $30 \text{ kWh}\cdot\text{kg}$ of water $^{-1}$, depending on time and location (Grover & Bruckner 1998). Additional water could be recycled from human metabolism (about 0.4 kg of transpiration water and respiration moisture a day per crew member) and human waste (Meyer & McKay 1989; Tikhomirov et al. 2007). It will also be a by-product of physicochemical processes such as on-site production of biofuels and materials (e.g., Zubrin et al. 1997). The systems described above are still in their infancy; increasing knowledge of Mars's resources, coupled to engineering effort, will likely support the development of more efficient water mining technologies and processes.

Strategies can also be developed to minimize water needs for cyanobacterial culture systems, in addition to recycling water from culture effluents. For the production of some products where cells lysis is not needed,

water requirements (as well as requirements for maintenance, nutrients and energy) could be reduced by immobilizing cyanobacteria within polymeric matrices. Such entrapment can preserve cyanobacteria's metabolic activities for prolonged periods of time, up to several years (Lukavský 1988; Hertzberg & Jensen 1989; Chen 2001). Water requirements could be further reduced by growing terrestrial cyanobacteria as biofilms, directly on the surface of Martian rocks, in a semi-closed environment where suitable temperature, pressure and moisture are provided. Such a growth system could be relatively close to the natural lifestyle of rock-dwelling cyanobacteria, noteworthy in terrestrial deserts (see, e.g., Friedmann & Ocampo 1976, de la Torre et al. 2003, and Warren-Rhodes et al. 2006 and Billi et al. 2013) but with more favorable moisture, UV protection and temperatures.

Finally, a reduced pressure at growth-permissive temperatures, as envisioned in the culture system, will foster water evaporation. However, if pN₂ sets the lower limit for atmospheric pressure and the culture system provides about a tenth of Earth's atmospheric pressure at sea level (see section "Atmospheric pressure and composition"), evaporation can easily be reduced by saturating the incoming air with water vapor (Kirsi Lehto, personal communication). Besides, as the system needs to be air-tight (apart from controlled gas exchange), water loss by evaporation can be minimized.

Solar energy and harmful radiations

On Earth, the solar flux is in large excess compared to the needs of cyanobacteria and green plants, which cannot use more than about 10–20 % of maximal sunlight on the surface (Way et al., 2011). Some cyanobacterium species such as *Arthrospira* spp. can utilize higher light densities but, at least for *A. platensis*, the radiation level for maximal photosynthesis activity is well below terrestrial mean day values—provided agitation intensity and cell density allow sufficient exposure (Hoshino et al., 1991). And on Mars? Even though Mars is on average 1.52 times farther from the Sun than Earth and consequently receives about 43 % as much sunlight at comparable latitude and time of day, this is in excess compared to photosynthesis needs, even in the case of a dust storm (McKay et al., 1993). The light flux needed for optimal photosynthesis of many cyanobacterial species is around 3×10^{19} photons·m⁻²·s⁻¹, which is only about 10 % of Mars's average ambient light flux (Lehto et al., 2007). At lower light levels photosynthesis is still possible,

albeit at lower efficiencies. It has for example been assessed that, at midday at vernal equinox, light levels on Mars are about 5000 times greater than the minimum required for photosynthesis (Cockell and Raven 2004).

It might be thought challenging to let photosynthetically active radiation (PAR light; 400–700 nm) reach cultures while protecting the latter from harsh radiation—namely UV radiation, solar energetic particles (SEP) and galactic cosmic rays (GCRs). First, the fraction of biologically effective UV radiation reaching the surface of Mars is much greater than that reaching the surface of Earth and includes UV-C radiation (> 200 nm). The models of Cockell et al. (2000) and Schuerger et al. (2003) predict a maximum flux of around $50 \text{ W}\cdot\text{m}^{-2}$ of $\text{UV}_{200-400 \text{ nm}}$ irradiance on the equatorial Martian surface at the mean orbital distance. Recent data from the Curiosity rover suggest lower values, with a maximum $\text{UV}_{200-380 \text{ nm}}$ irradiance of about $20 \text{ W}\cdot\text{m}^{-2}$ recorded at midday at Gale Crater (Gómez-Elvira et al., 2014). Despite being strongly germicidal on the surface, this radiation can be blocked by a few millimeters of Martian dust coverage (Mancinelli & Klovstad 2000; Dartnell & Patel 2014) or, to let PAR light in, by mere transparent covers such as glass filters.

Besides UV, ionising radiations of SEP and GCRs can reach the Martian surface and subsurface with high energies due to Mars's lack of intrinsic magnetic field and thin atmosphere. Pavlov et al (2012) and Dartnell et al. (2007) assessed absorbed dose rates at the surface to be in the range of 50–150 mGy·year⁻¹, and data from the Curiosity rover indicate an absorbed dose rate of 76 mGy·year⁻¹ at Gale Crater (Hassler et al., 2013). Could cells be protected from this radiation? Even though the primary cosmic radiation component decreases with shielding, secondary particles (lighter particles including neutrons and gamma-rays) form when radiation penetrate substrates and dose rates increase with shielding until the Pfotzer maximum, before decreasing due to energy loss, absorption and decay processes. From 76 mGy·year⁻¹ at the surface, for instance, they reach 96 mGy·year⁻¹ under 10 cm of Martian rock with a density of $2.8 \text{ g}\cdot\text{cm}^{-3}$ (Hassler et al., 2013). The design of culture chambers and the choice of material used for shielding should take into account their interactions with primary radiations on the Martian surface (see Le Postollec et al. 2009).

Approximately 5 m of Mars dirt would confer a protection to radiation equivalent to Earth's atmosphere (McKay et al., 1993). However, bacterial cells are overall much more radiation-resistant than human cells and such a

protection is unlikely to be needed. In addition, cyanobacteria are known to have high ploidy levels (Griese et al., 2011), pigments and specific morphological features which tend to increase radio-resistance. The resistance of cyanobacteria to photon irradiation (gamma-rays and X-rays) has been studied as early as in 1951 (Bonham and Palumbo 1951), and the high resistance of some species was observed in the early 1960s (Godward, 1962; Shields et al., 1961). In the following decade, the resistance of a wide range of species from various genera was tested. Resistance appeared to be highly variable among species, ranging from sensitive ones with D_{10} (dose required to reduce the viable number of cells by 90 %) below 1 kGy to highly resistant ones with D_{10} above 10 kGy (e.g., Asato 1971; Bruce 1964; Godward 1962; Kraus 1969; Kumar 1964; Shields et al. 1961). These results were confirmed and deepened by recent studies. *Synechococcus* and *Synechocystis* spp. were shown to be relatively sensitive to gamma rays, with D_{10} of about 0.3 kGy (Agarwal et al., 2008; Domain et al., 2004) and 0.7 kGy (Domain et al., 2004), respectively. Two *Anabaena* strains were shown to tolerate a 5 kGy gamma-ray dose without loss of survival, to have a GI_{50} (dose where growth is inhibited by 50 %) of 6-11 kGy and to survive doses of 15 kGy (Singh et al., 2010, 2013). *Arthrosphaera* sp. PCC 8005 cells were shown to be able to survive exposure to doses of at least 6.4 kGy of gamma irradiation, 1 kGy of He particle radiation and 2 kGy of Fe particle radiation (Badri et al., 2015). *Chroococcidiopsis* spp. cells were shown to withstand 2.5 kGy of X-ray irradiation in liquid culture with small to medium viability loss: 20 to 65 %, depending on the strain, with a D_{10} of 2-5 kGy (Billi et al. 2000). Viable *Chroococcidiopsis* spp. cells were also recovered after exposure to 15 kGy of X-rays (Billi et al. 2000) and to 12 kGy of gamma-rays (Verseux et al., 2017). Moreover, no significant DNA or membrane damage was detected after exposure to 1 kGy of He particle radiation, 2 kGy of Fe particle radiation, or 1 kGy of Si particle radiation (Verseux et al. 2017). A dose of 1 kGy roughly corresponds to a thousand years on the Martian surface, a timeframe way beyond that of division and repair of metabolically active cyanobacteria; cells are therefore not expected to be affected in BLSS cultures. The biological effect of ionizing radiation cannot be assessed based on the dose only: other parameters are to be taken into account—for instance, the composition of the radiation flux and its modification when interacting with the environment and the cells' properties—but the studies mentioned above show the orders of magnitude involved. Even if the effect of radiation on Mars was, at equivalent dose, more damaging by several orders of magnitude than the radiation flux used in these studies, the replication time and repair dynamics of even slow-

growing strains would be way below the time needed to receive a sterilizing dose. Consistently, it has been estimated that even vegetative cells of the bacterium *Escherichia coli* could survive the ionizing radiation dose that would be received after more than a thousand years on the surface of Mars (Dartnell et al., 2007). More generally, ionizing radiation on Mars is not expected to prevent microbial life (Dartnell et al. 2007; Horneck 2008).

Cultures could be buried inside regolith or covered with regolith-based material: although not expected to be needed for radiation protection, regolith shielding could be relevant for temperature control (taking advantage of regolith thermal insulating properties) and protection from the wind and dust. Manufacturing processes could be simple and cost-effective, taking for instance advantage of (i) the cement-like properties of unprocessed regolith mixed with water (McKay and Allen, 1996), (ii) the ubiquity of clay-like materials on Mars, (iii) the large fraction (about 40 % by weight of Viking 1 and 2 soil samples) of Martian soil represented by silicon dioxide (the basic constituent of glass), and (iv) the fact that plastics may be derived from local C and H (Zubrin & Wagner 2011). In this case, artificial lighting, mirrors or fiber optics could be used to bring PAR light to the cultures. Lighting could also be electrically powered, using for instance solar, wind or geothermal energy sources. Various studies have been performed regarding electrical lighting for plants in BLSS (easily adaptable to cyanobacterial culture), light-emitting diode (LED)-based systems being the most promising (Massa et al., 2007). However, even though electrical lighting would allow an accurate control on light intensity and photoperiods, energy and mass requirements could be greatly reduced—compared to electrical lighting—by using systems based on fiber-optics technology to harvest and transmit selected wavelengths from solar energy. Several of such systems have been developed in the context of space exploration, among others for the cultivation of microbial phototrophs (Mori et al., 1987) and plants (Jack et al., 2002; Nakamura et al., 2009). Equivalent system mass calculations showed a net benefit of using solar lighting rather than electric lighting in this context, under realistic mission assumptions (Drysdale et al., 2008). The cost effectiveness of such systems may be limited by temporary decreases in light availability (due to diurnal and seasonal light cycles, variable distance from the Sun and global dust storms), which drives a need for increasing collector size relative to culture areas, and may consequently be inadequate for growing plants in Mars BLSS (Massa et al., 2007). They could however represent a solution for growing cyanobacteria, which use light more efficiently and suffer less than plants from

temporary reduction of light and from changes in illumination patterns. That being said, the simplest and most cost-effective way of providing PAR could be to directly expose cultures to solar light while protecting them from lethal levels of radiation. A transparent but UV-blocking and thermal insulating material, which should also be resistant to Mars surface conditions in the long term, could be used. Radiation coming from straight above could also be stopped by regolith-based materials, while allowing light to come from the sides at adequate intensities for photosynthesis to occur (de Vera et al. 2014).

Heating will be needed to maintain liquid water and allow metabolism. Heating systems could be either directly based on solar energy, or on electricity generated by solar energy (e.g., using solar panels) and/or based on other power sources used within Martian outposts (e.g., wind, geothermal activity or fuels produced on site). This issue is largely documented elsewhere, as it applies to other components of human colonies such as habitats, and will not be detailed here.

Gravity

Would Mars's lower-than-Earth gravity affect cyanobacteria? The earliest microbiology experiments in space, reported in the early 1960s, did not show any effect of microgravity on individual cells (Zhukov-Verezhnikov et al., 1962). Consistently, theoretical studies suggested that microgravity would not directly affect cells of a diameter below 10 μm , in part due to the density uniformity and smallness of intracellular components (Pollard, 1965, 1967). Later, experiments on-board the orbital station Salyut 6 and biosatellites Kosmos 1887 and 2044 showed that unicellular algae were not affected in their development by microgravity (Sychev et al., 2001). However, a wide range of altered behavior and growth properties such as increased virulence, reduced lag phase, increased final cell population, increased productivity of secondary metabolites and increased conjugation rates have been reported in later microbiology experiments in spaceflight and simulated microgravity (see, e.g., Nickerson et al. 2000, Benoit & Klaus 2007 and Wilson et al. 2007), in various prokaryotes including cyanobacteria (Wang et al., 2006, 2004; Xiao et al., 2010). A well-supported hypothesis suggests that these effects are motility-dependent, with non-motile cells being the most affected. This might be explained by the reduced flow of metabolites and nutrients and from the reduced exchanges between bacteria and the environment (e.g., because mass-

driven convection does not occur), which result in a modified chemical environment around cells that alters biological responses (Horneck et al., 2010). However, microgravity was shown not to reduce N fixation abilities, photosynthetic O₂ production rates or growth in cyanobacteria (*e.g.*, Wang et al. 2006). Mars's reduced gravity (0.38 g) is consequently not expected to be an obstacle to cyanobacterium-based processes, especially if stirring generates a non-limiting flux of nutrients and metabolites. Studies aimed at confirming that reduced gravity do not alter cell processes of interest would however be useful.

Potential inputs from synthetic biology

If CyBLiSS allows to grow other microorganisms from resources available on site and thereby to create production processes which are almost independent of Earth, those processes may be made more efficient by using synthetic biology. This recent discipline could, indeed, increase the fitness of, and confer new functions to, the organisms in extraterrestrial outposts. Given its potential for enabling space exploration, synthetic biology has aroused NASA's interest (Cumbers and Rothschild, 2010; Langhoff et al., 2011; Menezes et al., 2014).

Synthetic biology for improving CyBLiSS-based system could serve three main purposes: (i) increasing microorganisms' resistance to conditions found beyond Earth, (ii) increasing their ability to resources available on site, and (iii) conferring them with new abilities (*e.g.*, the ability to produce useful compounds they don't produce naturally) (Figure 5.2).

How this could be done, and why it would be beneficial for long-term space missions, is described in more details in Verseux et al. (2016b). In short, engineered microorganisms could perform the functions they are selected for faster and using fewer resources. The system could also be simplified by reducing the number of organisms performing a given set of functions, by increasing organisms' versatility. A smaller set of compartments could thus be used, thereby reducing the variety of resource requirements and the set of

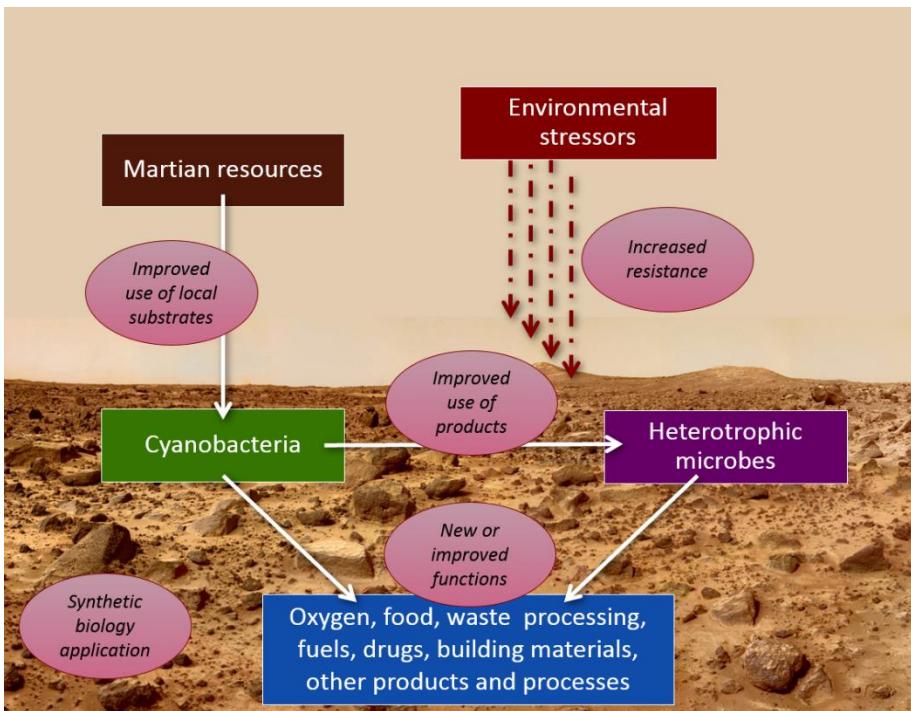


Figure 5.2: Simplified overview of the potential roles of synthetic biology in the development of Mars-specific, cyanobacterium-based BLSS. Reproduced from Verseux et al. (2016a).

possible failure causes. A few examples of potential uses of engineered organisms are given below.

Even though some edible cyanobacterial species such as *Arthrospira* spp. have excellent nutraceutical properties (e.g., Henrikson, 2009), they can currently not—as mentioned above—be used as a staple food due to their unpleasant and unvaried taste, lack of vitamin C and possibly essential oils, and low carbohydrate/protein ratios. These limitations could be addressed using synthetic biology (Ducat et al., 2011b). First, taste, smell and color molecules have already been, or could be, expressed in bacteria. Then, modifying the sugar, protein and lipid ratios, as well as introducing essential molecules (e.g., vitamin C) could be achieved using metabolic engineering and, more generally, nutraceutical properties could be improved by genetic

engineering. Preliminary work has been done in this direction; for instance, mutant strains of *A. platensis* have been selected that contained higher contents than the wild-type in essential amino acids, phycobiliproteins and carotenoids, among other nutrients (Brown, 2008b). Cyanobacteria could also be used for food complementation without being directly eaten: they can be engineered to secrete nutritional compounds, so used culture media could be harvested without lysing cells and added to food (Ducat et al., 2011b). Besides, as mentioned above, the possibility of engineering cyanobacteria to produce and secrete sugars has already been demonstrated. The use of plants could thus be restrained to applications where no large amounts are needed and where they could be grown within habitats (thus relieving the need for large-scale areas under highly controlled parameters): ornament and horticulture—which have beneficial psychological impact on crewmembers (Allen, 1991)—and occasional provision of comfort food.

Another vital resource of human bases will be energy. Solar, wind and nuclear energy are potential sources of on-site (or durable) energy, and these could be complemented by biofuels produced on Mars for, for instance, powering vehicles. Microorganisms are well-studied as biofuel producers. Some species studied for their abilities to generate biofuel precursors are heterotrophic and could have organic substrates generated by phototrophic microorganisms: for instance, yeasts are efficient ethanol producers and, even though usually relying on plant agricultural products as a carbon feedstock, cyanobacterial biomass (Aikawa et al., 2013; Möllers et al., 2014) or sugars secreted by cyanobacteria could be used as a substrate generated on-site. However, cyanobacteria could also be used to directly convert solar energy into biofuels, without relying on organic precursors: they can produce various energetic compounds such as alkanes and lipids (see for instance Quintana et al. 2011) and dihydrogen (Raksajit et al., 2012), which can in turn be used for reducing locally available CO₂ to hydrocarbons to produce fuel (Hepp et al., 1993). For both heterotrophic and autotrophic microorganisms, microbial engineering using synthetic biology tools and methods for producing energetic biofuel precursors which are not naturally produced by the hosts, as well as for increasing yields, is a very active field boosting an increasing number of achievements (e.g., Ducat et al., 2011; Hallenbeck, 2012; Peralta-Yahya et al., 2012; Radakovits et al., 2010; Zhang et al., 2011)

Engineered microorganisms could also be used for producing a pipeline of drugs on-site. In addition to the antibiotics, therapeutic peptides, antioxidants and other nutraceutical compounds that they naturally produce, microorganisms can be engineered to contain new metabolic pathways leading

to the production of various drugs. The most famous example is the production in yeast and bacteria of a direct precursor of artemisinin, an antimalarial drug (Martin et al., 2003; Ro et al., 2006), but much more is to come (see, e.g., Folcher and Fussenegger, 2012; Ruder et al., 2011).

Then, once all basic physiological requirements are covered (through BLSS processes, and drug and energy production), engineered microorganisms can be used for sustaining industrial processes. Metals could be extracted from Martian rocks by bioleaching, ways of engineering microorganisms to increase their abilities to extract (and possibly sort) element of interest on Mars has been suggested (Cockell, 2010, 2011). Engineered microorganisms could also be used for producing or improving building materials such as bioplastics (Hempel et al., 2011; Osanai et al., 2014) and concrete-like materials (Jonkers et al., 2010; De Muynck et al., 2010).

Other applications of synthetic biology can be considered in human extraterrestrial outposts. Some are similar to what is expected on Earth (see, e.g., Church et al. 2014, and Khalil and Collins 2010, for examples of potential future applications), but many biotechnologies will probably be specific to space exploration. Indeed, some processes will benefit from synthetic biology there whereas they can be more economically performed by other means on Earth, for example by chemical methods, by using the natural host or by simply harvesting rather than producing the targeted compounds. Besides, metabolic pathways might differ: some substrates that are cheap and abundant on Earth will be extremely hard to provide on Mars. The key point here is that once organic substrates can be obtained from on-site resources (e.g., using cyanobacteria) and that microorganisms can be grown on Mars at low costs (e.g., due to genetically increased resistance), metabolic engineering opens the way to a wide range of potential applications.

In summary, naturally evolved cyanobacteria or those engineered for increased resistance and abilities to use in situ resources could be used for processing on-site materials and turning them into forms available to other microorganisms. From this basis, synthetic biology could open the way to a wide range of applications including the production of vital resources, energy, drugs, building materials, industrial reagents and comfort goods—all starting from resources found on site. Other potential applications of synthetic biology for space exploration (including modifications to non-microorganisms) are discussed elsewhere (Cumbers and Rothschild, 2010; Langhoff et al., 2011; Menezes et al., 2014; Montague et al., 2012). Combined with physicochemical technologies (both completing each other and providing a safe redundancy for

vital processes), synthetic biology can thus lead to the development of complex, Earth-independent human bases on Mars and beyond.

Aim of Part II and chapter's description

The peculiar extremophilic and metabolic properties of cyanobacteria make some species highly promising candidates for key organisms in future biological life support systems, in support of foreseen crewed missions on Mars. A theoretical evaluation is described above but, for more details, the reader is referred to Verseux et al. (2016a, 2016b) and Rothschild (2016). Encouraging laboratory results were obtained and are described in Chapter 6.

Chapter 6

Cyanobacterium biomass as a substrate for heterotrophic growth on Mars⁵

Due to specific metabolic features, cyanobacteria could in theory be grown on Mars from local resources, with relatively low hardware requirements. Here we performed a quantitative assessment of the potential use of nutrients extracted from cyanobacterium biomass as a substrate for heterotrophic microorganisms of biotechnological interest, demonstrating its efficiency. Chemical analyses revealed the abundant nutrients available in this cyanobacterium-derived medium. We also assessed the potential problem represented by perchlorates in the Martian regolith and showed that those toxic salts are no game-changer. Overall, our results support the hypothesis that cyanobacteria could be used for processing local materials into a form which is available to other modules of biological life support systems, opening the way to Earth-independent resource production processes on Mars.

Introduction

Sending human to Mars in the coming decades is the stated goal of NASA (NASA, 2017) and some private industries (e.g., Musk, 2017).

While the first missions will likely be short-term (circa 3 years), a permanently or semi-permanently inhabited outpost, akin to polar stations, will be desirable to perform extensive research activity on site. Unfortunately, sustaining a permanent outpost on Mars by providing all life-support consumables from Earth seems unrealistic given launch costs, travel times and risks of failure. If humans are to spend considerable amounts of time on Mars, they may need to learn how to live “off the land” there.

A solution could be to rely on biological systems, as we often do on Earth. Microorganisms, for instance, could be extremely useful in this context;

⁵This chapter is in preparation for publication, in collaboration with Lynn Rothschild, Ivan G. Paulino-Lima, Salvatore Chiavarini, Eugenio Benvenuto, and Daniela Billi.

potential applications include the production of drugs, food, oxygen, biomaterials and various industrially useful chemicals, metal leaching, and food processing for taste improvement (see for instance Rothschild, 2016; Verseux et al., 2016a, 2016b).

Various systems relying on biology for producing and recycling resources of interest, referred to as bioregenerative life-support systems (BLSS), have consequently been proposed for spaceflight and planetary outposts (see for instance Godia et al., 2002; Lobascio et al., 2007; Nelson et al., 2010). However, if such systems rely exclusively on materials imported from Earth, their running time is limited and the amounts of elements present in the system can only decrease over time. For BLSS to be independent from Earth over extended periods of time, it is thus critical that BLSS are fed with materials available on Mars.

Mars contains water in different forms, solar energy, and likely all atoms needed to sustain life (e.g., Cockell and Raven, 2004; Meyer and McKay, 1989; Verseux et al., 2016a). Unfortunately, heterotrophic microorganisms rely on organic materials which are not expected to be abundant there (e.g., Ming et al., 2014). Similarly, no abundant form of fixed nitrogen has been identified (Mancinelli and Banin, 2003), and mineral nutrients may not be accessible to non-leaching microorganisms.

We previously hypothesized that specific cyanobacteria could be grown on Mars from the local mineral, atmospheric, and water resources due to their peculiar metabolic abilities. We proposed that they could then be used for feeding other organisms, thereby connecting biological life support systems to local resources (Chapter 5; Rothschild, 2016; Verseux et al., 2016a). In the work presented here, we show that soluble molecules extracted from cyanobacterium biomass can be a very efficient source of nutrients for two strains each of two heterotrophic species, *Escherichia coli* and *Bacillus subtilis*. We characterized those soluble nutrients to identify chemical species that could be metabolized by heterotrophic microorganisms. Finally, we assessed the challenge posed by perchlorates in the Martian ground. Our results strongly suggest the viability of cyanobacterium-based BLSS for future outposts on Mars.

Materials and methods

Bacterial cultures

Anabaena sp. PCC7120 was purchased from the Pasteur Culture Collection of Cyanobacteria (Paris, France) and grown under routine conditions at 25°C, in BG11 medium (unless specified otherwise), under a photon flux density of 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by fluorescent cool-white bulbs with a 16h/8h light/dark cycle.

Escherichia coli W (ATCC 9637) and *E. coli* K-12 MG1655 (700926) were purchased from the American Type Culture Collection (Manassas, VA). *Bacillus subtilis* 168 (BGSC 1A1) and *B. subtilis* SCK6 (BGSC 1A976) were purchased from the Bacillus Genetic Stock Center (Columbus, OH).

Prior to the experiment, *E. coli* and *B. subtilis* strains were grown in LB medium or on LB agar at 37°C, following standard procedures.

Lithotrophic growth of *Anabaena* sp. PCC 7120 with Mars regolith simulant

Samples of *Anabaena* sp. PCC7120 in exponential phase were washed twice and resuspended in Milli-Q water. One-milliliter samples were set aside for determination of chlorophyll *a* (Chl *a*) concentrations (see below) and 6-ml samples were added to 24 ml of Milli-Q water containing 0, 1 g, 2 g, or 5 g of regolith simulant (JSC Mars-1A, grain size < 1 mm; Orbitec, Madison, WI), in triplicates. Samples were then incubated under routine conditions.

After 6 weeks (or the day of inoculation, for determining initial concentrations), concentrations of Chl *a* were determined as follows. One-milliliter samples were centrifuged, supernatants were discarded and 1 ml of 90 % acetone in water was added to pellets. Samples were incubated overnight at room temperature, in the dark. Optical density measurements were performed and the concentrations (C) of Chl *a* were calculated as described in Jeffrey & Humphrey (1975): $C (\mu\text{g}\cdot\text{l}^{-1}) = 11.85 \text{ OD}_{664} - 1.54 \text{ OD}_{647} - 0.08 \text{ OD}_{630}$.

Cyanobacterium biomass as a nutrient source for heterotrophic bacteria

Cultures of *Anabaena* sp. PCC7120 grown in BG11 until early stationary phase were washed 3 times in Milli-Q water and pellets were dried at 65°C for

48h. Dry biomass was weighed, ground in liquid nitrogen, resuspended in Milli-Q water and submitted to several cycles of freezing (in liquid nitrogen) and thawing (at 60°C). Milli-Q water was added to reach a biomass concentration of 25 g·l⁻¹. Samples were incubated for 2h at room temperature, then stored at -20°C until the day of the experiment. Samples were then thawed and filtered using 0.22 µm filters (Millex-GS; MilliporeSigma, Darmstadt, Germany). The resulting filtrate is hereafter referred to as cyanobacterium-based medium.

Single colonies of *E. coli* MG1655, *E. coli* W, *B. subtilis* 168 and *B. subtilis* SCK6 were used to inoculate 3 ml of LB medium (10 g peptone, 5 g yeast extract, 10 g NaCl), then incubated overnight at 37°C under agitation. Cultures were then washed 3 times and resuspended in 3 ml of PBS. 20 µl were used to inoculate 1 ml of either PBS, Milli-Q water, LB medium, or cyanobacterium-based medium (each in triplicate for each strain).

Dilutions of droplets from inoculated PBS samples were spread on LB-agar to assess the initial concentration of *E. coli* cells. The other samples were incubated overnight at 37°C, under agitation. Samples were then serially diluted and spread on LB-agar plates and, after overnight incubation at 37°C, colonies were counted.

Additional assays were performed with *E. coli* W in various dilutions of the cyanobacterium-based medium.

Characterization of the cyanobacterium medium

Carbohydrate contents of the cyanobacterium-based medium were characterized by the phenol-sulfuric acid method (DuBois et al., 1956), using D-glucose as a standard. Nucleic acid concentrations were determined using either the Qubit dsDNA HS Assay Kit (for DNA) or the Qubit RNA HS Assay kit (for RNA), and a Qubit 2.0 Fluorometer (Invitrogen, Waltham, MA). Other elements were quantified by mass spectrometry at the Technical Unit for Environmental Characterization, Prevention and Recovery of ENEA's Casaccia Research Center.

Perchlorate growth assays

A perchlorate solution was prepared that contained 0.87 g·l⁻¹ calcium perchlorate (Sigma Aldrich) and 0.54 g·l⁻¹ magnesium perchlorate (Sigma-Aldrich, Saint-Louis, MO) in BG11. This composition was chosen so as to approximate the perchlorate contents of a solution prepared using 200 g·l⁻¹ of

Martian regolith (based on work by Olsson-Francis and Cockell [2010]) containing 0.6% wt perchlorate, as detected at the Phoenix landing site (Fang et al., 2015), 60% of which coming from calcium perchlorate and 40% from magnesium perchlorate (Kounaves et al., 2014).

Anabaena sp. PCC7120 was grown in perchlorate solution and in BG11, under routine conditions, and growth was monitored by taking OD₇₃₀ measurements twice a week.

Biomass was harvested and used for growing *E. coli* W, as described in subsection “Cyanobacterium biomass as a nutrient source for heterotrophic bacteria” above.

Results

Lithotrophic growth of *Anabaena* sp. PCC 7120 with Mars regolith simulant

Anabaena sp. PCC7210 grew in distilled water containing Orbitec's Mars-1A regolith simulant only (Figure 6.1). Chl *a* concentrations (and therefore

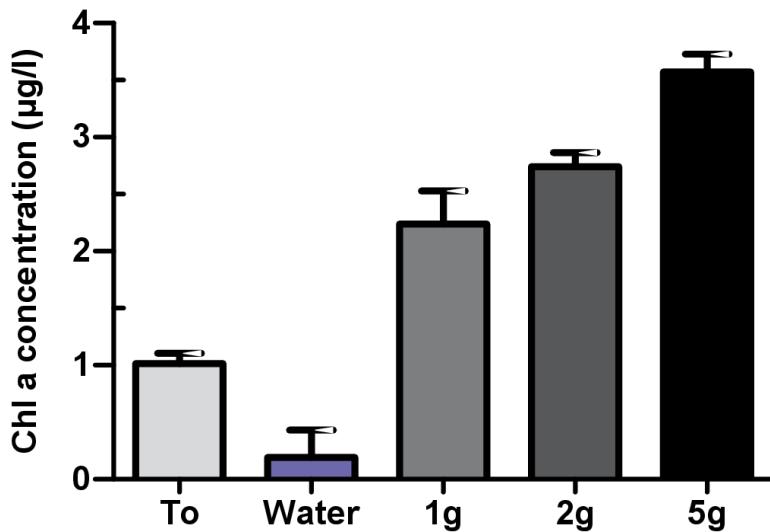


Figure 6.1. Growth of *Anabaena* sp. PCC7120 in Mars regolith simulant. Chl *a* concentrations at inoculation time (To) and after 6 weeks in bidistilled water with no regolith simulant (H_2O) or with Orbitec's Mars-1A regolith simulant (1, 2 or 5 g \cdot 30 ml $^{-1}$).

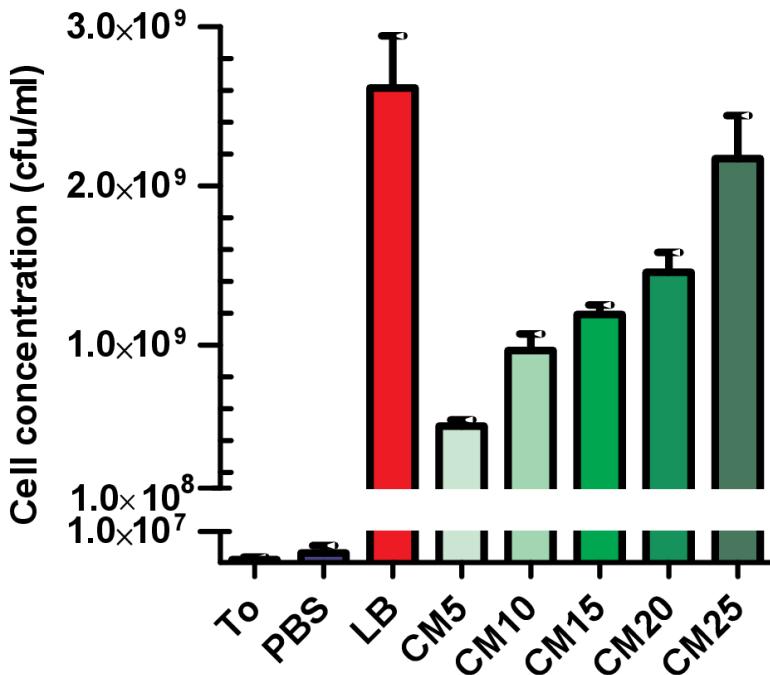


Figure 6.2. Growth of *Escherichia coli* W in PBS or cyanobacterium-based medium (see text for details) at concentrations before filtration ranging from 5 g·l⁻¹ (CM5) to 25 g·l⁻¹ (CM25).

cell concentrations) after 6 weeks increased with soil concentrations in the tested range, from 1 g·30 ml⁻¹ (cell populations doubled) to 5 g·30 ml⁻¹ (cell populations increased by a factor of about 3.5).

***Escherichia coli* and *Bacillus subtilis* can efficiently use cyanobacterium-based medium as a substrate**

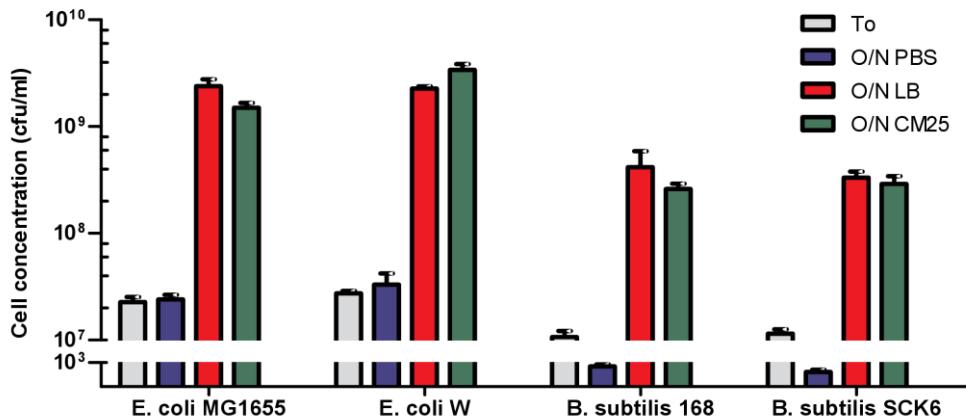


Figure 6.3. Growth of *E. coli* MG1655, *E. coli* W, *B. subtilis* 168, and *B. subtilis* SCK6 in PBS, LB broth or cyanobacterium-based medium at 25 g^{-1} before filtration (CM25).

A filtrate of ground *Anabaena* sp. PCC7120 used as the only source of nutrients (besides ambient atmosphere) supported efficient growth of two strains of *Escherichia coli* and two strains of *Bacillus subtilis* in Milli-Q water.

First, tests performed with *E. coli* W showed that concentration after overnight incubation increased with the concentration of cyanobacterium lysate over the tested range (5 to 25 g of dried biomass $\cdot\text{l}^{-1}$ before filtration) (Figure 6.2).

Then, a filtrate corresponding to 25 g of dried cyanobacterium biomass $\cdot\text{l}^{-1}$ before filtration (CM25) supported the growth of *E. coli* W up to more than $3 \cdot 10^9$ cells ml^{-1} , and *E. coli* K-12 MG1655 up to close to $1.5 \cdot 10^9$ cells $\cdot\text{ml}^{-1}$. Similarly, it supported the growth of *Bacillus subtilis* 168 and *B. subtilis* SCK6 up to a final concentration of more than $2.5 \cdot 10^8$ cells ml^{-1} . For all strains, the final cell density in CM25 is of the same order of magnitude as that obtained in LB medium (Figure 6.3).

Characterization of the cyanobacterium-based medium

Data on the composition of CM25 is given in Table 6.1.

Table 6.2. Composition of the cyanobacterium-based medium. Values are given for a medium prepared with $25 \text{ g} \cdot \text{l}^{-1}$ of biomass before filtration. When not specified otherwise, data was obtained by mass spectrometry.

Compounds		Concentration ($\text{mg} \cdot \text{l}^{-1}$)
Ammonium		13.3 ± 0.8
Phosphate		385 ± 45.0
Metal nutrients	Na	21.25 ± 3.8
	K	270 ± 15.3
	Mg	94.5 ± 18.3
	Ca	43.0 ± 8.3
	Fe	1.0 ± 0.3
	Mn	1.9 ± 0.6
	Zn	0.138 ± 0.011
	Cr	< 0.1
	Ni	0.017 ± 0.063
Free amino acids	Alanine	35.4 ± 16.6
	Glycine	28.3 ± 26.3
	Valine	15.9 ± 3.5
	Leucine	14.1 ± 4.6
	Isoleucine	7.0 ± 2.0
	Proline	2.2 ± 0.4
	Serine	1.3 ± 0.7
	Threonine	4.7 ± 1.8
	Methionine	0.3 ± 0.4
	Aspartic acid	0.3 ± 0.4
	Phenylalanine	4.5 ± 0.6
	Glutamic acid	28.7 ± 3.9
	Lysine	3.1 ± 3.2
	Tyrosine	1.2 ± 0.2
	Cysteine	0.1 ± 0.1

Nucleic acids ^a	DNA	9.0 ± 0.2
	RNA	66.3 ± 2.1
Carbohydrates ^{b,c}	1.10 x 10 ³ ± 45.09	
Monosaccharides	Xylose	0.6 ± 1.1
	Arabinose	0.3 ± 0.1
	Ribose	1.1 ± 0.9
	1,6-anhydro-glucose	0.7 ± 0.7
	Fructose	7.9 ± 1.8
	Glucose	9.5 ± 2.8
	Galactose	37.0 ± 1.8
Disaccharides	Sucrose	6.4 ± 1.9
	Lactose	1.6 ± 1.4
	Maltose	11.3 ± 6.7
	Trehalose	0.4 ± 0.2
Glycerylglycosides and hexoses-6-phosphate ^b	192 ± 46.2	
Putative trisaccharide ^d	174 ± 37.6	

^a Quantified using a Qubit 2.0 fluorometer and the associated assay kits.

^b Quantified using glucose as a standard.

^c Quantified using the phenol-sulfuric acid method.

^d Not identified. Present also in other cyanobacterium species (Billi, Salvatore et al., unpublished).

Perchlorate growth assays

Growth of *Anabaena* sp. PCC7120 was affected by the presence of ions from perchlorate salts expected if using regolith at 200 g·l⁻¹ as a source of nutrients. However, growth remained efficient, with cultures reaching an optical density at 730 nm above 4 (against 7 for controls) after three weeks (Figure 6.4A). Growth of *E. coli* W in nutrient solution prepared from cyanobacteria was not negatively affected by the fact that the latter was grown in presence of perchlorates (Figure 6.4B).

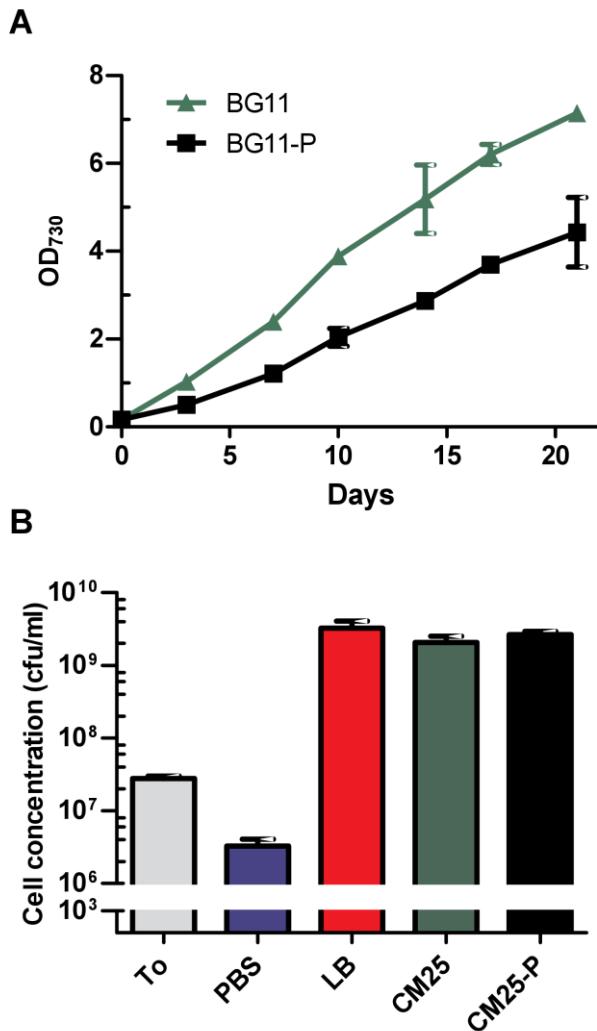


Figure 6.4. Effect of perchlorate solution on the growth of *Anabaena* sp. PCC7120 (A) and its lack of negative effect if subsequent used as a substrate for growing *E. coli* W (B). CM25: cyanobacterium-based medium at 25 g·l⁻¹ before filtration; CM25-P: cyanobacterium-based medium at 25 g·l⁻¹ before filtration (CM25) prepared from cyanobacteria grown in perchlorate solution.

Discussion

Some cyanobacterium strains could a priori be grown on Mars using local resources as substrates, with relatively low hardware requirements (Verseux et al., 2016a). Briefly, their rock-leaching abilities could allow them to obtain mineral nutrients from Martian regolith (Olsson-Francis and Cockell, 2010b), and their nitrogen-fixing and photosynthetic abilities, coupled to their abilities to thrive under low pressures of an atmosphere composed exclusively of nitrogen and carbon dioxide (Murugesan et al., 2015) would allow to vent in the ambient atmosphere after light pressurization and change in the CO₂-to-N₂ ratio. Finally, their resistance to ionizing radiation would prevent the need for strong radiation protection (e.g., Verseux et al. 2017): a simple cover filtering out UV and letting in photosynthetically active radiation would suffice. Alternatively, fiber optics and/or electric lighting could be used (see, e.g., Massa et al., 2007; Nakamura et al., 2009). Water is also widely available on Mars, under different forms, and could be harvested from various sources such as ground ice or atmospheric vapor (see, e.g., Rapp, 2007).

Cyanobacteria grown on Mars have been proposed as a basis for biological production processes on site which are almost independent of Earth (Chapter 5; Rothschild, 2016; Verseux et al., 2016a), by providing nutrients to other organisms. Here, we tested whether soluble compounds from lysed cyanobacterium biomass could be used as a nutrient source by heterotrophic microorganisms.

To test this approach, *Anabaena* sp. PCC7120 was grown, dried, lysed, and resuspended in Milli-Q water at concentrations ranging from 5 g·l⁻¹ to 25 g·l⁻¹. It was then filtered, and the filtrate was used to grow *Escherichia coli* W. *E. coli* cell counts after overnight incubation increased with increasing concentration of cyanobacterium lysate and, in the solution corresponding to 25 g·l⁻¹ before filtration (CM25), reached cell concentrations similar to those obtained with LB medium. Subsequently, *Escherichia coli* K-12 MG1655, *E. coli* W, *Bacillus subtilis* 168 and *B. subtilis* SCK6 were grown in CM25, where they reached cell concentrations in the same order of magnitude as those obtained with LB medium.

Analysis of CM25 revealed some of the available nutrients. Carbohydrates amount to approximately 1 g·l⁻¹ (with roughly 0.4 g·l⁻¹ of mono-, di- and trisaccharides, and hexose derivatives). If sugars were the only source of carbon, this concentration would not be satisfactory. It is, for instance, about a fourth of what is used in M9 minimal medium (assuming all carbohydrates

in CM25 can be metabolized by the grown heterotrophs). However, other molecules can be used efficiently as carbon sources. In LB medium, sugars actually represent a small fraction of the usable carbon, and amino acids have been suggested to be the main carbon sources (Sezonov et al., 2007). In our lysate, free amino acids account for about $0.2 \text{ g}\cdot\text{l}^{-1}$; peptides were not quantified but likely represent a much higher mass concentration. Similarly, the nitrogen sources are multiple. Heterotrophic bacteria can obtain nitrogen from various molecules including ammonium (Reitzer, 2003), amino acids and peptide chains (Coffin, 1989; Crawford et al., 1974), and nucleic acids (Paul et al., 1989). Although not all needed metal micronutrients were quantified, none is expected to be limiting. As an example, *E. coli* needs about $10^8 \text{ atoms}\cdot\text{cell}^{-1}$ of K and Mg, and $10^5 \text{ atoms}\cdot\text{cell}^{-1}$ of Ca, Zn and Fe (Finney and O'Halloran, 2003). The concentrations of the two metals most needed by bacteria, K and Mg, are approximately 7 mM and 4 mM in the lysate, while *E. coli* needs about 0.3 μM each to reach $2 \times 10^9 \text{ cells}\cdot\text{ml}^{-1}$. The elements quantified here thus suggest that the medium is rich in nutrients usable by heterotrophic bacteria. This is consistent with the densities reached overnight by all the four tested strains of heterotrophs.

Assuming growth rates and final biomass yields as in Olsson-Francis & Cockell (2010)'s experiments with a basaltic regolith simulant, growth rates would be roughly 0.3 day^{-1} and biomass at saturation $100 \text{ mg}\cdot\text{l}^{-1}$. Starting with a one-liter sample (or equivalent concentrated biomass), a 5-m³ culture could be obtained within 41 days, assuming cyanobacterium growth is the limiting factor. From a culture of this volume, approximately $3 \text{ l}\cdot\text{day}^{-1}$ of a very efficient growth medium for heterotrophic microorganisms could be produced.

Productivity could be further increased. First, the above estimations do not take into account the cyanobacterium growth-enhancing effects of a low-pressure/high-CO₂ atmosphere (see Murukesan et al. 2015) and of potential nutrients from recycled human waste products. Second, cyanobacterium productivity can be much increased by using a photobioreactor-like system optimizing medium agitation, light intensity, temperature and other factors. Then, simple treatments could modify the composition of cyanobacteria. An osmotic shock, for instance, increases sucrose contents in some species.

One may also consider using extracellular compounds. In this work, the broth in which cyanobacteria were grown was removed and cells were washed. Although this was necessary for a better characterization of the system, extracellular nutrients (e.g., substances secreted by living cells or released by dead cells) were eliminated. On Mars, besides molecules synthesized by

cyanobacteria, the extracellular medium may contain a wealth of metal nutrients if cyanobacteria are grown using regolith. Indeed, rock-dwelling cyanobacteria can help release metal nutrients from a wide range of rocks (see Olsson-Francis et al. 2012). *Anabaena cylindrica* has for instance been shown to release elements including K, Mg, Na, Ca, Fe, Mn, Ni and Zn from a Mars basalt analogue (Olsson-Francis and Cockell, 2010b).

If using extracellular compounds, one could even consider harvesting nutrients without destructing cyanobacterium cells, which may be beneficial to productivity. Substrates could for instance be secreted. This solution is being investigated in Lynn Rothschild's laboratory (at NASA Ames Research Center, Moffett Field, CA) where *Anabaena* sp. PCC7120 was engineered to secrete sucrose, which was then used as a carbon source to grow *Bacillus subtilis* (Rothschild, 2016). Prior to this, *Synechococcus elongatus* PCC7942 was engineered to produce and secrete either glucose and fructose, or lactate, then used as a substrate for *E. coli* (Niederholtmeyer et al., 2010). As for nitrogen: ammonium can be naturally released by some diazotrophic cyanobacteria, without cell lysis. For instance, extracellular ammonium can reach several mM in cultures of *Anabaena* spp. (mutants or wild-type, depending on species) relying on atmospheric N₂ as a sole nitrogen source (Spiller and Gunasekaran, 1990; Subramanian and Shanmugasundaram, 1986). What solution minimizes the cost-to-productivity ratio is still to be determined.

If using Martian regolith, the solution would a priori contain perchlorates, which have been quantified at several locations by surface instrumentation (Sutter et al., 2016). Those compounds, and their derivatives, are germicidal at temperatures relevant to cyanobacterium growth. In order to assess the extent of the issue, we performed growth assays where *Anabaena* sp. was grown in a solution containing the ions from perchlorate salts that would be present if using 200 g·l⁻¹ of regolith in water (Olsson-Francis and Cockell, 2010b), assuming concentrations of perchlorate ions of 0.6 % wt, similar to that found at the Phoenix landing site (Fang et al., 2015), coming at 60% from calcium perchlorate and 40% from magnesium perchlorate (Kounaves et al., 2014). Results showed that this environment affected growth, but did not prevent it entirely (optical density after three weeks was more than half that of controls). Besides, nutrients extracted from cyanobacteria grown with perchlorates were as much an efficient substrate for growing *E. coli* W as were nutrients from a control culture.

In summary, cyanobacteria could—thanks to their photosynthetic, rock leaching and nitrogen fixing abilities—be used for processing inorganic

compounds found on Mars into a form which is available to other microorganisms and possibly—although this is still to be demonstrated—to plants. Additional nutrients could come from the recycling of human waste. Finally, if some micronutrients (e.g., some metal ions) could not be mined or biologically synthesized on site, bringing them from Earth would only add negligible mass to the initial payload, as they are needed in trace amounts only.

Cyanobacteria could thus be a link between local resources and other organisms, and thereby a key element of Earth-independent biological life support systems on Mars.

SYNTHESIS AND CONCLUSIONS

This thesis mostly aimed at better understanding the effects of the space and Mars environments on cyanobacteria, using extremophile *Chroococcidiopsis* spp. as model organisms. Our main motivation was providing elements of answer to key astrobiology questions related to the search for life on Mars, and the possible exchange—natural or anthropogenic—of life between the blue and red planets. Besides those basic questions, applied aspects pertain to the potential use of cyanobacteria for biological life support systems beyond Earth.

In the first chapter, the background and motivations behind the work presented in Part I of this thesis were outlined. Environmental parameters found in space and on Mars, and of relevance to microorganisms, were described. It was explained that we currently don't know whether Mars ever harbored life but that efforts to find it out are extensive, with notably two rovers (ESA-Roscosmos's ExoMars and NASA's Mars 2020) foreseen in 2020. Research around the theory of lithopanspermia, according to which organisms might travel from one planet to another within rocks ejected by comet or asteroid impacts, was outlined. It was also proposed that some cyanobacteria—and noteworthy *Chroococcidiopsis* spp.—are relevant models to address such questions. An overview was given of the experiments, performed on Earth or in space, which demonstrate their resistance to some stress factors found beyond our atmosphere. The BOSS and BIOMEX experiments, part of the EXPOSE-R2 space mission and within which some *Chroococcidiopsis* samples were exposed to space and Mars-like conditions in low Earth orbit, were introduced. Then, the potential of cyanobacteria as a basis for long-term settlements beyond Earth was presented.

Before sending *Chroococcidiopsis* spp. to low Earth orbit as part of the EXPOSE-R2 space mission, ground tests were performed to validate the procedures foreseen for the flight. Some of the results obtained during those tests, and belonging to the BIOMEX project, were described in Chapter 2. We analyzed the endurance of cells and cellular components, as well as the modifications of their biosignatures, when mixed with mineral analogues of

Lunar or Martian regolith. We described how a very thin layer of minerals (for a total thickness, including 3–4 layers of cells and minerals, of about 15 µm) provided significant UV shielding and allowed for survival and biosignature detection under harsh conditions. We stated, for instance, that DNA was undetectable by PCR after exposure of *Chroococcidiopsis* sp. CCME 029 to 1.5×10^3 kJ·m⁻² of polychromatic UV or 10 kJ·m⁻² of UVC without minerals, but was still detectable after 8×10^5 kJ·m⁻² of polychromatic UV or 10 kJ·m⁻² of UVC if cells were mixed with an analogue of Lunar regolith. Moreover, we described how potential biomarkers, such as DNA, were still detectable after 570 MJ·m⁻² of polychromatic UV and Martian simulated atmosphere when mixed with analogues of Martian regolith, even though signals were attenuated by the treatment. Most importantly, the BIOMEX-related work described in Chapter 2—together with other results from Baqué et al. (2013a, 2013b, 2014, 2016)—suggested that the materials and methods foreseen for the EXPOSE-R2 space mission were appropriate.

A threat to potential, dormant microorganisms below Mars’s surface or undergoing lithopanspermia, and that could not be applied during the EXPOSE-R2 mission, is ionizing radiation over the long term. As part of the STARLIFE project, we consequently exposed hydrated and dried *Chroococcidiopsis* spp. cells to ionizing radiation with varying LET values (0.2 to 200 keV/µm), up to doses much higher than what could be received during past or current space missions. Some results of those experiments were presented, as well, in Chapter 2 (more data can be found in Verseux et al., 2017). We found that irradiation with up to 1 kGy of He or Si ions, 2 kGy of Fe ions, 5 kGy of X-rays, or 11.59 kGy of γ rays, did not eradicate *Chroococcidiopsis* populations or induce detectable damage to their DNA or plasma membranes. In other words, and although other factors (e.g., nature of the radiation flux and direct environment of the cell) should be taken into account, *Chroococcidiopsis* spp. can survive doses of ionizing radiation that it would receive in thousands of years on the surface of Mars, or on a rock in space ejected by a comet or asteroid impact.

Following the successful ground-based tests, the EXPOSE-R2 hardware, containing (among others) our *Chroococcidiopsis* samples, was launched to the ISS on July 23rd 2014, on-board the Progress 56 cargo spacecraft (Figure S1). Samples were exposed outside the International Space Station for close to 18 months. They underwent extreme temperature cycles (-15.0°C to +48.5°C), ionizing radiation (circa 0.5 Gy), vacuum for 22 months or a Mars-like atmosphere for close to 2 years and, for some samples, high doses of UV

(circa 500 kJ $\text{kJ}\cdot\text{m}^{-2}$, depending on location within the hardware). Besides, all samples remained in a dry state for 2.5 years before analysis. Upon landing, we analyzed cells' survival and biomolecules' preservation. Results were presented in Chapters 5 (for the BOSS project) and 6 (for BIOMEX). They demonstrated, once again, the high resistance of *Chroococcidiopsis* spp. to space and Mars conditions: all three selected strains survived exposure to all space and Mars-like experimental conditions in low Earth orbit. The simulations performed on the ground, in parallel, proved harsher. However, all samples also survived them, except for some planktonic samples of CCME 029 exposed to Mars-like conditions on the ground (without minerals, or with S-MRS if UV were included). Besides, the investigated biomarkers (various pigments, DNA, and morphological features) were still detectable in all conditions. The results from BOSS (Chapter 3) also demonstrated a higher resistance of biofilms than planktonic cells to conditions found beyond Earth, in part due to the strong protection offered by the few upper cell layers. As biofilms are a more common form of microbial occurrence than planktonic cells in nature, this could lead to a positive re-evaluation of microbial resistance to Mars and space environments. Results from BIOMEX (Chapter 4) also point out the protective role of minerals: even a very thin layer of minerals (for a total thickness, including cells and minerals, not exceeding 30 μm) of Martian mineral analogues led to an overall much higher detectability of biomarkers, and allowed for survival in otherwise lethal conditions (namely, Mars simulations on the ground, even in the presence of UV in the case of the clay-rich P-MRS). Taken as a whole, our results from the EXPOSE-R2 space mission are highly encouraging regarding the possibility of finding the marks of life on Mars, with the 2020 rovers or in subsequent missions.

Another important part of the work conducted during those doctoral years aimed at moving forward the concept of using cyanobacteria as a basis for biological life-support systems based on resources available beyond Earth. In addition to theoretical development, summarized in Chapter 5, we performed the experimental work described in Chapter 6. This work mainly aimed at testing the hypothesis, central to the whole project, that nutrients extracted from cyanobacterium biomass could indeed serve as an efficient substrate for heterotrophic microorganisms. It appeared to be the case, and with an unanticipated efficiency: soluble nutrients extracted from 25 $\text{g}\cdot\text{l}^{-1}$ of cyanobacterium biomass yielded a substrate similar to LB medium in terms of heterotrophic growth, even without significant optimization. Chemical

analyses revealed the abundant nutrients available in this cyanobacterium-derived medium. We also assessed the potential problem represented by perchlorates in the Martian regolith and showed that, although sometimes mentioned as an obstacle, those toxic salts are no game-changer. Our results thus reinforce the idea that cyanobacteria could be used for processing local materials into a form which is available to other modules of biological life support systems, opening the way to Earth-independent resource production processes on Mars.



Figure S1. The Progress 56 spacecraft, carrying EXPOSE-R2, arriving at the International Space Station. Picture by Oleg Germanovich Artemyev (Roscosmos).

REFERENCES

- Agarwal, R., Rane, S.S., and Sainis, J.K. (2008). Effects of ^{60}Co γ radiation on thylakoid membrane functions in *Anacystis nidulans*. *J. Photochem. Photobiol.* *91*, 9–19.
- Aikawa, S., Joseph, A., Yamada, R., Izumi, Y., Yamagishi, T., Matsuda, F., Kawai, H., Chang, J.-S., Hasunuma, T., and Kondo, A. (2013). Direct conversion of Spirulina to ethanol without pretreatment or enzymatic hydrolysis processes. *Energy Environ. Sci.* *6*, 1844–1849.
- Allen, J.L. (1991). Biosphere 2: The Human Experiment (New York: Penguin Books).
- Allen, C.C., Morris, R.V., Lindstrom, D.J., Lindstrom, M.M., and Lockwood, J.P. (1997). JSC Mars-1: Martian regolith simulant. In *Lunar and Planetary Science XXVII*, LPI Contribution No. 1593, (Houston, Texas).
- Allen, C.S., Burnett, R., Charles, J., Cucinotta, F., Fullerton, R., Goodman, R., Griffith, A.D., Kosmo, J.J., Perchonok, M., Railsback, J., et al. (2003). Guidelines and capabilities for designing human missions. In *NASA/TM-2003-210785*.
- Alvarez, A.J., Khanna, M., Toranzos, G.A., and Stotzky, G. (1998). Amplification of DNA bound on clay minerals. *Mol. Ecol.* *7*, 775–778.
- Arai, M., Tomita-Yokotani, K., Sato, S., Hashimoto, H., Ohmori, M., and Yamashita, M. (2008). Growth of terrestrial cyanobacterium, *Nostoc* sp., on Martian Regolith Simulant and its vacuum tolerance. *Biol. Sci. Sp.* *22*, 8–17.
- De Angelis, S., De Sanctis, M.C., Ammannito, E., Carli, C., Di Iorio, T., and Altieri, F. (2014). The Ma_Miss instrument performance, I: Analysis of rocks powders by Martian VNIR spectrometer. *Planet. Space Sci.* *101*, 89–107.
- Armstrong, J., Wells, L., and Gonzalez, G. (2002). Rummaging through Earth's attic for remains of ancient life. *Icarus* *160*, 183–196.
- Arvidson, R., Squyres, S., and Bell, J. (2014). Ancient aqueous environments at Endeavour crater, Mars. *Science* *343*, 1–8.
- Asato, Y. (1971). Photorecovery of gamma irradiated cultures of blue-green alga, *Anacystis nidulans*. *Radiat. Bot.* *11*, 313–316.
- Averner, M., Moore, B., Bartholomew, I., and Wharton, R. (1984). Atmosphere behavior in gas-closed mouse-algal systems: an experimental and modelling study. *Adv. Sp. Res.* *4*, 231–239.
- Badri, H., Monsieurs, P., Coninx, I., Wattiez, R., and Leys, N. (2015). Molecular investigation of the radiation resistance of edible cyanobacterium *Arthrospira* sp. PCC 8005. *MicrobiologyOpen* *4*, 187–207.
- Bahl, J., Lau, M.C.Y., Smith, G.J.D., Vijaykrishna, D., Cary, S.C., Lacap, D.C., Lee, C.K., Papke, R.T., Warren-Rhodes, K.A., Wong, F.K.Y., et al. (2011). Ancient origins determine global biogeography of hot and cold desert cyanobacteria. *Nat. Commun.* *2*, 163.
- Banin, A. (1989). Mars soil - A sterile regolith or a medium for plant growth? In *The Case for Mars III*, C.L. Stoker, ed. (San Diego, CA: Univelt), pp. 559–571.
- Baqué, M., Viaggiu, E., Scalzi, G., and Billi, D. (2013a). Endurance of the endolithic desert cyanobacterium *Chroococcidiopsis* under UVC radiation. *Extremophiles* *17*, 161–169.
- Baqué, M., de Vera, J.-P., Rettberg, P., and Billi, D. (2013b). The BOSS and BIOMEX space experiments on the EXPOSE-R2 mission: Endurance of the desert cyanobacterium

- Chroococcidiopsis* under simulated space vacuum, Martian atmosphere, UVC radiation and temperature extremes. *Acta Astronaut.* *91*, 180–186.
- Baqué, M., Scalzi, G., Rabbow, E., Rettberg, P., and Billi, D. (2013c). Biofilm and planktonic lifestyles differently support the resistance of the desert cyanobacterium *Chroococcidiopsis* under space and Martian simulations. *Orig. Life Evol. Biosph.* *43*, 377–389.
- Baqué, M., Verseux, C., Rabbow, E., de Vera, J.-P.P., and Billi, D. (2014). Detection of macromolecules in desert cyanobacteria mixed with a lunar mineral analogue after space simulations. *Orig. Life Evol. Biosph.* *44*, 209–221.
- Baqué, M., Verseux, C., Böttger, U., Rabbow, E., de Vera, J.-P.P., and Billi, D. (2016). Preservation of biomarkers from cyanobacteria mixed with Mars-like regolith under simulated Martian atmosphere and UV Flux. *Orig. Life Evol. Biosph.* *46*, 289–310.
- Bauermeister, A., Moeller, R., Reitz, G., Sommer, S., and Rettberg, P. (2011). Effect of relative humidity on *Deinococcus radiodurans*' resistance to prolonged desiccation, heat, ionizing, germicidal, and environmentally relevant UV radiation. *Microb. Ecol.* *61*, 715–722.
- Baumstark-Khan, C., and Facius, R. (2001). Life under conditions of ionizing radiation. In *Astrobiology: The Quest for the Conditions of Life*, G. Horneck, and C. Baumstark-Khan, eds. (Springer, New York), pp. 260–283.
- Beliaev, A.S., Romine, M.F., Serres, M., Bernstein, H.C., Linggi, B.E., Markillie, L.M., Isern, N.G., Chrisler, W.B., Kucek, L.A., Hill, E.A., et al. (2014). Inference of interactions in cyanobacterial-heterotrophic co-cultures via transcriptome sequencing. *ISME J.* *8*, 2243–2255.
- Benardini, J.N., Sawyer, J., Venkateswaran, K., and Nicholson, W.L. (2003). Spore UV and acceleration resistance of endolithic *Bacillus pumilus* and *Bacillus subtilis* isolates obtained from Sonoran desert basalt: implications for lithopanspermia. *Astrobiology* *3*, 709–717.
- Benoit, M.R., and Klaus, D.M. (2007). Microgravity, bacteria, and the influence of motility. *Adv. Sp. Res.* *39*, 1225–1232.
- Bergman, B., Johansson, C., and Soderback, E. (1992). The *Nostoc-Gunnera* symbiosis. *New Phytol.* *122*, 379–400.
- Bibring, J.-P., Langevin, Y., Mustard, J.F., Poulet, F., Arvidson, R., Gendrin, A., Gondet, B., Mangold, N., Pinet, P., Forget, F., et al. (2006). Global mineralogical and aqueous Mars history derived from OMEGA/Mars Express data. *Science* *312*, 400–404.
- Billi, D. (2009). Subcellular integrities in *Chroococcidiopsis* sp. CCME 029 survivors after prolonged desiccation revealed by molecular probes and genome stability assays. *Extremophiles* *13*, 49–57.
- Billi, D., Grilli Caiola M., Paolozzi, L., and Ghelardini, P. (1998). A method for DNA extraction from the desert cyanobacterium *Chroococcidiopsis* and its application to identification of ftsZ. *Appl. Environ. Microbiol.* *64*, 4053–4056.
- Billi, D., Friedmann, E.I., Hofer, K.G., Caiola, M.G., and Ocampo-Friedmann, R. (2000). Ionizing-radiation resistance in the desiccation-tolerant cyanobacterium *Chroococcidiopsis*. *Appl. Environ. Microbiol.* *66*, 1489–1492.
- Billi, D., Viaggiu, E., Cockell, C.S., Rabbow, E., Horneck, G., and Onofri, S. (2011). Damage escape and repair in dried *Chroococcidiopsis* spp. from hot and cold deserts exposed to simulated space and Martian conditions. *Astrobiology* *11*, 65–73.
- Billi, D., Baqué, M., Smith, H.D., and McKay, C.P. (2013). Cyanobacteria from extreme deserts to space. *Adv. Microbiol.* *3*, 80–86.

- Billi, D., Baqué, M., Verseux, C., Rothschild, L., and Vera, J.-P. de (2017). Desert cyanobacteria: Potential for space and Earth applications. In *Adaption of Microbial Life to Environmental Extremes*, H. Stan-Lotter, and S. Fendrihan, eds. (Springer International Publishing), pp. 133–146.
- Blüm, V., Gitelson, J., Horneck, G., and Kreuzberg, K. (1994). Opportunities and constraints of closed man-made ecological systems on the Moon. *Adv. Sp. Res.* *14*, 271–280.
- Boillot, F., Chabin, A., Buré, C., Venet, M., Belsky, A., Bertrand-Urbanik, M., Delmas, A., Brack, A., and Barbier, B. (2002). The Perseus Exobiology mission on MIR: Behaviour of amino acids and peptides in Earth Orbit. *Orig. Life Evol. Biosph.* *32*, 359–385.
- Boles, B. R., Thoendel, M., and Singh, P. K. (2004). Self-generated diversity produces “insurance effects” in biofilm communities. *Proc. Natl Acad. Sci.* *101*, 16630–16635.
- Bonham, K., and Palumbo, R.F. (1951). Effects of x-rays on snails, crustacea, and algae. *Growth* *15*, 155–168.
- Bosak, T., Knoll, A.H., and Petroff, A.P. (2013). The meaning of stromatolites. *Annu. Rev. Earth Planet. Sci.* *41*, 21–44.
- Böttger, U., de Vera, J.-P., Fritz, J., Weber, I., Hübers, H.-W., and Schulze-Makuch, D. (2012a). Optimizing the detection of carotene in cyanobacteria in a Martian regolith analogue with a Raman spectrometer for the ExoMars mission. *Planet. Space Sci.* *60*, 356–362.
- Boynton, W. V., Taylor, G.J., Evans, L.G., Reedy, R.C., Starr, R., Janes, D.M., Kerry, K.E., Drake, D.M., Kim, K.J., Williams, R.M.S., et al. (2007). Concentration of H, Si, Cl, K, Fe, and Th in the low- and mid-latitude regions of Mars. *J. Geophys. Res. Planets* *112*, E12.
- Brown, I.I. (2008a). Cyanobacteria to link closed ecological systems and in-situ resources utilization processes. In 37th COSPAR Scientific Assembly (Montréal, Canada), p. 383.
- Brown, I.I. (2008b). Mutant strains of *Spirulina (Arthospira) platensis* to increase the efficiency of micro-ecological life support systems. In 37th COSPAR Scientific Assembly (Montréal, Canada), p. 384.
- Brown, I.I., and Sarkisova, S. (2008). Bio-weathering of Lunar and Martian rocks by cyanobacteria: A resource for Moon and Mars exploration. In *Lunar and Planetary Sciences XXXIX*, pp. 1–2.
- Brown, I.I., Garrison, D.H., Jones, J.A., Allen, C.C., Sanders, G., Sarkisova, S.A., and McKay, D.S. (2008). The development and perspectives of bio-ISRU. In Joint Annual Meeting of LEAG-ICEUM-SRR (Cape Canaveral, Florida), p. 4048.
- Bruce, A.K. (1964). Extraction of the radioresistant factor of *Micrococcus radiodurans*. *Radiat. Res.* *22*, 155–164.
- Bryce, C.C., Horneck, G., Rabbow, E., Edwards, H.G.M., and Cockell, C.S. (2014). Impact shocked rocks as protective habitats on an anoxic early Earth. *Int. J. Astrobiol.* *14*, 115–122.
- Caldwell, B.J., Roma, P.G., and Binsted, K. (2016). Team cohesion, performance, and biopsychosocial adaptation research at the Hawaii Space Exploration Analog and Simulation (HI-SEAS). In 31st Annual Conference of the Society for Industrial and Organizational Psychology.
- Cady, L.S., Farmer, J.D., Grotzinger, J.P., Schopf, J.W., and Steele, A. (2003). Morphological biosignatures and the search for life on Mars. *Astrobiology* *3*, 351–268.
- Cao, G., Concas, A., Corrias, G., Licheri, R., Orru’, R., and Pisu, M. (2014). Process for the production of useful materials for sustaining manned space missions on mars through in-

- situ resources utilization. U.S. Patent No. 8,978,293. Washington, DC: U.S. Patent and Trademark Office
- Caro, G.M.M., Mateo-Martí, E., and Martínez-Frías, J. (2006). Near-UV transmittance of basalt dust as an analog of the Martian regolith: implications for sensor calibration and astrobiology. *Sensors* *6*, 688–696.
- Carpenter, J.D., Fisackerly, R., De Rosa, D., and Houdou, B. (2012). Scientific preparations for lunar exploration with the European Lunar Lander. *Planet. Space Sci.* *74*, 208–223.
- Carr, C.E., Rowedder, H., Lui, C.S., Zlatkovsky, I., Papalias, C.W., Bolander, J., Myers, J.W., Bustillo, J., Rothberg, J.M., Zuber, M.T., et al. (2013). Radiation resistance of sequencing chips for in situ life detection. *Astrobiology* *13*, 560–569.
- Carter, J., Loizeau, D., Mangold, N., Poulet, F., and Bibring, J.-P. (2015). Widespread surface weathering on early Mars: A case for a warmer and wetter climate. *Icarus* *248*, 373–382.
- Church, G.M., Elowitz, M.B., Smolke, C.D., Voigt, C.A., and Weiss, R. (2014). Realizing the potential of synthetic biology. *Nat. Rev. Mol. Cell Biol.* *15*, 289–294.
- Christensen, P.R., Bandfield, J.L., Hamilton, V.E., Ruff, S.W., Kieffer, H.H., Titus, T.N., Malin, M.C., Morris, R. V., Lane, M.D., Clark, R.L., et al. (2001). Mars Global Surveyor Thermal Emission Spectrometer experiment: Investigation description and surface science results. *J. Geophys. Res.* *106*, 23823–23871.
- Clark, B.C., Baird, A.K., Weldon, R.J., Tsusaki, D.M., Schnabel, L., and Candelaria, M.P. (1982). Chemical composition of Martian fines. *J. Geophys. Res. Solid Earth* *87*, 10059–10067.
- Clifford, S.M., Lasue, J., Heggy, E., Boisson, J., McGovern, P., and Max, M.D. (2010). Depth of the Martian cryosphere: Revised estimates and implications for the existence and detection of subpermafrost groundwater. *J. Geophys. Res.* *115*, E07001.
- Ciaravella, A., Scappini, F., Franchi, M., Cecchi-Pestellini, C., Barbera, M., Candia, R., Gallori, E., and Micela, G. (2004). Role of clays in protecting adsorbed DNA against X-ray radiation. *Int. J. Astrobiol.* *3*, 31–35.
- Cockell, C.S. (2008). The interplanetary exchange of photosynthesis. *Orig. Life Evol. Biosph.* *38*, 87–104.
- Cockell, C.S. (2010). Geomicrobiology beyond Earth: Microbe-mineral interactions in space exploration and settlement. *Trends Microbiol.* *18*, 308–314.
- Cockell, C.S. (2011). Synthetic geomicrobiology: Engineering microbe–mineral interactions for space exploration and settlement. *Int. J. Astrobiol.* *10*, 315–324.
- Cockell, C.S. (2014). Trajectories of Martian habitability. *Astrobiology* *14*, 182–203.
- Cockell, C.S. (2016). The similarity of life across the universe. *Mol. Biol. Cell* *27*, 1553–1555.
- Cockell, C.S., and Raven, J.A. (2004). Zones of photosynthetic potential on Mars and the early Earth. *Icarus* *169*, 300–310.
- Cockell, C.S., Schuerger, A., and Billi, D. (2005). Effects of a simulated Martian UV flux on the cyanobacterium, *Chroococcidiopsis* sp. 029. *Astrobiology* *5*, 127–140.
- Cockell, C.S., Catling, D.C., Davis, W.L., Snook, K., Kepner, R.L., Lee, P., and McKay, C.P. (2000). The Ultraviolet environment of Mars: Biological implications past, present, and future. *Icarus* *146*, 343–359.
- Cockell, C.S., Brack, A., Wynn-Williams, D.D., Baglioni, P., Brandstätter, F., Demets, R., Edwards, H.G.M., Gronstal, A.L., Kurat, G., Lee, P., et al. (2007). Interplanetary transfer of photosynthesis: an experimental demonstration of a selective dispersal filter in planetary island biogeography. *Astrobiology* *7*, 1–9.

- Cockell, C.S., Rettberg, P., Rabbow, E., and Olsson-Francis, K. (2011). Exposure of phototrophs to 548 days in low Earth orbit: Microbial selection pressures in outer space and on early Earth. *ISME J.* *5*, 1671–1682.
- Coffin, R.B. (1989). Bacterial uptake of dissolved free and combined amino acids in estuarine waters. *Limnol. Oceanogr.* *34*, 531–542.
- Corning, P. A. (2002). The re-emergence of “emergence”: a venerable concept in search of a theory. *Complexity* *7*, 18–30.
- COSPAR (2011). COSPAR Planetary Protection Policy [20 October 2002, as amended to 24 March 2011].
- Cottin, H., Kotler, J.M., Bartik, K., Cleaves, H.J., Cockell, C.S., de Vera, J.P.P., Ehrenfreund, P., Leuko, S., Ten Kate, I.L., Martins, Z., et al. (2015). Astrobiology and the possibility of life on Earth and elsewhere... *Space Sci. Rev.* *209*, 1–42.
- Cottin, H., Kotler, J.M., Billi, D., Cockell, C., Demets, R., Ehrenfreund, P., Elsaesser, A., D’Hendecourt, L., van Loon, J.J.W.A., Martins, Z., et al. (2017). Space as a tool for astrobiology: Review and recommendations for experimentations in Earth orbit and beyond *Space Sci. Rev.* *209*, 83–181.
- Crawford, C.C., Hobbie, J.E., and Webb, K.L. (1974). The utilization of dissolved free amino acids by estuarine microorganisms. *Ecology* *55*, 551–563.
- Crawford, I.A., Anand, M., Cockell, C.S., Falcke, H., Green, D.A., Jaumann, R., and Wieczorek, M.A. (2012). Back to the Moon: The scientific rationale for resuming lunar surface exploration. *Planet. Space Sci.* *74*, 3–14.
- Cumbers, J., and Rothschild, L.J. (2010). BISRU: Synthetic microbes for Moon, Mars and Beyond. In Astrobiology Science Conference 2010, LPI Contribution No. 1538 (League City, Texas), p. 5672.
- Dachov, T., Horneck, G., Häder, D.-P., Schuster, M., Richter, P., Lebert, M., and Demets, R. (2012). Time profile of cosmic radiation exposure during the EXPOSE-E mission: The R3DE instrument. *Astrobiology* *12*, 403–411.
- Dachov, T.P., Horneck, G., Häder, D.-P., Schuster, M., and Lebert, M. (2014). EXPOSE-R cosmic radiation time profile. *Int. J. Astrobiol.* *14*, 17–25.
- Dachov, T.P., Tomov, B.T., Matviichuk, Y.N., Dimitrov, P.G., and Bankov, N.G. (2016). High dose rates obtained outside ISS in June 2015 during SEP event. *Life Sci. Sp. Res.* *9*, 84–92.
- Dachov, T.P., Bankov, N.G., Tomov, B.T., Matviichuk, Y.N., Dimitrov, P.G., Häder, D.P., and Horneck, G. (2017). Overview of the ISS radiation environment observed during the ESA EXPOSE-R2 mission in 2014–2016. *Space Weather*. DOI: 10.1002/2016SW001580.
- Dahlgren, R., Shoji, S., and Nanzyo, M. (1993). Mineralogical Characteristics of Volcanic Ash Soils. In *Volcanic Ash Soils — Genesis, Properties and Utilization*, S. Shoji, and M. Nanzyo, eds. (Amsterdam: Elsevier Science Ltd), pp. 101–143.
- Dalton, B., and Roberto, F. (2008). Lunar regolith biomining: Workshop report. In NASA/CP-2008-214564.
- Danin, A., Dor, I., Sandler, A., and Amit, R. (1998). Desert crust morphology and its relations to microbiotic succession at Mt. Sedom, Israel. *J. Arid Environ.* *38*, 161–174.
- Dartnell, L.R. (2011). Ionizing radiation and life. *Astrobiology* *11*, 551–582.
- Dartnell, L.R., and Patel, M.R. (2014). Degradation of microbial fluorescence biosignatures by solar ultraviolet radiation on Mars. *Int. J. Astrobiol.* *13*, 112–123.

- Dartnell, L.R., Desorgher, L., Ward, J.M., and Coates, A.J. (2007). Modelling the surface and subsurface Martian radiation environment: Implications for astrobiology. *Geophys. Res. Lett.* *34*, L02207.
- Dartnell, L.R., Storrie-Lombardi, M.C., Mullineaux, C.W., Ruban, A. V., Wright, G., Griffiths, A.D., Muller, J.-P., and Ward, J.M. (2011). Degradation of cyanobacterial biosignatures by ionizing radiation. *Astrobiology* *11*, 997–1016.
- Dartnell, L.R., Page, K., Jorge-Villar, S.E., Wright, G., Munshi, T., Scowen, I.J., Ward, J.M., and Edwards, H.G.M. (2012). Destruction of Raman biosignatures by ionising radiation and the implications for life detection on Mars. *Anal. Bioanal. Chem.* *403*, 131–144.
- Demets, R., Schulte, W., and Baglioni, P. (2005). The past, present and future of Biopan. *Adv. Sp. Res.* *36*, 311–316.
- Demets, R., Bertrand, M., Bolkhovitinov, A., Bryson, K., Colas, C., Cottin, H., Dettmann, J., Ehrenfreund, P., Elsaesser, A., and Jaramillo, E. (2015). Window contamination on ExoP-ER. *Int. J. Astrobiol.* *14*, 33–45.
- Deng, M.D., and Coleman, J.R. (1999). Ethanol synthesis by genetic engineering in cyanobacteria. *Appl. Environ. Microbiol.* *65*, 523–528.
- Dexter, J., and Fu, P. (2009). Metabolic engineering of cyanobacteria for ethanol production. *Energy Environ. Sci.* *2*, 857.
- Direito, S.O.L., Marees, A., and Röling, W.F.M. (2012). Sensitive life detection strategies for low-biomass environments: optimizing extraction of nucleic acids adsorbing to terrestrial and Mars analogue minerals. *FEMS Microbiol. Ecol.* *81*, 111–123.
- Dodd, M.S., Papineau, D., Grenne, T., Slack, J.F., Rittner, M., Pirajno, F., O’Neil, J., and Little, C.T.S. (2017). Evidence for early life in Earth’s oldest hydrothermal vent precipitates. *Nature* *543*, 60.
- Domain, F., Houot, L., Chauvat, F., and Cassier-Chauvat, C. (2004). Function and regulation of the cyanobacterial genes *lexA*, *recA* and *ruvB*: LexA is critical to the survival of cells facing inorganic carbon starvation. *Mol. Microbiol.* *53*, 65–80.
- Dose, K., Bieger-Dose, A., Dillmann, R., Gill, M., Kerz, O., Klein, A., Meinert, H., Nawroth, T., Risi, S., and Stridde, C. (1995). ERA-experiment “space biochemistry.” *Adv. Sp. Res.* *16*, 119–129.
- Drysdale, A., Ewert, M., and Hanford, A. (2003). Life support approaches for Mars missions. *Adv. Sp. Res.* *31*, 51–61.
- Drysdale, A.E., Rutkze, C.J., Albright, L.D., and LaDue, R.L. (2004). The minimal cost of life in space. *Adv. Sp. Res.* *34*, 1502–1508.
- Drysdale, A., Nakamura, T., Yorio, N., Sager, J., and Wheeler, R. (2008). Use of sunlight for plant lighting in a bioregenerative life support system – Equivalent system mass calculations. *Adv. Sp. Res.* *42*, 1929–1943.
- DuBois, M., Gilles, K. a., Hamilton, J.K., Rebers, P. a., and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.* *28*, 350–356.
- Ducat, D.C., Way, J.C., and Silver, P.A. (2011). Engineering cyanobacteria to generate high-value products. *Trends Biotechnol.* *29*, 95–103.
- Dufour, D., Leung, V., and Lévesque, C.M. (2012). Bacterial biofilm: structure, function, and antimicrobial resistance. *Endod. Top.* *22*, 2–16.
- Edwards, H.G.M., Hutchinson, I.B., Ingley, R., and Jehlička, J. (2014). Biomarkers and their Raman spectroscopic signatures: a spectral challenge for analytical astrobiology. *Philos. Trans. A. Math. Phys. Eng. Sci.* *372*.

- Ehlmann, B.L., and Edwards, C.S. (2014). Mineralogy of the Martian surface. *Annu. Rev. Earth Planet. Sci.* *42*, 291–315.
- Ehlmann, B.L., Mustard, J.F., Murchie, S.L., Bibring, J.-P., Meunier, A., Fraeman, A.A., and Langevin, Y. (2011). Subsurface water and clay mineral formation during the early history of Mars. *Nature* *479*, 53–60.
- Eldridge, D., and Greene, R. (1994). Microbiotic soil crusts—a review of their roles in soil and ecological processes in the rangelands of Australia. *Soil Res.* *32*, 389–415.
- Fagliarone, C., Mosca, C., Ubaldi, I., Verseux, C., Baqué, M., Wilmotte, A., and Billi, D. (2017). Avoidance of protein oxidation correlates with the desiccation and radiation resistance of hot and cold desert strains of the cyanobacterium *Chroococcidiopsis*. *Extremophiles* *21*, 981–991.
- Fajardo-Cavazos, P., Waters, S.M., Schuerger, A.C., George, S., Marois, J.J., and Nicholson, W.L. (2012). Evolution of *Bacillus subtilis* to enhanced growth at low pressure: up-regulated transcription of des-desKR, encoding the fatty acid desaturase system. *Astrobiology* *12*, 258–270.
- Fang, D., Oberlin, E., Ding, W., and Kounaves, S.P. (2015). A common-factor approach for multivariate data cleaning with an application to Mars Phoenix mission data. *arXiv Prepr. arXiv1510.01291*.
- Farmer, J.D., and Des Marais, D.J. (1999). Exploring for a record of ancient Martian life. *J. Geophys. Res. Planets* *104*, 26977–26995.
- Filali, R., Lasseur, C., and Dubertret, G. (1997). MELiSSA: Nitrogen Sources for Growth of the Cyanobacterium Spirulina. In *Proceedings of the Sixth European Symposium on Space Environmental Control Systems*.
- Finney, L.A., and O'Halloran, T. V (2003). Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science* *300*, 931–936.
- Flemming, H. C., Neu, T. R., and Wozniak, D. (2007). The EPS matrix: the house of biofilm cells. *J. Bacteriol.* *189*, 7945–7947.
- Flemming, H.-C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* *8*, 623–633.
- Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., and Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* *14*, 563–575.
- Folcher, M., and Fussenegger, M. (2012). Synthetic biology advancing clinical applications. *Curr. Opin. Chem. Biol.* *16*, 345–354.
- Foley, I., Marsh, P., Wellington, E.M., Smith, A.W., and Brown, M.R. (1999). General stress response master regulator *rpoS* is expressed in human infection: a possible role in chronicity. *J Antimicrob Chemother* *43*, 164–165.
- Foster, T.L., Winans Jr., L., Casey, R.C., and Kirschner, L.E. (1978). Response of terrestrial microorganisms to a simulated Martian environment. *Appl. Environ. Microbiol.* *35*, 730–737.
- Foucher, F., Westall, F., Brandstätter, F., Demets, R., Parnell, J., Cockell, C.S., Edwards, H.G.M., Bény, J.M., and Brack, A. (2010). Testing the survival of microfossils in artificial Martian sedimentary meteorites during entry into Earth's atmosphere: The STONE 6 experiment. *Icarus* *207*, 616–630.
- Friedmann, E.I. (1980). Endolithic microbial life in hot and cold deserts. *Orig. Life Evol. Biosph.* *10*, 223–235.

- Friedmann, E.I., and Ocampo, R. (1976). Endolithic blue-green algae in the dry valleys: primary producers in the antarctic desert ecosystem. *Science* *193*, 1247–1249.
- Friedmann, I., Lipkin, Y., and Ocampo-Paus, R. (1967). Desert algae of the Negev (Israel). *Phycologia* *6*, 185–200.
- Fries, M.D., Bhartia, R., Beegle, L.W., Gursel, Y., and Mungas, G.S. (2010). Microscopic sample interrogation through multi-wavelength spectroscopy coupled with variable magnification imaging. *LPI Contrib.* *1538*, 5214.
- Giacomelli, G., Boscheri, G., Furfaro, R., Kacira, M., Lobascio, C., Patterson, L., Sadler, P., Pirolli, M., Remiddi, R., Story, D., et al. (2012). Bio-regenerative life support system development for Lunar/Mars habitats. In 42nd International Conference on Environmental Systems.
- Gitelson, J. (1992). Biological life-support systems for Mars mission. *Adv. Sp. Res.* *12*, 167–192.
- Gitelson, I., Lisovsky, G., and MacElroy, R. (2003). Manmade closed ecological systems (London and New York: Taylor & Francis).
- Godia, F., Albiol, J., Montesinos, J., and Pérez, J. (2002). MELISSA: a loop of interconnected bioreactors to develop life support in space. *J. Biotechnol.* *99*, 319–330.
- Godward, M.B.E. (1962). Invisible radiations. In *Physiology and Biochemistry of Algae*, R.A. Lewin, ed. (New York: Academic Press), pp. 551–566.
- Goesmann, F., Brinckerhoff, W.B., Raulin, F., Goetz, W., Danell, R.M., Getty, S.A., Siljeström, S., Mißbach, H., Steininger, H., and Arevalo Jr, R.D. (2017). The Mars Organic Molecule Analyzer (MOMA) instrument: characterization of organic material in martian sediments. *Astrobiology* *17*, 655–685.
- Golombek, M., Grant, J., Kipp, D., Vasavada, A., Kirk, R., Fergason, R., Bellutta, P., Calef, F., Larsen, K., and Katayama, Y. (2012). Selection of the Mars Science Laboratory landing site. *Space Sci. Rev.* *170*, 641–737.
- Gomez-Elvira, J., Armiens, C., Carrasco, I., Genzer, M., Gomez, F., Haberle, R., Hamilton, V.E., Harri, A.M., Kahanpaa, H., Kemppinen, O., et al. (2014). Curiosity's rover environmental monitoring station: Overview of the first 100 sols. *J. Geophys. Res. Planets* *119*, 1680–1688.
- Graham, J.M. (2004). The biological terraforming of Mars: planetary ecosynthesis as ecological succession on a global scale. *Astrobiology* *4*, 168–195.
- Greenberg, J.M., Li, A., Mendoza-Gómez, C.X., Schutte, W.A., Gerakines, P.A., and de Groot, M. (1995). Approaching the interstellar grain organic refractory component. *Astrophys. J. Lett.* *455*, L177.
- Griese, M., Lange, C., and Soppa, J. (2011). Ploidy in cyanobacteria. *FEMS Microbiol. Lett.* *323*, 124–131.
- Grilli Caiola, M., Ocampo-Friedmann, R., and Friedmann, E.I. (1993). Cytology of long-term desiccation in the desert cyanobacterium *Chroococcidiopsis* (Chroococcales). *Phycologia* *32*, 315–322.
- Grilli Caiola, M., Billi, D., and Friedmann, E.I. (1996). Effect of desiccation on envelopes of the cyanobacterium *Chroococcidiopsis* sp. (Chroococcales). *Eur. J. Phycol.* *31*, 97–105.
- Groemer, G., Sattler, B., Weisheitner, K., Hunger, L., Kohstall, C., Frisch, A., Józefowicz, M., Meszyński, S., Storrie-Lombardi, M., Bothe, C., et al. (2014). Field trial of a dual-wavelength fluorescent emission (L.I.F.E.) instrument and the Magma White rover during the MARS2013 Mars analog mission. *Astrobiology* *14*, 391–405.

- Grotzinger, J.P., Sumner, D.Y., Kah, L.C., Stack, K., Gupta, S., Edgar, L., Rubin, D., Lewis, K., Schieber, J., Mangold, N., et al. (2014). A habitable fluvio-lacustrine environment at Yellowknife Bay, Gale Crater, Mars. *Science* *343*, 1242777.
- Gusev, M. V., Baulina, O.I., Gorelova, O.A., Lobakova, E.S., and Korzhenevskaya, T.G. (2002). Artificial Cyanobacterium-Plant Symbioses. In *Cyanobacteria in Symbiosis*, A.N. Rai, B. Bergman, and U. Rasmussen, eds. (Boston: Kluwer Academic Pub.), pp. 253–312.
- Häder, D.-P., Richter, P., Schuster, M., Dachev, T., Tomov, B., Georgiev, P., and Matviichuk, Y. (2009). R3D-B2 – Measurement of ionizing and solar radiation in open space in the BIOPAN 5 facility outside the FOTON M2 satellite. *Adv. Sp. Res.* *43*, 1200–1211.
- Hallenbeck, P.C. (2012). *Microbial technologies in advanced biofuels production* (New York: Springer).
- Harrison, J.P., Gheeraert, N., Tsigelnitskiy, D., and Cockell, C.S. (2013). The limits for life under multiple extremes. *Trends Microbiol.* *21*, 204–212.
- Hassler, D.M., Zeitlin, C., Wimmer-Schweingruber, R.F., Ehresmann, B., Rafkin, S., Eigenbrode, J.L., Brinza, D.E., Weigle, G., Böttcher, S., Böhm, E., et al. (2013). Mars' surface radiation environment measured with the Mars Science Laboratory's Curiosity rover. *Science* *343*, 1244797.
- Hays, L.E., Graham, H. V., Des Marais, D.J., Hausrath, E.M., Horgan, B., McCollom, T.M., Parenteau, M.N., Potter-McIntyre, S.L., Williams, A.J., and Lynch, K.L. (2017). Biosignature preservation and detection in Mars analog environments. *Astrobiology* *17*, 363–400.
- Hedges, J.I., and Keil, R.G. (1995). Sedimentary organic matter preservation: an assessment and speculative synthesis. *Mar. Chem.* *49*, 81–115.
- Hecht, M.H., Kounaves, S.P., Quinn, R.C., West, S.J., Young, S.M.M., Ming, D.W., Catling, D.C., Clark, B.C., Boynton, W. V., Hoffman, J., et al. (2009). Detection of perchlorate and the soluble chemistry of martian soil at the Phoenix lander site. *Science* *325*, 64–67.
- Hempel, F., Bozarth, A.S., Lindenkamp, N., Klingl, A., Zauner, S., Linne, U., Steinbüchel, A., and Maier, U.G. (2011). Microalgae as bioreactors for bioplastic production. *Microb. Cell Fact.* *10*, 81.
- Hendrickx, L., and Mergeay, M. (2007). From the deep sea to the stars: human life support through minimal communities. *Curr. Opin. Microbiol.* *10*, 231–237.
- Hendrickx, L., De Wever, H., Hermans, V., Mastroleo, F., Morin, N., Wilmotte, A., Janssen, P., and Mergeay, M. (2006). Microbial ecology of the closed artificial ecosystem MELiSSA (Micro-Ecological Life Support System Alternative): reinventing and compartmentalizing the Earth's food and oxygen regeneration system for long-haul space exploration missions. *Res. Microbiol.* *157*, 77–86.
- Henrikson, R. (2009). *Earth food spirulina: how this remarkable blue-green algae can transform your health and our planet*. (Hana, Maui, HI: Ronore Enterprises, Inc.).
- Hepp, A., Landis, G., and Kubiak, C. (1993). A chemical approach to carbon dioxide utilization on Mars. In *Resources of Near Earth Space*, J.S. Lewis, M.S. Matthews, and M.L. Guerrieri, eds. (The Arizona Board of Regents).
- Hoffman, S.J., and Kaplan, D.I. (1997). Human Exploration of Mars : The Reference Mission of the NASA Mars Exploration Study Team.
- Horneck, G., Facius, R., Reichert, M., Rettberg, P., Seboldt, W., Manzey, D., Comet, B., Maillet, A., Preiss, H., Schauer, L., et al. (2003). HUMEX, a study on the survivability and

- adaptation of humans to long-duration exploratory missions, part I: lunar missions. *Adv. Space Res.* *31*, 2389–2401.
- Horneck, G., Facius, R., Reichert, M., Rettberg, P., Seboldt, W., Manzey, D., Comet, B., Maillet, A., Preiss, H., Schauer, L., et al. (2006). HUMEX, a study on the survivability and adaptation of humans to long-duration exploratory missions, part II: Missions to Mars. *Adv. Sp. Res.* *38*, 752–759.
- Horneck, G. (2008). The microbial case for Mars and its implication for human expeditions to Mars. *Acta Astronaut.* *63*, 1015–1024.
- Horneck, G., Stöffler, D., Ott, S., Hornemann, U., Cockell, C.S., Moeller, R., Meyer, C., de Vera, J.-P., Fritz, J., Schade, S., et al. (2008). Microbial rock inhabitants survive hypervelocity impacts on Mars-like host planets: First phase of lithopanspermia experimentally tested. *Astrobiology* *8*, 17–44.
- Horneck, G., Klaus, D.M., and Mancinelli, R.L. (2010). Space microbiology. *Microbiol. Mol. Biol. Rev.* *74*, 121–156.
- Horneck, G., Walter, N., Westall, F., Grenfell, J.L., Martin, W.F., Gomez, F., Leuko, S., Lee, N., Onofri, S., Tsiganis, K., et al. (2016). AstRoMap European Astrobiology Roadmap. *Astrobiology* *16*, 201–243.
- Hoshino, K., Hamochi, M., Mitsuhashi, S., and Tanishita, K. (1991). Measurements of oxygen production rate in flowing *Spirulina* suspensions. *Appl. Microbiol. Biotechnol.* *35*, 89–93.
- Hotchin, J., Lorenz, P., Markusen, A., and Hemenway, C. (1967). The survival of microorganisms in space, further rocket and balloon-borne exposure experiments. *Life Sci. Sp. Res* *5*, 1–6.
- Hotchin, J., Lorenz, P., and Hemenway, C.L. (1968). The survival of terrestrial microorganisms in space at orbital altitudes during Gemini satellite experiments. *Life Sci. Space Res.* *6*, 108–114.
- Hutchinson, I.B., Edwards, H.G.M., Ingleby, R., Harris, L., McHugh, M., Malherbe, C., Jehlicka, J., Marshall, C., and Parnell, J. (2014). Preparations for the launch of the EXOMARS Raman Laser Spectrometer — A review of recent studies which highlight the astrobiological and geological capabilities of portable Raman instrumentation. *LPI Contrib.* *1783*, 5093.
- Isenbarger, T.A., Carr, C.E., Johnson, S.S., Finney, M., Church, G.M., Gilbert, W., Zuber, M.T., and Ruvkun, G. (2008). The most conserved genome segments for life detection on Earth and other planets. *Orig. Life Evol. Biosph.* *38*, 517–533.
- Jack, D.A., Nakamura, T., Sadler, P., and Cuello, J.L. (2002). Evaluation of two fiber optic-based solar collection and distribution systems for advanced space life support. *Trans. ASAE* *45*, 1547–1558.
- Jeffrey, S.W., and Humphrey, G.F. (1975). New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochemical Physiology Pflanz* *167*: 191–194.
- Jiménez, C., Cossío, B.R., and Niella, F.X. (2003). Relationship between physicochemical variables and productivity in open ponds for the production of *Spirulina*: a predictive model of algal yield. *Aquaculture* *221*, 331–345.
- Jonkers, H.M., Thijssen, A., Muyzer, G., Copuroglu, O., and Schlangen, E. (2010). Application of bacteria as self-healing agent for the development of sustainable concrete. *Ecol. Eng.* *36*, 230–235.

- Jönsson, K.I., Rabbow, E., Schill, R.O., Harms-Ringdahl, M., and Rettberg, P. (2008). Tardigrades survive exposure to space in low Earth orbit. *Curr. Biol.* *18*, R729–R731.
- Josset, J.-L., Westall, F., Hofmann, B.A., Spray, J., Cockell, C., Kempe, S., Griffiths, A.D., De Sanctis, M.C., Colangeli, L., and Koschny, D. (2017). The Close-Up Imager onboard the ESA ExoMars Rover: objectives, description, operations, and science validation activities. *Astrobiology* *17*, 595–611.
- Kahn, B.A., and Stoffella, P.J. (1996). No evidence of adverse effects on germination, emergence, and fruit yield due to space exposure of tomato seeds. *J. Am. Soc. Hortic. Sci.* *121*, 414–418.
- Kanervo, E., Lehto, K., Stähle, K., Lehto, H., and Mäenpää, P. (2005). Characterization of growth and photosynthesis of *Synechocystis* sp. PCC 6803 cultures under reduced atmospheric pressures and enhanced CO₂ levels. *Int. J. Astrobiol.* *4*, 97–100.
- Keränen, M., Aro, E.M., and Tyystjärvi, E. (1999). Excitation-emission map as a tool in studies of photosynthetic pigment-protein complexes. *Photosynthetica* *37*, 225–237.
- Khalil, A.S., and Collins, J.J. (2010). Synthetic biology: applications come of age. *Nat. Rev. Genet.* *11*, 367–379.
- Kim, M., Zhang, Z., Okano, H., Yan, D., Groisman, A., and Hwa, T. (2012). Need-based activation of ammonium uptake in *Escherichia coli*. *Mol. Syst. Biol.* *8*.
- Keil, R.G., Montluçon, D.B., Prahl, F.G., and Hedges, J.I. (1994). Sorptive preservation of labile organic matter in marine sediments. *Nature* *370*, 549.
- Kim, W., Tengra, F.K., Young, Z., Shong, J., Marchand, N., Chan, H.K., Pangule, R.C., Parra, M., Dordick, J.S., and Plawsky, J.L. (2013). Spaceflight promotes biofilm formation by *Pseudomonas aeruginosa*. *PLoS One* *8*, e62437.
- Kimura, S., Tomita-Yokotani, K., Inoue, K., Sato, S., Arai, M., and Katoh, H. (2016). Space environmental tolerance of a terrestrial cyanobacterium, *Nostoc* sp. HK-01. (46th International Conference on Environmental Systems).
- Kirensky, L. V., Terskov, I.A., Gitelson, I.I., Lisovsky, G.M., Kovrov, B.G., and Okladnikov, Y.N. (1968). Experimental biological life support system. II. Gas exchange between man and microalgae culture in a 30-day experiment. *Life Sci. Space Res.* *6*, 37–40.
- Klingler, J.M., Mancinelli, R.L., and White, M.R. (1989). Biological nitrogen fixation under primordial Martian partial pressures of dinitrogen. *Adv. Space Res.* *9*, 173–176.
- Kounaves, S.P., Chaniotakis, N.A., Chevrier, V.F., Carrier, B.L., Folds, K.E., Hansen, V.M., McElhoney, K.M., O'Neil, G.D., and Weber, A.W. (2014). Identification of the perchlorate parent salts at the Phoenix Mars landing site and possible implications. *Icarus* *232*, 226–231.
- Kozyrovska, N., Lutvynenko, T.L., Korniichuk, O.S., Kovalchuk, M.V., Voznyuk, T.M., Kononuchenko, O., Zaetz, I., Rogutskyy, I.S., Mytrokhyn, O., Mashkovska, S.P., et al. (2006). Growing pioneer plants for a lunar base. *Adv. Sp. Res.* *37*, 93–99.
- Kral, T., Altheide, T.S., Lueders, A.E., and Schuerger, A.C. (2011). Low pressure and desiccation effects on methanogens: Implications for life on Mars. *Planet. Space Sci.* *59*, 264–270.
- Kraus, M.P. (1969). Resistance of Blue-Green algae to ⁶⁰Co gamma radiation. *Radiat. Bot.* *9*, 481–489.
- Kumar, H.D. (1964). Effects of radiations on blue-green algae II. Effects on growth. *Ann. Bot.* *28*, 555–564.

- Kurahashi-Nakamura, T., and Tajika, E. (2006). Atmospheric collapse and transport of carbon dioxide into the subsurface on early Mars. *Geophys. Res. Lett.* *33*, L18205.
- de la Torre, R., Sancho, L.G., Horneck, G., Ríos, A. de los, Wierzchos, J., Olsson-Francis, K., Cockell, C.S., Rettberg, P., Berger, T., de Vera, J.-P.P., et al. (2010). Survival of lichens and bacteria exposed to outer space conditions – Results of the Lithopanspermia experiments. *Icarus* *208*, 735–748.
- Langevin, Y., Poulet, F., Bibring, J.-P., and Gondet, B. (2005). Sulfates in the North Polar Region of Mars detected by OMEGA/Mars Express. *Science* *307*, 1584–1586.
- Langhoff, S., Cumbers, J., Rothschild, L.J., Paavola, C., and Worden, S.P. (2011). What are the potential roles for synthetic biology in nasa's mission? In NASA/CP-2011-216430 (Moffett Field).
- Lao, K., and Glazer, A.N. (1996). Ultraviolet-B photodestruction of a light-harvesting complex. *Proc. Natl. Acad. Sci.* *93*, 5258–5263.
- Layton, J.C., and Foster, P.L. (2003) Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in *Escherichia coli*. *Mol Microbiol* *48*, 254–267.
- Lee, C., and Brocks, J.J. (2011). Identification of carotane breakdown products in the 1.64 billion year old Barney Creek Formation, McArthur Basin, northern Australia. *Org. Geochem.* *42*, 425–430.
- Lehto, K., Kanervo, E., Stahle, K., and Lehto, H. (2007). Photosynthetic life support systems in the Martian conditions. In ROME: Response of Organisms to the Martian Environment (ESA AP-1299), C. Cockell, and G. Horneck, eds. (Noordwijk, The Netherlands: ESA Communications), pp. 151–160.
- Lehto, K.M., Lehto, H.J., and Kanervo, E.A. (2006). Suitability of different photosynthetic organisms for an extraterrestrial biological life support system. *Res. Microbiol.* *157*, 69–76.
- Le Postollec, A., Incerti, S., Dobrijevic, M., Desorgher, L., Santin, G., Moretto, P., Vandenabeele-Trambouze, O., Coussot, G., Dartnell, L., and Nieminen, P. (2009). Monte Carlo simulation of the radiation environment encountered by a biochip during a space mission to Mars. *Astrobiology* *9*, 311–323.
- Leuko, S., Neilan, B.A., Burns, B.P., Walter, M.R., and Rothschild, L.J. (2011). Molecular assessment of UVC radiation-induced DNA damage repair in the stromatolitic halophilic archaeon, *Halococcus hamelinensis*. *J. Photochem. Photobiol. B Biol.* *102*, 140–145.
- Leuko, S., Rettberg, P., Pontifex, A.L., and Burns, B.P. (2014). On the response of halophilic archaea to space conditions. *Life* *4*, 66–76.
- Leuko, S., Domingos, C., Parpart, A., Reitz, G., and Rettberg, P. (2015). The Survival and resistance of *Halobacterium salinarum* NRC-1, *Halococcus hamelinensis*, and *Halococcus morrhuae* to simulated outer space solar radiation. *Astrobiology* *15*, 987–997.
- Levin, G. V (2015). The curiousness of Curiosity. *Astrobiology* *15*, 101–103.
- Levine, E., and Thiel, T. (1987). UV-inducible DNA repair in the cyanobacteria *Anabaena* spp. *J. Bacteriol.* *169*, 3988–3993.
- Li, X., Danell, R.M., Brinckerhoff, W.B., Pinnick, V.T., van Amerom, F., Arevalo Jr, R.D., Getty, S.A., Mahaffy, P.R., Steininger, H., and Goesmann, F. (2015). Detection of trace organics in Mars analog samples containing perchlorate by laser desorption/ionization mass spectrometry. *Astrobiology* *15*, 104–110.
- Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature* *362*, 709–715.

- Liu, J., Bukatin, V.E., and Tsygankov, A.A. (2006). Light energy conversion into H₂ by *Anabaena variabilis* mutant PK84 dense cultures exposed to nitrogen limitations. *Int. J. Hydrogen Energy* *31*, 1591–1596.
- Liu, Y., Cockell, C.S., Wang, G., Hu, C., Chen, L., and De Philippis, R. (2008). Control of Lunar and Martian dust - Experimental insights from artificial and natural cyanobacterial and algal crusts in the desert of Inner Mongolia, China. *Astrobiology* *8*, 75–86.
- Lobascio, C., Lamantea, M., Cotronei, V., Negri, B., De Pascale, S., Maggio, A., Foti, M., and Palumberi, S. (2007). Plant bioregenerative life supports: The Italian CAB Project. *J. Plant Interact.* *2*, 125–134.
- Maggi, F., and Pallud, C. (2010). Space agriculture in micro- and hypo-gravity: A comparative study of soil hydraulics and biogeochemistry in a cropping unit on Earth, Mars, the Moon and the space station. *Planet. Space Sci.* *58*, 1996–2007.
- Lyon, D.Y., Monier, J.-M., Dupraz, S., Freissinet, C., Simonet, P., and Vogel, T.M. (2010). Integrity and biological activity of DNA after UV exposure. *Astrobiology* *10*, 285–292.
- Mancinelli, R.L. (2015). The affect of the space environment on the survival of *Halorubrum chaoviator* and *Synechococcus* (Nägeli): data from the Space Experiment OSMO on EXPOSE-R. *Int. J. Astrobiol.* *14*, 123–128.
- Mancinelli, R.L., and Banin, A. (2003). Where is the nitrogen on Mars? *Int. J. Astrobiol.* *2*, 217–225.
- Mancinelli, R.L., and Klovstad, M. (2000). Martian soil and UV radiation: microbial viability assessment on spacecraft surfaces. *Planet. Space Sci.* *48*, 1093–1097.
- Mancinelli, R.L., White, M.R., and Rothschild, L.J. (1998). Biopan-survival I: Exposure of the osmophiles *Synechococcus* sp (Nägeli) and *Haloarcula* sp. to the space environment. *Adv. Sp. Res.* *22*, 327–334.
- Marshall, C.P., and Marshall, A.O. (2010). The potential of Raman spectroscopy for the analysis of diagenetically transformed carotenoids. *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* *368*, 3137–3144.
- Martin, V.J.J., Pitera, D.J., Withers, S.T., Newman, J.D., and Keasling, J.D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* *21*, 796–802.
- Martín-Torres, F.J., Zorzano, M.-P., Valentín-Serrano, P., Harri, A.-M., Genzer, M., Kemppinen, O., Rivera-Valentin, E.G., Jun, I., Wray, J., Madsen, M.B., et al. (2015). Transient liquid water and water activity at Gale crater on Mars. *Nat. Geosci.* *8*, 357–361.
- Martinez-Frias, J., Amaral, G., and Vázquez, L. (2006). Astrobiological significance of minerals on Mars surface environment. *Rev. Environ. Sci. Bio/Technology* *5*, 219–231.
- Martins, Z., Cottin, H., Kotler, J.M., Carrasco, N., Cockell, C.S., De la Torre Noetzel, R., Demets, R., de Vera, J.P., D'Hendecourt, L., Ehrenfreund, P., et al. (2017). Earth as a Tool for Astrobiology—A European Perspective. *Space Sci. Rev.* *209*, 43–81.
- Massa, G.D., Emmerich, J.C., Morrow, R.C., Bourget, C.M., and Mitchell, C.A. (2007). Plant-growth lighting for space life support: A review. *Gravitational Sp. Biol.* *19*, 19–30.
- Mastrapa, R.M.E., Glanzberg, H., Head, J.N., Melosh, H.J., and Nicholson, W.L. (2001). Survival of bacteria exposed to extreme acceleration: Implications for panspermia. *Earth Planet. Sci. Lett.* *189*, 1–8.
- Mattimore, V., and Battista, J.R. (1996). Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. *J. Bacteriol.* *178*, 633–637.

- McEwen, A.S., Ojha, L., Dundas, C.M., Mattson, S.S., Byrne, S., Wray, J.J., Cull, S.C., Murchie, S.L., Thomas, N., and Gulick, V.C. (2011). Seasonal flows on warm Martian slopes. *Science* *333*, 740–743.
- McKay, C.P., Meyer, T.R., Boston, P.J., Nelson, M., and McCallum, T. (1993). Utilizing Martian Resources for Life Support. In *Resources of Near Earth Space*, J.S. Lewis, M.S. Matthews, and M.L. Guerrieri, eds. (The Arizona Board of Regents), pp. 819–843.
- McKay, D.S., and Allen, C.C. (1996). Concrete - A Practical Construction Material for Mars. In *Proceedings of the Fifth International Conference on Engineering, Construction, and Operations in Space*, (Albuquerque, NM), pp. 566–570.
- McKay, C.P. (1997). The search for life on Mars. *Orig. Life Evol. Biosph.* *27*, 263–289.
- McKay, C.P. (2010). An origin of life on Mars. *Cold Spring Harb. Perspect. Biol.* *2*, a003509.
- McKay, C.P., and Marinova, M. (2001). The physics, biology, and environmental ethics of making Mars habitable. *Astrobiology* *1*, 89–110.
- McKay, C.P., Stoker, C., Glass, B., Dave, A., Davila, A., Heldmann, J., Marinova, M., Fairen, A., Quinn, R., Zacyn, K., et al. (2013). The Icebreaker Life Mission to Mars: a search for biomolecular evidence for life. *Astrobiology* *13*, 334–354.
- McLennan, S.M., Anderson, R.B., Bell, J.F., Bridges, J.C., Calef, F., Campbell, J.L., Clark, B.C., Clegg, S., Conrad, P., Cousin, A., et al. (2014). Elemental geochemistry of sedimentary rocks at Yellowknife Bay, Gale crater, Mars. *Science* *343*, 1244734.
- McMahon, S., Parnell, J., Ponicka, J., Hole, M., and Boyce, A. (2013). The habitability of vesicles in martian basalt. *Astron. Geophys.* *54*, 17–21.
- McSween, H.Y. (1994). What we have learned about Mars from SNC meteorites. *Meteoritics & Planetary Sciences* *29*, 757–779.
- McSween, H.Y., Taylor, G.J., and Wyatt, M.B. (2009). Elemental composition of the Martian crust. *Science* *324*, 736–739.
- Menezes, A.A., Cumbers, J., Hogan, J.A., and Arkin, A.P. (2014). Towards synthetic biological approaches to resource utilization on space missions. *J. R. Soc. Interface* *12*, 20140715.
- Meyer, T.R., and McKay, C.P. (1989). The resources of Mars for human settlement. *J. British Interplanet. Soc.* *42*, 147–160.
- Meyer, T.R., and McKay, C.P. (1996). Using the Resources of Mars for Human Settlement. In *Strategies for Mars: A Guide to Human Exploration*, C.R. Stoker, and C. Emmart, eds. (San Diego, CA: American Astronautical Society), pp. 393–442.
- Meyers, P.A., and Ishiwatari, R. (1995). Organic matter accumulation records in lake sediments. In *Physics and Chemistry of Lakes*, P.A. Lerman, P.D.D.M. Imboden, and P.J.R. Gat, eds. (Springer Berlin Heidelberg), pp. 279–328.
- Miao, X., Wu, Q., Wu, G., and Zhao, N. (2003). Sucrose accumulation in salt-stressed cells of *agg* gene deletion-mutant in cyanobacterium *Synechocystis* sp PCC 6803. *FEMS Microbiol. Lett.* *218*, 71–77.
- Michalski, J.R., Cuadros, J., Niles, P.B., Parnell, J., Deanne Rogers, A., and Wright, S.P. (2013). Groundwater activity on Mars and implications for a deep biosphere. *Nat. Geosci.* *6*, 133–138.
- Mileikowsky, C., Cucinotta, F.A., Wilson, J.W., Gladman, B., Horneck, G., Lindegren, L., Melosh, J., Rickman, H., Valtonen, M., and Zheng, J.Q. (2000). Natural transfer of viable microbes in space. *Icarus* *145*, 391–427.
- Ming, D.W., Archer, P.D., Glavin, D.P., Eigenbrode, J.L., Franz, H.B., Sutter, B., Brunner, A.E., Stern, J.C., Freissinet, C., McAdam, A.C., et al. (2014). Volatile and organic

- compositions of sedimentary rocks in Yellowknife Bay, Gale crater, Mars. *Science* *343*, 124526.
- Moeller, R., Reitz, G., Berger, T., Okayasu, R., Nicholson, W.L., and Horneck, G. (2010). Astrobiological aspects of the mutagenesis of cosmic radiation on bacterial spores. *Astrobiology* *10*, 509–521.
- Moeller, R., Raguse, M., Leuko, S., Berger, T., Hellweg, C.E., Fujimori, A., Okayasu, R., and Horneck, G. (2017). STARLIFE-An international campaign to study the role of galactic cosmic radiation in astrobiological model systems. *Astrobiology* *17*, 101–109.
- Moissl-Eichinger, C., Cockell, C., and Rettberg, P. (2016). Venturing into new realms? Microorganisms in space. *FEMS Microbiol. Rev.* *40*, 722–737.
- Moll, D.M., and Vestal, J.R. (1992). Survival of microorganisms in smectite clays: implications for Martian exobiology. *Icarus* *98*, 233–239.
- Möllers, K.B., Cannella, D., Jørgensen, H., and Frigaard, N.-U. (2014). Cyanobacterial biomass as carbohydrate and nutrient feedstock for bioethanol production by yeast fermentation. *Biotechnol. Biofuels* *7*, 64.
- Montague, M., McArthur, G.H., Cockell, C.S., Held, J., Marshall, W., Sherman, L. a, Wang, N., Nicholson, W.L., Tarjan, D.R., and Cumbers, J. (2012). The role of synthetic biology for in situ resource utilization (ISRU). *Astrobiology* *12*, 1135–1142.
- Mori, K., Ohya, H., Matsumoto, K., and Furune, H. (1987). Sunlight supply and gas exchange systems in the microalgal bioreactor. *Adv. Space Res.* *7*, 47–52.
- Morris, R. V., Klingelhöfer, G., Bernhardt, B., Schröder, C., Rodionov, D.S., De Souza, P.A., Yen, A., Gellert, R., Evlanov, E.N., Foh, J., et al. (2004). Mineralogy at Gusev Crater from the Mössbauer spectrometer on the Spirit Rover. *Science* *305*, 833–836.
- Mueller, R.P., and Van Susante, P.J. (2011). A Review of Lunar Regolith Excavation Robotic Device Prototypes. In American Institute of Aeronautics and Astronautics Space 2011 Conference, Paper # 1073752, (Long Beach, CA).
- Muhlestein, D.J., Hooten, T.M., Koenig, R., Grossl, P., and Bugbee, B. (1999). Is Nitrate Necessary to Biological Life Support? In The International Conference on Environmental Systems (ICES) Meeting, (Denver, CO), pp. 2–5.
- Mora, M., Mahnert, A., Koskinen, K., Pausan, M.R., Oberauner-Wappis, L., Krause, R., Perras, A.K., Gorkiewicz, G., Berg, G., and Moissl-Eichinger, C. (2016). Microorganisms in confined habitats: Microbial monitoring and control of intensive care units, operating rooms, cleanrooms and the international space station. *Front. Microbiol.* *7*, 1573.
- Munteanu, A.-C., Valentina, M., and Adrian, U. (2015). Recent progress in understanding the molecular mechanisms of radioresistance in *Deinococcus* bacteria. *Extremophiles* *19*, 707–719.
- Murukesan, G., Leino, H., Mäenpää, P., Stähle, K., Raksajit, W., Lehto, H.J., Allahverdiyev-Rinne, Y., and Lehto, K. (2015). Pressurized Martian-Like pure CO₂ atmosphere supports strong growth of cyanobacteria, and causes significant changes in their metabolism. *Orig. Life Evol. Biosph.* *46*, 119–131.
- Musk, E. (2017). Making humans a multiplanetary species. *New Sp.* *5*, 46–61.
- Mustard, J.F., Murchie, S.L., Pelkey, S.M., Ehlmann, B.L., Milliken, R.E., Grant, J.A., Bibring, J.-P., Poulet, F., Bishop, J., Dobrea, E.N., et al. (2008). Hydrated silicate minerals on Mars observed by the Mars Reconnaissance Orbiter CRISM instrument. *Nature* *454*, 305–309.

- De Muynck, W., Verbeken, K., De Belie, N., and Verstraete, W. (2010). Influence of urea and calcium dosage on the effectiveness of bacterially induced carbonate precipitation on limestone. *Ecol. Eng.* *36*, 99–111.
- Mytrokhyn, O.V., Bogdanova, S.V., and Shumlyanskyy, L.V. (2003). Anorthosite rocks of Fedorivskyy suite (Korosten Pluton, Ukrainian Shield). *Curr. Probl. Geol.* Kyiv Natl. Univ. Kyiv *53*–57.
- Nakamura, T., Van Pelt, A.D., Yorio, N.C., Drysdale, A.E., Wheeler, R.M., and Sager, J.C. (2009). Transmission and distribution of photosynthetically active radiation (PAR) from solar and electric light sources. *Habitation* *12*, 103–117.
- Navarrete, J.U., Cappelle, I.J., Schnittker, K., and Borrok, D.M. (2012). Bioleaching of ilmenite and basalt in the presence of iron-oxidizing and iron-scavenging bacteria. *Int. J. Astrobiol.* *12*, 123–134.
- Nelson, M., Dempster, W.F., and Allen, J.P. (2008). Integration of lessons from recent research for “Earth to Mars” life support systems. *Adv. Sp. Res.* *41*, 675–683.
- Nelson, M., Pechurkin, N.S., Allen, J.P., Somova, L.A., and Gitelson, J.I. (2010). Closed ecological systems, space life support and biospherics. In *Environmental Biotechnology*, L.K. Wang, V. Ivanov, J.-H. Tay, and Y.-T. Hung, eds. (New York: Humana Press), pp. 517–565.
- Nicholson, W.L. (2009). Ancient micronauts: interplanetary transport of microbes by cosmic impacts. *Trends Microbiol.* *17*, 243–250.
- Nicholson, W.L., Fajardo-Cavazos, P., Fedenko, J., Ortiz-Lugo, J.L., Rivas-Castillo, A., Waters, S.M., and Schuerger, A.C. (2010). Exploring the low-pressure growth limit: evolution of *Bacillus subtilis* in the laboratory to enhanced growth at 5 kilopascals. *Appl. Environ. Microbiol.* *76*, 7559–7565.
- Nicholson, W.L., Krivushin, K., Gilichinsky, D., and Schuerger, A.C. (2013). Growth of *Carnobacterium* spp. from permafrost under low pressure, temperature, and anoxic atmosphere has implications for Earth microbes on Mars. *Proc. Natl. Acad. Sci. U. S. A.* *110*, 666–671.
- Nickerson, C.A., Mark Ott, C., Mister, S.J., Morrow, B.J., Burns-Keliher, L., and Pierson, D.L. (2000). Microgravity as a novel environmental signal affecting *Salmonella enterica* serovar Typhimurium virulence. *Infect. Immun.* *68*, 3147–3152.
- Niederholtmeyer, H., Wolfstädter, B.T., Savage, D.F., Silver, P.A., and Way, J.C. (2010). Engineering cyanobacteria to synthesize and export hydrophilic products. *Appl. Environ. Microbiol.* *76*, 3462–3466.
- Nixon, S.L., Cousins, C.R., and Cockell, C.S. (2013). Plausible microbial metabolisms on Mars. *Astron. Geophys.* *54*, 13–16.
- Olsson-Francis, K., and Cockell, C.S. (2010a). Experimental methods for studying microbial survival in extraterrestrial environments. *J. Microbiol. Methods* *80*, 1–13.
- Olsson-Francis, K., and Cockell, C.S. (2010b). Use of cyanobacteria for in-situ resource use in space applications. *Planet. Space Sci.* *58*, 1279–1285.
- Olsson-Francis, K., Simpson, A.E., Wolff-Boenisch, D., and Cockell, C.S. (2012). The effect of rock composition on cyanobacterial weathering of crystalline basalt and rhyolite. *Geobiology* *10*, 434–444.
- Osanai, T., Oikawa, A., Numata, K., Kuwahara, A., Iijima, H., Doi, Y., Saito, K., and Hirai, M.Y. (2014). Pathway-level acceleration of glycogen catabolism by a response regulator in the cyanobacterium *Synechocystis* species PCC 6803. *Plant Physiol.* *164*, 1831–1841.

- Pääbo, S., Poinar, H., Serre, D., Jaenicke-Després, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L., and Hofreiter, M. (2004). Genetic analyses from ancient DNA. *Annu. Rev. Genet.* 38, 645–679.
- Page, E., Monrozier, L.J., and Simonet, P. (1992). Adsorption of DNA on clay minerals: protection against DNaseI and influence on gene transfer. *FEMS Microbiol. Lett.* 97, 31–39.
- Parnell, J., Cullen, D., Sims, M.R., Bowden, S., Cockell, C.S., Court, R., Ehrenfreund, P., Gaubert, F., Grant, W., Parro, V., et al. (2007). Searching for life on Mars: selection of molecular targets for ESA's Aurora ExoMars mission. *Astrobiology* 7, 578–604.
- Parro, V., de Diego-Castilla, G., Rodríguez-Manfredi, J.A., Rivas, L.A., Blanco-López, Y., Sebastián, E., Romeral, J., Compostizo, C., Herrero, P.L., García-Marín, A., et al. (2011). SOLID3: A multiplex antibody microarray-based optical sensor instrument for in situ life detection in planetary exploration. *Astrobiology* 11, 15–28.
- Patel, M.R., Bérçes, A., Kerékgyárto, T., Rontó, G., Lammer, H., and Zarnecki, J.C. (2004). Annual solar UV exposure and biological effective dose rates on the Martian surface. *Adv. Sp. Res.* 33, 1247–1252.
- Paul, J.H., Jeffrey, W.H., David, A.W., Deflaun, M.F., and Cazares, L.H. (1989). Turnover of extracellular DNA in eutrophic and oligotrophic freshwater environments of southwest Florida. *Appl. Environ. Microbiol.* 55, 1823–1828.
- Pavlov, A.A., Vasilyev, G., Ostryakov, V.M., Pavlov, A.K., and Mahaffy, P. (2012). Degradation of the organic molecules in the shallow subsurface of Mars due to irradiation by cosmic rays. *Geophys. Res. Lett.* 39, L13202.
- Paulino-Lima, I.G., Janot-Pacheco, E., Galante, D., Cockell, C., Olsson-Francis, K., Brucato, J.R., Baratta, G.A., Strazzulla, G., Merrigan, T., McCullough, R., et al. (2011). Survival of *Deinococcus radiodurans* against laboratory-simulated solar wind charged particles. *Astrobiology* 11, 875–882.
- Paulino-Lima, I.G., Fujishima K., Navarrete J.U., Galante D., Rodrigues F., Azua-Bustos A., and Rothschild, L.J. (2017). Extremely high UV-C radiation resistant microorganisms from desert environments with different manganese concentrations. *J Photochem Photobiol B* 163, 327–336.
- Pavlopoulou, A., Savva, G., Louka, M., Bagos, P., Vorgias, C., Michalopoulos, I., and Georgakilas, A. (2016). Unraveling the mechanisms of extreme radio resistance in prokaryotes: lessons from nature. *Mutat. Res.* 767, 92–107.
- Pavlov, A.A., Vasilyev, G., Ostryakov, V.M., Pavlov, A.K., and Mahaffy, P. (2012). Degradation of the organic molecules in the shallow subsurface of Mars due to irradiation by cosmic rays. *Geophys. Res. Lett.* 39, L13202.
- Peralta-Yahya, P.P., Zhang, F., del Cardayre, S.B., and Keasling, J.D. (2012). Microbial engineering for the production of advanced biofuels. *Nature* 488, 320–328.
- Perchonok, M.H., Cooper, M.R., and Catauro, P.M. (2012). Mission to Mars: food production and processing for the final frontier. NASA Spec. ed, Annual Review of Food Science and Technology. NASA, Lyndon B. Johnson Space Center, Lyndon B. Johnson Space Center, Houston, TX.
- Peters, G.A., and Meeks, J.C. (1989). The Azolla-Anabaena Symbiosis: Basic Biology. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40, 193–210.
- Pollard, E.C. (1965). Theoretical studies on living systems in the absence of mechanical stress. *J. Theor. Biol.* 8, 113–123.

- Pollard, E.C. (1967). Physical determinants of receptor mechanisms. In Gravity and the Organism. The University of Chicago Press, Chicago, IL., S.A. Gordon, and M.J. Cohen, eds. (Chicago, IL: The University of Chicago Press), pp. 25–34.
- Ponti, M., Forrow, S.M., Souhami, R.L., D'Incàlci, M., and Hartley, J.A. (1991). Measurement of the sequence specificity of covalent DNA modification by antineoplastic agents using Taq DNA polymerase. *Nucleic Acids Res.* *19*, 2929–2933.
- Poughon, L., Farges, B., Dussap, C.G., Godia, F., and Lasseur, C. (2009). Simulation of the MELiSSA closed loop system as a tool to define its integration strategy. *Adv. Sp. Res.* *44*, 1392–1403.
- Poulet, F., Bibring, J.-P., Mustard, J.F., Gendrin, A., Mangold, N., Langevin, Y., Arvidson, R.E., Gondet, B., Gomez, C., Berthé, M., et al. (2005). Phyllosilicates on Mars and implications for early Martian climate. *Nature* *438*, 623–627.
- Preston, L.J., Melim, L.A., Polyak, V.J., Asmerom, Y., and Southam, G. (2014). Infrared spectroscopic biosignatures from Hidden Cave, New Mexico: possible applications for remote life detection. *Geomicrobiol. J.* *31*, 929–941.
- Pulz, O., and Gross, W. (2004). Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.* *65*, 635–648.
- Qiang, H., Zarmi, Y., and Richmond, A. (1998). Combined effects of light intensity, light-path and culture density on output rate of *Spirulina platensis* (Cyanobacteria). *Eur. J. Phycol.* *33*, 165–171.
- Quintana, N., Van der Kooy, F., Van de Rhee, M.D., Voshol, G.P., and Verpoorte, R. (2011). Renewable energy from Cyanobacteria: energy production optimization by metabolic pathway engineering. *Appl. Microbiol. Biotechnol.* *91*, 471–490.
- Rabbow, E., Rettberg, P., Barczyk, S., Bohmeier, M., Parpart, A., Panitz, C., Horneck, G., von Heise-Rotenburg, R., Hoppenbrouwers, T., Willnecker, R., et al. (2012). EXPOSE-E: An ESA astrobiology mission 1.5 years in space. *Astrobiology* *12*, 374–386.
- Rabbow, E., Rettberg, P., Barczyk, S., Bohmeier, M., Parpart, A., Panitz, C., Horneck, G., Burfeindt, J., Molter, F., Jaramillo, E., et al. (2014). The astrobiological mission EXPOSE-R on board of the International Space Station. *Int. J. Astrobiol.* *14*, 3–16
- Rabbow, E., Parpart, A., and Reitz, G. (2016). The Planetary and Space Simulation Facilities at DLR Cologne. *Microgravity Sci. Technol.* *28*, 215–229.
- Rabbow, E., Rettberg, P., Parpart, A., Panitz, C., Schulte, W., Molter, F., Jaramillo, E., Demets, R., Weiß, P., and Willnecker, R. (2017). EXPOSE-R2: The astrobiological ESA mission on board of the International Space Station. *Int. J. Astrobiol.* *8*.
- Radakovits, R., Jinkerson, R.E., Darzins, A., and Posewitz, M.C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryot. Cell* *9*, 486–501.
- Raksajit, W., Satchasataporn, K., Lehto, K., Mäenpää, P., and Incharoensakdi, A. (2012). Enhancement of hydrogen production by the filamentous non-heterocystous cyanobacterium *Arthospira* sp. PCC 8005. *Int. J. Hydrogen Energy* *37*, 18791–18797.
- Rapp, D. (2007). Human missions to Mars: Enabling technologies for exploring the Red Planet (Springer and Praxis Publishing Ltd).
- Reitzer, L. (2003). Nitrogen assimilation and global regulation in *Escherichia coli*. *Annu. Rev. Microbiol.* *57*, 155–176.
- Rettberg, P., Eschweiler, U., Strauch, K., Reitz, G., Horneck, G., Wänke, H., Brack, A., and Barbier, B. (2002). Survival of microorganisms in space protected by meteorite material:

- results of the experiment “EXOBIOLOGIE” of the PERSEUS mission. *Adv. Sp. Res.* **30**, 1539–1545.
- Rieder, R. (1997). The chemical composition of martian soil and rocks returned by the Mobile Alpha Proton X-ray spectrometer: Preliminary results from the X-ray mode. *Science* **278**, 1771–1774.
- Ro, D.-K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., et al. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440**, 940–943.
- Roach, L.H., Mustard, J.F., Murchie, S., Langevin, Y., Bibring, J.-P., Bishop, J.; Bridges, N., Brown, A., Byrne, S., Ehlmann, B.L., Herkenhoff, K., et al. (2007). CRISM Spectral Signatures of the North Polar Gypsum Dunes. In *Lunar and Planetary Science XXXVIII*, LPI Contribution No. 1338, (League City, Texas), p. 1970.
- Roldán, M., Thomas, F., Castel, S., Quesada, A., and Hernández-Mariné, M. (2004). Noninvasive pigment identification in single cells from living phototrophic biofilms by confocal imaging spectrofluorometry. *Appl. Environ. Microbiol.* **70**, 3745–3750.
- Rothschild, L.J., and Cockell, C.S. (1999). Radiation: microbial evolution, ecology, and relevance to Mars missions. *Mutat. Res. Mol. Mech. Mutagen.* **430**, 281–291.
- Rothschild, L.J. (2016). Synthetic biology meets bioprinting: enabling technologies for humans on Mars (and Earth). *Biochem. Soc. Trans.* **44**, 1158–1164.
- Rothschild, L.J., and Mancinelli, R.L. (2001). Life in extreme environments. *Nature* **409**, 1092–1101.
- Ruder, W.C., Lu, T., and Collins, J.J. (2011). Synthetic biology moving into the clinic. *Science* **333**, 1248–1252.
- Rull, F., Maurice, S., Hutchinson, I., Moral, A., Perez, C., Diaz, C., Colombo, M., Belenguer, T., Lopez-Reyes, G., and Sansano, A. (2017). The Raman Laser Spectrometer for the ExoMars rover mission to Mars. *Astrobiology* **17**, 627–654.
- Salisbury, F., Gitelson, J., and Lisovsky, G. (1997). Bios-3 : Siberian Experiments in Bioregenerative Life Support. *Bioscience* **47**, 575–585.
- Scalzi, G., Selbmann, L., Zucconi, L., Rabbow, E., Horneck, G., Albertano, P., and Onofri, S. (2012). LIFE Experiment: Isolation of Cryptoendolithic Organisms from Antarctic colonized sandstone exposed to space and simulated Mars conditions on the International Space Station. *Orig. Life Evol. Biosph.* **42**, 253–262.
- Schirmack, J., Böhm, M., Brauer, C., Löhmannsröben, H.-G., de Vera, J.-P., Möhlmann, D., and Wagner, D. (2014). Laser spectroscopic real time measurements of methanogenic activity under simulated Martian subsurface analog conditions. *Planet. Space Sci.* **98**, 198–204.
- Schopf, J.W. (2002). The fossil record: Tracing the roots of the cyanobacterial lineage. In *The ecology of cyanobacteria*, M. Potts and B.A. Whitton, ed. (Springer, Netherlands), pp. 13–35.
- Schuerger, A.C. (2015). Ultraviolet irradiation on the surface of Mars: Implications for EVA activities during future human missions. In *Planetary Protection Knowledge Gaps for Human Extraterrestrial Missions*, p. 1011.
- Schuerger, A.C., Mancinelli, R.L., Kern, R.G., Rothschild, L.J., and McKay, C.P. (2003). Survival of endospores of *Bacillus subtilis* on spacecraft surfaces under simulated martian environments: implications for the forward contamination of Mars. *Icarus* **165**, 253–276.

- Schulze-Makuch, D., Fairén, A.G., and Davila, A.F. (2008). The case for life on Mars. *Int. J. Astrobiol.* *7*, 117–141.
- Schulze-Makuch, D. (2013). Organic molecules in lunar ice: A window to the early evolution of life on Earth. In *Habitability of Other Planets and Satellites*, (Springer), pp. 115–125.
- Schulze-Makuch, D., Rummel, J.D., Benner, S.A., Levin, G., Parro, V., and Kounaves, S. (2015). Nearly forty years after Viking: Are we ready for a new life-detection mission? *Astrobiology* *15*, 413–419.
- Schwartzman, D., Caldeira, K., and Pavlov, A. (2008). Cyanobacterial emergence at 2.8 Gya and greenhouse feedbacks. *Astrobiology* *8*, 187–203.
- Sezonov, G., Joseleau-Petit, D., and D'Ari, R. (2007). *Escherichia coli* physiology in Luria-Bertani broth. *J. Bacteriol.* *189*, 8746–8749.
- Shi, L., Günther, S., Hübschmann T., Wick, L.Y., Harms, H., and Müller, S. Limits of propidium iodide as a cell viability indicator for environmental bacteria. *Cytometry A* *71*, 592–598.
- Shields, L.M., Durrell, L.W., and Sparrow, A.H. (1961). Preliminary observations on radiosensitivity of algae and fungi from soils of the Nevada test site. *Ecology* *42*, 440–441.
- Silverstone, S.E., and Nelson, M. (1996). Food production and nutrition in Biosphere 2: results from the first mission September 1991 to September 1993. *Adv. Sp. Res.* *18*, 49–61.
- Silverstone, S., Nelson, M., Alling, A., and Allen, J.P. (2003). Development and research program for a soil-based bioregenerative agriculture system to feed a four person crew at a Mars base. *Adv. Sp. Res.* *31*, 69–75.
- Silverstone, S., Nelson, M., Alling, A., and Allen, J.P. (2005). Soil and crop management experiments in the Laboratory Biosphere: An analogue system for the Mars on Earth® facility. *Adv. Sp. Res.* *35*, 1544–1551.
- Shirkey B., Kovarcik, D.P., Wright, D.J., Wilmoth, G., Prickett., T.F., and Helm, R.F. (2000) Active Fe-containing superoxide dismutase and abundant sodF mRNA in *Nostoc commune* (Cyanobacteria) after years of desiccation. *J Bacteriol* *182*, 189–197.
- Sims, M.R., Cullen, D.C., Bannister, N.P., Grant, W.D., Henry, O., Jones, R., McKnight, D., Thompson, D.P., and Wilson, P.K. (2005). The specific molecular identification of life experiment (SMILE). *Planet. Space Sci.* *53*, 781–791.
- Singh, S. (2014). A review on possible elicitor molecules of cyanobacteria: Their role in improving plant growth and providing tolerance against biotic or abiotic stress. *J. Appl. Microbiol.* *117*, 1221–1244.
- Singh, H., Fernandes, T., and Apte, S.K. (2010). Unusual radioresistance of nitrogen-fixing cultures of *Anabaena* strains. *J. Biosci.* *35*, 427–434.
- Singh, H., Anurag, K., and Apte, S.K. (2013). High radiation and desiccation tolerance of nitrogen-fixing cultures of the cyanobacterium *Anabaena* sp. strain PCC 7120 emanates from genome/proteome repair capabilities. *Photosynth. Res.* *118*, 71–81.
- Sinha, R.P., Kumar, H.D., Kumar, A., and Häder, D.P. (1995). Effects of UV-B irradiation on growth, survival, pigmentation and nitrogen metabolism enzymes in cyanobacteria. *Acta Protozool.* *34*, 187.
- Sinha, R.P., Richter, P., Faddoul, J., Braun, M., and Häder, D.-P. (2002). Effects of UV and visible light on cyanobacteria at the cellular level. *Photochem. Photobiol. Sci.* *1*, 553–559.
- Slade, D., and Radman, M. (2011). Oxidative stress resistance in *Deinococcus radiodurans*. *Microbiol. Mol. Biol. Rev.* *75*, 133–191.

- Smith, H.D., and McKay, C.P. (2005). Drilling in ancient permafrost on Mars for evidence of a second genesis of life. *Planet. Space Sci.* *53*, 1302–1308.
- Smith, H. (2016). Native fluorescence life detection instrument for planetary surfaces. In 41st COSPAR Scientific Assembly, Abstract B0. 6-9-16.
- Smith, H.D., Baqué, M., Duncan, A.G., Lloyd, C.R., McKay, C.P., and Billi, D. (2014a). Comparative analysis of cyanobacteria inhabiting rocks with different light transmittance in the Mojave Desert: a Mars terrestrial analogue. *Int. J. Astrobiol. FirstView*, 1–7.
- Smith, H.D., McKay, C.P., Duncan, A.G., Sims, R.C., Anderson, A.J., and Grossl, P.R. (2014b). An instrument design for non-contact detection of biomolecules and minerals on Mars using fluorescence. *J. Biol. Eng.* *8*, 16.
- Sorenson, J.A. (1986). Perception of radiation hazards. In *Seminars in Nuclear Medicine*, (Elsevier), pp. 158–170.
- Spiller, H., and Gunasekaran, M. (1990). Ammonia-excreting mutant strain of the cyanobacterium *Anabaena variabilis* supports growth of wheat. *Appl. Environ. Microbiol.* *53*, 447–480.
- Squyres, S.W., Arvidson, R.E., Bell, J.F., Calef, F., Clark, B.C., Cohen, B.A., Crumpler, L.A., de Souza, P.A., Farrand, W.H., Gellert, R., et al. (2012). Ancient Impact and Aqueous Processes at Endeavour Crater, Mars. *Science* *336*, 570–576.
- Stevenson, D.J. (2001). Mars's core and magnetism. *Nature* *412*, 214–219.
- Stevenson, B., and Waterbury, J. (2006). Isolation and identification of an epibiotic bacterium associated with heterocystous *Anabaena* cells. *Biol. Bull.* *210*, 73–77.
- Stewart, P.S., and Franklin, M.J. (2008). Physiological heterogeneity in biofilms. *Nat Rev Microbiol* *6*, 199–210.
- Stivalletta, N., Barbieri, R., and Billi, D. (2012). Microbial colonization of the salt deposits in the driest place of the Atacama Desert (Chile). *Orig. Life Evol. Biosph.* *42*, 187–200.
- Stoodley, P., Davies, D.G., and Costerton, J.W. (2002). Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* *56*, 187–209.
- Storrie-Lombardi, M.C., and Sattler, B. (2009). Laser-induced fluorescence emission (LIFE): in situ nondestructive detection of microbial life in the ice covers of Antarctic lakes. *Astrobiology* *9*, 659–672.
- Stromberg, J.M., Applin, D.M., Cloutis, E.A., Rice, M., Berard, G., and Mann, P. (2014). The persistence of a chlorophyll spectral biosignature from Martian evaporite and spring analogues under Mars-like conditions. *Int. J. Astrobiol.* *13*, 203–223.
- Subramanian, G., and Shanmugasundaram, S. (1986). Uninduced ammonia release by the nitrogen-fixing cyanobacterium *Anabaena*. *FEMS Microbiol. Lett.* *37*, 151–154.
- Sukhi, S.S., Shashidhar, R., Kumar, S.A., and Bandekar, J.R. (2009). Radiation resistance of *Deinococcus radiodurans* R1 with respect to growth phase. *FEMS Microbiol. Lett.* *297*, 49–53.
- Summons, R.E., Amend, J.P., Bish, D., Buick, R., Cody, G.D., Des Marais, D.J., Dromart, G., Eigenbrode, J.L., Knoll, A.H., and Sumner, D.Y. (2011). Preservation of martian organic and environmental records: Final report of the Mars Biosignature Working Group. *Astrobiology* *11*, 157–181.
- Sutter, B., Quinn, R.C., Archer, P.D., Glavin, D.P., Glotch, T.D., Kounaves, S.P., Osterloo, M.M., Rampe, E.B., and Ming, D.W. (2016). Measurements of oxychlorine species on Mars. *Int. J. Astrobiol.* 1–15.

- Sychev, V.N., Shepelev, E.Y., Meleshko, G.I., Gurieva, T.S., Levinskikh, M.A., Podolsky, I.G., Dadasheva, O.A., and Popov, V.V. (2001). Main characteristics of biological components of developing life support system observed during the experiments aboard orbital complex MIR. *Adv. Space Res.* *27*, 1529–1534.
- Sychev, V.N., Levinskikh, M.A., and Shepelev, Y.Y. (2003). The biological component of the life support system for a Martian expedition. *Adv. Sp. Res.* *31*, 1693–1698.
- Tarcea, N., Harz, M., Rösch, P., Frosch, T., Schmitt, M., Thiele, H., Hochleitner, R., and Popp, J. (2007). UV Raman spectroscopy—A technique for biological and mineralogical in situ planetary studies. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* *68*, 1029–1035.
- Taylor, G.R., Spizizen, J., Foster, B.G., Volz, P.A., Bücker, H., Simmonds, R.C., Heimpel, A.M., and Benton, E. V (1974). A descriptive analysis of the Apollo 16 microbial response to space environment experiment. *Bioscience* *505*–511.
- Taylor, S.R., and McLennan, S.M. (2009). Planetary Crusts: Their Composition, Origin and Evolution (Cambridge: Cambridge University Press).
- Thomas, D., Sullivan, S., Sprice, A., and Zimmerman, S. (2005). Common freshwater cyanobacteria grow in 100% CO₂. *Astrobiology* *5*, 66–74.
- Thomas, D.J., Boling, J., Boston, P.J., Campbell, K.A., McSpadden, T., McWilliams, L., and Todd, P. (2006). Extremophiles for ecopoiesis : Desirable traits for and survivability of pioneer Martian organisms. *Gravitational Sp. Biol.* *19*, 91–104.
- Trevors, J.T. (2012). Can dead bacterial cells be defined and are genes expressed after cell death? *J Microbiol Meth.* *90*, 25–8.
- Tikhomirov, A.A., Ushakova, S.A., Kovaleva, N.P., Lamaze, B., Lobo, M., and Lasseur, C. (2007). Biological life support systems for a Mars mission planetary base: Problems and prospects. *Adv. Sp. Res.* *40*, 1741–1745.
- Tokano, T. (ed.) (2005). Water on Mars and Life. Springer, Berlin.
- Trampuz, A., Piper, K.E., Steckelberg, J.M., and Patel, R. (2006). Effect of gamma irradiation on viability and DNA of *Staphylococcus epidermidis* and *Escherichia coli*. *J. Med. Microbiol.* *55*, 1271–1275.
- NASA (2017). National Aeronautics and Space Administration Transition Authorization Act of 2017. 115th Congress of the USA.
- Vago, J.L., Westall, F., Pasteur Instrument Teams, L.S.S.W.G., Coates, A.J., Jaumann, R., Koralev, O., Ciarletti, V., Mitrofanov, I., Josset, J.-L., De Sanctis, M.C., et al. (2017). Habitability on Early Mars and the search for biosignatures with the ExoMars Rover. *Astrobiology* *17*, 471–510.
- Vandenabeele, P., Jehlička, J., Vítek, P., and Edwards, H.G.M. (2012). On the definition of Raman spectroscopic detection limits for the analysis of biomarkers in solid matrices. *Planet. Space Sci.* *62*, 48–54.
- Vaniman, D.T., Bish, D.L., Ming, D.W., Bristow, T.F., Morris, R. V, Blake, D.F., Chipera, S.J., Morrison, S.M., Treiman, a H., Rampe, E.B., et al. (2014). Mineralogy of a mudstone at Yellowknife Bay, Gale crater, Mars. *Science* *343*, 1243480.
- de Vera, J.-P., Boettger, U., Noetzel, R.D.L.T., Sánchez, F.J., Grunow, D., Schmitz, N., Lange, C., Hübers, H.-W., Billi, D., Baqué, M., et al. (2012). Supporting Mars exploration: BIOMEX in low Earth orbit and further astrobiological studies on the Moon using Raman and PanCam technology. *Planet. Space Sci.* *74*, 103–110.

- de Vera, J.-P., Schulze-Makuch, D., Khan, A., Lorek, A., Koncz, A., Möhlmann, D., and Spohn, T. (2014). Adaptation of an Antarctic lichen to Martian niche conditions can occur within 34 days. *Planet. Space Sci.* *98*, 182–190.
- Verseux, C., Baqué, M., Lehto, K., de Vera, J.-P.P., Rothschild, L.J., and Billi, D. (2016a). Sustainable life support on Mars – the potential roles of cyanobacteria. *Int. J. Astrobiol.* *15*, 65–92.
- Verseux, C., Paulino-Lima, I., Baqué, M., Billi, D., and Rothschild, L. (2016b). Synthetic Biology for Space Exploration: Promises and Societal Implications. In *Ambivalences of Creating Life. Societal and Philosophical Dimensions of Synthetic Biology*, K. Hagen, M. Engelhard, and G. Toepfer, eds. (Springer-Verlag), pp. 73–100.
- Verseux, C., Baqué, M., Cifariello, R., Fagliacone, C., Raguse, M., Moeller, R., and Billi, D. (2017). Evaluation of the resistance of *Chroococcidiopsis* spp. to sparsely and densely ionizing irradiation. *Astrobiology* *17*, 118–125.
- Vítek, P., Jehlička, J., Edwards, H.G.M., and Osterrothová, K. (2009a). Identification of β-carotene in an evaporitic matrix—evaluation of Raman spectroscopic analysis for astrobiological research on Mars. *Anal. Bioanal. Chem.* *393*, 1967–1975.
- Vítek, P., Osterrothová, K., and Jehlička, J. (2009b). Beta-carotene—A possible biomarker in the Martian evaporitic environment: Raman micro-spectroscopic study. *Planet. Space Sci.* *57*, 454–459.
- Vítek, P., Edwards, H.G.M., Jehlička, J., Ascaso, C., Ríos, A.D.L., Valea, S., Jorge-Villar, S.E., Davila, A.F., and Wierzchos, J. (2010). Microbial colonization of halite from the hyper-arid Atacama Desert studied by Raman spectroscopy. *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* *368*, 3205–3221.
- Wacey, D., Saunders, M., Roberts, M., Menon, S., Green, L., Kong, C., Culwick, T., Strother, P., and Brasier, M.D. (2013). Enhanced cellular preservation by clay minerals in 1 billion-year-old lakes. *Sci. Rep.* *4*, 5841.
- Wadsworth, J., and Cockell, C.S. (2017). Perchlorates on Mars enhance the bacteriocidal effects of UV light. *Sci. Rep.* *7*, 4662.
- Wainwright, M., Wickramasinghe, N.C., Narlikar, J. V., and Rajaratnam, P. (2003). Microorganisms cultured from stratospheric air samples obtained at 41 km. *FEMS Microbiol. Lett.* *218*, 161–165.
- Wamelink, G.W.W., Frissel, J.Y., Krijnen, W.H.J., Verwoert, M.R., and Goedhart, P.W. (2014). Can plants grow on Mars and the moon: a growth experiment on Mars and moon soil simulants. *PLoS One* *9*, e103138.
- Wang, G., Chen, H., Li, G., Chen, L., Li, D., Hu, C., Chen, K., and Liu, Y. (2006). Population growth and physiological characteristics of microalgae in a miniaturized bioreactor during space flight. *Acta Astronaut.* *58*, 264–269.
- Wang, G., Li, G., Li, D., Liu, Y., Song, L., Tong, G., Liu, X., and Cheng, E.. (2004). Real-time studies on microalgae under microgravity. *Acta Astronaut.* *55*, 131–137.
- Warren-Rhodes, K.A., McKay, C.P., Boyle, L.N., Wing, M.R., Kiekebusch, E.M., Cowan, D.A., Stomeo, F., Pointing, S.B., Kaseke, K.F., Eckardt, F., et al. (2013). Physical ecology of hypolithic communities in the central Namib Desert: The role of fog, rain, rock habitat, and light. *J. Geophys. Res. Biogeosciences* *118*, 1451–1460.
- Wassmann, M., Moeller, R., Reitz, G., and Rettberg, P. (2011). Growth phase-dependent UV-C resistance of *Bacillus subtilis*: data from a short-term evolution experiment. *Arch. Microbiol.* *193*, 823–832.

- Way, J.C., Silver, P.A., and Howard, R.J. (2011). Sun-driven microbial synthesis of chemicals in space. *Int. J. Astrobiol.* *10*, 359–364.
- Weinstein, S., Pane, D., Ernst, L.A., Warren-Rhodes, K., Dohm, J.M., Hock, A.N., Piatek, J.L., Emani, S., Lanni, F., Wagner, M., et al. (2008). Application of pulsed-excitation fluorescence imager for daylight detection of sparse life in tests in the Atacama Desert. *J. Geophys. Res. Biogeosciences* *113*, G1.
- Weiss, B.P., Kirschvink, J.L., Baudenbacher, F.J., Vali, H., Peters, N.T., Macdonald, F.A., and Wikswo, J.P. (2000). A low temperature transfer of ALH84001 from Mars to Earth. *Science* *290*, 791–795.
- Westall, F., Loizeau, D., Foucher, F., Bost, N., Bertrand, M., Vago, J., and Kminek, G. (2013). Habitability on Mars from a microbial point of view. *Astrobiology* *13*, 887–897.
- Westall, F., Foucher, F., Bost, N., Bertrand, M., Loizeau, D., Vago, J.L., Kminek, G., Gaboyer, F., Campbell, K.A., Bréhéret, J.-G., et al. (2015). Biosignatures on Mars: what, where, and how? Implications for the search for Martian life. *Astrobiology* *15*, 998–1029.
- Wheeler, R.M. (2004). Horticulture for Mars. In ISHS Acta Horticulturae 642, N.E. Looney, ed. (Toronto, Canada), pp. 201–215.
- Wierzchos, J., Ascaso, C., and McKay, C.P. (2006). Endolithic cyanobacteria in halite rocks from the hyperarid core of the Atacama Desert. *Astrobiology* *6*, 415–422.
- Wilson, J.W., Ott, C.M., Höner zu Bentrup, K., Ramamurthy, R., Quick, L., Porwollik, S., Cheng, P., McClelland, M., Tsaprailis, G., Radabaugh, T., et al. (2007). Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. *Proc. Natl. Acad. Sci.* *104*, 16299–16304.
- Winters, Y.D., Lowenstein, T.K., and Timofeeff, M.N. (2013). Identification of carotenoids in ancient salt from Death Valley, Saline Valley, and Searles Lake, California, using laser Raman spectroscopy. *Astrobiology* *13*, 1065–1080.
- Wynn-Williams, D.D., and Edwards, H.G.M. (2000). Proximal analysis of regolith habitats and protective biomolecules in situ by laser Raman spectroscopy: Overview of terrestrial Antarctic habitats and Mars analogs. *Icarus* *144*, 486–503.
- Xiao, Y., Liu, Y., Wang, G., Hao, Z., and An, Y. (2010). Simulated microgravity alters growth and microcystin production in *Microcystis aeruginosa* (cyanophyta). *Toxicon* *56*, 1–7.
- Yang, C., Liu, H., Li, M., Yu, C., and Yu, G. (2008). Treating urine by *Spirulina platensis*. *Acta Astronaut.* *63*, 1049–1054.
- Zaets, I., Burlak, O., Rogutskyy, I., Vasilenko, a., Mytrokhyn, O., Lukashov, D., Foing, B., and Kozyrovska, N. (2011). Bioaugmentation in growing plants for lunar bases. *Adv. Sp. Res.* *47*, 1071–1078.
- Yen, A.S., Kim, S.S., Hecht, M.H., Frant, M.S., and Murray, B. (2000). Evidence that the reactivity of the martian soil is due to superoxide ions. *Science* *289*, 1909–1912.
- Zhang, F., Rodriguez, S., and Keasling, J.D. (2011). Metabolic engineering of microbial pathways for advanced biofuels production. *Curr. Opin. Biotechnol.* *22*, 775–783.
- Zhukov-Verezhnikov, N.N., Maiskii, I.N., Yazdovskii, V.I., Pekhov, A.P., Gyurdzhian, A.A., Nefed'eva, N.P., Kapichnikov, M.M., Podoplelov, I.I., Rybakov, N.I., Klemparskaya, N.N., et al. (1962). Results of first microbiological and cytological experiments on Earth satellites in space. *Artif. Earth Satell.* *11*, 47–71.
- Zubrin, R., and Wagner, R. (1996). The Case for Mars: The Plan to Settle the Red Planet and Why We Must (New York: Free Press).

Zubrin, R.M., Baker, D.A., and Gwynne, O. (1991). Mars Direct : A Simple, Robust, and Cost Effective Architecture for the Space Exploration Initiative. In 29th Aerospace Sciences Meeting, AIAA 91-0329.

LIST OF FIGURES

Figure 1.1 Phase diagram showing water's state as a function of pressure and temperature. The "Mars range" and "Earth range" boxes indicate the ranges of pressure and temperature found on Mars's and Earth's surfaces, respectively. While large amounts of water in liquid and solid states can be found on Earth's surface, liquid water is unstable on Mars's surface due to its low atmospheric pressures.

-15-

Figure 1.2 Rover of the ExoMars mission, a project involving the European (ESA) and Russian (Roscosmos) space agencies. Expected to be launched to Mars in 2020, its main scientific objectives will be to search for signs of life, study the evolution of Mars's geochemical environment, and track trace gases in the Martian atmosphere. Image: ESA. Available at exploration.esa.int/mars/53-910-exomars-rover (consulted on February 26th, 2018).

-19-

Figure 1.3 Electron micrograph of ultrathin section of a cell aggregate of *Chroococcidiopsis* sp. CCME 029, a cyanobacterium species used in a large fraction of the work presented here. The arrow points toward a division septum. Scale bar: 0.5 μm . Image from Billi et al. (2000).

-24-

Figure 1.4 Location of the EXPOSE-R2 platform, on the external platform of the Svezda module of the International Space Station. The structure visible to the right is solar panels, attached on their left side to the Station's main structure. The Earth and Sun are visible in the background. Image: NASA. Available at expose.cnes.fr (consulted on February 28th, 2018).

-33-

Figure 1.5 EXPOSE-R2 outside the International Space Station, during extravehicular activities. A cosmonaut and the Earth are visible in the background. Image: NASA. Available at eea.spaceflight.esa.int/attachments/spacestations/545a48ba741bf.jpg (consulted on February 27th, 2018).

-38-

Figure 2.1 Size distribution of the mineral mixtures determined by sieving. P-MRS has a smaller grain size, with a large fraction below 200 µm while S-MRS grains are more evenly distributed in the 0–1 mm range.

-46-

Figure 2.2 PCR-based evaluation of DNA persistence in dried *Chroococcidiopsis* under polychromatic UV and Mars-like atmosphere, after the “Mars” series of experiments, by random amplification of polymorphic DNA (RAPD) assay. A: PCR fingerprint from liquid culture (lane 1), altered fingerprint from dried cells without minerals exposed to 570 kJ/m² of UV and simulated Martian atmosphere (lane 2), and altered fingerprints from dried cells exposed to 570 MJ/m² of UV and simulated Martian atmosphere, and mixed with S-MRS (lane 3) or P-MRS (lane 4). B: PCR fingerprint from liquid culture (lane 1), and unaltered PCR fingerprint from dried cells exposed to simulated Martian atmosphere without minerals (lane 2) or in the presence of S-MRS (lane 3) or P-MRS (lane 4). Lanes M: DNA marker.

-52-

Figure 2.3 PCR-based evaluation of DNA persistence in dried *Chroococcidiopsis* sp. CCME 057, non-irradiated (NI), or exposed to doses of He ions or Fe ions up to 1 and 2 kGy (respectively), in the framework of the “Starlife” project, by random amplification of polymorphic DNA (RAPD) assay. Similarly unaltered band profiles were obtained for *Chroococcidiopsis* spp. CCME 057 and 029 exposed to all tested heavy ion and X-ray treatments (not shown). M: DNA ladder.

-54-

Figure 2.4 PCR-based evaluation of DNA persistence in dried *Chroococcidiopsis* spp. CCME 029 and 057, non-irradiated (NI) or exposed to γ irradiation, in the framework of the “Starlife” project, by random amplification of polymorphic DNA (RAPD) assay. Doses indicated above the lanes are those targeted prior to performing irradiation; the doses actually reaching samples were 11.59, 46.88, or 113.25 kGy. Similarly altered patterns were obtained from irradiated liquid samples (not shown). M: DNA ladder.

-55-

Figure 2.5 Confocal scanning laser microscope (CLSM) images with lateral projections showing mineral reflectance (white color; emission in the 490– 510 nm range after excitation at 488 nm) and autofluorescence of chlorophyll *a* (emission in the 555–609 nm range after excitation at 543 nm) and phycobiliprotein (emission in the 655–755 nm range after excitation at 635 nm) of dried *Chroococcidiopsis* sp. CCMEE 029 cells mixed with S-MRS (A, B) or P-MRS (C, D), after exposure to simulated Martian atmosphere alone (B, D) or in combination with 570 MJ/m² of polychromatic UV (A, C), as part of the “Mars” series of experiments. Scale bar: 10 µm.

-56-

Figure 2.6 Photosynthetic pigment autofluorescence in *Chroococcidiopsis* sp. CCMEE 029 from the “Mars” series of experiments, as revealed by CLSM-λscan performed by exciting samples with a 543-nm laser at 0.54 mW and collecting emission from 543 to 800 nm. Analyzed *Chroococcidiopsis* cells were either from liquid culture controls or dried cells mixed with P-MRS (A) or S-MRS (B) mineral analogues, unexposed (control) or exposed to all simulated Martian conditions (570 MJ/m² polychromatic UV and simulated Martian atmosphere) or simulated Martian atmosphere alone. Data points represent normalized fluorescence intensity at 653 nm ± standard error, for n ≥ 15 cells, as a function of emission wavelength.

-59-

Figure 2.7 Reflectance of the P-MRS (orange) and S-MRS (blue) mineral analogues, as revealed by CLSM-λscan performed by hitting minerals on a glass slide with 488-nm (plain lines) and 543-nm (dashed lines) lasers at 1 mW, and collecting emission of regions of interest from 488 to 800 nm and from 543 to 800 nm, respectively.

-60-

Figure 2.8 Damage to plasma membranes after 5 kGy of X-ray irradiation, as revealed by SYTOX Green. First row: *Chroococcidiopsis* sp. CCMEE 029 cells boiled for 15 min (A), non-irradiated, dried cells (B), dried, irradiated cells (C), and non-irradiated liquid samples (D). Second row: *Synechocystis* sp. PCC 6803 dried cells (E), dried, irradiated cells (F), and non-irradiated liquid samples (G). Photosynthetic pigments appear red, and cells with damaged membranes show green fluorescent nucleoids. Scale bar: 10 µm.

-61-

Figure 3.1 Location of *Chroococcidiopsis* samples part of the BOSS experiment in the EXPOSE-R2 facility. “Space” samples were located in Tray 1, compartments 3 and 4, and “Mars” samples in Tray 2, compartments 3 and 4, according to the EXPOSE-R2 terminology. B: biofilm; P: planktonic; 029, 057, 064: *Chroococcidiopsis* strains CCMEE 029, CCMEE 057 and CCMEE 064 (respectively). Each sample location had stacked sample carriers; samples in top carriers (t) were exposed to UV radiation, while samples in bottom carriers (b) were shielded from it. Compartment 4 of both trays also had a middle sample carrier, not shown here.

-79-

Figure 3.2 DNA damage in planktonic (P) and biofilm (B) samples of *Chroococcidiopsis* sp. CCMEE 064 (a), 029 (b), and 057 (c), as revealed by qPCR. For *Chroococcidiopsis* sp. CCMEE 064, only planktonic samples were included in the experiment. Bars show the number of amplifiable copies of the intact (amplifiable) target gene (encoding the 16S rRNA), normalized to the total mass of extracted DNA, for each sample. Data is shown as averages \pm standard deviations. Lab: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; GR: exposed to ground-based simulations; Space: exposed to space or space-like conditions; Mars: exposed to Mars-like conditions; L: liquid culture; P: planktonic, dried sample; B: biofilm, dried sample; t: exposed in the upper sample carrier of a two-layer stack; b: exposed in the lower sample carrier of a two-layer stack. Single asterisks indicate significant differences ($p < 0.05$) between planktonic and biofilm samples, based on planned Student t-tests uncorrected for multiple comparisons. Pairs of asterisks indicate significant differences ($p < 0.05$) after correcting for multiple comparisons using the Bonferroni method.

-86-

Figure 3.3 Confocal laser scanning microscope images of stained EPS in biofilm (b, c) and planktonic (c, d) samples of *Chroococcidiopsis* sp. CCMEE 057, confirming the higher abundance of lipids (but not polysaccharides) in biofilms than in planktonic samples. Lipids were stained using BODIPY FL C12 (a, b) and polysaccharides (α -mannopyranosyl and α -glucopyranosyl residues) using labelled concanavalin A (c, d). Scale bar: 7 μm .

-87-

Figure 3.4 Confocal laser scanning microscopy imaging of phycobiliprotein autofluorescence in biofilms (top row) and planktonic samples (bottom row) of *Chroococcidiopsis* sp. CCMEE 057, with lateral projections showing fluorescence along a plane perpendicular to the main images. Samples were excited at 635 nm, and emission was collected between 655 and 755 nm. Lab control: stored in the dried state in the laboratory during the mission; Flight Mars top: exposed to Mars-like conditions in the upper sample carrier of a two-layer stack; Flight Mars bottom: exposed to Mars-like conditions in the lower sample carrier of a two-layer stack; T: top part of the sample; B: bottom part of the sample. Scale bar: 10 μm .

-88-

Figure 3.5 Peak intensity (average fluorescence emission intensity at the CLSM optical section where it is maximum) of phycobiliprotein autofluorescence, as percentage of planktonic laboratory samples, in *Chroococcidiopsis* sp. CCMEE 057. Lab: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; Space: exposed to space or space-like conditions; Mars: exposed to Mars-like conditions; P: planktonic, dried sample; B: biofilm, dried sample; t: exposed in the upper sample carrier of a two-layer stack; b: exposed in the lower sample carrier of a two-layer stack. Similar results were obtained for chlorophyll a autofluorescence (not shown). Data is shown as averages \pm standard deviation. Asterisks indicate significant differences ($p < 0.05$) with the respective (P or B) Lab control, based on Dunnett multiple comparison tests.

-89-

Figure 4.1 Locations of the *Chroococcidiopsis* sp. CCMEE 029 samples loaded to the EXPOSE-R2 facility for the BIOMEX experiment. Cells were dried and mixed with a Moon regolith analogue (Lu), sandstone (Sa), or one of both Mars regolith analogues: S-MRS (S-M) or P-MRS (P-M). Results reported in this work pertain to samples in the “Mars” tray. Those were exposed to LEO ionizing radiations, extreme temperature cycles, a Mars-like atmosphere and, for samples in upper carriers (t), UV at the Martian range ($\lambda > 200$ nm). Samples in bottom carriers (b) were shielded from UV. Samples without minerals used as controls belong to another experiment (Billi et al., in preparation) and were located in the rightmost compartment of Tray 2.

-107-

Figure 4.2 DNA fingerprints obtained after random amplification of polymorphic DNA (RAPD) assay, from *Chroococcidiopsis* sp. CCMEE 029 cells without mineral (NM) or with mineral mixtures (P-MRS or S-MRS). The gel is distorted and similar patterns, from one lane to another, are of similar size (no steady reduction in fragments' size from left to right). M: DNA ladder; L: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; GR: exposed to ground-based simulations; t: exposed in the top-layer carrier of a two-layer stack; b: exposed in the bottom-layer carrier of a two-layer stack; P-MRS: mixed with the P-MRS regolith simulant; S-MRS: mixed with the S-MRS regolith simulant; NM: mixed with no mineral.

-112-

Figure 4.3 Heterogeneous association of cells with Mars regolith analogues, as revealed by confocal scanning laser microscope imaging. Minerals are visible through their reflectance (emission between 490 and 510 nm after excitation at 488 nm; shown in white) and dried *Chroococcidiopsis* sp. CCMEE 029 cells through the autofluorescence of phycobiliproteins and chlorophyll *a* (after successively exciting pigments with 543-nm and 635-nm lasers and collecting the emitted fluorescence in the 555–609 and 655–755 nm ranges, respectively; shown in orange) mixed with S-MRS (A) or P-MRS (B). Scale bar: 20 μ m.

-113-

Figure 4.4 Autofluorescence of photosynthetic pigments in dried *Chroococcidiopsis* sp. CCMEE 029, mixed or not with the P-MRS (A) or S-MRS (B) mineral mixtures, after excitation with a 543-nm laser at 0.54 mW. Samples were exposed (Flight and GR samples) or not (Lab samples) to Mars-like atmosphere, in combination (top samples) or not to UV. Lab: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; GR: exposed to ground-based simulations; top: exposed in top-layer carrier of a two-layer stack; bottom: exposed in the bottom-layer carrier of a two-layer stack; P-MRS: mixed with the P-MRS regolith simulant; S-MRS: mixed with the S-MRS regolith simulant; No mineral: mixed with no mineral. Data points represent fluorescence intensity normalized to the laboratory control without minerals (Lab, no mineral) at 653 nm. Values are average \pm standard error for $n \geq 15$ cells.

-114-

Figure 4.5 Phycobiliprotein autofluorescence in *Chroococcidiopsis* sp. CCME 029 mixed with P-MRS or S-MRS mineral mixtures, as revealed by CLSM imaging. Lab control: stored in the laboratory during the mission; Flight top: exposed to space conditions, including UV (top-layer carrier), in LEO; Flight bottom: exposed to space conditions, without UV (bottom-layer carrier), in LEO.

-115-

Figure 4.6 Fluorescence emission maxima at 648 nm (or 653 nm if indicated by an asterisk in the table) of photosynthetic pigments in *Chroococcidiopsis* sp. CCME 029, after excitation with a 543-nm laser at 0.54 mW. Those values correspond to peak values of curves shown in Figure 4.4. Lab: stored in the dried state in the laboratory during the mission; Flight: exposed in LEO, in the EXPOSE-R2 facility; GR: exposed to ground-based simulations; top: exposed in the top-layer carrier of a two-layer stack; bottom: exposed in the bottom-layer carrier of a two-layer stack; P-MRS: mixed with the P-MRS regolith simulant; S-MRS: mixed with the S-MRS regolith simulant; No mineral: mixed with no mineral. Two bars are indicated for P-MRS samples and correspond to two areas with differently preserved autofluorescence. Data are expressed as percentage of the emission intensity maximum of the Lab no mineral sample (\pm standard error for $n \geq 15$ cells).

-117-

Figure 5.1 Artist's rendering of a cyanobacterium-based biological life-support system on Mars. Artistic work by Sean McMahon. Reproduced from Verseux et al. (2016a).

-132-

Figure 5.2 Simplified overview of the potential roles of synthetic biology in the development of Mars-specific, cyanobacterium-based BLSS. Reproduced from Verseux et al. (2016a).

-165-

Figure 6.1 Growth of *Anabaena* sp. PCC7120 in Mars regolith simulant. Chl *a* concentrations at inoculation time (To) and after 6 weeks in bidistilled water with no regolith simulant (H_2O) or with Orbitec's Mars-1A regolith simulant (1, 2 or 5 g·30 ml⁻¹).

-174-

Figure 6.2 Growth of *Escherichia coli* W in PBS or cyanobacterium-based medium (see text for details) at concentrations before filtration ranging from 5 g·l⁻¹ (CM5) to 25 g·l⁻¹ (CM25).

-175-

Figure 6.3 Growth of *E. coli* MG1655, *E. coli* W, *B. subtilis* 168, and *B. subtilis* SCK6 in PBS, LB broth or cyanobacterium-based medium at 25 g·l⁻¹ before filtration (CM25).

-176-

Figure 6.4 Effect of perchlorate solution on the growth of *Anabaena* sp. PCC7120 (A) and its lack of negative effect if subsequent used as a substrate for growing *E. coli* W (B). CM25: cyanobacterium-based medium at 25 g·l⁻¹ before filtration; CM25-P: cyanobacterium-based medium at 25 g·l⁻¹ before filtration (CM25) prepared from cyanobacteria grown in perchlorate solution.

-179-

Figure S1 The Progress 56 spacecraft, carrying EXPOSE-R2, arriving at the International Space Station. Picture by Oleg Germanovich Artemyev (Roscosmos).

-188-

LIST OF TABLES

Table 1.1	Comparison of some of the environmental parameters on Mars and Earth's surfaces (modified from Graham [2004]) and Kanervo et al. [2005], extended with ionizing radiation data from Hassler et al. [2013] and Cockell et al. [2000]). More details, and implications for microorganisms, are given in the text.	-14-
Table 1.2	Examples of astrobiology-relevant environmental conditions survived by <i>Chroococcidiopsis</i> spp.	-25-
Table 1.3	Data illustrating the resistance of cyanobacteria to ionizing radiation, in perspective with dose rates on Mars's surface. See text for more details.	-27-
Table 1.4	Space exposure missions including cyanobacteria and performed (or started) prior to EXPOSE-R2. See text for details.	-32-
Table 2.1	Mineralogical composition of the S-MRS and P-MRS analogues of Martian regolith. The importance of each component is given, for both P-MRS and S-MRS, in weight percent of the analogue (modified from Böttger et al. 2012).	-45-
Table 2.2	Parameters obtained using the Planetary and Space Simulation facilities at DLR (Cologne, Germany) for ground-based simulations preformed in preparation for BIOMEX, and reported in this chapter (modified from version published in Baqué et al., 2014).	-47-
Table 2.3	Parameters of the four irradiation series reported in this study. All irradiations were performed as part of the "Starlife" project, except for irradiation with Si ions. C: <i>Chroococcidiopsis</i> ; S: <i>Synechocystis</i> . LET: linear energy transfer.	-48-
Table 2.4	Fluorescence emission at 563 nm and 653 nm of photosynthetic pigments in <i>Chroococcidiopsis</i> sp. CCME 029 cells from liquid culture and dried cells mixed with P-MRS or S-MRS, non-exposed (controls) or exposed to Mars simulations. Data are expressed as percent of the emission intensity at 653 nm of liquid culture (\pm standard error for $n \geq 15$ cells).	-59-

Table 3.1 Overview of exposure conditions. Flight: low Earth orbit (LEO), in the EXPOSE-R2 facility; MGR: ground-based facilities (mission ground reference); Lab: closet in the laboratory; Space: space (in LEO) or space-like conditions (in MGR); Mars: Mars-like conditions; top: upper sample carrier of a two-layer stack; bottom: lower sample carrier of a two-layer stack.

-82-

Table 3.2 Survival of desert strains of *Chroococcidiopsis* spp. following exposure in low Earth orbit (LEO) or ground-based simulations, or laboratory storage. Flight: exposed in LEO; GR: exposed to ground-based simulations; Lab: stored in the dried state in the laboratory during the mission; Space: exposed to space or space-like conditions; Mars: exposed to Mars-like conditions; top: exposed in the upper sample carrier of a two-layer stack (exposed to UV); bottom: exposed in the lower sample carrier of a two-layer stack (kept in the dark); ++ (green): colonies appeared after plating approximately 10^6 cells; + (orange): growth was observed after inoculating growth medium with circa 2×10^7 cells; - (red): no growth was observed.

-84-

Table 4.1 Mineralogical composition of the Phyllosilicate Mars Regolith Simulant (P-MRS) and the Sulfatic Mars Regolith Simulant (S-MRS). Modified from Böttger et al. (2012).

-105-

Table 4.2 Colony forming abilities of *Chroococcidiopsis* sp. CCMEE 029 samples following exposure to Mars-like conditions in low Earth orbit (Flight samples) and ground-based simulation (GR samples), or after storage in laboratory conditions (Lab). top: exposed in the top-layer carrier of a two-layer stack; bottom: exposed in the bottom-layer carriers of a two-layer stack; P-MRS: mixed with the P-MRS regolith simulant; S-MRS: mixed with the S-MRS regolith simulant; No mineral: mixed with no mineral; ++ (green): colonies appeared after plating approximately 10^6 cells; + (orange): growth was observed after inoculating growth medium with circa 2×10^7 cells; - (red): no growth was observed.

-111-

Table 5.1 Main sources of nutrients for cyanobacterium-based biological processes on Mars. Reproduced from Verseux et al. (2016b).

-131-

Table 6.1 Composition of the cyanobacterium-based medium. Values are given for a medium prepared with 25 g l^{-1} of biomass before filtration. When not specified otherwise, data was obtained by mass spectrometry.

-177-

ACKNOWLEDGEMENTS

A few years ago, as a Master's student, I emailed a famous researcher at NASA with the arrogant hope of doing an internship there. I was not even graduated and had very little to show as accomplishments. To my excitement and—I have to admit it—surprise, I had the honor of being opened the door to what I considered the most exciting lab in the world. Thank you, Lynn Rothschild, for your trust, for your mentorship, and for ushering me into the fascinating world of astrobiology.

I quickly understood that I wanted to keep carrying research in this field. I had read about a renowned Italian astrobiologist, whose projects were particularly exciting to me, and decided to try and carry a PhD in her lab. I had, again, the great surprise of being welcomed in spite of having little experience in the field and being unknown to her. Thank you, Daniela Billi. In your lab, I learned more about astrobiology than I could have hoped. Thank you as well for integrating me into the European astrobiology community. Lynn, Daniela, thank you for supervising me throughout those years. Thank you as well for putting up with my eccentricities. I know that the year I spent in a dome, in particular, had a cost for the lab; thank you for letting me grasp those unique opportunities anyway.

Moving into countries where you don't know anyone can be tough, but I had the chance of almost never feeling alone. Thank you Ivan, Kosuke, Kira, Ryan, Griffin, Jess, Evie, Jesica, the Rainbow community (with a special nod for Mike, Diana, Sean, and Andrea), and all of you who welcomed me to California when I was a lost kid with a weird accent. Thank you as well to Giorgia, Andrea, Marzia, Bruno, Alessandro, Emmanuele, and Riccardo, for your friendship in Rome. Thank you Claudia F, Claudia M, Clelia, Ilaria, Lisa, Serena, Francesca, Gianmarco, and all the others who came and went to the Billi's lab. Your cheerfulness was, at numerous times, of great support. I also much enjoyed the company of people from the surrounding labs: Bale, Fale, Laura, Angelo, Gabriele, and the many others. Still at Tor Vergata, I would

like to acknowledge the skilled assistance of Elena Romano and Emmanuela Viaggiu for the confocal analyses.

If you are not in the list above, it is not because you would have been forgotten, but because you deserve your own paragraph. Thank you, Mika, for welcoming me to Rome. Thanks for dragging me in front of movies and saving my culture, thanks for reminding me to rest and not take things too seriously, thanks for introducing me to people and places. Having you in the lab made everything much, much easier and, above all, pleasant and fun. I could not have hoped for a better co-PhD student, and I much value our friendship.

While the last time I have lived in France was more than four years ago, I have the undeserved luck of remaining close to fellow French people who, in spite of the distance and my (healing) tendency to neglect what is not related to work, preserved our friendship. Thank you to all of you, and particularly to Jérôme, Louis, and Yohann.

Taking part in the EXPOSE-R2 project was a unique opportunity. I am grateful to all of the people who welcomed me as a team member, and especially Jean-Pierre de Vera for his kindness, for welcoming me at DLR for Raman analyses, and for genuinely caring about the future of early-career astrobiologists.

Thanks to all of you who, although you were under absolutely no obligation to do so, provided advice and mentorship. Special thanks to Winfried Römer, Andrew Tolonen, and Rocco Mancinelli. Kirsi, thank you for your warm welcome in Turku. Silvano, thank you so much for your help in preparing my next steps. I owe you a lot; without you, I would not be finishing this section from Concordia Station, Antarctica. Thank you, Manuela Citterich, for your empathy and tutorship.

One of the past four years happened at HI-SEAS IV. I consequently want to thank my crewmates: Christiane (above all), Carmel, Tristan, Andrzej and Sheyna. You made the mission a great experience. Thank you as well to Mission Support, especially Kim, Bryan, Pete, Wendy, and Lucie.

I want to thank the anonymous reviewers who took the time to carefully read this thesis and give thoughtful comments.

Finally, and above everything, I thank my family. All of you, from cousins to grandparents, have always offered me all the support I needed. Special thanks to you, mum and dad, for the open mind and thirst for learning you have been carrying and made sure to transmit to your children. Thank you, Arthur and Juliette. All you taught me cannot be found in any book I know of, and yet this book would be the one that everyone should read.