



UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

Dipartimento di Scienze Ecologiche e Biologiche

Corso di dottorato di ricerca in Evoluzione Biologica e Biochimica XXIV Ciclo

**Resistance and survival of endolithic microorganisms in outer
space and Mars conditions simulated in space**

Coordinatore: Professoressa Laura Zucconi

Tutor: Professor Silvano Onofri

Candidato: Dottor Giuliano Scalzi

Indice

Prefazione	4
1. Introduction	5
1.1. What is astrobiology?	5
1.2. Life on Earth and beyond: there's a limit of Biosphere?	6
1.2.1. Boundaries of Biosphere	7
1.3. The dawn of the livings: Astrobiological Exposure Facilities.	8
1.3.1. EXPOSE-E facility	10
1.4. Model Organisms selected for EXPOSE-E LIFE experiments	12
1.5. The aim: Planetary Protection issues	12
2. Survival of rock-colonizing organisms after 1.5 year in outer space. EXPOSE-E special issue, Astrobiology (in press)	14
2.1. Introduction	15
2.2. Material and Methods	17
2.3. Results	22
2.4. Discussion	29
3. LIFE Experiment: Isolation of cryptoendolithic organisms from Antarctic colonized sandstone exposed to space and simulated Mars conditions on the International Space Station. (2012) Origins of Life and Evolution of Biospheres (accepted)	31
3.1. Introduction	32
3.2. Material and Methods	34
3.3. Results	39
3.4. Discussion	42

4. Evaluation of Procedures for the Collection, Processing, and Analysis of Biomolecules from Low-Biomass Surfaces 2011 Applied and Environmental Microbiology 77(9):2943-53.	45
4.1. Introduction	46
4.2. Material and Methods	49
4.3. Results	58
4.4. Discussion	64
5. Discussion	68
Bibliography	70

Prefazione

Questa tesi si basa sull'analisi della sopravvivenza, e dell'eventuale danno subito, di due specie di funghi neri meristemati e di comunità criptoendolitiche antartici successivamente all'esposizione per un anno e mezzo a condizioni spaziali reali e marziane simulate all'esterno della Stazione Spaziale Internazionale (ISS).

A seguito dell'esperimento, l'interesse del candidato si è esteso a quei microrganismi che, essendo presenti nelle "Spacecraft Assembly Facilities" (SAF), potrebbero potenzialmente contaminare sistemi extraterrestri considerati "incontaminati" dall'intervento antropico. Per ognuna delle tematiche affrontate, i risultati elaborati e discussi sono stati inviati a delle riviste peer-reviewed con IF per la valutazione e la pubblicazione. Ciascuno dei capitoli da 2 a 4 è in ogni sua parte conforme al manoscritto inviato ad una rivista (capitolo 2 in press, capitolo 3 accepted e capitolo 4 published), ad eccezione della bibliografia, la quale rappresenta il capitolo finale della tesi. Infine, nel capitolo 5 è presentata una discussione generale delle tematiche affrontate e dei risultati ottenuti e la loro rilevanza ai fini degli obiettivi dello studio.

1. Introduction

1.1 What is Astrobiology?

With the development of space technology, astrobiology has been established as a scientific discipline. Classical biological research has concentrated on the only example of “life” so far known, i.e. life on Earth. In contrast, astrobiology extends the boundaries of biological investigations beyond the Earth, to other planets, comets, meteorites and space at large. To date, the overriding objective of “exobiological” research is to attain a better understanding of the principles leading to the emergence of life from inanimate matter, its evolution, and its distribution on Earth and throughout the Universe (Klein 1986). To reach this goal, astrobiological research has focused on the different steps of the evolutionary pathway through cosmic history that maybe related to the origin, evolution and distribution of life.

Since it involves many disciplines, in this study emphasis will be laid to the results obtained through a biological approach.

The present thesis would in one hand discuss the bewildering resistance of microorganisms exposed to real space and Mars simulated conditions, and on the other hand clarify with a methodological approach, the new procedures to minimize the biological cross-contamination resulting from the exploration of the solar system.

1.2 Life on Earth and Beyond. There's a limit of Biosphere?

Despite an ever-expanding understanding of the limits of life in Earth's most extreme environments, little remains known about life's resolve when removed from Earth and the all-encompassing biosphere that cradles it. For example, can life resist to a transfer between planets?

Based on the wide distribution of cosmic dust, the theory of Panspermia (Arrhenius, 1903, 1908) postulates that microscopic forms of life, e.g. spores, can be propagated in space driven by radiation pressure from the sun (Robertson. 1937), thereby seeding life from one planet to another. Since its formulation, panspermia has been subjected to several criticisms with arguments such as (1) it cannot be experimentally tested (Lederberg, 1960), (2) it shunts aside the question of the origin of life (Dose, 1986; Allamandola et al., 1989; Haynes, 1990), and (3) spores will not survive long-time exposure to the hostile environment of space, especially vacuum and radiation.

Richter (1865) and Arrhenius (1903) first proposed the panspermia theory, which speculates about the transfer of life between planets. Although panspermia still remains little more than an idea and there is no evidence that it has occurred, the various steps required for the transfer of organisms from one planet to another have been the focus of experimental testing (Cockell, 2008). A variety of recent discoveries have shed new light on the likelihood of viable transfer in space such as (i) the detection of meteorites, some of lunar and some of Martian origin; (ii) the detection of organics and the still highly debated supposition of microbial fossils in one of the Martian meteorite; (iii) the probability of small particles of diameters between 0.5 μm and 1 cm boulder-sized rocks reaching escape velocities by the impact of large comets or asteroids on a planet, e.g., on Earth (Melosh 1988) or Mars (Vickery and Meloish 1996); (iv) the ability of bacterial spores to survive to a certain extent the shock waves of such a simulated impact (Horneck et al., 2001); (v) the high UV resistance of microorganisms at the low temperatures of deep space, tested at temperatures down to 10 K; (vi) the reported survival of bacterial spores over millions of years, if enclosed in amber or salt stocks, or in space over periods extending up to 6 years (Horneck et al., 1994); (vii) the paleogeochemical evidence of a very early appearance of life on Earth in the form of metabolically advanced microbial prokaryotic ecosystems leaving not more than approximately 0.4 Ga for the evolution of life from the simple precursor molecules to the level of a prokaryotic, photoautotrophic cell; (viii) the biochemical evidence of a common ancestor for all life forms on Earth; (ix) the likelihood of artificial or directed transport by probes sent to other planets (Crick and Orgel, 1973). Viable

transfer from one planet to another requires that life, probably of microbial nature, survives the following three steps: (i) the escape process, i.e. ejection into space, e.g., caused by a large impact on the parent planet; (ii) the journey through space, i.e. time scales in space comparable with those experienced by the Martian meteorites (approximately 1-15 Ma); and (iii) the landing process, i.e. non-destructive deposition of the biological material on another planet (Fig. 1).

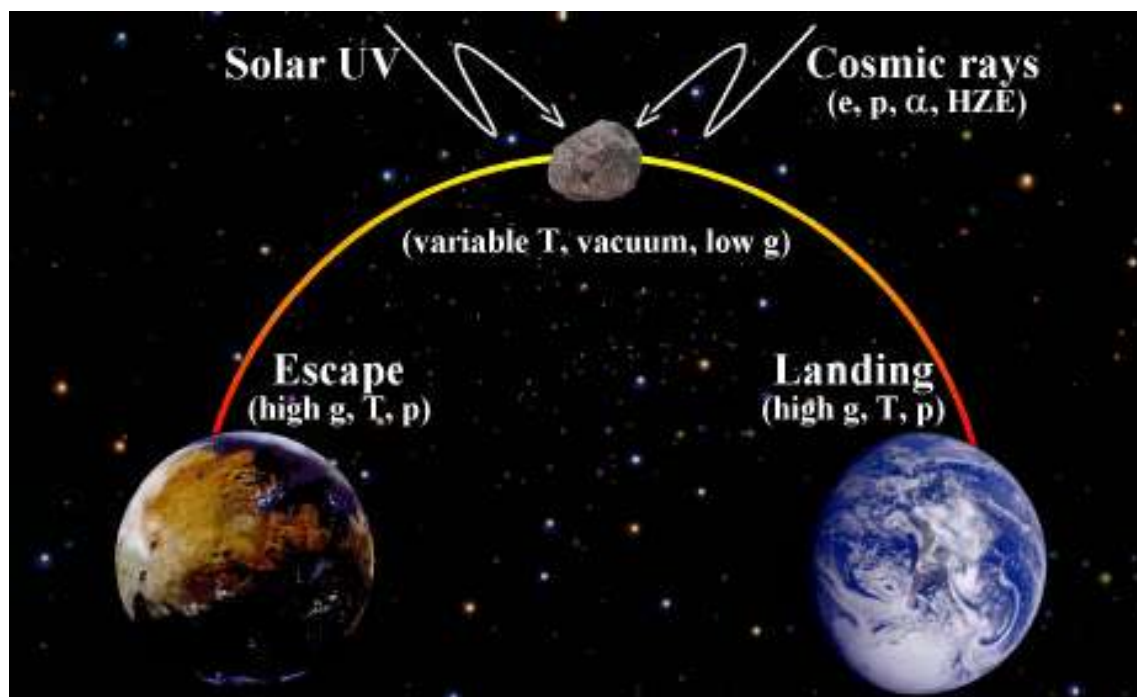


Fig. 1 Scenario of an interplanetary transfer of life in the solar system

Although it will be difficult to prove that life could be transported through our solar system, estimates of the chances for the different steps of the process to occur can be obtained from measurements, calculations, and experiments.

1.2.1 Boundaries of Biosphere

Distribution of air spora including their identity, behavior, movements and survival in the troposphere, as well as their impact on public health and agriculture are well understood (Horneck and Brack, 1992). They comprises viruses, bacteria, algae, microfungi, fungal spores of moss and fern, pollen, minute seeds, and protozoan cystis, up to the concentration of 100s-1000s per m^3 . It has been shown that spores as well as pigmented species are especially adapted to survive in inhospitable environment, characterized by low temperature, drought,

low pressure and solar radiation (Mancinelli and Shulls, 1978). With increasing height, the concentration of air spora rapidly falls off. In a few early experiments, using balloons or meteorological rockets with especially designed analysers, viable microorganisms were collected up to 77 km (Rogers and Meier, 1938; Imshenetsky et al., 1976, 1978, 1979). ***They collected predominantly black conidia and spores of fungi.*** It is assumed that pigmentation offers a selective advantage for the spores, because it protects them against the intense solar UV radiation. No viable microorganisms were collected from Earth orbit by use of collectors on Gemini 9A and 12. A pivotal role for the understanding of limits of life has been played by the progress of space technology.

1.3 The dawn of the livings: Astrobiological Exposure Facilities.

Space technology has provided opportunities to expose terrestrial specimens, such as prebiotic compounds, organic molecules and organisms, to this unique environment of space or to selected space conditions (Horneck and Brack 1992). Questions to be tackled include:

- the chemistry of precursors of life in space, e.g. in the interstellar medium or in comets;
- the role of the extraterrestrial short wavelength UV radiation, at present absorbed by the Earth's ozone layer, on prebiotic and biological evolution;
- the survival of microorganisms, when travelling in space, e.g. as blind passengers inside of meteorites (lithopanspermia hypothesis);
- the survival of microbial contaminants on space craft on outbound missions to other planets (planetary protection requirements);
- the limits of life.

During previous space missions, such as Gemini, Apollo 16 (Taylor et al. 1974), Spacelab 1 (Horneck et al. 1984a and b), Spacelab D2 (Horneck et al. 1994a; 1996), LDEF (Long Duration Exposure Facility) (Horneck et al. 1994b), MIR (Rettberg et al. 2002), EURECA (EUropean REtrievable CArrier) (Horneck et al. 1995) and several Foton missions (Horneck et al. 2001, Rettberg et al. 2004, Sancho et al. 2007) (Table 1), exposure of various microorganisms to selected or combined space conditions demonstrated the lethal effects of extraterrestrial short wavelength solar UV radiation, but also the enormous resistance of

selected species against LEO vacuum when UV shielded (Horneck 1998). Recent experiments on board of the BIOPAN facility extended the list of organisms surviving exposure to space vacuum in LEO with lichens (Sancho et al. 2007) and tardigrades (Jönsson et al. 2008). Both are eukaryotic and multicellular. The macroscopic lichens even survived UV-C exposure during their two-week flight in LEO.

Except for the two free flying satellite missions LDEF and EURECA that were planned to stay in orbit for approximately 1 year and with LDEF accidentally remaining in space for nearly 6 years, exposure of most exobiological experiments in space did not last longer than approximately 2 weeks (Foton, Spacelab, Gemini). With the availability of the International Space Station (ISS), more extended exposure experiments have become possible. The European Space Agency has provided the exposure facility EXPOSE for astrobiology experiments in Low Earth Orbit (LEO).

This technical availability has led to several studies, under real space conditions (Buecker and Horneck, 1970; Mancinelli and Klovstad, 2000; De la Torre et al., 2003) in spaceflight experiments (Horneck, 1993; Fajardo-Cavalzos et al., 2005; Sancho et al., 2007; De los Ríos et al., 2010; Horneck et al., 2010). The one of the main objectives of these astrobiological experiments has been to test whether different kinds of organisms can survive in the extremely hostile conditions of interplanetary space with particular attention to the space vacuum that causes dehydration of the samples and the high intensity of cosmic rays and solar extraterrestrial UV radiation, the latter being especially harmful to DNA. The survival capacity of exposed organisms is an interesting feature that can indirectly support or deny the old panspermia theory and recent revisions (Friedmann et al., 2001; Fajardo-Cavalzos et al., 2005).

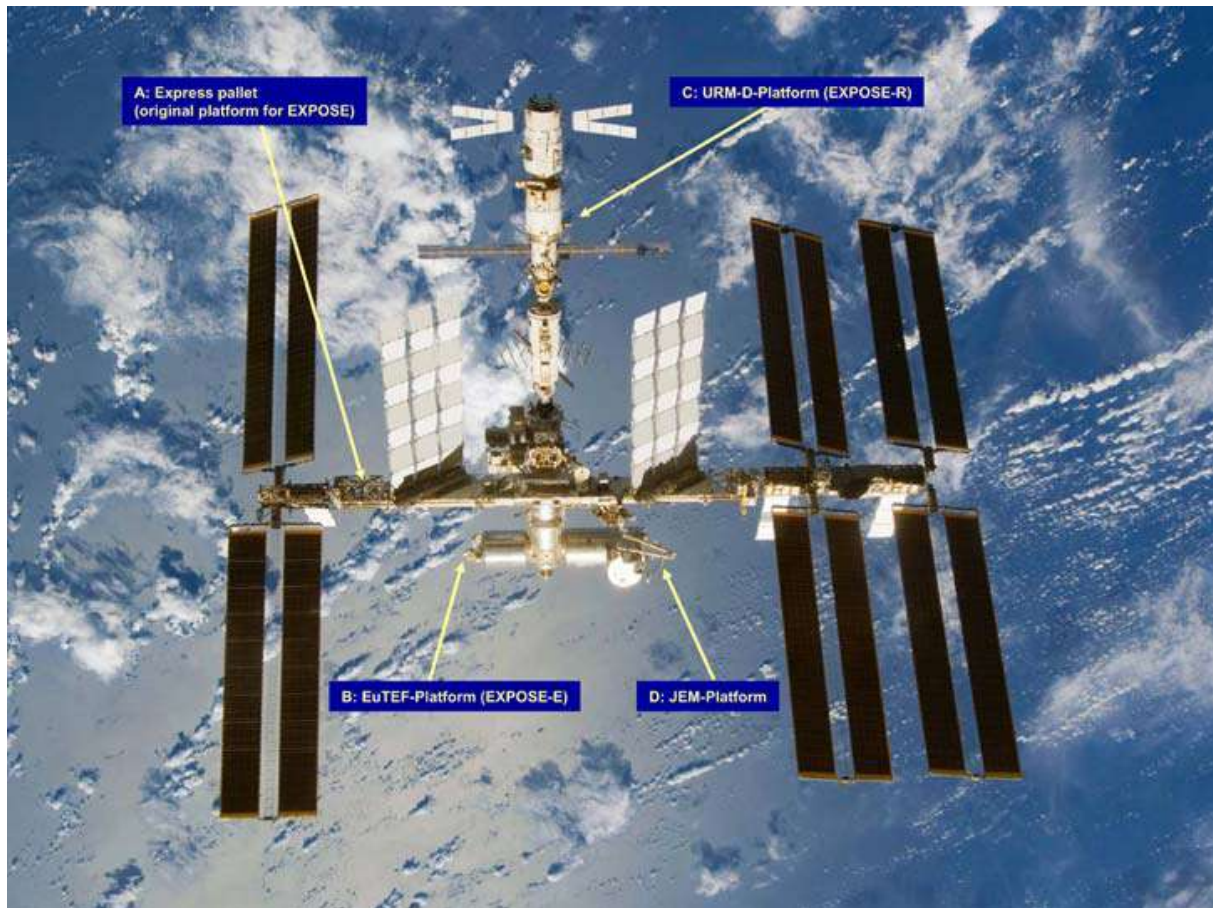


Fig. 2 International Space Station

1.3.1 EXPOSE Facility

Following an European Space Agency announcement of opportunity in 1996 for "Externally mounted payloads for 1st utilization phase" on the International Space Station (ISS), scientists working in the fields of astrobiology proposed experiments aiming at long-term exposure of a variety of chemical compounds and extremely resistant microorganisms to the hostile space environment. The ESA exposure facility EXPOSE was built and an operations' concept was prepared. The EXPOSE experiments were developed through an intensive pre-flight experiment verification test program. 12 years later, two sets of astrobiological experiments in two EXPOSE facilities have been successfully launched to the ISS for external exposure for 1.5 years. The present study will focus on EXPOSE-E Facility LIFE experiment, that will be described in depth in the next chapters.

Table 1. Experiments selected and accommodated in EXPOSE-E

Principal Investigator	Experiment	Topic of research
G. Horneck DLR (Germany)	PROTECT	To determine the resistance of spacecraft isolates to outer space for planetary protection purposes
D. Tepfer CNRS, Versailles (France)	SEEDS	To test plant seed as a terrestrial model for a Panspermia vehicle and as a source of universal UV screens
H. Cottin LISA-Créteil (France)	PROCESS	To study photochemical organic chemistry relevant to comets, meteorites, Mars and Titan
P. Rettberg DLR (Germany)	ADAPT	To study molecular adaptation strategies of microorganisms to different space and planetary UV climate conditions
S. Onofri Università degli studi della Tuscia di Viterbo (Italy)	LIFE	To study the resistance of lichens and lithic fungi at space conditions
D-P. Häder University of Erlangen (Germany)	R3D-2	Active radiation dosimetry (VIS, UV-A, UV-B, UV-C; LET spectra of cosmic radiation)
G. Reitz DLR (Germany) / F. Vanhaver SCK CEN (Belgium)	DOSIS/ DOBIES	Passive radiation dosimetry at the sample sites

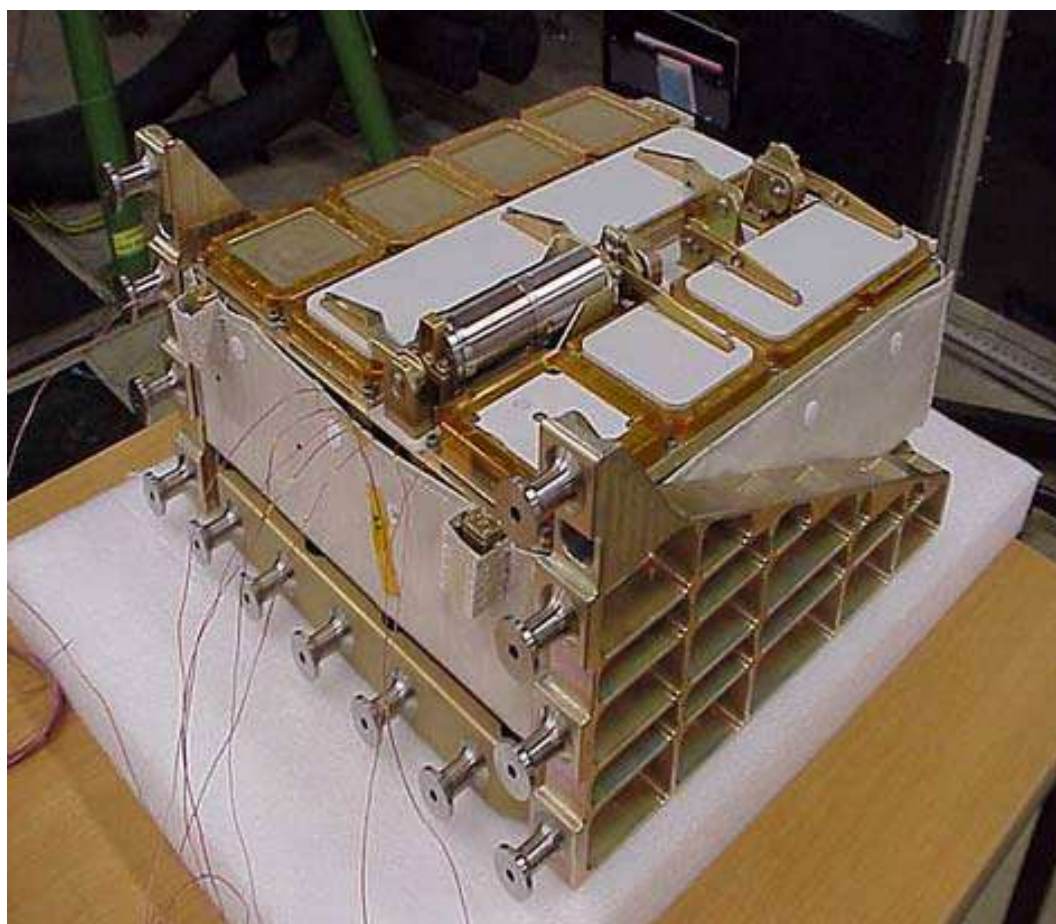


Fig. 3 EXPOSE Facility.

1.4 Model Organisms selected for EXPOSE-E LIFE experiments

In view of the harsh conditions of space, the choice of suitable test organisms is of great importance. Some authors, such as Horneck (1993), Horneck et al. (1994, 2001), and Mancinelli et al. (1998), have worked with bacterial endospores and halophiles, respectively, because of their known exceptionally high resistance to harsh terrestrial climatic conditions, while, in recent years, lichens have also been tested (Sancho et al., 2007, 2008; De la Torre et al., 2010). During the Foton M2 and Foton M3 missions, *Rhizocarpon geographicum*, as well as *Xanthoria elegans*, survived for 10 days in the LICHENS experiment, while *Aspicilia fruticulosa* survived for a 16 days journey in Low Earth Orbit (LEO) space, demonstrating resistance in a short term space experiment.

All the while, the lack of data in response to long term exposure in outer space remains a task of great interest.

In addition to the previous test systems mentioned, other model organisms have been elected as test samples to assess the possible survival in LEO space.

Since some of the most resilient life forms ever encountered are cryptoendolithic fungi and epilithic lichens originally discovered in the extremely cold, hyper-arid dry valleys of Antarctica (Friedman et al., 1982), and after positive results during Experiment Verification Tests (EVTs) (Onofri et al. 2008), Antarctic black meristematic fungi *Cryomyces antarcticus* (CCFEE 515), *Cryomyces minteri* (CCFEE 5187), as well as whole Antarctic cryptoendolithic communities have been exposed for 1.5 years in real space and Mars simulated conditions in the ESA EXPOSE-E facility.

The first two chapters of the present thesis will focus on the after-retrieval analyses of the LIFE test systems. In particular, it has been investigated the survival of the test systems as well the survivals of those organisms embedded in their natural substratum.

1.5 The aim: Planetary Protection issues

LIFE experiment as well as EXPOSE-type facilities have different implications in the future life detection missions, besides the understanding of the likelihood of lithopanspermia. Since the last 50 years, human activities in space exploration have become another potential source of spreading microorganisms between planets. Nearly 40 robotic missions have been launched with Mars as their destination (Horneck et al. 2007). In order to prevent the introduction of microbes from the Earth to another celestial body or vice versa, a concept of contamination

control has been elaborated by the Committee on Space Research (COSPAR) under consideration of specific classes of mission/target combinations, which have been recommended to be followed by each space-faring organization (COSPAR 2011). Lander missions to Mars require especially strict measures of cleanliness and partial sterility of the spacecraft (COSPAR 2011). The test systems that have been used in the LIFE experiment, though unlikely to be found on spacecraft, can be considered as models for demonstrating how life, if accidentally transferred from Earth to outer space, may resist and contaminate other celestial bodies and planets (i.e. Mars), thereby interfering with future life detection missions. Moreover EXPOSE itself it has been used as “testbed” for experiments in support of upcoming Planetary Protection mission.

To control the bioload of Spacecrafts and their related environments (Spacecraft Assembly Facilities), the fields of applied microbiology and molecular biology have made enormous technological advancements over the past two decades. The development of DNA-based methodologies has resulted in applications ranging from quantitative polymerase chain reaction (PCR) assays that are sensitive down to a single cell, to high-throughput methods that can simultaneously identify the widest possible range of biosignatures in a sample (Brodie et al. 2006; Sogin et al. 2006). Despite advances in the specificity and sensitivity of molecular biological technologies, however, the ability to efficiently collect (Bruckner and Venkateswaran 2007) and purify nucleic acids (La Duc et al. 2007b; Moissl et al. 2007b) from low-biomass environments remains a significant challenge for accurately describing the true microbial diversity of these samples. To this end, the third chapter of the present study will focus on suitable protocols and procedures involved with the collection, processing, and molecular analysis of contaminant biomolecules from clean-room surfaces.

2. Survival of rock-colonizing organisms after 1.5 year in outer space*

Abstract

Cryptoendolithic microbial communities and epilithic lichens have been considered as appropriate candidates for the scenario of Lithopanspermia, which proposes a natural interplanetary exchange of organisms by means of rocks that have been impact ejected from their planet of origin. So far, the hardiness of these terrestrial organisms in the severe and hostile conditions of space has not been tested over extended periods of time. A first long-term (1.5 years) exposure experiment in space was performed with a variety of rock-colonizing eukaryotic organism at the International Space Station on board of the European EXPOSE-E facility. Organisms were selected that were especially adapted to cope with the environmental extremes of their natural habitat. It was found that some – but not all - of those most robust microbial communities from extremely hostile regions on Earth are also partially resistant against the even more hostile environment of outer space, including high vacuum, temperature fluctuation, the full spectrum of extraterrestrial solar electromagnetic radiation and cosmic ionizing radiation. Although the reported experimental period of 1.5 years in space is not comparable with the time spans of thousands or millions of years believed to be required for Lithopanspermia, our data provide first evidence of the differential hardiness of cryptoendolythic communities in space.

Keywords: Astrobiology, Lithopanspermia, Radiation Resistance, Survival, Vacuum

*Silvano Onofri, Rosa de la Torre, Jean-Pierre de Vera, Sieglinde Ott, Laura Zucconi, Laura Selbmann, Giuliano Scalzi, Kasthuri J. Venkateswaran, Elke Rabbow, Francisco J. Sanchez Iñigo, and Gerda Horneck. EXPOSE-E special Issue, Astrobiology 2012 (in press)

2.1 Introduction

The Lithopanspermia hypothesis suggests that impact-ejected rocks could transfer living organisms through space from one planet to another. This scenario implies that rock-embedded organisms need to survive the following three phases: Firstly (phase-I), the ejection into space inside of rock fragments, caused by an impact of a cosmic projectile on one planet (Melosh, 1984); secondly (phase-II), the journey through space for a long time (hundreds, even thousands or millions, of years) (Gladman et al., 1996); and lastly (phase-III), the capture by and landing on another planet (Mileikowsky et al., 2000; Horneck et al., 2008; Nicholson, 2009). This hypothesis dates back to Lord Kelvin's Presidential address to the British Association in 1871 (Thomson, 1871). However, it was dismissed by most contemporary scientists because it was the general opinion that outer space would kill any living being exposed to it; and there was no way at that time to test it experimentally. Another severe criticism was that it anyhow just shifts the problem of the origin of life to another planet. Only in the last decade, after the detection of several meteorites that originated from Mars (Nyquist et al., 2001; Fritz et al., 2005; Shuster & Weiss, 2005; The Meteoritical Society, 2011), Lithopanspermia was again seriously considered (Sancho et al., 2007; Stöffler et al., 2007; Horneck et al., 2008; 2010; Nicholson, 2009; de la Torre et al., 2010).

Shock recovery experiments performed to test phase-I of Lithopanspermia showed that spores of *Bacillus subtilis* and the lichen *Xanthoria elegans* could survive pressures up to 40 GPa, which are comparable to those experienced by the Martian meteorites (Stöffler et al., 2007; Horneck et al., 2008). Space technology provided the opportunity to study a variety of biological specimens after exposure to space. Among the systems tested, bacterial spores (*B. subtilis*) and the lichens *Rhizocarpon geographicum* and *X. elegans* stood out due to their high resistance to the hostile space environment (Sancho et al., 2007; Horneck et al., 2010; de la Torre et al., 2010). However eukaryotes have never been studied after long-term exposures to space conditions.

To investigate the fate of lithic organisms and communities during long-term travel in space, we used ESA's EXPOSE-E facility (Rabbow et al., 2009; Rabbow et al., this issue). This facility was attached to the balcony of the Columbus module of the International Space Station (Fig. 1A). EXPOSE-E was designed to expose a variety of biological systems to selected parameters of space over time-spans of one year and more. All biological test

systems of the LIFE (Lichens and Fungi Experiment) experiment were rock-dwelling organisms from hostile regions: Antarctic cryptoendolithic (dwelling inside rocks) communities in their natural sandstone, microcolonial black cryptoendolithic fungi (*Cryomyces antarcticus* and *Cryomyces minteri*) isolated from Antarctic sandstone, and high mountain epilithic lichens (*R. geographicum* and *X. elegans*) (de Vera et al., 2003; 2008; Selbmann et al., 2005; Sancho et al., 2007; de la Torre et al., 2010). These lichens were selected as test systems due to their high resistance to space conditions demonstrated during short-term (10-16 days) exposures (LICHENS Experiment on ESA's FOTON M2 Mission 2005 and LITHOPANSPERMIA Experiment on ESA's FOTON M3 Mission 2007) (Sancho et al., 2007; de la Torre et al., 2010). The biological samples were accommodated in small chambers (1.4 cm in diameter) of the EXPOSE-E facility (Fig. 1B). During the space mission they were exposed either to the full space environment (vacuum from 10^{-7} to 10^{-4} Pa, fluctuations of temperature between -21.5 and +59.6 C°, cosmic ionizing radiation up to 190 mGy, and solar extraterrestrial electromagnetic radiation up to $6.34 \times 10^8 \text{ Jm}^{-2}$) or they were shielded from insolation. After 1.5 years in space, the samples were retrieved and their viability was investigated. During the mission the sun-exposed LIFE samples had been exposed to 1,879 eSch (estimated Solar Constant hours) (Rabbow et al. 2012).

2.2 Material and Methods

Experiment hardware and biological samples of the LIFE experiment on board of the International Space Station.

The EXPOSE-E facility is part of the European Technology Exposure Facility (EuTEF) (Fig. 1A), which was designed for testing different materials under selected parameters of space. EuTEF with EXPOSE-E accommodating the biological samples of the LIFE experiment (Fig. 1B), was launched on February 7, 2008 with Space Shuttle STS-122 to the International Space Station (ISS). On February 15, 2008 it was mounted onto the outside balcony of the Columbus module by Extravehicular Activity (EVA). EXPOSE-E was decommissioned on September 1, 2009, retrieved by EVA on September 2, 2009 and returned to Earth on September 12, 2009 with STS-128. During the 1.5 year mission, the samples were exposed to space vacuum (10^{-7} to 10^{-4} Pa) (Horneck et al., 2010), galactic cosmic radiation (≤ 190 mGy) (Berger et al., this issue) and to the full spectrum of solar extraterrestrial electromagnetic radiation ($\lambda > 110$ nm) with fluences of 9.19×10^5 Jm⁻² (below a 0.1% transmission neutral density filter) and 6.34×10^8 Jm⁻² (100% transmission insolated samples). All fluences were calculated for the biologically active UV range of $200 \text{ nm} < \lambda < 400 \text{ nm}$, and depending on the orientation of the ISS to the Sun. Temperature varied between -21.5 °C and +59.6 °C (Rabbow et al., 2012).

Figure 1

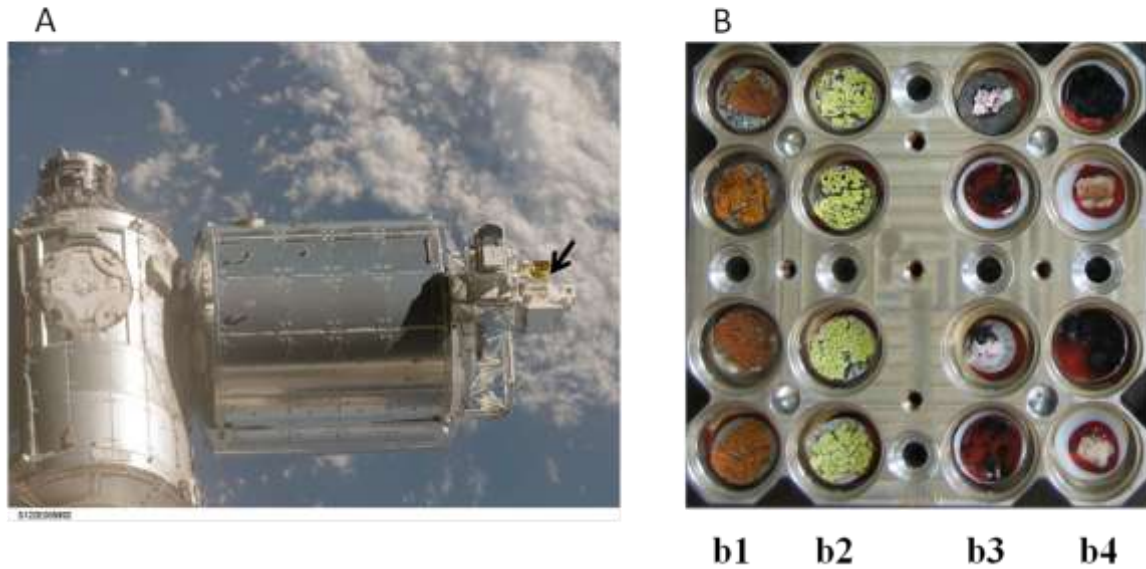


Fig. 1. Experiment hardware and biological samples of the LIFE experiment. **(A)** EXPOSE-E facility (arrow) attached to the Columbus module of the International Space Station (ISS) during orbital flight, accommodating the LIFE samples. **(B)** Samples of LIFE experiment accommodated in one of the compartments of EXPOSE-E facility (wells diameter 1.4 cm). Vertical rows show: *X. elegans* (b1), and *R. geographicum* (b2) on their natural rock habitat; dried cultures of the lichen fungus (mycobiont) of *X. elegans* (1st and 3rd sample from the top in b3); sandstone fragments colonized by a stratified cryptoendolithic microbial community (2nd and 4th samples from the top in b4), and the fungi *C. antarcticus* and *C. minteri* (from the top 2nd and last sample in b3, upper and 3rd sample in b4).

Test systems of the LIFE experiment

The lichen *Xanthoria elegans* was collected from alpine habitats between 2000 and 3000 m altitudes at Zermatt, Switzerland (45°59'N, 7°48'E) (de Vera et al., 2003; 2008); *Rhizocarpon geographicum* was collected from the Plataforma de Gredos, Spain (40°17'N, 5°14'W) at an altitude of 2020 m (de la Torre et al., 2010); sandstone fragments colonized by a stratified cryptoendolithic microbial community were collected by L. Zucconi at Battleship Promontory (76°54'37.6"S 160°55'27.5"E), Southern Victoria Land, Antarctica in January 2004, and the microcolonial black yeast-like fungi *Cryomyces antarcticus* CCFEE 515 and *Cryomyces minteri* CCFEE 5187, both dwelling cryptoendolithically, were isolated from sandstone collected in McMurdo Antarctic Dry Valleys (Selbmann et al., 2005). The survival of those cryptoendolithic organisms is of special interest in term of Lithopanspermia, because rocks may supply an additional external protection to face the impact-driven ejection into space (Horneck et al., 2008; Meyer et al., 2011) and transfer from one planet to another.

Viability assays of the LIFE test systems

The photosynthetic activity of the lichens *X. elegans* and *R. geographicum* was measured after reactivating the samples in a climatic chamber under the following controlled conditions: constant temperature of 10°C, 12 h light and 12 h dark cycles for 96 h (*X. elegans*) or 72 h (*R. geographicum*). Irradiation with photosynthetic active light was performed using a mercury lamp with a $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density. For rehydration samples were sprayed twice a day with deionized water. After reactivation, the activity of the PhotoSystem II (PSII) of the photobiont was measured by use of a Mini-PAM fluorometer (Heinz Walz GmbH), as described previously (Sancho et al., 2007; de Vera et al., 2008). Lichens were rewetted immediately before each measurement. The optimum quantum yield of chlorophyll *a* was determined by fluorescence measurements after 20 minutes of dark adaptation according to the equation:

$$F_v/F_m = (F_m - F_o) / F_m$$

with F_v = variable fluorescence yield, F_m = maximal fluorescence yield, and F_o = minimal fluorescence yield. This optimum quantum yield of PSII was taken as an indication of the PSII activity of the photobiont of the lichen system after the exposure to space parameters. The percentage ($n=2$ for space 100% insolated, and for space 0.1% insolated, and $n=4$ space dark samples) of PSII activity was determined from the ratio of the F_v/F_m of the same flight

sample before and after flight. Control data are given for the same sample measured before space flight (pre-flight) (Fig. 2A and B).

The survival of *C. antarcticus* and *C. minteri* (Fig. 3A and B) was determined from their colony forming ability as percentages of CFU (Colony Forming Units). Growth tests were performed by suspending fungal cells from rehydrated colonies in a 0.9% NaCl solution, inoculating them on Malt Agar (MA) Petri dishes (5 replicates), and incubating them at 15°C for 30 days. Control data were obtained from fresh colonies. Statistical analyses were performed by one-way analysis of variance (Anova) and pairwise multiple comparison procedure (Tukey test), carried out using the statistical software SigmaStat 2.0 (Jandel, USA). The means ($n=5$) $\pm$ s.d. are plotted. *, $P=0.001$; Power of performed test with $\alpha=0.050$: 1.000 (Fig. 3A and B).

The Propidium Monoazide (PMA) assay was used to check the integrity of the cell membranes after spaceflight. Fractions of DNA extracted from intact cells of cryptoendolithic fungi *C. antarcticus* and *C. minteri* colonies were compared with the fraction of DNA extracted from cells isolated from the space exposed colonized sandstone fragments. It was performed by adding PMA (Biotium, Hayward, CA) at a final concentration of 200 μ M to the re-hydrated fungal colonies or to powdered rock suspensions in PBS (Phosphate Buffer Saline) solution. PMA penetrates only damaged cell membranes, crosslinks then to DNA after light exposure and thereby prevents Polymerase Chain Reaction (PCR). Following DNA extraction and purification (Maxwell 16 automatised DNA extraction instrument, Promega, Madison, WI), quantitative PCR (Biorad CFX96 real time PCR detection system) was used to quantify the number of fungal Internal Transcribed Spacer (ITS) ribosomal DNA fragments present in both PMA treated and non-treated samples. For all reactions, 1 μ l of purified genomic DNA was added to 23 μ l of PCR cocktail containing 1X Power Sybr-Green PCR Master Mix (Applied Bios, Foster City, CA), as well as NS91 forward (5'-gtc cct gcc ctt tgt aca cac-3') and ITS51 reverse (5'-acc ttg tta cga ctt tta ctt cct c-3') primers, each at 0.02 M final concentration. These primers amplify a 203 bp product spanning the 18S/ITS1 region of rRNA encoding genes.

A standard Q-PCR cycling protocol consisting of a hold at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 20 s, and elongation at 72°C for 15 s, was performed. Fluorescence measurements were recorded at the end of each annealing step. At the conclusion of the 40th cycle, a melt curve analysis was performed by recording changes in fluorescence as a function of raising the temperature from 60-95°C in 0.5°C per 5

s increments. These protocols were applied to the processing of both fungal colonies, and cryptoendolithic sandstone samples.

Control data have been obtained for an identical sample stored on ground in the laboratory during the mission (Control). Because cryptoendolithic communities are not uniformly distributed within the rocks, different quantities of total fungal DNA were obtained from different rock samples (Fig. 3C, D and E).

Space data are given for samples shielded from extraterrestrial UV radiation (Space Dark), exposed to ($\lambda > 110$ nm) at a fluence of $9.19 \times 10^5 \text{ Jm}^{-2}$ beneath a 0.1 % transmission MgF_2 filter (Space 0.1% insolated), or at a higher fluence of $6.34 \times 10^8 \text{ Jm}^{-2}$ (Space 100% insolated). Statistical analyses were performed by one-way analysis of variance (Anova) and pairwise multiple comparison procedure (Tukey test), carried out using the statistical software SigmaStat 2.0 (Jandel, USA)*, $P=0.001$. **, $P>0.05$. Power of performed test with $\alpha=0.050$: 1.000. The means ($n=3$) and $\pm$ s.d. are plotted (Fig. 3C, D and E).

Confocal Laser Scanning Microscopy (CLSM) imaging: Viability of X. elegans analysed by LIVE/DEAD staining kit FUN 1

Adult lichen thallus, young thallus and the isolated mycobiont of *X. elegans* were stained by FUN 1 to determine their viability. A green and yellow color of the cells indicates that they still maintain vitality. A change from green to yellow color in the cytoplasm and from green to red color in the vacuoles is an indication of physiological activity expressed by accumulation of the dye in the vacuoles. Dead cells cannot be stained and therefore red crystals are not formed in the vacuoles.

Instruments used for imaging are: LSM 510 META of Carl Zeiss Mikroskopsysteme Jena GmbH with objective lenses 10 x and 40 x/oil emersion, scanning resolution 1024 x 1024 Pixel and time 64 μ s to 1,76 μ s, scan zoom 0.7x – 2.8, image bit depth 12 bit, experimental temperature condition 22°C, UV-Laser with emission wavelength 488/561/633nm and VIS-Laser HeNe with 633nm excitation and 5mW power, Filters with ChS1 679-754; Ch2 BP 505-550; Ch3 BP 575-615. For operation of the instrument, the Carls Zeiss Jena GmbH software system at the Institute of Genetics of the Heinrich-Heine-University Düsseldorf was used.

2.3 Results

First visual inspections assured that the “space samples” had not changed in shape and color compared to their pre-flight appearances. Tailored to each test system, different viability assays were applied: (i) photosynthetic activity of the lichenized alga (photobiont) of *X. elegans* and *R. geographicum* (Fig. 2A and B), (ii) colony forming ability of *C. antarcticus* and *C. minteri* (Fig. 3A and B), (iii) fraction of DNA amplified from intact cells of *C. antarcticus* and *C. minteri* and of cryptoendolithic communities inside sandstone fragments (Fig. 3C, D and E), and (iv) viability of *X. elegans* and the fungus of the lichen (mycobiont, cultured without the algal symbiont and dried) by means of vital staining (Fig. 4).

Among those space samples that were shielded from extraterrestrial insolation, but were exposed to space vacuum, cosmic radiation and temperature fluctuations (Space Dark in Figs. 2 and 3), the lichen *X. elegans* excelled by a PhotoSystem II (PSII) activity of ($99.35 \pm 0.59\%$) compared to the pre-flight data of the same samples (Fig. 2A). This high viability was not reached by the other space test systems kept in the dark during the mission: ($2.46 \pm 1.39\%$) for the PSII activity of the lichen *R. geographicum* (Fig. 2B), and ($8.04 \pm 3.05\%$) and ($0.13 \pm 0.07\%$) surviving cells for *C. antarcticus* and *C. minteri* respectively (Fig. 3A, and B). PMA assay showed ($98 \pm 4.67\%$) and ($8.14 \pm 0.35\%$) of DNA amplified from intact cells from colonies of *C. antarcticus* and *C. minteri* respectively (Fig. 3C, and D), in comparison to total extracted DNA; the percentage of DNA amplified from intact fungal cells in sandstone fragments was ($18 \pm 0.18\%$) (Fig. 3E).

Figure 2

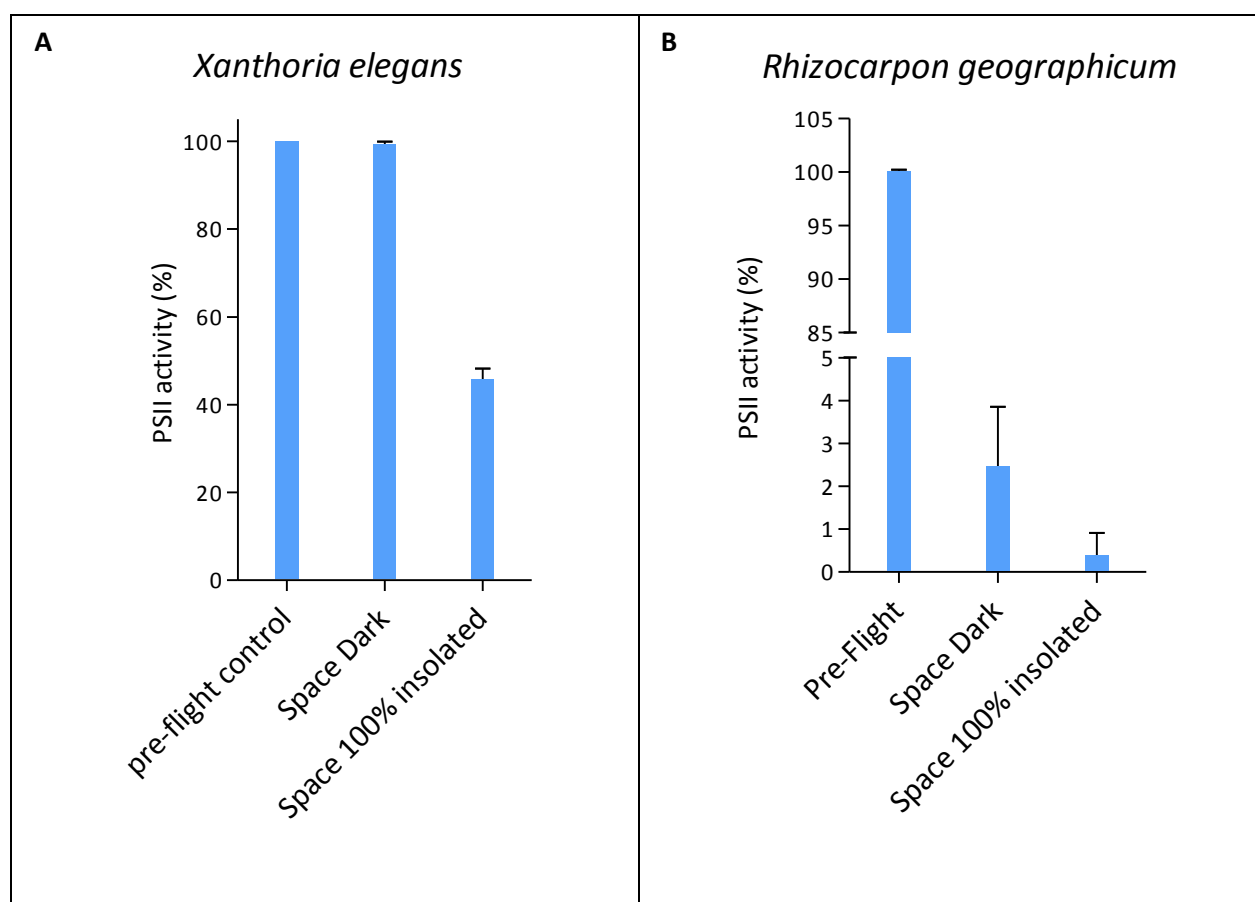
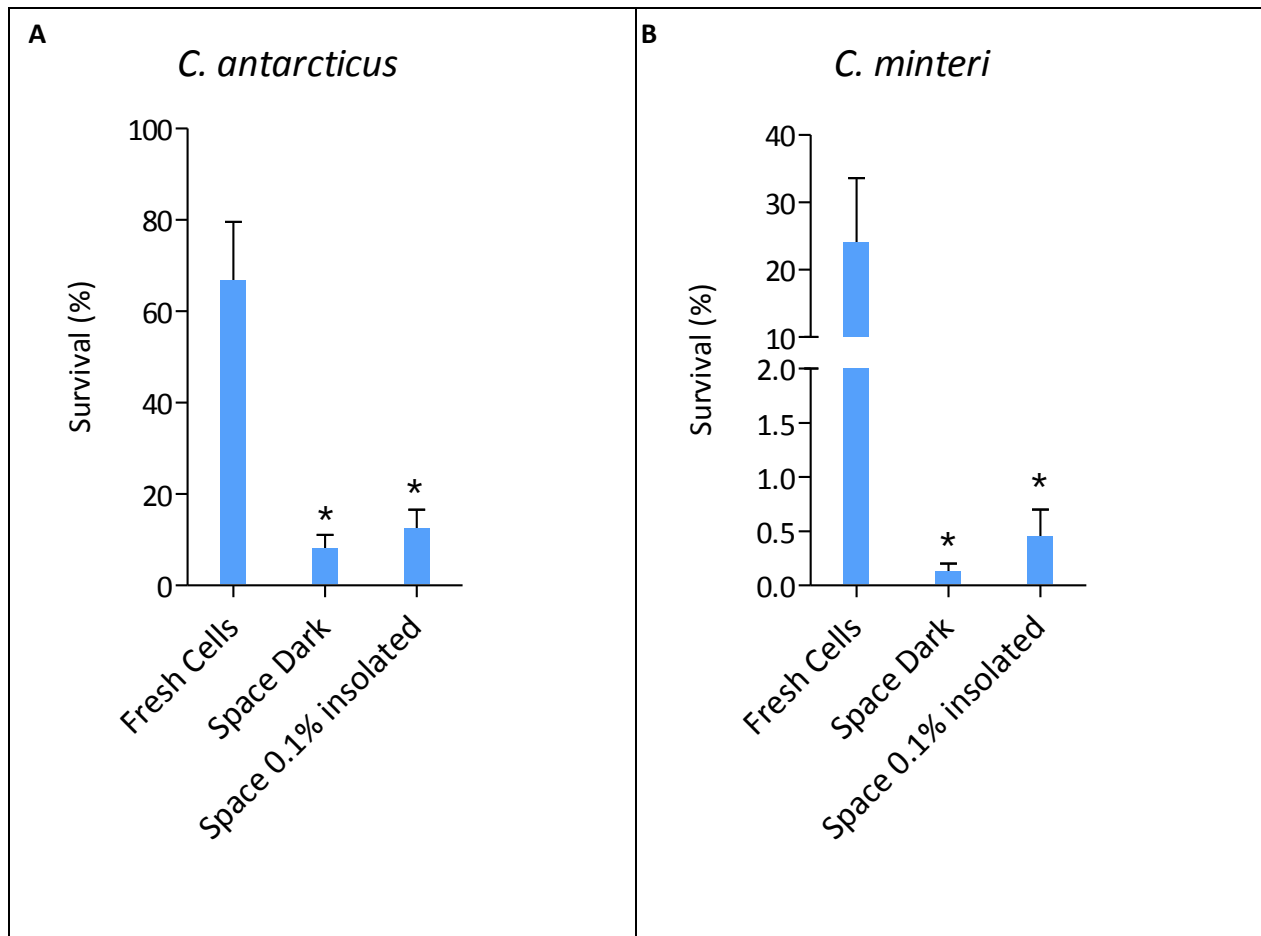
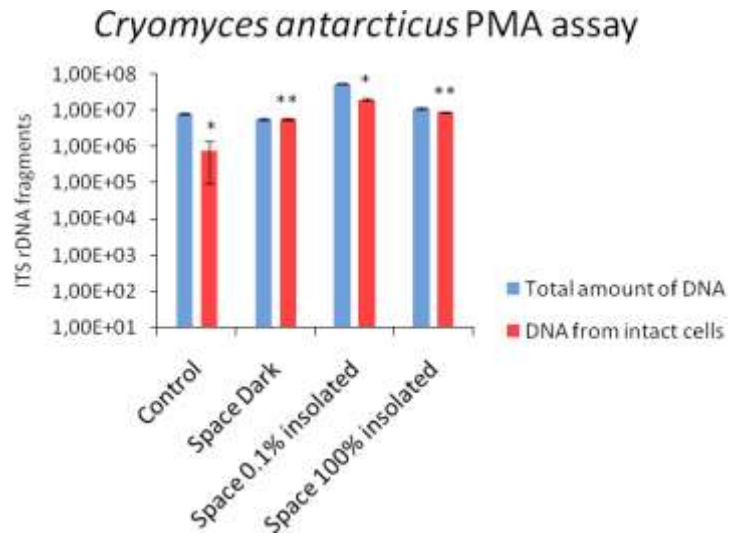


Fig. 2. Viability of the lichens (A) *X. elegans* and (B) *R. geographicum*, determined by fluorescence measurements of the photosynthetic activity of the photobiont. Control data are given for the same sample measured before space flight (pre-flight), as 100% viability. Space data are given for samples shielded from extraterrestrial UV-radiation (space dark), and insolated ($\lambda > 110$ nm) at a fluence of $6.34 \times 10^8 \text{ Jm}^{-2}$ (space 100% insolated).

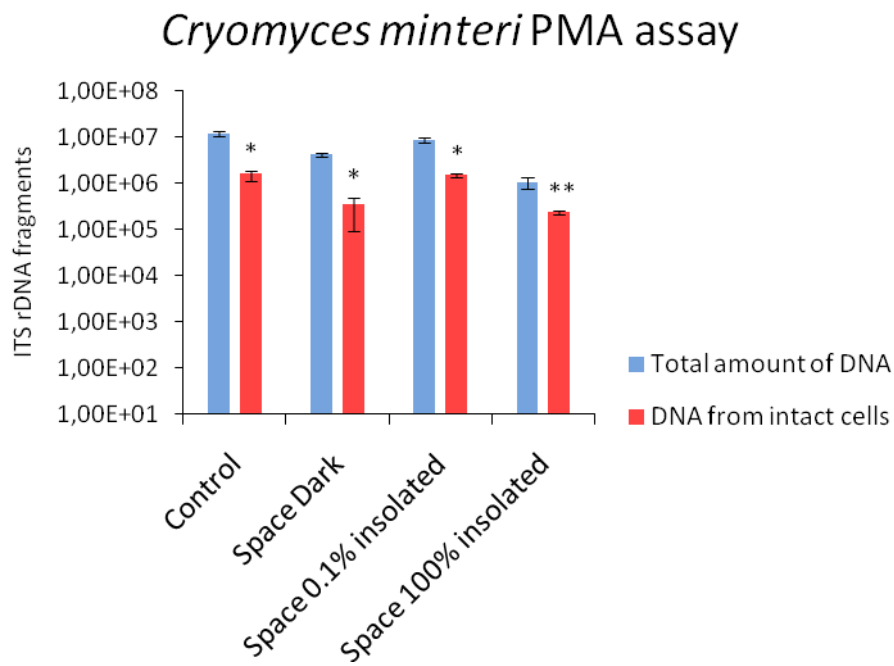
Figure 3



C



D



E

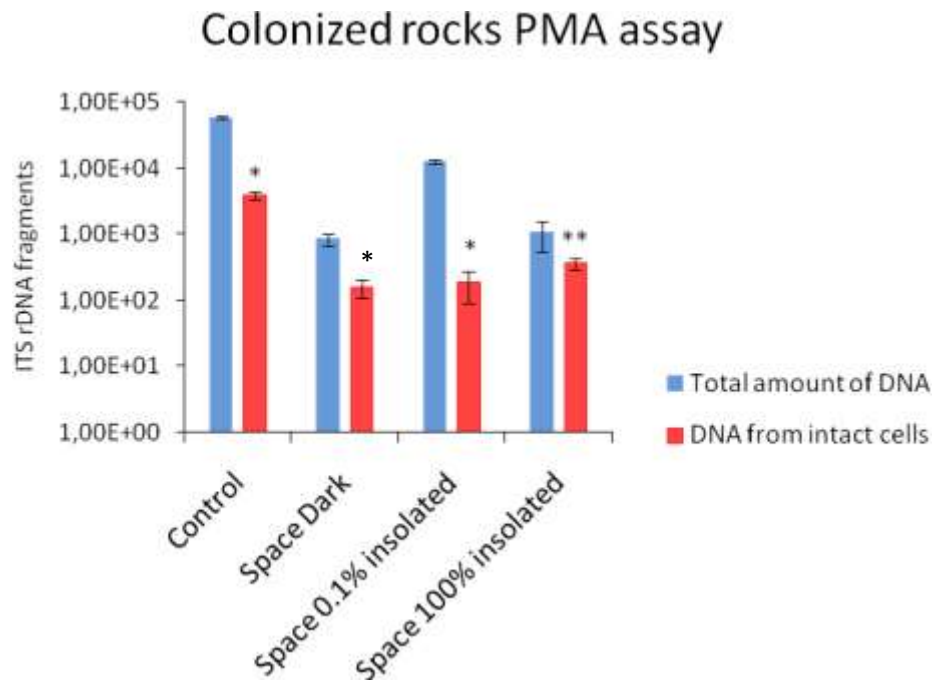


Fig. 3. Viability of the fungi (A), *C. antarcticus* and (B), *C. minteri*, as percentage of survival, determined as CFU (Colony Forming Units). Control data were obtained from fresh colonies. No CFU were obtained from the samples “Space 100 % insolated”. The means $\pm$ s.d. are plotted. *, $P=0.001$; Power of performed test with $\alpha=0.050$: 1.000.

Fraction of DNA amplified from intact cells of *C. antarcticus* and *C. minteri* (C and D) colonies, and from sandstone fragments (E) after different space conditions, compared to total extracted DNA. PMA penetrates only damaged cells’ membranes, crosslinking to DNA after light exposure, and thereby preventing PCR amplification. Following DNA extraction and purification, quantitative PCR was used to quantify the number of fungal ITS rDNA genes (203 bp) amplifiable in samples treated and non-treated with PMA. The means ($n=3$) and $\pm$ s.d. are plotted. Statistical significance was calculated using the Tukey test. *, $P=0.001$. **, $P>0.05$. Power of performed test with $\alpha=0.050$: 1.000.

The viability of culturable cells was not further decreased in the black fungi [(12.5 ± 4.11%) for *C. antarcticus* and (0.46 ± 0.24%) for *C. minteri*] that were insolated with the full extraterrestrial spectrum ($\lambda > 110$ nm) of the Sun, having received an UV-irradiation of $9.19 \times 10^5 \text{ Jm}^{-2}$ (Space 0.1% insolated, Fig. 3A, and B). The highest fraction of intact fungal cells (35 ± 0.15%) was accomplished by a sandstone sample that had received the full influx of solar electromagnetic radiation of $6.34 \times 10^8 \text{ Jm}^{-2}$ (Space 100% insolated, Fig. 3E). When comparing all test systems exposed to outer space, including the high influx of full solar extraterrestrial radiation ($6.34 \times 10^8 \text{ Jm}^{-2}$), again the lichen *X. elegans*, with a PSII activity of (45 ± 2.50%) (Fig. 2A) and *C. antarcticus* with (80 ± 0.82%) of DNA from intact cells (Fig. 3C), were the most resistant test systems of LIFE. However, black Antarctic cryptoendolithic fungi lost colony-forming ability after exposure to full insolation: no survivors were detected in the space 100 % insolated samples. Resistance of *X. elegans* and its mycobiont after exposure to the combined action of all space parameters tested – including full insolation - was confirmed by vital staining and Confocal Laser Scanning Microscopy. Fig. 4 shows the high capacity of the lichenized fungus (part of the symbiotic lichen association) in a young thallus (B), and of the lichen fungus of *X. elegans* in pure culture (C), to resist space exposure. The fungal cells in the cortex (Cx) of the young thallus were still vital, although a protecting mucilage layer had not been formed and the Parietin layer appeared to be very thin. The same features appeared to (C), the fungal cells of the mycelium.

FIGURE 4

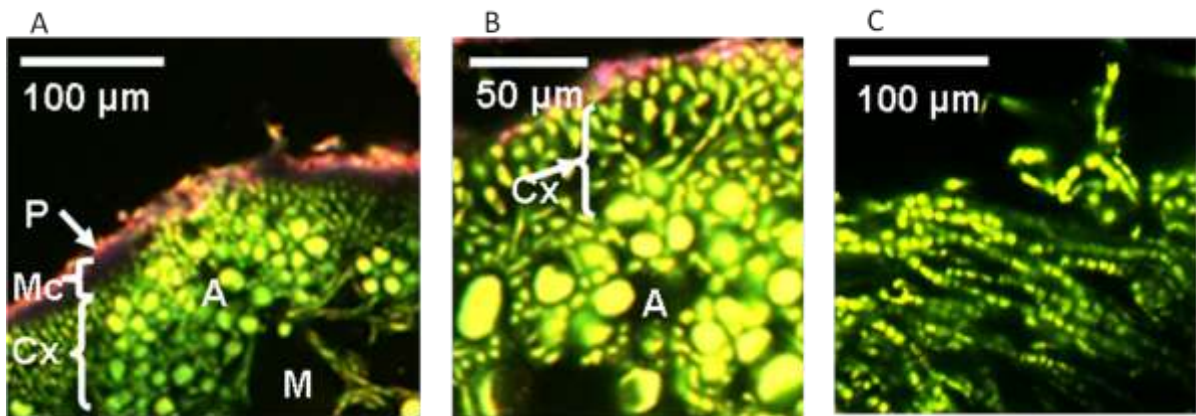


Fig. 4. Confocal Laser Scanning Microscopy (CLSM) imaging: Viability of *X. elegans* analyzed by LIVE/DEAD staining kit FUNI. (A), adult lichen thallus; (B), young thallus and (C), lichen fungus isolated in pure culture (mycobiont) of *X. elegans*, all dried and exposed to 100 % insolation and space vacuum. Green to yellow coloured cells are stained by FUNI, indicating vital cells. Turning from green to yellow and finally to red, indicates physiological activity expressed by the accumulation of the dye in the vacuoles. The high degree of maintained viability is due to a cortex Cx, a mucilage layer Mc and crystal deposits of Parietin P on the surface of the lichen. These layers are able to protect interior cells of the alga layer A and the medulla M.

2.4 Discussion

The LIFE experiment has provided for the first time data on the viability of rock-dwelling organisms and microbial communities after a long-term exposure to space parameters. These conditions cannot easily be simulated in the laboratory, if at all. The test systems, collected from hostile conditions, such as Antarctica and high mountain regions, are adapted to cope with high radiation intensities, arid phases, and extreme temperature fluctuations, somehow similar to those experienced in space. In *C. antarcticus*, for example, globular cells are enveloped in a thick melanised cell wall, protecting them from radiation and desiccation. Their meristematic way of producing colonies (i.e. dividing in all directions) further supports their resistance (Selbmann et al., 2005; Sterflinger, 2005; Onofri et al., 2008; 2009). Special protection against environmental extremes is also granted for the photobiont, the green alga *Trebouxia* sp. of the lichen *X. elegans*. In this symbiotic organization, the fungus forms a cortex with an upper layer incrustated with parietin and a mucilage layer enveloping the alga cells in a medulla matrix (Fig. 4A and B) (de Vera et al., 2003; 2008).

All organisms selected for the LIFE experiment are poikilohydric, i.e. they are able to dehydrate till most biochemical activities stop. In this state they are highly tolerant to stresses and may resume their metabolism once water becomes available again. Specifically, exposure to vacuum should inhibit any oxidative process related to their metabolism; this particular effect could protect against other damaging effects induced, e.g., by solar UV radiation, cosmic ray ions and temperature extremes.

The LIFE experiment has demonstrated that some – but not all - of those most robust microbial communities from extremely hostile regions on Earth are also partially resistant against the even more hostile environment of outer space. In this experiment the following species stood out as the most persistent survivors after 1.5 years in outer space: the black fungus *C. antarcticus* – as determined from PMA assay - and the symbiotic *X. elegans* - as determined from PSII activity- and its mycobiont - as determined by LIFE-DEAD staining. However, the CFU test did not yield any survivors of *C. antarcticus* flight samples that were exposed to the un-attenuated solar extraterrestrial spectrum (space 100 % insolated) and less than 10% survivors for the space dark samples. This means, even if the cell membrane seemed to be intact – as indicated by the PMA test -, the cells had lost their ability to grow and divide.

Earlier studies showed that the circumpolar and alpine red lichen *X. elegans* was able to retain its photosynthetic activity almost completely after 14 days in space (Sancho et al., 2007; de la Torre et al., 2010). This observation has been confirmed in this study for a much longer exposure time of 565 days in space, although this high viability was only observed, if the lichen had been shielded from solar electromagnetic irradiation, maintaining 45% PSII activity after 100% insolation exposure. Particularly, the mycobiont seems to play a fundamental role in maintaining the viability of the entire lichen system, because 565 days in space appeared not to have any effect on its physiological activity, even after 100% insolation (Fig. 4C). Interestingly, it has been earlier shown that *X. elegans* resisted also shock pressures, comparable to those experienced by the Martian meteorites during impact ejection (Horneck et al., 2008; Meyer et al., 2011), as required for phase-I of Lithopanspermia.

Although we have demonstrated that some rock-dwelling species are capable of partially withstanding the harsh environment of outer space – or certain parameters of it - for at least 1.5 years, the data are insufficient for drawing any consequences for the likelihood of Lithopanspermia. The possibility of surviving a much longer journey in space, as would be required for a natural travel from Mars to Earth or vice-versa, still remains an open question. So far, our studies have provided a first long-term experimental test on the survival of cryptoendolithic microbial communities in space, over a time period as long as currently available by space technology. These experiments demonstrate that outer space can act as a selection pressure on the composition of microbial communities (Cockell et al. 2011).

3. LIFE Experiment: Isolation of cryptoendolithic organisms from Antarctic colonized sandstone exposed to space and simulated Mars conditions on the International Space Station*

Abstract

Desiccated Antarctic rocks colonized by cryptoendolithic communities were exposed on the International Space Station (ISS) to space and simulated Mars conditions (LiFE - Lichens and Fungi Experiment). After 1.5 years in space samples were retrieved, rehydrated and spread on different culture media. Colonies of a green alga and a pink-coloured fungus developed on Malt-Agar medium; they were isolated from a sample exposed to simulated Mars conditions beneath a 0.1% T Suprasil neutral density filter or from a sample exposed to space vacuum without solar radiation exposure, respectively. None of the other flight samples showed any growth after incubation. The two organisms able to grow were identified at genus level by Small SubUnit (SSU) and Internal Transcribed Spacer (ITS) rDNA sequencing as *Stichococcus* sp. (green alga) and *Acarospora* sp. (lichenized fungal genus) respectively. The data in the present study support the possibility of eukaryotic life transfer from one planet to another by means of rocks and of survival in Mars environment.

Key words: *Antarctic colonized rocks, EXPOSE-E, International Space Station, Lithopanspermia, Lichens and Fungi Experiment.*

*Giuliano Scalzi, Laura Selbmann, Laura Zucconi, Elke Rabbow, Gerda Horneck, Patrizia Albertano and Silvano Onofri. Origins of Life and Evolution of Biospheres (Accepted for publication on December 21st 2011)

3.1 Introduction

The Lithopanspermia hypothesis suggests that impact-ejected rocks could transfer living organisms through space from one planet to another (Thomson 1871). This scenario implies that rock-embedded organisms need to survive (i) the ejection into space within rock fragments, (ii) the journey through space for a long time (hundreds up to thousands or millions of years) and (iii) the landing on another planet (Gladman et al. 1996; Mileikowsky et al. 2000; Horneck et al. 2008; Nicholson 2009).

Despite an ever-expanding understanding of the limits of life in the Earth's most extreme environments, little remains known about life's potential to survive when removed from Earth and the all-encompassing biosphere that harbors it. Scientific literature has numerous reports on microbial survival and proliferation in the most inhospitable environments that our planet has to offer (Rothschild and Mancinelli 2001; Venkateswaran et al. 2001; Canganella and Wiegel 2011). All the while, reports addressing the uppermost limits of microbial survival in near-Earth orbit (Horneck et al. 2010), in the interplanetary (Mileikowsky et al. 2000) or interstellar space are scarce (Valtonen et al. 2009). Most early studies of long-term survival in outer space have dealt with prokaryotes, mostly bacterial endospores (e.g., Horneck et al. 1994). Later on, studies were extended to eukaryotes exposed to either simulated space conditions or space during short-term flights, such as the experiments in the framework of ESA's Biopan missions (Sancho et al. 2007; de la Torre et al. 2010; Raggio et al. 2011). The lichens *Rhizocarpon geographicum*, *Xanthoria elegans* and *Aspicilia fruticulosa* were exposed to space environment for about 10 days; long term experiments in space on eukaryotic test organisms have been done for the first time within this LIFE experiment. To ascertain the uppermost extreme limits of life, it is important to start examining a wider spectrum of microbiota, including those rock-embedded communities from extreme environments such as Antarctic deserts, and expose them to space in their integrity, in their natural substrate.

Cryptoendolithic communities are among the most resistant life forms ever encountered, originally discovered in the extremely cold, hyper-arid Dry Valleys of Antarctica (Friedmann 1982). In structural cavities of Antarctic sandstones lichenized and non-lichenized fungi and algae (cryptoendoliths), bacteria and cyanobacteria, form the lichen-dominated cryptoendolithic community (Friedmann 1982). Their extreme environment is considered the

most similar terrestrial environment to Mars surface (Wynn-Williams and Edwards 2000; Onofri et al. 2004); it is characterized by very low temperatures, ranging from -20 to -50°C in winter, with annual average temperature below 0°C, wide thermal fluctuations, frequent freeze/thaw cycles, dryness due to the lack of snow or ice cover and rare precipitations (<100 mm Water Equivalent per year), high salt concentrations, low nutrient availability and high radiation including UV (Onofri et al. 2007). Molecular studies of environmental DNA extracts from these communities suggested the identity of an endolithic fungal phylotype with adjacent epilithically growing *Buellia* spp. (>97% similarity of SSU rDNA sequences; de la Torre et al. 2003). The same authors highlighted the microbial biodiversity in these communities comprising lichen mycobionts, free-living fungi, algae, as well as *Actinobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and some other unidentified bacterial phylotypes. Some new genera and species of black meristematic fungi have also been described, but most taxa have not yet formally described (Selbmann et al. 2005; Selbmann et al. 2008). The increasing knowledge about diverse eukaryotes thriving in such harsh conditions suggested us to test in a space flight experiment the survival of a whole rock community under space and simulated Martian conditions.

In this LIFE experiment, fragments of sandstone colonized by cryptoendolithic communities from the McMurdo Dry Valleys (Antarctica) were flown in the European EXPOSE-E facility attached to the exterior of the ISS. Their suitability was tested before flight in ground-based Experiment Verification Tests (Onofri et al. 2009). The samples were exposed for 18 months to the environment of space in Low Earth Orbit (LEO) and to simulated Mars conditions. Once returned to Earth they were subjected to both, molecular and cultivation-based assays to estimate survival and viability (Onofri et al. 2012) including growth ability.

3.2 Material and Methods

Colonized Sandstone

A sample of sandstone was collected under sterile conditions by L. Zucconi at Battleship Promontory (76°54'37.6"S 160°55'27.5"E), Southern Victoria Land, Antarctica in January 2004; it showed under a stereomicroscopic inspection a well developed and stratified cryptoendolithic community with typical color bands, including the green one, due to living organisms. They were stored under sterile conditions at -20°C. Well colonised sandstone fragments (11 mm wide, maximum 6 mm thick, meanly 437 mg) including significant portions of microbial biomass were removed under sterile conditions by striking the sandstone sample lengthwise with a rock hammer. Excised fragments were dehydrated at room temperature and glued, using Wacker silicone glue RTV-S 69, on sterile teflon disks (12 mm diameter). These fragments were successively accommodated in different trays of the EXPOSE-E facility.

EXPOSE-E facility

The European Space Agency (ESA) has recently developed the multi-user research facility EXPOSE-E as an exterior portion of the ISS (Rabbow et al. 2009) (Fig. 1). This facility is designed to foster and promote astrobiology research including studies on the survival of, and genotypic and phenotypic changes in, model organisms (plant seeds, bacterial spores, black meristematic fungi, lichens, and cryptoendolithic communities) when exposed to the outer space environment.

The EXPOSE-E facility is a multi-user facility having a box-shaped structure, accommodating samples in three different and separate compartments, called trays (Figure 1). Each tray is subdivided into four sample carriers composed of 16 wells disposed on two levels, to face either outward towards space and radiations or kept at the same conditions but in the darkness. The LIFE samples were exposed either in Tray 1 that was vented with access to space vacuum or in Tray 2 that was sealed and filled with a simulated Mars gas mixture. The carriers were covered by an optical filter system to control intensity and spectral range of solar UV irradiation. The conditions to which the Antarctic sandstone fragments were exposed are listed in Table 1. During the 1.5 year lasting mission the rock samples experienced space vacuum (10^{-7} to 10^{-4} Pa) (Horneck et al. 2010), cosmic radiation (≤ 190

mGy) (Berger et al. 2012) and the full spectrum of solar extraterrestrial electromagnetic radiation (for space exposed samples with a long pass cut-off at 110 nm, and for simulated Mars condition samples with a long pass cut off at 200 nm). The UV (200-400 nm) fluences reached $9.19 \times 10^5 \text{ Jm}^{-2}$ (below a 0.1% transmission neutral density filter) and $6.34 \times 10^8 \text{ Jm}^{-2}$ (100% transmission insolated samples). All fluences were calculated for the biologically active UV range of $200 \text{ nm} < \lambda < 400 \text{ nm}$ for the whole mission and depended on the orientation of the ISS to the sun. Temperature varied between $-21.5 \text{ }^{\circ}\text{C}$ and $+59.6 \text{ }^{\circ}\text{C}$ (Rabbow et al. 2012; Onofri et al. 2012).



Fig. 1 EXPOSE-E Facility before space flight.

Cultivability tests

Treated samples (only one third of the whole sample analyzed, meanly 140 mg), ground controls (mean weight 91,2 mg) and fresh colonized rocks (weight about 500 mg) were gently homogenized in sterile physiological solution (0.9% NaCl w/v) and plated on MA (Malt Agar and incubated at 15°C for 3 months. The MA medium was selected because it gave best growth for colonies from fresh colonized rock samples. Each test was performed in triplicate.

The organisms that were able to grow where isolated and stored in pure culture at 10°C.

Table 1 Exposure conditions during the 1.5 year lasting LIFE experiment.

Sample specification	Parameter
Space Dark	Space vacuum (10^{-7} to 10^{-4} Pa)
Space 0.1% insolated	Space vacuum and solar UV (>110 nm) screened with a MgF ₂ neutral density filter of 0.1% transmission
Space 100% insolated	Space vacuum and solar UV (>110 nm)
Mars Dark	1000 Pa CO ₂ gas mixture
Mars 0.1% insolated	1000 Pa CO ₂ and solar UV (>200 nm) screened with a Suprasil neutral density filter of 0.1% transmission
Mars 100% insolated	1000 Pa CO ₂ gas mixture and solar UV (>200 nm)
Ground Control	Dark, room temperature, 1 atm

DNA extraction and sequencing

DNA was extracted from colonies grown for 6 months at 10°C, using Nucleospin Plant Kit (Macherey nagel, Duren, Germany) according to the user's manual. Polymerase Chain

Reactions (PCR) were prepared in 25 µl tubes adding BioMix (BioLine GmbH, Luckenwalde, Germany), 5 µM of each primer and 40 ng of template. Internal Transcribed Spacers (ITS) were amplified using ITS1 and ITS4 primers (Table 2) for fungi with the following PCR protocol: an initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s, and an extension at 72°C for 30s. At the end of the last cycle, an additional extension at 72°C for 5 min was performed. Algal Internal Transcribed Spacer was amplified using ITS1T and ITS4T primers (Table 2) with the following protocol: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and an extension at 72°C for 2 min; an additional extension at 72°C for 7 min was performed. Algal SSU was amplified with a PCR spanning the whole rRNA Small Subunit region using primers Euk A and Euk B (Table 2) with the following protocol: an initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min, and a final extension at 72°C for 5 min. For SSU sequencing primers in Table 2 have been used. The DNA sequences were assessed by direct sequencing of the PCR product (Macrogen, South Korea).

Sequence assembling was performed by using the software ChromasPro v1.41 (2003-2007 Conor McCarthy School of Health Science, Griffith University, Southport, Queensland, Australia). Sequences were compared in the public domain (NCBI) using BLASTn algorithm.

Table 2 Nuclear SSU and ITS primers used for algal and fungal DNA amplification and sequencing (Katana et al. 2001; Moro et al. 2009)

Name	Sequence 5'-3'	Organism
EukA	AACCTGGTTGATCCTGCCAGT	Algae
898-919R	TAAATCCAAGAATTTACCTCT	Algae
1936-57R	GGTAGGAGCGACGGGCGGTGTG	Algae
CHLORO F	TGGCCTATCTTGTGCTGT	Algae
EukB	TGATCCTTCTGCAGGTTACCTAC	Algae
ITS1T	GGAAGGATCATTGAATCTATCGT	Algae
ITS4T	GGTTCGCTCGCCGCTACTA	Algae
ITS1	TCCGTAGGTGAACCTGCGG	Fungi
ITS4	TCCTCCGCTTATTGATATGC	Fungi

3.3 Results

The first test in the LIFE experiment was to observe the effects of dehydration. The growth ability of the flight samples, i.e. Antarctic sandstones hosting cryptoendolithic communities that were exposed to different conditions in space was compared to that of the dehydrated Ground Control and a fresh sample of sandstone stored at -20°C. Table 3 shows the colony formers after cultivation on MA. The highest colony numbers were obtained from the fresh samples: 52 ± 10.5 algae, 1 ± 0 black fungi, 90 ± 27 pink fungi and 12.6 ± 2.0 yeasts. The GC sample, which was stored for 1.5 years in the laboratory, showed only 20.56 ± 3.5 colonies of pink fungi.

A pink fungal colony with a pale pink mycelium (Fig. 2) composed of appressed hyphae of 4 μm in width was obtained from a flight sample that was exposed to space vacuum, but kept in the dark (Table 3: Space Dark).

A colony of a green unicellular alga (Fig. 3) grew from a flight sample that was exposed to simulated Mars atmosphere and the Martian spectrum of solar radiation $> 200 \text{ nm}$ beneath a suprasil neutral density filter (Table 3: Mars 0.1% insolated). All other flight samples did not result in any colonies after incubation (Table 3).

Macroscopic observations and microscopic morphological analyses showed that both, the pink fungus from the Space Dark sample as well as the green unicellular alga from the Mars 0.1% insolated sample seemed to be identical with those isolated from the fresh and the GC samples.

Table 3 Growth studies of the LIFE experiment after the 1.5 years lasting space mission:
Colonies formed after cultivation on MA (Flight samples and controls)

Exposure conditions	Colony formation				Total colonies/mg
	Algae	Black colonies	Filamentous pink fungi	Yeasts	
Space Dark	0	0	3*	0	0.021
Space 0.1% insolated	0	0	0	0	0
Space 100% insolated	0	0	0	0	0
Mars Dark	0	0	0	0	0
Mars 0.1% insolated	1*	0	0	0	0.007
Mars 100% insolated	0	0	0	0	0
Ground Control	0	0	20.5± 3.5	0	0.18
Fresh sample (dark,-20°C, 1 atm)	52 ± 10.5	1 ± 0	90 ± 27.0	12.66 ± 2.0	0.29

*Total CFU obtained from 3 replicates

To identify the fungal isolate, PCR amplification of ITS rDNA portions and sequencing were performed. The sequences obtained gave low identity (94%), with E-value = 0, in NCBI GenBank with the lichenized genus *Acarospora* A. Massal. The closest related sequence was



Fig. 2 Hyphal network of the mycelium isolated from a rock exposed for 1.5 years to space vacuum (10^{-7} to 10^{-4} Pa) in the dark (Space Dark sample), seen at the light microscope. Usually lichenized fungi, when separated from their photobionts, lose all their morphological peculiarities and grow as common mycelia.

that of *Acarospora rosulata* (accession number GU184116.1). Based on this data the fungus was identified at the genus level only.

To further identify the green alga, the same analyses (PCR amplification of ITS rDNA portions and sequencing) showed that the ITS sequences matched with different algal genera with similarities ranging from 95% to 97%, E-value = 0. They belonged to the Class *Trebouxiophyceae*, 5 algae showed similarities to the Order *Chlorellales*, and 1 to the Order *Prasiolales*, genus *Stichococcus* (97% identity). SSU sequencing showed a similarity ranging from 94% to 96%, E-value = 0, with different algal species in the genus *Stichococcus*, the closest related sequence (96%) was *S. deasonii* UTEX 1706 (accession number DQ275460).

This strain grew mostly as single cells or four-celled short uniseriate and unbranched, straight or sigmoid, filaments. However, the morphology of the cylindrical cells, 5 μm wide and 10 μm long on average, with broadly rounded poles, does not completely match the morphological description of the genus *Stichococcus* Nägeli (1849), in which only one unlobed parietal chloroplast is usually present, while our strain was almost always characterized by two chloroplasts.



Fig. 3 Green alga isolated from colonized sandstone exposed to simulated Mars conditions in space beneath a Suprasil neutral density filter with a cut off at 200 nm and 0.1 % transmission (Space 0.1% insolated). Phylogenetic analyses indicate that the alga belongs to the genus *Stichococcus*,

3.4 Discussion

LIFE is the first experiment where cryptoendolithic microorganisms within their natural substratum have been exposed for long time to space and simulated Martian conditions in Low Earth Orbit. After exposure to the conditions applied during the LIFE experiment, a few cryptoendolithic organisms grew after recovery, namely in one Space Dark sample (3 pink fungi) and in one Mars 0.1% insolated sample (1 green alga). The pink lichenized fungus seemed to belong to the genus *Acarospora* and the green alga seemed to belong to the genus *Stichococcus*. Due to the scarcity of algal ITS and SSU sequences in nucleotide databases, it was only possible to address the alga to that genus. Considering the generic level of the data obtained, an extended study based on morphology and multi-gene molecular phylogeny is in progress.

All other flight samples did not result in any growth under the conditions tested. It should be noted that the exposed samples were very small and the rock fragments used for growth tests exiguous. Due to the not uniform distribution of the organisms composing the community within the Antarctic sandstone fragments, it was not possible to know before flight which organisms were present in each subsample. This may explain the differences recorded in cultural tests between the Ground Control, where only pink fungi were isolated, and fresh samples, from which a variegated number of fungal and algal colonies (Table 3) were obtained. It is interesting to note that although no algal growth was observed in the ground control samples, one algal colony was isolated from one Mars 0.1% insolated sample. It should again be stressed that due to the small size of the analyzed samples, it was not guaranteed that all members of the rock community were represented. We have shown that at least two microorganisms, a fungus and an alga, of a rock colonizing community survived after 1.5 years in space.

Antarctic cryptoendolithic organisms have been suggested as good study model for survival in space because of their ability to cope with the complex interplay of extremely low temperatures, thermal fluctuations, arid phases and high radiation intensities (Selbmann et al. 2005; Ruisi et al. 2007; Selbmann et al., 2011). Moreover, it has been assumed that organisms living within rocks could enter a cryptobiotic state after being ejected into space (Onofri et al., 2012).

This feature is possibly aided by the presence of abundant extracellular polymeric substances (EPS) in many endolithic species. EPS production allows the community to survive long dry periods, it may be abundant in fungi (Selbmann et al. 2005), in lichen thalli (de Vera et al. 2004, de los Ríos et al. 2005), and in the whole cryptoendolithic community (de los Ríos et al. 2004). Previous studies ascertained that the presence of sugars such as glucose or buffer salts on the surface of *Bacillus subtilis* spores act as chemical protectants, highly increasing their survival in space (Horneck et al. 1994). Furthermore Mancinelli and Klovstad (2000) showed that under 1mm of Martian analog soil a *B. subtilis* monolayer did not exhibit loss of viability after exposure to 12.3 kJm^{-2} of UV radiation from deuterium source, and some protection was even afforded by a layer $12 \text{ }\mu\text{m}$ thick. The few rock-colonizing organisms that were isolated after retrieval of EXPOSE-E could have been survived because of: (i) the ability of cryptoendolithic organisms to tolerate desiccation and radiation and (ii) the shielding of even a small layer of rock material thereby preventing loss of viability after simulated Martian UV irradiation. However, the study showed also that most of the rock colonizing species did not survive after 1.5 years in space or in a simulated Martian climate. Nevertheless extremophilic endolithic Eukarya composing this Antarctic community may be suitable model organisms to investigate the resistance of life in the framework of *Lithopanspermia*. After astrobiology research has centered - at least in the past decades - almost exclusively on prokaryotic microorganisms, these recent findings of the LIFE experiment allow us to select models from a wider spectrum of living organisms.

4. Evaluation of Procedures for the Collection, Processing, and Analysis of Biomolecules from Low-Biomass Surfaces*

Abstract

To comprehensively assess microbial diversity and abundance via molecular-analysis-based methods, procedures for sample collection, processing, and analysis were evaluated in depth. A model microbial community (MMC) of known composition, representative of a typical low-biomass surface sample, was used to examine the effects of variables in sampling matrices, target cell density/molecule concentration, and cryogenic storage on the overall efficacy of the sampling regimen. The MMC used in this study comprised 11 distinct species of bacterial, archaeal, and fungal lineages associated with either spacecraft or clean-room surfaces. A known cellular density of MMC was deposited onto stainless steel coupons, and after drying, a variety of sampling devices were used to recover cells and biomolecules. The biomolecules and cells/spores recovered from each collection device were assessed by cultivable and microscopic enumeration, and quantitative and species-specific PCR assays. rRNA gene-based quantitative PCR analysis showed that cotton swabs were superior to nylon-flocked swabs for sampling of small surface areas, and for larger surfaces, biological sampling kits significantly outperformed polyester wipes. Species-specific PCR revealed differential recovery of certain species dependent upon the sampling device employed. The results of this study empower current and future molecular-analysis-based microbial sampling and processing methodologies.

* K. Kwan, M. Cooper, M. T. La Duc, P. Vaishampayan, C. Stam, J. N. Benardini, G. Scalzi, C. Moissl-Eichinger, and K. Venkateswaran. (2011) *Applied and Environmental Microbiology*, 77(9):2943-53

4.1 Introduction

The search for life and habitable conditions elsewhere in the solar system has led to a steady increase in the number of robotic probes and orbiters leaving Earth en route to other planetary systems. Planetary protection is a space science discipline that addresses concerns over the potential for forward contamination (i.e., the inadvertent contamination of extraterrestrial environments with terrestrial organic and biological matter) and backward contamination (i.e., the inadvertent exposure of the terrestrial biosphere to extraterrestrial material) during such endeavors (NASA 2005). Current guidelines for the assessment of biological contamination of spacecraft surfaces throughout assembly, testing, and launch operations (ATLO) are overseen by the International Committee on Space Research (COSPAR 2002) and are enacted by the National Aeronautics and Space Administration (NASA) to facilitate compliance with internationally agreed planetary protection policy, as developed by the Committee on Space Research (COSPAR 2002). Most spacefaring agencies (NASA included) also have their own policy that places specific implementation requirements on agency missions. Planetary protection-gearred approaches for monitoring of biological contamination of spacecraft and spacecraft assembly clean-room (SAC) surfaces at NASA are largely based on heritage data arising from studies supporting the Viking missions in the early 1970s. Scientists postulated that the probability of microorganisms surviving the journey to Mars and propagating upon arrival would be constrained by their hardiness with respect to the conditions they encountered. Therefore, NASA conservatively selected aerobic, heterotrophic mesophilic endospore-forming bacteria as the standard organisms by which to gauge the cleanliness of spacecraft and SAC surfaces. To be more specific, the enumeration of cultivable bacterial spores at the time of launch is compared to a maximum numerical limit. This mission-specific limit is derived from a combination of factors, including mission purpose, target planetary environment, and an assessed factor of risk that any organism transferred to said planetary environment would

result in “harmful (forward) contamination” (NASA 1967). From the Viking era to the present, NASA has employed cotton swabs to collect biological materials from spacecraft and SAC surfaces. Recently, however, it has been shown that the sole use of a cotton swabbing device provides an inaccurate assessment of the total burden and spectrum of microbial diversity associated with SAC surfaces (M. Cooper et al., unpublished data). In addition, the

National Research Council recently stated that NASA should “require the routine collection of (molecular) phylogenetic data to a statistically appropriate level to ensure that the microbial diversity of ATLO environments, and of all NASA spacecraft to be sent to Mars, is reliably assessed.” They also recommended that the space agency “require the systematic archiving of environmental samples for ATLO environments and for all spacecraft to be sent to Mars” (Committee on preventing the forward Contamination of Mars, National Research Council, 2006). While molecular analyses of microbial populations provide a more thorough phylogenetic assessment of spacecraft surfaces, such analysis regimens are not without pitfalls and limitations. Oftentimes, target biomolecules (e.g., rRNA genes, lipopolysaccharides) are not collected in great enough abundance to render downstream molecular analyses statistically significant and/or reproducible, thus requiring a more effective method for sample processing/concentration prior to analysis (La Duc et al., 2009; Vaishampayan et al., 2010). Furthermore, as sample collection protocols are modified to facilitate more effective downstream processing and analysis techniques, the efficiency and effectiveness of the entire procedure must be readdressed. Previous studies evaluating the effectiveness of sampling devices for the recovery of microorganisms from clean-room surfaces have used purified bacterial spores (Edmonds et al., 2009; Estil et al., 2009; Hodges et al., 2010; Hodges et al., 2006; Probst et al., 2010; Rose et al., 2004; Sanderson et al., 2002; Valentine et al., 2008) or pure cultures of vegetative cells (Dalmaso et al., 2008) as positive controls. However, such controls do not accurately represent the highly diverse microbial populations

which can be expected in nearly all environments, including clean rooms (Ghosh et al., 2010; La Duc et al., 2007; Probst et al., 2010; Steiglmeier et al., 2009). Additionally, whenever samples are collected for the purpose of molecular-analysis-based microbial monitoring, results will be affected not only by the presence of living cells but also by dead and decaying cells and spores, cellular debris, and indigenous biomolecules (e.g., nucleic acids, lipids, proteins, carbohydrates). Methodologies by which samples are collected, processed, and analyzed must be optimized to their respective targets to maximize the efficiency and accuracy of the final assessment. To this end, suitable protocols and procedures involved with the collection, processing, and molecular analysis of contaminant biomolecules from clean-room surfaces were evaluated and standardized. Variables such as differences in sampling materials, concentration of target biomolecules post sampling, the effect of cryostorage on extracted molecules, etcetera, were individually tested, employing a model microbial

community (MMC) that is relevant to low-biomass environments. To our knowledge, this is the first study to employ a complex MMC with relevant phylogenetic diversity for the testing and optimization of protocols for collecting, processing, and analyzing biomolecular samples from clean-room surfaces.

4.2 Material and Methods

MMC. Microbes previously reported to be present and/or dominant on spacecraft and SAC surfaces (Bruckner et al., 2008; La Duc et al., 2003; Moissl et al., 2008; Moissl et al., 2007; Moissl-Eichinger 2011; Newcombe et al., 2008) were mixed together to achieve the MMC. These microbial components include bacteria, archaea, fungi, aerobes, anaerobes, spores, and non-spore forming vegetative cells (Table 1). Whenever possible, strains isolated directly from spacecraft and SAC surfaces were included. *Deinococcus radiodurans* (ATCC 13939), *Cupriavidus metallidurans* CH34, *Micrococcus luteus* (ATCC 4698), *Clostridium sporosphaeroides* (DSM 1294), and *Methanobacterium formicicum* (DSM 1535), however, were procured from various culture collections (Table 1). All 11 strains used in this study were placed in the National Center for Agricultural Utilization Research (NRRL) culture collection, in Peoria, IL. The various steps involved in the standardization of procedures for MMC preparation, collection from surfaces, and subsequent processing and analysis are shown in Fig. 1.

Preparation of the MMC. All 11 strains in the MMC, with the exception of *Bacillus pumilus* SAFR-032, were streaked on appropriate culture media and incubated according to the growth conditions given in Table 1. *B. pumilus* spores were purified as previously described (Kempf et al., 2005). Briefly, a single purified colony was inoculated into liquid nutrient broth sporulation medium, and after 2 to 3 days of incubation at 32°C, cultures were examined in wet mounts on an Olympus BX-60 phase-contrast microscope to determine the level of sporulation. Once the number of free spores in the culture was greater than the number of vegetative cells, spores were purified by treatment with lysozyme and washing with salt and detergents (Kempf et al., 2005). Purified spores were resuspended in sterile deionized water, heat shocked (80°C for 15 min), and stored at 4°C in glass tubes. For each strain, a single isolated colony was picked, inoculated into 50 ml of suitable broth, and incubated under appropriate growth conditions (Table 1). Petroff-Hausser microscopic counting chambers were used to verify the cell density of each strain. To circumvent problems associated with clumping as a result of washing and centrifuging, appropriate volumes (precalculated, necessary to achieve 10⁵ cells/spores per MMC constituent) of cells or spores were removed from log-phase overnight broth culture and placed directly into 4 liters of phosphate-buffered saline (PBS, pH 7.2). After averaging 10 fields for each organism, the resulting MMC housed

1×10^6 cells and 1×10^5 (the standard error of the mean was used to assess variability) spores per ml (11 strains $\times 10^5$ cells/spores per ml each). Following rigorous mixing for more than an hour, 1.2-ml aliquots of the MMC were aseptically dispensed into thousands of cryogenic vials and those not immediately analyzed were stored at -80°C . Using the MMC as a positive control, prior to deposition onto stainless steel surfaces, a number of variables associated with sampling and processing were evaluated for their specific effects on the integrity and recovery of the MMC. The ramifications of the use of various concentrations (5 to 20%) of glycerol as a cryoprotectant, various buffered solutions (PBS or water) as suitable liquid media for deposition, ultrasonic energy to enhance the release of the MMC from sampling materials (Puleo et al., 1967), and a method for concentrating large sample volumes were tested, and results were comparatively analyzed.

Deposition of the MMC onto a surrogate spacecraft hardware surface. All stainless steel grade 304-020 (304 brushed stainless steel sheet, 20 gauge, 0.036 in. thick) coupons (25 cm^2) and sheets ($2,500 \text{ cm}^2$) were pre-cleaned with cleanroom-grade polyester wipes (Coventry 6209 c-prime; Tech Spray, L.P., Amarillo, TX). Coupons and sheets were subjected to ultrasonic cleaning with alkaline detergent soap (815 GD; Brulin, Indianapolis, IN) at 160°F , followed by a deionized-water rinse, drying, and a final rinse with 97% ethyl alcohol (Venkateswaran et al., 2004). Subsequently, coupons and sheets were rinsed with deionized water and dried with clean, dry nitrogen. This precision cleaning procedure was effective in removing any cultivable microbes and genetic materials associated with the metal surfaces. All precision-cleaned coupons and sheets were packaged individually in sterile containers until the time of use. Aliquots of the MMC (40 $\times$ 1.2 ml) were thawed, pooled, and concentrated via centrifugation (15 min, 4,000 $\times$ g) using the Amicon 50-kDa filtration system (catalog no.UFC905024; Millipore, Billerica, MA). Finally, to circumvent crystal formation resulting from PBS precipitation on metal surfaces, the MMC was washed and resuspended in sterile water to a final volume of 48 ml prior to deposition. Stainless steel coupons (25 cm^2) and sheets ($2,500 \text{ cm}^2$) were aseptically removed from their sterile packaging and placed into individual sterile petri dishes or trays, respectively. One-milliliter aliquots of the MMC were deposited onto either coupons (20 $\times$ 50 μl drops) or sheets (50 $\times$ 20 μl drops) using a multichannel, multidispensing electronic pipette (E8-200; Rainin, Oakland, CA). All coupons and sheets, including those serving as sampling blanks, were covered and allowed to dry for 24 h. To minimize bias associated with sample collection, care was taken to ensure that the operator in charge of deposition was not also in charge of sampling.

Sampling of MMC-spiked metal surfaces. (i) Small surface areas (25 cm²). Coupons were sampled with either cotton (catalog no. 806-WCL; Puritan Medical Products, Guilford, ME) or nylon flocked (catalog no. 552C.US; Copan Diagnostics Inc., Murrieta, CA) swabs, which were premoistened with sterile PBS immediately prior to sampling. Coupons were first sampled in a unidirectional horizontal manner while holding the swab at approximately a 30° angle to the surface. Swabs were rotated (ca. 120°) to present an area of the swab head that had not previously contacted the surface, and coupons were sampled in a unidirectional vertical manner. Finally, swabs were once again rotated (ca. 120°) and coupons were sampled in a unidirectional diagonal manner. Swab heads were aseptically severed from their shafts into individual 50-ml Falcon tubes containing 10 ml of sterile PBS. A negative control, whereby a swab was only premoistened with PBS, and a handling control, in which a swab was premoistened with PBS and exposed to the sampling environment, were also prepared.

(ii) Large surface areas (2,500 cm²). Stainless steel sheets were sampled with either ITW Alpha polyester wipes (catalog no. TX1009; Texwipe, Kernersville, NC) or biological sampling kits (BiSKit, catalog no. BIS-40001A; QuicksilverAnalytics Inc., Abingdon, MD). Wipes were rolled and placed into individual 50-ml glass test tubes containing 15 ml of PBS, which were autoclaved and allowed to cool prior to sampling (Kirschner 1979). Wearing sterile gloves, we removed the wipe from the tube with sterile forceps and unrolled it, leaving it folded in quarters. Sheets were first sampled in a unidirectional horizontal manner, after which time wipes were turned over and used to sample the sheets in a unidirectional vertical manner. Finally, the wipe was inverted to expose a fresh surface and the sheet was sampled in a unidirectional diagonal manner. By this method, 3/4 of one side of the wipe was used in sampling the surface. Negative and handling controls were prepared as detailed above for coupons. All wipes were placed into individual sterile glass 500-ml bottles for processing. BiSKits were moistened with 15 ml of sterile PBS, which was expelled from each unit and pooled to serve as the negative control. The collection bottle of each BiSKit was replenished with 15 ml of sterile PBS, and the entire module was inverted, allowing the macrofoam sponge to become saturated. The macrofoam sponge sampling portion of the BiSKit module was removed, and sheets were first sampled in a unidirectional horizontal manner. The BiSKit was then turned, and the sheets were sampled in a unidirectional vertical manner. Finally, the BiSKit was once again turned and sheets were sampled in a unidirectional diagonal manner. A handling control, in which a BiSKit was exposed to the environment but no surfaces, was also prepared.

TABLE 1. Various characteristics of MMC constituents

Sample no.	Micrrobe	Strain	Domain: phylum	Morphology	Culture conditions or reference ^a	Incubation time (h)	Other available source(s) ^b	Significance (reference(s))
1	<i>Aerobasidium pullulans</i>	28a.1	Eukarya: Ascomycota	Black yeast	TYG agar, 30°C	86	NRRL 58992	Eukaryotic representative, facultative anaerobic spore former, original isolate (16)
2	<i>Atherobacter radiocressiens</i>	50a.1	Bacteria: Gammaproteobacteria	Short rods	TYG agar, 32°C	60	NRRL B-59417	Resistant to desiccation and radiation, aerobic non-spore former, original isolate (16)
3	<i>Bacillus megaterium</i>	KL-197	Bacteria: Firmicutes	Rods	TYG agar, 32°C	38	NRRL B-59415	Frequently isolated from SAC (15, 16), facultative anaerobic spore former, vegetative cells of original isolate
4	<i>Bacillus pumilus</i>	SABR-032	Bacteria: Firmicutes	Spores	See reference 13	NA ^c	ATCC PTA-7603, NRRL B-30938	Strains showing extraordinary UV resistance, frequently isolated from SAC (13), facultative anaerobic spore former, spores of original isolate
5	<i>Deinococcus radiodurans</i>	ATCC 13939	Bacteria: Deinococcus-Thermus	Cocci, tetrad	TYG agar, 32°C	60	NRRL B-59418, DSM 20639	Strains showing extraordinary gamma radiation resistance, sequences retrieved from spacecraft surfaces (3), aerobic non-spore former, isolate procured from culture collection
6	<i>Morobacterium imperiale</i>	43v.1	Bacteria: Actinobacteria	Short rods	TYG agar, 32°C	60	NRRL B-59416	Extremely hardy, difficult to extract DNA from cells (17), aerobic non-spore former, original isolate
7	<i>Staphylococcus warneri</i>	82-4	Bacteria: Firmicutes	Cocci	TYG agar, 32°C	40	NRRL B-59414	Frequently isolated from SAC (16, 35), human associated, facultative anaerobic non-spore former, original isolate
8	<i>Mariproccus luteus</i>	ATCC 4698	Bacteria: Actinobacteria	Rods	Nutrient agar, 32°C	60	NRRL B-59413, DSM 20030	Common contaminant of indoor environments and SAC (16), air borne and human associated, aerobic non-spore former, original isolate
9	<i>Cupriavidus metallidurans</i>	CH34 ^d	Bacteria: Betaproteobacteria	Rods	TYG agar, 32°C	60	ATCC 43123	Ubiquitously isolated from SAC (22), heavy metal resistant, aerobic non-spore former
10	<i>Clostridium sporophtharoides</i>	DSM 1294	Bacteria: Firmicutes	Anaerobic rods	DSMZ medium 78, 37°C, anaerobic	48	ATCC 25781	Representative of obligate anaerobic spore formers found in SAC (30, 35), isolate procured from culture collection, vegetative cells
11	<i>Methanobacterium formicicum</i>	DSM 1535	Archaea: Euryarchaeota	Rods	DSMZ medium 119, 37°C, anaerobic	72	ATCC 33274	Representative of broad (uncultivated) archaeal diversity of SAC (21, 23), anaerobic non-spore former, isolate procured from culture collection

^a TYG, tryptone yeast extract glucose.^b ATCC, American Type Culture Collection; NRRL, USDA culture collection; DSM, German collection of cell cultures; PTA, patented strain.^c NA, not applicable since the spores were prepared prior to the investigation and the appropriate concentration from the original stock was used.^d Received from Natalie Leys, Hooft Onderzoeksinstituut Microbiologie at SCK-CEN, Antwerp, Belgium.

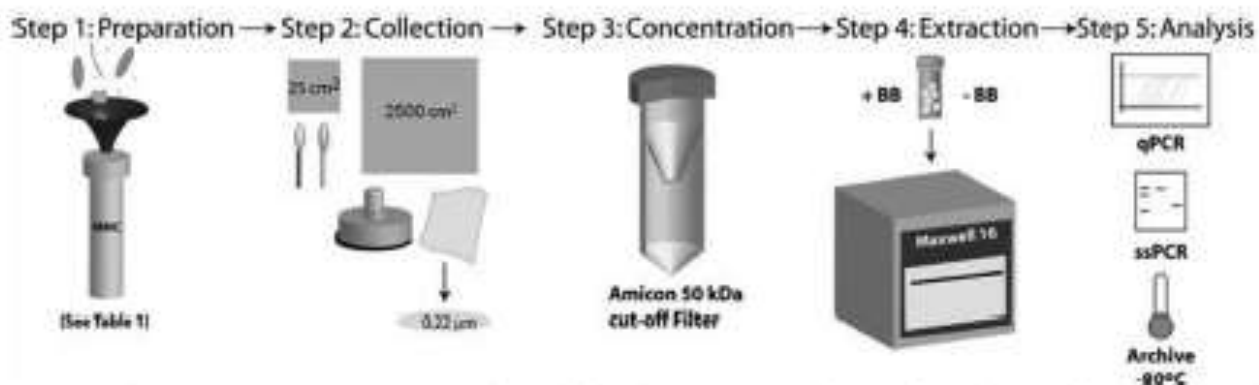


FIG. 1. Schematic representation of various procedures involved in the preparation of the MMC and its subsequent deposition onto metal surfaces, sample collection, processing, and analysis.

Sample processing. Each cotton or nylon-flocked swab was subjected to three rounds of biomolecule extraction processing. This consisted of vortex mixing (5 s, maximum speed), sonication in an ultrasonic bath (2 min, ~25 kHz), decanting into a fresh sterile 50-ml tube, and replenishment of 10 ml of sterile PBS. In the end, each swab head yielded a total of 30 ml of biomolecule extraction eluent, which was then concentrated to approximately 400 µl via two rounds of Amicon centrifugal filtration (15 min, 4,000 x g). Following the addition of 200 ml of sterile PBS, each polyester wipe containing bottle was vortex mixed (5 s, maximum speed) and sonicated in an ultrasonic bath (2 min, ~25 kHz). This was repeated three times, resulting in 600 ml of biomolecule extraction eluent, which was then subjected to membrane filtration (0.22-µm pore size). The 0.22-µm filter was aseptically transferred into a sterile 50-ml Falcon tube containing 10 ml of sterile PBS and vigorously agitated via vortex mixing (5 min, maximum speed). The resulting suspension was concentrated to approximately 400 µl via Amicon centrifugal filtration (15 min at 4,000 x g). Each BiSKit was subjected to three rounds of biomolecule extraction processing. The macrofoam sponge sampling portion of each BiSKit was mated with its respective sample collection module, and with appropriate force, sample volumes were expelled into the collection bottle in accordance with the manufacturer's instructions. Sample volumes were transferred into a 50-ml Falcon tube, and an additional 15 ml of sterile PBS was added to the BiSKit collection bottle, which was then reattached for subsequent extractions. The resulting suspension (ca. 45 ml) was concentrated to approximately 400 µl via Amicon centrifugal filtration (50 kDa, 15 min at 4,000 x g). The final concentrated biomolecule extract volume from each sampling device was resuspended in flowthrough from the final Amicon filtration to a total volume of 1.5 ml. Appropriate dilutions were then spread plated on suitable media, and CFU were enumerated after

incubation under prescribed conditions (Table 1). Total cell/spore counts were also estimated via light microscopy as previously described (Lawrence et al., 1991). Five hundred microliters of this biomolecule extract volume was subjected to bead beating homogenization (using Lysing Matrix E and the FastPrep-24 Homogenizer for 1 min at a rate of 5.0 motions per s; MP Biomedicals, Solon, OH) and subsequently combined with sample portions (500_1) that did not undergo bead beating (a total of 1 ml). This helped to ensure that the samples would contain DNA released from robust and labile cell types, as has been previously documented (La Duc et al., 2009). This combined milliliter then gave rise to 100 µl of purified DNA following extraction via the Maxwell 16 Automated DNA/ RNA Purification System (Promega, Madison, WI) in accordance with the manufacturer's instructions.

Sample analysis. (i) SYBR green quantitative PCR (qPCR). rRNA gene copy numbers were quantified in triplicate using a CFX-96 instrument (Bio-Rad, Hercules, CA). Standards were created using known amounts of full-length *Escherichia coli* 16S rRNA genes incorporated into suitable vector plasmids (Invitrogen, Carlsbad, CA), and standard curves were repeated for each qPCR. rRNA gene standards, spanning 10⁸ to 10² gene copies/µl, were generated by serially diluting *rrn*::pCR4-TOPO plasmids of known concentrations. Universal eubacterial primers (1369F and 1492R [Suzuki et al., 2000]) targeting the 16S rRNA gene and eukaryotic biased primers (NS91F and ITS51R [White et al., 1990]) targeting the internal transcribed spacer 1 region within the rRNA gene cluster, were employed for qPCR analysis. Each 25-µl reaction mixture consisted of 12.5 µl of Bio-Rad 2X iQ SYBR green Supermix, 10.5 µl of nuclease-free water (catalog no. W4502; Sigma, St. Louis, MO), 0.5 µl of forward primer 1369F (10 µM) or NS91F (10µM), 0.5 µl of reverse primer 1492R (10 µM) or ITS51R (10 µM), and 1 µl of template DNA. Purified rRNA gene amplicon standards of known concentrations and Sigma nuclease-free water negative controls were included in all experiments. Reaction conditions for 16S *rrn* qPCR were as follows: denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s and combined annealing and extension at 55°C for 35 s. Reaction conditions for eukaryotic *rrn* qPCR were as follows: denaturation at 95°C for 5 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 20 s, and extension at 72°C for 15 s and 80°C for 5 s.

(ii) Species-specific PCR (ssPCR). In order to assess the differential recovery of the various microbial phylotypes from both the stainless steel surfaces and sampling devices, ssPCR assays were engineered for each MMC strain. Species specific primer sequences are provided in Table 2, alongside detailed thermal cycling conditions for each strain. None of the resulting

primer combinations (Table 2) showed cross-reactivity with any of the other members of the MMC. Full-length (ca. 1,500-bp) rRNA gene amplicons were purified via gel excision and used as standards once concentrations were determined via microchannel based electrophoresis (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA) (Panaro et al., 2000). All amplicons detected at a concentration of less than or equal to 0.25 ng/μl fell below the limits of statistically significant detection and were ignored.

Statistical analyses, controls, and lower detection limits of assays employed. Care was taken to incorporate appropriate controls into each step of the sampling and analysis procedures discussed herein. Sterile, nuclease-free Sigma water and sterile PBS served as blanks to monitor the continued purity of reagents. The independent variables consisted of sampling device replicates (cotton and nylon-flocked swabs, BiSKits, and polyester wipes), their corresponding sampling blanks (active sampling of stainless steel sheets not seeded with the MMC), handling controls (sampling devices were exposed to the experimental environment), and negative controls (sampling materials with reagents). When needed, the MMC was used as a positive control with the medium blank serving as the corresponding negative control. Purified DNA arising from each MMC constituent served as a positive control for that strain's respective ssPCR assay, and samples that rendered no PCR amplification were spiked with positive- control DNA as a means of eliminating the possibility of false negatives resulting from inhibitory substances. The cell density of microbes or concentration of 16S rRNA genes housed within the MMC prior to deposition on the metal surface was considered to be 100%. The recovery percentage of colony counts, microscopic counts, total gene copy numbers, and species-specific gene copy numbers yielded by various sampling materials was calculated by dividing resulting values by the initial values representative of 100% (MMC prior to deposition). To elucidate subtle differences in resulting efficiencies of recovery from the sampling materials tested in this study (cotton versus nylon-flocked swabs; BiSKits versus polyester wipes), statistical analyses were carried out at all stages of analysis with the SYSTAT VER. 13.00.05 statistical software suite (SYSTAT Software, Chicago, IL). Briefly, routine Pearson's chi-square tests were performed to determine whether the distributions of resulting data sets were statistically normal, and paired Student t tests were employed to test the statistically significant similarity between the means of the results being compared.

TABLE 2. ssPCR conditions

Sample no., microbe, and primers	Primer sequences (5'-3') ^a	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles	Final extension	Amplicon size (bp)
1. <i>Aureobasidium pullulans</i> 28v1, ^b ssAP/ssAPr	AAT AAG GAT TGA CAG ATT GATAA GGT CTC GIT CGT TAT	94°C, 4 min	94°C, 50 s	61°C, 50 s	72°C, 50 s	35	72°C, 10 min	120
2. <i>Achromobacter nuckelsiens</i> 50v1, ^c ssARf/ssARr	GCC TTA TGG TTG TAA AGC/CTT ATT CTG CGA GTA ACG	94°C, 4 min	94°C, 50 s	53°C, 50 s	72°C, 50 s	35	72°C, 10 min	94
3. <i>Bacillus megaterium</i> KL-197, ^c ssBMf/ssBMr	GTG CTA CAA TGG ATG GTN/AAT CCG AAC TGA GAA TGG	94°C, 4 min	94°C, 50 s	52°C, 50 s	72°C, 50 s	35	72°C, 10 min	85
4. <i>Bacillus pumilus</i> SAFR-032, ^c 600f/980r	TGA AGC ACT TGA GAA ATT/TGC TGC AAA GAA AAT	No	95°C, 1 min	58°C, 30 s	72°C, 50 s	35	72°C, 10 min	380
5. <i>Deinococcus radiodurans</i> ATCC 13939, ^c ssDRf/ssDRr	CCA GAA GTC ACG AAT AAC/ATC CAG AAG CGA TAA ATC	94°C, 4 min	94°C, 50 s	54°C, 50 s	72°C, 50 s	35	72°C, 10 min	103
6. <i>Microbacterium imperiale</i> 47v1, ^c ssMf/ssMf	AAC GGC GTC TAA TAC TGG ATA TG A/AGC CCA TCC CAG ACC AAA	94°C, 4 min	94°C, 50 s	54°C, 50 s	72°C, 50 s	35	72°C, 10 min	83
7. <i>Staphylococcus warneri</i> 82-4, ^c ssSWf/ssSWr	ATA ACC TAC CTA TAA GAC T/ATC CAT CTA TAA GTG ACA	94°C, 4 min	94°C, 50 s	58°C, 50 s	72°C, 50 s	35	72°C, 10 min	124
8. <i>Micrococcus luteus</i> ATCC 4698, ^c ssMLf/ssMLr	TAA CCT GCC CTT AAC TCT/AAA CCG ATA AAT CTT TCC AA	94°C, 4 min	94°C, 50 s	55°C, 50 s	72°C, 50 s	35	72°C, 10 min	106
9. <i>Cupriavidus metallidurans</i> CH34m, ^d ssCMf/ssCMr	GGT GGA TGA TGT GGA TTN/ATC TCT GCT TCG TTA GTG	94°C, 4 min	94°C, 50 s	64°C, 50 s	72°C, 50 s	36	72°C, 10 min	86
10. <i>Clostridium sporosphaeroides</i> DSM 1294, ^c ssCSf/ssCSr	CAA GCA GTG GAG TAT GTG/CCT CGT TAG TTG GAT GTC	94°C, 4 min	94°C, 50 s	53°C, 50 s	72°C, 50 s	35	72°C, 10 min	84
11. <i>Methanobacterium formicicum</i> DSM 1535, ^c ssSMFf/ssSMFr	ATT GCT GGA GAT ACT ATT/GGG ATT ATA GGA TTT CAC	94°C, 4 min	94°C, 50 s	52°C, 50 s	72°C, 50 s	35	72°C, 10 min	89

^a All primers had an original concentration of 9 μ M.^b Reaction mixture (25 μ l/sample) consisted of 12.5 μ l 2 $\times$ BioMix (catalog no. 23012; BioLine Inc., Taunton, MA), 10.3 μ l nuclease-free water, 0.6 μ l forward primer, 0.6 μ l reverse primer, and 1 μ l template DNA.^c Reaction mixture (25 μ l/sample) consisted of 12.5 μ l 2 $\times$ BioMix (catalog no. 25012; BioLine Inc., Taunton, MA), 9.5 μ l nuclease-free water, 1 μ l forward primer, 1 μ l reverse primer, and 1 μ l template DNA.^d Reaction mixture (25 μ l/sample) consisted of 12.5 μ l 2 $\times$ BioMix (catalog no. 25012; BioLine Inc., Taunton, MA), 8.5 μ l nuclease-free water, 2 μ l MgCl₂ (50 mM), 1 μ l forward primer, 1 μ l reverse primer, and 1 μ l template DNA.

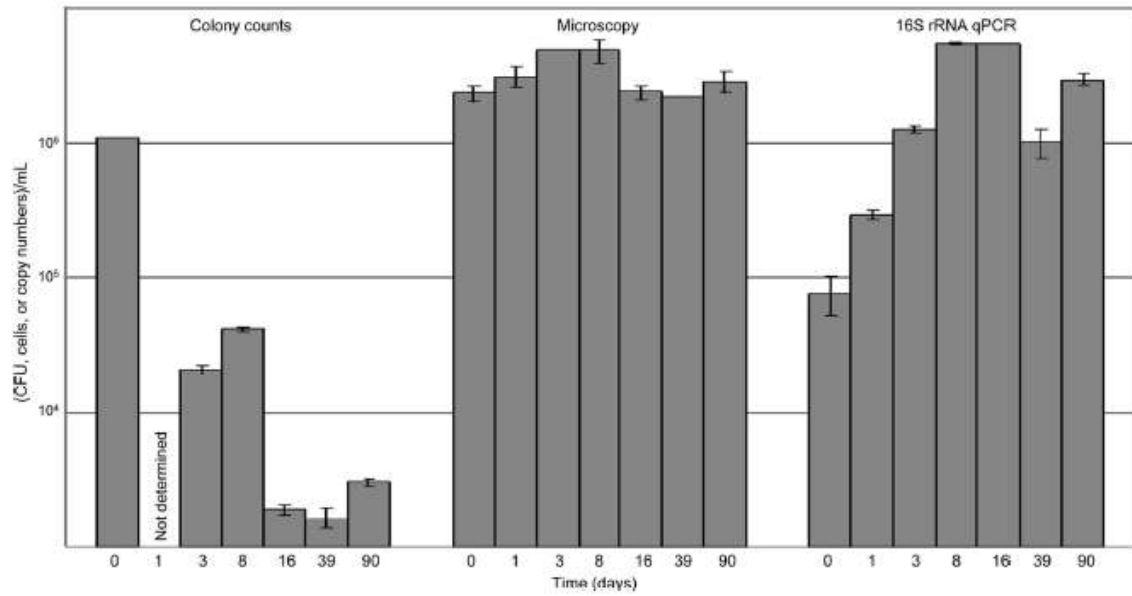


FIG. 2. Effect of low-temperature storage on MMC cellular cultivability and 16S rRNA gene integrity. Samples were stored at 80°C and processed at various intervals. CFU were counted after culture under appropriate conditions, total cells were enumerated microscopically with a Petroff-Hausser counting chamber, and 16S rRNA gene copy numbers were measured via qPCR. Error bars represent the standard error of the mean ($n = 10$).

4.3 Results

Effect of storage on the MMC. While storage for up to 90 days at -80°C had little or no effect on total cell/spore counts derived via Petroff-Hausser light microscopy counting ($\sim 10^6$ cells/spores per ml), the cultivability of MMC constituents decreased as much as 3 orders of magnitude (Fig. 2). When MMC aliquots were removed from -80°C storage at various time points (up to 90 days), subjected directly to DNA extraction protocols, and assessed via qPCR, the total DNA yield increased with increased storage time through 8 days, at which time it remained constant for up to 90 days at -80°C (Fig. 2).

Effects of sampling and processing variables on the MMC. Using the MMC as a positive control, several processing techniques were thoroughly evaluated prior to the testing and evaluation of the different surface sampling regimens. Glycerol concentrations of >5% hindered the centrifugal filtration of sample volumes, presumably due to the clogging of filter pores (data not shown). In addition, results stemming from the use of two distinct types of suspension media (water and PBS) for deposition of the MMC onto stainless steel revealed a greater recovery (150-fold) of MMC-borne DNA when cells were suspended in water. Scanning electron microscopy showed a high degree of salt crystal formation on metal coupons laden with PBS-suspended MMC, whereas those seeded with water-suspended MMC had no visible crystals (data not shown). In this study, ultrasonic energy, previously reported to facilitate the release of bacterial spores from sampling materials (Puleo et al., 1967), did not dramatically hinder the cultivation of MMC strains ($\sim 80\%$ cultivable after sonication), nor did it compromise the integrity of sample DNA molecules (see the supplemental material). Finally, following concentration via Amicon 50-kDa centrifugal filters, $\sim 60\%$ of the MMC DNA was recovered. However, additional washing of the filters did not result in a greater DNA yield.

Recovery of the MMC from stainless steel surfaces. (i) Small surface areas. The efficiency of MMC constituent recovery from 25-cm² stainless steel surface areas by cotton and nylon-flocked swabs was comparatively analyzed via qPCR (Fig. 3A). Using the mean MMC rRNA gene abundance as a point of comparison, cotton significantly outperformed nylonflocked swabs ($P = 0.00001$, $n = 30$) with recovery percentages of 32.8% and 4.3%, respectively. While the signal-to-noise (noise is contaminant DNA) ratio associated with cotton swab sampling blanks was high (19.6), nylon-flocked sampling blanks had a much

lower ratio (0.6). In addition, a wide range of values resulted among both cotton (10^4 to 10^5 *rrn* copies/25 cm²) and nylon-flocked (10^3 to 10^5 *rrn* copies/25 cm²) sampling replicates, possibly attributable to subtle variability in the composition of the swabbing matrices or the stainless steel surface.

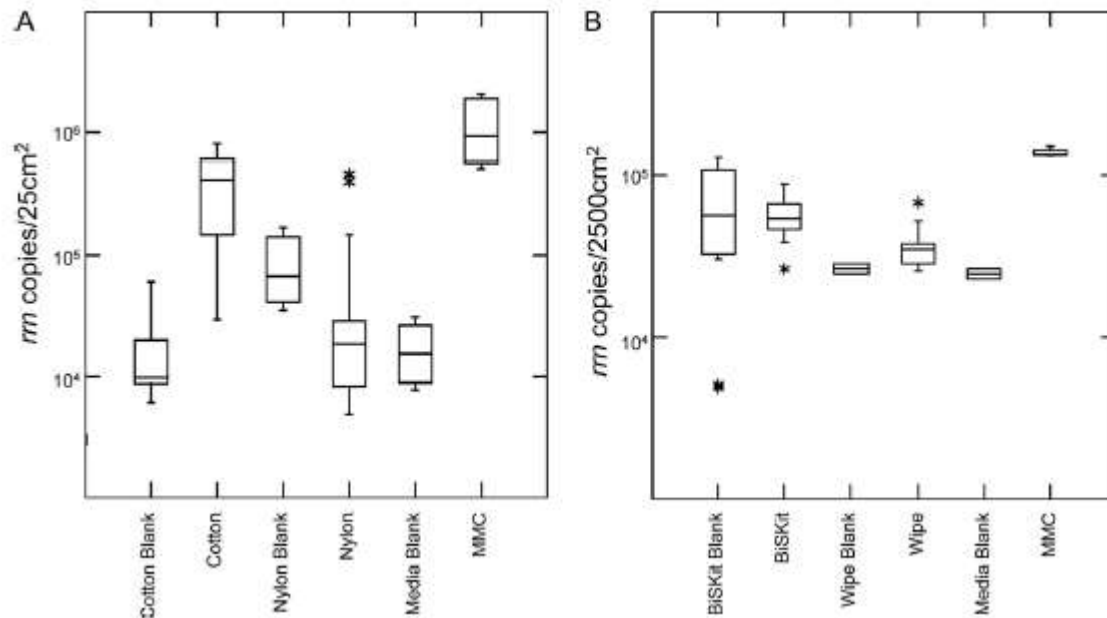


FIG. 3. Recovery of MMC constituents from metal surfaces by various sampling materials. Aliquots of the MMC were deposited onto metal surfaces, dried for 24 h, and collected with various sampling materials. Samples were analyzed via 16S rRNA qPCR, and results were visualized with box plots showing the mean and upper and lower quartiles for cotton and nylon-flocked swabs (A) and BiSKits and polyester wipes (B). Outliers (a greater-than-3-sigma deviation from the mean) are represented by asterisks. $n = 30$ measurements for each sampling material. Error bars depict the standard error of the mean in box plot format.

TABLE 3. Limits of detection of MMC constituents by ssPCR

Sample no.	Microbe	Presence of amplicons at initial inoculum concn (cells/ml) ^a of:						Detection limit (CFU/ml) ^b
		10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	
1	<i>Aureobasidium pullulans</i> 28v1	+	—	—	—	—	—	9.0 × 10 ⁶
2	<i>Acinetobacter radioresistens</i> 50v1	+	+	+	+	—	—	3.4 × 10 ³
3	<i>Bacillus megaterium</i> KL-197	+	+	+	+	+	—	2.1 × 10 ²
4	<i>Bacillus pumilus</i> SAFR-032	+	+	+	+	+	+	4.6 × 10 ¹
5	<i>Deinococcus radiodurans</i> ATCC 13939	+	+	+	—	—	—	1.6 × 10 ⁴
6	<i>Microbacterium imperiale</i> 47v1	+	+	+	+	—	—	5.6 × 10 ³
7	<i>Staphylococcus warneri</i> 82-4	+	+	+	—	—	—	4.3 × 10 ⁴
8	<i>Micrococcus luteus</i> ATCC 4698	+	+	+	—	—	—	4.5 × 10 ⁴
9	<i>Cupriavidus metallidurans</i> CH34	+	+	+	+	—	—	4.7 × 10 ³
10	<i>Clostridium sporosphaeroides</i> DSM 1294 ^c	+	+	—	—	—	—	7.7 × 10 ⁵
11	<i>Methanobacterium formicicum</i> DSM 1535 ^c	+	+	—	—	—	—	1.4 × 10 ⁵

^a The number of cells was determined via microscopic counting. After serial dilution, DNA from the reaction mixtures was extracted using the Maxwell 16 system. See Materials and Methods for details.

^b The PCR set was scored positive (+) when the amplified products were ≥0.25 ng/μl, as quantified by Bioanalyzer.

^c DNA, instead of whole cells, was serially diluted for this strain.

(ii) Large surface areas. The efficiency of MMC constituent recovery from 2,500-cm² stainless steel surface areas by BiSKit and polyester wipes was comparatively analyzed via qPCR (Fig. 3B). Using the mean MMC rRNA gene abundance as a point of comparison, BiSKit significantly outperformed wipes ($P = 0.00001$, $n = 21$) with recovery percentages of 41.9% and 26.7%, respectively. While the signal-to-noise (noise is contaminant DNA) ratio associated with polyester wipe sampling blanks was high (4.7), BiSKit sampling blanks had a much lower ratio (1.1). In addition, a wide range of values resulted among BiSKit sampling replicates (10^4 to 10^5 *rrn* copies/2,500 cm²), possibly attributable to subtle variability in the composition of the sampling matrices or the stainless steel surface.

ssPCR. Species-specific oligonucleotide primers targeting the rRNA genes of each of the MMC constituents were developed alongside PCR thermal cycling regimens to assess the recovery of each MMC strain. Target PCR amplicons ranged in size from 83 bp (*Microbacterium imperiale*) to 380 bp (*B. pumilus*, Table 2), which enabled qPCR analysis of each strain's rRNA gene. The detection limit of ssPCR for each MMC constituent was established by serially diluting intact vegetative cells (from 10^7 to 10^1 cells or spores/ml) and subsequently subjecting each dilution to DNA purification and appropriate ssPCR conditions. These limits have, to date, varied extensively (10^1 to 10^6 ; Table 3). Notably, the ssPCR conditions used for *B. pumilus* SAFR-032 spores were highly sensitive, capable of amplifying 380-bp products from spore densities as low as 4.6×10^1 spores/ml. The other aerobic spore former, *B. megaterium* KL-197, yielded specific PCR products at 10^2 cells/ml. In contrast, detection limits for the anaerobic species *C. sporosphaeroides*, and *M. formicicum* (10^5 cells/ml) were determined from serially diluted DNA rather than intact cells since these anaerobic cultures did not yield uniform cell densities below 10^5 cells/ml. Trends in the

differential recovery of MMC constituents achieved by various sampling devices were evaluated via standard and quantitative ssPCRs (Table 4). Traditional ssPCRs were performed with DNA extracts arising from each sampling replicate, and resulting amplicons were visualized with an Agilent 2100 Bioanalyzer. As was observed with general rRNA gene qPCR, cotton swabs proved more effective than nylonflocked swabs in collecting MMC-derived DNA from stainless steel surfaces (66.2% versus 48.1% occurrence, respectively; Table 4) following upon ssPCR analysis. Similarly, BiSKits outperformed polyester wipes in the recovery of MMC constituents from large surfaces (55.8% versus 40.3% incidence, respectively; Table 4). *B. megaterium* KL-197 was the only MMC strain whose rRNA gene was recovered from every sampling device replicate (Fig. 4 and Table 4). The rRNA genes of other desiccation-tolerant strains, such as *D. radiodurans* R1 and *Acinetobacter radioresistens* 50v1, were retrieved in the majority of swab and BiSKit sampling replicates (Fig. 4 and Table 4). *Aureobasidium pullulans* 28v1 and *Staphylococcus warneri* 82-4 were the only MMC constituents whose DNA was not recovered, regardless of the sampling matrix employed (Fig. 4 and Table 4). Coincidentally, the detection limits for these strains were among the highest within the MMC (9.0×10^6 and 4.3×10^4 , respectively). Finally, effective recovery of the *C. sporosphaeroides* rRNA gene was limited to both swabbing materials from small surface samples, as BiSKits and polyester wipes failed to yield amplifiable DNA from large surfaces (Fig. 4 and Table 4).

TABLE 4. Presence/absence of specific MMC species from several sampling material replicates

Microbe ^a	PCR amplicon size (bp)	Presence/absence of microbes ^b after sampling with:																											
		Cotton swab replicates							Nylon-flocked swab replicates							BiSKit replicates							Polyester wipe replicates						
		C1	C2	C3	C4	C5	C6	C7	N1	N2	N3	N4	N5	N6	N7	B1	B2	B3	B4	B5	B6	B7	W1	W2	W3	W4	W5	W6	W7
<i>Aureobasidium pullulans</i> 28v1	120	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Acinetobacter radioresistens</i> 50v1	94	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bacillus megaterium</i> KL-197	85	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bacillus pumilus</i> SAFR-032	380	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Deinococcus radiodurans</i> ATCC 13939	103	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Microbacterium imperiale</i> 47v1	83	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Staphylococcus warneri</i> 82-4	124	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Micrococcus luteus</i> ATCC 4698	106	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Cupriavidus metallidurans</i> CH34	86	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Clostridium sporosphaeroides</i> DSM 1294	84	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Methanobacterium formicicum</i> DSM 1535	89	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

^a *A. pullulans* and *S. warneri* were not detected in any of the sampling materials.

^b The PCR set was scored positive (+) when the amplified products were ≥ 0.25 ng/ μ l as quantified by the Agilent Bioanalyzer. The percent occurrence was calculated based on positive amplification from the total number of assays. For example, of the total of 77 samples (11 species $\times$ 7 replicates per sampling device), percent incidence is the percentage of 77 samples where DNA was detected.

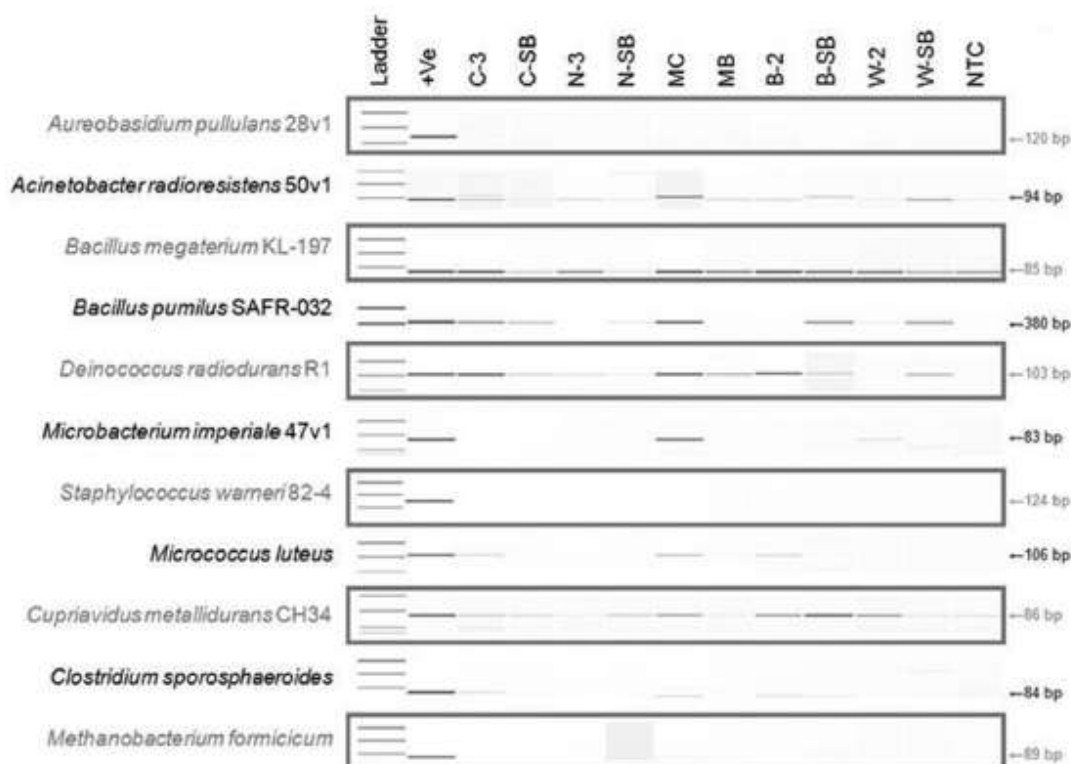


FIG. 4. ssPCR gel electrophoresis results for each MMC constituent. Selected sample replicates and sampling blanks of each sampling device, MMC aliquots, and media blanks were subjected to ssPCR for each MMC strain. ssPCR products were visualized via microchannel-based electrophoresis with an Agilent 2100 Bioanalyzer. Abbreviations: -Ve, positive control (DNA from cultured cells); C-3, cotton swab replicate 3; C-SB, cotton swab sampling blank; N-3, nylon-flocked swab replicate 3; N-SB, nylon-flocked swab sampling blank; MC, MMC (unseeded); MB, medium blank used for MMC; B-2, BiSKit replicate 2; B-SB, BiSKit sampling blank; W-2, polyester wipe replicate 2; W-SB, polyester wipe sampling blank; NTC, no-template control.

4.4 Discussion

The goal of this study was to evaluate and standardize protocols and procedures involved with the collection, processing, and molecular analysis of contaminant biomolecules from SAC surfaces. While several protocols are in place for the sampling of low-biomass surfaces (Brown et al., 2007; Brown et al., 2007; Buttner et al., 2001; Hodges et al., 2010; Lewandowski et al., 2010; NASA 1980, Probst et al., 2010), few employ molecular-analysis-based methods, and most experiment only with one or two test organisms. In this study, a consortium of bacterial spores and cells representing all 3 domains of microbial life was engineered as a proxy for the diverse, low-abundance microbial populations typical of clean-room environments. This MMC was used to test and optimize the numerous stages involved in molecular microbial diversity analysis. To the best of our knowledge, this is the first study to employ a complex MMC of diverse phylogenetic relevance to the testing and optimization of protocols for collecting, processing, and analyzing biomolecular samples from clean-room surfaces. Using the MMC as a positive control, a complex suite of sample handling and processing techniques was thoroughly evaluated prior to the testing and evaluation of different sampling regimens. The use of cryoprotectant chemicals (e.g., dimethyl sulfoxide, glycerol) is well known to protect microbial cells and allow increased viability and cultivability following prolonged storage at low temperatures (Li and Richey, 2004). However, as the MMC was intended to mimic the complex array of live, dead, and degraded material found on clean-room surfaces, no attempt to preserve the integrity of MMC organisms was made once the MMC was mixed. Instead, care was taken to protect the molecular analyses from the seemingly unavoidable presence of contaminant nucleic acid residues associated with cryoprotectant reagents. Furthermore, glycerol concentrations suitable for maintaining cellular cultivability (Li and Richey, 2004) impeded the centrifugal filtration of large sample volumes. Ergo, the use of such chemicals was intentionally avoided for this study and the MMC was stored at -80°C in PBS only. Similarly, while PBS was used for storing the MMC, it was found to hinder the recovery of MMC biomolecules. PBS and other buffered solutions may help prevent cellular death and degradation resulting from osmotic stress, but such protection comes with a cost: the precipitation and deposition of salt crystals onto the metal surfaces induce changes in surface chemistry and hinder the recovery of microbes (150-fold lower than with water-suspended MMC) (data not shown). Furthermore, such protection from drying and other osmotic stresses

is not representative of the conditions within cleanroom environments. Therefore, the MMC was harvested via centrifugal filtration from PBS and resuspended in sterile water (with accepted consequences for osmotically sensitive MMC strains) immediately prior to its deposition onto metal surfaces. For the purposes of this evaluative study and in accordance with previously published findings, it was important to consider the amount of time the MMC spent in a completely desiccated state while dried onto the metal surface. By applying tapelifting and/or polyvinyl alcohol coating techniques, effective removal of bacterial spores from metal surfaces was demonstrated, with recoveries as high as 90% for up to 18 months of desiccation (Tauscher et al., 2006). With regard to vegetative cells, however, previous unpublished studies have shown that the recovery of vegetative cells from metal surfaces was negatively influenced by increasing drying times on metal surfaces or swab heads (from a few to 24 h). In order to accurately mimic the conditions present in a humidity-controlled clean room (relative humidity of ~40 to 55%, facility dependent), the MMC was deposited and allowed to dry for 24 h on the metal surface prior to sample collection. There were several variables to examine with regard to the processing of collected MMC samples. Subjecting sampling devices to a sonic bath upon the addition of resuspension solution (sterile water or PBS) has been shown to promote the release of cells and spores from cotton, foam, and polyester matrices (Puleo et al., 1967). In this study, sonication did not dramatically hinder the cultivation of MMC strains (~80% cultivable after sonication), nor did it compromise the integrity of sample DNA molecules. As different sampling devices required different volumes of resuspension fluid (~45 to 600 ml) for optimum recovery, following sonication, the sample eluates underwent a centrifugal concentration step with Amicon filters. This concentration of cells, spores, and all other materials of biotic origin proved very effective and facilitated molecular biological downstream analysis, which is not possible at lower concentrations. The ~60% recovery of DNA achieved using Amicon centrifugal filters was superior to all other available protocols. Previously, DNA loss as high as 90% was reported when ~10 ml of sample was directly subjected to standard phenol-chloroform-based DNA extraction procedures, likely resulting from the direct processing of large sample volumes (~10 ml) without prior concentration (La Duc et al., 2009; Sambrook et al., 1989). Other studies have also confirmed the necessity of centrifugation or filtration of large sample volumes prior to downstream DNA processing and analysis (Buttner et al., 2001). The Amicon centrifugal filtration procedure advocated in this study proved effective and yielded reproducible results for sample volumes of up to 45 ml. When processing the ~600-ml samples collected with

polyester wipes, the entire volume was first subjected to 0.22- μ m membrane filtration and then further concentrated via Amicon centrifugal filtration. To achieve thorough and effective lysis of the hardest and most diverse microbial cell walls and outer membranes, both automated and manual alkaline lysis-based DNA purification procedures can be augmented with some form of mechanical abrasion (Sambrook et al., 1989). A simple bead-beating step prior to nucleic acid extraction protocols was necessary to lyse open the extremely resilient actinobacterial cells and even bacterial spores from environmental samples (La Duc et al. 2009, Wilson et al., 2002). However, bead beating or any other form of mechanical abrasion could inadvertently shear and/or significantly degrade large nucleic acid polymers from less resilient organisms (La Duc et al., 2009). To avoid biases in this regard, the samples collected in this study were split into two equivalent portions. One portion was subjected to bead beating prior to undergoing nucleic acid extraction, and the other portion was not subjected to mechanical abrasion prior to the purification of nucleic acids. Both portions were pooled together and subjected to automated DNA extraction as one sample volume (La Duc et al., 2009). As was previously recommended, automated methods are preferred to traditional manual phenol-chloroform-based DNA extraction techniques and were employed during this study so as to maximize reproducibility and avoid technician based variability (La Duc et al., 2009). Recently, a comparative evaluation of cotton swabs with other swab varieties concluded that nylon-flocked swabs were superior to cotton swabs for the collection of *Bacillus* sp. Spores from stainless steel and other surfaces (Probst et al., 2010). In a similar vein, the use of nylon-flocked swabs resulted in more-efficient recovery of cultivable contaminants from pharmaceutically relevant surfaces (Dalmaso et al., 2008). To date, the literature is devoid of reports similar to that of the present investigation, which focused on evaluating procedures for the effective and efficient collection and processing of not only intact microorganisms but a wide spectrum of biomaterials (from cells to nucleic acid remnants) from clean-room surfaces. This study found nylon-flocked swabbing material to be comparable to cotton for removing intact cultivable cells and spores from small ($\sim 25\text{-cm}^2$) metal surfaces ($\sim 1\%$ recovery). However, cotton swabs consistently yielded a higher recovery of rRNA gene copies than nylon flocked swabs (10-fold more). BiSKit sampling modules were superior (2-fold more) to NASA-certified polyester wipes for retrieving DNA molecules from larger surface areas, perhaps a consequence of the highly porous nature of the BiSKit macrofoam versus the tightly woven fibers within the polyester wipe. Even though all of the sampling devices of a given type originated from the same lot, both cotton and nylon-flocked

swabs suffered from remarkably high variability in resulting qPCR values for sampling blanks and across sample replicates (Fig. 3). Such inconsistency (up to 2 logs in the case of nylon flocked swab replicates) could be indicative of a notable degree of variation in sampling material composition within the same lot and merits further investigation. In addition, the signal-to noise ratios obtained with both nylon-flocked swabs and BiSKits were surprisingly low, suggestive of indigenous DNA associated with each sampling device. Though laborious, ssPCR proved invaluable in assessing the differential recovery of MMC constituents by the various sampling matrices tested. In addition, this molecular technique was not at all affected by the considerable levels of indigenous DNA associated with sampling materials. As was observed with general rRNA gene qPCR, cotton swabs and BiSKits were superior to their respective counterparts in collecting MMCborne DNA from metal surfaces. As could be expected, several of the MMC constituents most frequently detected via ssPCR were among those still cultivable after 90 days of storage at -80°C. These bacterial species were apparently the most resistant to desiccation and cryogenic storage. Additionally, it is not surprising that all sampling devices failed to recover ssPCR-amplifiable levels of DNA from *A. pullulans* 28v1 since the limit of detection of this eukaryotic strain was 9.0×10^6 gene copies. Since ~1,300-bp regions of the 18S-internal transcribed spacer gene cluster were able to be amplified with as little as $\sim 10^3$ CFU/reaction mixture (pure culture), it is apparent that ssPCR conditions must be further optimized to assess eukaryotic cells in this manner. These results stress the importance of selecting and tailoring sample collection and processing approaches based on the desired target within the sample and the level of detection/ resolution required. If the relevance of a given investigation is limited to the presence of intact, cultivable cells, then, based on other studies, nylon-flocked swabs would likely be the better sampling device. If, on the other hand, a survey is more interested in the entire spectrum of microbial diversity on a given surface, then the preferred sampling instrument would be either cotton swabs or BiSKits, depending on the surface area. The findings of this study tend to support those of previous work and hint at the need to readdress the use of standard methodologies over state-of-the-art, biomolecule-friendly sample collection and processing regimens.

5. Discussion

The subject of my thesis has provided for the first time data on the viability of rock-dwelling organisms and microbial communities after a long-term exposure to space parameters. Moreover, EXPOSE-E LIFE provided data on cryptoendolithic microorganisms within their natural substratum that have been exposed for long time to space and simulated Martian conditions in Low Earth Orbit.

The test systems, collected from hostile conditions, such as Antarctica and high mountain regions, are adapted to cope with high radiation intensities, arid phases, and extreme temperature fluctuations, somehow similar to those experienced in space. In *C. antarcticus*, for example, globular cells are enveloped in a thick melanised cell wall, protecting them from radiation and desiccation. Their meristematic way of producing colonies (i.e. dividing in all directions) further supports their resistance (Selbmann et al., 2005; Sterflinger, 2005; Onofri et al., 2008; 2009). Special protection against environmental extremes is also granted for the photobiont, the green alga *Trebouxia* sp. of the lichen *X. elegans*. In this symbiotic organization, the fungus forms a cortex with an upper layer incrustated with parietin and a mucilage layer enveloping the alga cells in a medulla matrix.

Although we have demonstrated that some rock-dwelling species are capable of partially withstanding the harsh environment of outer space – or certain parameters of it - for at least 1.5 years, the data are insufficient for drawing any consequences for the likelihood of Lithopanspermia. The possibility of surviving a much longer journey in space, as would be required for a natural travel from Mars to Earth or vice-versa, still remains an open question. This especially applies to organisms dwelling at the surface of rocks, like the lichen *X. elegans*, which would be fully exposed to the lethal spectrum of solar extraterrestrial UV radiation during a hypothetical interplanetary transfer. The only one data point at an exposure time of 1.5 years, resulting in a viability of $(45 \pm 2.50) \%$, as determined by PSII activity, does not allow any extrapolation over hundreds, thousands, or even millions of years, as would be required for Lithopanspermia (Gladman et al. 1996). The situation would be much more favorable for endolithic organisms, because they would be shielded from solar UV radiation by the surrounding rock material. However, the cryptoendolithic organisms tested in this study did not show a high survival rate in space, even if shielded from solar UV radiation. Our experiments demonstrate also that outer space can act as a selection pressure on

the composition of microbial communities (Cockell et al. 2011). Further studies are needed with more resistant communities. An example is the vagrant lichen species *Aspicilia fruticulosa* that – after 16 days in space - showed a complete recovery of space induced damage after 72 h of reactivation (Raggio et al. 2011).

Natural transfer of microorganism between planets via Lithopanspermia could have been occurring for millions of years (Gladman et al., 1996; Nicholson 2009). Since the last 50 years, human activities in space exploration have become another potential source of spreading microorganisms between planets. Nearly 40 robotic missions have been launched with Mars as their destination (Horneck et al., 2007). In order to prevent the introduction of microbes from the Earth to another celestial body or vice versa, a concept of contamination control has been elaborated by the Committee on Space Research (COSPAR) under consideration of specific classes of mission/target combinations, which have been recommended to be followed by each space-faring organization (COSPAR 2011). Lander missions to Mars require especially strict measures of cleanliness and partial sterility of the spacecraft (COSPAR 2011). The test systems that have been used in the LIFE experiment, though unlikely to be found on spacecraft, can be considered as models for demonstrating how life, if accidentally transferred from Earth to outer space, may resist and contaminate other celestial bodies and planets (i.e. Mars), thereby interfering with future life detection missions.

Since LIFE experiment has implication of planetary protection issues and since possible biological cross-contaminants could be organized in microbial communities, the other goal of this study was to evaluate and standardize protocols and procedures involved with the collection, processing, and molecular analysis of contaminant biomolecules from SAC surfaces. While several protocols are in place for the sampling of low-biomass surfaces, few employ molecular-analysis-based methods, and most experiment only with one or two test organisms. In this study, a consortium of bacterial spores and cells representing all 3 domains of microbial life was engineered as a proxy for the diverse, low-abundance microbial populations typical of clean-room environments. This MMC was used to test and optimize the numerous stages involved in molecular microbial diversity analysis. To the best of our knowledge, this is the first study to employ a complex MMC of diverse phylogenetic relevance to the testing and optimization of protocols for collecting, processing, and analyzing biomolecular samples from clean-room surfaces.

Bibliografia

Allamandola, L., Bar-Nun, A., Brock, T., Chang, S., Davies, R. E., Greenberg, J. M., Hochstein, L., Horneck, G., Huntress, W., Miller, S., Nealson, K. and Usher, D., In situ investigations, in *Exobiology in Earth Orbit* (edited by D. DeFrees, D. Brownlee, J. Tarter, D. Usher, W. Irvine and H. Klein). pp. 89.-99. NASA SP-500, NASA, Washington D-C.. 1989a.

Arrhenius, S., Die Verbreitung des Lebens im Weltenraum. Die Umschau 7,481-485, 1903.

Arrhenius, S., Das Werden der Welten. Akademische, Leipzig. 1908/*Worlds in the Making*. Harper and Row. New York, 1908.

Berger T., Hajek, M., Bilski, P., Vanhavere, F., Horwacik, T., Körner, C., and Reitz, G. (2012) Measurements of the dose due to ionizing radiation within the EXPOSE-E experiment applying passive radiation detectors. (this issue)

Brown, G. S., et al. 2007. Evaluation of a wipe surface sample method for collection of *Bacillus* spores from nonporous surfaces. *Appl. Environ. Microbiol.* **73**:706–710.

Brown, G. S., et al. 2007. Evaluation of vacuum filter sock surface sample collection method for *Bacillus* spores from porous and non-porous surfaces. *J. Environ. Monit.* **9**:666-671.

Bruckner, J. C., S. Osman, C. Conley, and K. Venkateswaran. 2008. Space microbiology: planetary protection, burden, diversity and significance of spacecraft associated microbes, p. 52–65. *In* M. Schaechter (ed.), *Encyclopedia of microbiology*. Elsevier, Oxford, United Kingdom.

Buttner, M. P., P. Cruz-Perez, and L. D. Stetzenbach. 2001. Enhanced detection of surface-associated bacteria in indoor environments by quantitative PCR. *Appl. Environ. Microbiol.* **67**:2564–2570.

Canganella F, Wiegel J (2011) Extremophiles: from abyssal to terrestrial ecosystems and possibly beyond. *Naturwissenschaften* 4:253-279

Cockell, C.S., Rettberg, P., Rabbow, E., and Olsson-Francis, K. (2011) Exposure of phototrophs to 438 days in low Earth orbit: microbial selection pressures in outer space and on early earth. *ISME J.* 10:1671-1682.

Committee on Preventing the Forward Contamination of Mars, National Research Council. 2006. Preventing the forward contamination of Mars. The National Academies Press, Washington, DC.

COSPAR. 2002. Planetary protection policy, October 2002, as amended, March 2008. COSPAR, Houston, TX.

COSPAR (2011) Planetary Protection Policy, October 2002, as amended 24 March 2011: <http://cosparhq.cnes.fr/Scistr/PPPPolicy%20%2824Mar2011%29.pdf>

Crick, F. H. C. and Orgel, L. E., Directed panspermia. *Iccmr.s* 19, 341-346. 1973.

Dalmaso, G., M. Bini, R. Paroni, and M. Ferrari. 2008. Qualification of high-recovery, flocked swabs as compared to traditional rayon swabs for microbiological environmental monitoring of surfaces. *PDA J. Pharm. Sci. Technol.* **62**:191–199.

De la Torre JR, Goebel BM, Friedmann EI, Pace NR (2003) Microbial Diversity of Cryptoendolithic Communities from the McMurdo Dry Valleys, Antarctica. *Appl Environ Microbiol* 215:3858-3867

de la Torre, R., Sancho, L., Horneck, G., de los Ríos, A., Wierzchos, J., Olsson-Francis, K., Cockell, C.S., Rettberg, P., Berger, T., de Vera, J.-P. P., Ott, S., Martinez Frías, J., Melendi, P.G., Lucas, M.M., Reina, M., Pintado, A., and Demets, R. (2010) Survival of lichens and bacteria exposed to outer space conditions – Results of the Lithopanspermia experiments. *Icarus* 208: 735-748.

De los Ríos A, Wierzchos J, Sancho LG, Ascaso C (2004) Exploring the physiological state of continental Antarctic endolithic microorganisms by microscopy. *FEMS Microbiol Ecol* 50:143–152

De los Rios A, Sancho LG, Grube M, Wierzchos J, Ascaso C (2005) Endolithic growth of two *Lecidea* lichens in granite from continental Antarctica detected by molecular and microscopy techniques. *New Phytol* 165:181–190

de Vera, J.P., Horneck, G., Rettberg, P., and Ott, S. (2003) The potential of lichen symbiosis to cope with extreme conditions of outer space. – I. Influence of UV radiation and space vacuum on the vitality of lichen symbiosis and germination capacity. *Int. J. Astrobiol.* 1: 285–293.

De Vera JP, Horneck G, Rettberg P, Ott S (2004) The potential of the lichen symbiosis to cope with the extreme conditions of outer space – II: germination capacity of lichen ascospores in response to simulated space conditions. *Adv Space Res* 33:1236–1243

de Vera, J.P., Rettberg, P., and Ott, S. (2008) Life at the limits: capacities of isolated and cultured lichen symbionts to resist extreme environmental stresses. *Orig. Life Evol. Biosph.* 38: 457–468.

Dose, K., Hypotheses on the appearance of life on Earth (review), *AdLj. Space Res.* 6(12), 181–186, 1986

Edmonds, J. M., et al. 2009. Surface sampling of spores in dry-deposition aerosols. *Appl. Environ. Microbiol.* 75:39–44.

Estill, C. F., et al. 2009. Recovery efficiency and limit of detection of aerosolized *Bacillus anthracis* Sterne from environmental surface samples. *Appl. Environ. Microbiol.* 75:4297–4306.

Friedmann EI (1982) Endolithic microorganisms in the Antarctic cold desert. *Science* 215: 1045–1053

Fritz, J., Artemieva, N. A., and Greshake, A. (2005) Ejection of Martian meteorites. *Meteoritics Planet. Sci.* 9/10: 1393-1411.

Ghosh, S., S. Osman, P. Vaishampayan, and K. Venkateswaran. 2010. Recurrent isolation of extremotolerant bacteria from the clean room where Phoenix spacecraft components are assembled. *Astrobiology* **10**:325–335.

Gladman, B. J., Burns, J. A., Duncan, M., Lee, P. and Levison, H. F. (1996) The exchange of impact ejecta between terrestrial planets. *Science* 271: 1387–1392.

Haynes, R. H., Etablierung von Leben auf dem Mars durch gerichtete Panspermie: Technische und ethische Probleme der Okopoesis. *Biol. Zmt.* II/ 109, 193-205, 1990.

Hodges, L. R., L. J. Rose, H. O’Connell, and M. J. Arduino. 2010. National validation study of a swab protocol for the recovery of *Bacillus anthracis* spores from surfaces. *J. Microbiol. Methods* **81**:141–146.

Hodges, L. R., L. J. Rose, A. Peterson, J. Noble-Wang, and M. J. Arduino. 2006. Evaluation of a macrofoam swab protocol for the recovery of *Bacillus anthracis* spores from a steel surface. *Appl. Environ. Microbiol.* **72**:4429– 4430.

Horneck G, Bücker H, Reitz G, Requardt H, Dose K, Martens KD, Mennigmann HD, Weber P (1984a) Microorganisms in the space environment. *Science* 225:226–228

Horneck G, Bücker H, Dose K, Martens KD, Bieger A, Mennigmann HD, Reitz G, Requardt H, Weber P (1984b) Microorganisms and biomolecules in space environment experiment ES 029 on Spacelab-1. *Adv Space Res* 4:19–27

Horneck, G. and Brack, A., Study of the origin. evolution and distribution of life with emphasis on exobiology experiments in Earth orbit. in .advances in Space Biology. (edited by S. L. Bonting). pp. 219-762. JAI Press. Greenwich, CT. 1992.

Horneck G, Eschweiler U, Rettberg P, Wehner J, Reitz G, Schott J-U, Willimek R, Strauch K, Dose K, Bieger- Dose A, Risi S, Kerz O, Klein A (1994a) Biological responses to extraterrestrial solar UV radiation and space vacuum, RD UVRAD, Sahm P, Keller MH, Schiewe B (eds) Proceedings of the German Spacelab Mission D-2, Norderney, 14–16 March, 1994, Wissenschaftliche Projektführung D2. DLR Köln, Germany

Horneck G, Bücken H, Reitz G (1994b) Long Term survival of bacterial spores in space. Adv Space Res 14:41–45

Horneck G, Eschweiler U, Reitz G, Wehner J, Willimek R, Strauch K (1995) Biological responses to space: results of the experiment “Exobiological Unit” of ERA on EURECA 1. Adv Space Res 16:105–118

Horneck G, Rettber P, Rabbow E, Strauch W, Seckmeyer G, Facius R, Reitz G, Strauch K, Schott J-U (1996) Biological dosimetry of solar radiation for different simulated ozone column thicknesses. J Photochem Photobiol B: Biol 32:189–196

Horneck G, Bucker H, Reitz G (1994) Long-term survival of bacterial spores in space. Adv Space Res 14:41-45

Horneck, G., Debus, A., Mani P., Spry J.A. (2007) Astrobiology exploratory missions and planetary protection requirements. In: *Complete Course in Astrobiology*, edited by G. Horneck and P. Rettberg, Wiley-VCH, Berlin New York, pp. 353-397.

Horneck, G., Stöffler, D., Ott S., Hornemann, U., Cockell, C.S., Moeller, R., Meyer, C., de Vera, J.-P., Fritz, J., Schade, S., and Artemieva, N. A. (2008) Microbial rock inhabitants survive impact and ejection from host planet: 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.

Imshenetsky, A. A., Pisarenko, N. F., Kuziurina, L. A. and Yakshina, V. M., Physiology of xerophylic microorganisms growing under Martian conditions, *L(h) Sci. Space Res.* 15, 47-52, 1977.

Imshenetsky, A. A., Lysenko, S. V. and Kazakov, G. A., Upper boundary of the biosphere, *Appl. Environm. Microbiol.* 35, 1-5. 1978.

Imshenetsky, A. A., Lysenko, S. V. and Lath, S. P., Microorganisms of the upper layer of the atmosphere and the protective role of their cell pigments, *Life Sci. Space Res.* 17, 105-109. 1979.

Jönsson KI, Rabbow E, Schill RO, Harms-Ringdahl M, Rettberg P (2008) Tardigrades survive exposure to space in low Earth orbit. *Curr Biol* 18(17):R729

Katana A, Kwiatowski J, Spalik K, Zakrys B, Szalacha E, Szymanska H (2001) Phylogenetic position of *Koliella* (Chlorophyta) as inferred from nuclear and chloroplast small subunit rDNA. *J Phycol* 37:443-451

Kempf, M. J., F. Chen, R. Kern, and K. Venkateswaran. 2005. Recurrent isolation of hydrogen peroxide-resistant spores of *Bacillus pumilus* from a spacecraft assembly facility. *Astrobiology* 5:391–405.

Klein, H. P.. Exobiology revisited. *Space Res.* 6(12), 187-192. 1986.

Kirschner, L. E., and J. R. Puleo. 1979. Wipe-rinse technique for quantitating microbial contamination on large surfaces. *Appl. Environ. Microbiol.* **38**:466–470.

La Duc, M. T., et al. 2007. Isolation and characterization of bacteria capable of tolerating the extreme conditions of clean-room environments. *Appl. Environ. Microbiol.* **73**:2600–2611.

- La Duc, M. T., W. Nicholson, R. Kern, and K. Venkateswaran. 2003. Microbial characterization of the Mars Odyssey spacecraft and its encapsulation facility. *Environ. Microbiol.* **5**:977–985.
- La Duc, M. T., S. Osman, and K. Venkateswaran. 2009. Comparative analysis of methods for the purification of DNA from low-biomass samples based on total yield and conserved microbial diversity. *J. Rapid Methods Autom. Microbiol.* **17**:350–368.
- Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell. 1991. Optical sectioning of microbial biofilms. *J. Bacteriol.* **173**: 6558–6567.
- Lederberg, J., *Exobiology : approaches to life beyond the Earth*, Science 132. 393400, 1960.
- Lewandowski, R., K. Kozłowska, M. Szpakowska, M. Stepinska, and E. A. Trafny. 2010. Use of a foam spatula for sampling surfaces after bioaerosol deposition. *Appl. Environ. Microbiol.* **76**:688–694.
- Li, X., and S. C. Ricke. 2004. Comparison of cryoprotectants for *Escherichia coli* lysine bioavailability assay culture. *J. Food Process. Preserv.* **28**:39–50.
- Mancinelli RL, Klovstad M (2000) Martian soil and UV radiation: Microbial viability assessment on spacecraft surfaces. *Planet Space Sci* 48:1093-1097
- Melosh, H. J. (1984) Impact ejection, spallation, and the origin of meteorites. *Icarus* 59: 234-260.
- Melosh J, Rickman H, Mileikowsky C (2009) Natural transfer of viable microbes in space from planets in the extra-solar systems to a planet in our Solar System and vice-versa, *Astrophys J* 690:210--215
- Meyer, C., Fritz, J., Misgaiski, M., Stöffler, D., Artemieva, N.A., Hornemann, U., Moeller, R., de Vera, J.-P., Cockell C., Horneck, G., Ott, S., and Rabbow, E. (2011) Shock experiments in support of the Lithopanspermia theory: The influence of host rock

composition, temperature, and shock pressure on the survival rate of endolithic and epilithic microorganisms. *Meteorit. Planet. Sci.* 46: 701–718.

Mileikowsky, C., Cucinotta, F., 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, Part 1: From Mars to Earth and Earth to Mars. *Icarus* 145: 391-427.

Moissl, C., J. Bruckner, and K. Venkateswaran. 2008. Archaeal diversity analysis of spacecraft assembly facilities. *ISME J.* **2**:115–119.

Moissl, C., M. T. La Duc, S. Osman, A. E. Dekas, and K. Venkateswaran. 2007. Molecular bacterial community analysis of clean rooms where spacecraft are assembled. *FEMS Microbiol. Ecol.* **61**:509–521.

Moissl-Eichinger, C. 2011. Archaea in artificial environments: their presence in global spacecraft clean rooms and impact on planetary protection. *ISME J.* **5**:209–219.

Moro CV, Crouzet O, Rasconi S, Thouvenot A, Coffe G, Batisson I, Bohatier J (2009) New design strategy for development of specific primer sets for PCR-based detection of Chlorophyceae and Bacillariophyceae in environmental samples. *Appl Environ Microbiol.* 2009 Sep;75(17):5729-33. Epub 2009 Jul 10

Nägeli C (1849) Gattungen einzelliger Algen, physiologisch und systematisch bearbeitet. Neue Denkschriften der Allg. Schweizerischen Gesellschaft für die Gesamten Naturwissenschaften 10(7):i-viii, 1-139, pls I-VIII

National Aeronautics and Space Administration. 1967. Harmful contamination: moon treaty United Nations treaty on principles governing the activities of states in the exploration and use of outer space, including the moon and other celestial bodies. Article IX, UN doc. A/RES/222/(XXI), TIAS no. 6347). United Nations Office for Outer Space Affairs, Vienna, Austria

National Aeronautics and Space Administration. 1980. NASA standard procedures for the microbiological examination of space hardware. Office of Space Science, National Aeronautics and Space Administration, Washington, DC.

National Aeronautics and Space Administration. 2005. Planetary protection provisions for robotic extraterrestrial missions. NPR 8020.12C, April 2005. National Aeronautics and Space Administration, Washington, DC.

Newcombe, D. A., M. T. LaDuc, P. Vaishampayan, and K. Venkateswaran. 2008. Impact of assembly, testing, and launch operations on the airborne bacterial diversity within a spacecraft assembly facility clean-room. *Int. J. Astrobiol.* 7:223–236.

Nicholson, W.L. (2009) Ancient micronauts: interplanetary transport of microbes by cosmic impacts. *Trends Microbiol.* 17: 243-250.

Nyquist, L. E., Bogard, D. D., Shih, C.-Y., Greshake, A., Stöffler, D., Eugster O., (2001) Ages and geological histories of Martian meteorites. *Space Sci. Rev.* 96: 105-164.

Onofri S, Selbmann L, Zucconi L, Pagano S (2004) Antarctic microfungi as models for exobiology. *Planet Space Sci* 52:229-237

Onofri S, Selbman L, de Hoog GS, Grube M, Barreca D, Ruisi S, Zucconi L (2007) Evolution and adaptation of fungi at boundaries of life. *Adv Space Res* 40:1657-1664

Onofri, S., Barreca, D., Selbmann, L., Isola, D., Rabbow, E., Horneck, G., de Vera, J.P.P., Hatton, J., and Zucconi, L. (2008) Resistance of Antarctic black fungi and cryptoendolithic communities to simulated space e Mars conditions. *Stud. Mycol.* 61: 99–109.

Onofri, S., Selbmann, L., Barreca, D., Isola, D., and Zucconi, L. (2009) Fungal survival in space conditions, new insights for lithopanspermia. *Plant Biosyst.* 143: S85–87.

Panaro, N. J., et al. 2000. Evaluation of DNA fragment sizing and quantification by the Agilent 2100 bioanalyzer. *Clin. Chem.* **46**:1851–1853.

Probst, A., R. Facius, R. Wirth, and C. Moissl-Eichinger. 2010. Validation of a nylon-flocked-swab protocol for efficient recovery of bacterial spores from smooth and rough surfaces. *Appl. Environ. Microbiol.* **76**:5148–5158.

Probst, A., et al. 2010. Diversity of anaerobic microbes in spacecraft assembly clean rooms. *Appl. Environ. Microbiol.* **76**:2837–2845.

Puleo, J. R., M. S. Favero, and N. J. Petersen. 1967. Use of ultrasonic energy in assessing microbial contamination on surfaces. *Appl. Microbiol.* **15**:1345– 1351.

Rabbow, E., Horneck, G., Rettberg, P., Schott, J.-U., Panitz, C., L’Afflito, A., von Heise-Rotenburg, R., Willnecker, R., Baglioni, P., Hatton, J., Dettmann, J., Demets, R., and Reitz G. (2009) EXPOSE, an astrobiological exposure facility on the International Space Station - from proposal to flight. *Orig. Life Evol. Biosph.* 39: 581-598.

Rabbow, E., Rettberg, P., Barczyk S., Bohmeier, M., Parpart, A., Panitz, C., Horneck, G., von Heise-Rotenburg, R., Hoppenbrouwers, T., Willnecker, R., Baglioni, P., Demets, R., Dettmann, J., and Reitz, G. (2012) EXPOSE-E, an ESA astrobiology mission 1.5 years in space (this issue).

Raggio, J., Pintado, A., Ascaso, C., De La Torre, R., De Los Ríos, A., Wierzechos, J., Horneck, G., and Sancho L.G. (2011) Whole lichen thalli survive exposure to space conditions: Results of Lithopanspermia experiment with *Aspicilia fruticulosa*. *Astrobiology* 11: 281-292.

Rettberg P, Rabbow E, Panitz C, Horneck G (2004) Biological space experiments for the simulation of Martian conditions: UV radiation and Martian soil analogues. *Adv Space Res* 33:1294–1301

Richter, H., Zur Darwinschen Lehre, Schmidts Jahrbuch Ges. Med. 126, 243-249, 1865.

Robertson, H. P., Dynamic effects of radiation on the solar system, Monthly Notices R. Astron. Soc. 97, 423-438, 1937.

Rose, L., B. Jensen, A. Peterson, S. N. Banerjee, and M. J. Srdino. 2004. Swab materials and *Bacillus anthracis* spore recovery from nonporous surfaces. Emerg. Infect. Dis. **10**:1023–1029.

Rothschild LJ, Mancinelli RL (2001) Life in extreme environments. Nature 409:1092–1101

Ruisi S, Barreca D, Selbmann L, Zucconi L, Onofri S (2007) Fungi in Antarctica. Rev Environ Sci Biotechnol 6:127–141

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sancho, L.G., de la Torre, R., Horneck, G., Ascaso, C., de los Rios, A., Pintado, A., Wierzchos, J., and Schuster, M. (2007) Lichens survive in space: Results from the 2005 LICHENS experiment. *Astrobiology* 7: 443–454.

Sanderson, W. T., et al. 2002. Surface sampling methods for *Bacillus anthracis* spore contamination. Emerg. Infect. Dis. **8**:1145–1151.

Sanger S, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. P Nat Acad Sci USA 74:5463–5467

Selbmann, L., de Hoog, G.S., Mazzaglia, A., Friedmann, E.I., and Onofri, S. (2005) Fungi at the edge of life, cryptoendolithic black fungi from Antarctic deserts. *Stud. Mycol.* 51: 1–32.

Selbmann L, De Hoog GS, Zucconi L, Isola D, Ruisi S, Gerrits van den Ende AHG, Ruibal C, De Leo F, Urzì C, Onofri S (2008) Drought meets acid: three new genera in a dothidealean clade of extremotolerant fungi. Stud Mycol 61:1–20

Selbmann L, Isola D, Zucconi L, Onofri S (2011) Resistance to UV-B induced DNA damage in extreme-tolerant cryptoendolithic Antarctic fungi: detection by PCR assays. *Fungal Biol.* 2011 Oct;115(10):937-44. Epub 2011 Mar 1

Shuster, D.L. and Weiss, B.P. (2005) Martian surface paleotemperatures from thermochronology of meteorites. *Science* 309: 594–597.

Sterflinger, K. (2005) Black yeasts and meristematic fungi: Ecology, diversity and identification. In *Yeast handbook: Biodiversity and ecophysiology of yeasts*, edited by C. Rosa and P. Gabor, Springer, New York, pp 505–518.

Stieglmeier, M., R. Wirth, G. Kminek, and C. Moissl-Eichinger. 2009. Cultivation of anaerobic and facultatively anaerobic bacteria from spacecraft-associated clean rooms. *Appl. Environ. Microbiol.* **75**:3484–3491.

Stöffler, D., Horneck, G., Ott, S., Hornemann, U., Cockell, C.S., Moeller, R., Meyer, C., de Vera, J.-P., Fritz, J., and Artemieva, N.A. (2007) Experimental evidence for the potential impact ejection of viable microorganisms from Mars and Mars-like planets. *Icarus* 186: 585-588.

Suzuki, M. T., L. T. Taylor, and E. F. DeLong. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. *App. Environ. Microbiol.* **66**:4605–4614.

Tauscher, C., A. C. Schuerger, and W. L. Nicholson. 2006. Survival and germinability of *Bacillus subtilis* spores exposed to simulated Mars solar radiation: implications for life detection and planetary protection. *Astrobiology* **6**:592–605.

Taylor GR, Spizizen J, Foster BG, Volz PA, Bücker H, Simmons RC, Heimpel AM, Benton EV (1974) A descriptive analysis of the Apollo 16 microbial response to space environment experiment. *BioScience* 24:505–511

The Meteoritical Society (2011). Meteoritical Bulletin Database. Available on line at <http://www.lpi.usra.edu/meteor/metbull.php>

Thomson, W. (Lord Kelvin) (1871). The British Association Meeting at Edinburgh. Inaugural Address of Sir William Thomson, LL.D., F.R.S., President. *Nature* 4: 262-270.

Vaishampayan, P., S. Osman, G. Andersen, and K. Venkateswaran. 2010. Molecular methods to monitor microbial community composition of the phoenix spacecraft assembly clean room. *Astrobiology* 10:499–508.

Valentine, N. B., et al. 2008. Evaluation of sampling tools for environmental sampling of bacterial endospores from porous and nonporous surfaces. *J. Appl. Microbiol.* 105:1107–1113.

Valtonen M, Nurmi P, Zheng JQ, Cucinotta FA, Wilson JW, Horneck G, Lindegren L, Melosh J, Rickman H, Mileikowsky C (2009) Natural transfer of viable microbes in space from planets in the extra-solar systems to a planet in our Solar System and vice-versa, *Astrophys J* 690:210–215

Venkateswaran K, Satomi M, Chung S, Kern R, Koukol R, Basic C, White D (2001). Molecular microbial diversity of a spacecraft assembly facility. *Syst Appl Microbiol* 24:311-320

Venkateswaran, K., S. Chung, J. Allton, and R. Kern. 2004. Evaluation of various cleaning methods to remove *Bacillus* spores from spacecraft hardware materials. *Astrobiology* 4:377–390.

White, T. J., T. D. Bruns, S. B. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* N. Innis, D. Gelfand, J. Sninsky, and T. White (ed.), *PCR protocols and applications—a laboratory manual*. Academic Press, New York, NY.

Wilson, K. H., et al. 2002. High-density microarray of small-subunit ribosomal DNA probes. *Appl. Environ. Microbiol.* **68**:2535–2541.

Wynn-Williams DD, Edwards HGM (2000) Antarctic ecosystems as model for extraterrestrial surface habitats. *Planet Space Sci* 48:1065–1075

Ringraziamenti

Vorrei ringraziare innanzitutto i miei genitori e mio fratello, per il supporto morale e l'interesse nei confronti della mia ricerca.

Un ringraziamento particolare va al mio tutor, Professor Silvano Onofri, per avermi sostenuto con particolare enfasi in questi tre anni e per avermi aiutato a chiarire tutte le questioni scientifiche e tecniche inerenti al mio progetto di tesi.

A Caterina Ripa, Sara Magrini e Eleonora Egidi va una profonda gratitudine nell'avermi fatto affrontare il progetto con più leggerezza, la loro presenza in questi tre anni è stata fondamentale.

Devo poi ringraziare Kasthuri Venkateswaran, Myron La Duc, Parag Vaishampayan e Christina Stam, che mi hanno aiutato durante il periodo di internato al Jet Propulsion Laboratory, e per aver creduto in me anche nei momenti più difficili.

Inoltre un particolare ringraziamento va alla Dott.ssa Laura Selbmann e alla Professoressa Laura Zucconi per avermi aiutato nella stesura dei manoscritti con commenti ed integrazioni, il loro aiuto è stato ineguagliabile.

Inoltre un ringraziamento va ai miei amici più stretti, Giovanni Fusco, Matteo Iacobini, Riccardo Albanese e Antonello Casciola per avermi aiutato sempre, consigliato e sostenuto, anche se non scientificamente, durante questo percorso.

Un altro ringraziamento va a Giuseppe Cirillo, Roberto Caporossi, Leonardo Digiesi e Andrea Fiaschetti, per avermi sopportato in casa durante questi 3 anni di dottorato, ed aver condiviso dei momenti indimenticabili.