

Viability of the lichen *Xanthoria elegans* and its symbionts after 18 months of space exposure and simulated Mars conditions on the ISS

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Abstract: The lichen *Xanthoria elegans* has been exposed to space conditions and simulated Mars-analogue conditions in the lichen and fungi experiment (LIFE) on the International Space Station (ISS). After several simulations and short space exposure experiments such as BIOPAN, this was the first long-term exposure of eukaryotic organisms to the hostile space conditions of the low Earth orbit (LEO). The biological samples were integrated in the EXPOSE-E facility and exposed for 1.5 years outside the ISS to the combined impact of insolation, ultraviolet (UV)-irradiation, cosmic radiation, temperatures and vacuum conditions of LEO space. Additionally, a subset of *X. elegans* samples was exposed to simulated Martian environmental conditions by applying Mars-analogue atmosphere and suitable solar radiation filters. After their return to Earth the viability of the lichen samples was ascertained by viability analysis of LIVE/DEAD staining and confocal laser-scanning microscopy, but also by analyses of chlorophyll *a* fluorescence. According to the LIVE/DEAD staining results, the lichen photobiont showed an average viability rate of 71%, whereas the even more resistant lichen mycobiont showed a rate of 84%. Post-exposure viability rates did not significantly vary among the applied exposure conditions. This remarkable viability is discussed in the context of particular protective mechanisms of lichens such as anhydrobiosis and UV-screening pigments.

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Introduction

Challenging effects of global transitions and a vast range of terrestrial ecological niches resulted in an enormous capacity of life to resist hostile environmental conditions and a broad range of adaptive mechanisms. To assess this resistance in the context of space conditions, numerable exposure- and simulation-experiments were conducted with micro-organisms originating from extreme arid, alpine and polar regions, including bacteria, biofilms, cryptoendolithic fungi and lichen symbioses (Onofri *et al.* 2004; de Vera *et al.* 2004a, b; de Vera *et al.* 2008; Hájek *et al.* 2012; Sadowsky & Ott 2012; Baqué *et al.* 2013).

The study presented focuses on the lichen *Xanthoria elegans* (Link) Th.Fr. Lichens are symbiotic associations of photoautotrophic green algae or cyanobacteria (photobiont, PB) and heterotrophic fungi (mycobiont, MB). Lichen symbioses are characterized by high resistance to severe environmental conditions (Lange & Kappen 1972; Kappen 1985; Hawksworth 1988; Lange 1990). Lichens developed a broad range of anatomical, morphological and physiological adaptations (Lange & Kappen 1972; Ott & Sancho 1991; Sadowsky &

Ott 2012; Meeßen *et al.* 2013a, b). Moreover, all lichens are poikilohydric with the ability for anhydrobiosis (also called anabiosis), an inactive state of latent life while being desiccated (Crowe *et al.* 1992). Additionally, they frequently produce high amounts of secondary lichen compounds (Huneck & Yoshimura 1996) which may provide protection against ultraviolet (UV) irradiation and/or excess photosynthetically active radiation (Solhaug & Gauslaa 1996; Nybakken *et al.* 2004; Kranner *et al.* 2005). These adaptive traits enable the lichen symbiosis to colonize all biomes on Earth and adapt to the harsh environmental conditions of extreme habitats such as deserts, alpine and polar regions (aridity, cold, freeze–thaw cycles, high insolation) where they occasionally form the dominant vegetation (Kappen *et al.* 1996; Kappen & Schroeter 1997; Kappen 2000; Sadowsky & Ott 2012). Owing to these adaptations, lichens found some attention in astrobiological research (de Vera *et al.* 2003, 2004a, 2008, 2010; de la Torre *et al.* 2007, 2010a, b; Sancho *et al.* 2007; Stöffler *et al.* 2007; Horneck *et al.* 2008; de Vera & Ott 2010; Raggio *et al.* 2011; Onofri *et al.* 2012; Sánchez *et al.* 2014) and were supposed to be astrobiological model organisms (Sancho *et al.* 2008).

Prior to the lichen and fungi experiment (LIFE) on the International Space Station (ISS) the lichen species *X. elegans* was investigated in several experiments not only under real (LITHOPANSPERMIA on BIOPAN 6; de la Torre et al. 2010b; Raggio et al. 2011) but also under simulated space conditions that included stressors as desiccation, low Earth orbit (LEO)-vacuum of 10^{-5} Pa, sub-zero temperatures, solar irradiation, cosmic radiation and shock pressure impacts (de Vera et al. 2003, 2004a, b; Stöffler et al. 2007; Horneck et al. 2008; Sancho et al. 2008; de Vera & Ott 2010; Meyer et al. 2011). The studies demonstrated the high resistance of *X. elegans* towards hostile conditions and assessed the limits of life for extremotolerant organisms. Entire *X. elegans* thalli were neither significantly damaged by the combined influences of UV radiation (UVR) ($\lambda > 160$ nm; 2.8 W m^{-2} , 16 h) and vacuum up to 10^{-3} Pa (de Vera et al. 2003) nor by the combined influences of UVR ($\lambda = 200$ nm; 3.8 GJ m^{-2}) and vacuum up to 10^{-5} Pa (de Vera et al. 2004b), with survival rates ranging between 95 and 50%, depending on the stressors applied. Additionally, *X. elegans* symbiont cells survived hypervelocity impacts up to 45 GPa (Horneck et al. 2008). Based on the knowledge achieved in simulation experiments and 10 days LEO-exposure (BIOPAN 6) on the resistance of *X. elegans* to hostile conditions the next step was to test its ability to survive a long-term exposure to the combined conditions including the cosmic radiation of LEO-space. Consequently, it was chosen for LIFE to be exposed to real space and simulated Mars conditions for about 18 months (559 days). LIFE was intended to test the ability of the micro-organisms to resist the space conditions of LEO and to assess the habitability of Mars' atmosphere for micro-organisms. Recent literature discusses the putative habitability of micro-niches on Mars (Westall 2013). It was one of the objectives of LIFE to study this possibility.

LIFE is the first experiment to expose lichens and – in parallel – its isolated MB for long-term duration (> 1 year) to LEO-space and to Mars-analogue conditions. The samples were exposed in the EXPOSE-E facility which was specifically designed for astrobiological research (Rabbow et al. 2012) and located on the European Columbus Module of the ISS.

The scientific objective of the present study is to analyse the viability of both *X. elegans* symbionts after LEO-space exposure and simulated Martian environmental conditions, to investigate differences between the exposure conditions, to characterize the extend and pattern of potential damages, to correlate the present results to previous studies and to assess the contribution of lichen-specific protective mechanisms to post-exposure viability. Both, the space-exposed samples as well as the Mars simulation samples experienced three irradiation regimen at the ISS: one sub-set was exposed to the full spectrum of solar and cosmic irradiation, including ionizing radiation of different sources (refer to Berger et al. 2012), for a second sub-set the insolation was filtered down to 0.1%, whereas a third sub-set was kept dark in the interior of the EXPOSE-E facility. A corresponding set of samples was exposed to conditions simulating as similar as possible the environmental conditions of the ISS exposure experiment,

according to the data received from the EXPOSE-E facility at the ISS. The simulation was performed at the DLR in Cologne, herein referred to as Mission Ground Reference (MGR; Rabbow et al. 2012).

Preliminary results of LIFE were already presented (Onofri et al. 2012), while the present study focuses on the viability of *X. elegans* as an entire thallus and the effects of the long-term exposure. Post-flight viability of the lichen samples was examined by LIVE/DEAD analysis, using FUN-1[®] staining and confocal laser-scanning microscopy (CLSM) analysis. The two-colour fluorescent cell-dye method allows the assessment of viability by membrane integrity and metabolic activity (Millard et al. 1997). In addition, chlorophyll *a* fluorescence analyses confirmed the viability of the lichen's PB by providing insight to the photosynthetic capacity in its photosystem II (Lange et al. 1989; Schroeter et al. 1992).

The Lithopanspermia hypothesis (based on a proposal by Thomson, 1871) was revisited recently (Sancho et al. 2007; Stöffler et al. 2007; Horneck et al. 2008, 2010; de la Torre et al. 2010b) especially after several meteorites originating from Mars had been found and studied (Melosh 1984; Shuster & Weiss 2005). The hypothesis implies the transfer of organisms on their host rock through space, and the seeding of life on a new planet, compelling the organisms to survive the impact-driven ejection, an interplanetary travel and an atmospheric re-entry after being captured by a planet's gravity. In the context of this hypothesis, several organisms were tested on their ability to survive shock pressures (Horneck et al. 2008), space conditions and a re-entry (de la Torre et al. 2010a; de Vera et al. 2010; Raggio et al. 2011; Onofri et al. 2012). Since the lichens *X. elegans*, *Rhizocarpon geographicum* and *Circinaria gyrosa* were part of this series of experiments (space exposure experiments LITHOPANSPERMIA, LICHENS I, LICHENS II and STONE at BIOPAN 4–6 on FOTON 2–3, 2002–2005; Sancho et al. 2007; de la Torre et al. 2010b), the present experiment on *X. elegans* contributes to the debate on the likelihood of lithopanspermia by revealing the long-term survival of symbiotic eukaryotes to the conditions of space exposure.

Material and methods

Lichen samples

X. elegans (Link) Th. Fr. (1860) is a cosmopolitan lichen colonizing various harsh habitats, including alpine (up to 7000 m a.s.l. in Himalaya), maritime and continental Antarctic inland sites (Øvstedal & Lewis Smith, 2001). It is frequent on volcanic, silicate and limestone rock, at nitrophilic sites, and on anthropogenic substrata like concrete. *X. elegans* is usually exposed to high levels of insolation (Solhaug & Gauslaa 1996, 2004; de Vera 2005). The thalli of *X. elegans* used in the present study were collected in June 2008 at Col du Sanetsch, Valais, Switzerland (46°21'48"N, 07°17'51"E, at 2140 m a.s.l.) and adjacent collection sites (Zermatt, 46°00'N, 07°71'E, at 1950 m a.s.l.), air-dried and frozen at -20°C until further use. Samples originating from these collection sites were used in

Table 1. Experimental parameters of the LIFE experiment. The irradiance at the sample site over the full spectrum are shown as average of the three distinct slots used for *Xanthoria elegans* samples (*) as calculated by Redshift (Redshift Protocol 2011). The irradiance for UVC (100–280 nm), UVB (280–315 nm), UVA (315–400 nm) and PAR (300–700 nm) are given in MJ m⁻². Please mind the overlap of UVA and PAR. The last row shows the UV-fluence_(200–400 nm) as calculated by Rabbow et al. (2012). Data are calculated for the middle of the sample site (Red Shift Protocol 2011).

Space conditions			Mars-analogue conditions		
10 ⁻⁴ –10 ⁻⁷ Pa			10 hPa		
208 ± 8 mGy			215 ± 16 mGy		
Top layer, MgF ₂ window (110 nm <λ> 1 mm)		Bottom layer	Top layer, quartz window (200 nm <λ> 1 mm)		Bottom layer
Full insolation 4873 MJ m ⁻² *	0.1% neutral density insolation 6.30 MJ m ⁻² *	Dark exposure 0 MJ m ⁻²	Full insolation 4799 MJ m ⁻² *	0.1% neutral density insolation 5.90 MJ m ⁻² *	Dark exposure 0 MJ m ⁻²
UVC: 13.8	UVC: 0.009	UVC: –	UVC: 17.8	UVC: 0.010	UVC: –
UVB: 41.2	UVB: 0.040	UVB: –	UVB: 46.8	UVB: 0.037	UVB: –
UVA: 236	UVA: 0.23	UVA: –	UVA: 249	UVA: 0.21	UVA: –
PAR: 2185	PAR: 2.55	PAR: –	PAR: 2227	PAR: 2.44	PAR: –
634 MJ m ⁻²	0.92 MJ m ⁻²	0 MJ m ⁻²	475 MJ m ⁻²	0.63 MJ m ⁻²	0 MJ m ⁻²

astrobiological studies before (de Vera *et al.* 2003, 2004a, b, 2007, 2008, 2010; Stöffler *et al.* 2007; Horneck *et al.* 2008).

Methods

Sample handling

Thalli were drilled off their stone substrata with a sample of diameter 7 mm. A maximum quantum yield (Fv/Fm) measurement of photosystem II (mini-PAM, Walz, Germany) was performed before further processing to ensure the photosynthetic activity as a measure of the PB's vitality. Samples for the exposure experiment at the ISS and for the MGR were air-dried on orange silica gel and glued into the sample slots of the EXPOSE-E facility. The slots were sealed by an MgF₂ or a quartz window with respect to the experimental conditions, for details refer to Rabbow *et al.* (2012).

After the exposure the ISS samples were removed from the EXPOSE-E facility at the DLR Cologne and mounted carefully in glass flasks separately, re-distributed to the investigators (including the MGR samples) and stored at –20 °C until further investigation.

Exposure parameters

The LIFE applied six experimental conditions to overall 24 lichen and fungi samples (Table 1): 12 samples were exposed to space vacuum (tray 1) and 12 to a Mars-analogue atmosphere (tray 2). On the one hand tray 1 exposed its samples to 10⁻⁴–10⁻⁷ Pa LEO space vacuum and provided them three intensities of solar radiation (dark, 0.1% neutral density filtered and full insolation with a wavelength range of 110 nmλ1 mm; samples covered with MgF₂ windows). On the other hand, tray 2 supplied simulated Mars conditions with a 10³ Pa atmosphere (consisting of 95.3% CO₂, 2.7% N₂, 1.6% Ar, 0.15% O₂ and ~370 ppm water vapour) and also three intensities of solar irradiation (dark, 0.1% neutral density filtered and full insolation with a wavelength range of

200 nmλ1 mm; samples covered by quartz windows). The reduced solar intensities were realized by additional neutral density filters, covering half of the sample slots of the trays' top layer and reducing the samples irradiation by the factor of 1000 while the dark-exposed samples were situated directly below the insulated top layer (Rabbow *et al.* 2012). Fully insulated samples accumulated an average of 4800 MJ m⁻² photon energy input; 0.1% neutral density filtered samples accumulated 6.3 MJ m⁻² photon energy in space vacuum or 5.9 MJ m⁻² in Mars-analogue conditions (Table 1). Ionizing radiation was measured by passive detectors and its quality was evaluated and related to the sample positions (Berger *et al.* 2012; Dachev *et al.* 2012; Schuster *et al.* 2012). The samples experienced 208 ± 8 mGy (tray 1) and 215 ± 16 mGy (tray 2) of ionizing radiation from different sources (galactic cosmic rays, South Atlantic Anomaly, secondary radiation produced in the hardware, refer to Berger *et al.* 2012.) The temperature ranged from –21.7 to +43 °C, with a one-time maximum of 61 °C for a few hours (Rabbow *et al.* 2012). The EXPOSE-E facility was mounted outside the Columbus Module and its lids and valves were opened between 20th February 2008 and 20th August 2009, exposing the samples for 559 days. Owing to off-nominal events during the mission about 20% of the expected environmental data were lost. To assure a precise analysis of the samples' exposure conditions the solar irradiation was recalculated (RedShift Report 2011) leading to the average accumulated irradiance (as experienced by the samples) shown in Table 1.

LIVE/DEAD staining

The LIVE/DEAD staining was performed with the fluorescent dye FUN-1[®] (Molecular Probes, Oregon, USA). The dye contains fluorophores excitable at 488 nm and changing their emission properties when metabolized. By up-taking the dye each cell achieves fluorescent labelling, detectable in the green channel (band-pass 505–550 nm), while only metabolically

active cells obtain a bright yellow to red emission with a vacuolar deposit of the dye (band-pass 575–615 nm, for details refer to Millard *et al.* 1997). For the quantification of active and inactive cells parts of the samples were wetted, cross-sectioned into slices of 80–160 μm with a cryotome (Reichert-Jung, Germany) and immediately dyed on a microscope slide. The FUN-1[®] solution was composed of 20 μM FUN-1[®] in HEPES buffer (pH 7; 1.0 μl in 1 ml). The dyeing protocol followed the producer's manual, except an altered and prolonged incubation time with respect to the algal cell wall which was found to slow down the uptake of staining solution. The dyed samples were examined by CLSM (LSM 510 Meta, Zeiss, Germany), to verify the metabolic activity of the MB and the PB of *X. elegans*. For analysis, 6–15 pictures were taken for each sample with a 1.0–2.0 pinhole opening and amplifier values according to the fluorescence signal intensity (400–800; equal for the red and green channel). The measured emission for the picture composition was according to the emission maxima of the fluorophores: green channel (signals of inactive cells) and red channel (signals of metabolically active cells). Besides these channels, PB cells were additionally marked by their chlorophyll auto-fluorescence for better discrimination, displayed in a blue channel (band-pass 660–750 nm).

The CLSM-pictures were analysed for metabolically active (interpreted as alive) and metabolically inactive (interpreted as dead) cells by manual counting, using an ImageJ counting tool (cell counter, ImageJ, W. Rasband, NIH, USA). Manual counting was necessary due to PB cell artefacts. These cells which showed bright yellow fluorescence active cells but no vacuolar deposits are misinterpreted by software-aided cell counters as active cells.

Owing to the expenses of space missions the sample quantity in space experiments is limited. Therefore not more than four replicates were available for the dark exposed and two replicates for the 0.1% neutral density filtered as well as for the fully insolated samples. The LIVE/DEAD staining procedure of each replicate included 7–29 technical replicates, gained by analysing and evaluating different sections and spots of each sample. The vital control samples were collected together with the ISS and MGR samples and stored at $-20\text{ }^{\circ}\text{C}$ until use.

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was assessed by a Mini-PAM fluorimeter (Walz, Germany) according to Jensen (2002). The maximum quantum yield (Fv/Fm) as a measure for the activity of the photosystem II is calculated as the ratio of variable fluorescence (Fv) over maximum fluorescence (Fm).

The analysis of the 16 ISS and the 16 MGR lichen samples followed three different protocols of sample re-activation prior to Mini-PAM measurement.

a) Standard protocol, applied directly before and after flight/MGR simulation: the lichen thalli were provided with 200 μl sterile water and the chlorophyll *a* fluorescence was measured 10 min afterwards. This is repeated four times to the maximum water supply of 800 μl .

b) First re-activation protocol: 200 μl water application twice and measurements after 0, 1, 2, 3, 24 and 48 h. The samples which had been exposed in Mars-analogue conditions were additionally measured after 72 and 96 h.

c) Second re-activation protocol: for the samples 100 and 116 (space vacuum conditions) the first re-activation was repeated after a day of air-drying, in order to emulate the wet/dry cycle of lichens in their natural environment. Again the measurements took place after 0, 1, 2, 3, 24, 48, 72 and 96 h.

Statistical analyses

Testing the data for significant difference was done with GraphPad InStat 3 software, applying an unpaired *t*-test with Welch correction, ANOVA with parametric or non-parametric testing or Mann–Whitney test with two-tailed *P* value. Correlation coefficient and standard deviations were calculated with MS excel 2010.

Abbreviations (exposure parameters)

Space: samples were exposed in space vacuum conditions; Mars ac: samples were exposed in Mars-analogue conditions; full ins.: samples experienced full solar irradiation; 0.1% ins.: solar irradiation of the samples was reduced to 0.1% of the full insolation (neutral density filter); dark exp.: samples experienced no direct insolation.

Results

After the exposure experiment both, the ISS and MGR samples appeared unaltered in colour and structure in the first visual inspections compared with untreated control samples. The viability of the lichen samples was analysed by different methods, with regard to the different cell types (PB and MB) and the structure of the lichen thallus. The LIVE/DEAD staining with FUN-1[®] was well feasible for the hyphal cells of the MB and appropriate for the coccal green algal PB. Additionally, photosynthetic activity of the PB was analysed by chlorophyll *a* fluorescence and related to the LIVE/DEAD staining results.

LIVE/DEAD staining (FUN-1[®]/CLSM)

In all samples metabolically active MB and PB cells were observed when incubated with FUN-1[®]. The samples displayed a reproducible staining pattern in CLSM analyses. The space exposed samples (ISS) and the samples exposed to simulated space conditions (MGR) as well as the control samples (stored at $-20\text{ }^{\circ}\text{C}$, dark), showed equal reactions regarding up-take, and metabolization of FUN-1[®] and fluorescence signal quality. Figures 1 and 2 show the number of metabolically active PB and MB cells, respectively, displayed as percentage of total cells counted and according to the six different experimental conditions on the ISS and in the MGR. The number of replicates is given in Table 2.

The percentage rate mean values of active PB cells ranged from 43% (MGR 0.1% ins. Mars ac and MGR full ins. Mars ac) to 83% (ISS full ins. space) and for active MB cells

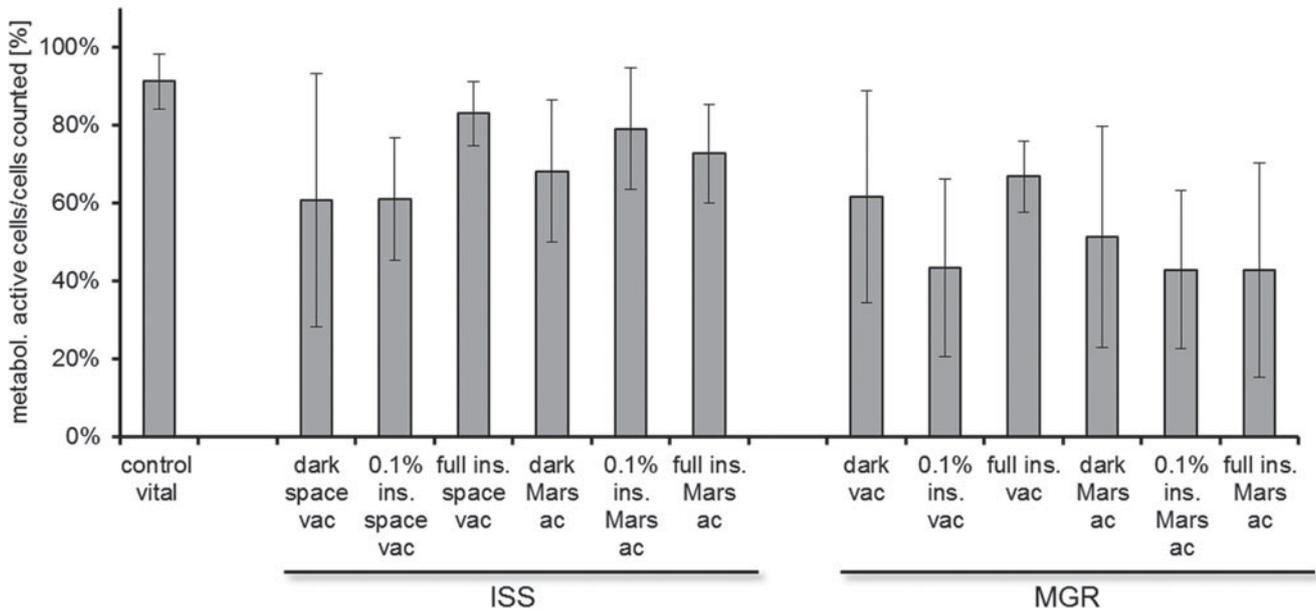


Fig. 1. LIVE/DEAD staining: metabolically active photobiont cells as percentage of cells counted, mean viability values for each sample position/experimental condition in EXPOSE-E plus untreated control group.

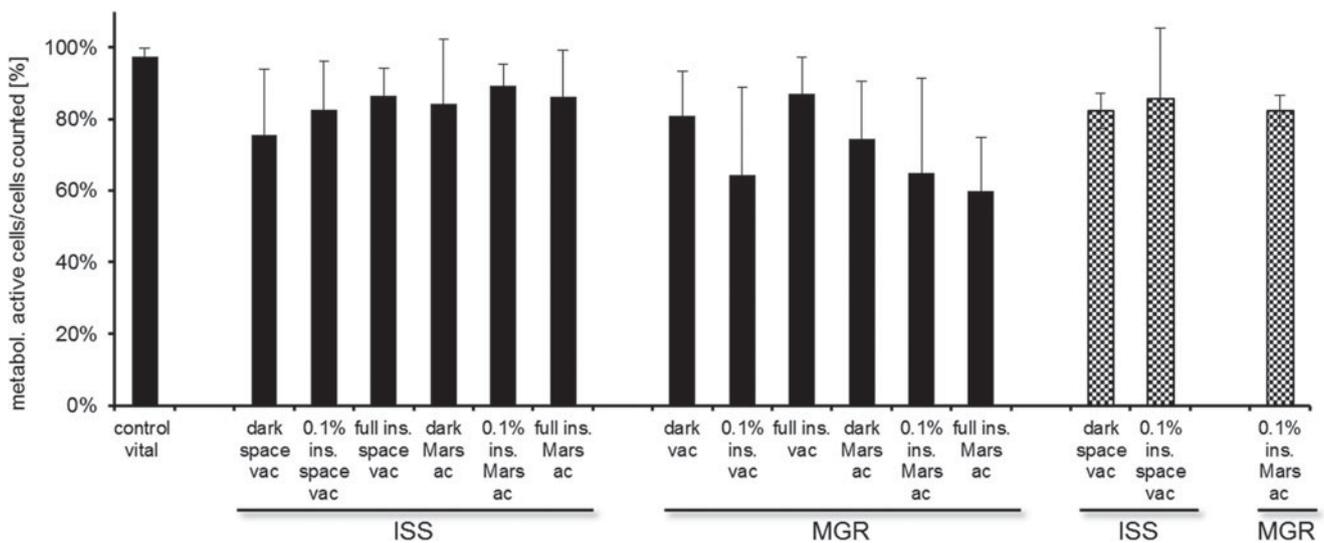


Fig. 2. LIVE/DEAD staining: metabolically active mycobiont (MB) cells as percentage of cells counted, mean viability values for each sample position/experimental condition in EXPOSE-E plus untreated control group. Squared columns show isolated non-lichenized MB samples.

from 60% (MGR full ins. Mars ac) to 89% (ISS 0.1% ins. Mars ac).

MB hyphae appeared to be more resistant towards extreme conditions compared with PB cells which can be recognized to be deformed or collapsed in some sections. The space exposed samples showed a higher survival rate of clustered PB cells in the anatomical centre of the lichen thallus or below thick cortical structures as well as apothecia (Fig. 3). The samples revealed a remarkably high number of metabolically active cells with over-all mean values of 61% for active PB cells and of 78% for MB cells. The MB always showed higher viability percentages than the PB, while the loss of viability of both symbionts is correlated to each other under all experimental

conditions applied. Samples of the isolated non-lichenized MB were tested with the 0.1% neutral density insolation filtering and as dark exposed samples. A mean value of 83% metabolically active hyphae of the mycelium (non-lichenized MB) has been detected, which is comparable to the percentage rate mean values of active MB cells in the entire lichen thallus (mean value of 79%).

In order to evaluate the specific effect of the experimental conditions applied, the viability rates of PB, lichenized MB and non-lichenized MB were correlated to the different experimental conditions. The combined ISS and MGR viability rates of PB and MB in dependency to the three intensities of solar irradiation are illustrated in Fig. 4. There is no significant

Table 2. Cell viability: the results obtained from the EXPOSE-E samples sorted by the six different conditions applied on ISS and during MGR. Legend: space: samples experienced space vacuum; Mars ac: samples experienced Mars analogue conditions; full ins.: full insolation; 0.1% ins.: 0.1% neutral density filtered insolation; dark exp.: samples experienced no direct irradiation MV: mean value of vital cells as percentage of total cells counted, uses mean values of technical replicates; SD: standard deviation; n_{tech} , n_{biol} : number of technical and biological replicates; control vital: untreated *Xanthoria elegans* control samples, stored at -20°C .

	ISS				MGR			
	MV in %	SD	n_{tech}	n_{biol}	MV in %	SD	n_{tech}	n_{biol}
<i>Photobionts</i>								
Control vital	91.3	7.0	19	6	91.3	7.0	19	6
Dark exp., space	60.8	32.5	22	4	61.7	27.2	23	4
0.1% ins., space	61.2	15.8	7	2	43.5	22.9	14	2
Full ins., space	83.1	8.2	14	2	66.8	9.2	10	2
Dark exp., Mars ac	68.3	18.2	27	4	51.5	28.4	27	4
0.1% ins., Mars ac	79.1	15.5	13	2	43.0	20.2	7	1
Full ins., Mars ac	72.7	12.7	20	2	42.9	27.6	13	2
<i>Mycobionts</i>								
Control vital	97.4	2.3	14	6	97.4	2.3	14	6
Dark exp., space	75.7	18.4	29	4	80.8	12.6	20	4
0.1% ins., space	82.7	13.5	8	2	64.2	24.8	13	2
Full ins., space	86.6	7.7	14	2	87.1	10.3	10	2
Dark exp., Mars ac	84.3	18.0	23	4	74.3	16.4	29	4
0.1% ins., Mars ac	89.2	6.2	10	2	64.9	26.6	7	1
Full ins., Mars ac	86.2	13.1	16	2	59.8	15.2	15	2

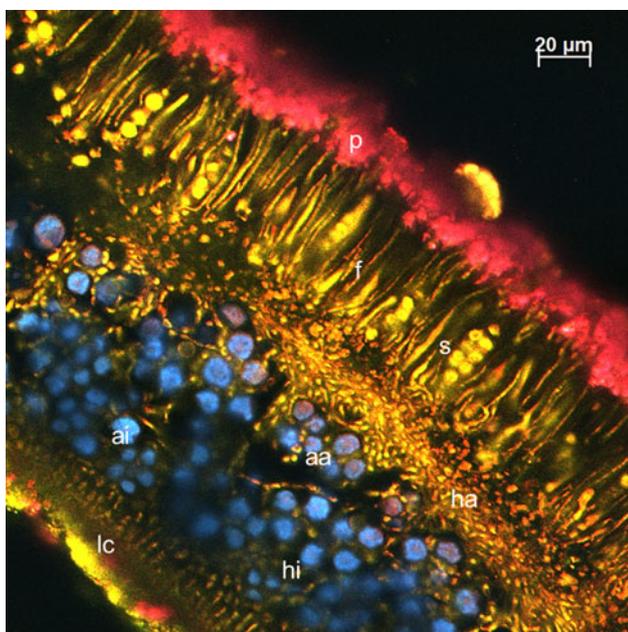


Fig. 3. *Xanthoria elegans* (cross-section) stained with FUN-1[®], documented by CLSM, ISS LIFE sample (full insolation space vacuum). p: parietin encrusted upper cortex layer; f: apothecia (fruiting body) with s: spores; fungal hyphae (MB): ha: active (vital) hyphae, hi: inactive hyphae; algal cells (PB): aa: active (vital) algae, ai: inactive algae; lc: lower cortex.

difference in the viability rates in reference to the insolation conditions. The three orders of magnitude higher energy input to unfiltered ISS or MGR samples – compared to the 0.1% neutral density filtered samples – did not elicit a significant

change of the survival rates. The dark exposed samples did not show higher viability rates while the untreated control samples showed a significantly higher amount of metabolically active cells for both MB and PB.

Additionally, the results of ISS and MGR dark-exposed samples were assembled to depict the differences between Mars-analogue and space vacuum conditions (Fig. 5). No significant differences were observed. All MGR samples showed a clear decrease in metabolic activity, compared with the control and but also compared with the ISS samples, with a minimum of 36% PB viability in one MGR sample. These results coincide with the results of the chlorophyll *a* fluorescence analyses (see below), indicating a possible negative effect of MGR-exposure on viability.

The ISS lichen samples revealed a minor decrease in activity for the cells exposed in Mars-analogue conditions. The mean values of the viability rates of the MB showed a 5.5% higher viability under Mars-analogue conditions, compared with the space vacuum MB samples. The difference is significant (two-tailed *P* value 0.0212, Mann–Whitney test). The difference of the PB viability rates resulted in an insignificant increase of 4% in favour of the Mars-analogue atmosphere. The MGR vacuum samples showed an elevated level of metabolic activity compared with those cells exposed under Mars-analogue conditions. The MB mean viability rate was 7.2% higher and the PB showed even 9.5% higher viability in the MGR vacuum, compared with the MGR Mars-analogue atmosphere. The different amount of metabolically active cells between ISS and MGR samples was tested for its significance (Mann–Whitney test). The difference is significant for the MB (two-tailed *P* value: 0.0152) and very significant for the PB (two-tailed *P* value: 0.0043).

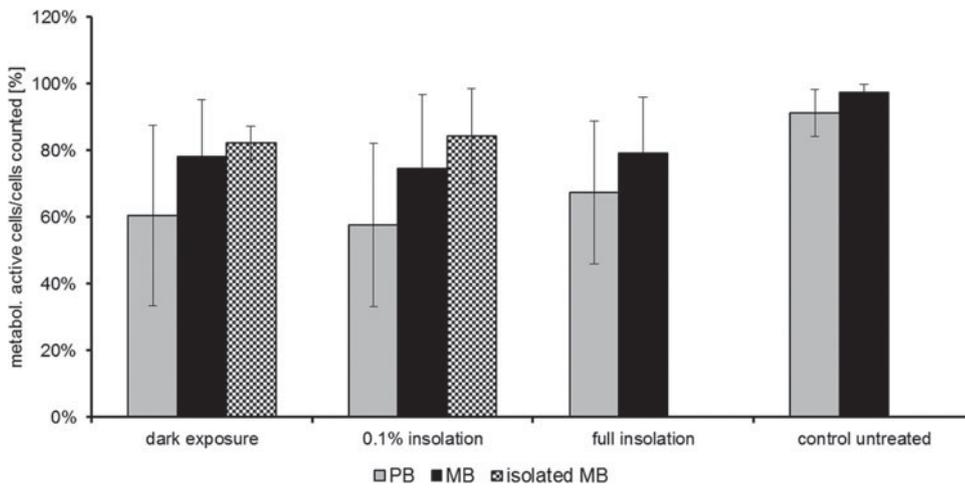


Fig. 4. Viability of ISS and MGR samples related to received photon/EM fluence (110/200 nm λ 1 mm); viability assessed by Fun-1[®] LIVE/DEAD staining and analysed by CLSM. No significant differences occurred related to the different insolation applied.

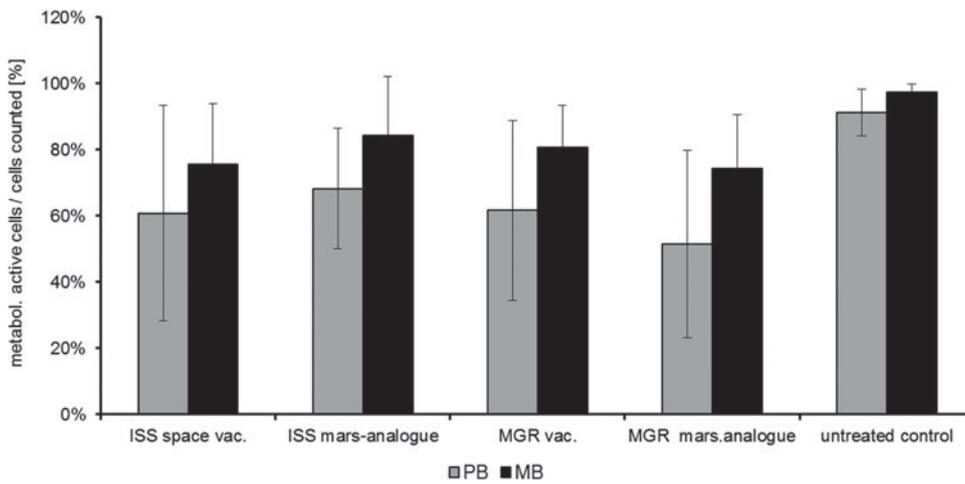


Fig. 5. Mars-analogue atmosphere versus space vacuum conditions: mean viability rates of photobiont cells and mycobiont hyphae in *X. elegans* with reference to the atmospheric conditions; dark-exposed samples.

DNA damage and cell decay caused by ionizing radiation of different sources has not been quantified yet. The measured accumulated doses of ionizing radiation (200–231 mGy) were mainly due to protons of the radiation belt which hit the ISS when it passed through the South Atlantic Anomaly (Dachev *et al.* 2012). The doses are not likely to explain viability loss of samples, because 50% of irradiated lichen algae survived doses of 90–480 Gy (10–54 kR) while fungal hyphae seemed even less sensitive to radiation (Gannutz 1972). The results of the LIVE/DEAD analyses indicate that after 1.5 year exposure in LEO a total average of 71% of the PB and 84% of the MB cells revealed metabolic activity. A less pronounced decrease of viability of MB and PB cells occurred under ISS Mars-analogue conditions. This is confirmed by a higher PS II activity of the PB exposed in ISS Mars-analogue conditions according to chlorophyll *a* fluorescence measurements as presented below.

Chlorophyll a fluorescence

Preliminary results of chlorophyll *a* fluorescence measurements on the post-flight viability of *X. elegans* PB have been presented before by Onofri *et al.* (2012) including also results of the lichen *R. geographicum*. Complementary, the following analysis focuses on the correlation between chlorophyll *a* fluorescence (as a marker of the PB's vitality) and the previously described results of the LIVE/DEAD analyses with regard to the experimental conditions applied and the characteristics of *X. elegans*.

The lichen samples (ISS and MGR) were measured prior to the exposure (pre-flight) and after their return (post-flight) according to a standardized measuring protocol. The mean values of the natural pre-flight values and initial post-flight measurements are given in Fig. 6. The highest post-flight Fv/Fm (= maximum quantum yield of photosystem II) value

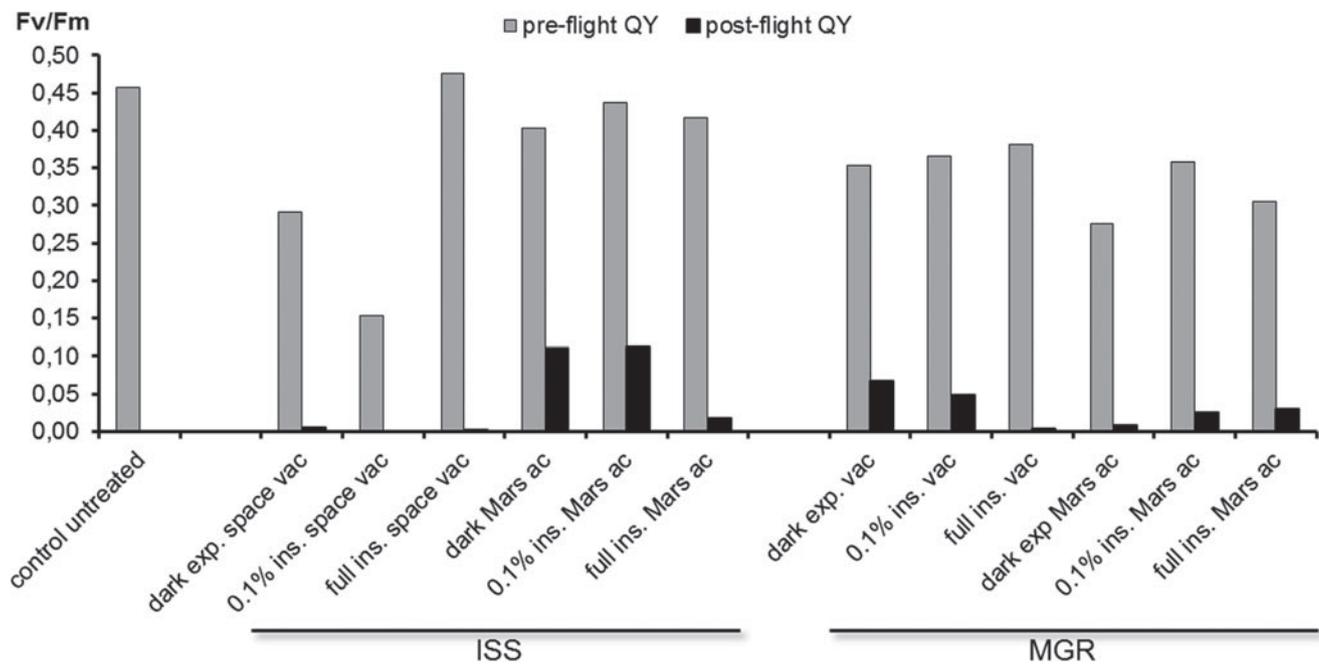


Fig. 6. Chlorophyll *a* fluorescence data of pre- and post-flight photosynthetic activity (Fv/Fm) as measured after standard protocol for each sample (ISS and MGR). The short wetting time was not sufficient for the strongly desiccated samples.

which was achieved by a single sample was 0.238 (MGR dark exp. space), followed by 0.148 and 0.129 (both ISS dark exp. Mars ac) representing 37–40% of their pre-flight Fv/Fm values. Most of the samples showed none or just a minimum of activity, defined as $Fv/Fm < 0.1$ in the initial post-flight measurement.

After the measurements described above, an additional re-activation was carried out two times. The first re-activation procedure enabled the strongly desiccated lichen (due to the effect of long-term space vacuum) to take up the allocated water and re-activate in a time range of 48 and 96 h. In this experiment, the number of examined samples was reduced, due to the finite sample quantity and in order to conserve sample material for different experiments to follow (e.g. DNA analyses, anatomical studies). The eight samples selected (four ISS; four MGR) showed different effects in response to the prolonged water availability. After 48 h of re-activation, four of the analysed samples indicated post-flight photosynthetic activity of the PB. Two out of these four samples revealed high Fv/Fm values (Fig. 7(a): 0.543: ISS dark exp. Mars ac; 0.621: ISS 0.1% ins. Mars ac) while two samples gave values indicating moderate photosynthetic activity (Fig. 7(a): 0.392: ISS dark exp. space and Fig. 7(b): 0.335: MGR 0.1% ins. Mars ac). The other four samples showed very low Fv/Fm values (Fig. 7(a): 0.106: ISS full ins. space; Fig. 7(b): 0.105: MGR dark exp. space, 0.000: MGR full ins. space and 0.039: MGR dark exp. Mars ac). Three out of the four samples which were exposed under Mars-analogue conditions showed increased Fv/Fm values after 96 h of re-activation (Fig. 7(a): 0.602: ISS dark exp. Mars ac; 0.641: ISS 0.1% ins. Mars ac; Fig. 7(b): 0.444: MGR 0.1% ins. Mars ac). One of the MGR samples revealed no photosynthetic activity after the prolonged re-activation period (Fig. 7(b): MGR dark exp. Mars ac).

The second re-activation procedure (Figs. 7(c): ISS samples; 7(d): MGR samples), following the same protocol as used in the first re-activation, was performed after the four space vacuum exposed samples of the first re-activation (ISS and MGR) had been desiccated again, because cycles of drying and wetting mimic the lichens' poikilohydric lifestyle. The Fv/Fm value increased by 56% for the ISS dark exp. sample. For other space vacuum samples the Fv/Fm values increased but were still remained on a low level (Fig. 7(c): 0.234: ISS full ins.; Fig. 7(d): 0.207: MGR dark exp.) after 96 h of re-activation. The MGR full ins. revealed no residual photosynthetic activity (Fig. 7(d)).

Four samples can be regarded as viable, according to the chlorophyll *a* fluorescence measurement, two samples showed reduced activity, and only two samples failed the re-activation, both of MGR. In six tests, the prolonged re-activation procedure resulted in higher Fv/Fm values, compared with the short-term activation pre- and post-flight measurements.

For three of the samples, there was a clear correlation between the re-activation procedure (wetting time, interrupted by desiccation) and a gain of measurable photosynthetic activity (Fig. 8). Thus, it is concluded that a prolonged time of re-activation procedure of multiple water supply and intermediate desiccation results in re-activation of photosynthetic activity, even for samples appearing inactive in the initial post-flight measurement. Compared with the ISS samples the MGR samples always showed reduced Fv/Fm values.

Correlation of viability data by LIVE/DEAD staining and chlorophyll a fluorescence

1. Compared with the ISS samples the MGR samples showed reduced viability in LIVE/DEAD staining and in PS II activity.

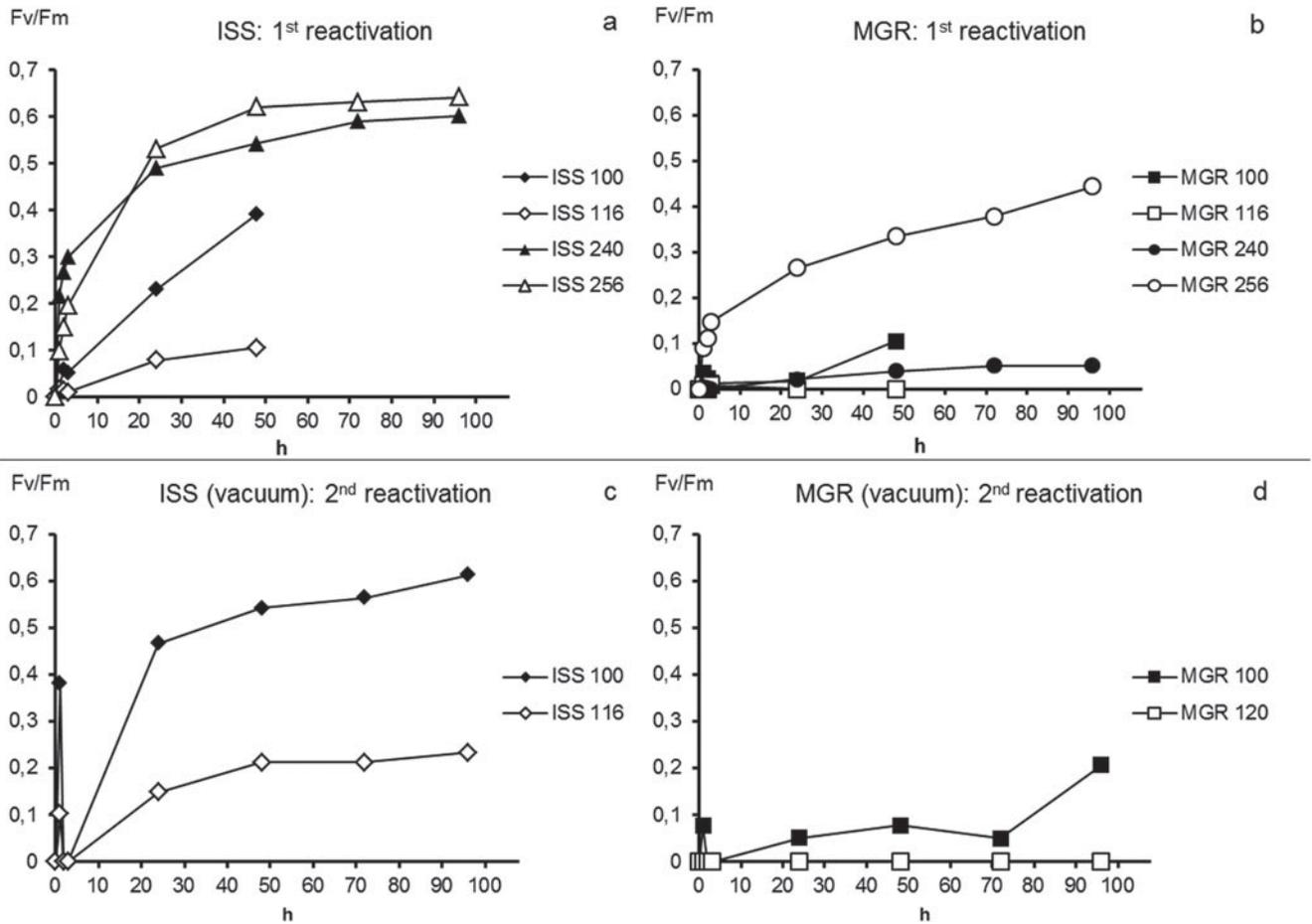


Fig. 7. Photosynthetic activity (as maximum quantum yield, Fv/Fm) in *Xanthoria elegans* after LIFE exposure according to the prolonged re-activation protocols (a) ISS samples, first re-activation; space vacuum: dark-exposed (100), full insolation (116); Mars-analogue conditions: dark exposed (240), full insolation (256). (b) MGR samples, conditions (simulated) and sample numbers as in (a). (c) ISS samples, second re-activation of the space vacuum samples, dark exposed (100), full insolation (116). (d) MGR samples, second re-activation of the vacuum samples, dark exposed (100), full insolation (120).

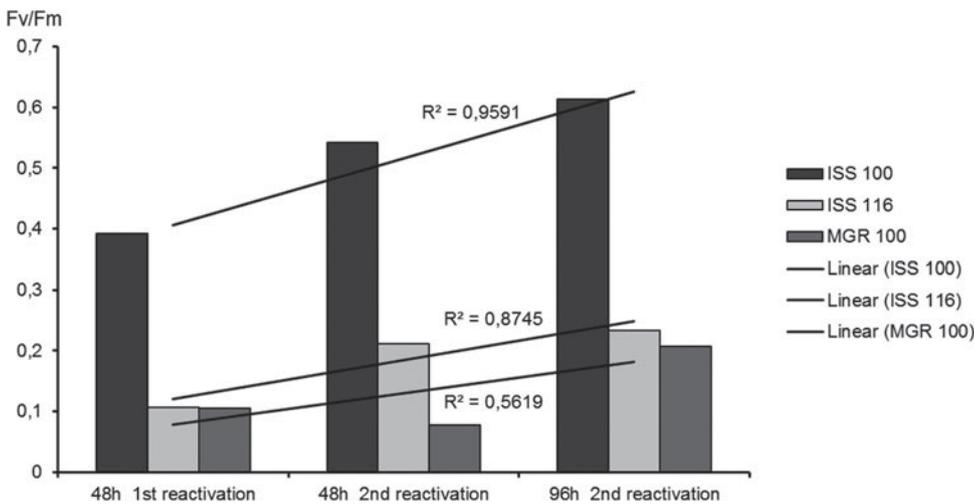


Fig. 8. First and second re-activation of the ISS and MGR space vacuum samples: samples show positive linear correlations of reactivation time (h) and maximum quantum yield of PS II (Fv/Fm). Samples dried between the re-activation (wetting) in ambient conditions.

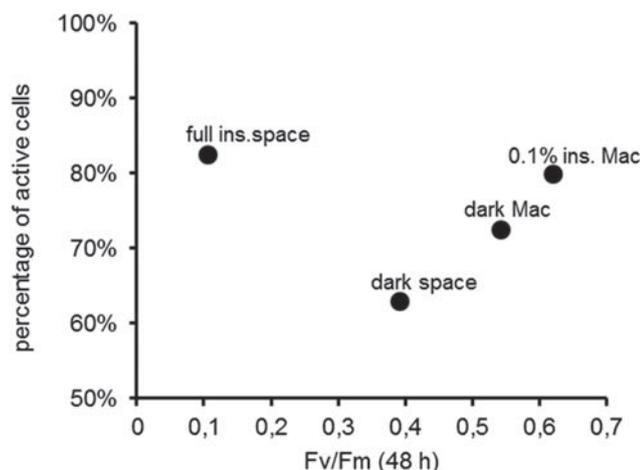


Fig. 9. ISS samples: correlation between the Fv/Fm values (chlorophyll *a* fluorescence) and cell viability assessed by LIVE/DEAD staining. The viability of the analysed samples is correlated for the both methods, except the space vacuum full insolation sample, which showed high metabolic activity combined with lower Fv/Fm values than samples of filtered insolation or dark exposure. full ins., full insolation; 0.1% ins., 0.1% insolation; dark, dark exposure; space, space vacuum; Mac, Mars-analogue conditions.

2. A prolonged re-activation procedure is mandatory to properly re-activate the photosynthetic activity. This might be due to the intense desiccation in space vacuum and the time which might be needed for cellular repair mechanisms.
3. The duration of the re-activation and the maximum quantum yield of photosystem II (Fv/Fm) are correlated (Fig. 8).
4. The ISS samples exposed under Mars-analogue conditions revealed higher viability rates, compared with the ISS space vacuum samples (Figs. 5 and 7(a)), and needed less re-activation time (Fig. 7(a)). According to the ISS sample results Mars-analogue conditions provided improved survival conditions for the anabiotic lichens.
5. The measured Fv/Fm values of the ISS samples can be correlated to the amount of PB cells detected as metabolically active in the LIVE/DEAD staining for three out of four ISS samples (Fig. 9: four samples after 48 h of first re-activation). Higher Fv/Fm values correlated to higher PB vitality rates in the LIVE/DEAD staining analysis. Only one sample (ISS full ins. space) showed a low Fv/Fm (maximum quantum yield) value, but high metabolic activity of PB cells in LIVE/DEAD staining analysis, indicating that the photosynthetic activity can be reduced without a loss of the overall metabolic activity in the PB cells.

Discussion

To fathom the limits and limitations of life it is fundamental to conduct real space and Mars-analogue exposure experiments. LIFE tested the potential of lichens' survival under

non-terrestrial conditions, as intense solar radiation, lack of atmospheric shielding, LEO-cosmic-radiation, vacuum which causes extreme desiccation, different atmosphere and a temperature-regime including rapid freeze/thaw cycling (Berger *et al.* 2012; Rabbow *et al.* 2012). The Mars-analogue conditions compartment of LIFE was intended to assess the possibility of microbial life (extinct or recent) in putative micro-niches on Mars.

According to the LIVE/DEAD viability analyses, the symbionts of *X. elegans* achieved remarkable high viability rates with a total average of 71% (PB) and 84% (MB) after 18 months of space exposure on the ISS. These results were substantiated by the chlorophyll *a* fluorescence analysis, illustrating a profound recovery of photosynthetic activity if properly re-activated. Besides these data obtained from LIVE/DEAD and chlorophyll *a* fluorescence analyses, *X. elegans* also revealed its remarkable post-flight viability and the capability of continuous proliferation in culture-based assays of the PB (Brandt *et al.* in preparation).

The post-flight viability of *X. elegans* is not reached by other organisms exposed in LIFE, including the lichen *R. geographicum* which re-gained not more than $2.5 \pm 1.4\%$ of its pre-flight PS II activity, compared with 75% for some *X. elegans* samples (ISS dark exp. space; Onofri *et al.* 2012). Additionally, Antarctic sandstone samples which were colonized by cryptoendolithic micro-organisms (fungi, algae and yeasts among others) and exposed in parallel to both lichens, produced three fungal colonies from the dark exposed space vacuum sample and one green algal colony from the 0.1% insolated Mars-analogue conditions sample (Scalzi *et al.* 2012), although qPCR propidium monoazide assays indicated quite high rates of non-damaged cryptoendolithic fungal cells (Onofri *et al.* 2012).

The high survival rates of *X. elegans* might be caused by its particular adaptation properties. Unlike the cryptoendolithic organisms, *X. elegans* does not depend on shielding by rock, but is well adapted to exposed habitats (Nybakken *et al.* 2004; de Vera *et al.* 2008) characterized by high insolation (Nybakken *et al.* 2004), extreme temperatures and occasionally higher altitudes. The adaptations of the lichens to the environmental conditions in their original habitat comprise different strategies of stress avoidance (Kappen 2000; Meeßen *et al.* 2013b) and damage repair (Eker *et al.* 1994; Petersen *et al.* 1999; Buffoni Hall *et al.* 2003). However, this study focuses on the anhydrobiotic (anabiotic) state of desiccated lichens and on the potential to avoid damage caused by insolation with wavelengths >110 nm (space vacuum) and >200 nm (Mars-analogue conditions), respectively.

LIVE/DEAD staining and chlorophyll *a* fluorescence analyses form a comparable pattern of both vital MB and PB in the samples examined. The only exception is the PB of the ISS fully insolated space vacuum sample which failed to reach its pre-flight Fv/Fm values in the chlorophyll *a* fluorescence measurements, revealing a deficit in photosynthetic activity, although the LIVE/DEAD staining analysis indicated a high average quantity of metabolically active cells (about 83%). The chlorophyll *a* fluorescence data of *X. elegans*

(ISS samples) correspond to the results presented by Onofri *et al.* (2012), indicating a full recovery (about 99%) of the photosynthetic activity for all ISS samples, except the space vacuum samples exposed to full solar irradiation which reached only 45% of the pre-flight Fv/Fm values. This is consistent with the present study indicating 50% of the pre-flight Fv/Fm value for this sample. The viability rates of each sample's PB and MB appear to be coupled to each other, this is confirmed by a linear correlation between the viability rates of MB and PB (coefficient of correlation is 0.95). PB and MB reacted likewise to the environmental condition applied with a proportionally higher rate of damage in the PB.

Owing to the narrow sample number available in LIFE, and its subsequent partition on various analytical methods, the influence of individual sample characteristics might be considered. Intersample variety might also be caused by different pre-flight vitality of individual lichen samples as well as by slightly differing thallus orientation during exposure or individual thallus structures (Meeßen *et al.* 2013b).

Anhydrobiosis: vacuum and freezing

Their poikilohydric nature enables lichens to endure unfavourable environmental conditions in a state of anhydrobiosis, 'latent life', mainly induced by desiccation (Crowe *et al.* 1992). Anhydrobiosis provides enormous desiccation tolerance (Hoekstra *et al.* 2001; Schlenzog *et al.* 2003; Rebecchi *et al.* 2007) and supports the importance of the poikilohydry as a fundamental life strategy of the lichen symbiosis in response to hostile conditions (Kranter *et al.* 2008). Although the non-lichenized PB is discussed to be more sensitive to intense insolation and desiccation than the MB (de la Torre *et al.* 2002; de Vera *et al.* 2008; de Vera & Ott 2010; de Vera 2012), the results of the present study for lichenized PB reported its high survival and re-activation potential after 18 months in anhydrobiosis under space and Mars conditions.

Considering space vacuum of 10^{-4} – 10^{-7} Pa as well as 10^3 Pa of the Mars-analogue atmosphere for 18 months, the lichens experienced a strong, long-term desiccation. Normally, the re-activation of air-dried lichens by water-uptake takes 5–20 min (Lange *et al.* 1989; Dyer & Crittenden 2008), depending on the species and the dry state. The prolonged re-activation time (48 or 96 h) of the ISS and MGR samples appears to be due to the longer time needed to moisten sufficiently after such extreme desiccation events, to re-start metabolism, to run repair processes and to re-initiate photosynthetic activity in the PB. This is supported by the correlation between elongated re-activation procedures with rising Fv/Fm values (Fig. 8). Consistently, the samples exposed in Mars-analogue atmosphere took half of the time to regain their photosynthetic activity. However, the experiment demonstrates the protective potential of the anhydrobiotic state, permitting re-activation after long-term desiccation by LEO-vacuum conditions (Sánchez *et al.* 2014). This is consistent with former simulation experiments (de Vera *et al.* 2003, 2004b, 2010, 2013) and storage experiments, in which

desiccated thalli of *X. parietina* (stored at -20°C), appeared fully viable after 13 years (Honegger 2003) while micro-fungi survived 13 years in liquid nitrogen (Smith 1982). Desiccated lichens also survived shock freezing in liquid nitrogen, preparation for scanning electron microscope (SEM) analyses and low-temperature SEM examination (20 kV electron beam, high vacuum, Honegger 2003). Moreover, isolated PB revealed high resistance to freezing (Hájek *et al.* 2012; Sadowsky & Ott 2012).

To conclude, concerning anhydrobiosis neither vacuum nor freezing appears to be the limiting factor for survival for lichen symbionts. Anhydrobiosis includes de-activation of photosystem II (Lange *et al.* 1989), avoiding the accumulation of reactive oxygen species (ROS), which harm essential cell functions (Wieners *et al.* 2012; Kranter *et al.* 2005). The repeated freezing and thawing cycles can be tolerated by lichens in their natural habitat in a diurnal course (Kappen 1985). The temperature fluctuations during exposition ranged between $+43$ and -21.7°C and do not exceed the range of temperatures lichen may experience in the natural habitat. The freezing resistance of *X. elegans* was not sufficiently tested by the conditions in LIFE.

The ISS Mars-analogue samples showed slightly higher viability rates in LIVE/DEAD analysis compared with the ISS space vacuum samples. Additionally, the respective Fv/Fm values were higher after shorter re-activation for samples exposed in Mars-analogue conditions. Depending on the length of the desiccation event the re-activation time needed was more prolonged, as indicated by the time-dependent increase of Fv/Fm in the (space) vacuum samples. The LIVE/DEAD staining results for the PB indicated less difference between the viability of Mars-analogue and space vacuum samples than the chlorophyll *a* fluorescence data pointed to, indicating that Fv/Fm measurements should not be the only test for viability of the PB in the lichen thallus.

Solar irradiation/cortex layer

In the present study, ISS and MGR lichen samples, including the isolated MB samples, lost vitality not related to the insolation applied according to the LIVE/DEAD staining analyses. This is consistent with space condition simulation experiments conducted by de Vera *et al.* (2003), which revealed no decrease in viability for *X. elegans* thalli irradiated with UV (200–400 nm) to a dose of 10 kJ m^{-2} applied in 4 h, and a decrease to a minor extent after UVR $>160\text{ nm}$ up to doses of 150 kJ m^{-2} , combined with vacuum conditions. The UVR doses of the fully insolated ISS samples accumulated to about 634 MJ m^{-2} within 559 days (Rabbow *et al.* 2012), not exceeding UVR-resistance of the entire lichen thalli. The high resistance to insolation of the photosynthetic partner (algae) might largely be caused by the secondary metabolite parietin deposited in the upper cortex layer (Solhaug & Gauslaa 1996), by the algal cell clustering in the PB layer, characteristic for the genus *Trebouxia* (Schaper 2003) and the protection by the layered anatomical structure of the lichen thallus, mainly formed by the MB (Henssen & Jahns 1974).

The UV and blue light screening abilities of the secondary metabolite parietin have been discussed by several authors, mainly with respect to UV-B irradiation (e.g. Solhaug & Gauslaa 1996; Wynn-Williams *et al.* 2002; Nybakken *et al.* 2004). For PAR insolation parietin encrusted cortices were found to screen 81% of blue, 27% of green and 1% of red wavelength ranges (Solhaug *et al.* 2010).

The production of the screening secondary lichen metabolites can be enhanced in the symbiotic state (Brunauer & Stocker-Wörgötter 2005) and the amount of parietin produced has been described to be effected by UV-B in lichens colonizing irradiated sites (Solhaug *et al.* 2003; Nybakken *et al.* 2004). The parietin encrusted cortex of *X. elegans* constitutes 8% of the total thallus thickness (Meeßen *et al.* 2013b) hence the additional quantity of secondary metabolites is adding to the insolation protection of the sensitive PB, more than e.g. sole cortex thickness (Dietz *et al.* 2000; Gauslaa & Solhaug 2004). In the anhydrobiotic (desiccated) state, the shrinkage of the lichen thallus causes a higher density of the secondary metabolite parietin, enhancing the screening and protective effect, making UV and blue light screening most efficient (Ertl 1951; Solhaug *et al.* 2010).

The difference in viability occurring in the two different methods used may be caused by direct UV-induced damage of the photosynthetic key components (e.g. D1 protein, Rubisco; Holzinger & Lütz 2006).

The Mars-analogue insolation included a 200 nm cut-off filter, shielding the samples (Mars ac) from vacuum-UVR ($\lambda < 200$ nm). Missing vacuum-UVR insolation might explain the slightly higher viability of the ISS Mars ac samples (LIVE/DEAD staining results). However, the viability rates of all ISS samples analysed were not correlated to the irradiation received. Even the dark exposed samples did not show increased viability in LIVE/DEAD staining analysis. The small increase in the viability rates of ISS Mars-analogue samples is rather due to the Mars-analogue atmosphere.

The samples studied were collected from slightly different high alpine sites. According to the intense UV-B irradiation at the different sites (Nybakken *et al.* 2004) the parietin production has been stimulated in slightly different amounts or might have been even altered seasonally (Solhaug & Gauslaa 2004; Gauslaa & McEvoy 2005). As a consequence, different viability rates of ISS or MGR insolated samples might also be caused by different concentration of parietin in the upper cortex layer.

Ionizing radiation

To assess and quantify the impact of cosmic radiation on the samples seems to be difficult, because of the relatively low accumulated doses (200–231 mGy), the complex composition of the radiation and the shielding provided by the EXPOSE-E hardware or the ISS (Berger *et al.* 2012). Ionizing radiation was mainly delivered by protons of the South Atlantic Anomaly (Dachev *et al.* 2012) and galactic cosmic heavy ions (Berger *et al.* 2012). It can be discussed, that the dark-exposed samples suffered from secondary radiation produced in the hardware and the final energy deposit of accelerated protons (Hausmann

2013 personal communication). Due to the sparsely differentiation of MB and PB cells (Carlile 1995) and the lichens' modular structure, single dead cells can be spared and replaced, not affecting the lichens' entire structure. Surviving cells are able to recover from radiation showing normal growth (Gannutz 1972).

MGR samples

According to both methods the viability of the MGR samples is significantly reduced compared with the ISS samples, although the MGR samples were not subjected to cosmic radiation sources above Earth level. This might be an indication to the disparity in viability of MGR and ISS samples, caused by inevitable differences in sample handling between ISS and MGR.

Mars habitability

The *X. elegans* samples exposed on the ISS in Mars-analogue conditions showed slightly higher viability and clearly higher photosynthetic activity compared with the space vacuum-exposed samples. The freezing temperatures of Mars were not tested in LIFE. As all samples were in anhydrobiosis (desiccated) for the duration of the experiment their ability to thrive in Mars micro-niches remains open.

Lithopanspermia hypothesis

The Lithopanspermia hypothesis is i.e. based on a proposal by Thomson (1871), suggesting that life could survive interplanetary travel. It emphasizes the role of the rock material, protecting the micro-organism on their transfer through space, e.g. by shielding against UV irradiation (Nicholson *et al.* 2000; Benardini *et al.* 2003; Cockell 2008; Horneck *et al.* 2008). Despite further modifications of the hypothesis, its principle deals with the interplanetary transfer of micro-organisms, seeding life from one planet to another.

One of the basic objectives of LIFE was to obtain results on the ability of the organisms tested to survive the interplanetary transfer phase. The results of the experiments with *X. elegans* align with the results of the simulation studies and the short-term exposure on BIOPAN 5 and 6 (Sancho *et al.* 2007, 2008; de la Torre *et al.* 2010b). The survival abilities of *X. elegans* appear not to be exhausted even by the conditions applied at the LIFE project. Though the 1.5 year mission duration is much shorter than the estimated length of a hypothetical interplanetary transfer e.g. 2.6 Myr for some Mars meteorites (Clark 2001), the results presented indicate that *X. elegans* might be able to survive a longer duration in space or might be a promising test subject for a Directed Lithopanspermia as proposed by Crick & Orgel (1973).

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