Reactivation of microbial strains and synthetic communities after a spaceflight to the International Space Station : corroborating the feasibility of essential conversions in the MELiSSA Loop

Reactivation of microbial strains and synthetic communities after a spaceflight to the ISS: corroborating the feasibility of essential conversions in the MELiSSA loop

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Abstract

To sustain human deep space exploration or extra-terrestrial settlements where no resupply from Earth or other planets is possible, technologies for *in-situ* food production, water, air, and waste recovery need to be developed. The Micro-Ecological Life Support System Alternative (MELiSSA) is such Regenerative Life Support System (RLSS) and builds on several bacterial bioprocesses.

However, alterations in gravity, temperature and radiation associated with the space environment can affect survival and functionality of the microorganisms.

In this study, representative strains of different carbon and nitrogen metabolisms with application in the Micro Ecological Life Support System Alternative (MELiSSA), were selected for launch and Low Earth Orbit (LEO) exposure.

An edible photoautotrophic strain (*Arthrospira* sp PCC 8005), a photoheterotrophic strain (*Rhodospirillum rubrum* S1H), a ureolytic heterotrophic strain (*Cupriavidus pinatubonensis* 1245), and combinations *of Cupriavidus pinatubonensis* 1245 and autotrophic ammonia and nitrite oxidizing strains (*Nitrosomonas europaea* ATCC19718, *Nitrosomonas ureae* Nm10 and *Nitrobacter winogradskyi* Nb255) were sent to the International Space Station for 7 days. There, the samples were exposed to 2.8 mGy, a dose 140 times higher than on Earth, and a temperature of 22 ± 1°C.

Upon return to Earth, the cultures were reactivated and their growth and activity compared to terrestrial controls stored under refrigerated (5 ± 2°C) or room temperature (22 ± 1°C and 21 ± 0°C) conditions. Overall, no difference was observed between terrestrial and ISS samples. Most cultures presented lower cell viability after the test, regardless of the type of exposure, indicating a harsher effect of the storage and sample preparation than the spaceflight itself. Post-mission analysis revealed the successful survival and proliferation of all cultures except for *Arthrospira,* which suffered from the pre-mission depressurization test. These observations validate the possibility of launching, storing and reactivating bacteria with essential functionalities for microbial bioprocesses in RLSS.

Keywords: LEO, oxygenic photosynthesis, anoxygenic photosynthesis, nitrogen recovery, nitritation, nitratation

Introduction

Human space exploration missions of long or permanent duration require autonomy from terrestrial resupply. Hence, extensive regeneration of consumables and waste to provide food, water and breathable air is necessary (Barta and Henninger 1994). A Regenerative Life Support System (RLSS) enables recycling the waste in a simplified ecosystem that can incorporate physicochemical and biological processes. The use of microbial bioprocesses has specific advantages that can improve the self-sufficiency of the system and allow a higher diversity of sustainable conversions, due to the diversification in the microbial metabolism (Council 1997). The conversion of organic compounds into CO_2 can be performed aerobically or anaerobically by heterotrophic bacteria, and can find use in the treatment of carbon enriched waste streams, such as fecal material, food waste and urine (Wheeler 2003). Autotrophic bacteria can be utilized for the fixation of CO_2 into edible biomass or the oxidation of inorganic elements, such as ammonium into nitrite and nitrate. Phototrophic bacteria utilize light as an energy source for biomass production that is rich in proteins and valuable nutrients. Their presence in a RLSS can provide dietary supplement for the astronauts (Escobar and Nabity 2017; Hendrickx *et al.* 2006; Hendrickx and Mergeay 2007).

One example of microbial utilization in a RLSS is the Micro-Ecological Life Support System Alternative (MELiSSA) designed for the European Space Agency (Hendrickx *et al.* 2006). At first, anaerobic thermophilic conversion of organic waste into volatile fatty acids (VFAs), hydrogen gas, CO_2 and ammonium (NH_4^+) is performed in the first compartment (CI) of the MELiSSA loop (Lissens *et al.* 2004). The removal of the soluble organic compounds from the filtrate of CI is carried out in the compartment CII via anoxygenic photosynthesis by the edible purple non-sulphur bacterium *Rhodospirillum rubrum* S1H (Mastroleo *et al.* 2009a). The conversion of ammonium into nitrate, which is the preferred form of nitrogen for the hydroponic plant compartment (CIVb), takes place in the nitrification compartment (CIII) with autotrophic nitrifiers such as, e.g., *Nitrosomonas europaea* ATCC19718 and *Nitrobacter winogradskyi* Nb255 (Clauwaert *et al.* 2017). With a richer community, urine can be treated in CIII (De Paepe *et al.* 2018). In the case of composing the community synthetically, a specific ureolytic autotroph (e.g., *Nitrosomonas ureae* Nm10) to convert the urea into ammonium or heterotroph (e.g., *Cupriavidus pinatubonensis* 1245) to convert the urea into ammonium and to oxidize organic compounds in urine will be necessary. The production of edible biomass and O_2 is obtained in compartment CIVa with the photosynthetic cyanobacterium *Arthrospira sp.* PCC8005 and in CIVb, the plant compartment (Olsson-Francis and Cockell 2010).

The ultimate goal of RLSS technologies is its utilization in space. This will expose the microoranisms to an extreme environment in which altered gravity, possibly temperature fluctuations and higher levels of radiation can significantly affect microorganisms (Horneck *et al.* 2010). Yet, the effect of space on microorganisms suitable for RLSS conversion have been limited to some functionalities, tested at different conditions. Several phototrophs were tested for outer space conditions, and both algae (*Chlorella* and *Rosenvingiella*) and cyanobacteria (*Gloeocapsa)* survived 548 days outside the International Space Station (ISS) (Cockell *et al.* 2011). The CIVb *Arthrospira* sp. PCC8005 has not been included in spaceflights experiment yet. The CI*R. rubrum* grown on agar plates was sent twice to the International Space Station, which demonstrated its survival and indicated that radiation has a higher impact than microgravity (Mastroleo *et al.* 2009b). The ureolytic heterotroph *C. pinatubonensis* and key nitrogen cycle bacteria also survived a 44 days space flight on board of the unmanned satellite FOTON M-5 (Lindeboom *et al.* 2018). *N. urea* and its synthetic communities have never been exposed to LEO conditions.

The aim of this study is to elucidate the reactivation of metabolically diverse microorganisms for a potential use in the MELiSSA Life Support System, after exposure to the same LEO environment, launch and storage conditions.

An edible autotrophically grown cyanobacteria (*Arthrospira* sp PCC 8005), an heterotrophically grown purple non-sulphur bacterium (*Rhodospirillum*

rubrum S1H), a ureolytic heterotrophic strain (*Cupriavidus pinatubonensis* 1245) and combinations of *Cupriavidus pinatubonensis* 1245 and autotrophic ammonia and nitrite oxidizing strains (*Nitrosomonas europaea* ATCC19718*, Nitrosomonas ureae* Nm10, *Nitrobacter winogradskyi* Nb255) were selected. The cultures were sent to the ISS for 7 days, where they were exposed to 2.8 mGy, a dose 140 times higher than on Earth at an average temperature of 22 ± 1 °C. To maximise the resolution of the experiment and identify the effect of LEO exposure, the transfer times between the laboratories at the launch base were optimized compared to previous experiments, which presented a storage and travel time amounting to 2/3 of the total experimental time (Lindeboom *et al.* 2018) and control cultures were also transferred to the launch site.

Upon return on Earth, the culture's reactivation was tested for cell viability (cell number or ATP quantification) and ability to proliferate (growth or activity preservation). LEO exposed cultures were compared with terrestrial control cultures, exposed to 0.02 mGy background radiation dose and various test temperatures, namely refrigerated controls (5 ± 2 °C) and controls stored at room temperature (21 ± 0°C) and spare samples preserved at the launch site in Baikonur (22 ± 1 °C). Pre-storage values were also compared to the post-storage, elucidating for the first time the role of storage over LEO exposure.

Materials and Methods

Sample preparations and hardware

Overview of the cultures origin and cultivation is presented in S1. All cultures were prepared at Belgian Nuclear Research Centre (SCK●CEN) Aug 25th, 2015. The nitrifying cultures were prepared mixing equal volumes of each strain. The culture of C. pinatubonensis intended for the nitrifying cultures was pelleted and resuspended in PBS beforehand. Overview of the cultures is presented in Table 1, while the final concentration of the nitrogen species present in the media are reported in Figure S1. Aliquots of the cultures were then divided in quadruplicates in polypropylene cryotubes, with silicone washer seal and internal thread (VWR International, Radnor, USA). Each cryotube contained 5 mL of liquid culture. The cryotubes were then stored in the dark in a 1.5 L polypropylene plastic Biojar (Air Sea Containers, Birkenhead, UK) (Figure 1a). The lid and the bottom of the Biojar were equipped with 1 track-etch detector each (Harzlas TD-1, Fukuvi Chemical Industry CO. LTD., Fukui-city, Japan) (Figure 1b) and 32 cryotube lids were equipped with 144 thermoluminescent detectors (MTS-6, MTS-7, MCP-6 and MCP-7, Radcard, Kraków, Poland) (Figure 1c). The track-etch and thermoluminescent detectors were provided and analyzed respectively by the radiation dosimetry departments of the Nuclear Physics Institute of the Czech Academy of Sciences in Prague (Czech Republic) and SCK•CEN. Additionally, stand-alone temperature loggers (iButton, HQMatics, Dokkum, The Netherlands) were added to the payload to monitor the temperature over the full mission.

To ensure the tightness of the cryotubes, the samples intended for LEO exposure (ISS) and spare samples preserved at the launch site in Baikonur (G) were subjected to a depressurization test the day of their preparation (Aug 25th, 2015), as reported in S1.2.

Low Earth Orbit flight and ground storage conditions

The samples were hand-carried in phase change material boxes (series 22 PCM) to RSC-Energia (Moscow, Russia) on Aug 27th, 2015, and subsequently sent to the launch site (Baikonour, Kazakhstan). Here, samples destined for the ISS were placed inside the capsule and launched on Sept 2th, 2015, while the controls for terrestrial refrigerated (G-5) and room (G-21) temperature exposure were sent back from Baikonur to SCK●CEN, where they arrived on Sept 4th, 2015. Spare samples (G) were preserved at the launch site and returned together with the ISS samples from Sept 2nd, 2015 to Sept 12th, 2015. ISS samples were launched on Sept 2nd and installed in the Columbus module of the ISS on Sept 5th. The ISS samples were discharged on Sept 11th and landed back on Earth on Sept 12th. Total ISS permanence was 9.84 days. Sample analysis was performed one day after the recovery of ISS samples in the lab, on Sept 13th, 2015. Overview of the temperature and radiation profilis presented in Figure 2.

The ground control G (22 ± 1°C) was selected as the reference for the nitrification tests of the ISS samples (22 ± 1°C), unless otherwise mentioned. The terrestrial storages in a temperature-controlled environment experienced a refrigerated temperature of 5 ± 2°C (G-5) and a room temperature of 21 ± 0°C (G-21) (Figure 2a).

The thermoluminescent detectors showed that, during the 9.84 days mission, the ISS samples were exposed to a total radiation absorbed dose in water

of 2.4 mGy (Figure 2b). 0.4 mGy were added to the overall dose, due to the radiation with high ionization density (heavy nuclei and high energy neutrons) measured by the track-etch detectors (Figure 2c). Hence, the total absorbed dose in water received by the ISS samples is 2.8 mGy, more or less 140 times higher than the 0.02 mGy measured for the terrestrial controls G, G-5 and G-21.

Post-mission characterization and reactivation.

Upon retrieval, the nitrogen composition of the storage media and the purity of the nitrifying cultures was assessed as described in S1.3 and S1.4. Despite the culture preparation in sterile conditions, the development of heterotrophic populations was observed for all the nitrifying cultures, but not for the *Arthrospira* sp. and *R. rubrum* and *C. pinatubonensis* cultures. Overview of the Operational Taxonomic Units (OTUs) detected is presented in Figure S2, and Table S1.

Post-mission cultures were characterized evaluating the cell viability and ability to proliferate.

Arthrospira sp. and *R. rubrum* and *C. pinatubonensis*'s cell viability was assessed with flow cytometric photosynthetic potential evaluation (FL3/FL4 ratio), live/dead ratio and plate count quantification, respectively. Their ability to proliferate was checked monitoring the growth after subculture in liquid medium. Pulse Amplitude Modulated analysis (PAM) was additionally performed on *Arthrospira* sp's cultures. Detailed materials and methods are presented in S1.5.

Due to the slow growth of autotrophic bacteria, cell viability for the nitrifying cultures was assessed measuring intracellular ATP (adenosine triphosphate). The ability to proliferate was checked evaluating the presence of nitrifying activity, monitoring over time the concentration of ammonia, nitrite and nitrate. Overview of the materials and methods utilized is described in S1.6.

Results

Photoautotrophic stain *Arthrospira* sp PCC 8005 was irreversibly affected by a pre-flight depressurization test.

The pH of *Arthrospira* sp**.** media before the test was 10.3. Post-storage pH measurements on ISS, G, G-5 and G-21 revealed a similar endpoint pH, on average 10.2 ± 0.0.

Flow cytometry count on the *Arthrospira* sp. mother culture gave a FL3/FL4 ratio around 0.5, indicating a healthy physiological state of the cells before the experiment. A similar ratio was observed in the G-5 samples. G-21 showed a ratio of 1, sign of a partially impaired photosynthetic system, while ISS and G presented a ratio higher than 3, indicating a fully damaged system (Figure 3a). Pulse Amplitude Modulated analysis (PAM) measurements of the photosynthetic efficiency of G-5 and G-21 showed Fv/Fm values close to 0.6, namely 0.521, indicating a healthy culture. Fv/Fm values could not be determined for ISS and G samples. *Arthrospira* sp. samples ISS and G could not resume growth, while G-21 and G-5 reached and OD_{750} after 15 days of cultivation (Figure 3b).

Since *Arthrospira* sp. PCC8005 is highly resistant to ionizing radiation (Badri *et al.* 2015), it is suspected that the effect observed on the flight and ground control samples are not related to the space experiment but rather to samples preparation pre-flight. Indeed, the samples that did not resume the growth (ISS and G) were the ones subjected to the depressurization test. Hence, the same treatment was performed on freshly grown cultures of *Arthrospira* sp. Their ability to propagate was further evaluated.

Prior to the depressurization procedure, the pH of the culture (split in 20 tubes) was set at 10.3. Immediately after the depressurization, the pH increased to 11.5 and then stabilized to pH 10.6 after about 1 min, when reaching the equilibrium with ambient air. After 20 days of storage, estimated theoretical time between sample preparation on Earth and activating experiment on ISS, of these samples in the dark, the pH was 10.4 and the OD_{750} 0.984. The subculture of the samples after 20 days yielded an OD_{750} of 0.294 at a pH of 9.9 after 4 weeks of cultivation, indicating a lack of growth.

Rhodospirillum rubrum.

The pH measurements of *R. rubrum* after the storage revealed a mild acidification of the media in all the samples, with no difference among conditions (ISS, G, G-5, G-21), from a pH of 7.8 to an average value of 7.0 ± 0.1. The mother culture of *R. rubrum* presented a fraction of live cells of

ca. 20%. After the storage, a value 10 times lower was observed in all the samples (Figure 3c).

No difference among samples (ISS, G, G-5, G-21) was observed during growth and all the cultures reached stationary phase after 3 days (Figure 3d).

Cupriavidus pinatubonensis.

The storage medium of *C. pinatubonensis* was adjusted upon sample preparation to a pH of 6.8. After the test, no variation was detected among the ISS, G and G-21 cultures, which presented an average pH of 7.0 ± 0.1, 6.9 ± 0.0 and 6.9 ± 0.0, respectively. A mild acidification to a pH of 6.7 ± 0.1 was observed in the G-5 cultures.

C. pinatubonensis's viable cell number was similar among samples, with a logCFU mL^{-1} of 9, irrespective of the treatment (Figure 3e). The Illumina analysis confirmed that both for the ISS and the G sample no other strains were present (Figure S2). The growth observed after the storage was similar among ISS, G, G-21 and G-5, as all samples reached the stationary phase 1 day after the subculture (Figure 3f).

Ureolytic communities.

Even though the reactivation and heterotrophic of *C. pinatubonensis* (C) was demonstrated in the previous section, its ureolytic activity was also evaluated, for comparison with the cultures including the autotrophic ureolytic strain *N. ureae* (U). Overview of the rates is presented in Table S2. Since urease is a widely distributed enzyme, the heterotrophic contaminant identified in the samples U, CEW and CUW could possibly contribute to ureolysis. Nonetheless, C showed the highest activity (1.77 ± 0.06 and 1.72 ± 0.04 mg-N L-1 h-1 for ISS and G, respectively) and the ureolytic rate in samples U and the ureolytic rate in samples U, where no Cupriavidus was detected, was on average 7 to 11 times lower than C for ISS and G, respectively. C showed the highest activity (42.48 ± 1.44 and 41.28 ± 0.96 mg-N L^{-1} d^{-1} for ISS and G, respectively) and the ureolytic rate in samples U was on average 7 to 11 times lower than C for ISS and G, respectively. Ureolysis was observed also in all the samples where *C. pinatubonensis* was mixed with other bacteria (Figure 4a).

The combinations CEW and CUW, where the relative abundance of *C. pinatubonensis* was between 50-55 and 61-63 %, according to the Illumina results (Figure S2), showed G and ISS ureolytic rates of 547.2 ± 115.2 and 417.6 ± 52.8 mg-N L^{-1} d^{-1} for CEW, and of 168.0 ± 24.0 and 273.6 ± 91.2 mg-N L^{-1} d^{-1} for CUW. The presence of *N. ureae* in the latter community did not seem to have a beneficial effect on the total ureolysis.

Overall, the comparison between ISS and G samples did not show a significant impact of LEO conditions in any of the cultures tested (p value > 0.05).

Nitrifying communities.

An overview of the pH and nitrogen species after the test in the ISS and G nitrifying cultures is presented in Figure S1. A general acidification of the medium from the initial pH of 7.5 to 6.7-5.8 was observed in all the cultures containing AOB. The samples with *N. europaea* (E) presented the lowest average pH (5.8 ± 0.3), which could indicate active ammonia oxidation during the transportation and on flight.

Overview of the ATP concentration and Equivalent Active Cells (EAC), assuming 10^{-18} mol ATP equals 1 EAC, is presented in Figure S3. Absolute ATP measurements showed that all the mother cultures presented EAC concentration in the range between 10^7 and 10^8 cells ml^{-1}. Despite the proliferation of heterotrophic contaminant, the comparison of the EAC value measured for the mother cultures shows a decrease in EAC after the experiment in all the cultures, except for EW. The comparison between ISS and G revealed mostly equal EAC levels, with the exception of E, whose ISS samples displayed 1.7 times higher ATP concentration. Comparisons of the samples stored at different temperature (G-5 and G-21) revealed mostly similar ATP levels. Samples that present higher EAC levels at G-5 are W and EW with 1.6 and 2 times higher values in G-5, while higher ATP levels in the G-21 cultures was observed only for CEW.

The ammonia and nitrite oxidation rates are presented in Table S2. The OTUs table presented in in Figure S1 shows that the only AOB present belong to the Nitrosomonas genus, suggesting no contamination of ancillary AOB. The cultures in which ammonia oxidation is performed by *N. europaea*

displayed a significantly lower activity (p value < 0.05) in G samples compared to ISS and high variability among replicates during the activity test. Hence, the back-up cultures stored under refrigerated (G-5) and temperature controlled (G-21) conditions were also analyzed for E, EW, and CEW. These cultures could all be reactivated in the first week of test. Overall, the ammonia oxidation activity of the cultures with *N. europaea* as key AOB displayed no significant difference between ISS, G-5 and G-21 (p value > 0.05) (Figure 4b). Compared to the mother culture, E showed a 5-fold decrease in activity, while EWC and EW presented comparable rates. This indicates that neither the relative abundance of the AOB nor the presence of heterotrophic contaminant is responsible for the decrease in activity of the sample E compared to the mother culture.

The cultures with *N. ureae* had a more stable response to reactivation among replicates already during the first ammonia oxidation activity test. The rates achieved by U and CUW were comparable to the ones of the mother cultures. Similar to the cultures with *N. europaea*, the ISS and G rates were not affected by the storage conditions for *N. ureae*.

Nitrite oxidation after the ISS and ground storage could be observed in all cultures tested (Figure 4c). All the sequences associated with NOB belonged to Bradyrhizobiaceae, suggesting no contamination of ancillary NOB (Figure S1). The consortium W, which did not present any AOB, showed the highest activity loss compared to the mother culture, showing 6 and 3 times lower rates than ISS and G, respectively. W represent also the only culture in which the G rates were 2 times higher than the ISS rates (p value < 0.05). The samples which presented *Nitrosomonas spp.* instead, did not display any significant difference among ISS and G cultures (p value > 0.05).

Discussion

The storage exposed the samples to stable conditions.

Results from the characterization of the storage conditions reveal that the samples were exposed to very stable conditions throughout the experiment and especially during the flight itself (Figure 1), which is not always feasible during LEO flight experiments (Byloos *et al.* 2017; Cockell *et al.* 2011; Lindeboom *et al.* 2018). The higher standard deviation measured for the ISS samples originates from the lower temperature experienced during the launch and landing activities, while the one registered for the G-5 samples was caused by the 5 hours needed to cool down from room temperature, as shown in Figure 1a. Hence, the results observed were not caused by thermic fluctuations. This allows the analysis of the storage and radiation independently from the effect of temperature.

Overall, the radiation experienced by the cultures showed no significant difference in dose between the different tube positions. All samples were exposed to a higher dose than the terrestrially preserved controls (ca 140 times), which could have impacted the performance of the ISS samples. A systematic difference between the different detector types, associated with the different sensitivity towards radiation with high ionization density (MCP detectors are commonly less sensitive than MTS), low energy neutrons (measured only by the 6-type detector) or heavy ions and high energy neutrons (measured by the track-etch detectors), was also observed. This expected variability indicates that the use of the different detector types can provide a better insight in the quality of the radiation field.

Photoautotrophic stain *Arthrospira* sp PCC 8005 was irreversibly affected by a pre-flight depressurization test.

This mission represents the first spaceflight for *Arthrospira* sp. PCC8005, although it was already exposed to high doses of ionizing radiations (i.e., more than 6000 Gy) in lab-scale simulations, without significant consequences (Badri *et al.* 2015). The disruption of the photosynthetic system and the inability to propagate after the mission was observed in both the ISS and G samples. This is therefore not a result of the higher radiation dose experienced, but rather of the pre-flight assembly procedures. This hypothesis finds confirmation in the results of the follow-up depressurization test, which had a dramatic impact on the growth of terrestrially preserved *Arthrospira* sp. The off-gassing of CO_2 probably induced a harmful or deadly increase in pH, from which the culture was not able to recover. The results obtained highlight the need to transition from standardized sample preparation procedures, which do not take into account the microbial response, towards individualized procedures based on the type of microorganism. Additionally, the media selected can promote either microbial growth (if substrate is available) or inhibition (generated by pH fluctuations), further indicating the need for case-specific choices.

R. rubrum resumed growth after storage despite post-mission biomass deterioration.

R. rubrum showed a 10-fold decrease in live cells in all the cultures after 13 days of storage. A mild acidification of the media was also observed after the experiment, possibly contributing to the poor culture health observed for *R. rubrum*. A longer storage time will be required for cultures intended for distant human outposts or as back-up, which can possibly affect permanently their ability to recover. The development of optimized storage conditions and media (i.e., higher buffer capacity) will be crucial for real-life application. The utilization of different techniques, such as freezing with proper cryoprotectants, will allow the preservation of heterotrophic strains for a longer period (Kerckhof *et al.* 2014). Nonetheless, it could grow after sub-culture in fresh media and reached a steady state after few days of cultivation. This was the third spaceflight for MELiSSA CII compartment bacterium, *R. rubrum*, following MESSAGE-2 and BASE-A spaceflight experiments, but the first including *R. rubrum* in liquid clture, confirming its survival in different culture conditions (Mastroleo *et al.* 2009b).

C.pinatubonensis was not affected by LEO exposure.

C. pinatubonensis prove to be unaffected by the experimental conditions tested. It displayed similar cell count as before the space mission (PM samples), was able to resume growth in fresh medium and also showed the highest urea hydrolysis rate. This might indicate that, at least for some conversions, the use of *C. pinatubonensis* might be preferred due to its robustness. Nonetheless, conversion of nitrogen to undesired by-products should be carefully evaluated as heterotrophic strains might perform them with higher speed. This was for example observed evaluating the nitrate removal under anoxic conditions (Figure S4). In the culture of *C. pinatubonensis*, complete depletion of nitrate was observed within the 24 h of experiment in both ISS and ground controls, while all the other cultures still presented residual NO_3^- in the media.

Autotrophic nitrogen conversions were not affected by LEO exposure but showed biomass deterioration after storage.

The cultures associated with nitrogen metabolism mostly showed lower EAC values after the space mission and terrestrial storage, indicating a general decay of the biomass, despite the development of heterotrophic contaminations. Overall, the temperature (G-5 vs G21) did not seem to influence the EAC values, while ISS samples yielded mostly similar EAC concentrations than G storage. This suggest that the presence of contaminants most likely originated before the cultures were split for the different storage conditions, which seems to find confirmation in the similar sequencing profile of the G and ISS samples (Figure S2). Although autotrophic bacteria can release in the media small organic compounds (soluble microbial products), which can function as organic substrate for heterotrophic growth, the EAC results indicate that, in minimal medium without added organic substrates, the proliferation of heterotroph contaminants was limited and generally the biomass decayed. AOB and NOB were never outcompeted by the heterotrophic populations and were active in all the samples analyzed. This co-existence points towards the use of microbial synthetic communities combining multiple metabolisms. Nitrifying communities have been previously exposed to LEO orbit conditions and all metabolic functions could be recovered. However, the previously reported experimental set up foresaw longer storage and travel time amounting to 2/3 of the total experimental time, with one month passing from the landing of the sample to the activity tests (Lindeboom *et al.* 2018). Hence, the effect of long storage time and spaceflight could not be completely differentiated, as for instance, AOB activity has been recovered after 5 months of terrestrial storage at 4 and 20 °C (Vlaeminck *et al.* 2007).

Conclusions

In conclusion, this study presents for the first time the results on key bacteria and synthetic communities for RLSS exposed to the same LEO environment. All cultures except for *Arthrospira* could be successfully prepared and exposed to LEO conditions, proving that they could be efficiently preserved and reactivated to an extent comparable to terrestrially stored cultures. Furthermore, the results validate the storage in liquid medium for space mission.

The confirmation that microorganisms involved in RLSS can withstand space conditions for a mission lasting longer than the time required to reach the Moon eliminates one of the main uncertainties regarding its applicability in space, and promotes the development of additional studies and technologies.

CULTURE	CODE	Phototrophic production of edible biomass		Production of nitrate in urine (CIII)				
		Autotrophic/ oxygenic photosynthesis (CIVa)	Heterotrophic/ anoxygenic photosynthesis (CII)	Ureolysis	Ammonia oxidation	Nitrite oxidation	OD ISS	OD G
Arthrospira sp PCC8005	A	X	-	-	-	-	1.06	1.09
Rhodospirillum.rubrum S1H	R	-	X	-	-	-	0.92	0.92
Cupriavidus pinatubonensis strain '1245'	C	-	-	X	-	-	1.85	1.85
*Nitrosomonas europaea strain 'Winogradsky (ATCC19718) **	E	-	-	-	X	-	0.10	0.10
*N. ureae strain Nm10 **	U	-	-	X	X	-	0.05	0.05
Nitrobacter winogradskyi strain 'Nb255'	W	-	-	-	-	X	0.07	0.14
*N. europaea + N. winogradskyi **	EW	-	-	-	X	X	0.07	0.07
*Cupriavidus pinatubonensis *+ N. europaea + N. winogradskyi +*	CEW	-	-	X	X	X	0.14	0.14
*N. ureae + N. winogradskyi **	UW	-	-	X	X	X	0.05	0.05
*Cupriavidus pinatubonensis *+ N. ureae + N. winogradskyi +*	CUW	-	-	X	X	X	0.10	0.10

Table 1. Overview of the cultures, the nitrogen-related metabolic activities and OD recorded upon sample preparation. Contamination was observed at the end of the tests in the cultures marked with '*'. Column headers refer to the relevant MELiSSA compartment number (CII, CIII and CIVa) for every conversion.

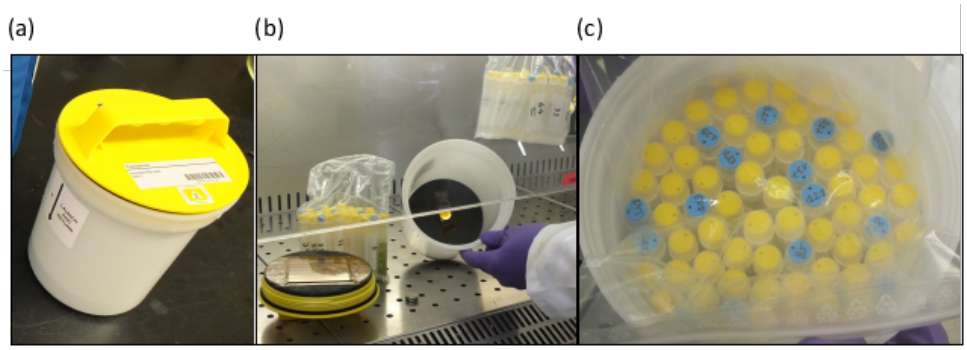

FIG 1: Pictures of (a) the Biojar, (b) iButton temperature loggers inside the container and (c) cryotubes containing the samples. The blue cap indicates the presence of thermoluminescent detectors.

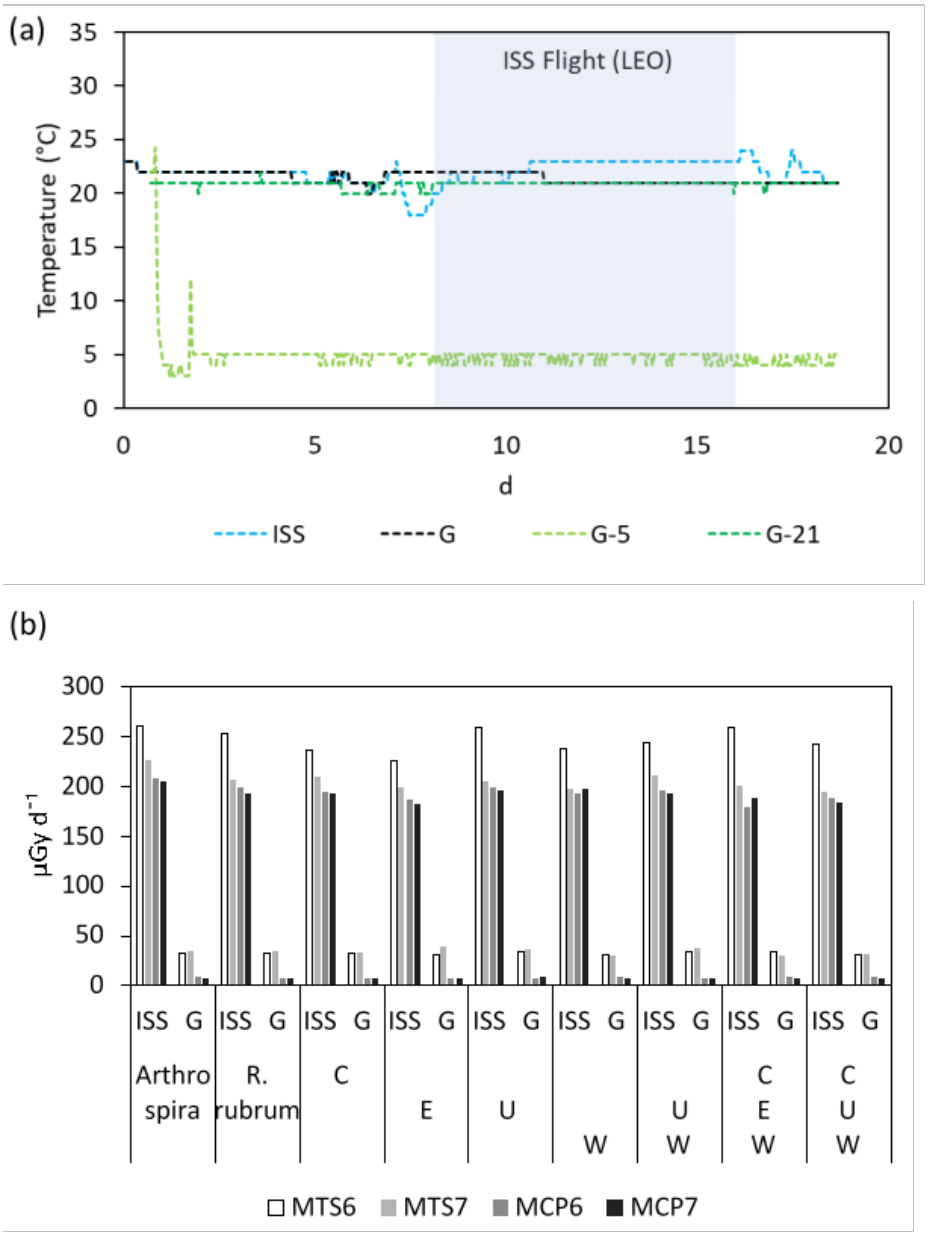

FIG 2. Temperature profile (°Celsius) from the iButton (a) and radiation absorbed dose in water measured with MTS6, MTS7, MCP6 and MCP7 detectors for the ISS and G samples (b).

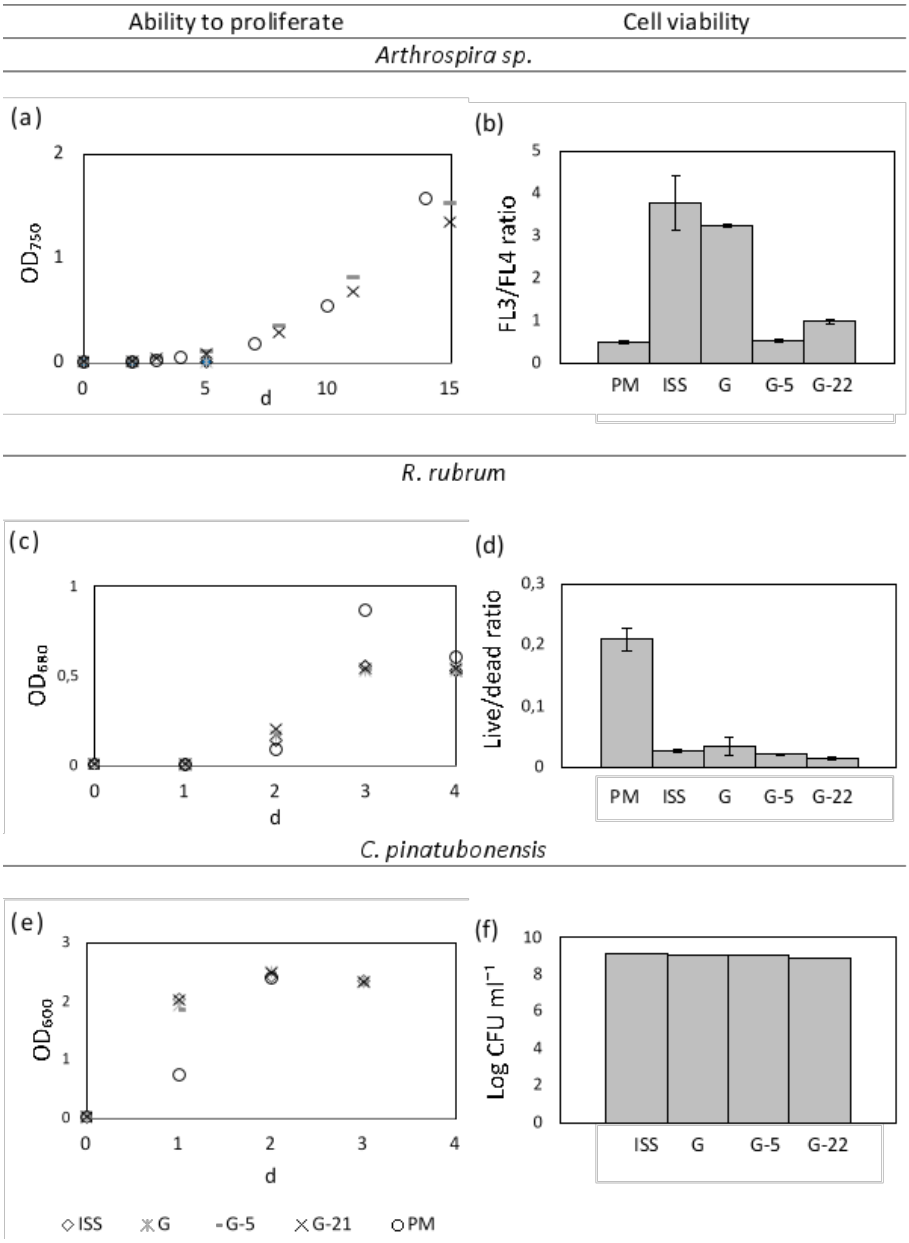

FIG 3. Overview of the cell viability for pre-mission (PM), LEO exposed samples (ISS), spare terrestrial samples preserved at the launch site in Baikonur (G), terrestrial refrigerated samples (G-5) and samples stored at room temperature (G-21), evaluated with FL3/FL4 ratio (a) and growth curve (b) for *Arthrospira sp.* live/dead cell count (c) and growth curve (d) for *R. rubrum* and CFU counts (e) and growth curves (f) for *C. pinatubonensis*.

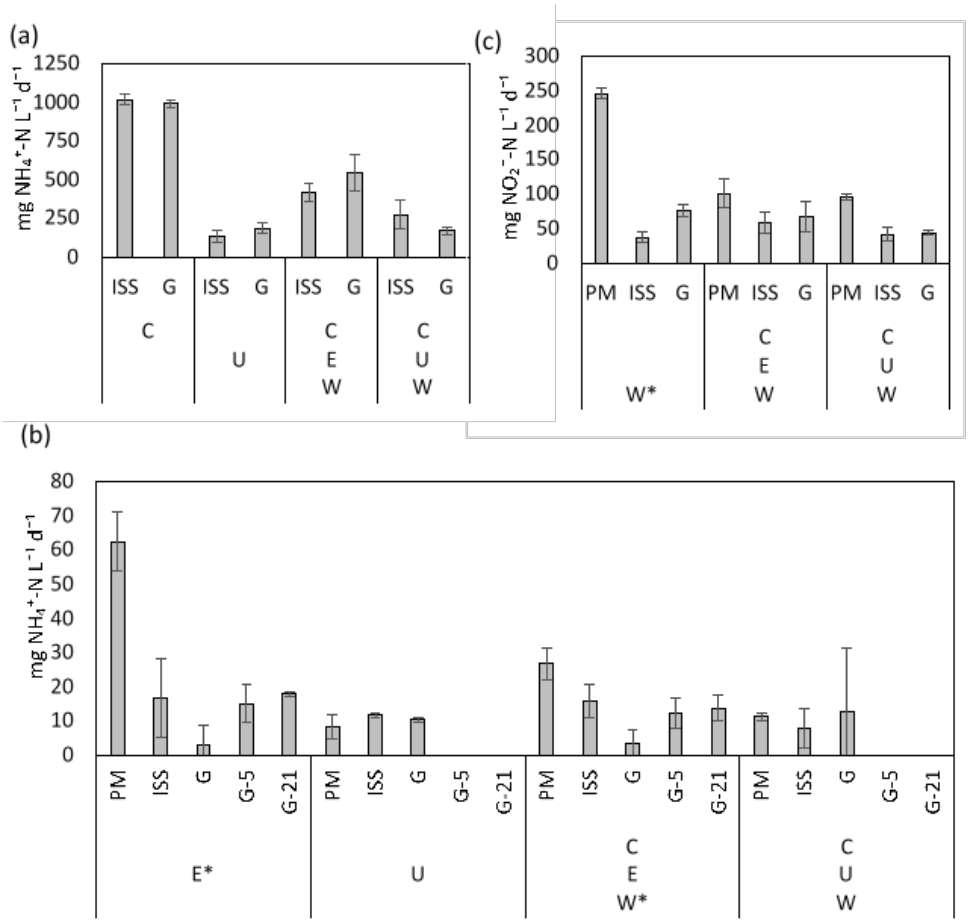

FIG 4: Overview of the ureolysis (a), ammonia oxidation (b) and nitrite oxidation (c) rates as mg-N L^{-1} d^{-1} for the pre-mission (PM), LEO exposed samples (ISS), spare terrestrial samples preserved at the launch site in Baikonur (G), terrestrial refrigerated samples (G-5) and samples stored at room temperature (G-21). The rates of the cultures not present in the graphs were not determined. The cultures marked with '*' present a significant difference between ISS and G samples.

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