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Survival limits of psychrotolerant microorganisms with relevance for planetary protection of the icy moons

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Investigating the survival limits of extremophilic microorganisms exposed to simulated space conditions can shed light on the ability of terrestrial microorganisms to survive and propagate on other planetary bodies. Although microbes can be found in all environmental niches on Earth, this study focuses on psychrophilic and psychrotolerant microorganisms (prokaryotes and eukaryotes) which have been isolated from locations of interest such as icy moon analogue environments (e.g. Canadian high arctic, Antarctica) and cleanrooms, which might be relevant for forward planetary protection. Our research aimed to reproduce conditions for microorganisms on spacecraft travelling to the outer solar system which could contaminate the icy moon's subsurface oceans. The microorganisms were grown under oligotrophic conditions in minimal media supplemented with only a single carbon source and exposing them to extreme conditions, in terms of

temperature fluctuations, in terms of freeze and thaw cycles, and radiation, as they occur during the space travel to the outer solar system. Our results in combination with future metagenome data and phenotype prediction tools will allow the identification of planetary protection relevant microorganisms in spacecraft assembly cleanrooms and on spacecraft and support the development of a target-oriented planetary protection constraints for missions to the icy moons.

This article is part of the theme issue 'Planetary Protection for sustainable space exploration'.

1. Introduction

Exposing extremophilic and non-extremophilic microorganisms to simulated space conditions has been extensively researched [1–4]. This knowledge plays a vital role in understanding the survival limits of a range of microbial species and will give insight into how and if such organisms could survive on other planetary bodies. Assessing the bioburden of a spacecraft prior to launch has been performed since the first robotic mission to Mars in the 1970s [5]. Such evaluations were the reasons for which the Viking missions (American space probes which landed on Mars in 1976) were one of the first to include heat sterilization to reduce the bioburden [6]. Spacecrafts are assembled in cleanrooms, sterilized through the use of heat, chemicals and/or radiation, to reduce the microbial counts to target limits [7,8]. In addition, cleanrooms are monitored frequently for bioburden to guarantee effective measures and to determine which microbial species could potentially contaminate spacecraft, thereby providing knowledge on contamination risk management and microbial survival [9– 11].

The Committee on Space Research (COSPAR), and here its panel on planetary protection is tasked with developing a planetary protection policy and providing implementation guidelines for compliance with the UN Outer Space Treaty of 1967 [12], to protect the Earth (backward contamination) and celestial bodies from harmful contamination (forward contamination). In regards to icy moon exploration, the panel has focused on psychrophilic/psychrotolerant microorganisms capable of growing between 20 and −20°C, and therefore posing a risk of microbial contamination for icy moon environments. Having identified microorganisms capable of growing until −20°C and to entrust tight guidelines for forward planetary protection of the icy moons, the planetary protection policy has adopted a −28°C temperature threshold for replication [13]. Therefore, the assembly and preparation of spacecraft which will travel to icy moons should adhere to the updated policy. This is even more relevant since one of the organisms investigated in this study was isolated in a spacecraft assembly facility (SAF) cleanroom. It is essential to follow planetary protection policies to avoid the unwanted contamination of sites of astrobiological interest.

In the context of this paper, we define icy moons as celestial bodies orbiting a planet of the outer solar system which have an icy surface with a subsurface liquid ocean. Currently, two missions are being conducted, including ESA's JUICE and NASA's Europa Clipper mission, which at the time of writing are both heading for the Jupiter system. These missions have been prepared based on knowledge gained by the Cassini–Huygens mission to the Saturn system which has helped to understand the geological characteristics of the icy moons [14,15]. These celestial bodies are of interest owing to their peculiar environmental characteristics, which could harbour life [16]. On Enceladus, a moon of Saturn, the environmental conditions, potentially suitable for life as we know it on Earth are determined by a subsurface ocean below a 30 to 40 km thick ice layer [17]. Furthermore, plume analysis from the Cassini mission has shown how organic material is being continuously ejected from the subsurface [14]. This cryovulcanism effect has also been shown to take place on Europa, one of Jupiter's moon, which harbours a subsurface ocean under a thicker ice layer of approximately 100 km [18].

Despite difficulties in accessing the subsurface ocean characteristics of the icy moons, the kilometre-thick ice layer would protect microbes against cosmic radiation. Researchers have shown how even a less thick ice layer would protect from ionizing and non-ionizing radiation on Enceladus [19,20]. Simulation studies to investigate microbial survival, have been suggested to be performed to close knowledge gaps ahead of future space missions including temperature, desiccation, UV-C and polychromatic UV radiation, low (Mars-relevant) pressure and synergistic effects of biocidal factors [13,21].

Psychrotolerants are not only of concern for planetary protection, but also for the food industry as they can grow at locations where food is being stored and cause potential harm after food ingestion [22,23]. At the same time, however, these microorganisms have also proven to be very useful to the food industry as the contain enzymes capable of catalysing reactions at low temperatures which are interesting for biotechnology and food processing applications [24,25]. Based on the aforementioned reasons, this study wanted to investigate at least one organism from each domain of life. The selection criteria of the chosen microorganisms were based on growth temperature (capable of growing at subzero temperatures), growth on solid media, visible growth within 48 h, and the capability to grow in minimal media supplemented with a single carbon source different from glucose. This is because carbon sources have been identified in carbonaceous chondrites which have arrived on Earth and which could have arrived on the icy moons as well [26]. Furthermore, icy moon subsurface ocean composition measurements have suggested a mineralogic composition containing carbon sources [27]. Literature research yielded a list of possible microbial candidates, table 1, including the bacteria *Planococcus halocryophilus* Or1 (DSM 24743) [28], *Chromohalobacter sarecensis* LV4 (DSM15547) [29], *Paenisporosarcina antarctica* N-05 (DSM21991) [32], *Psychromonas boydii* 174 (DSM17665) [33], *Cryobacterium flavum* Hh8 (DSM26475) [34] and *Virgibacillus arcticus* Hal1 (DSM19574) [35]. The archaea *Halorubrum luteum* CGSA15 (DSM23812) [36] and *Halorhabdus tiamatea* SARL4B (DSM18392) [37] also fulfilled these criteria, as well as the yeast species *Rhodotorula frigidalcoholis* JG1b [38] and *Rhodotorula mucilaginosa* Toulouse J8, A2-5 (DSM114276). The experimental methodologies and subsequent results are presented without the inclusion of the archaeal species and the bacterial species *V. arcticus*, *C. flavum* and *P. boydii*, owing to their inability to grow in our minimal media and owing to their low growth rates.

Given the promising nature of both the (subsurface-)environment of the icy moons to support life and of the microbial candidates isolated from analogue environments on Earth, we developed several research questions to be tackled. These include: Can Earth-based life survive on the icy moons? Can Earth-borne microorganisms survive a trip to the icy moons by hitch-hiking on a spacecraft? What are the risks to planetary protection? We believe the microbial candidates chosen for this research to be suited for answering these research questions. Furthermore, this research would help in gaining knowledge to develop strategies to tackle planetary protection concerns related to icy moon exploration.

2. Methods

(a) Growth in minimal media to determine optimal carbon source

The bacteria and yeast species were ordered from DSMZ (Leibniz-Institute DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), with the exception of *R. frigidalcoholis* which was kindly provided by Prof. Dr. Whyte from McGill University, Montreal, Canada. The organisms were first grown on their respective liquid and solid optimal media to determine viability, table 2. The organisms were then inoculated in M9-complete minimal salts media containing the following: 47.8 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.6 mM NaCl, 3.7 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.02 mM $Fe(III)Cl_3$, with 0.2% (w/v) of differing carbon sources. This was performed to evaluate the growth, or lack thereof, in M9 and to determine which carbon source would be chosen for the experiments.

Table 1. Characteristics of the microorganisms investigated in the study. The table includes information of the organism type, growth characteristic, isolation location, aerobic use, organic use of the candidate organisms initially screened, as well as relevant characteristics.

organism	domain of life	growth condition	isolation location	gram	motility	spore formation	aerobic or anaerobic growth	carbon source utilization	pigment	other characteristics
Planococcus halocryophilus Or1 (DSM 24743)	bacteria	psychrophilic	high Arctic permafrost	positive [28]	positive [28]	negative [28]	aerobe [28]	acetate, alanine, arginine, cellobiose, dextrin, fructose, galactose, gelatin, gluconate, glucosamine [28]	positive [28]	—
Chromohalobacter sarecensis LV4 (DSM 15547)	bacteria	mesophilic/ moderate halophile	Saline lake Laguna Verde, Bolivia	negative [29]	positive [29]	negative [29]	aerobe [29]	asparagine, histidine, L-alaninamide, lactose, ribose, trehalose, xylitol [30]	positive [29]	heterotroph [29]
Paenisporosarcina antarctica N-05 (DSM 21991)	bacteria	psychrophilic	Antarctic soil	positive [31]	negative [31]	positive [30]	facultative anaerobe [32]	glucose [31], alpha-ketobutyric acid [32]	positive [31]	—
Psychromonas boydii 174 (DSM 17665)	bacteria	psychrophilic	sea-ice core, Point Barrow, Alaska, USA	negative [33]	negative [33]	unknown	facultative anaerobe [33]	D-mannose, D-xylose [33], acetate, aspartate, cellobiose, citrate, fructose, glucose, glutamate, lactate [30]	positive [30]	gas vacuole [33]
Cryobacterium flavum Hh8 (DSM 26475)	bacteria	psychrophilic	permafrost in Tibet	positive [34]	negative [34]	negative [34]	aerobe [34]	fructose, ribose, cellobiose,	positive [34]	—

(*Continued.*)

Table 1. (*Continued.*)

organism	domain of life	growth condition	isolation location	gram	motility	spore formation	aerobic or anaerobic growth	carbon source utilization	pigment	other characteristics
								arabinose, mannose, glucose, galactose, glycerol, starch, sodium gluconate [34]		
Virgibacillus arcticus Hal1 (DSM 19574)	bacteria	psychrophilic	permafrost Canadian high arctic	positive [35]	positive [35]	elliptical endospores [35]	facultative anaerobe [35]	glucose, fructose, mannose, melibiose, trehalose, mannitol, casein, gelatin, lactose, xylitol [35]	positive [35]	—
Halorubrum luteum CGSA15 (DSM 23812)	Archaea	mesophilic/ halophilic	water of Lake Chagannor, China	negative [36]	positive [36]	unknown	aerobe [36]	L-aspartic acid, pyruvate, glycerol, DL-lactate, L-malate, fumarate, citrate, glycine, L-alanine, L-glutamate [36]	positive [36]	—
Halorhabdus tiamatea SARL4B (DSM 18392)	Archaea	mesophilic/ halophilic	Shaban deep, Northern Red Sea	negative [37]	negative [37]	negative [37]	anaerobe [37]	glucose, xylose, fructose, galactose, proline, esculin [37]	negative [37]	—
Rhodotorula frigidalcoholis JG1b	Eukarya	eurypsychrophilic	upper-elevation McMurdo Dry	not applicable	negative [38]	negative [38]	aerobe [39]	D-glucose, sucrose, raffinose, galactose, trehalose, maltose,	positive [38]	—

(*Continued.*)

Table 1. (*Continued.*)

organism	domain of life	growth condition	isolation location	gram	motility	spore formation	aerobic or anaerobic growth	carbon source utilization	pigment	other characteristics
			Valleys of Antarctica					melezitose, L-sorbose, L-rhamnose, D-xylose, DL-arabinose, D-ribose, glycerol, galactitol, D-mannitol, and xylitol [39]		
Rhodotorula mucilaginosa Toulouse J8, A2-5 (DSM114276)	Eukarya	mesophilic/ psychrotolerant	isolated from a SAF cleanroom	not applicable	negative [40]	negative [40]	aerobe [40]	unknown	positive [40]	resistance to a number of antifungals [40]

The carbon source choice was made based on the shortest time to reach late-exponential growth phase with a cell concentration of at least 10^7 cells ml^{-1}. The evaluated carbon sources were D-alanine ($C_3H_7NO_2$) purchased from Sigma-Aldrich, D-gluconic acid sodium salt ($C_6H_{11}NaO_7$) purchased from Fluorochem, D-glucose monohydrate ($C_6H_{12}O_6$) purchased from Merck and only used as control, L-glutamic acid ($C_5H_9NO_4$) purchased from Sigma-Aldrich, DL-glyceric acid 20% in water ($C_3H_6O_4$) purchased from Fluorochem and glycerol ($C_3H_8O_3$) purchased from Sigma-Aldrich. All experiments in this study were performed at least in biological triplicates. Relative survival (N/N_0) described in each figure was determined for each experiment as the number of colony-forming units (CFUs) of the microorganisms grown in media (N_0) divided by the CFUs of the microorganisms surviving the exposed conditions (N).

(b) Exposure of the organisms to desiccation

The bacteria and the yeast species were grown aerobically to late exponential phase in M9-complete with their respective carbon source (see table 2) and 200 µl aliquots of each grown culture were placed on sterile 1 cm glass discs. The discs were left to dry at room temperature for 24 h with a relative humidity of 40% ± 10% under sterile conditions in a clean bench. The discs which were exposed to desiccation under anoxic conditions for the same amount of time in an anoxic chamber (Coy Laboratory Products Inc., MI, USA, with O_2< 5 ppm, relative humidity 13 ± 0.5%) containing forming gas (5% H_2 in N_2). Following drying, the samples which were meant for cold-exposure were transferred to a sterile plastic box and stored in freezers at −20°C or −80°C. The samples exposed to cold temperatures and anoxic conditions following drying, were transferred in a Trex box Transport and Exposure Box as per Beblo-Vranesevic *et al*. [42], inside the anoxic chamber, which was then sealed and stored at −20°C or −80°C for the desiccation timepoints. Survival was determined by dilution plating for colony counts where the discs were re-suspended in a 5 ml Eppendorf tube containing 1 ml of sterile PBS (4 g NaCl, 3 g KH_2PO_4, 7 g NA_2HPO_4 in 1000 ml MQ) which was lightly vortexed for ca. 30 s before an aliquot was taken.

(c) Exposure to freezing

The bacterial species and yeast species were grown aerobically to late exponential phase in M9 with the respective carbon source. Aliquots of 1 ml were transferred to sterile 1 ml Eppendorf tubes. The tubes were placed in cryo-boxes and stored at −80°C for the selected timepoints. To determine the survival, one tube per replicate was thawed at 25°C for 1 h prior to dilution plating.

(d) Exposure to freezing and thawing

To determine the survival of the bacteria and the yeast species to freezing and thawing, 4 ml of late exponential phase cultures, grown in M9, was transferred to sterile 5 ml Eppendorf tubes. The tubes were placed inside a cryo-box and placed in a −80°C freezer for 1 h. The samples were then thawed for 1 h at 25°C and an aliquot of 100 µl was used for dilution plating. Following the thawing time, the 5 ml Eppendorf tubes containing the samples were placed again at −80°C for 1 h. The cycle was repeated until no survival was detected. No survival was then determined to be the lack of CFU identification for three cycles in a row.

(e) Exposure to UV radiation

To determine the limits of survival to UV radiation, the organisms grown in M9 were irradiated with UV-C (254 nm) radiation. An aliquot of 2700 µl from each organism grown to late

Table 2. Growth characteristics of the initial candidate organisms selected to study. Summary of data related to media type, growth time on solid and liquid media, growth temperature and growth in minimal media with preferred carbon source. *Not possible to determine NaCl concentration.

organism	growth media	total NaCl concentration in media	incubation time on solid media	incubation time in liquid media	minimal growth temperature (°C)	optimal growth temperature (°C)	maximal growth temperature (°C)	growth in minimal media (carbon source)
Planococcus halocryophilus Or1 (DSM 24743)	Tryptic Soy Broth	*	48 h	48 h	−10 [28]	25 [28]	37 [28]	yes (L-alanine)
Chromohalobacter sarecensis LV4 (DSM 15547)	Marine Broth (6% w/v NaCl)	6% (4.06% added)	24 h	3–4 days	0 [29]	30 [29]	45 [29]	50% MB-50% M9 (D-gluconic)
Paenisporosarcina antarctica N-05 (DSM 21991)	Marine Broth	1.94%	48 h	4–5 days	0 [32]	18 [32]	23 [32]	no
Psychromonas boydii 174 (DSM 17665)	Marine Broth	1.94%	10–15 days	4–5 days	0 [33]	6 [33]	10 [33]	no
Cryobacterium flavum Hh8 (DSM 26475)	Polypeptone media (DSMZ Media 513)	*	7–8 days	20 days	0 [34]	10 [34]	19 [34]	no
Virgibacillus arcticus Hal1 (DSM 19574)	Marine Broth	1.94%	48 h	no growth (60 days)	0 [35]	25 [35]	30 [35]	no
Halorubrum luteum CGSA15 (DSM 23812)	Natronobacteria media (DSMZ Media 371)	20%	3–4 days	20 days	17 [36]	35 [36]	41 [36]	no
Halorhabdus tiamatea SARL4B (DSM 18392)	Halobacteria media (DSMZ media 372)	20%	30 days	no growth (60 days)	15 [37]	45 [37]	55 [37]	no
Rhodotorula frigidalcoholis JG1b	universal media for yeasts	*	48 h	48 h	−10 [38]	25 [38]	30 [38]	yes (L-glutamic)

(*Continued*.)

Table 2. (*Continued.*)

organism	growth media	total NaCl concentration in media	incubation time on solid media	incubation time in liquid media	minimal growth temperature (°C)	optimal growth temperature (°C)	maximal growth temperature (°C)	growth in minimal media (carbon source)
	(DSMZ media 186)							
Rhodotorula mucilaginosa Toulouse J8, A2-5 (DSM114276)	universal media for yeasts (DSMZ media 186)	*	48 h	48 h	5	25 [41]	30	yes (L-glutamic)

exponential phase was placed in a closed sterile UV transmissible quartz cuvette (0.5 cm path-length; Hellma GmbH & Co. KG, Muelheim, Germany). The cuvettes with the samples were placed 32.5 cm below the UV lamp and contained a magnetic stirrer which allowed the culture to be mixed during irradiation for all the cells to receive the same homogenous amount of UV fluence. The *C. sarecensis* cultures which were not grown in 100% M9 were washed twice in PBS to remove media traces which could potentially absorb UV and could therefore lead to artefactual protection. The washing was performed by placing the grown cultures in 50 ml Falcon tubes, centrifuging the tubes at 2500 × g for 10 min, removing the supernatant and filling the tube with the same volume of PBS. The UV-C lamp was a low-pressure Hg lamp (Model NN 8/15; Heraeus, Berlin, Germany). Prior to each irradiation experiment, the lamp was allowed to warm up for at least 30 min and the fluence was measured. The fluence was determined by the use of a UVX radiometer with a 254 nm sensor (UVP Ultra-Violet Products, Cambridge, United Kingdom). The exposure time was calculated by dividing the target dose (in J m^{-2}) by the fluence rates reported on the radiometer (in μW cm^{-2}).

The exposure to polychromatic (200–400 nm) Mars-like UV radiation was performed in the same fashion. Irradiation was performed by using the UV 500S irradiation source (Dr. Hoenle AG; UV-Technologie, Germany). The fluence rate of the lamp was determined prior to each experiment, after 30 min to allow the lamp to warm up, by using the Bentham DMc150 transportable spectroradiometer (Bentham Instruments Ltd., Reading, United Kingdom) as per Rabbow *et al.* [1].

3. Exposure to X-ray radiation

The different bacteria and yeast species were exposed to ionizing radiation by growing the cultures to late exponential phase and placing the samples in the Gulmay RS 225A (Gulmay Medical Ltd.) X-ray source. The output of the X-ray source was set to 200 kV and 15 mA. The samples were placed at a 10 cm distance below the x-ray source and were irradiated with 30 Gy min^{-1} ± 5 Gy min^{-1} until the target dose was reached. The irradiation dose in Gy min^{-1} was determined with a UNIDOS dosimeter (PTW Freiburg, Germany).

All exposure experiments, with the exception of specific desiccation storage conditions, were performed at room temperature.

The $D_{10}/F_{10}/E_{10}$ (dose, fluence, exposure) values, representing 10% survival, were calculated by plotting the relative survival in logarithmic scale (log natural) in SigmaPlot 13.0 with the following equation:

$$D_{10} = \frac{\ln(0.1) - \ln(\exp(b[0]))}{b[1]},$$

where b[0] and b[1] are the coefficients of the linear regression curve plotted by SigmaPlot version 14.5.

All figures were created with the use of the data visualization library Seaborn version 0.12.1 [43], an interface of Matplotlib [44]. When stated in the figure description, the polynomial regression lines were plotted with the following regression function:

```
def polynomial_regression(x, y, degree):
coeffs = np.polyfit(x, np.log(y), degree)
coeffs[-1] = 0
return np.exp(np.polyval(coeffs, x)
degree = x
```

4. Results

Having selected the organisms for the experiments and having confirmed their growth in minimal media supplemented with a single carbon source, the organisms were exposed to the described extreme conditions. Owing to the extreme length in desiccation survival of the organisms to conditions such as desiccation, values reported in the results reflect the latest data at the time of publication. Despite reporting a defined value we believe specific species investigated could be able to tolerate a further extended extreme condition exposure. Details on species and experiment termination have been reported in the figure captions when relevant.

The summary of the growth media and conditions tested are listed in table 2. Microbial species including *P. boydii*, *C. flavum*, *V. arcticus*, *H. luteum* and *H. tiamatea* were excluded from further investigations as they did not satisfy all growth criteria for this study. The summary of the maximum limit for survival of the species, selected for investigation, after exposure to the different conditions is listed in table 3 and the summary of the maximum survival for the species exposed to desiccation is listed in table 4. These values refer to the highest exposure dose or exposure time for which viable colony counts were detected. Higher exposure times and doses were performed to confirm no viable cells.

When the bacteria and yeast species were exposed to desiccating conditions, all differences in limits of survival became apparent. The survival limits changed for each species depending on the availability of oxygen and on the temperature during the desiccation exposure. For example, *R. mucilaginosa* which has a survival limit of 100 days when desiccated at 20°C, but an increased survival of greater than 246 days at −20°C and greater than 156 days at −80°C, figure 1. Increasing survival with decreasing temperatures can also be seen with *C. sarecensis* which was able to tolerate 13 days of desiccation at 20°C, 19 days at −20°C and 22 days at −80°C. By contrast, both *P. halocryophilus* and *R. frigidalcoholis* showed the same survival limit across the three temperature ranges. Nonetheless, there seems to be a general trend showing increased desiccation survival at lower temperatures. Despite similar survival limits, when *P. halocryophilus* and *R. frigidalcoholis* were exposed to desiccation under anoxic conditions, survival decreased faster when the samples were exposed to desiccation at temperatures above 0°C. Therefore, also under anoxic conditions, a decrease in exposure temperature increases the survival trends of both prokaryotic and eukaryotic microorganisms.

Growing the microorganisms in minimal media and exposing them to freezing conditions at −80°C was conducted to evaluate the survival limits of the microorganisms when grown under nutrient-limiting conditions and in environments with temperatures closer to the ones on the surfaces of Enceladus and Europa. For this reason, after the bacterial species were grown in M9-complete, they were frozen at −80°C and stored until pre-defined timepoints to evaluate survival through time. The results summarized in figure 2*A* highlight how the microorganisms which were the most tolerant to this condition are the two yeast species, *R. mucilaginosa* surviving up to 230 days and *R. frigidalcoholis* up to 728 days. We speculate that since cell reductions for these time points are low, both yeast species would be capable of surviving more extended periods of time. By contrast, the bacterial species *C. sarecensis* and *P. halocryophilus* were only able to tolerate 6 and 30 days of desiccation (under all tested conditions), respectively.

Understanding the survival of the organisms when exposed to freezing and thawing without additional cryoprotectants might not only give an indication to their survival through seasonal changes, but also to spacecraft rotation and position in the solar system and thereby being exposed to the extreme low temperatures of space and the heat of the sun. Our results show how also for this condition, the most tolerant organisms are the yeast species compared with the two bacterial species, figure 2B. With *C. sarecensis* surviving only two cycles of freeze and thaw and *P. halocryophilus* 25 cycles. In comparison *R. frigidalcoholis* was capable of surviving 38 cycles and *R. mucilaginosa* more than 300. Furthermore, based on the survival curve of *R. mucilaginosa* we expect it to survive many additional cycles.

Table 3. Survival characteristics of the organisms selected for study. Summary of maximal survival fluences, doses and exposure times of the selected bacteria and yeast species. The table highlights the maximum extent to which survival was detected when the organisms were exposed to desiccation, UV-C and polychromatic UV radiation, X-ray radiation and freezing and thawing cycles. *Survival values for *P. antarctica* were determined after growth in Marine Broth. #In brackets E_{10} value, exposure time in days to which there is 90% of sample lethality.

organism	extent of desiccation survival (days)	UV-C F_{10} fluence (J m^{-2})	maximum UV-C survival fluence (J m^{-2})	polychromatic UV F_{10} fluence (J m^{-2})	maximum polychromatic UV fluence (J m^{-2})	X-ray D_{10} dose (Gy)	maximum X-ray dose (Gy)	maximum number of freeze and thaw cycles	maximum amount of days to freezing
Planococcus halocryophilus Or1	60	30	150	297	1250	158	1000	20	70 (18#)
Chromohalobacter sarecensis LV4	13	487	2000	5189	12 500	249	1000	2	6 (1.3#)
Paenisporosarcina antarctica N-05	nd	nd	nd	nd	2000*	nd	nd	2*	6*
Rhodotorula frigidalcoholis JG1b	123	382	1450	5757	17 000	770	3000	38	>728 (6769#)
Rhodotorula mucilaginosa ESA	>70	429	1500	10 478	20 000	838	2500	>350	>250 (234#)
								ongoing	ongoing

Table 4. Survival characteristics of the organisms selected for study to desiccation at different temperatures with oxic and anoxic conditions. Rows show maximum exposure time, in days, to which each organism was capable of tolerating desiccating conditions.

organism	extent of desiccation survival at room temperature (days)	extent of desiccation survival at −20°C (days)	extent of desiccation survival at −80°C (days)	extent of anoxic desiccation survival at room temperature (days)	extent of anoxic desiccation survival at −20°C (days)	extent of anoxic desiccation survival at −80°C (days)
Planococcus halocryophilus Or1	61	61	49	61	61	61
Chromohalobacter sarecensis LV4	13	19	22	nd	nd	nd
Rhodotorula frigidalcoholis JG1b	123	123	123	123	123	196
Rhodotorula mucilaginosa ESA	101	246	156	nd	nd	nd

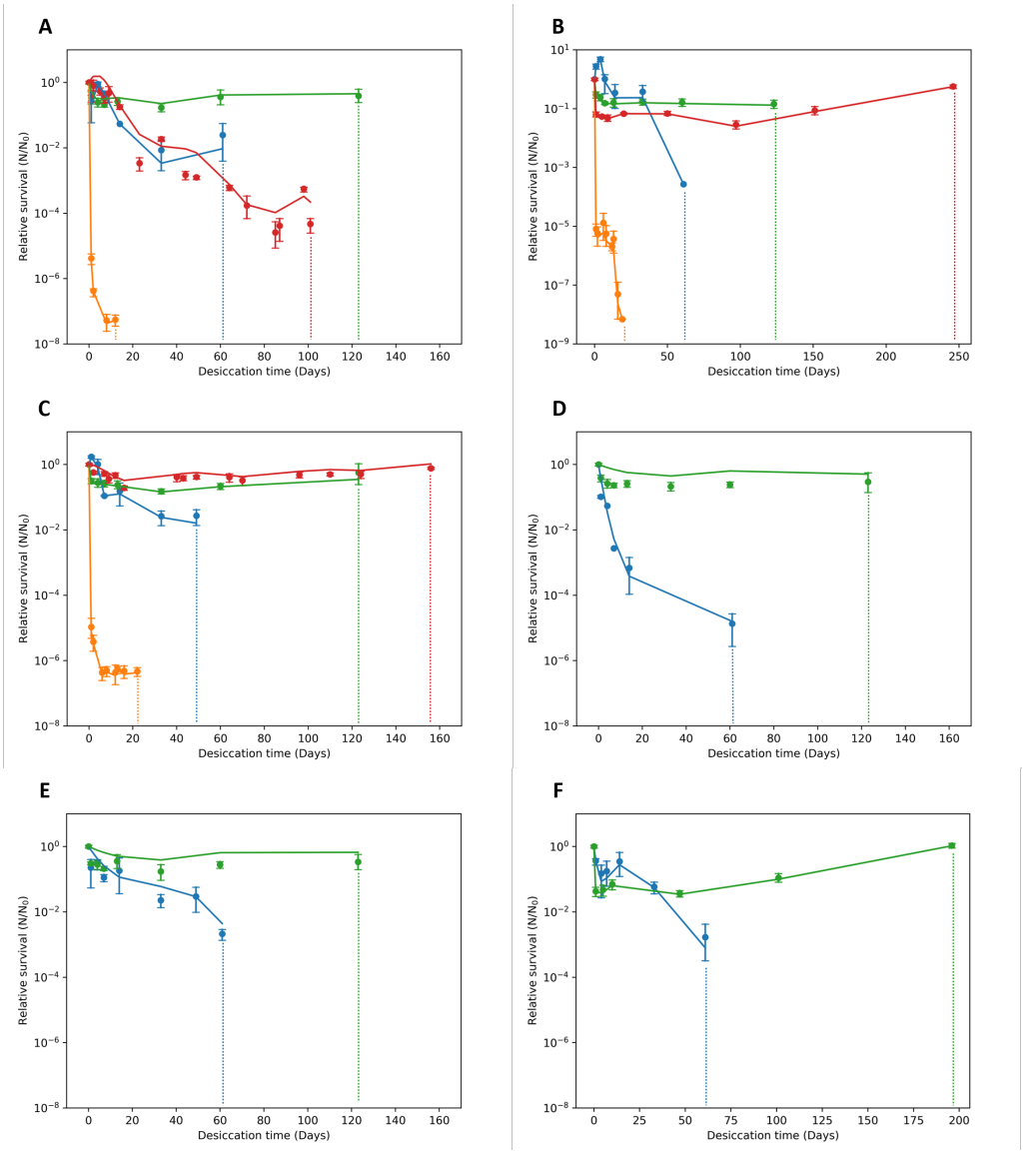

Figure 1. Survival to desiccation increases with lower temperatures. Desiccation survival of *P. halocryophilus* (blue), *C. sarecensis* (orange), *R. frigidalcoholis* (green) and *R. mucilaginosa* (red) under oxic and anoxic conditions and at different temperatures including: oxic at 20°C (A), oxic at −20°C (B), oxic at −80°C (C), anoxic at 20°C (D), anoxic at −20°C (E) and anoxic at −80°C (F). The coloured solid curves represent the polynomial regression for the survival of each plotted organism. Vertical dotted lines represent the end of an experiment owing different factors: sample limitations *P. halocryophilus* (A and C) *R. frigidalcoholis* (A), no CFUs *C. sarecensis* (all subfigures), *R. mucilaginosa* (A), *P. halocryophilus* (B, D, E and F), and time limitations *R. mucilaginosa* (B and C), *R. frigidalcoholis* (all subfigures). *C. sarecensis* survival was not detected under any anoxic desiccation condition. *R. mucilaginosa* survival under anoxic desiccation was not performed owing to limited time.

An important (and somewhat surprising) observation is that the eukaryotic microorganisms were generally more tolerant to freezing and thawing than the prokaryotes. However, both for UV-C and polychromatic UV radiation, *C. sarecensis* was equally tolerant as both yeast species, figure 3. When exposed to UV-C radiation, *P. halocryophilus* was able to tolerate up to 150 J m^{-2} in media, *C. sarecensis* up to 2000 J m^{-2} and *R. frigidalcoholis* up to 1450 J m^{-2} and *R. mucilaginosa* up to 1500 J m^{-2}. A similar trend can be seen with polychromatic UV light as *P. halocryophilus* was able to tolerate up to 1250 J m^{-2}, *C. sarecensis* up to 12 500 J m^{-2}, *R. frigidalcoholis* up to 17 000

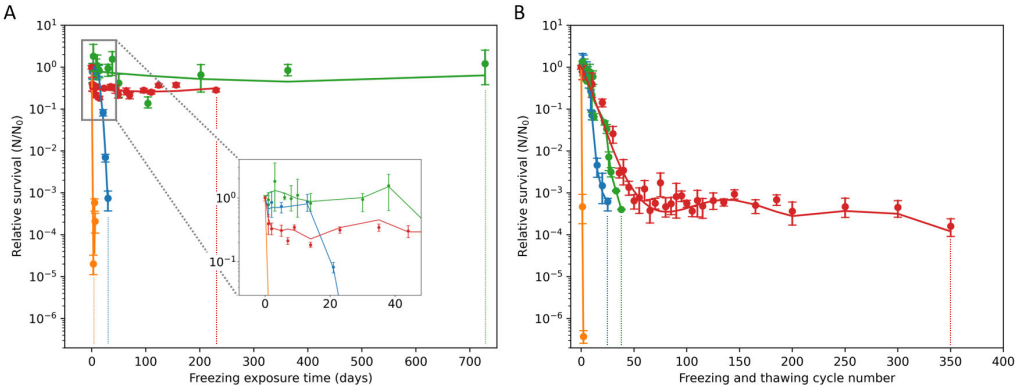

Figure 2. Yeasts are tolerant to freezing and thawing. Survival of *P. halocryophilus* (blue), *C. sarecensis* (orange), *R. frigidalcoholis* (green) and *R. mucilaginous* (red) to freezing at −80°C (A) and to freezing and thawing (B). The coloured solid curves represent the polynomial regression lines of the survival of each plotted organism. Determining the survival of *R. mucilaginosa* exposed to freezing conditions (A) as well as *R. frigidalcoholis* to freeze and thaw (B) is an ongoing experiment. Vertical dotted lines represent the end of an experiment. In this figure for *C. sarecensis*, *P. halocryophilus* and *R. frigidalcoholis* (B) survival was not detected further. For *R. mucilaginosa* and *R. frigidalcoholis* (A), the dotted lines represent the latest survival data at the time of submission.

J m^{-2} and *R. mucilaginosa* up to 20 000 J m^{-2}. The F_{10}/D_{10} values which is the dose to which there is 90% lethality, shows a similar relationship, see table 3.

When exposed to ionizing radiation, however, a more detrimental effect was seen on the survival of *C. sarecensis*, as this species was possibly not able to tolerate the penetrating effects as the eukaryotic microorganisms, figure 4. *C. sarecensis* was able to tolerate up to 1250 Gy, *P. halocryophilus* up to 1000 Gy while *R. frigidalcoholis* was able to tolerate up to 3000 Gy and *R. mucilaginosa* up to 2500 Gy.

(a) Assumed radiation doses at Europa and Enceladus

Despite the fact that no lander with radiation measurements to date has arrived on the surface of Europa or Enceladus, measurements from orbit and from telescopes can provide useful information concerning the radiation doses. For example the ionizing radiation energy reaching Europa from Jupiter is in between 10 kiloelectron volt (KeV) and 100 megaelectron volt (MeV), instead on Enceladus galactic cosmic ray energies are greater than 3 gigaelectron volt (GeV) [20]. Paulino-Lima *et al.* [45] showed that the Earth solar constant for total UV (200–400 nm) is 116.16 W m^{-2}. By using the following equation, we can approximate the solar constant for Europa and Enceladus.

$$UV\ solar\ constant\ for\ planet\ X\ = Earth\ Solar\ constant\ \times \left(\frac{1}{d^2}\right),$$

where d is the distance in AU of the target from the Sun. With Jupiter being 5.2 AU and Saturn being 9.5 AU from the Sun. If we assume that the two moons are the same distance from the Sun as their respective home planets, we can determine that the UV solar constant for Europa is circa 4.3 W m^{-2} and circa 1.29 W m^{-2} for Enceladus. With these values and our survival data we can estimate how long the organisms would be capable of surviving on the surface of Enceladus or Europa when only exposed to polychromatic UV radiation in the 200–400 nm range, table 5.

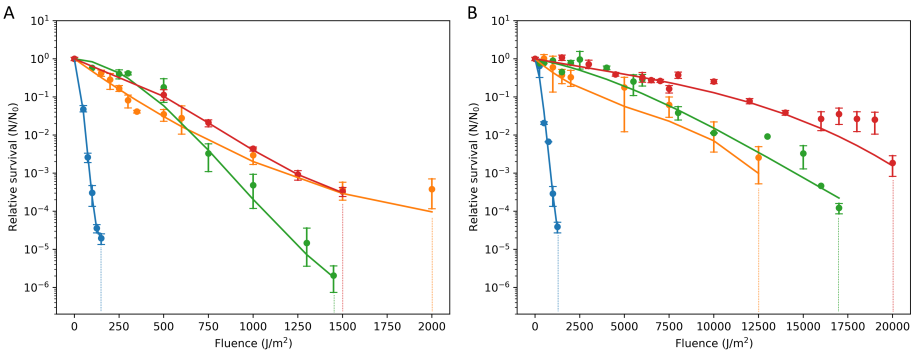

Figure 3. UV wavelength favours different organisms. Survival of *P. halocryophilus* (blue), *C. sarecensis* (orange), *R. frigidalcoholis* (green) and *R. mucilaginous* (red) to UV-C (254 nm) (A) and polychromatic UV (200–400 nm) (B) radiation. All the organisms were exposed to a fluence ranging from 50 to 2000 J m^{-2} for UV-C and from 100 to 20 000 J m^{-2} for polychromatic UV. The coloured solid curves represent the polynomial regression for the survival of each plotted organism. Vertical dotted lines represent the end of an experiment where no further survival was detected.

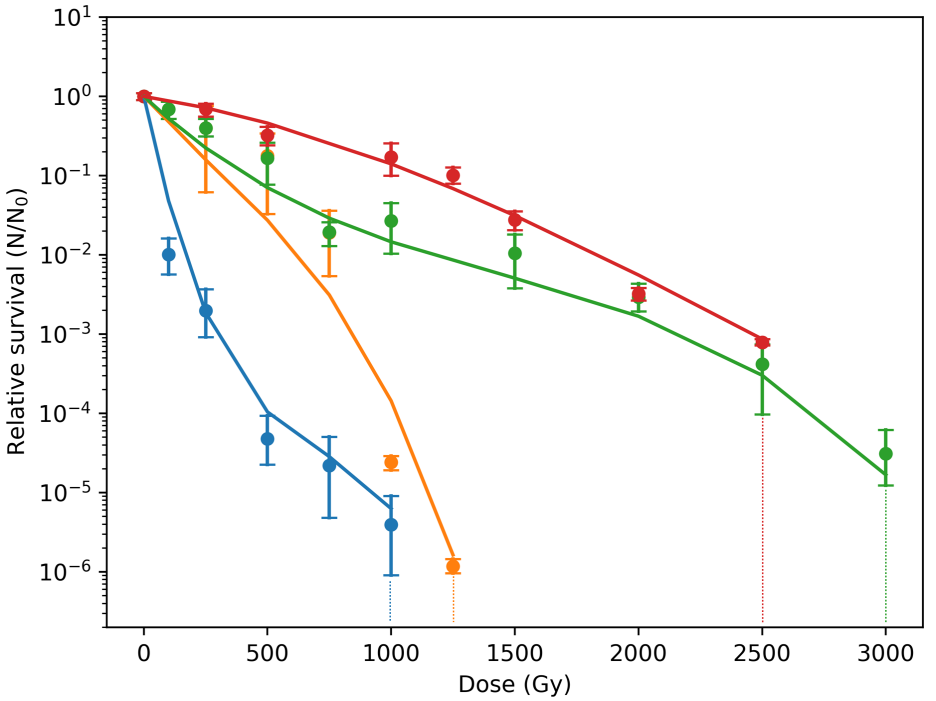

Figure 4. Eukaryotic microorganisms tolerate ionizing radiation more than the prokaryotes. Survival of *P. halocryophilus* (blue), *C. sarecensis* (orange), *R. frigidalcoholis* (green) and *R. mucilaginous* (red) to ionizing radiation from 50 to 3000 Gy. The coloured solid curves represent the polynomial regression for the survival of each plotted organism. Vertical dotted lines represent the end of an experiment where no further survival was detected.

5. Discussion

Evaluating the survival of the microbial species exposed to simulated space conditions is important to evaluate the risk of contamination of sites of interest in the solar system. Our results unexpectedly highlight how the investigated psychrotolerant eukaryotic microorganisms are more tolerant to space-like conditions than psychrotolerant prokaryotes. Nonetheless,

Table 5. Estimated polychromatic UV (200–400 nm) radiation survival timelines for the organisms investigated in this study on the surface of Europa and Enceladus.

species	maximum polychromatic UV survival dose (J m^{-2})	survival time on surface of Europa assuming solar constant of 4.3 W m^{-2}	survival time on surface of Enceladus assuming solar constant of 1.29 W m^{-2}
R. mucilaginosa	20 000	1.29 h	4.3 h
R. frigidalcoholis	17 000	1.09 h	3.66 h
C. sarecensis	12 500	48 min	2.69 h
P. halocryophilus	1250	4.8 min	16 min

yeasts have been already highlighted for their unique tolerances to extreme conditions especially related to the extreme environments in which they have been isolated from [46] and in relation to Mars and icy moons [47]. Despite extensive research having focused on yeasts as model organism for investigating the survival to space-like conditions, limited research is linked to yeasts of the *Rhodotorula* species [48–50]. Furthermore, we believe our results to be useful to identify species of interest when assessing the bioburden of cleanrooms and SAFs and for targeted sterilization techniques. Especially since the strain of *R. mucilaginosa* investigated in this study was isolated in a SAF (table 2) and other *Rhodotorula* species have been identified in the International Space Station (ISS) which is of concern for planetary protection [51,52]. Studies such as this provide a framework for assessing microbial survival under extreme conditions and informing stakeholders to maintain, expand and update planetary protection measures during missions to sites of astrobiology interest including the surface and subsurface of icy moons.

(a) Growth in minimal media

To evaluate the growth on icy moon-like conditions, we used minimal media composition that was previously developed in our group to evaluate the growth of the organisms with a single carbon source [53]. Despite the exact composition of the icy moon subsurface ocean being unknown, the growth in minimal media would support the results produced by having the microorganisms grown in oligotrophic conditions. Therefore, one of the selection criteria for the organism to be evaluated in this study was the capability to grow in minimal media with a single carbon source. In addition, we aimed to study microorganisms that can reach the late exponential growth phase within 96 h to ensure feasibility in the frame of this project. Therefore, from the list highlighted in table 2 the organisms selected were four including *P. halocryophilus*, *C. sarecensis*, *R. frigidalcoholis* and *R. mucilaginosa*, which were capable of growing in our desired conditions. Despite *R. mucilaginosa* not being a psychrophile and not isolated from an analogues site, it was chosen to be investigated owing to the fact that it was isolated in the cleanroom of a SAF and it is an organism which can be part of the human microbiome, making it of importance for future long-term crewed missions [40]. Earlier research has investigated microbial communities with media simulating the subsurface ocean of Europa and pressure to evaluate growth [54]. Importantly, community growth and growth of single microorganisms can be different, whereby communities of different bacterial species can support each other whilst single organism growth do not do this [55].

(b) Desiccation

Evaluating the survival to desiccation in this study was not only important to understand the potential survival of the organisms in SAFs and spacecraft surfaces, but also to environments

such as the icy moons. Regular isolation campaigns in cleanrooms and SAFs identify microorganisms on surfaces and in the air [11,56]. In addition, isolation studies are also performed in spacecraft interiors such as inside the ISS [57,58]. Therefore, despite strict sterilization techniques, microorganisms make their way to space. Undoubtedly, evaluating the survival of more microbial species to desiccation is needed to guide in updating sterilization techniques, risks for planetary protection and public health (hospitals and public transport). This is especially important when considering that the strain of the yeast *R. mucilaginosa*, used in this study and known to cause disease in immunocompromised patients, was isolated in the cleanroom of a SAF where conditions might be suitable for selection to desiccation [59,60].

Our results highlighted how yeast species are more tolerant than bacterial species to desiccation, and how the decrease in temperature can aid survival for the organisms tested in this study. Despite challenges in maintaining cell homeostasis at lower temperatures, psychrophilic microorganisms are well adapted to these conditions. Characteristics to which they have adapted is maintaining an adequate membrane fluidity to perform the needed metabolic functions at low temperatures [61]. It has been suggested that psychrophiles can increase the concentration of unsaturated fatty acids of to the phospholipids maintain membrane fluidity at low temperatures [62]. Perhaps this suggests the increase in desiccation survival of *R. mucilaginosa* at oxic conditions at −20°C and *R. frigidalcoholis* at anoxic conditions at −80°C. However, research with mesophilic organisms such as *E. coli* have shown that there is an increase in saturated fatty acids during desiccation to maintain membrane viscosity at low water activity [63,64]. This suggests that the traits highlighted pertain to mesophiles and psychrophiles only, especially since *C. sarecensis* had poor desiccation tolerance regardless of the temperature. Perhaps traits associated to desiccation survival are linked to environment, and *C. sarecensis* being isolated from the soil where it has not evolutionarily been selected to withstand these conditions [29].

(c) Freezing and thawing

Survival to freezing and thawing conditions is another characteristic important for survival of the organisms on spacecraft surfaces exposed to space conditions. The results we have presented are very meaningful from a planetary protection point of view, as the only organism (*R. mucilaginosa*) which is also associated with the human microbiome is the one capable of surviving the most the freezing and thawing conditions. During the journey to the Jupiter system, targeting the icy moons, ESA's JUICE (Jupiter Icy Moons Explorer) spacecraft will be exposed to temperatures ranging from 250°C when performing the Venus flyby, to −230°C when farthest from the Sun [65]. Nonetheless, we acknowledge the limitation of this results in not having tested the sublimation/re-sublimation process which would take place during freezing and thawing conditions under the vacuum of space. In addition, the survival of the bacterial species *P. halocryophilus* to freeze and thaw has been evaluated, which also provides interesting results and conclusions in relation to Mars habitability [66]. Despite the study by [66] evaluating the survival to freeze and thaw at a comparatively reduced ΔT (°C) and with an increased salt concentration, the results provide a valuable comparison of 70 cycles of freeze and thaw as well as can highlight how microorganism survival can differ under different experimental approaches. Understanding if Earth-borne microorganism can tolerate these conditions is critical in evaluating the risk for forward contamination of the icy moons. Our results have shown how all organisms tested in this study are capable of tolerating at least two cycles of freezing and thawing.

In standard laboratory practice, storage of microorganisms is performed at very low temperatures such as −80°C with glycerol or other compounds used as cryoprotectants to prevent intracellular ice crystal formation and damage to the cell [67]. Cryoprotectants are not available in the natural environment where the investigated organisms were isolated from. Nevertheless, the organisms of this study have the ability to produce cryoprotectants

or make use of compatible solutes to avoid damage from freezing [68–70]. What must be noted, however, is that despite having these abilities, it is not known whether the microorganisms have the time to produce sufficient cryoprotectants during freezing exposure in our experimental set-up, especially since the time taken for the sample tubes to reach 0°C was approximately only 10 min per cycle (electronic supplementary material, fig. S1). The high-latitude freezing and thawing cycles that take place during seasonal shifts in the natural environment where the organisms where isolated from, differ greatly from our experiment conditions [71]. Undoubtedly, freezing the microorganism at −80°C and thawing at 25°C is not what they are exposed to in the natural environment. Nonetheless, given the psychrophilic/psychrotolerant nature of these organisms, it was expected that they would be better adapted compared with other mesophilic microorganisms.

Exposure to freezing conditions at low temperatures without additional cryoprotectants can be a challenge. As shown in our results, we expected *C. sarecensis* and *P. halocryophilus* to tolerate years of storage at −80°C in minimal media such as the yeast species instead of only 6 and 70 days of freezing, respectively. In addition, as research has shown, *E. coli* (a mesophilic organism) was capable of surviving six cycles of freezing and thawing, more than the investigated *C. sarecensis* and *P. antarctica* [72]. However, in this experiment *E. coli* was exposed to freezing at −18°C and thawing at 4°C, milder conditions compared with our simulation studies. Nonetheless, this provides a comparison of the survival characteristics of psychrophilic/psychrotolerant with mesophilic microorganisms. In addition, our results highlight the extreme conditions to which the selected yeast species are capable of withstanding compared with the selected bacterial species. At the same time, the results highlight the importance of explorative studies to identify such extreme microbial characteristics.

(d) UV radiation and X-ray

Ionizing and non-ionizing radiation are two other extreme aspects of space travel and icy moon environment. Evaluating the tolerances of the microorganisms to these environmental conditions is critical to highlight the risks for planetary protection and survival limits. Our results suggest that if the microorganisms evaluated in this study would survive the hitchhike on spacecraft such as JUICE, they would be able to tolerate the polychromatic UV conditions from as little as 5 min to 4.3 h. Despite other extreme environmental conditions coming into play on the surface of the icy moons, the risk of the organism contaminating the surface is not negligible. Furthermore, the presence of fissures or cold-geysers [73,74] on the surface of the icy moons could increase the risk of subsurface ocean contamination; however, it must be emphasized that the extent and time scale of material exchange between the icy moon's surface and subsurface are unknown and strongly dependent on the specific location on the moon in both [21].

Even though the microorganism were isolated from environments with high UV flux [28,29,38], they did not all tolerate the same radiation-exposure conditions.

Despite demonstrating sensitivity in the freeze and thaw experiments, *C. sarecensis* was able to tolerate similar UV fluence as the yeast species, when exposed to both UV-C and polychromatic UV radiation. Literature research yielded no information on the presence of pigments in *C. sarecensis*. Instead the genus of *Rhodotorula* was found to be able to produce pigments including torularhodin, torulene, β-carotene and γ-carotene, precursors of carotenoids [75]. In particular *R. mucilaginosa* through the use of torularhodin can, to an extent, prevent damage induced by UV radiation by scavenging oxygen and peroxyl radicals [76]. The importance of carotenoid pigments has also been found in other yeast species. Previous research has shown that when comparing the survival of albino and wild-type *Sporobolomyces ruberrimus* and *Cystofilobasidium capitatum* yeast against UV-B (280–315 nm) exposure, the albino strains were more sensitive to the radiation [77]. Furthermore, research has shown that *R. frigidalcoholis* is capable of overexpressing the genes related to β-carotene precursor when at 0°C. The authors

suggest this is the case owing to the ability of the pigments to modulate the cellular membrane fluidity and to protect both from solar irradiation and γ radiation, both sources being present in the isolation environment [39]. This ability is also shared by bacterial species such as *P. halocryophilus*, investigated in this study [78].

6. Conclusion

The microorganisms evaluated in this study have been shown to tolerate a range of extreme environmental conditions, primarily selected to replicate the conditions on the icy moons. However, these extreme conditions can also be found in other locations of the solar system. Understanding the survival limits of such organisms will help to identify species of interest for exploring mechanisms of survival on non-terrestrial bodies, as well as to determine risks for planetary protection. Undoubtedly, experiments such as these require a long time to be conducted: exemplified by the 700 day sampling of *R. frigidalcoholis* to freezing, or the 196 day sampling for exposure to desiccation. Interestingly, the yeast species were found to be much more tolerant to different conditions replicating icy moons than the bacterial species. These results add valuable information to the planetary protection community as they will help with improving the guidelines for sterilization and identification/characterization of microorganisms. Combining the results of this study with future metagenome data from SAFs could aid in the development of tools allowing the identification of microorganisms relevant for forward planetary protection during spacecraft assembly. Nonetheless, we acknowledge limitations in the experimental approach including: a reduced number of microbial species, exclusion of archaea from further investigations, relatively short growth periods, choice of organisms capable of colony formation, effects of vacuum, lower temperatures during exposure to extreme conditions as well as a combination of these, which despite being desirable were not feasible in the frame of this project. However, we believe these results show the extent to which Earth-based life can survive in extreme environments such as the ones of the icy moons. Especially in the age of missions such as ESA's JUICE and NASA's Europa Clipper, the maintenance of planetary protection guidelines and approaches, as well as identifying risks such as the ones in this study, is imperative to avoid the contamination of icy moon environments.

Data accessibility. Raw data used to generate figures are available in open access format on the Dryad database [79]. Supplementary material is available online [80].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. T.Z.: formal analysis, investigation, methodology, writing—original draft; K.B.-V.: methodology, resources, supervision, writing—review and editing; M.IdJ.: supervision, writing—review and editing; M.G.N.: supervision, writing—review and editing; P.R.: methodology, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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References

1. Rabbow E *et al*. 2012 EXPOSE-E: an ESA astrobiology mission 1.5 years in space. *Astrobiology* **12**, 374–386. (doi:10.1089/ast.2011.0760)

2. Beblo-Vranesevic K, Galinski EA, Rachel R, Huber H, Rettberg P. 2017 Influence of osmotic stress on desiccation and irradiation tolerance of (hyper)-thermophilic microorganisms. *Arch. Microbiol.* **199**, 17–28. (doi:10.1007/s00203-016-1269-6)
3. Beblo-Vranesevic K *et al*. 2020 Impact of simulated martian conditions on (Facultatively) anaerobic bacterial strains from different mars analogue Sites. *Curr. Issues Mol. Biol.* **38**, 103–122. (doi:10.21775/cimb.038.103)
4. Beblo-Vranesevic K, Piepjohn J, Antunes A, Rettberg P. 2022 Surviving mars: new insights into the persistence of facultative anaerobic microbes from analogue sites. *Int. J. Astrobiol.* **21**, 110–127. (doi:10.1017/s1473550422000064)
5. Puleo JR, Fields ND, Bergstrom SL, Oxborrow GS, Stabekis PD, Koukol RC. 1977 Microbiological profiles of the viking spacecraft. *Appl. Environ. Microbiol.* **33**, 379–384. (doi:10.1128/aem.33.2.379-384.1977)
6. Schuerger AC, Mancinelli RL, Kern RG, Rothschild LJ, McKay CP. 2003 Survival of endospores of *Bacillus subtilis* on spacecraft surfaces under simulated martian environments: implications for the forward contamination of Mars. *Icarus* **165**, 253–276. (doi:10.1016/s0019-1035(03)00200-8)
7. La Duc MT, Kern R, Venkateswaran K. 2004 Microbial monitoring of spacecraft and associated environments. *Microb. Ecol.* **47**, 150–158. (doi:10.1007/s00248-003-1012-0)
8. Velbel MA *et al*. 2022 Planning implications related to sterilization-sensitive science investigations associated with mars sample return (MSR). *Astrobiology* **22**, S112–S164. (doi:10.1089/AST.2021.0113)
9. Hendrickson R, Urbaniak C, Minich JJ, Aronson HS, Martino C, Stepanauskas R, Knight R, Venkateswaran K. 2021 Clean room microbiome complexity impacts planetary protection bioburden. *Microbiome* **9**, 238. (doi:10.1186/s40168-021-01159-x)
10. Koskinen K *et al*. 2017 Microbial biodiversity assessment of the european space agency's exomars 2016 mission. *Microbiome* **5**, 143. (doi:10.1186/s40168-017-0358-3)
11. Malli Mohan GB, Stricker MC, Venkateswaran K. 2019 Microscopic characterization of biological and inert particles associated with spacecraft assembly cleanroom. *Sci. Rep.* **9**, 14251. (doi:10.1038/s41598-019-50782-0)
12. COSPAR. 2024 Panel on planetary protection (PPP). See https://cosparhq.cnes.fr/scientific-structure/panels/panel-on-planetary-protection-ppp/.
13. Spry JA *et al*. 2024 Planetary protection knowledge gap closure enabling crewed missions to mars. *Astrobiology* **24**, 230–274. (doi:10.1089/ast.2023.0092)
14. Cable ML *et al*. 2021 The science case for a return to enceladus. *Planet. Sci. J.* **2**, 132. (doi:10.3847/psj/abfb7a)
15. Spilker LJ. 2019 Cassini-Huygens' exploration of the Saturn system: 13 years of discovery. *Science* **364**, 1046–1051. (doi:10.1126/science.aat3760)
16. Altair T, de Avellar MGB, Rodrigues F, Galante D. 2018 Microbial habitability of europa sustained by radioactive sources. *Sci. Rep.* **8**, 260. (doi:10.1038/s41598-017-18470-z)
17. Iess L *et al*. 2014 The gravity field and interior structure of enceladus. *Science* **344**, 78–80. (doi:10.1126/science.1250551)
18. Melosh HJ, Ekholm AG, Showman AP, Lorenz RD. 2004 The temperature of europa's subsurface water ocean. *Icarus* **168**, 498–502. (doi:10.1016/j.icarus.2003.11.026)
19. Hendrix AR, House CH. 2023 Low effective ultraviolet exposure ages for organics at the surface of enceladus. *Commun. Earth Environ.* **4**, 485. (doi:10.1038/s43247-023-01130-8)
20. Pavlov AA, McLain H, Glavin DP, Elsila JE, Dworkin J, House CH, Zhang Z. 2024 Radiolytic effects on biological and abiotic amino acids in shallow subsurface ices on Europa and Enceladus. *Astrobiology* **24**, 698–709. (doi:10.1089/ast.2023.0120)
21. Rettberg P *et al*. 2019 Biological contamination prevention for outer solar system moons of astrobiological interest: what do we need to know? *Astrobiology* **19**, 951–974. (doi:10.1089/ast.2018.1996)
22. Gounot AM. 1986 Psychrophilic and psychrotrophic microorganisms. *Experientia* **42**, 1192–1197. (doi:10.1007/BF01946390)
23. Nakamura A, Takahashi H, Kondo A, Koike F, Kuda T, Kimura B, Kobayashi M. 2022 Distribution of psychrophilic microorganisms in a beef slaughterhouse in Japan after cleaning. *PLoS One* **17**, e0268411. (doi:10.1371/journal.pone.0268411)

24. Jin S, Wang Y, Zhao X. 2022 Cold-adaptive mechanism of psychrophilic bacteria in food and its application. *Microb. Pathog.* **169**, 105652. (doi:10.1016/j.micpath.2022.105652)
25. Joseph B, Kumar V, Ramteke PW. 2019 Chapter 47 - Psychrophilic enzymes: potential biocatalysts for food processing. In *Enzymes in food biotechnology* (ed. M Kuddus), pp. 817–825. Academic Press. (doi:10.1016/B978-0-12-813280-7.00047-5)
26. Kimura J, Kitadai N. 2015 Polymerization of building blocks of life on europa and other icy moons. *Astrobiology* **15**, 430–441. (doi:10.1089/ast.2015.1306)
27. Reynard B, Sotin C. 2023 Carbon-rich icy moons and dwarf planets. *Earth Planet. Sci. Lett.* **612**, 118172. (doi:10.1016/j.epsl.2023.118172)
28. Mykytczuk NCS, Foote SJ, Omelon CR, Southam G, Greer CW, Whyte LG. 2013 Bacterial growth at -15 °C; molecular insights from the permafrost bacterium *Planococcus halocryophilus* Or1. *ISME J.* **7**, 1211–1226. (doi:10.1038/ismej.2013.8)
29. Quillaguamán J, Delgado O, Mattiasson B, Hatti-Kaul R. 2004 Chromohalobacter sarecensis sp. nov., a psychrotolerant moderate halophile isolated from the saline Andean region of Bolivia. *Int. J. Syst. Evol. Microbiol.* **54**, 1921–1926. (doi:10.1099/ijs.0.63153-0)
30. Barberán A, Caceres Velazquez H, Jones S, Fierer N. 2017 Hiding in plain sight: mining bacterial species records for phenotypic trait information. *mSphere* **2**, e00237-17. (doi:10.1128/mSphere.00237-17)
31. Reddy GSN, Manasa BP, Singh SK, Shivaji S. 2013 *Paenisporosarcina indica* sp. nov., a psychrophilic bacterium from a glacier, and reclassification of *Sporosarcina* antarctica Yu *et al*., 2008 as *Paenisporosarcina* antarctica comb. nov. and emended description of the genus *Paenisporosarcina*. *Int. J. Syst. Evol. Microbiol.* **63**, 2927–2933. (doi:10.1099/ijs.0.047514-0)
32. Yu Y, Xin YH, Liu HC, Chen B, Sheng J, Chi ZM, Zhou PJ, Zhang DC. 2008 *Sporosarcina* antarctica sp. nov., a psychrophilic bacterium isolated from the Antarctic. *Int. J. Syst. Evol. Microbiol.* **58**, 2114–2117. (doi:10.1099/ijs.0.65838-0)
33. Auman AJ, Breezee JL, Gosink JJ, Schumann P, Barnes CR, Kämpfer P, Staley JT. 2010 *Psychromonas boydii* sp. nov., a gas-vacuolate, psychrophilic bacterium isolated from an arctic sea-ice core. *Int. J. Syst. Evol. Microbiol.* **60**, 84–92. (doi:10.1099/ijs.0.007773-0)
34. Liu Q, Liu H, Wen Y, Zhou Y, Xin Y. 2012 *Cryobacterium flavum* sp. nov. and *Cryobacterium luteum* sp. nov., isolated from glacier ice. *Int. J. Syst. Evol. Microbiol.* **62**, 1296–1299. (doi:10.1099/ijs.0.033738-0)
35. Niederberger TD, Steven B, Charvet S, Barbier B, Whyte LG. 2009 *Virgibacillus arcticus* sp. nov., a moderately halophilic, endospore-forming bacterium from permafrost in the Canadian high Arctic. *Int. J. Syst. Evol. Microbiol.* **59**, 2219–2225. (doi:10.1099/ijs.0.002931-0)
36. Hu L, Pan H, Xue Y, Ventosa A, Cowan DA, Jones BE, Grant WD, Ma Y. 2008 *Halorubrum luteum* sp. nov., isolated from Lake Chagannor, Inner Mongolia, China. *Int. J. Syst. Evol. Microbiol.* **58**, 1705–1708. (doi:10.1099/ijs.0.65700-0)
37. Antunes A, Taborda M, Huber R, Moissl C, Nobre MF, da Costa MS. 2008 *Halorhabdus tiamatea* sp. nov., a non-pigmented, extremely halophilic archaeon from a deep-sea, hypersaline anoxic basin of the Red Sea, and emended description of the genus *Halorhabdus*. *Int. J. Syst. Evol. Microbiol.* **58**, 215–220. (doi:10.1099/ijs.0.65316-0)
38. Goordial J *et al*. 2016 Improved high-quality draft genome sequence of the *Eurypsychrophile Rhodotorula* sp. JG1b, isolated from permafrost in the hyperarid upper-elevation McMurdo Dry Valleys, Antarctica. *Genome Announc.* **4**, e00069-16. (doi:10.1128/genomeA.00069-16)
39. Touchette D, Altshuler I, Gostinčar C, Zalar P, Raymond-Bouchard I, Zajc J, McKay CP, Gunde-Cimerman N, Whyte LG. 2022 Novel Antarctic yeast adapts to cold by switching energy metabolism and increasing small RNA synthesis. *ISME J.* **16**, 221–232. (doi:10.1038/s41396-021-01030-9)
40. Hof H. 2019 *Rhodotorula* spp. in the gut - foe or friend? *GMS Infect. Dis.* **7**, Doc02. (doi:10.3205/id000042)
41. DSMZ. 2024 *Rhodotorula* mucilaginosa DSM 13621. See https://www.dsmz.de/collection/catalogue/details/culture/DSM-13621.
42. Beblo-Vranesevic K *et al*. 2017 The responses of an anaerobic microorganism, Yersinia intermedia MASE-LG-1 to individual and combined simulated Martian stresses. *PLoS One* **12**, e0185178. (doi:10.1371/journal.pone.0185178)
43. Waskom ML. 2021 seaborn: statistical data visualization. *JOSS* **6**, 3021. (doi:10.21105/joss.03021)

44. Hunter JD. Matplotlib: A 2D Graphics Environment. *Comput. Sci. Eng.* **9**, 90–95. (doi:10.1109/MCSE.2007.55)
45. Paulino-Lima IG, Pilling S, Janot-Pacheco E, de Brito AN, Barbosa JARG, Leitão AC, Lage C de AS. 2010 Laboratory simulation of interplanetary ultraviolet radiation (broad spectrum) and its effects on *Deinococcus radiodurans*. *Planet. Space Sci.* **58**, 1180–1187. (doi:10.1016/j.pss.2010.04.010)
46. Segal-Kischinevzky C, Romero-Aguilar L, Alcaraz LD, López-Ortiz G, Martínez-Castillo B, Torres-Ramírez N, Sandoval G, González J. 2022 Yeasts inhabiting extreme environments and their biotechnological applications. *Microorganisms* **10**, 794. (doi:10.3390/microorganisms10040794)
47. Leo P, Onofri S. 2023 Yeasts in the era of astrobiological research. *J. Indian Inst. Sci.* **103**, 699–709. (doi:10.1007/s41745-023-00378-5)
48. Koike J, Oshima T, Koike KA, Taguchi H, Tanaka R, Nishimura K, Miyaji M. 1992 Survival rates of some terrestrial microorganisms under simulated space conditions. *Adv. Space Res.* **12**, 271–274. (doi:10.1016/0273-1177(92)90182-w)
49. Onofri S, Barreca D, Selbmann L, Isola D, Rabbow E, Horneck G, de Vera JPP, Hatton J, Zucconi L. 2008 Resistance of Antarctic black fungi and cryptoendolithic communities to simulated space and Martian conditions. *Stud. Mycol.* **61**, 99–109. (doi:10.3114/sim.2008.61.10)
50. Pulschen AA, de Araujo GG, de Carvalho ACSR, Cerini MF, Fonseca L de M, Galante D, Rodrigues F. 2018 Survival of extremophilic yeasts in the stratospheric environment during balloon flights and in laboratory simulations. *Appl. Environ. Microbiol.* **84**, e01942-18. (doi:10.1128/AEM.01942-18)
51. Daudu R, Parker CW, Singh NK, Wood JM, Debieu M, O'Hara NB, Mason CE, Venkateswaran K. 2020 Draft genome sequences of *Rhodotorula mucilaginosa* strains isolated from the international space station. *Microbiol. Resour. Announc.* **9**, e00570-20. (doi:10.1128/MRA.00570-20)
52. Wilson L *et al*. 2025 Characterization of virulence-related phenotypes of *Candida parapsilosis* and *Rhodotorula mucilaginosa* isolated from the international space station (ISS). *Life Sci. Space Res.* **45**, 16–24. (doi:10.1016/j.lssr.2025.01.002)
53. Domínguez-Andrés J *et al*. 2020 Growth on carbohydrates from carbonaceous meteorites alters the immunogenicity of environment-derived bacterial pathogens. *Astrobiology* **20**, 1353–1362. (doi:10.1089/ast.2019.2173)
54. Del Moral Jimenez A. 2024 Microbial-Based Proxies for Europa's Habitability and Biosignature Detection. PhD, The Open University.
55. Stubbendieck RM, Vargas-Bautista C, Straight PD. 2016 Bacterial communities: interactions to scale. *Front. Microbiol.* **7**, 1234. (doi:10.3389/fmicb.2016.01234)
56. Moissl-Eichinger C. 2012 Extremophiles in spacecraft assembly clean rooms. In *Adaption of microbial life to environmental extremes: novel research results and application* (eds H Stan-lotter, S Fendrihan), pp. 231–261. Vienna, Austria: Springer Vienna. (doi:10.1007/978-3-211-99691-1_10)
57. Moissl-Eichinger C, Rettberg P, Pukall R. 2012 The first collection of spacecraft-associated microorganisms: a public source for extremotolerant microorganisms from spacecraft assembly clean rooms. *Astrobiology* **12**, 1024–1034. (doi:10.1089/ast.2012.0906)
58. Checinska A *et al*. 2015 Microbiomes of the dust particles collected from the international space station and spacecraft assembly facilities. *Microbiome* **3**, 50. (doi:10.1186/s40168-015-0116-3)
59. Jarros IC *et al*. 2020 Microbiological and virulence aspects of *Rhodotorula mucilaginosa*. *EXCLI J.* **19**, 687–704.
60. Hirano R, Mitsuhashi T, Osanai K. 2022 *Rhodotorula mucilaginosa* Fungemia, a rare opportunistic infection without central venous catheter implantation, successfully treated by liposomal amphotericin B. *Case Rep. Infect. Dis.* **2022**, 7830126. (doi:10.1155/2022/7830126)
61. Hassan N, Anesio AM, Rafiq M, Holtvoeth J, Bull I, Haleem A, Shah AA, Hasan F. 2020 Temperature driven membrane lipid adaptation in glacial psychrophilic bacteria. *Front. Microbiol.* **11**, 824. (doi:10.3389/fmicb.2020.00824)

62. D'Amico S, Collins T, Marx JC, Feller G, Gerday C. 2006 Psychrophilic microorganisms: challenges for life. *EMBO Rep.* **7**, 385–389. (doi:10.1038/sj.embor.7400662)
63. Scherber CM, Schottel JL, Aksan A. 2009 Membrane phase behavior of *Escherichia coli* during desiccation, rehydration, and growth recovery. *Biochim. Biophys. Acta* **1788**, 2427–2435. (doi:10.1016/j.bbamem.2009.08.011)
64. Greffe VRG, Michiels J. 2020 Desiccation-induced cell damage in bacteria and the relevance for inoculant production. *Appl. Microbiol. Biotechnol.* **104**, 3757–3770. (doi:10.1007/s00253-020-10501-6)
65. DLR. 2023 *JUICE - an unprecedented journey to Jupiter*. DLR (German Aerospace Center). See https://www.dlr.de/en/latest/news/2023/02/juice-an-unprecedented-journey-to-jupiter (accessed 10 September 2024).
66. Heinz J, Schirmack J, Airo A, Kounaves SP, Schulze-Makuch D. 2018 Enhanced microbial survivability in subzero brines. *Astrobiology* **18**, 1171–1180. (doi:10.1089/ast.2017.1805)
67. Tedeschi R, De Paoli P. 2011 Collection and preservation of frozen microorganisms. In *Methods in biobanking* (ed. J Dillner), pp. 313–326, vol. **675**. Totowa, NJ, USA: Humana Press. (doi:10.1007/978-1-59745-423-0_18)
68. Ali P, Shah AA, Hasan F, Hertkorn N, Gonsior M, Sajjad W, Chen F. 2020 A glacier bacterium produces high yield of cryoprotective exopolysaccharide. *Front. Microbiol.* **10**, 3096. (doi:10.3389/fmicb.2019.03096)
69. Rapuano R, Graziano G. 2022 Some clues about enzymes from psychrophilic microorganisms. *Microorganisms* **10**, 1161. (doi:10.3390/microorganisms10061161)
70. Son Y, Min J, Shin Y, Park W. 2023 Morphological and physiological adaptations of psychrophilic *Pseudarthrobacter psychrotolerans* YJ56 under temperature stress. *Sci. Rep.* **13**, 14970. (doi:10.1038/s41598-023-42179-x)
71. Sawicka JE, Robador A, Hubert C, Jørgensen BB, Brüchert V. 2010 Effects of freeze-thaw cycles on anaerobic microbial processes in an Arctic intertidal mud flat. *ISME J.* **4**, 585–594. (doi:10.1038/ismej.2009.140)
72. Gunnarsdóttir R, Müller K, Jensen PE, Jenssen PD, Villumsen A. 2012 Effect of long-term freezing and freeze-thaw cycles on indigenous and inoculated microorganisms in dewatered blackwater. *Environ. Sci. Technol.* **46**, 12408–12416. (doi:10.1021/es3018489)
73. Helfenstein P, Porco CC. 2015 Enceladus' geysers: relation to geological features. *Astron. J.* **150**, 96. (doi:10.1088/0004-6256/150/3/96)
74. Shibley NC, Laughlin G. 2021 Do oceanic convection and clathrate dissociation drive europa's geysers? *Planet. Sci. J.* **2**, 221. (doi:10.3847/psj/ac2b2c)
75. Ochoa-Viñals N, Alonso-Estrada D, Pacios-Michelena S, García-Cruz A, Ramos-González R, Faife-Pérez E, Michelena-Álvarez LG, Martínez-Hernández JL, Iliná A. 2024 Current advances in carotenoid production by *Rhodotorula* sp. *Fermentation* **10**, 190. (doi:10.3390/fermentation10040190)
76. Moliné M, Flores MR, Libkind D, Carmen Diéguez MDC, Farías ME, van Broock M. 2010 Photoprotection by carotenoid pigments in the yeast *Rhodotorula mucilaginosa*: the role of torularhodin. *Photochem. Photobiol. Sci.* **9**, 1145–1151. (doi:10.1039/c0pp00009d)
77. Moliné M, Libkind D, Del Carmen Diéguez M, van Broock M. 2009 Photoprotective role of carotenoids in yeasts: response to UV-B of pigmented and naturally-occurring albino strains. *J. Photochem. Photobiol. B Biol.* **95**, 156–161. (doi:10.1016/j.jphotobiol.2009.02.006)
78. Raymond-Bouchard I, Chourey K, Altshuler I, Iyer R, Hettich RL, Whyte LG. 2017 Mechanisms of subzero growth in the cryophile *Planococcus halocryophilus* determined through proteomic analysis. *Environ. Microbiol.* **19**, 4460–4479. (doi:10.1111/1462-2920.13893)
79. Zaccaria T, Beblo-Vranesevic K, de Jonge MI *et al*. 2025 Data from: Survival limits of yeasts and other extremophilic microorganisms with relevance for planetary protection of the icy moons. Dryad Dryad Repository. (doi:10.5061/dryad.70rxwdc7r)
80. Zaccaria T, Beblo-Vranesevic K, de Jonge MI, Netea MG, Rettberg P. 2025 Supplementary material from: Survival limits of psychrotolerant microorganisms with relevance for planetary protection of the icy moons. Figshare. (doi:10.6084/m9.figshare.c.8210669)