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RESEARCH ARTICLE

Succession of the fungal community of a spacecraft assembly clean room when enriched in brines relevant to Mars

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Abstract

Spacecraft can carry microbial contaminants from spacecraft assembly facilities (SAFs) to the cold arid surface of Mars that may confound life detection missions or disrupt native ecosystems. Dry hygroscopic sulphate and (per) chlorate salts on Mars may absorb atmospheric humidity and deliquesce at certain times to produce dense brines, potential sources of liquid water. Microbial growth is generally prohibited under the non-permissive condition of extremely low water activity in the frigid potential brines on Mars. Here we challenged the microbial community from samples of the Jet Propulsion Laboratory SAF with the extreme chemical conditions of brines relevant to Mars. Enrichment cultures in SP medium supplemented with 50% $MgSO_4$ or 20% $NaClO_3$ were inoculated from washes of SAF floor wipes. Samples were taken for each of the first four weeks and then at six months after inoculation to follow changes in the SAF microbial community under high salinity for long periods. Metagenomic DNA extracts of community samples were examined by Illumina sequencing of 18S rRNA gene sequences using fungal primers. The fungal assemblage during the first month of enrichment was predominantly common Ascomycetes, primarily Saccharomyete yeasts. Basidiomycetes were detected, mainly in the Microbotryomycetes and Tremellomycetes. Fungi were much less abundant in enrichment cultures at 50% $MgSO_4$ than at 20% $NaClO_3$. After 6 months of enrichment, few fungi remained. Microbes persisting from the JPL SAF microbial community in aged cultures enriched at extreme salinities might be the most capable of subsequently surviving and proliferating at the near surface of Mars. The SAF fungal assemblage did not survive and proliferate as well as the SAF bacterial community.

Contents

Introduction

Natural environments on celestial bodies that might support life need to be protected from contamination by visiting spacecraft (Irwin and Schulze-Makuch, 2001; Rummel *et al.*, 2014). The goal is to reduce the chances of confounding life detection missions due to terrestrial life carried by spacecraft. Should native life exist in another world, microbes capable of colonizing the alien environment could jeopardize microbial communities. Extensive protocols are used to reduce the bioburden on robotic spacecraft sent to Mars, the ocean worlds or any body where life is reasonably plausible. Spacecraft are assembled in clean rooms to reduce biocontamination. Air is filtered, workers don personal protective equipment and airlocks are used to limit the transport of dust and microbes into the facility. Studying the microbes in spacecraft assembly facilities (SAFs), which are by location the most likely cells to be carried by spacecraft, will help us understand the probability that relevant microbial assemblages could survive and proliferate in another world. Further, describing the lifestyles and mechanisms by which microbes are successful under environmental conditions that are analogues of Mars and the ocean worlds, suggests adaptations that might be expected from native life on these worlds.

The environments of Mars and the ocean worlds are characterized by extreme chemical and physical conditions, including very low temperatures (Mancinelli *et al.*, 2004; Davila *et al.*, 2010; Rummel *et al.*, 2014). Since liquid water is necessary for canonical life, its scarcity on Mars and other bodies reduces the extent of special regions that are potentially habitable. High concentrations of salts can substantially depress the freezing point of water, thereby maintaining liquid water at temperatures as low as −70°C, expanding the extent of special regions. However, high solute concentrations can lower water activity (a_w) to the point that only organisms with rare adaptations are able to survive and proliferate. Even the modest salinity of the ocean (2% NaCl; $a_w = 0.97$) greatly limits the microbial taxa capable of colonization (Grant, 2004). In saturated NaCl, with an a_w of 0.75, only extremely halotolerant organisms survive. Driving freezing points to a range where liquid water could currently persist near the surface of Mars requires high enough concentrations of (per)chlorate salts to lower the a_w below ∼0.6, the known limit of life (Grant, 2004; Schneegurt, 2012; Rummel *et al.*, 2014; Primm *et al.*, 2017; Pál and Kereszturi, 2020; Rivera-Valentín *et al.*, 2020). Growth at record low a_w previously has been demonstrated with fungi in high sugars and salts (Pitt and Christian, 1968; Williams and Hallsworth, 2009).

The microbial communities in SAFs are closely monitored by planetary protection teams to understand and reduce bioburden on spacecraft. Halotolerant fungi in SAFs appear to be in greatly lower abundance than halotolerant bacteria (Moissl *et al.*, 2007; Plemenitaš *et al.*, 2014; Venkateswaran *et al.*, 2014; Checinska *et al.*, 2015; Weinmaier *et al.*, 2015; Hendrickson *et al.*, 2017). In one study, fungi were found to be ∼2% of the culturable microbial community (Hendrickson *et al.*, 2017), while in another, cultivable fungi on SAF dust particles were ∼1 log unit lower in abundance (10^3–10^4 CFU g^{-1} dust) than that of bacteria (Checinska *et al.*, 2015). It is interesting to note that archaea, often associated with hypersaline environments, were nearly absent in SAFs. The bacterial assemblages in SAFs have been shown to be quite diverse, both by cultivation and molecular analyses (La Duc *et al.*, 2003; 2009; 2012, 2014; Moissl *et al.*, 2007, 2008; Bashir *et al.*, 2016; Danko *et al.*, 2021; Hendrickson *et al.*, 2021; Carte *et al.*, 2024). Substantial halotolerance has been observed for SAF isolates, suggesting that these dry environments select for salinotolerant microbes (Moissl-Eichinger *et al.*, 2013; Venkateswaran *et al.*, 2014; Smith *et al.*, 2017; Zanmuto *et al.*, 2018). Wipe samples from SAFs cultivated at very high salinities (50% $MgSO_4$ or 20% $NaClO_3$) enriched for a microbial community that appeared to retain the functional groups needed to perpetually maintain biogeochemical cycles under these analogue chemical conditions (Carte *et al.*, 2024).

Fungi are widely found in aquatic and terrestrial hypersaline environments (Butinar *et al.*, 2005; Evans *et al.*, 2013). Marine salterns (Butinar *et al.*, 2005; Cantrell *et al.*, 2006; Nayak *et al.*, 2012), sabkhas (Borut, 1960; Al-Musallam *et al.*, 2011), salt flats (Evans *et al.*, 2013) and even the Dead Sea (Guiraud *et al.*, 1995; Steiman *et al.*, 1995) are known to harbour populations of salinotolerant fungi. Hypersaline fungal communities are dominated by Ascomycetes, mainly *Aspergillus*, *Eurotium* and *Penicillium*. *Alternaria*, *Chaetomium*, *Cladosporium*, *Eurotium*, *Fusarium* and

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Hortaea are commonly observed, along with *Mucor*, *Rhizopus* and *Ulocladium*. Melanized xerophilic yeasts are commonly considered the most halotolerant fungi (Gunde-Cimerman *et al*., 2000). Since cosmopolitan genera, such as *Aspergillus* and *Penicillium*, dominate hypersaline fungal assemblages, it has been suggested that these represent a halotolerant subset of common soil fungi in these locales. Ranzoni (1968) concluded that there are no fungi characteristic of hypersaline and arid environments, a suggestion that has been echoed by other researchers (Guiraud *et al*., 1995; Grishkan *et al*., 2003; Evans *et al*., 2013).

Halophilic fungi (requiring high salt for growth) tend to be far less common than halophilic bacteria (Grant, 2004; Evans *et al*., 2013; Plemenitaš *et al*., 2014). Fungi from hypersaline environments typically are halotolerant (able to grow at high salinities) but grow better at lower salinities (Hujslová *et al*., 2010; Nayak *et al*., 2012; Evans *et al*., 2013). Broad tolerance to salinity, without halophilicity, with slower growth at high salinities, supports the suggestion that fungal communities in hypersaline environments are not highly specialized. Fungi use mechanisms similar to those of bacteria to survive in brines, including tight membranes, ion efflux pumps and the accumulation of compatible solutes to balance internal osmotic potential with that of the medium (Plemenitaš *et al*., 2014; Leo and Onofri 2023; Suryanarayanan and Ravishankar, 2023). In addition, fungi reinforce their cell wall to withstand increased turgor pressure and resist salt uptake (Gunde-Cimerman and Zalar, 2014).

The current study examines the fungal assemblages that formed when wipe samples from SAF surfaces were enriched in medium containing either 50% $MgSO_4$ or 20% $NaClO_3$. Community DNA extracts, weeks and months after inoculation, were the subject of next generation sequencing of rRNA genes to identify phylogenetic groups. A companion study describing the bacterial assemblages in these enrichments reported substantial diversity (Carte *et al*., 2024). Here we report that the fungal assemblage was not as diverse, being dominated by yeasts and rich in common Ascomycetes.

Methods

Sampling of SAF

Sterile polyester wipes (Texwipe; Kernersville, NC) were moistened with 15 ml of sterile water and used to swab three 1-m^2 surfaces of high-traffic floors in the Aseptic Assembly Facility at NASA Jet Propulsion Laboratory (JPL), a certified ISO 5 clean room, during the assembly of the Mars 2020 Sample Caching System hardware (Fig. 1). A fourth sterile wipe was used as a procedural control. During sampling, a fresh pair of sterile gloves was worn for each sample collection. The wipes were

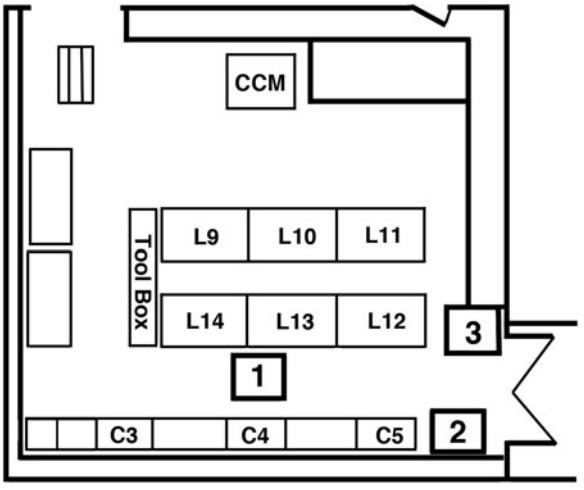

Fig. 1. *Map of the aseptic assembly facility in the JPL SAF showing the locations of three wipe samples of high-traffic areas of the floor.*

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packaged in sterile polypropylene tubes with screw caps and shipped overnight in a cool container from JPL to Wichita State University. Upon arrival, the wipes were wetted with 30 ml of a sterile chaotropic solution (0.1% Na pyrophosphate) to dislodge microbes from the wipes. After 10 min, liquid was squeezed from the wipes within the tubes using a sterile syringe plunger. The extracts then were used to inoculate enrichment cultures and for direct DNA extractions.

Enrichment cultures

Enrichment cultures were maintained in selective salt plains (SP) medium containing (per liter): NaCl, 1.0 g; KCl, 2.0 g; $MgSO_4{\cdot}7H_2O$, 1.0 g; $CaCl_2{\cdot}H_2O$, 0.36 g; NaBr, 0.23 g; $FeCl_3{\cdot}6H_2O$, 1.0 mg; trace minerals, 0.5 ml; yeast extract, 10.0 g; tryptone, 5.0 g; glucose, 1.0 g (Caton *et al*., 2004), supplemented with either 50% (w/v) $MgSO_4$ (2.0 M; $a_w = 0.94$) or 20% (w/v) $NaClO_3$ (1.9 M; $a_w = 0.91$). While not specific for fungi, as it contains less sugar than the fungal media of Emmons (1963) or Sabouraud (1892), SP medium is eutrophic and rich in amino acids. Flasks (100 ml) were inoculated with aliquots (1 ml) of the fluids extracted from SAF wipes and maintained on an Innova rotary shaker (125 rpm; 1-in stroke dia; New Brunswick Scientific, Edison, NJ) at room temperature for a month. Samples (6 ml) were taken weekly during this incubation for community analyses. The enrichment cultures were then removed from the shaker, wrapped with parafilm to limit evaporation and stored static for an additional five months, before sampling for community analyses.

DNA extraction and molecular analyses

Crude DNA extracts were made from aliquots (6 ml) of samples, isolates and enrichment cultures using a freeze-thaw technique (Caton *et al*., 2004). Cells were collected by serial microcentrifugation for 5 min at 14 000 × ***g***. Pellets were resuspended in 300 µl of sterile water before six cycles of freezing in liquid N_2 and thawing at 80°C, with vigorous vortex mixing every other cycle. Homogenates were clarified by microcentrifugation for 10 min at 14 000 × ***g*** and the final supernatant heated for 5 min at 80°C. Extracts were stored at −20°C before PCR amplification. Direct extracts from wipe samples before enrichment yielded insufficient DNA for reliable PCR amplification and community analyses as expected, since samples with extremely low biomass typically require specialized extraction methods.

Crude community DNA extracts from enrichment culture samples were used for Illumina sequencing (miSeq v2 Nona PE-250bp) of 18S rRNA genes by a commercial vendor (University of Kansas Center for Genomics). Each sample in the multiplex reactions produced ~ 30 000 reads and the FASTQ files were demultiplexed for forward and reverse reads. Metagenomic 18S sequence libraries were analysed on the Galaxy platform (Afgan *et al*., 2018) following the microbial analysis package workflow, incorporating tools designed for MOTHUR (Schloss *et al*., 2009). Forward and reverse reads were combined to create contigs, generating an average read length of 334 nucleotides. Chimeras were found using chimera.vsearch and removed using remove.seqs in MOTHUR on the Galaxy platform. These were aligned using SILVA alignment v.138 and classified in reference to SILVA taxonomy v.138.

Results

Succession of SAF fungal communities enriched in 20% $NaClO_3$

Three wipes from SAF floor sampling were used to inoculate microbial enrichments in SP medium supplemented with 20% (w/v) $NaClO_3$. Fungi were detected by Illumina sequencing of PCR-amplified 18S rRNA genes for all wipes at all time points. Prior to enrichment, direct extraction of wipe samples did not yield sufficient DNA for amplification of fungal sequences, as observed previously for bacterial analyses (Highlander *et al*., 2023; Carte *et al*., 2024). The overall number of sequences obtained in the fungal libraries from the wipe samples were far fewer (<1%) than those obtained from the analysis of bacteria in these enrichment cultures (Carte *et al*., 2024). The greatest number of fungal sequences were obtained

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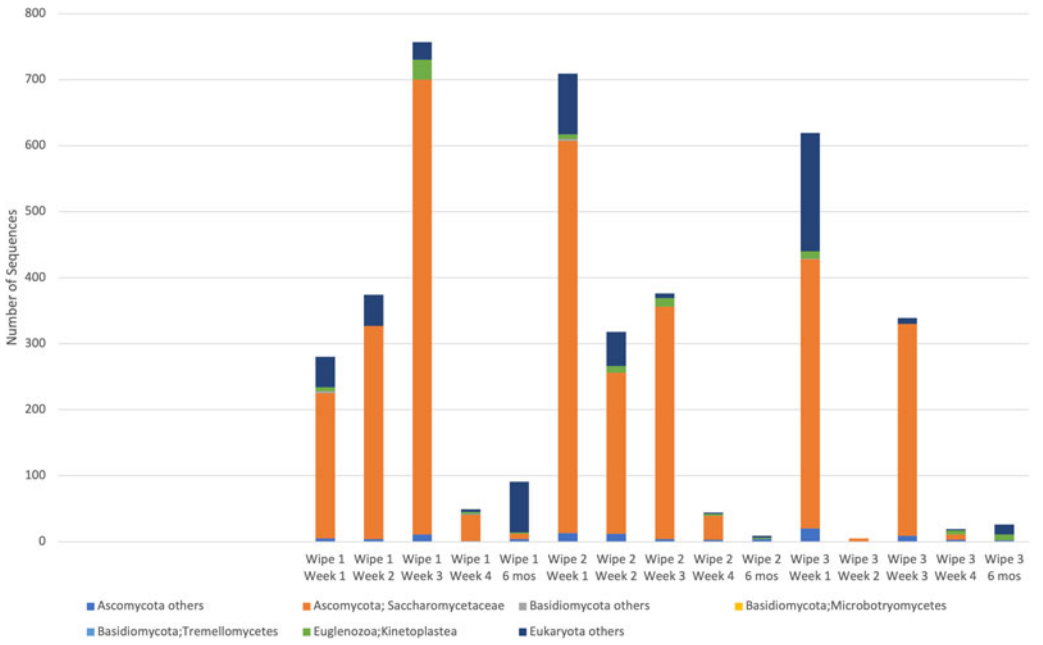

Fig. 2. *Abundance of fungal families observed by pyrosequencing and phylogenetic analyses of 18S rRNA genes from metagenomic extracts of SAF wipe assemblages after 1 wk, 2 wk, 3 wk, 4 wk, and 6 mo of enrichment in SP medium supplemented with 20% (w/v; ~1.9 M) $NaClO_3$.*

during the first three weeks of enrichment in 20% $NaClO_3$ (Fig. 2). After six months of enrichment, ~4- to 10-fold fewer sequences were obtained than after three weeks of enrichment.

The families of fungi obtained are shown as a percentage of the total assemblage, to illustrate successional changes over time for each wipe (Fig. 3). Certain features of the assemblage were relatively

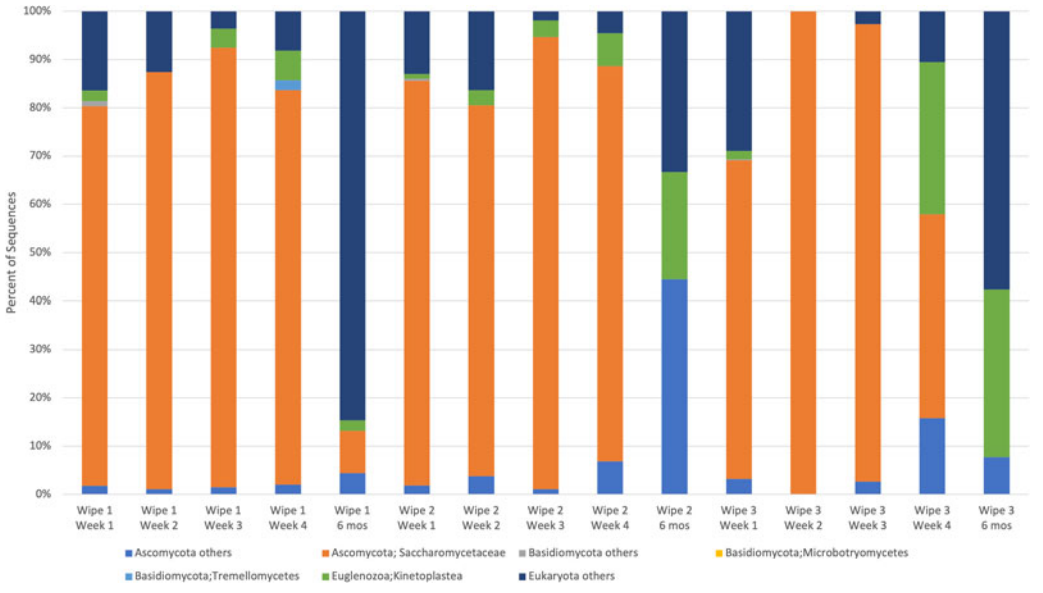

Fig. 3. *Relative abundance of fungal families observed by pyrosequencing and phylogenetic analyses of 18S rRNA genes from metagenomic extracts of SAF wipe assemblages after 1 wk, 2 wk, 3 wk, 4 wk, and 6 mo of enrichment in SP medium supplemented with 20% (w/v; ~1.9 M) $NaClO_3$.*

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consistent among the three wipes. For the first month of enrichment at 20% $NaClO_3$, *Saccharomycetaceae* within the Ascomycota were dominant. The remaining assemblage was mainly other Ascomycota. Basidiomycota were practically absent in these enrichments. Non-fungal Eukarya, including Kinetoplastida in the Euglenozoa, were more numerous. The assemblages remaining after six months of enrichment in 20% $NaClO_3$ exhibited a decidedly different structure. Ascomycota were greatly reduced, particularly the *Saccharomycetaceae* that were so dominant during the first month of enrichment. Overall numbers of fungal sequences were greatly reduced, but libraries were enriched for non-fungal Eukarya including Euglenozoans, although these were still in low abundance.

The number of OTUs observed and the Chao estimates of OTUs decreased during the 6-mo enrichment period (Table 1). The reductions in OTUs were between 68 and 86%, with the subspecies level being most impacted. Good's coverage was relatively low at the highest sequence identity level (subspecies at 99%) but was high at the family level (88% identity). Diversity based on the Non-parametric Shannon Index was greatest at the 99% identity level and was above 3.0 through the genus level of identity (94%). The Shannon Evenness Index showed small increases during the enrichment period, with the family level of identity (88%) exhibiting the greatest increase in evenness.

Succession of SAF fungal communities enriched in 50% $MgSO_4$

Three wipes from SAF floor sampling were used to inoculate microbial enrichments in SP medium supplemented with 50% (w/v) $MgSO_4$ and fungi were again detected by Illumina sequencing of PCR-amplified 18S rRNA genes. Very few fungal sequences were obtained overall, with only a few samples (mainly from Wipe 3) showing substantial amplification (Fig. 4). The observed fungal assemblages were noticeably less consistent between wipes and time points than those observed from the 20% $NaClO_3$ enrichments. There were apparently large shifts in the fungal sequences present at different time points during the first month of enrichment (Fig. 5). Wipe 3 was nearly entirely Basidiomycota for the first three weeks of enrichment. There were shifts within the Basidiomycota among Microbotryomycetes, Tremellomycetes and others during this time. The other wipes also showed greater contributions from Basidiomycota in the fungal assemblages of the $MgSO_4$ enrichments than in those of the $NaClO_3$ enrichments. After six months of enrichment in 50% $MgSO_4$, few fungal sequences were detected. These libraries were mainly composed of Ascomycota and non-fungal unclassified Eukarya, although these were in low abundance.

Far fewer fungal OTUs were detected (~5 to 20%) in the 50% $MgSO_4$ enrichments (Table 2) than in the 20% $NaClO_3$ enrichments. There was less speciation, with the number of OTUs at the level of subspecies being close to the number of OTUs at the family level. Good's coverage also was generally

Table 1. *Diversity indices for the enrichment cultures in 20% $NaClO_3$*

Sequence identity	99%			97%			94%			88%		
Parameter	Wk 1	Wk 4	6 Mo	Wk 1	Wk 4	6 Mo	Wk 1	Wk 4	6 Mo	Wk 1	Wk 4	6 Mo
OTUs	2961	338	424	1689	263	274	940	186	176	269	104	87
Coverage	0.68	0.52	0.47	0.86	0.67	0.65	0.93	0.79	0.76	0.98	0.90	0.87
Chao1	7380	636	1489	1482	306	300	661	186	176	155	54	58
Lower CI	5814	368	569	1246	191	202	545	104	94	118	41	39
Upper CI	9485	1171	1535	1805	551	509	840	295	249	241	93	121
npShannon	5.43	4.56	4.6	4.53	3.99	3.61	3.76	3.36	3.05	2.11	2.28	2.36
Inv Simpson	11.48	10.97	123.37	10.30	6.82	10.34	7.90	5.56	8.15	3.41	4.46	13.78
Shannon even	0.70	0.78	0.80	0.67	0.76	0.78	0.63	0.71	0.75	0.46	0.56	0.71

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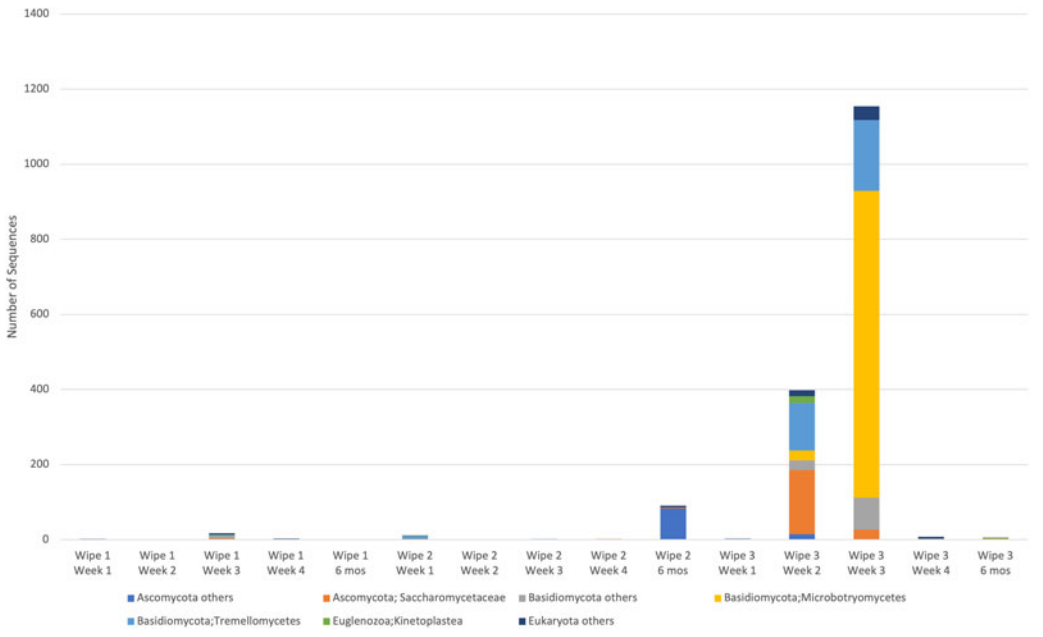

Fig. 4. *Abundance of fungal families observed by pyrosequencing and phylogenetic analyses of 18S rRNA genes from metagenomic extracts of SAF wipe assemblages after 1 wk, 2 wk, 3 wk, 4wk, and 6 mo of enrichment in SP medium supplemented with 50% (w/v; ~2.0 M) $MgSO_4$.*

lower but showed more variability. The number of OTUs detected decreased over time in the 20% $NaClO_3$ enrichments, but this trend was not observed in the 50% $MgSO_4$ enrichments. The greatest number of OTUs were observed after 4 weeks, especially at the higher levels of sequence identity. The Non-parametric Shannon Diversity Index showed a dip at 4 weeks as well. Overall, the assemblage

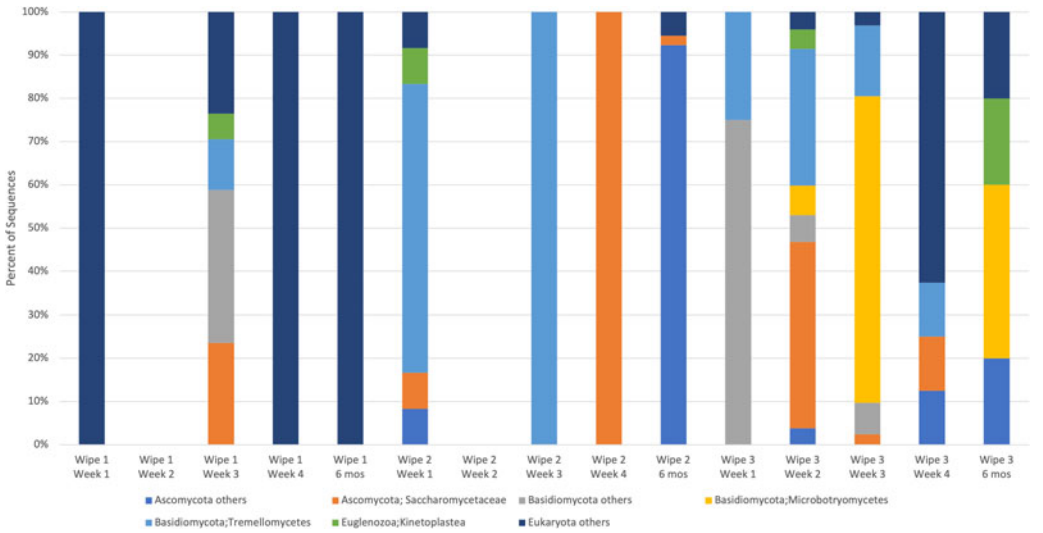

Fig. 5. *Relative abundance of fungal families observed by pyrosequencing and phylogenetic analyses of 18S rRNA genes from metagenomic extracts of SAF wipe assemblages after 1 wk, 2 wk, 3 wk, 4wk, and 6 mo of enrichment in SP medium supplemented with 50% (w/v; ~2.0 M) $MgSO_4$.*

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Table 2. *Diversity indices for the enrichment cultures in 50% $MgSO_4$*

Sequence identity	99%			97%			94%			88%		
Parameter	Wk 1	Wk 4	6 Mo	Wk 1	Wk 4	6 Mo	Wk 1	Wk 4	6 Mo	Wk 1	Wk 4	6 Mo
OTUs	99	461	107	73	247	81	60	132	60	42	45	38
Coverage	0.29	0.84	0.13	0.60	0.63	0.46	0.99	0.99	0.99	0.73	0.65	0.54
Chao1	248	979	233	75	205	90	58	125	41	25	25	23
Lower CI	106	706	112	40	157	50	31	84	27	17	18	15
Upper CI	664	1403	556	187	294	205	156	214	89	64	50	63
npShannon	4.50	2.56	3.68	3.49	1.81	2.90	3.04	1.42	2.23	2.52	0.65	1.62
Inv Simpson	74.01	5.27	139.77	20.78	4.04	42.96	6.29	3.30	13.03	6.13	1.47	6.28
Shannon even	0.94	0.84	0.99	0.90	0.76	0.96	0.83	0.75	0.92	0.79	0.64	0.85

was less diverse in the 50% $MgSO_4$ enrichments than in the 20% $NaClO_3$ enrichments. Shannon evenness was high indicating that the OTUs represented were in approximately equal abundance, substantially more even than the fungal assemblage enriched at 20% $NaClO_3$.

Discussion

Fungi exhibit characteristics that increase their potential to survive or proliferate on celestial bodies (Selbmann *et al*., 2005; Evans *et al*., 2013; Blachowicz *et al*., 2019). Fungal spores are remarkably tough, vegetative cells tend to ferment under anaerobic conditions and many fungi exhibit growth tolerances to high salinity and low temperatures. Growth was observed previously with fungi at near-record low a_w in high sugar syrups (Pitt and Christian, 1968). Fungi are found in low abundance in SAFs, seemingly insignificant (Moissl *et al*., 2007; Plemenitaš *et al*., 2014; Venkateswaran *et al*., 2014; Checinska *et al*., 2015; Weinmaier *et al*., 2015; Hendrickson *et al*., 2017). However, their resiliency suggests that fungi remain a particular concern in regard to forward planetary protection (Leo and Onofri, 2023).

The diversity of the fungal assemblage in SAFs appears to be very much lower than that of the bacterial assemblage. Fungal spores and hyphae have been observed on fallout dust particles in SAFs (Checinska *et al*., 2015; Yuan *et al*., 2017; Mohan *et al*., 2019). The isolates tended to be limited to common moulds and yeasts of the Ascomycota, such as *Aspergillus, Cladosporium, Penicillium* and *Saccharomyces*, and the Basidiomycota in the Tremellomycetes. Of 26 genomes sequenced from SAF fungal isolates, 9 genera were detected comprising 13 species, with *Aspergillus* and *Penicillium* being most common (Chander *et al*., 2022). From a Chinese SAF, 13 families of fungi were isolated, with Tremellomycetes being most common (Yuan *et al*., 2017). Analyses of SAF fungal assemblages using molecular techniques gave similar results but also uncovered greater diversity. An analysis of dust microbiomes from SAF and International Space Station (ISS) samples detected 153 OTUs by ITS sequencing of metagenomic extracts, with Ascomycetes being most prominent, including Dothideomycetes, Eurotiomycetes and Saccharomycetes (Checinska *et al*., 2015). Basidiomycetes also were detected, including Cytobasidiomycetes, Microbotryomycetes and Tremellomycetes. A study of an SAF in Japan and the ISS by ITS sequencing suggested that ~50 OTUs of fungi were present in the SAF, with fewer than 10 detected for the ISS, including *Cladosporium* (90% of sequences), *Alternaria* and *Aspergillus* (Satoh *et al*., 2011). The molecular analysis of samples from a Chinese SAF detected ~50 different species, including *Debaryomyces* and *Pichia* (Yuan *et al*., 2017). Fungal assemblages in SAFs appear to be dominated by common Ascomycetes with relatively limited diversity. It does not seem that the SAF assemblages are rich in fungi found only in specialized environments.

The environment of clean rooms used as SAFs is maintained in a similar way as clean rooms used for surgery and pharmaceutical production. Bacteria dominate the microbial communities of these

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clean rooms, with fungi accounting for less than 1% of the microbes detected (Sandle, 2011). In a vaccine preparation facility, fungi were detected at 300–800 CFU m^{-3} of air (Utescher *et al*., 2007), comparable to levels reported in SAFs (Checinska *et al*., 2015; Hendrickson *et al*., 2017). Hospital buildings had somewhat lower counts of airborne fungi with <100 CFU m^{-3} (Ekhaise *et al*., 2008; Saadoun *et al*., 2008; Kim *et al*., 2010). *Aspergillus, Cladosporium, Penicillium* and *Saccharomycetes* were most common among these clean rooms, not unlike the assemblages observed in SAFs (Utescher *et al*., 2007; Ekhaise *et al*., 2008; Saadoun *et al*., 2008; Nagano *et al*., 2009; Kim *et al*., 2010; Sandle, 2011). A global survey of indoor fungi by ITS sequencing found that the fungal genera observed were mainly cosmopolitan, with few differences at the class level (Amend *et al*., 2010). Diversity was correlated with latitude and building function did not have a significant effect on the fungal assemblages. Ascomycetes dominated the indoor environments detected by ITS sequencing and by cultivation. This supports the suggestion that the assemblage of fungi in clean rooms is not specialized, as observed in the current report for SAFs and as previously suggested for fungal assemblages in natural hypersaline environments (Ranzoni, 1968; Guiraud *et al*., 1995; Grishkan *et al*., 2003).

The Ascomycota detected in SAF enrichment cultures of the current report were common *Saccharomycetaceae* and *Trichomaceae* (*Aspergillus*, *Cladosporium*, *Eurotium* and *Penicillium*), which appear to exhibit high halotolerance (Grishkan *et al*., 2003; Gostinčar *et al*., 2010; Gonsalves *et al*., 2012; Zajc *et al*., 2017). Ascomycetes are reported to be the dominant fungi in the harshest environments of the Antarctic dry valleys (Coleine *et al*., 2020). Halotolerance and xerophilicity of *Eurotium* have been reported for isolates from salterns and the Dead Sea (Andrews and Pitt, 1987; Abdel-hafez *et al*., 1989; Kis-Papo *et al*., 2003; Butinar *et al*., 2005). Yeasts in the *Saccharomycetaceae* and Basidiomycota (Microbotryomycetes and Tremellomycetes) were the most abundant fungi in SAF enrichment cultures. These groups are commonly reported from marine, saltern and other hypersaline environments, often exhibiting high salinity tolerances in culture (Singh and Raghukumar, 2014; Zajc *et al*., 2017). Many yeasts from natural hypersaline environments are melanized, being especially halotolerant (Gunde-Cimerman *et al*., 2000; Zajc *et al*., 2017), but those were in low abundance in our SAF enrichment cultures. Less work has been done with athalassohaline fungal cultures like those of the current study, which are not predominantly NaCl. An extensive study of yeasts from diverse environments demonstrated high tolerances to salts including $MgCl_2$, $MgSO_4$, KCl, and NaBr (Zajc *et al*., 2014). Tolerance to 0.5 M $MgClO_3$ has been reported for a strain of *Penicillium* (Espeso *et al*., 2019). Both $MgSO_4$ and $NaClO_3$ were used for the enrichments in the current study.

The fungal assemblages in the SAF enrichments of the current study changed over time and differed between brines and wipe samples. Saccharomycetes were dominant in the eukaryotic assemblage in early stages of the enrichment but were succeeded mainly by non-fungal eukaryotes in low abundance at 6 months of enrichment. This may be interpreted in terms of the success strategies of these organisms. The Saccharomycete yeasts tend to be fast growing when nutrients of high quality are present and abundant, a trait that would be expected for r-strategists, which are successful due to their rapid growth rate. After 1 month of aeration by rotary shaking, the culture flasks were left static for the next 5 month, without adding nutrients. This change should favour organisms that are characteristically K-strategists, being permanent members of a community, relying on adaptability and versatile nutritional requirements for success. In the bacterial community of these SAF enrichments, actinobacteria were most successful after 6 mo, as these are generally K-strategists (Carte *et al*., 2024). Initially, there were dominating blooms of staphylococci, which are fast-growing r-strategists. We might expect that the harsh, relatively low-nutrient environment of Mars would select K-strategists, in the same way that these enrichment cultures did.

For the 50% $MgSO_4$ enrichment culture of Wipe 3, there was a bloom of Basidiomycete yeasts, also r-strategists, which was succeeded by non-fungal Eukaryotes in low abundance. It is interesting to note that during this yeast bloom from Wipe 3 in weeks 2 and 3 of enrichment, bacterial numbers fell dramatically (Carte *et al*., 2024). By 6 mo, the bacterial community had recovered its former diversity and

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abundance. Changes to the fungal assemblage in the Wipe 3 enrichment culture were different than those observed in the enrichment cultures from the other two wipes. Since the wipe samples were taken from the same SAF environment, only meters apart, the suggestion is that the successional process had a substantial stochastic element, as was observed for the bacterial community. There were far fewer fungi detected in the enrichment cultures at 50% $MgSO_4$ than in those at 20% $NaClO_3$. The molarities of ions were similar as was the a_w of the two brines. The reason for the difference between salts is not clear and this was not observed for the bacterial community. During the SAF enrichments, both bacterial and fungal community members were followed by design. The base SP medium used was not as rich in sugar as typical fungal media (Sabouraud, 1892; Emmons, 1963). With higher concentrations of simple sugars, the bloom of *r*-strategist yeasts may have been greater and lasted longer, but ultimately the K-strategists would be expected to succeed them. Given the limited abundance and diversity of fungi after 6 month of enrichment, it seems that the SAF assemblage was not rich in K-strategists, being succeeded mainly by non-fungal Eukaryotes and actinobacteria.

Fungi exhibit remarkable tolerances to harsh environmental conditions that are present on solar system bodies such as hypersalinity. Their resistant perennating structures give them additional survivability under conditions non-permissive for growth. Fungi currently hold the record for growth at the lowest a_w (Pitt and Christian, 1968; Williams and Hallsworth, 2009). Therefore, discussions of planetary protection need to include salinotolerant fungi (Leo and Onofri, 2023). Finding fungi capable of long-term survival and proliferation in concentrated $MgSO_4$ and $NaClO_3$ brines, demonstrates that fungi may be carried by spacecraft and further have the potential to influence life detection missions or native microbial communities. This concern should be tempered by the observation that fungal K-strategists were not successful end members of SAF enrichments in brines relevant to Mars. Further, heterotrophic fungal metabolism is relatively limited and fungi do not fill critical roles in biogeochemical cycles. The end members of the bacterial community in these SAF enrichments included representatives of each functional group needed to maintain biogeochemical cycles for the microbial community (Carte *et al.*, 2024), and therefore pose a greater threat to forward planetary protection than fungi.

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