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RESEARCH ARTICLE

Dehydrated thin film media to rapidly estimate bioburden for planetary protection flight implementation

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Abstract

Planetary Protection (PP) is the practice of safeguarding solar system bodies from terrestrial biological contamination and screening the Earth against potentially harmful extraterrestrial biological contamination. On Earth, cleanrooms and spacecraft surfaces are assayed using swabs and wipes that are then heat shocked for 15 min at 80°C to select for spores. The samples are further processed using the pour-plate method and Petri plates (TSA plates), with trypticase soy agar (TSA) serving as the growth medium. This sampling and processing procedure, called the NASA Standard Assay (NSA), is used by PP engineers around the world. Recent years have seen an increase in the incorporation of state-of-the-art technology, such as membrane filtration, into the NSA, with a push for implementing environmentally friendly technology into day-to-day activities. Dehydrated thin film media, such as Petrifilm Rapid Aerobic Count (RAC) plates, suit these goals as an alternative method to TSA plates. RAC plates show bacterial growth (and distinguish colonies from foreign particles such as bubbles) faster than TSA plates due to the incorporation of chromogenic colour indicators in the media. RAC plates also possess a much smaller environmental footprint than TSA plates, and are designed to evaluate even some of the challenging-to-detect environmental organisms, including spreaders that fill over 25% of the plate area in only a few hours. With these benefits in mind the PP Group at the NASA Jet Propulsion Laboratory took on the task of comparing RAC plates directly to TSA plates within the context of the NSA. Not only were the RAC plates able to detect surface environmental samples and *in vitro* spiked samples equivalent to NSA-processed TSA plates, but spreader organisms were countable on RAC plates at culture densities 10- to 100-fold greater than on TSA plates. In addition, RAC plates showed a robust, linear detection capability when challenged with membrane filter incorporation and organisms were easily acquired from RAC plates for archiving or post-processing experiments including MALDI-TOF bacterial identification. With their ease of use, small footprint, and both rapid and accurate bioburden measurements, RAC plates have the potential to overcome limitations posed by current PP culturing protocols.

Contents

Introduction

Since the ratification of the 1967 Outer Space Treaty, Planetary Protection (PP), employing policies developed by the Committee on Space Research, has guarded against the transfer of biological contamination to celestial bodies, known as forward contamination, or the transfer of adverse extraterrestrial biologics to Earth, referred to as backward contamination (United Nations, 1967; Meltzer, 2011; Rummel and Pugel, 2019). PP is implemented in many ways. Spacecraft are assembled in cleanrooms and sampled by PP engineers to enumerate microbial burden, and techniques such as heat microbial reduction are performed to decrease bioburden present on hardware (Chung *et al*., 2008). The National Aeronautics and Space Administration (NASA) also establishes PP requirements through the release of NASA Procedural Requirements documents that apply to NASA Centers, contractors and grant recipients (Science Mission Directorate, 2008; Seasly, 2020). These PP policies and procedures are increasing in importance as outer planet missions reach farther into the solar system and closer to icy satellites, which potentially harbour life in their subsurface water (Beauchamp and Belz, 2013).

With the increased need for PP implementation, the addition of state-of-the-art technology into current PP spacecraft bioburden evaluation methods is a recent research priority (Hendrickson *et al*., 2020; Pratt and Smith, 2020). PP spacecraft bioburden evaluation is performed via a protocol known as the NASA Standard Assay (NSA) (NASA, 2010). In the NSA, once samples are acquired from spacecraft and associated surfaces (using cotton swabs or polyester wipes), they are suspended in sterile water (for swabs) or buffered solution (for wipes) followed by sonication for two minutes. Samples then undergo heat shock at 80°C for 15 min to enable the detection of primarily aerobic, mesophilic, cultivable spores and heat-shock survivors (Moissl-Eichinger *et al*., 2015; Rettberg *et al*., 2019; Wood *et al*., 2021). Portions of each sample are poured into Petri plates followed by the growth medium, tryptic soy agar (TSA). The plates are incubated at 32°C for a period of 72 h, during which colony forming units (CFUs) are counted every 24 h. Often, additional steps must be taken to ensure accurate organism quantification. For instance, the presence of air bubbles or debris in the growth media is difficult to distinguish from CFUs, necessitating a dissecting microscope for further resolution. Additionally, bacterial spreaders, colonies that grow rapidly to result in overlapping growth and often cover greater than 25% of the plate area, are routinely encountered in environmental samples (particularly in non-cleanroom spacecraft assembly facilities), decreasing plate count accuracy by overlapping other colonies. Finally, PP engineers often manually prepare TSA media for Petri plates, plate the samples, and subsequently count and analyse tens of thousands of spacecraft and cleanroom samples during a typical flagship-class mission, the most complex mission class in the NASA portfolio. Manually preparing TSA plates and plating the samples is not only labour- and cost-intensive but also generates considerable waste (for Mars 2020, approximately 11.9 kilograms, 26.18 pounds, of waste[1] per

1This number is based on the TSA plate weight of 1 g TSA, 15 g petri plate, and 20 g water (which equates to 36 g total per plate). Then, an estimate of 330 plates needed per week estimated from Mars 2020 over 6 years of work is used to determine the number.

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week) and takes up a substantial amount of physical bench and incubator space. With an ever-increasing worldwide participation in life-detection missions of outer planets, a more compact, efficient and sustainable PP culture media is necessary.

Designed to overcome several of the challenges with agar media outlined above, Rapid Aerobic Count (RAC) plates (3 M Company, St. Paul, MN) are ready to use, thin film culture devices for the isolation of bacteria from a variety of food and environmental surfaces (Chandrapati and Nordby, 2014, 2015; Bird *et al.*, 2016). The presence of incorporated chromogenic indicators leveraging the oxidative and fermentative pathways of microbes makes these culture devices particularly useful to easily visualize and enumerate CFUs. Additionally, the underlying technology in RAC plates helps overcome the interpretation challenges associated with extracellular molecules produced by certain spore formers that result in a spreading morphology on agar plates (Kinsinger *et al.*, 2005).

In the present study, we investigate the effectiveness of RAC plates compared to TSA plates to qualify the RAC plates for use in the NSA on future missions requiring PP implementation. To study the dynamic range of performance on the RAC plates, 10 to 250 CFUs were comparatively assessed on RAC and TSA plates, with and without the inclusion of a membrane filtration processing step prior to plating. To test the range of microorganisms that the RAC plates can measure, microbial spreaders were evaluated on RAC and TSA plates using three organisms with demonstrated spreading morphology, *Bacillus subtilis* (Kinsinger *et al.*, 2005), *Paenibacillus lactis* (Scheldeman *et al.*, 2004), and a natural *Bacillus* isolate from a Mars Perseverance assembly facility; *Bacillus atrophaeus* served as a non-spreader control (Maturin and Peeler, 2001). Finally, direct comparison of RAC plates to TSA plates was investigated through side-by-side environmental sampling using swabs. The implementation of RAC plates into the NSA will not only save future flight missions time through more efficient culturing and counting of microorganisms but will also provide a more environmentally-friendly approach through reduced waste and a smaller footprint.

Materials and methods

Bacterial cultures

B. atrophaeus spores were obtained from Mesa Laboratories (Bozeman, MT). *B. subtilis* was obtained from a Mars Exploration Rover (MER) PP sampling event prior to MER rover egress from hardware final assembly (closeout) and archived at −80°C in glycerol. *P. lactis* was obtained from a Phoenix Mars Lander tendril plate sampling event and archived at −80°C in glycerol. Mars 2020 Isolate 1736-9-I, a *Bacillus* of unknown species, was obtained from a Mars 2020 cleanroom countertop and stored at −80°C on Microbank PL.170 cryobeads from Pro-Lab Diagnostics (Round Rock, TX). Growth of organisms from frozen stock cultures was facilitated by streaking the spores onto TSA plates and incubating for 24 h at 32°C before harvesting a single colony for further experiments. Detailed microbial archive storage information for the organisms used in this study is included in Supplementary Table S1.

CFU detection accuracy

To make a 1:1 comparison between TSA plates and RAC plates minus NSA processing or membrane filtration, a starting concentration of 1.9×10^7 spores ml^{-1} was diluted into DI water (3 M, 2020) to a final concentration of 10, 20, 100 and 250 spores ml^{-1} of *B. atrophaeus* spores (Boseman, MT). Then, 1 ml of each concentration of spores was immediately pipetted directly onto RAC plates or onto TSA plates. Counting occurred at 24, 48 and 72 h on both RAC and TSA plates. TSA plates were then manually counted using a darkfield colony counter and RAC plates were counted using the Petrifilm Plate Reader Advanced (3 M Company, St. Paul, MN). A flow chart illustrating the experiment process from start to finish is shown in Fig. 1, and the result from this experiment is shown in Fig. 2. This experiment was also performed to evaluate dehydrated thin film media accuracy relative

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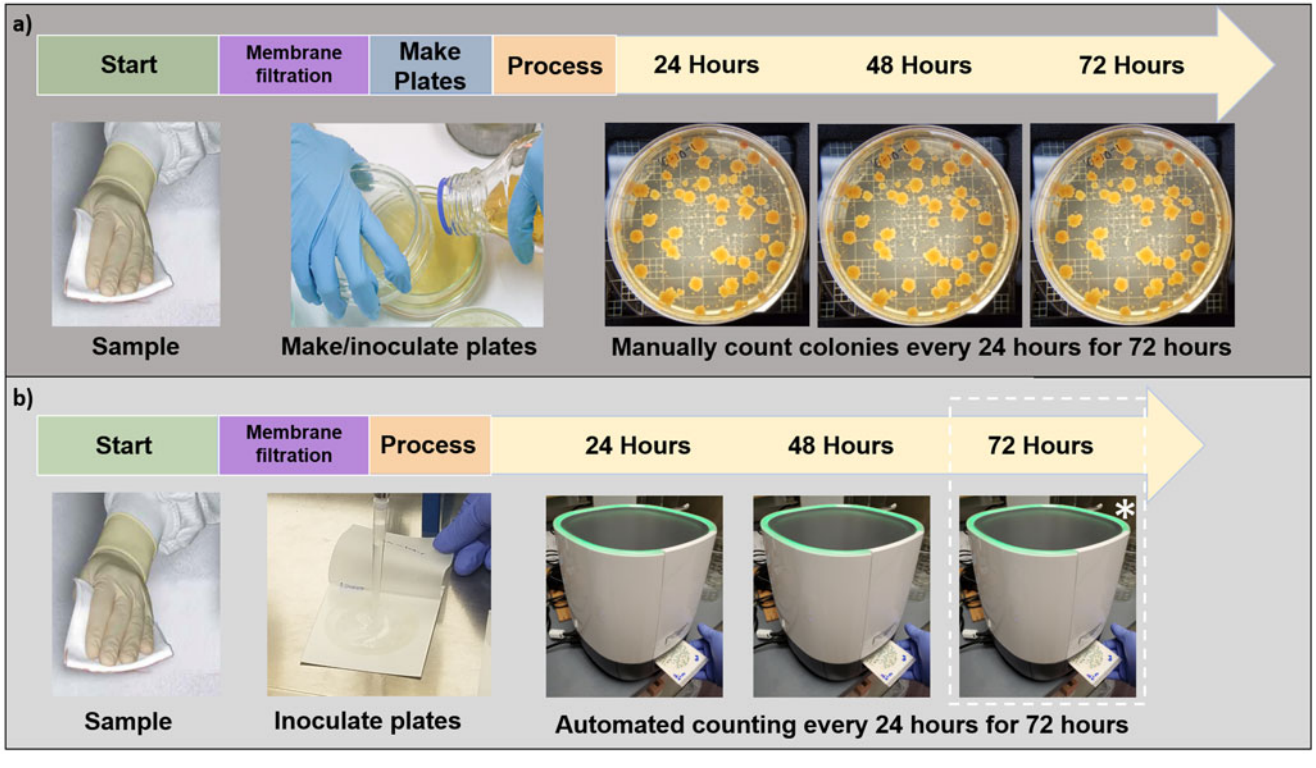

Figure 1. *The process flow to directly compare RAC and TSA plates. Differences between the two experiments include (a) needing to pour plate TSA and make the plates for each NSA, where (b) RAC plates are pre-made. TSA plates are also manually counted for the NSA (a), and RAC plates are automatically counted using the Petrifilm Plate Reader Advanced (b). *The RAC plates were counted for 72 h to directly compare RAC and TSA plates in this experiment (the results for which can be seen in Fig. 2), but RAC plates should be evaluated at only 24–48 h due to chromogenic indicators that highlight small colonies from dirt or background media.*

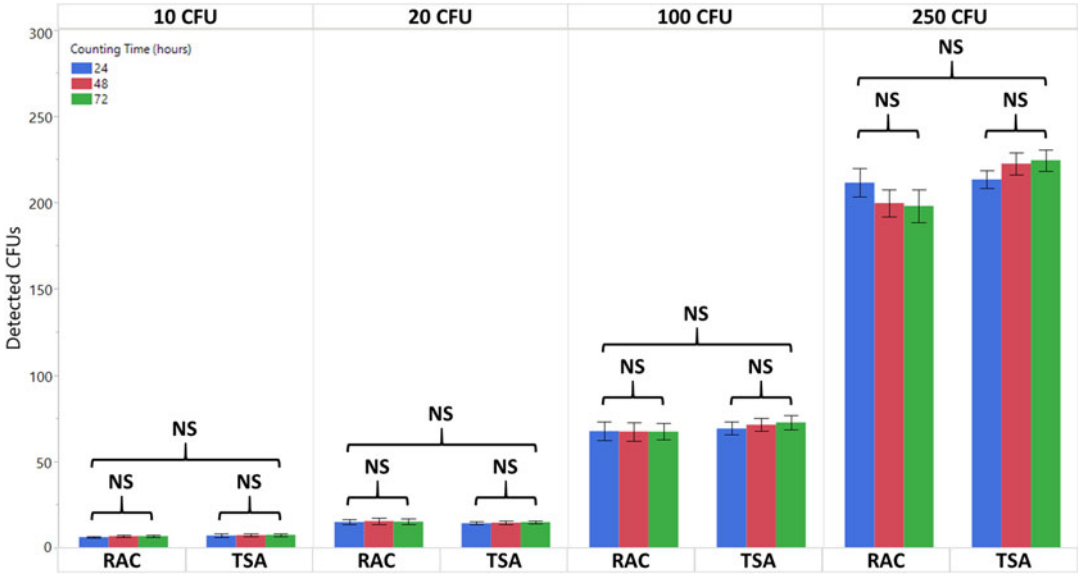

Figure 2. *10, 20, 100, 250 CFUs of* B. atrophaeus *spores were counted at 24, 48 and 72-h on both RAC and TSA plates. At each CFU target, no significant change was detected over time regardless of the plate type. In addition, a small difference in CFUs (between 0.4 and 27 average) was detected on the plates at each time point when comparing plate types with each other. (NS, not significant with* $P > 0.05$*). 18 replicates were used for each sample, and negative controls included with the plates incorporated blank TSA and RAC plates (which did not grow colonies). Manual counting of 250 CFU plates versus automated counting using the Petrifilm plate reader took an average of 88.51 s versus 6.27 s using the reader. By comparison, manual counting of 10 CFU samples versus automated counting using the Petrifilm plate reader took an average of 4.60 s versus 6.75 s using the reader.*

to TSA pour plates. The definition of accuracy that we are using, according to the National Institute of Standards and Technology, is the 'closeness of the agreement between the result of a measurement and the value of the measurand' (Taylor and Kuyatt, 1994); in this case, the value of the measurand is the target CFU count.

Membrane filtration

A membrane filtration procedure has been used for PP flight implementation beginning with the Europa Clipper mission (Stott *et al*., 2022). The option to use this method in lieu of pipetting spacecraft or cleanroom samples directly into empty petri dishes increased the pour fraction (the ratio of processed sample volume to acquired sample volume) as well as decreased the waste produced from flight mission sample processing by minimizing the number of plates needed for PP bioburden evaluation.

Figure 3 illustrates the membrane filter setup along with a flow-chart of the membrane filtration procedure. The membrane filtration equipment includes a 3-place Pall laboratory manifold with hose barb cap, end cap and manifold valves (Port Washington, NY). The equipment also includes manifold standard adaptors, 150 ml magnetic filter funnels, Whatman 0.2 μm cellulose acetate membrane filters (Cytiva, Marlborough, MA), sterile forceps, DI water, 1/4" inner diameter Tygon E-3603 (Saint-Gobain, Malvern, PA) laboratory tubing, and a 1 l glass filter flask. The membrane filtration setup consisted of the following: The Pall laboratory manifold was first connected to the top of a filter flask using Tygon tubing and a rubber stopper. The barbed end of the filter flask was attached to a vacuum pump via Tygon tubing. The 150 ml magnetic filter funnels were placed into each of the three manifold openings via the manifold standard adaptors, and subsequently, the 0.2 μm Whatman filters were placed inside the magnetic filter

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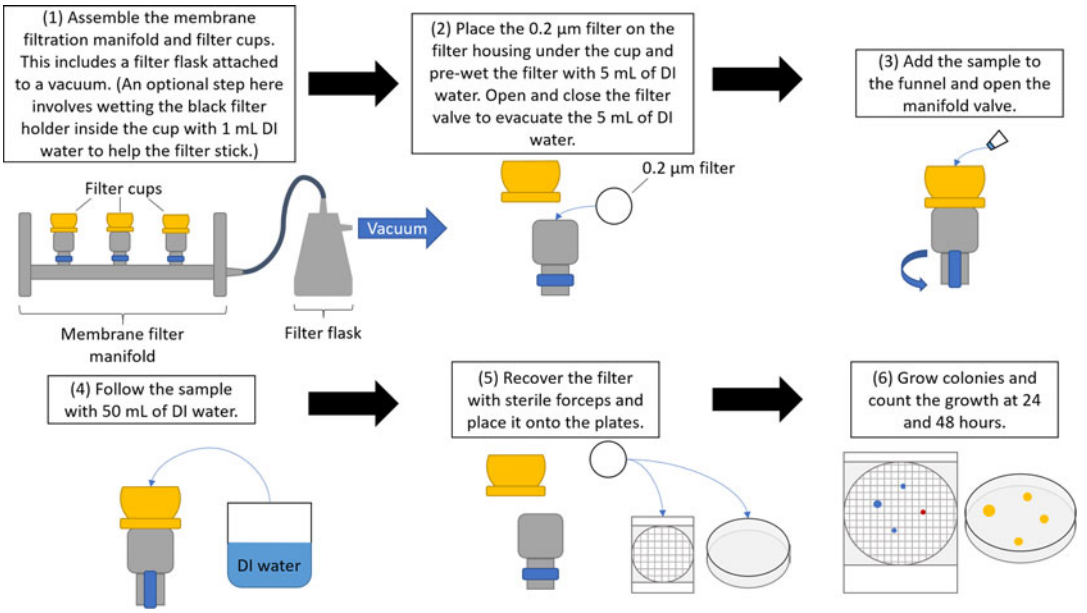

Figure 3. *The membrane filtration setup (upper-left) along with the membrane filtration procedure used in the experiments. A protocol similar to this is currently used on Europa Clipper Planetary Protection sample processing (Stott* et al.*, 2022).*

funnels using sterile forceps. To begin filtration, 5 ml of sterile DI water was run through each funnel to wet the filters. Next, sample volumes were added to the funnel; see CFU Detection Linearity and Environmental Samples for Enumeration, for further methods description.

CFU detection linearity

During membrane filtration, after wetting the filter, 50 ml of sterile DI water was added to each funnel. To that volume, 1 ml of a known concentration of spores was added and subsequently filtered. Finally, the funnels were rinsed with an additional 50 ml of sterile DI water. Using sterile forceps, the membrane filters were removed from the funnels and placed onto RAC plates by lifting the top film from each plate and lowering the filter onto the centre of the plate. 1 ml of sterile DI water was added to the top of the filter to hydrate each RAC plate. The RAC top film was then lowered and a Petrifilm Flat Spreader (6425, 3 M Company, St. Paul, MN) was used to distribute the sample evenly before placing the plates in an incubator. The membrane filter RAC plates were counted using the Petrifilm Plate Reader Advanced at 24 and 48 h. Figure 4 illustrates the ability of RAC plates to detect organisms with the addition of membrane filters.

Environmental samples for enumeration

Environmental samples were obtained from JPL facilities (non-cleanroom facilities were used due to ease of access) using a cotton-tipped swab (806-WC, Puritan, Guilford, ME) as follows: swabs were moistened with sterile DI water before sampling a 25 cm^2 surface (National Aeronautics and Space Administration, 2010; Probst *et al*., 2010; Benardini and Venkateswaran, 2016). Following sampling, the swab head was aseptically broken off from the shaft and transferred into a test tube containing 10 ml sterile DI water. Twenty high-contact surfaces were sampled and subsequently combined into five sets (with each set originating from a separate location) of four samples, where the four swabs in each set were vortexed and pooled, resulting in a total volume of 40 ml. This 40 ml sample was then processed

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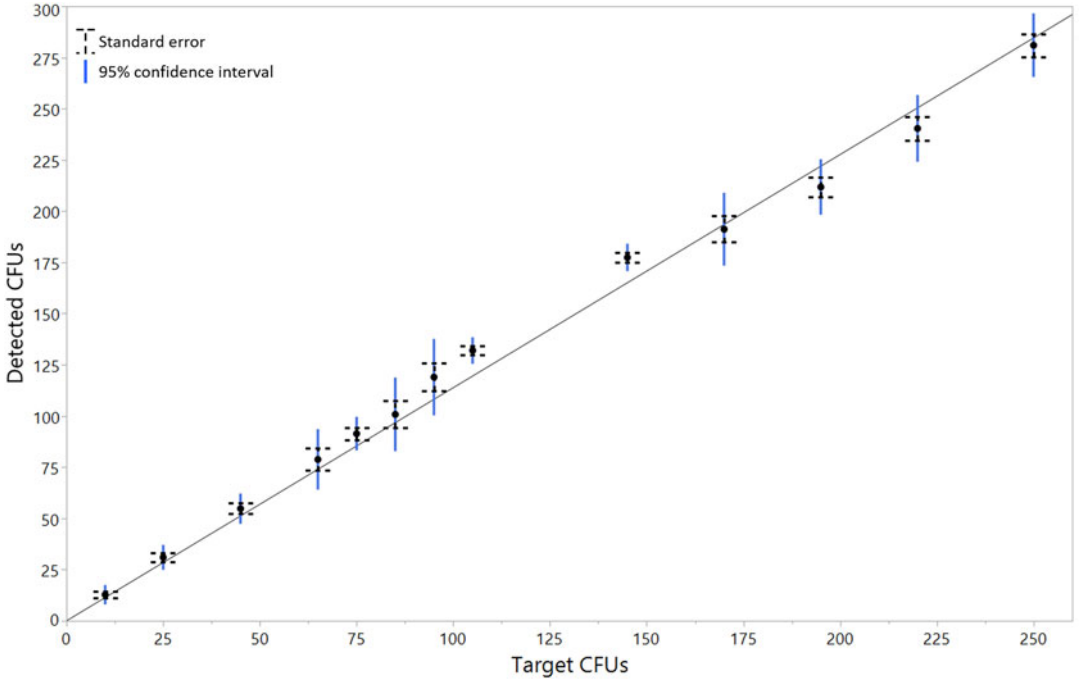

Figure 4. *The ability of RAC plates to support the growth of a range of* B. atrophaeus *spores from 10–250 CFUs captured on 0.2* µm *membrane filters. The* x*-axis represents the CFUs added to the membrane filter, and the* y*-axis represents the number of CFUs acquired from the filters. The error bars shown are* ± *SEM (dashed lines) and the 95% confidence interval (blue solid lines) with a sample size of at least 5 for each point. 5 replicates were used for each sample, and a table with the data is available in Table S2. Negative controls included DI water filtered and placed on RAC plates.*

per the NSA (NASA, 2010). The samples were each sonicated for two minutes, divided into two 20 ml aliquots, and treated as follows: one set of samples was subjected to heat shock (80°C±2, for 15 min) while the other set was processed without a heat shock step. Each 20 ml sample was then split into two 10 ml aliquots and processed via membrane filtration for both RAC or TSA plates. *B. atrophaeus* (100 µl of a 10^4 spore ml^{-1} population) was pipetted onto a membrane filter before placing on TSA and RAC plates as a positive control, with an observation of growth considered a passing result. Similarly, no growth on a negative control (DI water processed through membrane filtration and followed by filter placement on TSA and RAC plates) was considered a passing result. A handling control was also included, which consisted of exposure of a DI water-moistened swab to air in the same facility where a surface sample was collected. As with the positive and negative controls, the handling control was processed through membrane filtration, and the filter was placed on both TSA and RAC plates; no growth was considered a passing result. The plates were incubated at 32°C for 48 h and evaluated at 24 and 48 h. The results from these samples can be seen in Table 1.

Genus and species identification with MALDI-TOF (M-T)

Bacterial isolates from sampling events are often archived (identified and frozen) for PP flight implementation. The microbial repository allows researchers to recognize future Earth-sourced microbial contaminants (e.g., from extraterrestrial samples analysed robotically on planets such as Mars). A subset of colonies on RAC plates from the environmental sampling experiment were harvested via inoculating loops and streaked for further isolation on TSA plates. This was done to demonstrate viability for

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Table 1. *The table of MALDI-TOF results stemming from organisms grown on RAC plates*

Sample	Location	Likely species	Database
Sample 4, Heat Shock, Isolate 1	Pooled between computer mouse and buttons, light switch and floor	Bacillus aerophilus (2.44)	PP
Sample 4, Heat Shock, Isolate 2	Pooled between computer mouse and buttons, light switch and floor	Brevibacterium _sp._IN_308 (2.31)	PP
Sample 5, Heat Shock, Isolate 1	Pooled between cabinet door, keyboard keys, floor, markers	Bacillus foraminis (2.46)	PP
Sample 5, Heat Shock, Isolate 2	Pooled between cabinet door, keyboard keys, floor, markers	Bacillus licheniformis _M E R_13 6 (2.52)	PP
Sample4, Heat Shock, Isolate 3	Pooled between computer mouse and buttons, light switch and floor	Bacillus_subtilis _M_465 (2.4)	PP
Sample 3, Non-Heat Shock, Isolate 1	Pooled between refrigerator handle, chair, table and hand rail	Prestia (Bacillus) megaterium _Ph_03A1.1.1 (2.56)	PP
Sample 3, Non-Heat Shock, Isolate 2	Pooled between refrigerator handle, chair, table and hand rail	Domibacillus enclensis (1.99)	-
Sample4, Non-Heat Shock, Isolate 1	Pooled between computer mouse and buttons, light switch and floor	Bacillus licheniformis_ MER_13 6 (2.35)	PP
Sample 5, Heat Shock, Isolate 3	Pooled between cabinet door, keyboard keys, floor, markers	Bacillus licheniformis (2.11)	BDAL
Sample 5, Heat Shock, Isolate 4	Pooled between computer mouse and buttons, light switch and floor	Bacillus licheniformis (2.23)	BDAL

Identification scores were considered using the following ranges: 1.7–2.0, probable genus identification; 2.0–2.2, highly probable genus identification & probable species identification; 2.2–3.0, highly probable species identification. Despite the usage of both the Bruker stock MALDI-TOF database as well as the Planetary Protection archive database only the two bottom rows (Sample 5) returned with higher scores using the Bruker stock database compared to the Planetary Protection archive database. This suggests that RAC plates are suitable for post-growth processing steps within current Planetary Protection assays and protocols. ($n = 4$ samples were used for the MALDI evaluation, and the highest scores were selected of each sample set for the scores reported below.) Growth did not occur in samples 1 or 2, which are not shown in the table. Multiple laboratory sample locations were pooled to increase the likelihood of a positive test in this experiment. Controls, like with the NASA Standard Assay, included a handling control for both TSA and RAC plates.

post-RAC culture isolate identification and downstream organism archiving capability. Following 24 h of culturing on TSA plates, organism identification was performed via a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometer (MALDI Biotyper CM System, Bruker Daltonics, Billerica, MA). The Bruker Real-Time Classification software was used to compare spectra generated from each isolate to both a custom JPL PP database (Seuylemezian *et al*., 2018) and the manufacturer provided Bruker Daltonics, Inc. (BDAL) spectral library containing a total of 7281 MSPs (Main

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Spectrum). Identification scores were considered as follows: 1.7–2.0, probable genus identification; 2.0–2.2, highly probable genus identification & probable species identification; 2.2–3.0, highly probable species identification. Table 1 shows the scores used for identification from MALDI-TOF, and Supplementary Table S2 demonstrates the growth record of environmental samples on RAC plates.

RAC plate versus TSA plate spreader enumeration

Spreaders, large bacterial colonies that may grow to 25% or more of a culture plate, cause decreased resolution when evaluating bacterial colony growth (Maturin and Peeler, 2001). In the context of PP flight implementation, spreaders are occasionally found in cleanroom environments, and can potentially contribute to spacecraft surface contamination. Thus, increased resolution in enumerating spreaders is value added to determine the cleanliness of these spacecraft assembly environments. Four different microorganisms were tested to compare the ability of RAC plates and TSA plates to enumerate different densities of spreaders. *P. lactis, B. subtilis*, Mars 2020 Isolate 1736-9-I (a *Bacillus*), and *B. atrophaeus* (as a non-spreader control) were first thawed and streaked onto TSA plates before incubating for 24 h at 32°C. A single colony was subsequently harvested and mixed into 10 ml of TSA before incubating at 37°C and shaking at 100 RPM overnight. The cells were washed in PBS before eight serial dilutions in sterile DI water were performed and 1 ml from each dilution was plated onto each plate type, TSA and RAC. Plates were incubated at 32°C for 72 h. The spreaders on plates were checked at 24, 48, and 72 h for each plate type. Spreaders were identified in the dilutions where a single colony grew to over 25% of the plate area. Figure 5 illustrates the ability of RAC plates and TSA plates to enumerate spreaders commonly found in PP flight samples. The ability of TSA plates and RAC plates to enumerate spreaders at different dilutions (densities) was evaluated.

Data analysis and statistics

Data analysis and statistical analysis was performed using JPL 14 by SAS Institute, Inc. (Cary, NC). Experimental data were evaluated as mean ± standard error of the mean (SEM), and replicate measurements were used where error bars are shown. To evaluate differences between two independent groups a student's t-test was used, and for the comparison of multiple groups a one-way ANOVA analysis with post-hoc Tukey's multiple comparison test was used. $P < 0.05$ was accepted as statistically significant unless otherwise specified.

Results

Cfu detection accuracy

Following an initial optimization of the plated volume using NSA-specific procedures showed that 1 ± 0.25 ml should be added to RAC plates (Supplementary Figure S1), the comparable CFU detection ability of RAC and TSA plates for *B. atrophaeus* spores was evaluated (Fig. 2). 1 ml of *B. atrophaeus* spores were plated on both RAC and TSA plates without membrane filtration by performing a set of serial dilutions to target 10, 20, 100 and 250 CFU per plate. CFUs were then evaluated at 24, 48 and 72 h following the start of incubation – similar to the current procedure used in the NSA. While minor CFU differences were observed over time for both RAC and TSA plates, the differences in the CFUs acquired were statistically similar regardless of the time point or the plate type.

CFU detection linearity

RAC plates were evaluated for their ability to support the growth of spores concentrated onto 0.2 μm Whatman filters via membrane filtration, across a range of 10–250 CFUs. The objective was to evaluate the linearity of RAC plates to measure up to 250 CFUs to match the FDA's recommended maximum

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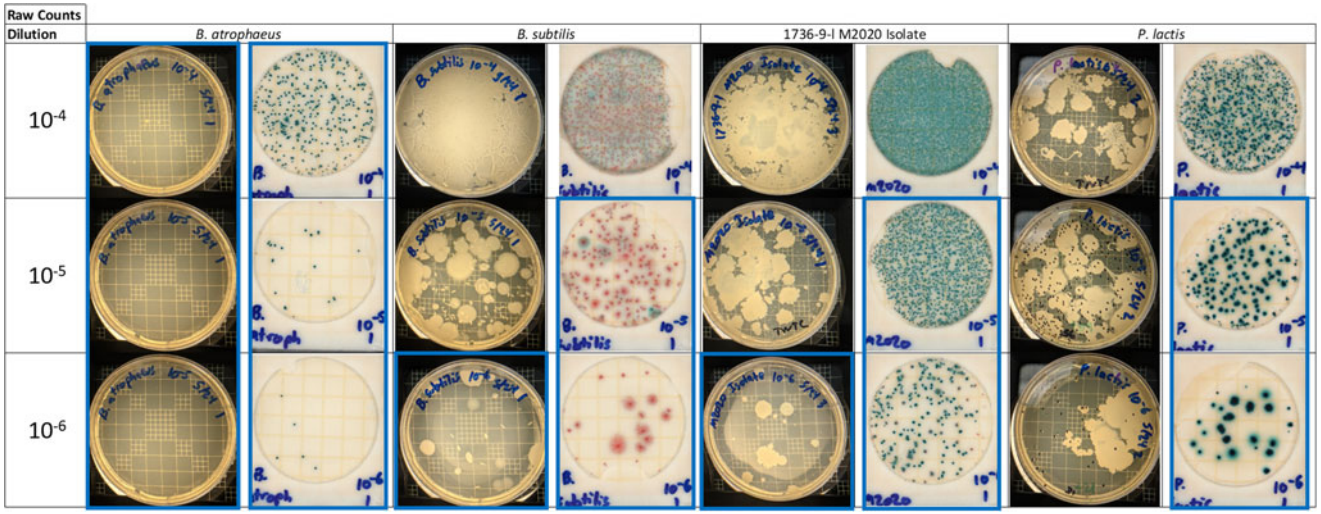

Figure 5. *RAC plates (right), TSA plates (left). Appearance of organisms exhibiting spreader morphology. The images for* B. atrophaeus*, B. subtilis, and 1736-9-I M2020 Isolate are following incubation at 37°C for 24 h. (*P. lactis *is pictured after 48 h of incubation at 37°C as spreading did not occur until 48 h.) RAC plates were able to culture distinct colonies at higher dilutions (densities) than TSA plates for all 3 spreader organisms – with B. atrophaeus acting as a non-spreader control for reference. RAC enumerated at* 10^{-5} *versus* 10^{-6} *for TSA with B. subtilis,* 10^{-4} *versus* $>10^{-6}$ *for TSA with 1736-9-I M2020 Isolate, and* 10^{-4} *versus* $>10^{-6}$ *for* P. lactis*. 3 replicates were performed for each organism, and negative controls for each experiment included blank RAC and TSA plates. The dilutions where individual spreader colonies were distinguishable are highlighted by a blue box surrounding the image.*

for aerobic plate counts (Maturin and Peeler, 2001). This study was necessary to incorporate membrane filtration since the membrane filters have a growth area of just over half that of RAC plates: only 17.35 cm^2, compared to 30 cm^2 culture area for RAC plates. In other words, this test ensures that up to 250 CFUs can still be evaluated in a more crowded, smaller surface area. Overall, despite the smaller growth area of the membrane filters, the data were still highly linear from 10 to 250 CFUs with an R^2 variance of 0.9909 and an intersect at 0 CFUs (Fig. 4). These results were also acquired through counting the plates at 24 and 48 h.

Comparison between RAC and TSA plates using environmental samples

RAC and TSA plates were together evaluated for recovery of environmental isolates to complement the previous *in vitro* comparison experiments in a non-cleanroom indoor environment. This was accomplished by collecting and processing swab samples from JPL facilities (high-contact surfaces) using the NSA (as described in the methods) to emulate the PP approach used for flight missions. Swabs from multiple surfaces were pooled to increase the likelihood that positive samples return in this assay. The heat shock step enabled the detection of aerobic, mesophilic, cultivable spores that can survive 80°C for 15 min and can grow at 32 °C on TSA. The non-heat shock samples enabled the detection of aerobic, mesophilic, cultivable bacteria at 32 °C on TSA that may or may not exhibit thermoduric properties. Overall, five out of ten total samples resulted in growth at 24 h of incubation on both RAC and TSA (Supplementary Table S2). As expected, a very low bioburden, often in a fractional positive range (<1 CFU 1 ml^{-1}), was observed with a comparable recovery between the two plate types. RAC plates demonstrated the ability to recover this low-level bioburden. Extended incubation up to 48 h resulted in the coalescence of colonies and spreader morphology for both RAC and TSA plates.

Genus and species identification with MALDI-TOF

To determine if the RAC plates maintain viability of the isolates for archiving purposes, various morphologically unique colonies were selected from RAC plates that had growth in the environmental sampling experiment. These colonies were subsequently isolated on TSA and identified taxonomically using the Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometer (Table 1). The output resulted in a secure genus identification of *Bacillus* among a majority of the isolates. Moreover, there was a highly probable species identification including *Bacillus aerophilus*, *Bacillus foraminis*, *Bacillus licheniformis* (matching an isolate from the Mars Exploration Rover mission), *Bacillus subtilis* (matching an isolate from the InSight mission), and *Prestia (Bacillus) megaterium* (matching an isolate from the Phoenix mission). All plates but one produced MALDI-TOF scores above 2.0, which is good enough to identify a genus. Of the nine identified colonies, seven were identified primarily from the PP MALDI database stemming from past-flight mission archived organisms over the Bruker BDAL database (Seuylemezian *et al.*, 2018). These results demonstrate that RAC plates can support the growth and maintain viability of a PP-relevant set of microorganisms.

Evaluation of organisms with spreader morphology

Organisms with spreader morphology pose a unique enumeration challenge. By coalescing together and often taking up 25% or more of the growth area of the plate (Maturin and Peeler, 2001), these types of organisms make quantification particularly difficult (or impossible). Therefore, the ability of RAC and TSA plates to detect spreader organisms occasionally isolated from spacecraft or in spacecraft assembly facilities was examined. Three spore species isolated from spacecraft assembly cleanrooms and from three separate missions were chosen: *P. lactis*, *B. subtilis*, and Mars 2020 Isolate 1736-9-I (a *Bacillus* of unknown species). The spreading morphology exhibited by the test organisms was first verified via observation every 24 h for a total of 72 h after culturing on TSA. Next, the

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organisms were diluted and plated from stationary phase growth, in TSB. While the initial starting population was not known, the goal was to determine relative differences in TSA and RAC plate ability to determine spreader growth based on guidance from FDA BAM Chapter 3 (Maturin and Peeler, 2001). As can be seen from Fig. 5, the evaluation of a greater density of spreaders is improved on RAC plates compared to the TSA plates. RAC plates were able to culture distinct colonies at higher dilutions (densities) than TSA plates for all three spreader organisms (10^{-5} versus 10^{-6} for TSA with *B. subtilis*, 10^{-4} versus $>10^{-6}$ for TSA with 1736-9-I M2020 Isolate, and 10^{-4} versus $>10^{-6}$ for *P. lactis*).

Discussion

In this study, Petrifilm RAC plates, which are dehydrated, thin-film media, were evaluated for inclusion into the PP NSA as a spacecraft and cleanroom bioburden spore culturing method alternative to TSA plates. While TSA plates have been a mainstay in the NSA for over 50 years, recent NASA guidance for PP aims to modernize the field by incorporating state-of-the-art technology while at the same time reducing the field's carbon footprint. RAC plates provide a number of characteristic advantages over TSA plates, including a smaller footprint, decreased plate preparation time, less waste (see the supplementary data for a detailed calculation based on Mars Science Laboratory), more rapid colony appearance on plates (with the assistance of chromogenic indicators), and automated counting. These many characteristic advantages, alongside the experimental results within this manuscript, combine to demonstrate that dehydrated, thin-film media is a viable spore growth evaluation mechanism update to the NSA.

The first question answered in this manuscript asked whether the RAC plates can detect CFU with the accuracy and precision of TSA plates for 10, 20, 100 and 250 target *Bacillus atrophaeus* CFUs at 24, 48 and 72-h incubation periods. An initial experiment testing the plated volume of spores on RAC plates versus TSA plates showed that 1 ± 0.25 ml is needed specifically for the NSA as too little volume will not completely hydrate the plate and too much will cause the sample to run off of the plate (Supplementary Figure S1 and Supplementary Figure S2). Using 1 ml per plate, RAC plates were shown in this study to detect *B. atrophaeus* spores, the biological indicator organism for the NSA, up to 72 h at the same rate as TSA plates (Fig. 2). The precision of the assays can be determined from the standard deviation, as ISO 5725-4 defines precision as 'the closeness of agreement between independent test results obtained under stipulated conditions' (International Organization for Standardization, 2020). An average of 11.34 standard deviation was measured for RAC and a similar 9.15 average standard deviation was measured with TSA plates. Both RAC plates and TSA plates additionally produced statistically equivalent CFUs (using a one-way ANOVA with post hoc Tukey's multiple comparison test and $P < 0.05$) regardless of the measurement time in the assay. While the CFU counts increased over time in some places and decreased in others, perhaps due to RAC being counted automatically and TSA being counted manually, the difference in plate counts over time for each assay was statistically insignificant. Finally, RAC plates with automated counting capabilities demonstrated consistent counting times from 10 CFUs to 250 CFUs per plate at 6.75 and 6.27 s, respectively (Supplementary Figure S3). Manual counting TSA plates took 4.60 s for 10 CFU plates and 88.51 s for 250 CFU plates, showing that time saved in counting thin film media like RAC plates increases as the colony counts increase. An additional experiment also demonstrated that the Petrifilm Plate Reader Advanced matched manual colony counts for all timepoints and CFUs (except for 200 CFUs counted at 24 h, Supplementary Figure S4). The increased counting efficiency along with the matching colony counts between RAC and TSA plates for 10, 20, 100 and 250 CFUs up to 72 h show that RAC plates are not only capable of detecting CFUs equivalent to the NSA's TSA plates, but can also be more efficient in terms of evaluation time.

RAC plates were also compatible with membrane filtration, which has been used in the NSA since the start of the Europa Clipper mission, and it carries many advantages, including the ability to process large sample sizes on the order of hundreds of millilitres or more, to the ability to filter out very small numbers of organisms in a sample (Lee *et al.*, 2007). As membrane filtration is the PP processing

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method of the future, RAC plates were therefore evaluated for their ability to detect *B. atrophaeus* from 10 to 250 CFUs with the inclusion of membrane filters into the plates (Fig. 4). RAC plates could detect CFUs linearly for the entire CFU target range with an R^2 variance of 0.9909 and the intersection of the line going through the origin (0 CFUs). The 95% confidence intervals for the data points in Fig. 4 also show that with the inclusion of membrane filtration, RAC plates can evaluate organisms down to 0 CFU. The ability to detect from 0 to 250 CFU is significant particularly because – although most PP spacecraft sampling results return less than 10 CFUs – cleanroom samples occasionally return with up to 250 CFUs (or TNTC, too numerous to count).

Cleanroom and laboratory facilities can additionally return with spreader samples, single colonies that rapidly grow to 25% of the plate area or more. RAC plates were able to enumerate spreaders at more concentrated dilutions than TSA plates for common spreader organisms *B. subtilis* and *P. lactis*, while moreover evaluating a previously unknown spreader *Bacillus* sampled directly from a Mars 2020 spacecraft assembly cleanroom at a more concentrated dilution than TSA plates as well. The ability of RAC plates to capture spreaders, which are often found in spacecraft facility sampling events, in 10-fold or greater concentrations than TSA plates (Fig. 5) will allow PP engineers to more accurately characterize the cleanliness of spacecraft assembly sites. RAC plates were also evaluated for their ability to detect slow-growing extremophile organisms normally difficult to detect in the NSA on TSA plates (Wood *et al.*, 2021).

Finally, RAC plates demonstrated the ability to recover low-level bioburden from environmental samples (Supplementary Table S2) using the NSA to mimic swab samples taken from spacecraft hardware. RAC plates were equally able to distinguish heat-shocked organisms from non-heat-shocked organisms along with traditional TSA plates even with bioburden present at less than 1 CFU per ml. An important note is that RAC plates are designed to detect aerobic organisms, and therefore are a logical comparison to TSA plates; should fungi or anaerobes be desired for detection and analysis there are other thin film media available for use. This study's focus is entirely on improving long-standing weaknesses in TSA plate culture within the NSA, and therefore the fungal and anaerobic plates are a future research topic; however, fungi and anaerobes are possible for analysis as a recent work analysed the potential of thin film media to evaluate fungi for built environments such as the International Space Station (Simpson *et al.*, 2022).

Supporting post-growth microbial identification of organisms, a common, critical PP flight support activity, was also performed successfully (Table 1). Organisms were harvested from RAC plates and run through MALDI-TOF analysis for identification. Comparison to the stock Bruker MALDI-TOF library along with additional spacecraft MALDI-TOF isolate data going back to 1975 (Seuylemezian *et al.*, 2018) identified *Bacillus aerophilus*, *Bacillus foraminis*, *Bacillus licheniformis*, *Bacillus subtilis* and *Prestia (Bacillus) megaterium* in the analysis. The identification data also shows that RAC plates can detect slow-growing (potential extremophile) organisms (Tiago *et al.*, 2006; Tourney *et al.*, 2008; Mahariawan *et al.*, 2020) normally difficult to detect in the NSA on TSA plates (Wood *et al.*, 2021). Harvesting organisms from thin film media is also an important activity as future PP organism detection methods will likely employ metagenomics as a flight activity (Wood *et al.*, 2021). Overall, these results suggest the applicability of dehydrated thin film media for monitoring environmental samples in flight missions.

Conclusion

This study reports the capability of RAC plates as an accurate and effective method for measuring spacecraft and cleanroom bioburden within PP's NSA. RAC plates matched the detection efficiency of TSA plates in measuring the growth of 10, 20, 100 and 250 CFU of *Bacillus atrophaeus* for 24, 48 and 72 h. RAC plates also linearly detected *Bacillus atrophaeus* from 10 to 250 CFU with the addition of membrane filtration, a bioburden technique recently employed by PP. The R^2 variance for RAC plate linear detection was 0.9909 with an intersection at the origin, or 0 CFUs. As a variety of organisms, including spreaders, appear in PP spacecraft and cleanroom sampling events, the ability of RAC

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plates to at least match TSA plates in detecting a range of common environmental bioburden was assessed as well. RAC plates not only detected *P. lactis*, *B. subtilis*, and Mars 2020 Isolate 1736-9-I (a *Bacillus* of unknown species) at the same concentration as TSA plates, but they additionally enumerated spreaders from all three species at a 10 to 100 CFU/ml^{-1} greater culture density than TSA plates. The ability of RAC plates to enumerate environmental samples was additionally determined through wet swab sampling of twenty high-contact surfaces using the NSA as a protocol for both sampling and processing. Enumeration of the environmental samples at 48 h resulted in the detection of minimal bioburden in the fraction positive range, less than 10 CFU ml^{-1}, or no growth on either TSA or RAC plates. The RAC plates are suitable for enumerating bioburden as part of the NSA with or without membrane filtration. Moreover, the small footprint, fast growth through incorporation of chromogenic indicators, and decreased waste compared to TSA plates, provide additional environmental and logistical advantages.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S1473550423000149.

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Conflict of interest. The authors declare that they have no competing interests.

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