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RESEARCH ARTICLE

Microbial protocols for spacecraft: 2. Biocidal effects of Delrin and nylon in sealed compartments may enhance bioburden reductions in planetary spacecraft

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Abstract

Interplanetary spacecraft are assembled with thousands of parts composed of many diverse materials. Little is known on whether any of the spacecraft materials are biocidal to the typical microbiomes that develop on spacecraft during pre-launch processing. During ongoing experiments to examine the interactive effects of solar UV irradiation, solar heating, ionizing radiation, and vacuum, we observed that bacterial spores of three *Bacillus* spp. were killed when incubated within small vacuum chambers for 5 days – without exposure to the aforementioned factors. Eight potential spacecraft materials were tested within the vacuum chambers for biocidal activities against spores of *B. atrophaeus* ATCC 9372, *B. pumilus* SAFR-032 and *B. subtilis* 168. All three species were fully inactivated (i.e., no survivors detected) by machined parts manufactured from Delrin®; a thermoplastic polyacetal polymer. Although not tested here, it is known that Delrin can off-gas formaldehyde, and thus, we hypothesize that this volatile organic compound (VOC) was responsible for the biocidal activity of the material. Knowledge of the biocidal nature of routinely used spacecraft materials might offer diverse methods to inactivate deeply embedded or shielded microbiota within spacecraft via the release of biocidal VOCs.

Contents

Introduction

Interplanetary spacecraft are developed to explore diverse planetary bodies within the Solar System. Some spacecraft are targeted for landing on planetary bodies with important astrobiological science goals. Europa, Enceladus and Mars are the three most important targets for astrobiological exploration because liquid water is believed to be present within subsurface oceans (i.e., Europa and Enceladus),

surface brines (Mars) and subsurface ices (Mars) (Spohn and Schubert, 2003; Rummel *et al*., 2014; Walker and Schmidt, 2015). However, many of these targets have restrictions on exploration because of the perceived risks to the science goals due to forward contamination posed by microorganisms found on spacecraft surfaces. Planetary protection protocols (Frick *et al*., 2014) are typically initiated to mitigate against the risks of forward contamination of sensitive sites like Special Regions on Mars (see Kminek *et al*., 2010; Rummel *et al*., 2014).

During preliminary research on the interactive effects of solar UV irradiation, solar heating, ionizing radiation (IRAD) and vacuum, we developed a small Ionizing Radiation Vacuum Chamber (IVC) system composed of commercially available parts from numerous vendors. All of the parts were initially believed to be inert and non-reactive to the cleaning, sterilization, and experimental protocols used in the project. However, in the first series of experiments in which multiple IVC units were included, all spores of three *Bacillus* spp. were found to be completely inactivated (i.e., no survivors recovered from aluminium coupons during the assays) by exposure to sealed IVC units over 5 days – without exposure to the aforementioned factors. Results suggested that at least one material within the IVC system was biocidal to the endospores of all three bacteria.

The primary objective of the current project was to characterize the biocidal effects of eight materials used within the IVC systems on spore survival. A secondary objective of the project was to confirm that a standard Most Probable Number (MPN) assay (described below) was removing most – if not all – of the *Bacillus* spp. spores from the aluminium coupons used as a proxy for a structural spacecraft material. Biocidal activity of any treatment requires confidence that the extraction process is highly efficient, and thus, a reduced number of recovered spores or cells represents a true estimate of the biocidal nature of the treatment.

Methods

Biological samples

Spores of three different *Bacillus* species were selected based on their tolerance to space conditions, UV-resistance, or relevance for space exploration: (1) *Bacillus atrophaeus* ATCC 9372, (2) *Bacillus pumilus* SAFR-032, and (3) *Bacillus subtilis* 168. Spore suspensions of *B. atrophaeus* were purchased from Mesa Labs (Lakewood, CO, USA). Stock spore suspensions of *B. pumilus* and *B. subtilis* were prepared using a liquid sporulation medium as described by Mancinelli and Klovstad (2000).

The bacterial spore suspensions of *B. atrophaeus* 9372, *B. subtilis* 168 and *B. pumilus* SAFR-032 were used to prepare the working suspensions by adding the spores into a 0.5 critical micelle concentration of Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) solution (i.e., the final concentration of the stock Triton X-100 solution was 0.006875%). Spore concentrations were adjusted to $\sim 2 \times 10^7$ spores ml^{-1} by optical density (at 600 nm) with a spectrophotometer (Geneysis 30, Thermo-Fisher Scientific, Madison, WI, USA). Estimates of actual spore densities were determined by pipetting 100 μl aliquots of the spore-Triton mixtures directly into a test tube containing 9.9 ml sterile deionized water (SDIW) and then serially diluting the stock solution (1:9) seven times in SDIW. Each of the lowest six dilutions was dispensed into two columns of a 96-well plate containing growth media (cat. no. 087722C, Thermo-Fisher Scientific), incubated at 30°C for 48 h, and examined for bacterial growth using a MPN method described by Schuerger *et al*. (2003, 2005, 2006).

Sample preparation on aluminium coupons

Uncoated aluminium coupons (54 mm × 17 mm × 0.5 mm) were purchased from Seton (quote 26233681, Seton, Chicago, IL, USA). Coupons were cut in half with tin-snips, heat-sterilized at 130°C for 24–72 h, and then doped with 100-μl aliquots of one of the spore suspensions such that each coupon received $\sim 2 \times 10^6$ spores of individual *Bacillus* spp. in a drop ~ 1 cm in diameter (when dried). Spore suspensions were allowed to settle 18 h overnight and dried in a NuAire Class II,

Type A2 biosafety cabinet (model NU-440-600, NuAire, Inc., Plymouth, MN, USA) for 3–4 h under a stream of sterile air. The deposition protocol (Schuerger *et al.*, 2003; Schuerger, 2022) yielded uniform monolayers of spores. The qualities of the monolayers were confirmed by either stereo light microscopy or by scanning electron microscopy (SEM) (e.g., see Schuerger *et al.*, 2003; Schuerger, 2022). Surface textures of the coupons, spore densities viewed with SEM, and recovery efficiencies for the MPN and polyvinyl alcohol (PVA) protocols were previously reported (Schuerger *et al.*, 2005; Tauscher *et al.*, 2006; Schuerger, 2022).

All survival data were compared to what are called $T = 0$ *controls* that represent recovered numbers of spores from coupons immediately after the monolayers were dried; and thus, served as the most accurate starting population of viable spore numbers on the coupons. The $T = 0$ controls are represented by dashed lines in the figures below.

IVC design

IVCs (Figs. 1 and 2) were developed for a separate project to hold ten aluminium coupons doped with diverse bacterial species. The goal of the other ongoing research was to characterize the effects of IRAD under vacuum (10^{-2} hPa) and exposure to IRAD (e.g., protons, electrons, neutrons, X-rays or δ-rays). The IVC units were constructed to hold the bacteria under vacuum while being irradiated. During preliminary experiments, entire populations of non-irradiated controls were inactivated for all three *Bacillus* spp. after 5 days in the sealed IVC units at 1013 hPa and 24°C. Results suggested that one or more of the materials within the IVC units were biocidal against the *Bacillus* spp. The research below was designed to determine the cause of the biocidal activity.

Most parts for the IVC units were purchased from Ideal Vacuum Products, LLC (Albuquerque, NM, USA), except as indicated below. Some parts required a design phase and a custom quote from Ideal Vacuum Products. A dissembled IVC system is depicted in Fig. 1. Within each IVC internal void space, one Microbial Sample Holder (MSH) was designed to hold 10 ea, Al-coupons upon which spores of the three *Bacillus* spp. were applied separately. Once all coupons were secured to the upper surface of the MSH-Delrin component, the MSH was inserted into the IVC internal void space, secured with a nylon allen-wrench bolt, the IVC top secured to the base unit and the system sealed until used at an IRAD source. A fully assembled IVC unit is depicted in Fig. 2 (shown here with Al-coupons mounted to a black polycarbonate MSH unit).
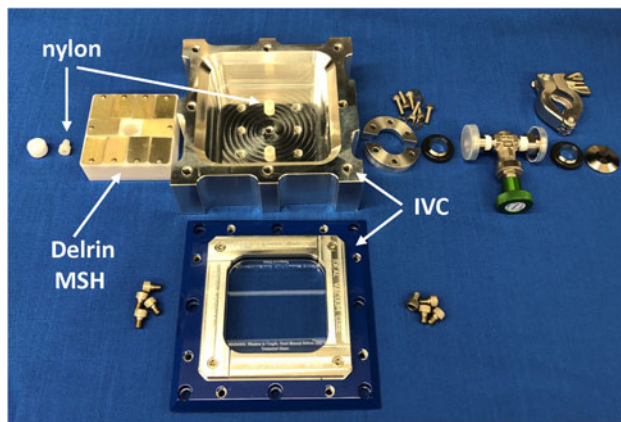

Fig. 1. *Disassembled Ionizing Radiation Vacuum Chamber (IVC) system with one Delrin Microbial Sample Holder (MSH) and three nylon screws. Ten aluminium 6061 microbial coupons were mounted on the upper surface of the Delrin MSH. Once mounted, the Delrin MSH would be inserted into the void space in the base IVC unit. The IVC system top was 15.2 × 15.2 cm in both the left/right and top/down dimensions. The internal void space was 372 cm³.*
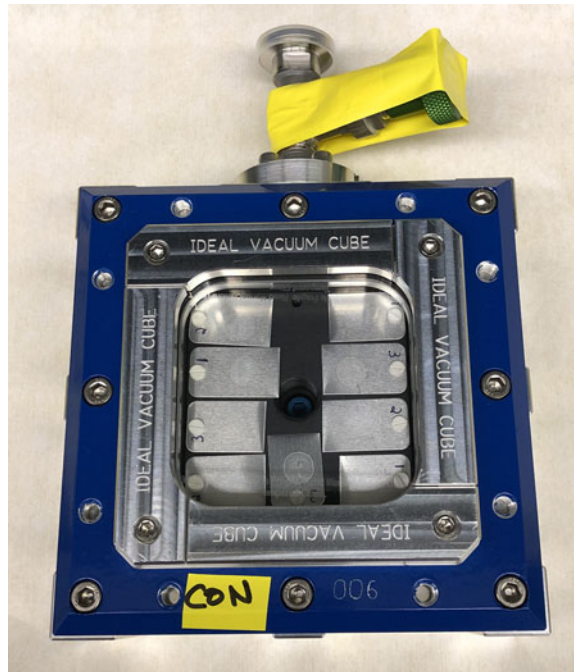
Fig. 2. *Fully assembled Ionizing Radiation Vacuum Chamber (IVC) system. Black polycarbonate is shown here as the MSH material holding nine aluminium 6061 coupons. Each coupon is doped with ∼2 × 10⁶ spores in 1-cm wide bacterial monolayers. Thus, in this photo, there are three replicates each of three* Bacillus *spp. mounted on a polycarbonate MSH unit in a sealed IVC system.*

Two different MSH units were custom made using either Delrin or black polycarbonate. The Delrin and polycarbonate MSH units were machined by EMF, Inc. (Merritt Island, FL, USA) from stocks obtained from McMaster-Carr (see Table 1; Orlando, FL, USA) with a design developed by our team. Initially, Delrin was the preferred material due to its greater resistance to degradation by liquid sterilizing solutions like ethanol and isopropanol. Table 1 gives the vendor names and part numbers for all materials within the IVC system not obtained from Ideal Vacuum Products.

Testing the effect on spore viability of various materials used in IVC assembly

Each biocidal assay tested the individual materials (Table 1) within sterilized IVC units by placing only that material on the floor of the internal void space within an IVC unit and transferring Al-coupons doped with bacterial spores to the same surface. The internal void spaces within the IVC units were surface sterilized with sterile cotton wipes saturated in 70% isopropanol. The sterilized IVC units were allowed to dry in a forced-air biosafety hood for at least 30 min prior to initiating an assay. Once the materials and *Bacillus*-doped coupons were placed within an IVC unit, the tops were carefully attached, screwed down, and stored on a lab bench at 24°C for 5 days.

After incubation, spores were recovered from the coupon surfaces and resuspended by one of two methods. The samples were either processed according to (1) a standard MPN protocol (Schuerger *et al.*, 2003, 2005, 2006; Schuerger, 2022) or (2) a polyvinyl alcohol (PVA) spore recovery method (Lindberg and Horneck, 1991; Tauscher *et al.*, 2006). In brief, for the standard MPN protocol, coupons were placed in 50 cc conical tubes (Fisher Scientific) containing 20 ml DI water and 1 g sterile silica sand and agitated for 2 min with vortexing. Samples were then serially diluted, and the lowest six dilutions pipetted into 96-well plates (two columns of wells per dilution). Each well contained 180 μl of trypticase soy broth (Difco, Fisher Scientific) and 20 μl of the diluted spore suspension. Plates were incubated for 48 h and then scored for bacterial growth using a MPN method (Mancinelli and Klovstad, 2000).

Table 1. *Parts tested in the biocidal assays described herein*

Part name	Part no.	Vendor	Number used in IVC
IVC unit (Fig. 1)	See Supplemental data	Ideal Vacuum Products	1
Delrin	8573K128	McMaster-Carr	1
Polycarbonate	85625K48	McMaster-Carr	1
Nylon bolt (Lg)	95868A735	McMaster-Carr	4
Nylon screws (Sm)	94735A716	McMaster-Carr	10
PTFE screws	94701A611	McMaster-Carr	10
Polycarbonate screws	93140A403	McMaster-Carr	10
GAF-1 (Gafchromic HD-V2) radiation film	947344	Ashland Specialty Ingredients	1
GAF-2 (HD-V2 radiation film plus graphite SEM stubs mounting film)	HD-V2 (#947344) + PELCO tabs (16084-3)	Ashland Specialty Ingredients + Ted Pella	1

IVC, Ionizing Radiation Vacuum Chamber; Lg, large bolt/screw; Sm, small bolt/screw; PTFE, Teflon; GAF-1, Gafchromic HD-V2 radiation film; SEM, scanning electron microscopy.

For the PVA assay, an aqueous 10% (w/v) solution of PVA was used to coat bacterial spores after the 5-day incubations. Each sample received 125 µl of PVA solution, pipetted such that the PVA covered the entire monolayer without disrupting the monolayer with the pipet tip. The PVA-coated coupons were maintained – until dry – inside square petri plates and incubated at 37°C for approximately 5 h and then stored at room temperature overnight in petri plates until processed the following day.

Dried PVA coatings were peeled from coupons with sterile forceps and placed inside 50 cc conical tubes containing 20 ml SDIW and 1 g silica sand. Samples were shaken for 10 s to dissolve the PVA and then serially diluted following the same protocols as the standard MPN assay described above. Each IVC unit contained three replicates of each bacterial strain, and each experiment was performed twice ($n = 6$).

Three separate assays were completed in which similar materials were grouped. In all cases, empty IVC units with no other materials – but containing aluminium coupons with replicates of the three *Bacillus* spp. – served as the controls for each assay.

Experiment-1 compared the biocidal effects of the Delrin and black polycarbonate MSH units, Gafchromic H2-V2 radiation film (GAF-1), Gafchromic H2-V2 radiation film attached to the Al-coupons with PELCO graphite tabs (Table 1) used to secure samples to SEM stubs (GAF-2), and an empty IVC unit. Assays were maintained for 5 days at 24°C and the coupons with *Bacillus* spores were assayed with the standard MPN protocol.

Experiment-2 compared the biocidal effects of small bolts and screws used within the IVC units which included the following materials: large nylon screws (4 ea), small nylon screws (10 ea), PTFE teflon screws (10 ea), polycarbonate screws (10 ea) and an empty IVC unit (Table 1). Assays were maintained for 5 days at 24°C and the coupons with *Bacillus* spores were assayed with the standard MPN protocol.

Experiment-3 was designed to determine if significant loss of recovered numbers of spores was correctly assigned to biocidal effects of the test treatment, or if some of the decrease in the number of recovered spores could be attributed to spore adherence to the surface of the coupons. Previously, Tauscher *et al.* (2006) demonstrated very high removal rates of *B. subtilis* spores from similar Al-coupons with a PVA assay. The PVA versus MPN assay would indicate if spores were being completely removed from the Al-coupons using the standard MPN assay. Experiment 3 consisted of a cohort of Al-coupons doped with *B. atrophaeus* ATCC 9372, (2) *B. pumilus* SAFR-032, or (3) *B.*

subtilis 168 placed within empty IVC units (no Delrin), incubated at 24°C for 5 days, and then assayed with either the standard MPN or PVA protocols.

Statistics

Data were analysed with the Statistical Analysis System (SAS) version 9.4 (SAS Institute, Inc., Cary, NC, USA). Data were log10 transformed to induce homogeneity of treatment variances and analysed with ANOVA and protected least-squares mean separation tests (LSM; $P \leq 0.05$). However, because ANOVA cannot process zeroes when log-transformations are used, an arbitrary low value of 0.01 was assigned to Excel spreadsheet cells in which no detectable *Bacillus* spores were observed (i.e., numbers of spores were below the detection limit of the MPN assay). All original data for each assay are given as Tables S1–S3 for assays, respectively, (1) Delrin and Polycarbonate MSH units, (2) small screws and bolts used within the IVC/MSH assemblies and (3) the MPN versus PVA extraction protocols.

Results

Of all of the materials tested, Delrin was the most biocidal to spores of all three *Bacillus* spp. after only 5 days incubation within the IVC units (Fig. 3). Furthermore, no viable spores of all three *Bacillus* spp. exposed to Delrin were detected in the MPN assays. In contrast, spores within the empty IVC units were, in general, not significantly different from the $T = 0$ controls (dashed lines in Figs. 3–5), and similar to many of the other materials tested ($P > 0.05$; $n = 6$).

The second most biocidal component of the IVC system was the large nylon screws (part # 9568A735; McMaster-Carr; Table 1) as shown in Fig. 4 ($P \leq 0.05$; $n = 6$). Viable populations of spores were reduced by ∼2.5–3 logs for *B. atrophaeus* 9372, *B. pumilus* SAFR-032 and *B. subtilis* 168. In addition, a slight reduction in the recovered spores for *B. atrophaeus* and *B. pumilus* was observed for polycarbonate screws (part # 93140A403; McMaster-Carr; Table 1) (Fig. 4). Similar to the data presented in Fig. 3, most materials that exhibited minor reductions in spore numbers were similar to each other and similar to the $T = 0$ controls ($P > 0.05$) (Fig. 4).

Lastly, in a direct MPN versus PVA comparison on the efficiencies of recovering spores from Al-coupons, the numbers between the two assays were not significantly different for either the IVC-alone or the Delrin-IVC assays ($P > 0.05$; $n = 6$; Fig. 5). The MPN/PVA comparison within the empty IVC units was not significantly different from each other or from the $T = 0$ controls (i.e., dashed line in Fig. 5) for any of the three *Bacillus* spp. tested ($P > 0.05$). Furthermore, no viable spores were detected in the MPN assays when Delrin was present within the IVC units confirming the biocidal effects of Delrin against all three *Bacillus* spp. presented in Fig. 3. Interestingly, one positive well in the 96-well plates used in the PVA assays for each individual *Bacillus* sp. exhibited growth after incubating the 96-well plates for 48 h at 30°C. Although single positive MPN wells were observed in the Delrin-PVA treatments in each *Bacillus* sp. dataset (i.e., thus creating average responses in plots for Fig. 5), the overall effects were not significant when compared statistically ($P > 0.05$).

Results indicated that (1) the MPN and PVA assays yielded similar results, and thus, are considered confirmatory of each other; (2) Delrin was the most biocidal of all materials tested and that there was no evidence that spores remained attached to the coupons in the MPN assays; (3) large nylon screws used within the IVC units also exhibited biocidal activity against all three *Bacillus* spp. tested but the responses were not as severe as Delrin; (4) most other materials were similar to the $T = 0$ controls except where indicated by slight significant losses of recovered spores; and (5) all responses were similar among *B. atrophaeus*, *B. pumilus,* and *B. subtilis* spores with no species being dramatically different from the other two species.

Discussion

As planetary spacecraft are designed and assembled, information on the potential biocidal effects of materials on the microbiomes on surfaces would be useful to predict the risks of forward contamination
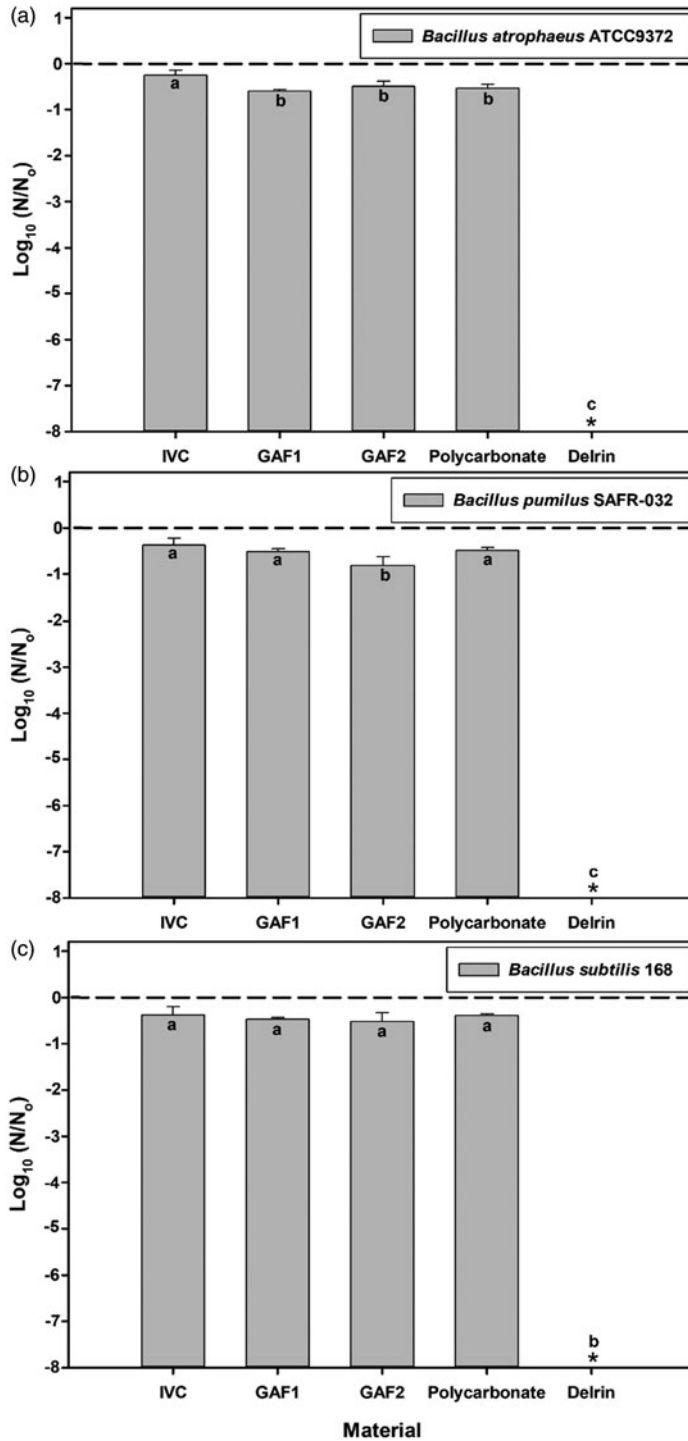
Fig. 3. *Effects of Delrin, polycarbonate, and two Gafchromic film configurations (see text) on the survival of three* Bacillus *spp. Materials were incubated within sealed IVC units for 5 days at 24°C. ANOVA and protected LSmeans tests indicated that Delrin was completely biocidal for* Bacillus *atrophaeus 9372 (a),* B. pumilus *SAFR-032 (b), and* B. subtilis *168 (c) (P < 0.0001; n = 6). Treatments followed by similar letters were not significantly different; each* Bacillus *sp. was analysed separately.*
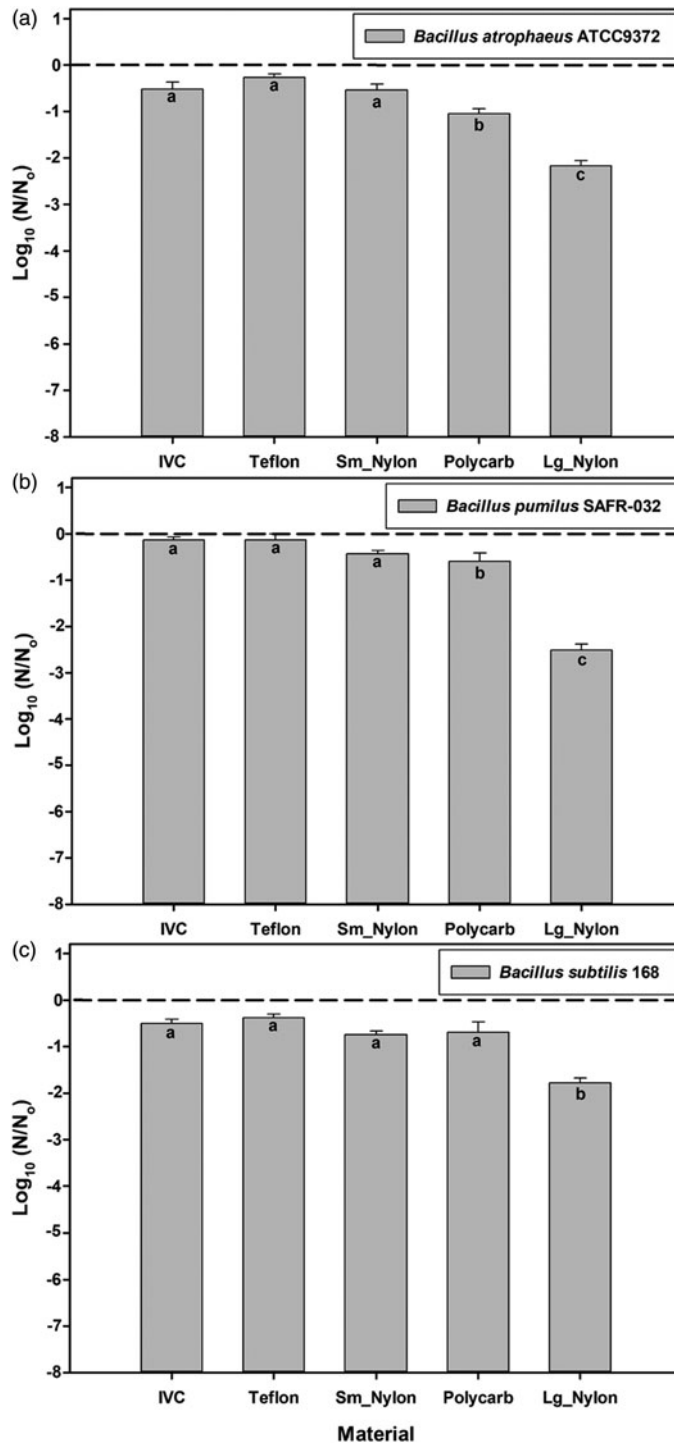
Fig. 4. *Effects of four types of screws on the survival of three* Bacillus *spp. incubated within IVC units for 5 days. ANOVA and protected LSmeans tests indicated that the large nylon screws (Lg-Nylon) induced the greatest biocidal effect of the materials tested against* Bacillus atrophaeus *9372 (a),* B. pumilus *SAFR-032 (b), and* B. subtilis *168 (c) (*P < 0.05; n = 6). *Treatments followed by similar letters were not significantly different; each* Bacillus *sp. was analysed separately.*
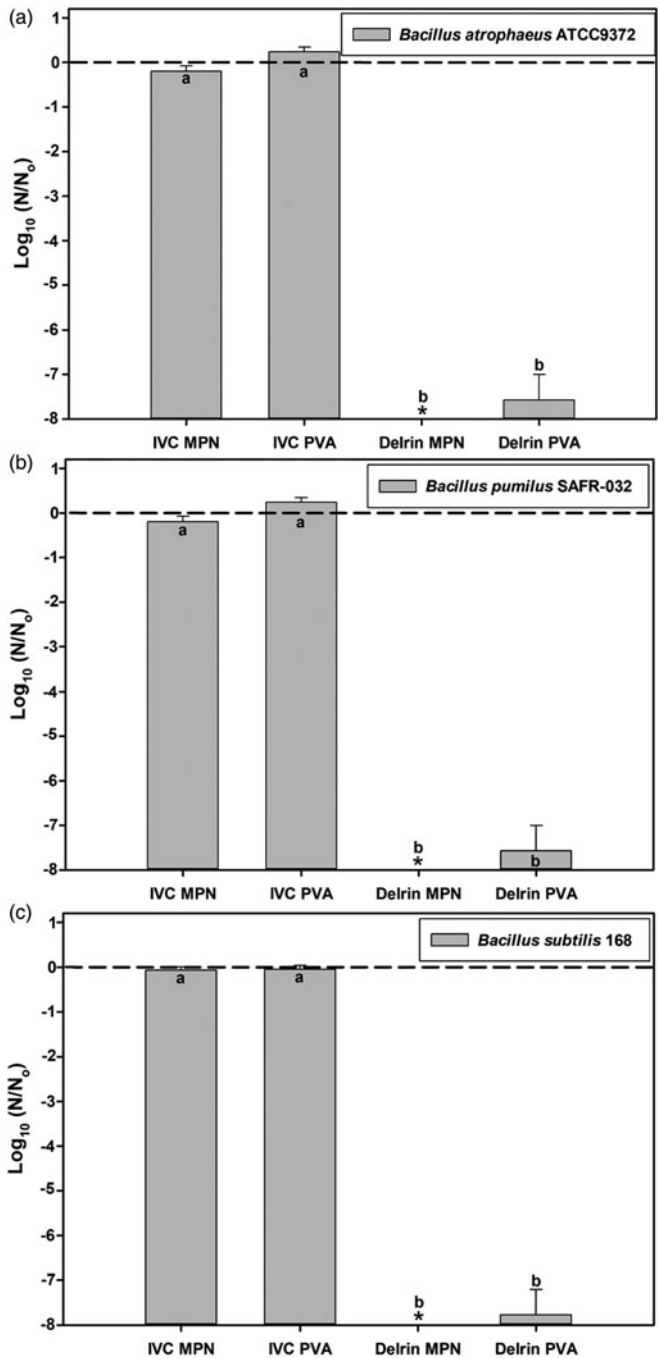
Fig. 5. *Recovery rates for the MPN and the PVA assays for three* Bacillus *spp. applied individually to Al-coupons. ANOVA and protected LSmeans tests indicated that the MPN and PVA assays yielded similar (i.e., not significantly different) recovery rates for* Bacillus atrophaeus *9372 (a),* B. pumilus *SAFR-032 (b), and* B. subtilis *168 (c) (P > 0.05; n = 6). Treatments followed by similar letters were not significantly different; each* Bacillus *sp. was analysed separately. The Delrin-MPN assays yielded no detectable viable spores. In contrast, the bars for the Delrin-PVA assays appear to be larger than the Delrin-MPN assays, but in fact were not significantly different (P > 0.05). Only one positive MPN well for each bacterium was responsible for the bars present in the Delrin-PVA assays.*

of key planetary locations. Here we report on the biocidal effects of two materials on three *Bacillus* spp. when the materials were incubated adjacent to endospores in sealed compartments. By far, Delrin was the most biocidal of all materials tested. One type of nylon screw was also observed to suppress survival of endospores by 2–3 logs. In general, all other materials had either no effect or only a very minor effect on the recovery of viable *Bacillus* spp. endospores from coupons. Although not tested here, it is likely that volatile formaldehyde was given off by Delrin during heat-sterilization of the material (see Colwell, 1970; Kusy and Whitley, 2005; Archodoulaki *et al.*, 2007; Williams *et al.*, 2011; Spinei *et al.*, 2021) causing the death of endospores over short incubation times of 5 days. The biocidal volatile organic compound (VOC) from nylon screws is not known. The biocidal nature of formaldehyde is well established (e.g., Rogers *et al.*, 2007; Guo *et al.*, 2021).

There are three direct applications of the data presented here. First, if some materials are biocidal to spacecraft bacteria or microbiomes (e.g., Delrin, nylon screws), said materials could be used proactively to reduce bioburdens if the biocidal VOCs do not negatively impact other mission constraints (e.g., VOCs damaging sensitive electronics). For example, in a sealed compartment in a large spacecraft like the Europa Clipper (currently underdevelopment at the Jet Propulsion Lab (JPL), Pasadena, CA, USA for flyby missions to Europa), biocidal VOCs might be used effectively to reduce spacecraft bioburdens that are typically hard to reach by liquid sterilization protocols. In fact, biocidal materials deemed to pose no risk to spacecraft might be useable in *preplanned* sealed compartments during early assembly.

Second, Delrin effectively inactivated 100% of spores of three *Bacillus* spp. after only 5 days of exposure, and thus, materials like Delrin could be used to fumigate complex assemblies of spacecraft components over short-periods of time. After an effective time-step is reached, the materials could then be removed from the components or subsystems during final assembly.

Third, knowing the biocidal effects of materials routinely used within spacecraft (e.g., amounts of the materials used and exposure times to achieve >6 log reductions in surface bioburdens) will permit spacecraft engineers to better model and predict the surviving bioburdens at launch. For example, it is plausible that bioburdens on Mars landers were significantly lower than the published numbers (e.g., the Mars InSight lander had an estimated 1.5×10^5 spores for the entire lander; Hendrickson *et al.*, 2020) if materials like Delrin and nylon screws were used within sealed chambers.

Furthermore, the importance of the MPN versus PVA comparison cannot be over emphasized. The PVA assay is a direct removal assay that has been previously shown to remove spores from aluminium coupons at nearly 99% efficiency (Tauscher *et al.*, 2006). In contrast, the MPN assay used here and described previously by Mancinelli and Klovstad (2000) and Schuerger *et al.* (2003, 2005, 2006) relies on a turbulent liquid removal process that might fail to recover all spores if they are tightly bound to the coupons. The observation that the standard MPN and PVA assays were in agreement (Fig. 5) suggests that the MPN data presented in Figs. 3 and 4 (and in Schuerger *et al.*, 2003, 2005, 2006) are accurate representations of all spores present on the coupons during the assays. Thus, the biocidal effects of multiple treatments are likely due to biocidal effects and not spore attachment effects (i.e., stickiness) on coupons.

In summary, materials that produce biocidal VOCs may provide another sterilization protocol for reducing or eliminating bioburdens on spacecraft surfaces. Additional research into the biocidal effects of spacecraft materials should be pursued to further the understanding on how off-gassing VOCs affect microbial survival on spacecraft. Such biocidal VOC-emitting components might be used proactively to reduce launched bioburdens in sealed compartments.

Author contributions. ACS envisioned, wrote and edited the paper as presented above; he was the lead on data analysis. ACS, PS and RTT conducted experiments, assisted in data analysis and co-wrote and co-edited the manuscript.

Conflict of interest. The authors have no financial, commercial or other relationship conflict of interest with any organization listed in the text.

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