Quantification of Encapsulated Bioburden in Spacecraft Polymer Materials by Cultivation-Dependent and Molecular Methods

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Abstract
Bioburden encapsulated in spacecraft polymers (such as adhesives and coatings) poses a potential risk to jeopardize scientific exploration of other celestial bodies. This is particularly critical for spacecraft components intended for hard landing. So far, it remained unclear if polymers are indeed a source of microbial contamination. In addition, data with respect to survival of microbes during the embedding/polymerization process are sparse. In this study we developed testing strategies to quantitatively examine encapsulated bioburden in five different polymers used frequently and in large quantities on spaceflight hardware. As quantitative extraction of the bioburden from polymerized (solid) materials did not prove feasible, contaminants were extracted from uncured precursors. Cultivation-based analyses revealed <0.1–2.5 colony forming units (cfu) per cm² polymer, whereas quantitative PCR-based detection of contaminants indicated considerably higher values, despite low DNA extraction efficiency. Results obtained from this approach reflect the most conservative proxy for encapsulated bioburden, as they give the maximum bioburden of the polymers irrespective of any additional physical and chemical stress occurring during polymerization. To address the latter issue, we deployed an embedding model to elucidate and monitor the physiological status of embedded Bacillus safensis spores in a cured polymer. Staining approaches using AlexaFluor succinimidy 488 (AF488), propidium monoazide (PMA), CTC (5-cyano-2,3-dioctyl tetrazolium chloride) demonstrated that embedded spores retained integrity, germination and cultivation ability even after polymerization of the adhesive Scotch-Weld 2216 B/A. Using the methods presented here, we were able to estimate the worst case contribution of encapsulated bioburden in different polymers to the bioburden of spacecraft. We demonstrated that spores were not affected by polymerization processes. Besides Planetary Protection considerations, our results could prove useful for the manufacturing of food packaging, pharmacy industry and implant technology.

Introduction
In course of space exploration, the potential danger to contaminate celestial bodies with terrestrial microorganisms (or vice versa) has received growing attention. The goal of planetary protection is to mitigate such risks, which are most severe in case of missions to planets where conditions are favorable for life. In order to reduce the risk of contamination and ensure the scientific value of possible life detection missions, the Committee on Space Research (COSPAR) has issued specific regulations for spacecraft cleanliness [1]. Spacecraft under planetary protection limitations are constructed in clean rooms and maintained under bioburden control to diminish risks of microbial contamination. Sampling of the spacecraft-associated microbiome (including microbial heat shock resistant bioburden) is employed to study the microbial diversity of the cleanroom environment, spacecraft elements, and the assembled spacecraft (e.g. [2,3,4,5,6,7,8]). Airborne contamination is collected by active sampling (the most commonly used samplers in the pharmaceutical and medical device industry are impaction and centrifugal samplers [9]), while bioburden on surfaces can be detected by the use of swabs, wipes, or similar devices [10,11,12]. However, these sampling procedures cannot cover all sources for microbiological contamination. A potential contamination pathway that escapes such standard controls is the bioburden encapsulated in polymers, which are widely applied in the construction of spacecraft [13].
For modern spacecraft hardware lighter but often more porous composite materials are preferred over those heavy stainless steel materials used in the Viking era. Beside metal-based materials, spacecraft hardware comprises silicones (e.g. in electronic components, optics, and solar cells), other polymeric materials (e.g. parachutes, airbags, thermal covers, coatings, paints, and wires) and carbon-polyesters used for structures. Due to their porous structure these new materials increase the risk of an accidental contamination of extraterrestrial environments [14].

Bacterial spores embedded or encapsulated in these pores are – to a certain degree – protected from stresses like space vacuum and radiation during space travel and from heating during entry into a planet’s atmosphere [15,16,17,18,19,20,21,22,23]. Such sheltered bioburden has to be considered, since materials degrade, and some parts of a spacecraft intended to land on a planet’s surface may break (e.g. the head shield, the back shell, parts of the active descent system such as engines). In case such impacts do not result in complete sterilization of spacecraft hardware due to entry heating, they may have some potential to inoculate microbes into favorable environments of a planet’s bottomset beds [24,25]. The risk of bioburden enclosure in polymers is not equal for all materials. Structural materials like polyimides are cured at high temperature to observe if sedimentation or polymerization occurred. The samples were incubated up to 24 h at room temperature to observe if sedimentation or polymerization occurred.

Materials and Methods

Bacterial Strains and Cultivation Conditions

Bacterial strains were purchased from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Bacillus safensis DSM 19292T was selected as the standard spore model for most experiments due to its high resistance to various environmental stresses and as an obvious contamination source of spacecraft and clean room environments (B. safensis was isolated from the surface of the Mars Odyssey Orbiter spacecraft and also the assembly-facility surfaces at Jet Propulsion Laboratory and Kennedy Space Center [27]). Available spore preparations revealed a high number of germinable spores, (17.6%) in contrast to Geobacillus stea thermophilus (< 5%) used only for visualization of spores in the embedding model (see below). Hence, the number of viable but non-germinable spores (VBNG) was kept low. Spore suspensions were prepared by lysozyme (200 ng/μL) and DNase treatment followed by heat shock (15 min, 80°C) as described previously [28,29]. For the experiments, B. safensis was incubated for 2–5 d at 32°C on soybean casein digest agar (Becton Dickinson, Heidelberg, Germany). Spores of Geobacillus stea thermophilus DSM 5934T were collected from agar medium and incubated in 65% 2-propanol for 3 h (at room temperature), followed by two wash cycles with deionized water. G. stea thermophilus was incubated 2–5 d at 55–60°C on soybean casein digest agar.

Polymer Materials

The analyzed materials for flight hardware construction and their preparation are listed in Table 1. For each material, the most suitable solvent for dissolution or dilution of the uncured polymer was determined by mixing precursors with different solvents (at concentrations of 10–50 wt.-%) and vigorous vortexing. The samples were incubated up to 24 h at room temperature to observe if sedimentation or polymerization occurred.

Surface-embedding of Spores in Scotch-weld 2216 B/A

B. safensis or G. stea thermophilus spores were washed twice in acetone and diluted to obtain an acetone spore suspension of ~1×10^5 colony forming units (CFU)/mL. One drop of the suspension was spread on the surface of water-soluble polymer (polyvinyl alcohol (PVA)) and dried for 2 d at room temperature. 1–2 g of freshly prepared Scotch-Weld 2216 B/A (Table 1) were placed on top of the dried spore suspension on PVA. The polymer was allowed to cure for 7 d at room temperature. The water soluble PVA was peeled off or washed from the sample after curing, leaving embedded spores concentrated in a layer proximal to the surface of the material.

Scanning Electron Microscopy (SEM) and Dual Beam Focused Ion Beam (FIB)

Samples of inoculated surfaces of the adhesive Scotch-Weld 2216 B/A were prepared on a dual-beam FIB/SEM workstation (FEI Helios Nanolab) and investigated by field emission scanning electron microscopy (SESEM) using a Hitachi SU-4800 instrument operated at 1 kV. Prior to inserting the sample in the FIB, a thin Au layer was sputter-deposited on the surface of the sample to protect the polymer in the subsequent preparation steps. FIB scanned the specimen in a grid to create an image. FIB cut out a 10 μm by 10 μm test area with an ion beam perpendicular to the specimen surface, which was then sectioned by the ion beam in 1 μm steps. Images were taken by an SEM vertically oriented to the ion beam. FESEM images of PVA, and inoculated and non-inoculated Scotch-Weld 2216 B/A allowed visualization of embedded spores in the adhesive. FIB/SEM images verified the partial embedding of the spores necessary for further staining approaches (see Fig. 1).
Table 1. List of materials analyzed, their preparation, and the solvent most suitable for dilution of the uncured precursors.

| Name (Supplier) | Type | Utilization in spacecraft | Preparation | Solvent used for extraction | Density (g/cm³) |
|-----------------|------|---------------------------|-------------|-----------------------------|----------------|
| Scotch-Weld 2216 B/A (3M, France) | Epoxy adhesive | screw locks, bonding | component A (hardener): component B (base) = 7.5 (w/w)\(^1\) | 2-propanol | 1.32 |
| SG121FD (MAP, France) | Silicone coating | Thermal control paint | base : catalyst = 86:14 (w/w) | MAP SG121FD thinner | 1.41 |
| Solithane 113 (Specialty Polymers & Services, USA) | Urethane resin | conformal coating, screw locks, bonding, insulation | S113 : C113–130 (hardener) = 100:70 (w/w)\(^2\) | ethanol | 1.07 |
| ESP 495 (ACC Silicones/EADS Astrium) | Silicone adhesive | Adhesive for thermal protection system | – | acetone | 1.07 |
| Dow Corning 93–500 (Ellsworth Adhesives GmbH, Germany) | Silicone encapsulant | Sealing and bonding | encapsulant ; curing agent = 10:1 (w/w)\(^3\) | acetone | 1.08 |

\(^1\)mixed thoroughly for 5 min.
\(^2\)mixed thoroughly for 1–2 min.

doi:10.1371/journal.pone.0094265.t001

Fluorescence-based Detection of Surface-embedded Spores and Confirmation of their Identity after Re-growth

Detection of (partially) embedded, intact spores in Scotch-Weld 2216 B/A was performed by staining spores with AlexaFluor succinimidyl ester 488 (AF488, MoBiTec, Gottingen, Germany) prior to the embedding procedure and with propidium monoazide (PMA, Biotium, Hayward, CA, USA) subsequent to embedding. AF488 is a fluorescent dye that can be applied for the visualization of many vegetative cells [30] as well as spores [29]. For labeling, spores were exposed to 100 μM ATTO-dye labeling buffer (ATTO-TEC GmbH, Siegen, Germany), to yield unprotonated amino groups of proteins, and 10 μg AF488 were added. Reaction between dye and proteins was enabled at RT in the dark on a shaker for 30 min. Stained spores were washed three times with sterile water to remove unspecific staining. PMA selectively enters wall-compromised cells and binds to DNA [31] as has also been demonstrated for heat-inactivated B. safensis spores [29]. After treatment of spores with 10 mM dithiothreitol (DTT) at 65 °C for 15 min (to increase permeability), they were stained with 50 μM PMA in the dark for 50 min. Cross-linking of PMA with DNA was enabled via halogen light (500 W) exposure for 3 min of samples kept on ice. The labeling procedure was completed by three serial washing steps. Cy5 (cyanine dye 5) was applied as a surface marker, by adding 2 μL of an aqueous dye stock solution (30 μM) on top of the spore preparation.

50 mM CTC (5-cyano-2,3-dioctyl tetrazolium chloride, Polysciences Europe GmbH, Eppelheim, Germany) was used to visualize metabolic activity of B. safensis during its germination process. Spore germination was provoked by incubation in growth medium or glucose solution (at 32 °C, approx. 1–2 h; either in tubes or for embedding models covered with growth media and incubated horizontally). Slightly attached and not properly embedded spores were removed by sonication at maximum intensity (120 W, 35 kHz) for 10 min in a 50 mL falcon tube filled with 35 mL of sterile distilled water to guarantee evaluation of embedded or encapsulated spores. Fluorescently labeled spores were monitored via confocal laser scanning microscopy (CLSM) as described previously [29], using an inverse Laser Scanning Microscope (LSM 510-Meta confocal microscope, CLSM, Zeiss, Munich, Germany). Fluorescence signals of PMA or CTC (excitation 488 nm, detection 505–530 nm) and Cy5 (excitation 633 nm, detection 650 nm) were detected in the multi-track mode to avoid cross-talk phenomena. Z-stacks were scanned with a 50% overlap of each 0.4 μm section. CLSM images were analyzed, arranged and 3D projections were made with the Zeiss LSM Image Browser Software.

CLSM monitored embedding models were subjected to cultivation dependent analysis to support conclusions drawn from fluorescence staining of embedded spores. Scotch-Weld 2216 B/A surfaces were cleaned with 70% (v/v) ethanol to minimize contaminations caused by non-sterile CLSM procedures. For cultivation 1 mL tryptic soy agar was poured on model surfaces and incubated at 32 °C for 4 d. Grown colonies were identified (Genceat Life Technologies, Regensburg, Germany) by their PCR amplified rrnB-16S and gyrB gene sequences [27]. Bacteria-specific primers 9 bF (GRGATCCTGGCTCAG) [32] and 1406uR (ACGGGCGGTGTGTRCAA) [33] (1.25 ng/μL each) were used for rrnB-16S gene PCR containing 200 μM dNTP mix, 2 U of Taq DNA polymerase in Buffer Y with MgCl₂ (10x). DNA templates were amplified in 10 cycles of denaturation
Survival of *B. Safensis* Spores in Uncured Polymer Precursors

To determine the possible sporicidal effects of the uncured polymer precursors, a *B. safensis* spore suspension (10^5 cfu/mL) was washed twice in its respective solvent (depending on the material tested, see Table 1). 0.5 g of freshly-prepared polymer was mixed with the final spore pellet and incubated 0–60 min at room temperature. After adding 1 mL of solvent to the polymerspore mixture and vigorous mixing, the resulting suspension was serially diluted in sodium chloride peptone buffer. The appropriate dilutions were subjected to heat shock (80°C, 10 min, according to specifications of US Pharmacopeia [37]) and pour-plated on soybean casein digest agar. For comparison the cultivable spore number after washing and resuspending in solvent without polymer was determined. For these experiments, growth conditions were always identical for experimental and control samples. It was not attempted to optimize recovery conditions for *B. safensis* as this organism served as model to comparatively evaluate the effect on spore survival of the polymer precursors. Recovery of viable spores after incubation was always lower than the inoculum, which is to be expected due to losses during the experimental procedures, but generally high enough to reasonably expect spores to survive in polymer precursors.

Recovery of *B. Safensis* Spores Grown in the Presence of Nutrient Agar

Components of the polymer materials were prepared as shown in Table 1, diluted in the appropriate solvent (0.1–0.5 g/mL, depending on the solubility of the precursors), and inoculated with *B. safensis* spores to reach a final spore concentration of 10^7 cfu/mL. *B. safensis* spores (10^5 cfu/mL) in solvent only (without the polymer precursors) served as a positive control. Samples were subjected to heat shock (80°C, 10 min) or incubation at room temperature for 2.5 min. PCR sequences were compared with GenBank deposited sequences (http://www.ncbi.nlm.nih.gov/genbank/) using the basic local alignment search tool (blast; http://blast.ncbi.nlm.nih.gov/Blast.cgi) [36].

**Calculation of the Encapsulated Bioburden in Precursors Corrected for the Recovery Efficiency**

To calculate the corrected value for mean encapsulated bioburden (CME), the recovery efficiency was included in the determined colony number.

Recovery efficiency (RE) was calculated by

\[
RE = \frac{MR}{MI}
\]

with a standard deviation of \(sd(RE) = \sqrt{\left(\frac{sd(MR)}{MR}\right)^2 + \left(\frac{sd(MI)}{MI}\right)^2}\).

Here: \(MR\) = mean recovered spores from the polymer precursor, \(MI\) = mean inoculated spores, and calculated standard deviations \(sd(MI)\) and \(sd(MR)\).

Tests for bioburden were repeated 4–10 times for each material, yielding mean and standard deviation of the number of colonies recovered from a certain volume of material (ME, sd(ME)).

Mean encapsulated bioburden \(CME = \frac{ME}{RE}\), with standard deviation:

\[
sd(CME) = \frac{sd(ME)}{RE} \sqrt{\left(\frac{sd(ME)}{ME}\right)^2 + \left(\frac{sd(RE)}{RE}\right)^2}
\]

The values given in this report are conservative values, calculated by \(CME + 1sd(CME)\). When 0 cfu were recovered, calculations were performed with 1 cfu, and CME was given as less than (<) the resulting value.

Cultivation-based Determination of Bioburden in Kaolin

To determine the bioburden of kaolin (filler material of Scotch-Weld 2216 B/A), samples of kaolin in 3 grain sizes (ASP 200, 600, and 900) were obtained from a supplier in Germany (Quarzwerke, Frechen, Germany). Of each grain size, 10 g were suspended in 90 mL sodium chloride peptone buffer (Merck kGaA, Darmstadt, Germany, prepared according to manufacturer’s instructions) and subjected to heat shock (80°C, 10 min) or incubation at room temperature in parallel. Of each sample, 0.1 mL in 10 replicates was spread-plated on R2A agar, and plates were incubated for 7 d at 30–35°C. The respective contribution of kaolin to the weight of the adhesive was taken into account (8.9% for ASP 200, and 11.4% for ASP 600 and ASP 900 each) to determine the total bioburden of Scotch-Weld 2216 B/A.

DNA Extraction from Kaolin and Purification

DNA was extracted from kaolin (filler in Scotch-Weld 2216 B/A) with the Precellys Bacterial/Fungal DNA kit (Peqlab, Erlangen, Germany). 0.1 g kaolin (of three grain sizes: ASP 200, 600 and ASP 900; in triplicates) was suspended in 200 μL Tris-EDTA (TE) buffer. Suspensions were transferred to a 2 mL Precellys tube and processed as indicated by the manufacturer. The extracted DNA (solved in 50 μL Elution Buffer) was purified by precipitation overnight at −20°C with the same volume of ice-cold 2-propanol (abs.), followed by washing with ice-cold ethanol (70% v/v) and including a heat activation step before pour-plating 1 mL aliquots on soybean casein digest agar or R2A agar (Sigma-Aldrich; Steinheim, Germany). Plates were incubated 5–7 d at 30–35°C, followed by 5–7 d at 55–60°C to detect mesophilic and thermophilic spore-formers, respectively. In total, 45–80 cm^2 of each material was investigated. If colonies were detected, they were isolated and visualized microscopically after Gram-staining.
drying of the pellet for 3 h. DNA was resuspended in 15 μL PCR-grade water and stored at −20°C till further use. DNA-free extraction blanks for qPCR experiments were prepared by suspension of a mixture of ASP 200, ASP 600 and ASP 900 (0.035 g each, triplicates) in 1 mL of sterile DNaseI buffer (100 mM Tris-Cl, pH 7–7.5; 25 mM MgCl2; 5 mM CaCl2). 2 μL of DNaseI (1 mg/mL) were added to the kaolin suspension to digest present DNA by incubation at 37°C for 1 h. The enzyme was inactivated at 90°C for 1 h, and removed by washing three times with sterile water. After 2 d of drying at room temperature, the extraction blanks were subjected to the DNA extraction and purification procedure as described above.

DNA Extraction from Polymer Precursors

Samples were prepared by dissolving 5 g Solithane 113 in 10 mL ethanol (abs.). Ethanol inoculated with R. sphaeroides spores (2×10^6) was used as a positive control, and the same volume of ethanol was processed as extraction negative control. Samples were concentrated by centrifugation (8,600 x g, 1 min) and resuspension of the pellet in 100 μL TE buffer; however, the formed precipitates remained undissolvable. For decoating of spores, 500 μL decoating buffer (50 mM Tris Base (pH >9.5), 1% (w/v) sodium docecyl sulfate (SDS), 8 M urea, 50 mM dithiothreitol (DTT), 10 mM Na2EDTA) were added, followed by incubation at 60°C for 90 min with shaking [38]. Samples were washed three times with Sodium chloride-Tris-EDTA (STE) buffer (10 mM Tris-Cl (pH 8), 10 mM Na2EDTA, 150 mM NaCl) and once with Lysis buffer (Precellys Bacterial/Fungal DNA kit, Peqlab). Afterwards, pellets were resuspended in 300 μL Lysis buffer and stored overnight at −20°C. After homogenization of the samples and degradation of cells, following the manufacturer’s instructions as described above (see DNA extraction from kaolin), the suspensions were transferred to pegGold PhaseTrap columns (Peqlab) after short centrifugation (12,000 x g, 20–30 sec). The same volume of 24:25:1 (v:v:v) chloroform:phenol:isoamyl-alcohol was added and carefully mixed, followed by centrifugation at 1,400 x g rpm, 5 min, at room temperature. Subsequently, the supernatant was transferred into a new tube and mixed with 0.057 vol. 7 M ammonium-purification procedure as described above.

Quantitative PCR (qPCR)

Reaction mix for each sample (final volume 20 μL) consisted of 10 μL SYBR Green mix (Qiagen, Hilden, Germany), 1 μL forward (338 hF, ACTTCTACGAGGCGACGAC) and reverse primer (517 rR, GWATATTACCCCGGCGKGTG; 25 ng/μL each) [33,39], 7 μL H2O and 1 μL template (DNA sample or extraction blank or water (negative control)). Prepared qPCR suspensions were gently mixed and tubes were loaded into the Rotor Gene 6000 Cycler. The PCR program was adapted from the SYBR Green protocol (Qiagen) with a hot start step for polymerase activation at 95°C for 15 min and 40 cycles of 94°C for 15 sec, 60°C 30 sec, 72°C 30 sec. qPCR amplification was terminated with a melt cycle from 72°C to 95°C. For analysis of the amplification results, the Rotor Genes 6000 software was used. 1 μL of the rRNA-16S gene PCR product of Bacillus atrophaeus was included as a standard (10^3 to 10^6 rRNA-16S gene copies). Since DNA templates extracted from kaolin interfered with the qPCR, one standard dilution was mixed with a DNA-free extraction blank from kaolin in order estimate the effect on the amplification process. The inhibitory effect in kaolin samples was calculated according to differences between the kaolin extraction blank + standard and the pure standard. This value was used to correct kaolin sample values without an inhibiting effect. Values were equalized to 1 g kaolin and the estimated copy numbers were divided by a mean of all known bacterial rRNA-16S gene copy numbers per cell ([4.1 rRNA-16S gene copies per cell; source: ribosomal database rrnDB [40,41]].

Results

General Remarks and Test Strategy

The test strategy to determine encapsulated bioburden was developed with Scotch-Weld 2216 B/A as an example polymer, because it combined several relevant features. The physical structure of the cured polymer was found suitable for electron and epifuorescence microscopy, and it contained an additional component of filler material, which needed to be investigated. The precursors of the polymer could be dissolved in 2-propanol.

Two test models were developed and afterwards applied to different polymers when feasible.

Determination of Structural Integrity and Viability of Encapsulated Endospores in Cured Polymer (Test Model I)

A surface embedding model was newly developed as a method to allow easy localization of intentionally embedded spores in the cured polymer block, and to facilitate the access for microscopic and electron microscopic techniques. Inoculated spores were partially or completely embedded to a depth of several spore diameters. Hence, they were directly accessible for analytical examination without the necessity of sectioning or fractioning to disrupt the polymer. This preparation method allowed application of a range of detection methods as shown in Results Section 2. This test model was only applied to Scotch-Weld 2216 B/A.

Investigation of Uncured Polymer Precursors to Quantitatively Estimate the Worst Case Bioburden (Test Model II)

While Test Model I allowed localization and characterization of experimentally embedded spores in high numbers, any inherent bioburden of the polymers would be too dispersed to allow detection by microscopic methods in situ. Chemical or physical disintegration of the polymers would have to be applied to extract intrinsic encapsulated microorganisms from cured polymers. In contrast to the experiments performed on lucite [26], solvent dissolution of the cured spacecraft relevant polymers investigated here, was not feasible. Mechanical degradation of the polymer was considered possible, but exceedingly difficult to control with regard to uniformity of the procedure and mechanical spore disintegration. Hence, no advantage was seen in milling or grinding procedures. As an innovative approach, determination of the bioburden of the uncured polymer precursors was pursued. During the terminal step of polymerization (curing) an increase in polymer bioburden is obviously impossible, while physical and chemical processes during polymerization might even reduce viable microorganisms. Hence, bioburden in uncured polymer was considered the most conservative proxy for encapsulated bioburden in cured polymer. As all uncured polymer precursors were found to be soluble in suitable organic solvents, the test model was applicable to all the spacecraft relevant polymers investigated here.
For all uncured polymers, survival of inoculated spores of the \textit{B. safensis} model organism during a 60 min exposure period was verified. This was performed at high spore concentrations (10^7 cfu/mL) to allow dilution in buffer and to minimize the effect of solvent and polymer residues in the plating assay.

To achieve maximum sensitivity in determination of inherent bioburden, polymer precursors were diluted in solvent as little as possible and pour-plated in agar without transfer to an aqueous diluent. For all investigated polymers germination and colony growth of inoculated spores (\textit{B. safensis} 10^2 cfu/mL) in presence of residual polymer and solvent was verified. This also allowed determination of the recovery efficiency for the model organism for each given material and method. Recovery results were used to specify the amount of un-inoculated material that had to be extracted to generate significant figures for estimation of intrinsic endospore burden per cm\(^3\) of cured polymer.

Application of Test Model I: Detection and Viability Determination of Surface-embedded Spores in Cured Scotch-Weld 2216 B/A

Preliminary experiments had shown that ultra-thin sectioning using a microtome followed by transmission electron microscopy (TEM) was not feasible for the material due to the spongy structure of Scotch-Weld 2216 B/A. Instead, surface-embedding followed by scanning electron microscopy was found to be the method of choice to qualitatively demonstrate the presence and accessibility of embedded spores in Scotch-Weld 2216 B/A. Polymerization of the adhesive Scotch-Weld 2216 B/A on a layer of spores on the inoculated surface of water-soluble poly vinyl alcohol (PVA) yielded a surface with fully embedded, partially embedded and loosely attached spores as shown in Fig. 1A. To visualize the effect of encapsulation and polymerization on structure and integrity of \textit{G. stearothermophilus} and \textit{B. safensis} spores, scanning electron microscopy (SEM) in combination with focused ion beam (FIB) electron microscopy were employed.

![Figure 2. CLSM images of Test Model I.](https://example.com/image.png)
SEM images of the sample surface confirmed the embedding status ([Fig. 1]). Non-inoculated surfaces were smooth (as seen in [Fig. 1A to the right of the embedded spores] without spore structures. Double beam FIB/SEM analysis confirmed the varied depth of surface-embedding as indicated in the SEM analysis. Spores embedded to the depth of several spore diameters could be visualized ([Fig. 1 C]). The double beam FIB/SEM images also showed the presence of filler material in the polymer, which obstructed the clear outlines of the faint shapes of embedded spores.

While the electron-microscopic study allowed visualization of spores and their arrangement with respect to the polymer matrix, for viability determination physiological tests had to be performed. Samples of Scotch-Weld 2216 B/A with surface-embedded *B. safensis* spores were overlaid with nutrient agar after cleaning the surface with 70% ethanol to remove loosely attached spores and inactive vegetative contaminants. Several colonies developed in the agar, indicating that (partially) embedded spores of *B. safensis* were still cultivable after the curing process.

Application of standard cultivation technique and physiological fluorescent dye staining followed by fluorescence and confocal laser scanning microscopy to surface-embedded samples clearly illustrated the high potential of this preparation method for analysis of polymer-embedded spores.

In order to systematically evaluate the depth to which spores could be introduced into the adhesive by the embedding procedure, spores were stained with AF488 prior to embedding. As a surface marker, Cy5 was employed. Full Cy5 fluorescence was present above the adhesive surface in the liquid layer and disappeared on and below the surface. This allowed exact localization of partially embedded spores in Scotch-Weld 2216 B/A ([Fig. 2A]). Controls without embedded spores and stained with Cy5 and AF488 afterwards revealed only scattered locations of AF488 signals, which could be clearly distinguished from spore shapes and spore label intensity. Staining quality and CLSM channel configurations were adjusted and judged for labelled spores in suspension, labelled spores on polymer surface, as well as embedded and encapsulated labelled spores. A slight decrease in label fluorescence could be detected for embedded and encapsulated spores. AF488-prestained spores were successfully detected to a depth of 9.2 µm by CLSM ([Fig. 2B]). In addition, PMA-staining after the embedding procedure was performed as described in [29] to analyze the effect of polymerization on spore integrity in situ. Spores could be stained even after being (partially and fully) embedded in Scotch-Weld 2216 B/A in a layer of approximately 3 µm from the surface ([Fig. 2C–E]). Notably, the process of embedding did not significantly change the percentage of PMA-stainable (disrupted) spores compared to the non-embedded control, indicating that most spores were unaffected by the embedding process (Table 2). This was confirmed by successful CTC staining, indicating an active oxidative metabolism of (partially) embedded spores of *B. safensis* after reactivation by nutrient supplement ([Fig. 2F]). Colonies that appeared after covering half-embedded spores with nutrient agar were *B. safensis* colonies, as revealed by *mB*-16S (99.9% identity) and gyrB gene sequencing ([96.40% identity to type strain deposited sequences in GenBank: http://www.ncbi.nlm.nih.gov/genbank/]).

**Application of Test Model II for Scotch-Weld 2216 B/A:**

**Survival of *B. safensis* Spores in Polymer Precursor**

The possible sporidial effect of the uncured polymer precursors was tested by inoculating Scotch-Weld 2216 B/A as described in Materials and Methods. Cultivable spore recovery directly after inoculation was 59% ([Fig. 3]). The number of retrievable cultivable spores remained stable over the incubation period of 60 min (not shown). An inhibitory effect of the solvent (2-propanol) used for dilution of the material after the incubation period had been experimentally excluded in preliminary experiments.

**Application of Test Model II for Scotch-Weld 2216 B/A:**

**Recovery Efficiency of Inoculated Spores from Polymer Precursors in Presence of Organic Solvents**

Colony formation of activated spores in the pour-plate medium was only slightly inhibited by the presence of Scotch-Weld 2216 B/A and solvent residues. The recovery efficiency of spores amounted to 89% ([Fig. 3], which was higher, but in the same order of magnitude as survival in the precursors. This discrepancy was due to the difference in experimental procedure to determine survival and recovery, respectively. In survival determination, a very high inoculum was used, which was consecutively diluted before plating, which may have caused some loss of spores. The recovery efficiency (ratio of recovered cfu in nutrient medium in presence and absence of polymers) was used to correct the value for total intrinsic bioburden.

**Application of Test Model II for Scotch-Weld 2216 B/A:**

**Encapsulated Viable Intrinsic Bioburden of Uninoculated Polymer Precursors**

A recovery efficiency of >10% was arbitrarily specified as acceptance criterion for extraction of natural encapsulated bioburden from polymer precursors because the quantity of the material studied was high (87 g or 66 cm² for Scotch-Weld 2216 B/A; Table 3; investigated in 80 separate samples). In the total amount of Scotch-Weld 2216 B/A investigated, 0 cfu were found on the agar plates (Table 3). Thus, contamination of Scotch-Weld 2216 B/A with spores cultivable under the given conditions was calculated to be below 0.1 cfu/cm³ (Table 3).

**Application of Test Model II for Scotch-Weld 2216 B/A:**

**Molecular Determination of Contaminants in Polymer Precursors**

Quantitative PCR (qPCR) was applied to estimate the total natural bioburden of the polymer materials, including both dead and potentially viable cells. However, DNA extraction from polymer precursors proved very difficult. Although Scotch-Weld 2216 B/A could be dissolved properly in 2-propanol, remaining residues and clay particles bound biomaterial effectively, so that a separation of polymer and biomolecules (incl. cells and DNA) was impossible. This resulted in a very poor recovery of spore signatures, which were added as positive control (<0.1% DNA extraction efficiency).

**Application of Test Model II for Scotch-Weld 2216 B/A:**

**Contribution of the Filler Material Kaolin to the Overall Bioburden Determined by Molecular Methods and Cultivation**

Scotch-Weld 2216 B/A contains about 30% (w/w) of kaolin as a mineral filler material (see fiber-like inclusions in [Fig. 1C]). Bioburden of kaolin was determined separately, since dissolution by solvents was impossible. In addition, due to its natural origin, this filler was suspected to be a source of elevated microbial contamination. Kaolin ASP 200, 600 and 900 (3 different size) were separately tested for microbial contamination by cultivation on three different media as well as by DNA extraction and qPCR. As kaolin could be suspended in water, DNA
estimation as the number of contaminants based on molecular methods can only be a rough estimate, that the calculated number of microbial cell is unknown for unidentified microbes. Furthermore, this method also detects DNA from dead cells, permitting no possible sporicidal effect of the polymer precursors was tested as described in Materials and Methods. Recovery directly after growth of 102 cfu/cm3, was determined by the different methods as summarized in Table 1. Bioburden determination of uncured polymer precursors was performed following Test Model II.

Application of test model II for other polymers: survival of B. safensis spores in uncured polymer precursors. The possible sporicidal effect of the polymer precursors was tested as described in Materials and Methods. Recovery directly after inoculation was >10% for all tested polymer materials (Fig. 3), ranging from 13% in ESP 495 to 60% for DC 93–500. The number of cultivable spores remained stable over the incubation period three of the selected materials, whereas in the uncured silicone coating MAP SG121FD, a reduction of cultivable spores (to 0.03% of the initial inoculum) within 60 min of exposure indicated a pronounced sporicidal effect (Fig. 5). An inhibitory effect of the solvent (SG121FD thinner) used for dissolution of MAP SG121FD after the incubation period could be experimentally excluded.

Application of Test Model II for other Polymers: Recovery Efficiency of Spores from Polymer Precursors in Presence of Organic Solvents

Colony numbers of B. safensis spores were slightly reduced by the presence of polymers and solvents in the solid growth medium in all cases, but colony counts exceeded 10% (Fig. 3), ranging from 24% in MAP SG121FD to 63% in DC 93–500. As noted before, higher recovery efficiencies compared to survival ratios may be due to loss of spores during the serial dilutions performed for survival determination. In general, survival and recovery was in the same order of magnitude.

Application of Test Model II for other Polymers: Encapsulated Cultivable Bioburden of Uninoculated Polymer Precursors

The number of colonies recovered from the unspiked polymer precursors by solvent dilution and incubation in solid nutrient medium was very low in all cases. In 87 g (81 cm3) Solithane 113, 1 cfu was recovered, giving a corrected bioburden of 0.1 cfu/cm3. The detected colony consisted of gram-positive, rod-shaped cells with visible endospores. One colony was also recovered from 64 g (59 cm3) DC 93–500. However, as the isolate did not survive heat shock treatment when suspended in solvent, it was very likely a secondary contamination of vegetative organisms. Bioburden of DC 93–500 was determined to be below 0.1 cfu/cm3. Investigation of 48 g (45 cm3) ESP 495 yielded 2 cfu of spore-forming organisms, one of which appeared under elevated temperature (55–60 °C) incubation. Although 4 more colonies were recovered, those consisted of vegetative organisms, sensitive to the extraction procedure and were considered secondary contaminations. Thus, the corrected intrinsic bioburden of ESP 495 amounted to 0.3 cfu/cm3. In MAP SG121FD coating, results varied for the two analyzed batches. 5 cfu were recovered after incubation under mesophilic conditions in the first batch of SG121FD (18 g or 13 cm3 investigated). Gram-staining of these colonies showed gram-positive, rod-shaped cells, indicating spore-forming organisms. No colony was detected in the second batch of SG121FD, of which a higher amount was analyzed (71 g or 50 cm3). It should be taken into consideration that cultivability of B. safensis spores
was rapidly reduced within 60 min of exposure in SG121FD (Fig. 5). The spore-forming organisms, which were detected in one of the batches of SG121FD could have been more resistant to the inhibitory effects of the silicone coating than B. safensis.

Application of Test Model II for other Polymers: Molecular Determination of Contaminants in Uninoculated Polymer Precursors

Solithane 113 was selected in addition to Scotch-Weld 2216 B/A to attempt the application of molecular methods. When attempting to extract DNA from Solithane 113, similar obstacles arose as for Scotch-Weld 2216 B/A. Although diluted in ethanol and using an adapted DNA extraction protocol, uncharacterized polymer residues masked targeted biomolecules for subsequent molecular analyses. 2×10⁶ spores were added as a positive control, resulting in the detection of approx. 10⁴ rrnB-16S gene copies via qPCR (less than 0.5% extraction efficiency; in case the average spore contains more than a single gene copy, the extraction efficiency would be even lower, as expected. In addition, polymer components in the DNA suspension caused interference with the qPCR signal, which had to be adjusted by application of a correction factor. Nevertheless, Solithane 113 samples revealed a higher contamination with rrnB-16S genes than positive controls (Fig. 6). Since the recovery efficiency for the total natural bioburden from Solithane 113 is unknown, the true bioburden cannot be estimated. However, a minimum contamination of approx. 4×10³ gene copies per 5 g material (500/g or 535/cm³) can be envisaged.

Discussion

Planetary protection ensures the validity of future life detection missions on foreign celestial bodies by biological control of spacecraft and its assembling environment in clean room facilities [42]. Since the Viking mission era, spacecraft surface associated bacterial spore burden has been assessed by standard cultivation protocols [43,44,45,46]. Recent approaches were based again on cultivation-level [47], or applied new promising molecular methods, since cultivation is limited to standard laboratory conditions and bears the risk to ignore the majority of microorganisms in a sample [48,7,49,50]. The knowledge about polymer encapsulated bioburden, however, remains sparse, most likely due to difficulty to retrieve the organisms from cured polymers. Milling of cured polymers was considered impractical as it involves mechanical shear forces and local heating, which are very difficult to control and their effect on encapsulated cells or spores is unknown [51,52]. Comparable hurdles have been described by [14] and [26] during recovery and detection of encapsulated bioburden in silicone or Plexiglas and had to be overcome by adapted extraction and permeabilization protocols. While the polymers used in the aforementioned studies were selected specifically to study encapsulation models, the aim of the present study was to find test models that can be used to characterize the intrinsic bioburden of spacecraft-relevant polymers. The test models developed for Scotch-Weld 2216 B/A, and applied to ESP 495, DC 93–500, MAP SG121FD and Solithane 113 can be used for varied test strategies to characterize and

| Table 3. Contamination of the polymer materials with bacterial endospores determined by dilution in solvent and cultivation (corrected values calculated with recovery efficiency). |
| --- |
| Material | Amount of material investigated (cm³) | Total spore-forming colonies detected (cfu) | Encapsulated bioburden (cfu/cm³), corrected |
| --- |
| Scotch-Weld 2216 B/A | 66 | 0 | <0.1 |
| MAP SG121FD (batch 1/batch 2) | 13/50 | 5/0 | 2.4/0.3 |
| Solithane 113 | 81 | 1 | 0.1 |
| ESP 495 | 45 | 2 | 0.3 |
| DC 93–500 | 59 | 0 | <0.1 |

![Figure 4](image1.png) Microbial contamination per cm³ Scotch-Weld 2216 B/A contributed by filler material kaolin (3 grain sizes ASP 200, 600 and 900), as determined by cultivation (cfu = without heat shock, black bars; cultivable spores = heat-shock-survivors, gray bars) and by qPCR (total contaminants (rrnB-16S gene copies, white bars). Standard deviations are not shown as the data is only meant to give a broad orientation. For a statistically sound analysis a much higher sample number would need to be investigated. doi:10.1371/journal.pone.0094265.g004

![Figure 5](image2.png) Survival of B. safensis spores in uncured MAP SG121FD during 0–60 min of incubation. doi:10.1371/journal.pone.0094265.g005
estimate the intrinsic bioburden of polymers. The models lend themselves to investigate not only the most frequently used polymers for spacecraft but also other polymers used in food and pharmaceutical industry or medical devices.

We first developed a surface-embedding model that allowed easy access to the inoculated spores for assessment of the effect of polymerization on encapsulated B. saperdus spores in Scotch-Weld 2216 B/A as an exemplary polymer. Without any disruptive extraction from the polymer, embedding effects could be studied with a minimum of preparation artifacts. Fluorescently labelled spores, which could be detected to a depth of 3 μm, showed spore coat integrity and respiratory metabolism in presence of provided nutrients and some of them were able to germinate and form colonies. Thus, it cannot be assumed that the polymerization process is damaging to spores, although this result is expected to vary with different polymeric materials as well as with spore structure. For materials that lend themselves to application of the described or similar methods, our developed encapsulation model in combination with molecular staining techniques can be a valuable tool to assess how the germination ability of spores is affected by polymerization and gives additional information about embedded spores to the approach with Alexa-FISH presented by Mohapatra and La Duc [26].

Although the model presented by Mohapatra and La Duc was pioneering work and yielded interesting data on spiked spores, it could not be applied to quantitatively estimate the intrinsic bioburden of polymers. The intrinsic bioburden of polymers is expected to vary for a number of reasons: i) they are composed of widely different materials, ii) some include materials of natural origin (e.g. fillers), iii) manufacturing conditions for the polymers are diverse and iv) uncured materials may have different effects on microorganisms. For estimation of intrinsic bioburden in a broad range of polymers a method for lot-specific quantitative estimation of the intrinsic bioburden was sought. We developed a test model that combines cultivation-based and molecular methods for quantitative bioburden determination. Since biocompatible disruption or dissolution of the cured polymers was found to be impossible in this study, uncured polymer precursors were diluted in solvents before polymerization. This was considered the most conservative and also the least sporidical approach. Inoculated endospores (shown to be insensitive to the employed diluents) could be recovered with reasonable effectiveness. Hence, the strategy applied herein was shown to be applicable to determine the maximum number of cultivable intrinsic spores in a wide range of polymers.

A low contamination with encapsulated cultivable endospores was found for all investigated polymer materials, in the range of < 0.1 spores/cm³ to a maximum of 2.4 spores/cm³ in one batch of MAP SG121FD. SG121FD was the only tested material, for which a sporidical effect on inoculated model spores was observed over incubation periods of ≥60 min. Thus, the risk of germinable spor contamination for this material was considered to be low.

The bioburden of polymer precursors is specific for each material and can vary from batch to batch (as shown for MAP SG121FD). For practical application a number of batches would have to be tested, and any change in the manufacturer or the manufacturing process of the polymer material would necessitate re-evaluation of its bioburden.

As for previous attempts of bioburden evaluation [43,44,45], the limitation of this method was the use of cultivation-based detection methods, which are known to detect only a fraction of the total number of viable organisms present in a sample of natural origin [53,54,55]. However, if the recovered spores are unable to germinate under optimal conditions, the probability for germination and propagation on a possibly hostile planetary surface might be considered minute, even if phenomena like superdormant spores are regarded [56]. Nevertheless, for planetary protection purposes, dead and not-yet-culturable organisms need to be considered as well, since they might interfere with life or biomarker detection methods. Therefore, determinations of the molecular bioburden were considered an important complement to cultivation-based assays. However, DNA extraction from the polymer precursors of Scotch-Weld 2216 B/A proved difficult, because DNA was bound by the polymer particles diluted in solvent as described to be the case for silicones, too [14]. Hence, promising high throughput omics technologies (e.g. transcriptomics and proteomics) could not be applied beyond our qPCR and microscopy based approaches. Due to the low extraction efficiency, quantitative data could not be obtained for Scotch-Weld 2216 B/A, but for its filler kaolin, which amounted to ~10^3 cells/cm³ (estimation from rrnB-16S gene copies determined via qPCR). This amount exceeded by far the number of microorganisms that could be detected by cultivation in the same material. However, a general conclusion for bioburden of this filler is difficult to draw, since kaolin is a natural resource originating in clay mines, which are exposed to various environmental influences, and harbor a remarkable diversity of extremophiles like iron oxidizing and reducing bacteria (dark iron oxides and sulfides are major discoloring impurities of white kaolin [57]), and acidophilic archaea and bacteria (sulfidic mine waters [58]). Additionally, mining and processing of kaolin is not performed under sterile conditions and may vary for different manufacturers. Nevertheless, this result was in accordance with our expectations, as molecular methods include DNA from dead organisms, as well as from viable and viable-but-nonculturable organisms. The high numbers of potentially viable microbial contaminants (also found in previous studies by other authors, e.g. [59], indicate that fillers of polymers should not be dismissed in studies of encapsulated bioburden.

**Recommendations for Future Studies**

We recommend the application of the presented methods to other materials for which an evaluation of encapsulated bioburden is important. Such materials may be other adhesives, coatings (silicone) and bulk polymers (polyurethane, silicone and polyimide), honeycomb panels, heat shields (Norcoat Liège cork) and components like wires and connectors [60]. Since these materials are quite diverse, in each case a risk analysis is recommended to decide, if viable bioburden is expected to be present at all. For composite materials like wires and connectors it is important to investigate whether bioburden resides in the polymeric insulation.
materials, in between the insulating layers, or on the wires themselves. The testing strategy will have to be adjusted accordingly, and a special set of methods for evaluating its bioburden will have to be posed.

The method of choice for components where dissolvable precursors are available is Test Model II, resulting in the most conservative evaluation for embedded spores. A suitable surface embedding model where added microorganisms are easy to detect and analyse in various depths of embedding as presented in our Test Model I could be helpful for evaluating polymerization effects on the viability of other microbes.

Presented physiological markers with fluorescent activity are quick and applicable given that the material itself does not show severe auto-fluorescence. If the material has to be considered a potential contamination risk and especially if auto-fluorescence is preexisting [26], a separate analysis (e.g., qPCR) of certain components as performed for the kaolin filler might be appropriate. As revealed by our study, separate sterilization of such natural components would be a good alternative in order to reduce the viable encapsulated bioburden. In addition, it might also be important to phylogenetically, metabolically and physiologically characterize the detected bioburden to estimate its risk for planetary protection.

Conclusion

With polymeric components of spacecraft sent from earth to other celestial bodies, but also with the ubiquitous use of polymer materials in food and pharmaceutical industry, the question of their possible internal bioburden has been raised. Here, we have investigated methods that can be applied to estimate the number of persistent microorganisms encapsulated in different polymeric materials. The methods, which were presented as a composite strategy by use of viability staining of embedded microorganisms, classic cultivation procedures, and molecular detection techniques to evaluate encapsulated bioburden of spacecraft model hardware, are not only of interest for the research field of planetary protection. For food [61] and pharmaceutical industry [62,63,64] and clinical equipment [65], an evaluation of cleanliness and sterility is also of great importance, since these fields are confronted by new threats like multi-resistant bacteria and pathogens [66]. Bioburden embedded in polymeric materials is of interest wherever plastic materials are used. Survival of bacterial endospores embedded in such materials is of significance when the huge amount of plastic materials is considered, that is rotting in dumps or littering the surface of the earth and the oceans [67]. These materials do not only leach chemicals but may also harbor microorganisms that get released and proliferate.

Our protocols proved feasible for five typical spacecraft polymers, and we can state that the overall cultivable endospore burden inside the analyzed polymers was low with <0.5 spores/cm² for most of the investigated materials. Furthermore, beyond cultivation and molecular-based methods, this study shows the potential of physiological staining methods like AF488, PMA and CTC to evaluate embedded spores in polymers. The combination of these dyes demonstrated for the first time the capability of spores to withstand mechanical forces during embedding in and curing of a polymer.

Acknowledgments

The authors wish to thank Kim Ho Plan and Stephan Rütten (DWI, Aachen) for FESEM imaging, and Alexander Heijl (RWTH Aachen) for FIB/SEM imaging. Reinhard Wirth and Veronika Menath are gratefully acknowledged for fruitful discussions and for technical assistance, respectively. We would also like to thank Gerhard Kminek for valuable input to the manuscript. Free samples of ESP 495 were kindly provided by EADS Astrium. AJP was supported by the German National Academic Foundation (Studienstiftung des deutschen Volkes).

Author Contributions

Conceived and designed the experiments: CM KH. Performed the experiments: A. Bauermeister AM AA A. Boker NF CW AJP. Analyzed the data: A. Boker AM AJP CM KH. Contributed reagents/materials/analysis tools: A. Boker. Wrote the paper: A. Boker AM CM KH.

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