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Low-Temperature Decontamination with Hydrogen Peroxide or Chlorine Dioxide for Space Applications

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The currently used microbial decontamination method for spacecraft and components uses dry-heat microbial reduction at temperatures of >110°C for extended periods to prevent the contamination of extraplanetary destinations. This process is effective and reproducible, but it is also long and costly and precludes the use of heat-labile materials. The need for an alternative to dry-heat microbial reduction has been identified by space agencies. Investigations assessing the biological efficacy of two gaseous decontamination technologies, vapor hydrogen peroxide (Steris) and chlorine dioxide (ClorDiSys), were undertaken in a 20-m^3 exposure chamber. Five spore-forming *Bacillus* spp. were exposed on stainless steel coupons to vaporized hydrogen peroxide and chlorine dioxide gas. Exposure for 20 min to vapor hydrogen peroxide resulted in 6- and 5-log reductions in the recovery of *Bacillus atrophaeus* and *Geobacillus stearothermophilus*, respectively. However, in comparison, chlorine dioxide required an exposure period of 60 min to reduce both *B. atrophaeus* and *G. stearothermophilus* by 5 logs. Of the three other *Bacillus* spp. tested, *Bacillus thuringiensis* proved the most resistant to hydrogen peroxide and chlorine dioxide with D values of 175.4 s and 6.6 h, respectively. Both low-temperature decontamination technologies proved effective at reducing the *Bacillus* spp. tested within the exposure ranges by over 5 logs, with the exception of *B. thuringiensis*, which was more resistant to both technologies. These results indicate that a review of the indicator organism choice and loading could provide a more appropriate and realistic challenge for the sterilization procedures used in the space industry.

When a spacecraft visits regions of the solar system which may potentially hold biological interest, Planetary Protection (1967 Outer Space Treaty) precautions must be observed to protect any present and future life detection activities (2). Planetary protection precautions require that flight systems must be assembled, tested, and launched under conditions to control the forward contamination of pristine extraterrestrial environments by terrestrial microorganisms and indeed backward contamination during Earth return missions.

Spacecraft and their components are constructed and assembled in high classification clean room facilities (30, 38) similar to that used in medical (12, 16, 42) and industrial pharmaceutical (31) applications. Although it might be expected that the contaminating organisms of these facilities will be the result of human activity (30), the highly desiccated and nutrient-limited environment of spacecraft assembly clean rooms demonstrate selective pressure toward oligotrophic organisms that can persist in the environment (30). Of these organisms, spore-forming bacteria are perhaps the best suited to persistence and survival (21, 26). For example, spore-forming *Bacillus* species are the most commonly isolated (26), but other aerobic and anaerobic bacterial species have also been detected, namely, *Staphylococcus*, *Acinetobacter*, *Micrococcus*, *Clostridium*, and *Streptococcus* spp. (36–38), as well as eukaryotic organisms such as yeasts and fungi (37). The continual microbial monitoring of the spacecraft assembly clean rooms has led to the characterization of two new *Bacillus* species: *Bacillus odysseyi* (27) and *Bacillus nealsonii* (41).

Since microorganisms that survive within spacecraft assembly facilities can potentially contaminate spacecraft components and thus ultimately their destinations, the bioburden needs to be reduced to safe levels to satisfy the tightly regulated Planetary Protection requirements before deployment (2). This cannot be done by sterile manufacture alone. Regular assays of spacecraft allows a baseline contamination level to be calculated which must be controlled and reduced by a decontamination process.

The current validated decontamination process used in the space industry is dry-heat microbial reduction (DHMR) (14), which has been used on spacecraft and their components since the Viking lander missions in 1975. The spacecraft and components are heated to >110°C within a sealed dry oven for extended periods of time (e.g., up to 50 h for one cycle) (8, 40). This method has provided effective and repeatable decontamination which has been validated using thermocouples and biological indicator(s) (BI). The kinetics of heat inactivation of microorganisms is well established and therefore demonstrates a high degree of sterility assurance.

The use of heat-sensitive components such as used on the Beagle 2 Lander ruled out the use of DHMR, and so alternative technologies such as sporicides, gamma irradiation, and gas plasma (8, 11, 32) were used. These technologies had to be accompanied by evidence of their efficacy to fulfill National Aeronautics and Space Administration (NASA) requirements for this mission (32); the methods were not validated for continued use in the space industry. There has been much interest in developing alternative and validated low-temperature methods that would enable both larger modules and small components to be decontaminated. Such methods must operate at a low temperature (<60°C), be compatible with a number of different materials used within the space

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industry, leave no significant levels of residues, and be able to be carried out safely without uncontrolled exposure to the decontamination technology operators. The technology should also be scalable to allow the decontamination of various sizes of objects, from small components to entire spacecraft.

Low-temperature decontamination is widely used within the medical-device, pharmaceutical, and laboratory sectors (1, 3, 5, 13, 18, 25). Gaseous hydrogen peroxide (10, 17, 20, 29) and chlorine dioxide technologies (6, 10, 19, 34, 39) have been shown to be effective against a wide range of bacterial and viral organisms.

This investigation set out to assess alternatives to dry-heat microbial reduction by investigating low-temperature decontamination technologies for the surface decontamination of heat-sensitive spacecraft components. The technologies were selected after an extensive literature review and scoring matrix to determine the most appropriate for this application. Two of the technologies tested for their biological efficacy, material compatibility, and residue formation are described here.

MATERIALS AND METHODS

Technology selection. A technical review of current gaseous decontamination technologies used in laboratories, pharmaceutical environments, and health care settings was carried out to determine the most suitable technologies to be used here (data not included). The technologies selected were methods utilizing vapor hydrogen peroxide (VHP; Steris, Inc., United Kingdom) and gaseous chlorine dioxide (ClO_2 [ClorDiSys], supplied by Primatec, United Kingdom).

Microorganisms. Commercially available biological indicators preloaded with $>10^6$ spores of *Bacillus atrophaeus* ATCC 9372 (SGM Biotech, USA) and *Geobacillus stearothermophilus* ATCC 7953 (Steris) sealed within Tyvek and Mylar pouches were selected for use after a prestudy trial (data not shown).

In addition, three naturally occurring organisms (NOO), previously isolated from spacecraft clean room assembly facilities—*Bacillus megaterium* (2c1 European Space Agency [ESA] organism reference [clean room isolate]), *Bacillus safensis* (DSM 19292), and *Bacillus thuringiensis* (E24 ESA organism reference [clean room isolate])—were chosen and supplied in spore suspensions (DLR, Germany). Biological indicators using these spore suspensions were produced using each NOO spore suspension; 10 μl of $>10^8$ spores/ml of each spore suspension was pipetted and dried (22 ± 3°C for 2 h) onto clean stainless steel coupons (Grade 316; M-Tech Diagnostics, United Kingdom). After drying the coupons were then heat sealed into Tyvek and Mylar pouches (SGM Biotech) and kept at 4 ± 2°C for <48 h prior to exposure. These NOO biological indicators were exposed to three cycles of the most efficacious cycle from each of the decontamination technologies, determined from the results of the commercial BI exposure.

Exposure chamber. All processes were undertaken in the environmental chamber facility of the Health Protection Agency (HPA), Porton Down, United Kingdom. The facility consisted of an ante room connected to a sealable room with an internal volume of 20.7 m^3. The anteroom was used for preparation and collection of samples.

The gaseous decontamination generators were connected to the chamber using locking gas ports on the exterior wall. A fan (100 liters/min; CED, United Kingdom) was placed into the chamber (at floor level) to assist with the mixing of the decontaminant. A viewing window from chamber to ante room had two glove ports beneath, which enabled BI to be placed into phosphate-buffered saline (PBS) at specific time points to stop the exposure to the decontaminant.

The chamber was set up with sensor probes to detect the concentration of the gaseous decontaminant, the relative humidity, and the temperature in the immediate vicinity of the BI coupons. These sensor probes were used to ascertain when the maximum concentration of gaseous decontaminant had been achieved for the VHP and ClO_2 generators. The VHP probes were self-contained units, VHP ARD system sensing units, which incorporated a Fluke digital thermometer, a Digitron relative humidity probe, and an ATI VHP electrochemical sensor. The ClO_2 sensor was integral to the generator; gas from the chamber was returned to the sensor (Optex model AF26-S15; Primatec) from the sample area by tubing. A separate probe containing the relative humidity and temperature sensors (Vaisala model HMD4DY; Primatec) was connected to the generator and placed in the chamber. All probes were calibrated prior to use.

TABLE 1 Decontamination cycle parameters

Technology	Generator	Decontaminant concn (ppm)	Exposure period (min)	Temp (°C)
VHP	ARD-1000	750	20	35
	ARD-1000	625	50	35
	ARD-1000	500	45	35
ClO_2	Minidox M	396	60	25

Generators. The generator supplied by Steris used during the study was a VHP-1000ARD generator. This generator dehumidified the air within the chamber to 20% during the dehumidification phase. The conditioning phase was then begun when 35% liquid hydrogen peroxide (Steris) was injected into the chamber to achieve the required concentration of VHP in the air. The VHP concentration was maintained above the set point during the decontamination phase for the required period of time. The aeration phase allowed the removal of VHP from the chamber to a safe limit for entry to remove the exposed coupons. An external heater (Dragon Two; Delonghi, United Kingdom) was placed in the chamber to increase the temperature to 35°C during the study.

A ClorDiSys Minidox M generator was supplied for the study (PrimaTec, United Kingdom). The generator operated by entering a preconditioning phase where a humidifier was used to increase the relative humidity within the chamber to >65%. The conditioning phase held the relative humidity for a preset time. During the charge phase the generator produced and injected the chlorine dioxide gas into the chamber. The gas concentration was held at a predetermined level within the chamber in the exposure phase. The aeration phase was used to remove the chlorine dioxide gas from the chamber.

The generators were operated by trained HPA staff. The VHP generator was installed and programmed with multiple cycles (Table 1) by the company engineers. The two different technologies were studied at different temperatures with VHP at 35°C and ClO_2 at 25°C due to problems with condensation at the higher temperature on the photometer lens in the ClO_2 generator (external to the chamber and therefore at a lower temperature) causing the decontamination cycle to continually abort. This problem was ameliorated but not totally resolved by reducing the chamber's temperature to 25°C, but it caused time delays that only allowed one ClO_2 concentration to be studied in the investigation. The VHP generator was also operated outside of the chamber but did not suffer from the same problems because the sensors were within the chamber. The sampling points were chosen after a short study using each generator was undertaken (data not shown).

Experimental procedure. For each sterilization cycle, 18 of each type of commercially produced biological indicator coupons (Steris and SGM Biotech, Ltd.) were prepared. Fifteen of the coupons were attached by their pouches to a supporting frame that rested on a table inside the test chamber within reach of the glove ports. Three coupon pouches were retained as the unexposed controls within the laboratory. The frame was then placed within a sealed box, held at positive pressure to the exposure chamber. When the peak decontaminant concentration was reached, the box was opened, exposing the BI and starting the exposure period.

The three unexposed BI controls of each organism, representing the zero time point, were opened and placed into universal tubes (Sterilin, United Kingdom) containing 5 ml of PBS and 0.1% Tween 80 (VWR,

TABLE 2 Comparison of values for each microorganism exposed to VHP and ClO_2 technologies

Organism	Decontaminant (concn [ppm])	D value (mg/liter)
G. stearothermophilus	VHP (500)	585.4
	VHP (625)	493.3
	VHP (750)	159.8
	ClO_2 (396)	726.7
B. atrophaeus	VHP (500)	92.7
	VHP (625)	76.9
	VHP (750)	48.4
	ClO_2 (396)	924.4
B. megaterium	VHP (750)	45.8
	ClO_2 (396)	757.8
B. safensis	VHP (750)	68.6
	ClO_2 (396)	627.8
B. thuringiensis	VHP (750)	175.4
	ClO_2 (396)	2.38×10^4

United Kingdom) and four glass beads (3 mm in diameter; VWR) using sterile disposable forceps (SLS, United Kingdom).

At each of the additional five time points, the BI within the Tyvek pouches ($n = 3$) were aseptically deposited into universal tubes containing 5 ml of PBS and 0.1% Tween 80 and four glass beads (3 mm in diameter) using sterile disposable forceps. Previous experiments determined that there was no loss of viability to the BI caused by absorption of hydrogen peroxide into the containers even over extended periods of opening (5 min maximum opening) (data not included). The order in which the BI were taken during each sampling period was alternated (i.e., in one cycle organism A would be taken before organism B, and then in the next cycle organism B would be taken before organism A) to achieve an even exposure for each organism over the set of triplicate cycles. The chamber was aerated after the final coupon had been taken. The samples were removed to the laboratory where they were processed within 1 h after the end of the exposure period.

The universals were ultrasonicated (5 min) within a water bath (Branson series 5510; 42 KHz, input power of 185 W) to aid removal of the spores from the coupons. The universal tubes containing the coupons were then removed and vortexed (5 min) (Heidolph Multireax; SLS), after which a further 5 ml of sterile water (Aguettant, United Kingdom) was added, and the samples were vortexed for an additional 5 min.

Serial dilutions (1 in 10) to 10^{-4} were prepared using sterile water. Aliquots (100 μl) of the appropriate dilution were pipetted and spread onto Trypticase soy agar (TSA [bioMérieux, Marcy l'Etoile, France]) in duplicate. The plates were incubated at the recommended temperature (60°C for *G. stearothermophilus* and 37°C for all four other organisms used) for 48 h, after which the colonies were enumerated; the samples were then refrigerated from 2 to 6°C, while the plates were incubated. If no organisms were detected after plating 100 μl of the neat sample dilution, then the entire sample was filtered through a 0.2-μm-pore-size Cyclopore track etched membrane (Whatman, USA). Once the sample had been filtered, the filter was placed directly onto a TSA plate and incubated at the appropriate temperature for 48 h. A total of nine BI were processed for each time point (i.e., three replicates per run × three runs).

Data analysis. The data were expressed in terms of the survival fraction, which was calculated as the proportion of organisms recovered after a set exposure period divided by the unexposed population: survival fraction = N/N_0. These data were processed in SigmaPlot 10.0, where the data was log transformed and then graphically analyzed. Average values ($n = 9$, three replicates per run × three runs) were plotted with error bars representing geometric standard deviations.

To avoid biasing the results by the low levels of spore recovery from the later sample points, an arbitrary cutoff point for the data was used for regression calculation. Any data set(s) with no detectable spores in six of the nine filtered samples and only one spore in the remaining three samples was treated as having no spores detectable and was removed from the regression calculation. A linear regression was plotted to calculate the D values (time taken for a 1-log reduction in spore numbers) (Table 2).

RESULTS

Exposure to VHP caused a rapid inactivation of *B. atrophaeus* spores with a 6-log reduction in survival fraction within 6 min and a 5-log reduction in *G. stearothermophilus* in 20 min (Fig. 1). A steady decrease in the survival of *B. thuringiensis* spores was witnessed over the 20-min exposure period, with a 6-log reduction in the survival fraction by the end of the exposure period.

Exposure to ClO_2 caused a steady reduction in the survival fraction of *G. stearothermophilus* of ~5 logs in 60 min (Fig. 2). The survival fraction of *B. atrophaeus* also reduced by 5 logs at 60 min. *B. thuringiensis* numbers were not significantly reduced during the 60-min exposure period.

The D values (Table 2) for *G. stearothermophilus* decreased from 585.4 (500 ppm) to 159.8 s (750 ppm). A greater D value of 726.7 s was observed when ClO_2 at a concentration of 396 ppm was used. With *B. atrophaeus* the D value was 48.4 s at 750 ppm and 92.7 s at 500 ppm. The D value for *B. atrophaeus* exposed to ClO_2 was 924.4 s. *B. megaterium* and *B. safensis* had D values that closely matched that of *B. atrophaeus* when exposed to VHP (750 ppm) and that of *G. stearothermophilus* when exposed to ClO_2. The D values for *B. thuringiensis* were greater than those for both *B. atrophaeus* and *G. stearothermophilus* when exposed to both VHP and ClO_2, which were 175.4 s and 6.6 h, respectively.

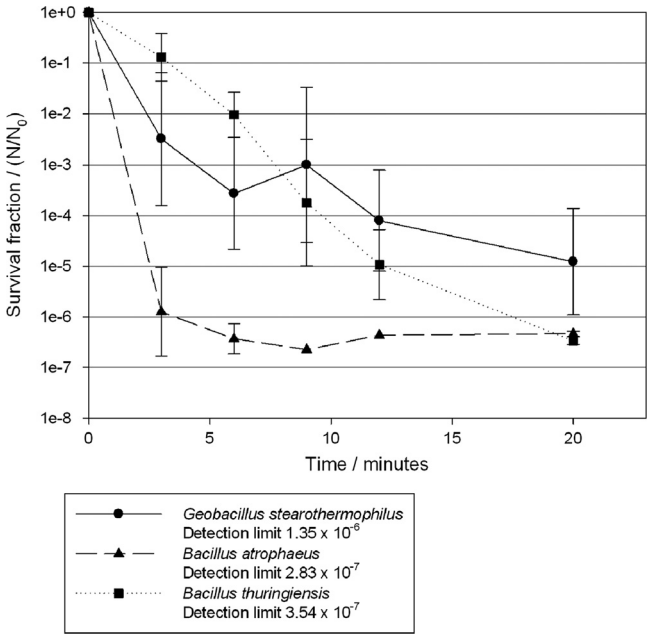

FIG 1 Survival fractions of *G. stearothermophilus* (●), *B. atrophaeus* (▲), and *B. thuringiensis* (■) after exposure to VHP at a concentration of 750 ppm for 20 min.

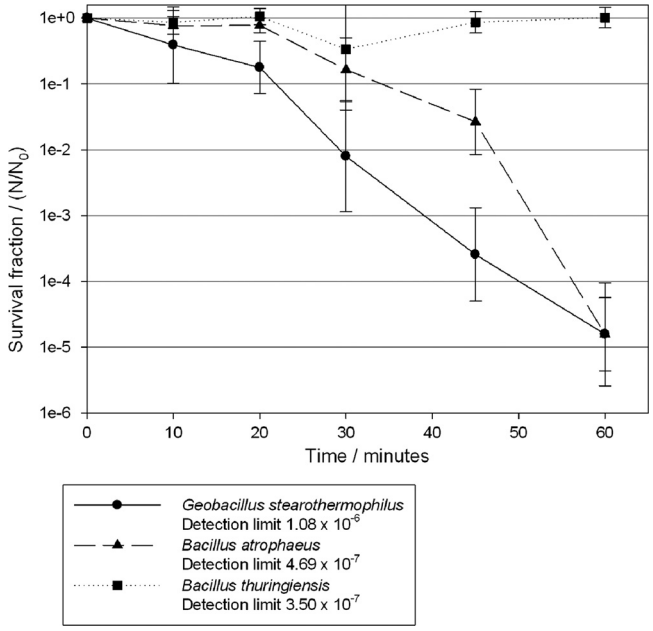

FIG 2 Survival fractions of *G. stearothermophilus* (●), *B. atrophaeus* (▲), and *B. thuringiensis* (■) after exposure to ClO_2 at a concentration of 396 ppm for 60 min.

DISCUSSION

Prior to the launch of spacecraft bound for planetary bodies, the microbial load must be monitored, controlled, and potentially reduced in order to satisfy Planetary Protection guidelines to ensure any risks of forward contamination to other celestial bodies are minimized (2). While the current validated method uses DHMR, the increase in the number of thermolabile materials being used in spacecraft today has led to a requirement for the development of alternative low-temperature surface decontamination technologies. We investigated here the biological efficacy of two low-temperature gaseous decontamination technologies using a range of biological spore indicators.

The VHP and ClO_2 systems achieved a 5-log reduction in the recovery of the biological indicators used within 20 and 60 min, respectively (Fig. 1 and 2). The results are expressed in graphs as survival fractions, allowing linear regressions to be drawn and D values to be calculated. As indicated in Materials and Methods, these results were adjusted to take account of the low numbers of spores that were recovered from a small number of samples that may otherwise have led to a bias and skewing of the D values. D values were first used for heat sterilization and describe this process by first-order kinetics (4). However, gaseous disinfection is a more complex process that requires a decontaminant to penetrate into a biofilm of microorganisms dried onto a surface and to cause irreversible damage to these spores. The use of D values is a simplified description of the inactivation kinetics, and the use of D values produced from the linear regression may lead to an incomplete decontamination procedure. Therefore, it is recommended that the D values should be used as guidelines for the overestimation of exposure periods rather than exact times. For example, the D value for *B. thuringiensis* was 175.4 s compared to that for *G. stearothermophilus* (159.8 s). However, after a 20-min exposure there was a difference of >1 log in the survival fractions of the two organisms, with *G. stearothermophilus* exhibiting greater survival (Fig. 2) as the rate of killing for *G. stearothermophilus* slowed over the last few time points.

This retention of viability in a small subsection of spores that remain resistant to gaseous disinfectants is a phenomenon known as "trailing" (33). Various explanations for this include: (i) the presence of a subpopulation of hyper-resistant spores, (ii) the occlusion of spores by layering or other factors, and/or (iii) the possibility of cross-contamination. However, each of these explanations can be regarded as a product of the experimental situation and has limited relevance to a natural situation wherein contamination on space hardware will be of a much lower magnitude in terms of density (26, 36, 37).

We have demonstrated here that *G. stearothermophilus* spores were the more resistant of the two commercially available indicators for VHP, whereas *B. atrophaeus* was more resistant to ClO_2 (Table 2). These results are in line with the recommendations from the respective companies, *G. stearothermophilus* for VHP (Steris) (22, 24) and *B. atrophaeus* for ClO_2 (ClorDiSys) (28), for organisms to be used as biological indicators to validate their processes, respectively. Two of the naturally occurring organisms, *B. megaterium* and *B. safensis*, demonstrated lower resistance to the decontamination technologies compared to the recommended biological indicators. However, *B. thuringiensis* exhibited a level of resistance comparable to that of *G. stearothermophilus* when exposed to VHP, with respective D values of 175.4 and 159.8 s. In the case of ClO_2, *B. thuringiensis* exhibited a greatly increased resistance compared to *B. atrophaeus*, with D values of 6.6 h and 924.4 s, respectively (Fig. 2). The resistance of *B. thuringiensis* to ClO_2 gas has previously been demonstrated (15), a study wherein 10^6 spores of *B. thuringiensis* were dried onto paper, wood, and epoxy surfaces and then exposed to ClO_2 (5,400 ppm) in a sealed container for 720 min. In this case, there was a single injection of ClO_2 in the exposure chamber, and the concentration decreased with time. A minimum of 10,800 ppm of ClO_2 was required to completely inactivate the spores on paper and wood (15). Microbial reduction using VHP has previously been demonstrated to be dependent on the initial microbial loading on coupons, e.g., MS2 coliphage at concentrations of 10^{10} PFU (33). However, the initial loading with *B. thuringiensis* in the present study was considerably lower (10^6 PFU). These results indicate that further work is required to determine the mode of resistance of *B. thuringiensis* and to determine whether it is species or, indeed, strain specific.

There was an increase in the rate of killing after the first 20-min period for *B. atrophaeus* and *G. stearothermophilus* exposed to ClO_2, which may be explained by the mode of operation of the generator (Fig. 2). The ClO_2 technology uses an external humidifier to raise the humidity within the chamber to >65% during the preconditioning phase prior to ClO_2 injection. The increase in the humidity above that normally found in the chamber may allow the water vapor to microcondense onto surfaces and penetrate into a dried population of microorganisms. Chlorine dioxide readily dissolves in water (28); if this water has condensed onto the surfaces and surrounds the spores, then there will be greater penetration into the coupons and a quicker kill. In the present study the biological indicators were kept within a positively pressurized box during the conditioning phase and only exposed at the peak ClO_2 concentration. This suggests that the initial slow reduction in survival fraction may be a lack of penetration of water vapor during the preconditioning and conditioning phases, followed by

absorption of the ClO_2 into the dried spore population on the coupons.

While the VHP system produced more rapid kills, for example, with *G. stearothermophilus* (D value of 159.8 s) compared to the ClO_2 system (D value of 726.7 s), respectively, the kill time for the ClO_2 system was still within the expected range (PrimaTec, unpublished data). Indeed, the concentrations of ClO_2 used in a decontamination cycle are normally far higher (5 to 30 mg/liter) than the concentration used here (1.1 mg/liter, 396 ppm) (9, 15, 19, 23), and the higher levels decrease the kill time for the biological indicators (19). However, the increased ClO_2 concentration could potentially lead to greater damage of sensitive spacecraft materials through the deposition of chemical residues (based on current material compatibility and residue analysis data).

Biological indicators are widely used to demonstrate the efficacy of decontamination cycles (35, 43). Microbiological indicators are produced with 10^6 microorganisms dried within a 1-cm^2 area (7). This high point loading is not representative of environments where the level of contamination may be lower, e.g., in spacecraft assembly clean rooms where the density of microorganisms on surfaces is at very low levels, i.e., approximately 0 to 4 CFU/cm^2 (26, 36, 37). A biological indicator could be produced using the standard organism *G. stearothermophilus* or using *B. thuringiensis* spores, which both have comparable levels of resistance to 750 ppm of VHP in terms of D values but with a reduced loading concentration. Therefore, a more appropriate biological indicator for this setting may contain a lower loading of spores, i.e., 10^3 or 10^4, dried onto a larger area (10 cm^2). This indicator would then present a realistic but stringent challenge for gaseous decontamination technology. The combination of the decontamination cycle at the highest concentrations shown here and the actual low surface contamination in spacecraft assembly facilities shows that the D values produced within the present study can be used as effective guidelines to ensure a safe decontamination.

In conclusion, we have demonstrated that low-temperature gaseous decontamination technologies can be used as an appropriate alternative to the existing decontamination procedure of DHMR and, on the basis of the biological efficacy and material compatibility results, VHP has been chosen by the European Space Agency as an alternative to DHMR.

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