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Research Article

A Field-Based Cleaning Protocol for Sampling Devices Used in Life-Detection Studies

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Abstract

Analytical approaches to extant and extinct life detection involve molecular detection often at trace levels. Thus, removal of biological materials and other organic molecules from the surfaces of devices used for sampling is essential for ascertaining meaningful results. Organic decontamination to levels consistent with null values on life-detection instruments is particularly challenging at remote field locations where Mars analog field investigations are carried out. Here, we present a seven-step, multi-reagent decontamination method that can be applied to sampling devices while in the field. *In situ* lipopolysaccharide detection via low-level endotoxin assays and molecular detection via gas chromatography–mass spectrometry were used to test the effectiveness of the decontamination protocol for sampling of glacial ice with a coring device and for sampling of sediments with a rover scoop during deployment at Arctic Mars-analog sites in Svalbard, Norway. Our results indicate that the protocols and detection technique sufficiently remove and detect low levels of molecular constituents necessary for life-detection tests. Key Words: Organic decontamination—Sterilization—Life detection—Biosignature contaminants—Mars analog—Ice coring. Astrobiology 9, 455–465.

Introduction

Organic decontamination of sampling tools and storage materials is crucial for life-detection, habitability, and ecological investigations of extremophiles, which live in the most inhospitable niches on Earth and, potentially, on Mars and elsewhere. Molecular compositions and distributions are key observations used to identify and decipher fingerprints of extant and extinct life. When investigations are confronted with trace-level quantities of organic molecules and low biological activity, unwanted signals from earlier sample acquisition and handling (forward contamination) can compromise meaningful observations and irreversibly alter the pristine environments that are sought to be characterized. Ultrasensitive analytical detection limits have been developed for essential biomolecules and monomers associated with life on Earth [*e.g.,* adenosine triphosphate (ATP), amino acids, nucleic acids, pigments, lipopolysaccharides, lipids and their fossil hydrocarbons]. However, the ubiquitous nature of natural and synthetic organics on Earth challenges the thoroughness of decontamination and sampling protocols for extreme terrestrial and planetary research.

Minimizing forward contamination of extraterrestrial bodies with Earth-derived biological materials is particularly important to the search for extraterrestrial life (Rummel, 2001; Mancinelli, 2003). Disinfecting space hardware to minimize forward contamination by Earth biology has been investigated (Venkateswaran *et al.*, 2004). However, decontaminating spacecraft hardware to low threshold levels as determined for ultrasensitive organic-compound or life-detection instrumentation developed for flight is necessary for authenticating martian observations in upcoming missions such as the ESA-NASA ExoMars and future Mars landers and sample return missions. The increasing sensitivity

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of terrestrial laboratory analytical techniques will make these issues even more important for future sample return missions. Similarly, molecular-level decontamination of sampling devices and avoidance of cross contamination between sampling steps are critical for any astrobiology field study in Mars analog environments on Earth (Christner *et al.*, 2005).

The stringent requirement for an effective blank in molecular life-detection studies necessitates both sterilization (destruction of vegetative microorganisms and spores) and organic decontamination (removal of remnant organic molecules from cells, other samples, storage and handling, lubricants, detergents, etc.). It is critically important to recognize that sterilization procedures have varying degrees of effectiveness and do not necessarily remove organic contaminants. High-temperature destruction of microbes (via steam autoclaving) or organics (*i.e.*, via dry oxidation in air at $>450^{\circ}C$ for an extended period of time), ultraviolet sterilization, and plasma cleaning [*e.g.,* chemical oxidation with hydrogen peroxide at high temperature (Crow and Smith, 1995)] may effectively decontaminate surfaces for some laboratory and flight hardware components, but these techniques are not applicable for studies in remote field sites or for specific flight hardware. In such cases, chemical and physical cleaning techniques are the only practical options.

However, both chemical and physical approaches can be problematic. In particular, chemicals selectively remove biological and nonbiological organics based on the reagent composition, but these can also damage and alter the surfaces of the sampling devices. Moreover, impure reagents (*e.g.,* phthalates or other plasticizers from reagent bottles) and chemicals or particulates shed from applicators (*e.g.,* plastic or cellulose fibers from swabs and wipes) during the cleaning and testing processes can add other contaminants. Lastly, complete decontamination is almost impossible to achieve because the environment in terrestrial Mars analog field studies is not controllable, and biological materials are ubiquitously present (*e.g.,* in aerosols). The challenges faced in achieving sterile, organic-clean surfaces have prompted the development of various cleaning protocols that are usually tailored to specific research needs. The reagents and techniques commonly used to disinfect and oxidize organics are explored below, and the development of a new protocol for field application that removes organics from the surfaces of sampling devices is presented. Disinfectants that sterilize surfaces are also used in this protocol to promote organic removal.

Existing decontamination methods

Chemical disinfectants commonly used in medical facilities, pharmaceutical clean rooms, and environmental laboratories (*e.g.,* acids, alcohols, quaternary ammonium compounds, phenols, amphoteric surfactants, aldehydes, hypochlorites, and hydrogen peroxide) induce different reactions with cellular materials, unbound organics, and inorganic materials (McDonnell and Russell, 1999; Penna *et al.*, 2001). The resultant biocidal effectiveness for bacteria, fungi, viruses, and spores differs for each reagent. *Bacillus* and *Clostridium* spores are the microbes most resistant to disinfectants (McDonnell and Russell, 1999, and references therein) and have been the focus of sporicide studies (*e.g.,* Setlow, 2006). It is important to note that disinfectants are usually chosen based on preserving the integrity of instruments and on the type of disinfection required for the specific application. Although sterilization does not necessarily remove organics, it is clearly advantageous to disinfect sampling devices prior to organic decontamination, since many disinfectants modify and break down cell walls and membranes and react with biomolecules so as to make them more susceptible to other types of removal, like solvation. For instance, ethanol or isopropanol are effective disinfectants for vegetative bacteria, viruses, and fungi [but not spores (McDonnell and Russell, 1999)], and are also suitable solvents for many biomolecules and oils. Similarly, many disinfectants are oxidizing agents that effectively break down nonrefractory organics (free and biologically bound biomolecules) and thus also serve as decontamination agents for removal of a variety of organics.

The application of 30% hydrogen peroxide (H_2O_2) solution for cleaning spacecraft hardware is a good example of a sterilant/decontamination agent. In liquid form, H_2O_2 is in general an effective sterilant (McDonnell and Russell, 1999) and is safe for most metals, but it has variable sporicidal (Kempf *et al.*, 2005) and bacteriocidal properties. H_2O_2 tends to be more effective against Gram-positive bacteria compared to Gram-negative bacteria (McDonnell and Russell, 1999). Moreover, catalase and peroxidase enzymes can render H_2O_2 inactive by breaking it down into O_2 and H_2O (Pardieck *et al.*, 1992). In contrast, oxidation of organic matter proceeds when *OH radicals formed from H_2O_2 attack aromatic, $-CH_2-$, $-CO-$, $-COOH$, $-OH$, $-NH_2$, and other polar functional groups. Thus, H_2O_2 causes oxidation of proteins and peroxidation of lipids in cell membranes, and it breaks down nucleic acids. As such, H_2O_2 is an effective sterilant; but, more importantly, it aids in decontaminating surfaces through the degradation of biomolecules to smaller polar products, which are readily rinsed off by the aqueous H_2O_2 solution or other polar solvents.

Breakdown of more-complex macromolecules in humic, lignin, or kerogenous materials by H_2O_2 yields water-soluble carboxylic acids that can be oxidized further (Goldstone *et al.*, 2002). For this reason, H_2O_2 is also commonly used by geochemists to oxidize organic matter in soils and sediments (Mikutta *et al.*, 2005); however, sodium hypochlorite (NaOCl) and disodium peroxodisulfate ($Na_2S_2O_8$) have been shown to be more effective for these applications. All three of these oxidizing reagents have been shown to be most reactive with polar organic molecules, such as recently produced biomolecules, and to have little effect on the aliphatic hydrocarbons (Mikutta *et al.*, 2005).

Similar oxidative reagents have been applied to deep subsurface ice cores acquired with drilling fluids [*e.g.,* Vostok (Christner *et al.*, 2005, and references therein)]. After extensive investigation into methods for removal of exterior microbes and nucleic-acid contaminants from the outer ice halo of ancient ice, Rogers *et al.* (2004) and Christner *et al.* (2005) concluded that sequential rinsing with a 5% sodium hypochlorite (NaOCl) solution combined with surface melting and removal was the most effective post-sampling decontamination treatment. Many of the above described decontamination protocols have either been tested solely in laboratory studies (*i.e.,* flight hardware) or in post-sampling decontamination (*i.e.,* ice cores and soil); for *in situ* field studies in terrestrial Mars analog settings, no suitable protocols are as yet available.

Here, we describe the development of a new cleaning protocol for removing organics from surfaces of sampling devices used in habitability and life-detection studies. The protocol was designed for, but is not limited to, application in remote field locations. Supporting data show that the new protocol, which involves a "cocktail" of reagents, can achieve reproducible, below-detection-limit levels of organics. In this study, the presence of bacterial lipopolysaccharide (LPS) was used as an operationally defined marker for organics. LPS is a component of Gram-negative bacteria and ubiquitous in nature (Maeda and Taga, 1979; Herbert, 1990; Trent *et al.*, 2006). LPS is commonly used as an estimate for microbial biomass. Due to LPS involvement in inflammatory responses, these molecules have been classed as bacterial endotoxins, and several tests have been devised for their rapid and sensitive detection. The presence of LPS is exclusively used as a measure of cleanliness of equipment in the food and pharmaceutical industries (Roslansky and Novitsky, 1991) for the purposes of evaluating health risks in accordance with guidelines set by the U.S. Food and Drug Administration and assessing biological contamination in controlled laboratories. In the present study, testing for bacterial LPS concentrations on surfaces of field-deployed sampling devices was undertaken with use of the *Limulus* Amebocyte Lysate (LAL) assay in which surface samples were collected on dry, sterile, LPS-free, polyester-tipped swabs for analysis. The LAL investigations were supplemented with tests for a broader suite of organic compounds detected via gas chromatography–mass spectrometry.

Materials and Methods

Cleaning protocol development was carried out during the 2005 and 2006 field seasons of the Arctic Mars Analog Svalbard Expeditions (AMASE) at various geological settings (Steele *et al.*, 2008). Cleaning was conducted primarily on the deck of a ship in ambient weather conditions that were comparable to those of deployment sites onshore. Occasionally, cleaning was done on the glacier or at the rock outcrop before deployment. The protocol development involved tests on two sampling devices: a manually deployable coring device (Mark V, Kovacs Enterprises, Inc., USA) and the sampling scoop on the robotic arm of the Jet Propulsion Laboratory's Cliffbot rover (Huntsberger *et al.*, 2007). Following each cleaning, decontamination of surfaces was gauged based on LPS concentrations determined via the LAL assay for LPS collected on polyester-tipped swabs wiped over surfaces. Tests for the presence of other organic compounds by gas chromatography–mass spectrometry were reserved for the final protocol test. Details of the two analytical approaches are discussed below.

Briefly, a simple two-step cleaning of the coring device during AMASE 2005 did not sufficiently remove cellular remnants as demonstrated by the high LPS concentrations/cm^2 (2–3 orders of magnitude above the LAL detection limits of <0.002 endotoxin units/cm^2 or EU/cm^2, data not shown). In this two-step cleaning, we used bleach-saturated wipes (Clorox, containing 5% sodium hypochlorite) that were premoistened with 100% distilled water (Fisherbrand). The bleach residue was then removed with more pre-moistened, distilled-water wipes.

In a second test, four steps were employed. We added additional reagents and emphasized mechanical action in each step. Bleach was replaced with an alternative disinfectant (CIDecon disinfectant wipes containing active ingredients 0.27500% glutaraldehyde, 0.01375%, o-phenylphenol, and 0.00275% tertiary amylphenol, which are fixatives that kill but preserve cells). Organic solvents (100% alcohols and gas chromatography–grade dichloromethane) were used to wash away dead cells and free organics. All surfaces were wiped repeatedly. Specifically, surfaces were wiped separately and sequentially with the following commercial cloths: (i) Fisherbrand 100% distilled-water wipes, (ii) CIDecon disinfectant wipes, then (iii) Contec wipes saturated with 70% isopropanol in distilled water. The inner barrel surface was then (iv) rinsed with ethanol and dichloromethane (pure, gas chromatography–grade solvents), and the outer barrel surface was wiped with ethanol applied to a sterile polyester wipe (SterileLP, ITW Texwipe). All solvents applied to wipes were gas chromatography grade without modification. However, even after this four-step method, LPS remnants ($>0.2\,EU/cm^2$ for triplicate swabs of different regions) were measured on inner and outer barrel surfaces.

The continual presence of remnant LPS on surfaces cleaned with the above two methods implied that other biological and organic materials were not removed. Forward contamination by suspect organics could compromise astrobiological samples that contain trace-level organic biosignatures; therefore, these two cleaning methods were deemed unacceptable for trace organic and life-detection studies. Based on these tests, a more extensive decontamination and validation protocol was developed and extensively tested during AMASE 2006.

Seven-step cleaning protocol

The novel protocol developed in this study involved both chemical and physical removal of particulates and organics via a seven-step procedure (Table 1). Concerns for adverse effects on the sampling device materials were considered for both devices but not thoroughly investigated herein. All solutions used (reagents 4–7 in Table 1: distilled/deionized water, 5% sodium hypochlorite, 30% H_2O_2, 70% ethanol) were prefiltered with sterile, single-use polycarbonate filter units (Fisherbrand, $0.2\,\mu m$) to assure particulate-free reagents. Blank tests on these filters showed that organics were not leached from the filters into the aqueous solutions; thus, the filtering did not affect the decontamination protocol.

The goal of step #1, which involves the use of distilled water wipes, is to remove particulate contamination, such as dust, sediment, and residues from storage and prior sampling. Step #2 is the initial disinfecting step that aims at fixing and breaking up cell material. The isopropanol wipes of step #3 provide additional disinfection but also remove organics and residuals from the earlier wipes. In steps #4–7, all surfaces were cleaned with woven polyester, clean-room–grade wipes saturated with various reagents in order to decrease the level of particulates and have a better control on reagent application. The water wipe and rinse of step #4 removes organic and inorganic residuals from the three earlier steps. Although NaOCl and H_2O_2 are known to be strong sterilants and sporicides (Russell, 1990; Sabli *et al.*, 1996; Young and Setlow, 2003), steps #5 and #6 effectively chlorinate and oxidize (Alimova *et al.*, 2005) remaining organics, which makes them more readily soluble in ethanol. Thus, the final ethanol

Table 1. Seven-Step Cleaning Protocol

Step	Reagent and application	Source	Action*
1	Distilled water pre-saturated wipe	Fisherbrand premoistened wipes	Wipe
2	Disinfectant pre-saturated wipe (containing glutaraldehyde, o-phenylphenol, and tertiary amylphenol)	CIDecon disinfectant wipe	Wipe
3	Isopropanol (70%) pre-saturated wipe	Contec	Wipe
4	Distilled water saturated clean-room wipe†	18 mega-Ω filtered water‡	Wipe and rinse
5	Sodium Hypochlorite (5%) saturated clean-room wipe†	Laboratory grade (VWR)‡	Wipe
6	Hydrogen Peroxide (30%) saturated clean-room wipe†	Perhydrol‡	Wipe
7	Ethanol saturated clean-room wipe†	Laboratory grade (VWR)‡	Wipe and rinse

*All surfaces were wiped twice with repeated rubbing action: inside, from center to each end, and outside, from top to blades.
†Clean-room wipe: woven polyethylene-filament, clean-room class 100 wipe (SterileWipe LP, TEXWIPE).
‡Filtered with Nalgene single-use, sterile filter units with 0.2 μm filters.

wiping (step #7) removes remnant organic contaminants by solvation. Dichloromethane or other less polar solvent for aliphatic hydrocarbons was not included in the developed protocol because of possible reaction and degradation of the epoxy-based barrel of the coring device.

Wiping was done in parallel bands as much as possible to avoid contact between a clean surface and a wipe that had already collected contaminants. Surface wiping was carried out from top to bottom (for the corer), from inside to the outside (for the scoop) and for both devices from the "cleanest" to the "dirtiest" parts. The contaminated wipes were folded repeatedly to provide a clean surface and avoid multiple use of the same wipe surface. For each cleaning step, multiple wipes (usually 2–3) were used.

During cleaning, powder-free nitrile gloves (nonsterile) were worn and exchanged frequently. Every attempt was made to avoid direct contact of the gloves with the sampling devices to minimize contamination with nitrile compounds (organics with cyanide functional groups). Instead, the coring device was held in place with heavy-duty aluminum baking trays (washed with Simple Green detergent and rinsed with distilled/deionized water) and heavy-duty aluminum foil (not washed). The trays and aluminum foil were previously baked at 500°C in circulating air for 8 h to combust residual trace organics. In contrast, the rover scoop was held in place by the mobile rover arm (Huntsberger *et al.*, 2007). Aqueous and ethanol rinse wastes were collected in the aluminum trays for proper disposal. After cleaning, both sampling devices were wrapped in baked, heavy-duty aluminum foil prior to transport to the field.

Coring device deployment and ice sampling

The first sampling device tested was a 14-inch diameter, 1.15-meter Mark V coring device made from glass filament–wound epoxy composite with stainless steel blades and screws at the base (Kovacs Enterprises, Inc.). The metal blades and screws were removed and sequentially cleaned by sonication in distilled/deionized water, 5% sodium hypochlorite, 30% H_2O_2, 70% ethanol (reagents 4–7 listed in Table 1). This was followed by a rinsing with dichloromethane (gas chromatography grade) to ensure removal of petroleum and other organic residues. After reassembly and barrel cleaning via the seven-step protocol described above, the foil-wrapped barrel was additionally wrapped in sterile lab bench pads for cushioning and added protection during transport to the sample site.

Alpine glacial ice samples were acquired from the central part of Friedrichsbreen, Bockfjorden (UTM coordinates: 33x 0461401 8820574). The barrel was unwrapped on site, and different 25 cm^2 regions were swabbed outside and inside the barrel in duplicate for tests of cell-wall lipopolysaccharide abundance with dry, single-use, sterile, Dacron polyester-fiber-tipped, polyethylene-stemmed swabs (ATP-free swabs by Copan, Inc., which have no detectable LPS as determined via the LAL assay described below). A consistent swabbing technique was employed, which involved rotation of the swab and a back and forth motion once over the surface. The barrel was also swabbed for organic contaminants with three cotton-tipped, wood-stemmed swabs, the tips predipped in dichloromethane (gas chromatography grade). Cotton-tipped wood swabs were required for organic tests because plastic swabs, such as those used for LPS tests, are not compatible with many solvents, especially chlorinated solvents. Most of the plant organics inherent to new unused cotton-tipped swabs had been previously extracted from the swabs by soaking them twice in a Soxhlet extractor in a solution of dichloromethane:methanol (2:1, v/v, gas chromatography–grade solvents) for 24 h each time.

During the manually powered ice coring, the barrel top was covered in aluminum foil to prevent contamination of the top surface of the ice core. While on the glacier, the acquired ice core was immediately extruded from the barrel onto new aluminum foil and broken into sections using a chisel and hammer that were both cleaned via the seven-step protocol. Since the hammer was more difficult to clean due to its rough, worn surfaces, it was also wrapped multiple times in baked heavy-duty aluminum foil and rewrapped, as necessary, to ensure that material from the hammer did not fall onto ice samples. Care was taken not to rip the foil on the hammer. The inside of the barrel (post-sampling) as well as the ice-core top, side, and interior (after sectioning into pieces with cleaned tools) surfaces were swabbed for both LPS and organic analyses, as described above, to track forward and backward contamination. Blanks were collected for both analyses by exposing a set of swabs to air at the field site for 30 s, which is equivalent to the duration that core and equipment were exposed to the external environment before

sampling. Positive controls were also collected, which included the skin of the person swabbing and snow algae adjacent to the coring site. The LPS swabs were re-inserted into their original containers, while the cotton-tipped swabs were stored in glass vials (previously washed with Simple Green detergent, rinsed with distilled/deionized water, and baked at 500°C for 8 h) and returned to the ship and laboratory, respectively, for analysis.

Rover deployment and sediment sampling

A second sampling device used for the decontamination tests was the mechanical scoop of the Jet Propulsion Laboratory's Cliffbot rover, which was made of carbon-fiber 3D composite with a stainless steel serrated edge and screws. At the first rover site, the Cliffbot scoop was cleaned in the field just prior to deployment via the standard field-deployment Jet Propulsion Laboratory cleaning method (Terry Huntsberger, personal communication). This cleaning technique involved wiping of internal surfaces with 2–3 sterile water wipes soaked with an aqueous solution of 30% hydrogen peroxide (reagent 6 in Table 1) while at the field site. Tests for LPS concentrations were conducted on the interior scoop surface (two different 25 cm^2 regions) two times—before and after sampling outcrop or sediments—to establish a baseline comparison to the seven-step procedure tested during the second rover deployment. The LPS tests were conducted in the same manner as described above—swabbing surfaces with LPS-free, polyester-fiber swabs and analyzing the LPS abundance on the swabs via the LAL assay (described below).

For a second rover site (Huntsberger *et al.*, 2007), the scoop was cleaned on the deck of the ship prior to field deployment via the seven-step protocol (Table 1) and was then wrapped in baked aluminum foil for transport. The aluminum foil cover was manually removed at the field site, and two different 25 cm^2 sections of the scoop interior were tested for LPS concentrations prior to collecting a sample. After the outcrop sample was transferred to a storage container, the scoop interior surface was retested for LPS concentrations. In addition, the surface of the sampled rock formation was also swabbed with a polyester-tipped swab (again two regions of 25 cm^2) to determine the natural level of LPS present on the exposed rock surfaces and provide a baseline for environmental LPS levels.

LAL analyses

The polyester swab tips were manually broken off (within the original swab container), removed with baked tweezers, and transferred into prepackaged sterile polypropylene tubes where they were mixed with 1 ml endotoxin-free, LAL reagent water (Charles River Laboratories, product No. W110). A 100 μl aliquot of each sample was analyzed for the concentration of cellular LPS with use of a single-use, disposable Charles River-Endosafe PTS cartridge containing LAL assay for 5–0.05 EU/ml detection and a miniaturized handheld instrument called Lab-On-a-Chip Application Development Portable Test System (LOCAD-PTS; by Charles River Laboratories) similar to that used for meeting forward contamination, *i.e.,* "planetary protection," tests on NASA's Mars Exploration Rovers. LPS measurements were made in endotoxin units per ml for each aliquot. An endotoxin unit (EU) is currently defined by the U.S. Food and Drug Administration as the endotoxin activity, as measured by the LAL assay, of 100 pg of reference standard endotoxin (RSE) from *Escherichia coli* isolate EC-6 (U.S. Food and Drug Administration, 1987, 1991). EU per milliliter measurements were converted into EU per unit area swabbed. The Endosafe-PTS cartridges have built-in positive product controls that contain endotoxin. Spike recoveries on this endotoxin control are used to monitor for LAL inhibition and enhancement due to extraneous particulates and chemicals in samples.

In general, 1 EU approximates to 10^5 cells of a single strain of *E. coli*, where each bacterium typically consists of approximately 2×10^6 LPS molecules, *i.e.,* 10^{-15} g LPS (Raetz, 1986). Extrapolation from EU to cell numbers is difficult with environmental samples, which are often made up of a variety of species or strains, each expressing various amounts of LPS (*e.g.,* Neidhardt, 1987). Assuming that the surface chemicals swabbed were similar to RSE EC-6, the lower detection limit of 0.05 EU/25 cm^2 (or 0.2 pg/cm^2) would represent less than four cell-number equivalents per cm^2. Cell-number equivalents/cm^2 were calculated based on the above assumption.

Organic analyses

The cotton-tipped swabs from each sampling of surface organics and blanks were extracted with dichloromethane: methanol (2:1, v/v) via sonication. The extracts were condensed, transesterified at 100°C for 1 h by using a prepared 5% solution of acetyl chloride (>99%, Sigma Aldrich) in methanol, then extracted with hexane:chloroform (4:1, v/v) 3 times without neutralization (method of Masood *et al.,* 2005, modified with the addition of chloroform). Since some organics will not easily hydrolyze with the above method (*e.g.,* some carbohydrate-bound lipids), the methylated extracts were separated into neutral (fractions I) and polar fractions (fractions II) on a silica gel column with hexane: dichloromethane (1:1, v/v) and methanol, respectively. Fraction II was subjected to a second, more thorough hydrolysis with 0.5 *N* KOH (Fisherbrand, certified ACS reagent) in methanol at 70°C overnight (16 h), extracted with hexane: chloroform (4:1), dried under N_2, then silylated with 50% N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (Pierce Biotechnology) in acetonitrile (99.9% Acros Organics) at 70°C overnight in N_2-flushed sealed vials. All solvents used for organic analyses were gas chromatography grade (Fisher Optima or GCResolve) unless specified. All glassware was baked at 500°C for 8 h.

Both fractions were analyzed via gas chromatography–mass spectrometry in full scan mode (50–650 Da). An Agilent 5973N MSD was fitted with an Agilent 6890 GC equipped with a 5%-phenyl-methylpolysiloxane capillary column (HP-5MS; 30 m×25 μm i.d., 0.25 μm film thickness) and He carrier gas. Samples in 50 μl hexane (fraction I) or as neat derivatives in 100 μl solvent (fraction II) were injected in pulsed splitless mode into a 300°C injection port. The gas chromatograph (GC) oven was programmed from 50°C (held for 1 min) to 300°C (at 3°/min), and then held for 10 min at 300°C. Total ion chromatogram (TIC) results from blank swab controls were subtracted from the sample TIC results.

Results

Semi-quantitative results that we used to assess the thoroughness of our cleaning method included LAL assay for LPS

concentrations (as EU per unit area) and organic compound detection via gas chromatography–mass spectrometry.

The LAL results on (a) the decontaminated coring device prior to deployment, (b) the exterior ice-core surfaces and (c) the coring device after coring, and (d) the blanks and positive controls are shown in Table 2. The data reveal that the seven-step cleaning protocol (Table 1) effectively removed all target molecules to values within or below the detection limits of $<0.002\,EU/cm^2$ [determined by the area swabbed ($25\,cm^2$) and the detection limits of the LAL assay cartridges (0.05 EU/ml) used in the Endosafe PTS instrument]. After coring, both the ice core exterior and interior (after sectioning) were also below detection. The only surfaces that showed LPS values slightly above detection were the interior surfaces of the corer barrel and the top surface of the ice core itself. Notably, both positive controls (of snow algae and the analyst) yielded 3 to 5 orders of magnitude greater LPS concentrations.

Lipopolysaccharide concentrations were measured on freshly cleaned surfaces (pre-sampling) and after collection and storage of a sample (post-sampling). The LPS concentrations on the rover scoop surfaces after the first cleaning test employing H_2O_2 only, revealed 2 orders of magnitude higher values ($0.186\,EU/cm^2$) than after the seven-step procedure cleaning, which brought the values down to below detection ($<0.002\,EU/cm^2$; Table 3). For the seven-step cleaning protocol, the LPS levels on swabs collected post-sampling of the inside of the scoop increased by 2 orders of magnitude ($0.167\,EU/cm^2$) relative to pre-sampling levels. LPS levels on the scoop surface after sampling were roughly equivalent to those detected on the rock surfaces, which indicates backward contamination of the scoop by the rock sample. Post-sampling LPS concentrations on the scoop after the simple H_2O_2 cleaning were another order of magnitude higher (up to $2.43\,EU/cm^2$; Table 3), which demonstrates again that simple H_2O_2 cleaning was not effective in removing organic signals detected via the LAL assay.

The coring device, ice-core surfaces, and blanks were tested for organics. Low–molecular weight (LMW) saturated fatty acids (as methyl esters; $C_{16:0}$–$C_{24:0}$), and carbohydrate derivatives were identified in fraction I of all samples (*e.g.*, Fig. 1A, Table 4), including the blanks, which also served as swab controls (Fig. 1B). The only other major compounds identified as part of fraction I were a set of high–molecular weight (HMW) saturated fatty acids ($C_{25:0}$–$C_{34:0}$) observed in swabbed top surface of the ice core (Fig. 1C, Table 4). Interestingly, in this atypical sample, the HMW fatty acids were nearly twice as abundant as the LMW fatty acids, yet even-carbon-chain lengths dominated both sets of fatty acids. All other extraneous compounds were not identified but also observed in blanks.

In contrast to fraction I, fraction II yielded quantitatively and qualitatively similar compositions for all samples, including blanks (not shown). In general, the gas chromatograph–mass spectrometer (GC-MS) response for fraction II was 1–5 times greater than for fraction I. Fraction II was dominated by plant-derived steroid and pentacyclic terpenoids biomarkers. Traces of $C_{14–24}$ even-chain-length saturated and unsaturated fatty acids (as *tert*-butyldimethylsilyl derivatives) were also observed. Detection limits for fatty acids were estimated to be $1\,ng/cm^2$ for swabbed surfaces.

Discussion

The data presented above show that decontamination with one or two reagents (*i.e.*, H_2O_2; NaOCl and water) was insufficient at removing LPS from sampling-device surfaces (both ice-coring device or scoop surface). These procedures were similar to earlier published decontamination protocols (McDonnell and Russell, 1999; Penna *et al.*, 2001; Rogers *et al.*, 2004; Venkateswaran *et al.*, 2004; Christner *et al.*, 2005; Kempf *et al.*, 2005; Mikutta *et al.*, 2005). Notably, some of these prior investigations aimed at post–sample-acquisition decontamination (*e.g.*, soil and ice core) as opposed to pre-sampling approaches (*e.g.*, flight hardware). The scoop results demonstrate that wiping with H_2O_2 alone is insufficient at reducing biosignature measurements to null values (these surfaces were up to 2 orders of magnitude "dirtier" than those cleaned with the seven-step protocol). Positive results for LPS after cleaning with these simplified procedures in-

Table 2. LPS Concentrations Detected on the Cleaned Barrel of the Coring Device (Pre- and Post-Deployment), on the Various Ice-Core Surfaces, and for the Positive Controls

Sample	*LPS concentration (mean EU/cm²)*	*Coefficient of variation (%)**	n*	*Cell-number equivalent/cm²*†
Precoring				
Blank (swab control)	<0.002	NV	2	<4
Positive control (analyst)‡	0.015	14.6	2	30
Coring device barrel interior and blades	<0.002	NV	3	<4
Coring device barrel exterior	<0.002	NV	2	<4
Ice core				
Ice-core exterior side surface	<0.002	NV	2	<4
Ice-core top surface	>0.002	5.4	2	>4
Ice-core interior after break	<0.002	NV	2	<4
Postcoring				
Coring device barrel interior and blades	<0.003	9.9	2	<6
Positive control (snow with algae)‡	0.790	—	1	1580

*NV, no variation; *n*, number of swabs analyzed for each measurement.
†Assuming that the surface chemicals swabbed were similar to RSE EC-6 (see text).
‡Sample was diluted by 100× in order to test within the detection limits using the LAL assay.

TABLE 3. CONCENTRATIONS OF LPS ON THE INTERIOR SURFACE OF THE CLIFFBOT SCOOP, BEFORE AND AFTER CLEANING, AND POST-SAMPLE-ACQUISITION INCLUDING POSITIVE CONTROL

Sample	*LPS concentration (mean EU/cm²)*	*Coefficient of variation (%)*	n*	*Cell-number equivalent/cm²†*
H_2O_2-only cleaning				
Scoop pre-sampling	0.186	0.04	2	372
Scoop post-sampling (test 1‡)	2.430	30.3	3	4860
Scoop post-sampling (test 2‡)	1.890	142	3	3780
Seven-step cleaning				
Scoop pre-sampling	<0.002	—	—	<4
Scoop post-sampling	0.167	1.70	2	334
Positive control (rock sample)	>0.200	—	—	>400

**n*, number of swabs analyzed for each measurement.
†Assuming that the surface chemicals swabbed were similar to RSE EC-6 (see text).
‡The scoop was used to sample sedimentary outcrops.

dicate that some biological and organic materials were not removed, a result that could compromise trace organic and life-detection studies and prompted us to pursue method development for extensive decontamination and validation protocol.

In addition, LAL assays show that levels of LPS following sediment sampling by the Cliffbot rover were still high (up to 10^3 greater), which corroborated that "in-between-sample cleaning" or "single-use sampling devices" are crucial for both terrestrial Mars analog sample acquisition studies and for design and applications of flight and analytical hardware in future Mars missions. Furthermore, post-sampling, high LPS concentrations on surfaces indicate backward contamination and the need for decontamination measures between samplings to avoid cross contamination of astrobiological samples.

The cocktail of reagents in the seven-step protocol act both to sterilize and decontaminate the surfaces of sampling

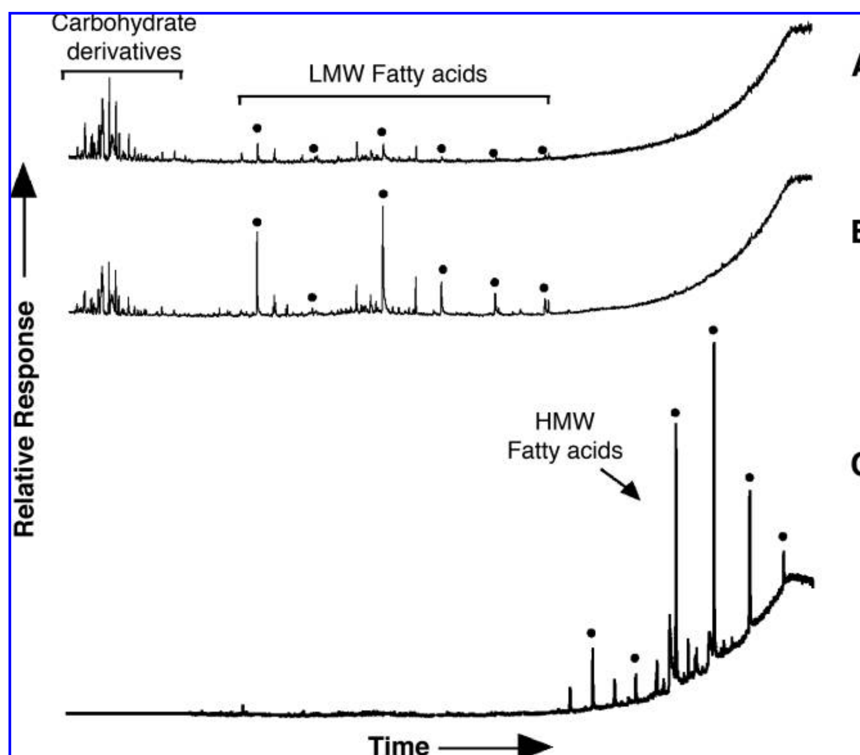

FIG. 1. Total ion chromatograms from gas chromatography–mass spectrometry analyses of fraction I of swab extracts. (**A**) Organics observed on the interior surfaces of the coring device after cleaning but before coring include carbohydrate derivatives and low–molecular weight (LMW) fatty acids (as methyl esters; $C_{16:0}$–$C_{24:0}$). (**B**) Organics detected in the swab control were qualitatively the same as those in (A) but slightly more abundant, most likely due to incomplete extraction of the swabs before use. (**C**) Organics from the ice-core top surface are shown as sample TIC minus the TIC for the blank. High–molecular weight (HMW; $C_{25:0}$–$C_{34:0}$) fatty acids are also present in the top ice surface and are similar to those observed in snow algae and cryoconites (Eigenbrode, unpublished). Dots indicate even carbon numbers for fatty acid chains.

Table 4. Groups of Organic Molecules Detected in Fraction I Via Gas Chromatography–Mass Spectrometry for the Coring Device after Cleaning and Ice-Core Surfaces

Sample	*Carbohydrate derivatives**	*$C_{14–24}$ fatty acids**	*$C_{28–34}$ fatty acids**	*TIC of Figure 1*
Precoring				
Blank (swab control)†	+	+	—	1C
Coring device barrel interior and blades	+	+	—	1A
Coring device barrel exterior	+	+	—	1A‡
Ice core				
Ice-core exterior side surface	+	+	—	1A‡
Ice-core top surface	+	+	++	1B
Ice-core interior after break	+	+	—	1A‡
Postcoring				
Coring device barrel interior and blades	+	—	—	1A‡

*+, presence of compounds; ++, stronger relative response of HMW fatty acids compared to LMW fatty acids in the same sample.
†Background is defined by the qualitative composition of the blank.
‡Figure 1A is representative of multiple samples.

devices and thus contributes to its effectiveness. Through the course of method development, however, the need for a combination of mechanical and chemical action became apparent. Most likely, the physical action assisted in the removal of contaminants adhered to the surfaces. In the final, seven-step protocol, each surface was wiped at least 14 times. The wide range of disinfectants (glutaraldehyde, phenylphenol and tertiary amylphenol, alcohol, sodium hypochlorite, and hydrogen peroxide) applied in the seven-step protocol was necessary to ensure the destruction of chemically resistant strains of both vegetative cells and spores.

The LAL assay results from the coring device cleaning tests show that the seven-step cleaning protocol removed all detectable traces of cell wall material (as LPS). Thus, it is likely (though not tested) that other biological macromolecules, such as DNA, proteins, complex carbohydrates, lipids, were also removed. The post-cleaning GC-MS results for organics present on the swab extracts are consistent with this conclusion, since the only sample with detectable traces above blank levels was from the top surface of the ice sample—a location where organic biosignatures, such as HMW fatty acids (Table 4) identified in surface life (in cryoconites, wind-blown debris, snow algae, etc.), are expected to be strong. The relatively abundant and diverse array of compounds present in the blanks and their affinity to other plant molecular signatures (Simoneit, 2002; Vichi *et al.*, 2007) indicate that the cotton/wood swabs, though thoroughly extracted before use, were the source of the high backgrounds and degraded the test's sensitivity.

The organic molecules in the swab extracts of the corer surfaces were largely comparable in composition and quantity to extracts for the blank swab control, which indicates that the organics in these samples were derived from the cotton and wood of the swab. Fraction I of swab extracts from the ice-core top surfaces showed significantly higher quantities and larger variations in composition compared to all other samples, which indicates detectable quantities of ice-derived organics, such as wind-blown debris and snow algae (Fig. 1A, 1B). The GC-MS results are consistent with relative differences in LPS concentrations except for the traces of LPS detected in the interior core barrel after coring. The correlative swab extracts for organics from the same surface showed no detectable signal above GC-MS background. This discrepancy is due to the lower sensitivity and selectivity of gas chromatography–mass spectrometry organic analysis compared to the LAL assay for LPS.

The presence of lipopolysaccharides on interior core barrel surfaces after sampling the ice is particularly interesting. Measured values are just above detection limit ($<0.003\,EU/cm^2$), which indicates backward transfer of ice-derived cell wall material to the core barrel. Diverse life has been documented below (Skidmore *et al.*, 2000; Abyzov *et al.*, 2001; Bhatia *et al.*, 2006; Mader *et al.*, 2006; Kastovska *et al.*, 2007) and within (Catranis and Starmer, 1991; Karl *et al.*, 1999; Priscu *et al.*, 1999; Abyzov *et al.*, 2001; Sheridan *et al.*, 2003; Christner *et al.*, 2003; Miteva and Brenchley, 2005) glacial ice. Life on glacier surfaces includes snow algae (Müller *et al.*, 1998; Benning *et al.*, 2006) and cryoconite or snow-pack microbial communities (Christner *et al.*, 2003; Gunde-Cimerman *et al.*, 2003; Amato *et al.*, 2007). In the accreted Vostok lake ice, Karl *et al.* (1999) detected ~100 pg of LPS per liter ice. The backward transfer of the smallest detectable amount of LPS from the sampled ice to the ice coring barrel, as found in this study, is consistent with earlier findings of microorganisms, including Gram-negative bacteria (the producers of LPS), within glacial ice at other sites. It also indicates that the seven-step protocol sufficiently decontaminates sampling device surfaces so that traces of life in extreme environments, like the Arctic ice and rocks, can be detected and quantified in a contamination-free mode.

The seven-step protocol, though highly practical and applicable for most laboratory studies, still poses challenges when tested in the field in terms of complexity of the cleaning steps and keeping materials clean in the field. During AMASE, the availability of instruments on board the research ship and at deployment sites allowed this protocol to be developed and tested. Despite the successful tests carried out in the field so far, reducing the seven-step protocol to fewer steps will be a focal point of our future research. The current method targets removal of a wide variety of organic contaminants that may not be characterized before cleaning. Reduction in the number of steps will likely depend on the abundances and types of organic contaminants present and the material being cleaned.

Field-based applications of this protocol will be particularly crucial for cleaning and decontamination of sampling devices that are not single use (*i.e.,* for successive sample acquisitions). Decontamination between sample collections is important when organic concentrations in samples are already very low, which makes the sample susceptible to forward contamination, and when sample devices have been used for more-complex sample matrixes with higher organics or bioload, which can contribute to backward or sample-to-sample contamination (*e.g.,* successive rock sampling with a rover scoop). Sequential sampling with a device will require a thorough and stringent decontamination protocol after each sampling step. The possibility of utilizing single-use sampling devices that are pre-cleaned according to the seven-step protocol may be a compelling alternative to cleaning devices on site between sample collections. Single-use sampling devices may be specifically suitable for planetary applications.

Future adjustments to the newly developed decontamination protocol will entail a switch to polyester clean-room wipes throughout the whole decontamination process, as this will be more conducive to other simplifications. Efforts will also be made to reduce the number of steps and further decrease background levels of organics. Cotton/wood swabs for organic analyses, which attributed to diverse organics in blanks, will be replaced with organic-free glass fiber wipes held with forceps. The effectiveness of the multi-step decontamination protocol will also be cross-correlated in future deployments with other life-detection techniques, including measurements of a broader range of organics identifiable from swab and rinse extracts via gas and liquid chromatography. In particular, tests for LPS and gas chromatography–amenable organics will be supplemented with measurements of total ATP, which will allow investigators to exploit more fully the potential of cellular activity or remnants.

The Charles River Endosafe LOCAD-PTS unit capable of quantifications of LPS concentration was tested on the International Space Station in March 2007, and similar units are likely to be used for future ESA and NASA Mars missions. Although such units are not yet widely used in the terrestrial Mars-analog research community or in flight hardware decontamination testing, the data presented above show that the combination of a thorough cleaning protocol with the low-level LPS detection capabilities of such units is invaluable with regard to a clean sample-device treatment—both prior to deployment and between sampling events—that could be applied to terrestrial field-based astrobiology research and future planetary missions.

Application of the seven-step protocol was successful in decontaminating epoxy-resin fiberglass, stainless steel, and carbon-fiber 3D composite materials of two sampling devices to null levels deemed necessary for our life-detection tests. Further, there were no obvious adverse effects on the sampling equipment, LAL assay, and organic chemical tests. Although the protocol was designed to remove the most reactive reagents, incomplete removal of glutaraldehyde, sodium hypochlorite, and H_2O_2 could adversely affect other analytical tests. In addition, chemical reagents used here are not compatible with all sampling hardware materials. Thus, the seven-step protocol in its entirety is not appropriate to all life-detection studies; however, it does lay the groundwork by which decontamination protocols can be tailored and tested for specific field, lab, and planetary investigations.

Conclusions

Our results show that the combination and step-wise application of disinfectants with oxidative and solvation properties for organic decontamination are effective at removing cellular remnants and other organic traces to levels necessary for life-detection studies. The validation of this seven-step protocol—specifically for ice sampling—allows us to proceed with confidence in terrestrial analog investigations of icy environments; thus, this protocol may be applicable to future astrobiology missions to icy regions of Mars, Europa, and Enceladus.

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Author Disclosure Statement

No competing financial interests exist.

Abbreviations

AMASE, Arctic Mars Analog Svalbard Expedition; ATP, adenosine triphosphate; EU, endotoxin unit; GC, gas chromatograph; GC-MS, gas chromatograph–mass spectrometer; HMW, high molecular weight; LAL, *Limulus* Amebocyte Lysate; LMW, low molecular weight; LOCAD-PTS, Lab-On-a-Chip Application Development Portable Test System; LPS, lipopolysaccharide; RSE, reference standard endotoxin; TIC, total ion chromatogram.

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