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Glacial ice cores: a model system for developing extraterrestrial decontamination protocols

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Abstract

Evidence gathered from spacecraft orbiting Mars has shown that water ice exists at both poles and may form a large subsurface reservoir at lower latitudes. The recent exploration of the martian surface by unmanned landers and surface rovers, and the planned missions to eventually return samples to Earth have raised concerns regarding both forward and back contamination. Methods to search for life in these icy environments and adequate protocols to prevent contamination can be tested with earthly analogues. Studies of ice cores on Earth have established past climate changes and geological events, both globally and regionally, but only recently have these results been correlated with the biological materials (i.e., plant fragments, seeds, pollen grains, fungal spores, and microorganisms) that are entrapped and preserved within the ice. The inclusion of biology into ice coring research brings with it a whole new approach towards decontamination. Our investigations on ice from the Vostok core (Antarctica) have shown that the outer portion of the cores have up to 3 and 2 orders of magnitude higher bacterial density and dissolved organic carbon (DOC) than the inner portion of the cores, respectively, as a result of drilling and handling. The extreme gradients that exist between the outer and inner portion of these samples make contamination a very relevant aspect of geomicrobiological investigations of ice cores, particularly when the actual numbers of ambient bacterial cells are low. To address this issue and the inherent concern it raises for the integrity of future investigations with ice core materials from terrestrial and extraterrestrial environments, we employed a procedure to monitor the decontamination process in which ice core surfaces are painted with a solution containing a tracer microorganism, plasmid DNA, and fluorescent dye before sampling. Using this approach, a simple and direct method is proposed to verify the authenticity of geomicrobiological results obtained from ice core materials. Our protocol has important implications for the design of life detection experiments on Mars and the decontamination of samples that will eventually be returned to Earth.

Keywords: Exobiology; Ices; Mars; Experimental techniques

1. Introduction

NASA and ESA currently have scheduled missions to examine the martian surface and ice caps for liquid water and life, with the first sample return missions from Mars scheduled to launch as early as 2014 (Hubbard et al., 2002; http://nssdc.gsfc.nasa.gov/planetary/mars_2003_05.html). A major concern in such missions is the forward contamination of Mars with microbes and biological molecules transported on spacecraft from Earth, which might then be misinterpreted as evidence for extraterrestrial life, but more importantly, could irreversibly alter the pristine nature of the martian environment (Barengoltz, 2000; Rummel, 2000, 2001; Mancinelli, 2003). Assessing procedures used in Solar System exploration and sample return missions is the responsibility of The Planetary Protection Advisory Committee, originally established by NASA to directly address concerns raised in the United Nations Outer Space Treaty of 1967 (Rummel, 2001). The measures taken by NASA to control the forward contamination of Mars during the Viking missions seemed appropriate at the time. However, nearly 30 years of deliberation and an increased knowledge of the tenacity of life have made it necessary to reevaluate appropriate protocols

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Table 1
Recent ice-based biological studies of glacial and subglacial environments

Origin of ice core or glacial samples	Investigators	Techniques employed	Decontamination procedure
Vostok Station, Antarctica	Abyzov (1993), Abyzov et al. (1998, 2001)	Culture-based, physiological, and microscopy	Device that melts sample from the ice core interior
Ellesmere Island, Canadian Arctic	Dancer et al. (1997)	Culture-based	Flame-sterilized ice axes
Hans Tausen ice cap, Greenland	Willerslev et al. (1999)	16/18S rDNA-based identification	Band saw removal of ice core exteriors
Vostok Station, Antarctica	Priscu et al. (1999)	Geochemical, 16S rDNA-based identification, and microscopy	Washing procedure
Vostok Station, Antarctica	Karl et al. (1999)	Geochemical, physiological, microscopy, and flow cytometry	Washing procedure
Dye 3 and GISP2, Greenland	Castello et al. (1999)	RNA-based identification	Aseptic subcoring of ice samples and UV irradiance
Nonpolar and polar ice core samples	Christner et al. (2000, 2001, 2003)	Culture-based, 16S rDNA-based identification, physiological, and microscopy	Sampling device that melts sample from the ice core interior and washing procedures
GISP2, Greenland	Sheridan et al. (2003), Miteva et al. (2004)	Culture-based, 16S rDNA-based identification, and flow cytometry	Aseptic subcoring of cleaned ice samples
Ice made in the laboratory[a]	Rogers et al. (2004)	Culture and PCR based analysis	Washing with sodium hypochlorite, ethanol, HCl, NaOH, and H_2O_2, UV-irradiance, and aseptic subcoring

[a] "Sham" cores constructed to test various methods of ice core decontamination.

for the protection of planets and satellites from contamination by earthly microorganisms. The implementation of appropriate procedures to decontaminate spacecraft materials and authenticate biological results is therefore of prime importance to these scientific endeavors. Developing suitable approaches to conduct these future studies can directly benefit from the experience gained from the study of terrestrial glacial and subglacial environments, which confront similar contamination issues.

Ice cores from glacial ice on Earth have provided detailed paleorecords of temperature, precipitation, chemistry and gas composition of the lower atmosphere, volcanic eruptions, solar variability, sea surface productivity, anthropogenic emissions, and a variety of other climate and biogeochemical indicators. These data have proven to be invaluable in our understanding of climate processes and for testing theories that can predict future climatic changes. Though this research has integrated many disciplines, the biological material (i.e., plant fragments, seeds, pollen grains, fungal spores, and microorganisms) preserved within the ice itself has only recently received attention (Table 1). As a result of this new information, we now believe that glaciers and large subglacial lakes sealed below kilometers of ice (e.g., Subglacial Lake Vostok) contain viable life and biological molecules that persist over extended periods of time (Priscu and Christner, 2004).

The foremost technical obstacle in ice core studies is the implementation of appropriate procedures to decontaminate samples used in microbiological, molecular-based, and biogeochemical analyses. Ice core drilling requires the use of a drilling fluid (typically *n*-butyl acetate or kerosene and a densifier) to prevent lithostatic pressure from causing plastic collapse of the borehole at drilling depths >300 m of ice (Talalay and Gundestrup, 2002a, 2002b). These fluids are not sterilized or pasteurized previous to use, contain no bactericides, and kerosene-based fluids contain known bacterial substrates. Ice cores are further handled during collection and archiving, with no special considerations for removing or preventing additional biological contamination. Therefore, the major concern in a microbiological study with ice core materials is that the sample was adequately decontaminated. While it has been argued that glaciated terrestrial environments may serve as an appropriate analog for preserving life that exists or did exists on Mars (Christner et al., 2000; Priscu and Christner, 2004), the information gained in the development of strategies to decontaminate ice core materials is directly applicable for evaluating biologically clean approaches crucial to the search for life and biosignatures in martian ice samples.

The issue of microbiological decontamination of ice core materials has been addressed by a number of investigators who have independently tested and implemented procedures in their laboratories to aseptically obtain samples (Table 1).

While considerable effort has been put forth to develop clean sampling technology, proving that a positive result is not an artifact is nevertheless very difficult without experimental evidence to confirm the removal of extraneous cells and molecules. To address this issue and the inherent concern it raises for the integrity of future investigations with ice materials from both terrestrial and extraterrestrial sources, we employed quantitative decontamination procedures in our laboratory to verify the authenticity of results obtained. Herein we report the efficacy of these procedures on earthly ice and discuss their relevance to life

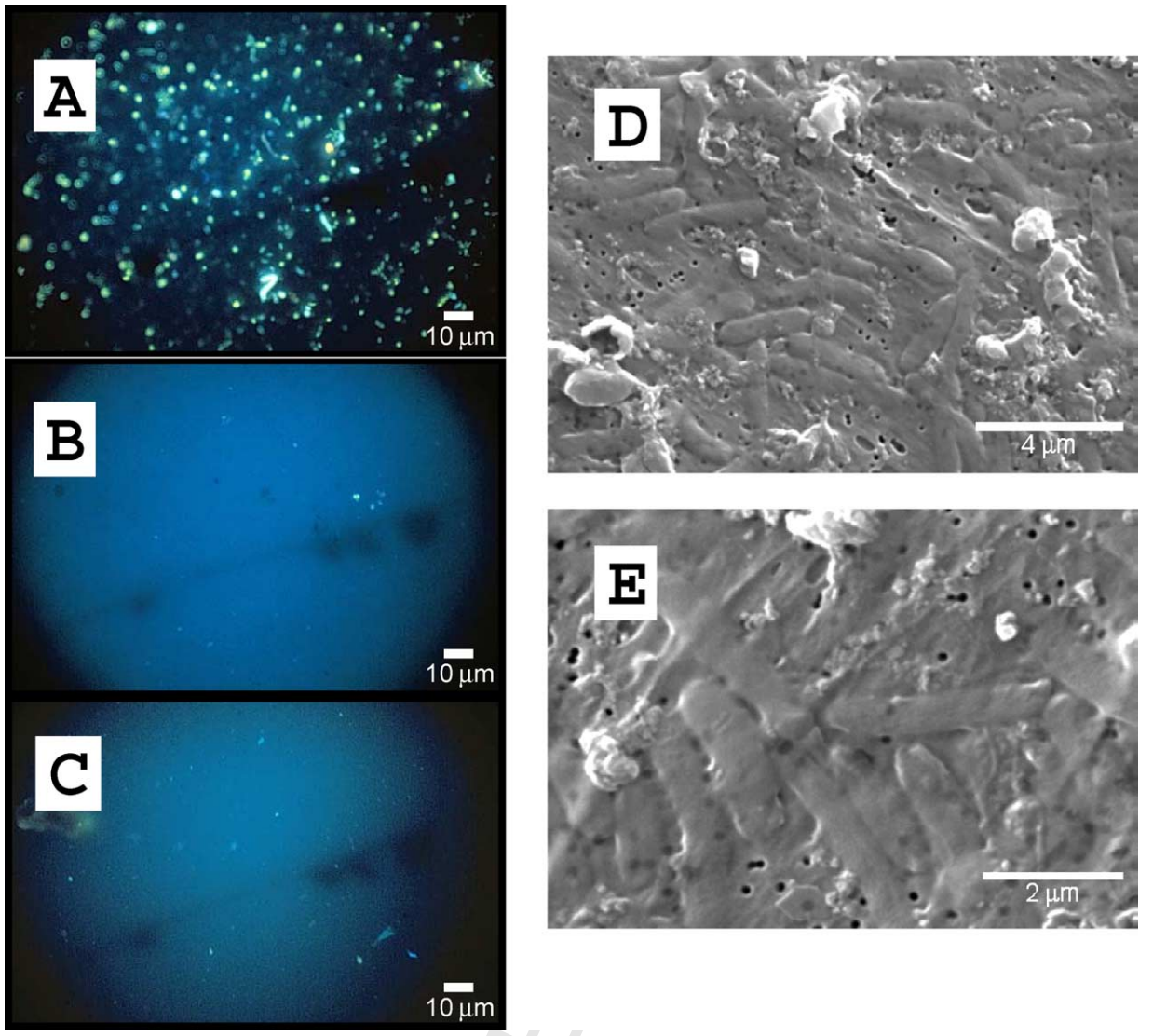

Fig. 1. Epifluorescence images of apparently prokaryotic cells in successive samples collected after the exterior 0.47 cm was removed (A), and from interior samples collected after 0.57 cm (B) and 0.82 cm (C) of the original core radius was removed from Vostok core 1577. The cells were stained with SYBR-Gold, a DNA specific probe (A)–(C). Scanning electron micrographs of bacterial contaminants on the outer portion of the core (D), (E).

detection missions that will sample icy environments on Mars.

2. Ice core decontamination and sampling

In initial sampling trials with ice samples from the Vostok ice core, microscopic analysis established that rod-shaped cells (~3 µm long and ~1 µm wide) were abundant on the core exterior (Fig. 1A, 1D, and 1E), which apparently resulted from microbial contamination present in the drilling fluid or were introduced during subsequent handling.

These cells were morphologically distinct from those observed in melted fractions from the core interior, the vast majority of which were cocci 0.7–1.4 µm in diameter. Direct cell counts of collections from the ice core exterior verified that the numbers of cells were up to 3 orders of magnitude higher than those observed from the core interior (Table 2).

Due to the extreme gradient of cells from the exterior to the interior of the ice core, it was essential to confirm that the sampling method was effective in removing external cellular and molecular contamination. Based on strategies developed to examine cellular contamination during drilling and sampling of permafrost samples (e.g., Shi et al., 1997; Willerslev et al., 2003, 2004a, 2004b), we amended our protocol to include an intentional contamination of the ice core before sampling with the microorganism *Serratia marcescens*. In addition, a target DNA sequence and fluorescent dye (rhodamine 6G) were also included in the tracer mixture (Table 3), which provided a means to also monitor the removal of macromolecular and molecular contamination during the sampling procedure.

After the tracer mixture was applied, the exterior portion (5 mm of the radius; 10 mm of the diameter) of the ice core was physically removed by scraping with a microtome

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Table 2
The numbers of cells per milliliter (cell ml^{-1}) in melt water collected from the outer surface and innermost portion of samples from the Vostok ice core

Core ID[a]	Core age[b] (yrs.)	Ice core exterior			Ice core interior			Ratio from outside to inside
		Cumulative average cell $ml^{-1} \pm$ SD[c]	% error[d]	Core radius removed (cm)	Cumulative average cell $ml^{-1} \pm$ SD	% error[d]	Total core radius removed (cm)	
V179	7.12E+03	6.54E+03 ± 7.26E+01	1.1	0.52	1.01E+02 ± 2.17E+01	22	1.50	3.21E+01
V763	5.05E+04	4.77E+03 ± 1.86E+02	3.9	0.40	1.13E+02 ± 2.21E+01	19	0.78	4.21E+01
V1577	1.14E+05	3.02E+05 ± 3.55E+02	0.1	0.47	3.79E+02 ± 5.25E+01	14	0.82	7.98E+02
V2091	1.53E+05	1.83E+05 ± 1.81E+03	1.0	0.71	4.23E+01 ± 1.08E+01	25	1.26	4.33E+03
V2490	2.02E+05	2.34E+04 ± 3.99E+02	1.7	0.69	8.88E+01 ± 2.00E+01	23	1.01	2.64E+02
V2749	2.37E+05	4.81E+03 ± 1.08E+02	2.3	0.63	1.82E+02 ± 2.99E+01	16	1.30	6.47E+01
V3015	2.98E+05	2.36E+04 ± 8.21E+02	3.5	0.59	3.49E+01 ± 1.59E+01	45	0.93	6.78E+02
V3197	3.60E+05	2.84E+03 ± 1.28E+02	4.5	0.74	3.35E+01 ± 9.80E+00	29	1.16	8.49E+01
V3351	NA	1.17E+04 ± 3.61E+02	3.1	0.31	6.81E+01 ± 1.35E+01	20	0.37	1.72E+02
V3540[e]	NA	2.41E+04 ± 4.90E+02	2.0	0.48	3.70E+02 ± 3.79E+01	10	0.67	6.49E+01
V3566[e]	NA	2.94E+04 ± 5.67E+02	1.9	0.38	4.29E+02 ± 2.27E+01	5.3	0.56	6.84E+01

SD—standard deviation from the mean. NA—not available.
[a] Ice core samples are designated by the depth of recovery from below the surface. For example, V179 was recovered from 179 m below the surface (mbs).
[b] Based on Petit et al. (1999) time scale.
[c] Numbers reflect the background level of cells on core exteriors due to contamination from drilling fluid, subsequent handling, and archiving.
[d] The percent error is the standard deviation divided by the arithmetic mean.
[e] Ice core samples not in stratigraphic order or of meteoric origin (i.e., derived by accretion of water from Lake Vostok onto the bottom of the ice sheet; Jouzel et al., 1999).

Table 3
Tracers used for monitoring ice core sampling

Tracer	Specifics	Concentration applied	Rationale	Detection limit
Cell	*Serratia marcescens*	5×10^7 cells	$\sim 10^5$ cells ml^{-1} observed on core exteriors	1 cfu ml^{-1}
Macromolecule	4.3kb DNA plasmid: pTOPO-ITS-*Naegleria*[a] Mol. wt. $\sim 2.6 \times 10^6$	2 ng (4.4×10^8 molecules)	If $\sim 10^5$ cells ml^{-1} on exterior, 10^5–10^6 16S rDNA copies are present	500 molecules ml^{-1} (round of PCR)$^{-1}$ (theoretical)
Molecule	Rhodamine 6G: Mol wt. 479.0	100 mg l^{-1}	DOC values 2 log higher on core exterior than core interior	0.001 mg l^{-1}

cfu—colony forming units of *S. marcescens* on tryptic-soy agar media.
[a] Plasmid DNA is pCRII-TOPO vector (Invitrogen) containing ~400 nucleotide cloned DNA insert (Pélandakis et al., 2000) that would not be expected to be present in the sample.

blade to remove the contaminated exterior of the sample and expose previously unhandled ice. In a biologically-clean environment, the scraped ice samples were thoroughly washed with 95% ethanol and then rinsed with sterile deionized H_2O, to disinfect and remove another ~5 mm layer of the ice core. Initial rinsing with 4 °C sterile deionized H_2O resulted in extensive core fracturing, due to the temperature difference between the ice (−10 °C) and water (4 °C). Such fractures can lead to complete sample disintegration and also provide a means of entry for surface contaminants to penetrate into the core interior. Ethanol had the advantage that it remained liquid at −20 °C resulting in no ice core fracturing during rinsing, and the ethanol was easily diluted subsequently to nontoxic concentrations by rinsing with 4 °C sterile deionized H_2O. As measurements of dissolved organic carbon (DOC) are sensitive to the presence of ethanol, samples for these analyses were decontaminated using the scraping protocol described in Appendix A. The cleaned ice core was incubated at 22 °C until at least 15 mm of the original core radius had melted away, and the remaining ice was allowed to melt completely in the dark at 4 °C. A detailed description of the sampling protocol appears in Appendix A; Fig. 2 shows schematically the removal of external portions of the ice core during each step of the decontamination procedure.

3. Assessment of the decontamination procedure

The outermost portion of each core was contaminated with a total of 5×10^7 cells of *S. marcescens* (Table 3), which is a concentration equivalent to the cell numbers naturally occurring on core exteriors (Table 2). The effectiveness of removing viable cells of *S. marcescens* from the ice surface is shown in Fig. 3A, with 5.7×10^4 and 1.6×10^5 colony forming units (cfu) ml^{-1} of *S. marcescens* recovered from

the melted ice scraped from the exterior of samples 179 and 2749, respectively.

Physical scraping of the ice exterior followed by washing with ethanol and deionized H_2O removed 0.7–0.9 cm of the core radius and 25–35% of the ice mass. This procedure yielded a sample in which no viable cells of *S. marcescens* were recovered. Direct epifluorescent counts of cells in washes and melt water recovered during sampling revealed that the number of cells ml^{-1} plateaus after 1.3–1.5 cm of the core radius is removed (Fig. 3A), indicating that removal of at least 1.5 cm of core radius eliminates surface contamination. *S. marcescens* cells from logarithmic growth are rod-shaped cells that have a length of 2.5–3 μm and a width of 1–1.5 μm. These test cells are characteristically much larger than those observed in ice core samples; cells of this size were never observed in samples from the core interior.

Although ethanol is an effective disinfectant, it does not destroy nucleic acids. It was therefore necessary to demonstrate that our washing and melting procedure was able to remove extraneous DNA, as the inability to do so would hinder PCR-based approaches for characterizing the genetic nature (i.e., phylogenetic identification using 16S rDNA sequencing) of microbial populations in the ice. The macromolecular plasmid DNA sequence was amplified from the outermost collected portion of the ice sample, but amplicons were not generated in samples where at least 1 cm of the core radius was removed, even when a sensitive nested PCR approach was employed (Fig. 3C). Since $\sim 10^8$ copies of the plasmid was placed on the external surface of each core (Table 3), which is equivalent to the number of 16S rRNA gene copies anticipated in a population of $\sim 10^7$ cells (Klappenbach et

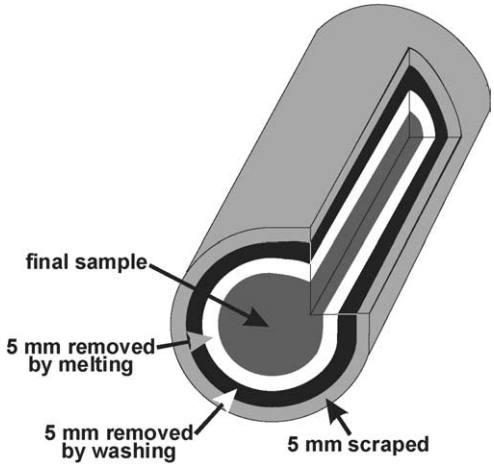
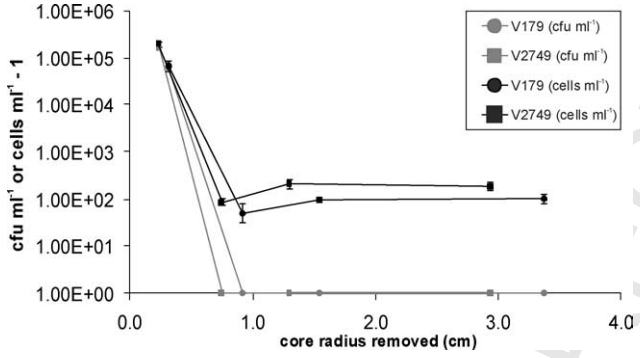
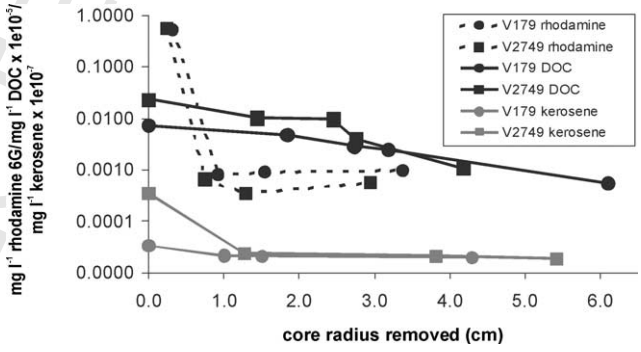


Fig. 2. Schematic illustrating the removal of ice core veneers during each step of the decontamination procedure.



(A)



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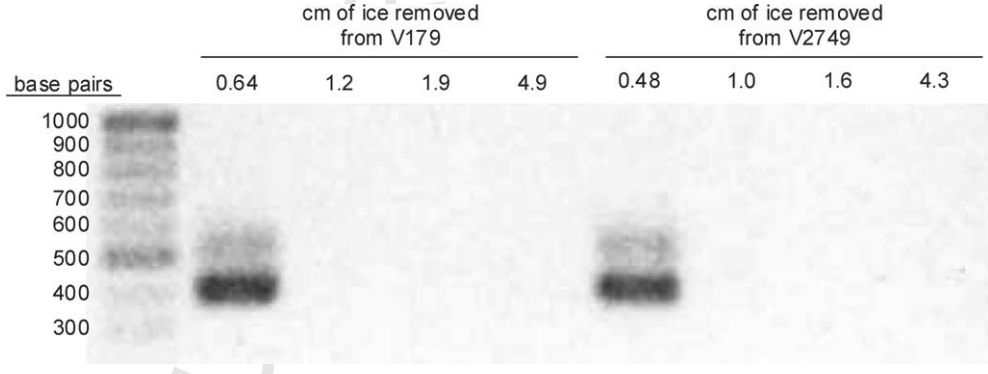

(C)

Fig. 3. Removal of cells and molecules from the surface of Vostok ice cores 179 and 2749 during sampling. (A) Concentration of culturable *Serratia marcescens* (cfu ml^{-1}) and the total number of directly counted cells in the fractionated wash and melt water collected during sampling. The *y*-axis is denoted cfu ml^{-1} or cell ml^{-1} to permit plotting zero data logarithmically. The *y*-axis error bars denote standard deviation from the mean. (B) Concentration of rhodamine 6G ($mg\,l^{-1}$), DOC ($mg\,l^{-1}$), and kerosene ($mg\,l^{-1}$) measured with the removal of core radius during sampling. (C) Negative image of the ethidium bromide-stained 1% agarose gel used to separate PCR amplified plasmid sequence from collected samples. Amplification was conducted as detailed in the text.

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al., 2001), it is reasonable to conclude that our decontamination method was effective in removing DNA present on the core surface. While the use of a known plasmid sequence is a robust control for nucleic acid contamination, the presence of *S. marcescens* on the core exterior serves as a secondary check for the persistence of DNA released from lysed cells. Thus, if the results from a PCR-amplified 16S rDNA library yielded sequences that were 100% identical to the 16S rDNA sequence of the *S. marcescens* strain used in the tracer mixture, we would conclude that the sample was contaminated. It is important to acknowledge that PCR-based approaches to detect DNA in low biomass and ancient samples are highly susceptible to contamination from the laboratory environment and via molecular biology reagents. Therefore, in addition to the methods described herein to verify the removal of DNA contamination from the sample, it is equally vital that investigations of this nature follow verifiable guidelines to ensure that positive PCR amplifications are not artifactual (e.g., Cooper and Poinar, 2000; Hofreiter et al., 2001; Willerslev et al., 2004a).

The concentration of kerosene and rhodamine 6G fall below detectable levels after ~1 cm of the core radius is removed (Fig. 3B), implying that penetration of fluid contaminants was minimal. The gas chromatograph-mass spectrometry (GCMS) results for hydrocarbons on the outermost section of core 2749 was 3450 mg l^{-1}, but fell below concentrations detected in the blank after 1.27 cm of the core exterior was removed. Even though core 179 was recovered without the use of drilling fluid, a hydrocarbon concentration of 322 mg l^{-1} nevertheless existed on the outermost portion of the ice. The mass spectra generated from the exterior of core 2749 (Fig. 4E) mirrors the spectral signature obtained from Jet-A kerosene (Fig. 4A), whereas the spectra generated from the exterior of core 179 has mass 71 ion peaks from hydrocarbons with a longer retention time (Fig. 4C) and could be a result of substances other than kerosene-based hydrocarbons.

Diesel fuel is used extensively in drilling operations to run mechanical equipment and it is possible that the hydrocarbon signatures detected on core 179 may have resulted from secondary contamination of ice cores during handling.

The concentration of DOC in the outermost portion of core 2749 was more than 3 times higher than that of core 179 (Fig. 3B), which was expected due to the high level of kerosene observed on the surface of the core 2749 sample. From outside to inside, the DOC concentration decreased 14- and 21-fold for core 179 and 2749, respectively, but did not plateau at a single uniform value. As discussed above, the inability to detect kerosene after >1 cm of the core surface was removed demonstrated that ice in the core interior was not contaminated by drilling fluid. Together, these data indicate that the internal DOC levels measured are not due to drilling fluid penetration into the core, but are a reflection of the actual variability of DOC concentrations that exist within these samples. It is not surprising to find such fluctuations in the level of DOC, as glacial ice is a heterogeneous mixture containing a nonuniform dispersal of impurities. The heterogeneous distribution of two and three-grain boundaries in an ice sample (Nye, 1992; Price, 2000) and the heterogeneous distribution of sediment inclusions (Jouzel et al., 1999; Petit et al., 1999) can explain the variation in DOC concentrations within adjacent portions of the same ice core.

4. Discussion and conclusions

The inclusion of biology into ice coring research has required developing effective approaches toward decontamination and methods to validate the results obtained. Since the outer portion of these cores have up to 3 orders of magnitude higher bacterial density than the innermost sample (Table 2), the extreme gradients which exist make contamination a very relevant aspect of microbiological investigations with ice cores, particularly when the actual ambient numbers of bacterial cells are low. We developed a reliable, multifaceted protocol that can be used to monitor the microbiological, molecular and geochemical contamination of ice cores. Importantly, this protocol provides us with a quantitative tool to verify the authenticity of results obtained and eliminate samples that fail to pass the decontamination procedure. The contrasting drilling techniques used to recover core 179 (borehole BH5) and 2749 (borehole 5G) at Vostok Station allow the effect of drilling fluid on the microbiological and geochemical results to be compared. The crystals in the sample from 179 m are small (1–2 mm; Lipenkov et al., 1989), crystal sizes for depths >2083 m have not been published but are likely to be relatively larger at 2749 m, and ice from below 3539 m has a mean crystal size >20 cm to 1 m (Jouzel et al., 1999). Our protocol shows that samples are free of drilling fluid, microbial, and macromolecular contamination when at least 3 cm of the core diameter is removed. Hence, crystal structure appears to have little impact on the permeation of our experimental contaminants. Importantly, all of our studies were conducted on cores containing no visible physical fractures (tested with cross-polarized light and microscopy). The sample from 762 m is from what is known as the "brittle zone," where gases begin to form clathrates. Coring this brittle ice is difficult and often yields core of poor quality with visible fractures. We purposely selected a section of this core that contained no visible fractures making our depth comparisons comparable. Presumably, the penetration of contaminants in ice samples with physical fractures will show different results than we present here.

Our data are consistent with previous studies documenting the penetration of contaminants into ice core materials which include (i) electrical conductivity measurements showing that impurities diffuse about 10 mm into the ice over 10 years at −20 °C (Schwander et al., 1983), (ii) 1000-fold lower lead concentrations after <2 cm of the core radius was removed (Boutron and Patterson, 1986), and (iii) drilling fluid penetration analysis that indicates hydrophobic

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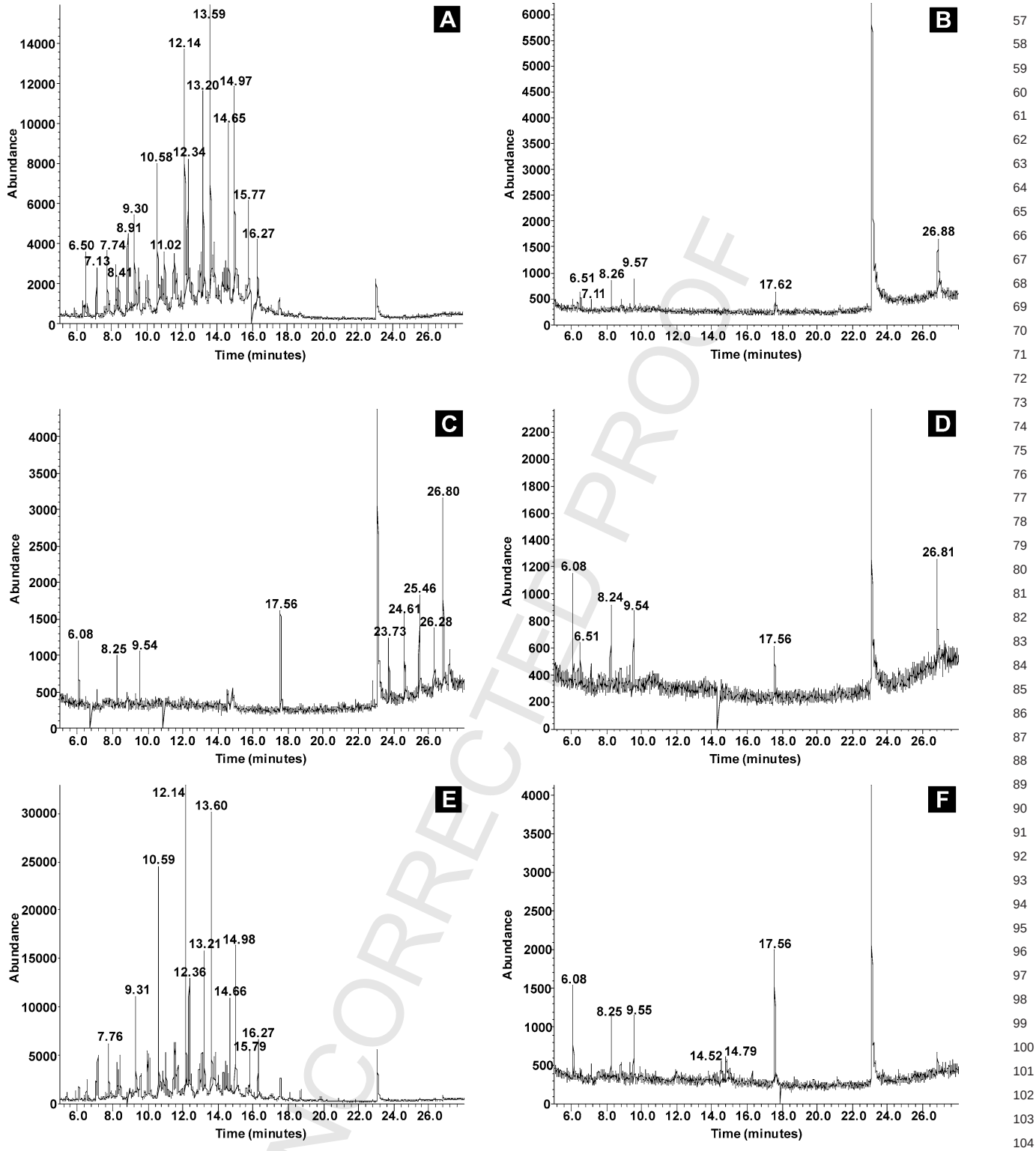

Fig. 4. GCMS spectrum from organic phase extraction of (A) Jet-A fuel compared to spectrum from exterior and interior samples of Vostok ice samples 179 and 2749. (B) is a deionized water blank. (C) is the outermost scraped 1.0 cm of the exterior radius of core 179 and (D) is an internal sample of 179 after 1.0 cm was removed and includes the ice present to a depth of 1.5 cm from the original core surface. (E) The outermost scraped 1.27 cm of the exterior radius of core 2749 and (F) an internal sample of 2749 after 1.27 cm was removed and includes the ice present to a depth of 3.8 cm from the original core surface.

butyl acetate permeates <5 mm into the interior of glacial ice cores (Gosink et al., 1994). Previous reports of microbial life in the Vostok ice core by Karl et al. (1999), Priscu et al. (1999), and Christner et al. (2001) used ice core decontamination procedures (Table 1) that either washed and removed approximately 40% of the ice core (~3 cm from the diame-

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ter) or used a melting device (see Christner et al., 2000) that collected sample from the ice core interior (>2 cm internal to the ice core exterior). The data we present here shows that the removal of ~3 cm from the diameter of Vostok ice cores yields contaminated free ice in terms of dissolved organics, DNA, and microbial cells. Hence, our data corroborate the microbial and geochemical results published by these authors.

A number of approaches have been proposed to directly or indirectly detect extraterrestrial life, including microscopic observation of biogenically precipitated minerals (McKay et al., 1996; Friedmann et al., 2001; Thomas-Keprta et al., 2001), investigating stable carbon isotopic distributions (Mojzsis and Arrhenius, 1998), examining amino acid homochirality (Bada, 2001), measuring elemental abundance of biogenic elements (Conrad and Nealson, 2001), and physiological analysis of biological redox signatures (Crawford et al., 2002). The failure of the Viking GCMS to detect any organic compounds in the martian soil was a strong incentive to explore a nonbiological explanation for the positive result obtained from the labeled release experiment (Klein, 1978). It has since been documented that Viking's GCMS, which was claimed to have part per billion sensitivity (Biemann et al., 1977), could not detect organics in dry valley Antarctic soils that contained viable bacteria (Levin and Straat, 1981) and required $>3.0 \times 10^7$ cells per gram of soil to achieve its detection threshold (Glavin et al., 2001). If fossilized or extant life is present in the martian ice caps or frozen subsurface, it is expected to be of low density, which will require extremely sensitive instruments to identify diminutive signatures of life. As such, these methods will be particularly vulnerable to even trace amounts of residual terrestrial microbial and organic contamination. To our knowledge, the cell densities in the Vostok ice core (Fig. 3A and Table 2) are among the lowest reported in any natural environment on Earth. Thus, studying microorganisms and biosignatures in Antarctic glacial ice not only provides a physical analog of potential niches for the persistence or preservation of life on Mars, but also at concentrations relevant to those anticipated in the martian environment.

According to the recommendations of the U.S. National Academy of Sciences Space Studies Board, spacecraft destined for the martian surface without life detection instrumentation (category IVa missions) are permitted 300 spores per square meter and 3×10^5 spores per vehicle (National Research Council, 1992; DeVincenzi et al., 1996). These spacecraft are assembled within ultra clean facilities (Class 10–100,000) in which great lengths are taken to maintain an environment that discourages microbial persistence and growth. Nevertheless, spores, viable nonsporulating microorganisms, and DNA persevere on spacecraft and in the clean facilities where these instruments are constructed and housed (Venkateswaran et al., 2001; La Duc et al., 2003). Landers with life detection instrumentation (category IVb missions) are expected to have a microbial burden commensurate with those of the 1976 Viking landers (National Research Council, 1992), which were exposed to dry heat for 30 h at 111.7 °C, but were not then accessible to microbial assays after heat treatment (National Research Council, 1974, 2000; DeVincenzi et al., 1996). While this stringent treatment is likely to kill the vast majority of microorganisms, it is interesting to note that an archaea was recently described that not only survives moist heat (a substantially more effective method of sterilization than dry heat) for 24 h at 121 °C, but is capable of cell division at this temperature (Kashefi and Lovley, 2003). Even if such a heat treatment is effective in sterilizing the spacecraft and its internal components, nucleic acids, proteins, amino acids, and other terrestrial organic contamination can persist and may result in the misinterpretation of life detection experiments (Barengoltz, 2000). Unless innovative technologies and procedures are developed that can definitively remove such contamination, it is inevitable that probes and rovers landing on the martian surface will carry a concealed and unwanted payload; namely earthly microorganisms and terrestrial organic molecules of biological origin. Despite this inevitable contamination, the present Mars missions are scheduled and the issue of preventing the forward contamination of Mars may be a moot point, as it may be already colonized with earthly microbes seeded from previous explorations to the surface. While the planned Mars sample return missions are considered by some to be too risky at this point (DiGregorio, 2001), if they are implemented, it will be vital to develop rigorous decontamination procedures and methods to prove that terrestrial contamination has not been introduced. Hence, similar considerations for verifying the elimination of contamination in studies of samples from terrestrial glacial and subglacial environments are apropos to discussions of life detection experiments in situ and in the samples that will ultimately be returned to Earth.

The use of a tracer that consists of viable microorganisms and biological macromolecules may not be acceptable in all extraterrestrial circumstances, and alternative abiotic mixtures that mimic the physical and chemical properties of cells and macromolecules should be investigated. For example, we have observed in fractionated melt water removed progressively during ice core sampling that disinfection and removal of <1.5 cm of the core radius may be sufficient to kill extraneous cells, but it is still possible for macromolecules to persist. Therefore, we can only be confident that a sample has been adequately decontaminated if it can be demonstrated that all three components (i.e., cells, macromolecules, and the smaller fluorescent organic molecule) of our tracer mixture are not detectable. Nonbiological water-soluble chemical tracers and fluorescent microspheres have been used to monitor microbial contamination in deep marine sediment cores and permafrost (House et al., 2003; Juck et al., 2004), and similar mixtures are worth considering as potential tracer compounds for life detection experiments in extraterrestrial environments and sample return missions. To definitively rule out the possibility of contamination, it will be essential to sequentially test every sample,

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outside to inside, to demonstrate that terrestrial microorganisms and molecules have not infiltrated the inner sample. This could be confirmed by the absence of a surface-applied tracer in the inner sample.

Sampling of the martian subsurface will require drilling and core recovery strategies analogous to those used in terrestrial studies, although substantially more complicated logistically. Examples of such anticipated efforts include the Robotic Shallow Mission and Deep Sub-surface Mission, which will attempt to core depths of ~300 m and ~3 km, respectively (Mancinelli, 2003). Evidence for H_2O ice in permafrost at high latitudes and in both the northern and southern ice caps of Mars (Boynton et al., 2002; Mitrofanov et al., 2003; Titus et al., 2003; Bibring et al., 2004) implicate the polar regions as the most likely and convenient environments to detect extant life or the biochemical preservation of past life. In contrast to sampling the deep subsurface, biological investigations of ice in the polar regions (such as the high latitude layered ice areas that the Phoenix 2008 mission will analyze) will not require a major invasive drilling effort, as these environments are relatively accessible from the surface or sediment-rich margins of the ice caps. A number of reports have documented microbial survival for hundreds of thousands of years in glacial ice (Abyzov et al., 1998; Christner et al., 2003; Sheridan et al., 2003; Miteva et al., 2004) and for millions of years in permafrost (Shi et al., 1997), so it is not unreasonable to suppose microbial longevity over 10^5–10^7 years, the estimated time the martian ice caps have existed (Herkenhoff and Plaut, 2000). However, the most favorable place to search for existing martian life in the near surface is in regions that have experienced recent or periodic melting, such as the environments contiguous to the ice caps, where it is predicted that the discharge of liquid water occurs during high obliquity (Pathare and Paige, 1998; Jakowski et al., 2003). There is also evidence that liquid water exists for brief periods in mid to low latitudes (Malin and Edgett, 2000; Christensen, 2003; Arvidson et al., 2004), but it seems less likely that surface environments at these lower latitudes can sustain a permanent environment for life. The proposed 2008 Phoenix NASA mission will land between latitudes 65° and 75° N, and will operate for up to 150 sols during the northern summer (http://phoenix.lpl.arizona.edu). The key objective of the mission is to examine samples of surface and subsurface soil and ice, returning a wealth of data concerning near-surface ices, the potential habitability of the soil, and the history of climate as written into the icy soils. To ensure environmental protection of the polar environment, which has a higher probability of habitability, it is especially important that this mission and other future missions to the icy regions of Mars include methods to prevent and detect terrestrial contamination.

The contamination concerns raised herein are particularly relevant to any discussion of life detection in samples from the ice caps or permafrost in the martian polar regions. While our view may seem alarmist, the issue of terrestrial contamination is an important and legitimate matter. Although implementing a verifiably clean sampling strategy for extraterrestrial life detection experiments will entail adding an additional technological barrier to an already difficult, expensive, and precarious mission, it will be necessary to convince the scientific and lay communities that evidence consistent with a martian life signature is unequivocal and not just the result of contamination caused by a lack of foresight.

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Appendix A

A.1. Bacterial strain, plasmid DNA, and fluorescent dye

Serratia marcescens (MSU strain) was cultured in trypticsoy broth or on 1.5% agar solidified media (Difco Inc.) and used as the indicator strain. Cell suspensions were quantified by directly counting cells with a Nikon Optiphot epifluorescent microscope, equipped with B-2E (FITC), Y-2E (Texas Red), and EF4UV (DAPI) filter cubes. Culture stocks were preserved in a solution of 15% glycerol and stored at −80 °C. The plasmid DNA construct consists of the pCRII-TOPO vector (Invitrogen) with a 400 bp insert amplified from the 5.8S rRNA gene and internal transcribed spacers of *Naegleria fowleri* (Pélandakis et al., 2000), a eukaryotic pathogen which is the primary cause of amoebic meningoencephalitis. The plasmid was transformed into competent *Escherichia coli* JM 109 cells (Promega), plasmid-containing colonies were selected based on resistance to ampicillin, and plasmid DNA was prepared using the QIAprep Spin Miniprep Kit (Qiagen) and quantitated using Pico Green nucleic acid stain (Molecular Probes, Inc., cat. no. P-7589) on a Biotek FL600 microplate fluorometer. Rhodamine 6G was purchased from Sigma (cat. no. R 4127) and kept as a stock solution of 1000 mg l^{-1}.

The above components were combined in a 1.1 ml solution containing 4.5×10^7 cells ml^{-1} of *S. marcescens*, 2 ng of plasmid DNA, and 100 mg l^{-1} (final concentration) of rhodamine 6G. The rationale and detection limits for the constituents are summarized in Table 3. This solution was spread evenly on all ice core surfaces with a sterile latex-gloved hand. The visible presence of rhodamine on ice surfaces ensured that the entire core was contaminated.

A.2. Ice core source

Core section 179 (178.00–179.00 m below surface, mbs) originated from a borehole at Vostok Station (Antarctica) designated BH5. The BH5 borehole is shallow, and was therefore drilled without the aid of drilling fluid. Sections 763 (762.00–762.32 mbs), 1577 (1576.00–1576.33 mbs), 2091 (2090.30–2090.60 mbs), 2490 (2489.00–2489.31 mbs), 2749 (2748.43–2748.94 mbs), 3015 (3014.71–3015.00 mbs), 3197 (3196.28–3196.51 mbs), 3351 (3350.05–3350.50 mbs), 3540 (3539.62–3539.99 mbs), and 3566 (3564.99–3565.32 mbs) were recovered from the deep 5G borehole. Ice core samples are referred to and designated by the depth relative to the surface (i.e., V179 represents the ice sample recovered from 178.00–179.00 mbs). The age of each ice core sample appears in Table 2. The physical and chemical properties of the Vostok ice core have been studied extensively (e.g., Petit et al., 1999; Priscu et al., 1999; Cuffey and Vimeux, 2001; Caillon et al., 2003).

The drilling fluid used by the Russian-based drilling crew in the 5G borehole is commercial grade kerosene or jet fuel (trade names: Jet-A, TC-1, JP-8), which has a complex combination of C9 to C16 hydrocarbons produced by the distillation of crude oil and includes other trace components typical of high end jet fuel such as toluene, ethylbenzene, and 1,2,4-trimethylbenzene. For core depths between 1–2700 m, the fuel mixture was supplemented with the halogenated solvent CFC-11. Following the Montreal Protocol and subsequent ban on CFCs, the freon derivative Foran 141b was used as a densifier (V. Lipenkov, personal communication).

Ice cores were obtained from the United States National Ice Core Laboratory (Denver, CO) and airfreighted overnight to Montana State University under close supervision. The temperature during shipping never exceeded −15 °C. Ice cores are archived at Montana State University in a walk in freezer at −20 °C, sealed in polyethylene tubing and stored in cardboard core tubes.

A.3. Ice core sampling

The outermost layer of ice (at least 5 mm of the core radius, 10 mm of the core diameter; Fig. 2) was scraped with a 95% ethanol-rinsed stainless steel microtome blade in a −10 °C cold room and the removed ice scrapings were collected for analysis as described below. Ice samples were then transferred to a Class 100 Purifier Horizontal Clean Bench (Labconco Corporation, model 36125), housed separately within a −20 °C walk in freezer, which was precleaned with a solution of 95% ethanol and exposed to 20 min of ultraviolet (UV-C) irradiation. Sections of the scraped ice cores were held with sterile tongs within the laminar flow hood and completely rinsed with 500 ml of −20 °C 0.2 μm-filtered 95% ethanol, and then rinsed with 500 ml of 4 °C, sterile, organic-free, deionized H_2O (EASYpure™UV/UF, Barnstead Thermolyne Corp.). The deionized H_2O wash was collected for analysis. The remaining ice cores were placed in sterile polypropylene containers and transferred to a positive pressure glove box (Analytical Balance Chamber, model 830-ABC, Plas-Labs, Inc.) at 22 °C that was precleaned with a solution of 0.25% sodium hypochlorite and evacuated 3-times with 0.2 μm-filtered zero air. Ice samples were placed in sterile 16 cm stainless steel strainers and incubated under these conditions until at least 15 mm of the original core radius had melted away. The melt water generated during this procedure was collected for analysis. The remaining ice was sealed within a sterile polypropylene container and allowed to melt completely in the dark at 4 °C, which took as long as 72 h.

Samples for dissolved organic carbon (DOC) and kerosene analysis were decontaminated separately from the sections of cores used for biological analysis. The outer portion of the ice core surface was removed at 1–2 mm intervals using a sterile razor blade inside a −20 °C walk-in freezer. For kerosene measurements, razor blades were cleaned with acetone and dried thoroughly. Successive scrapings were collected in acid washed combusted glass Shimadzu TOC vials to monitor the DOC content from the outside to the interior of the ice cores. For analysis of kerosene, ice shavings were collected in precombusted glass vials and kept frozen at −20 °C until extraction.

A.4. Viable and direct cell counts

The number of culturable cells of *S. marcescens* was determined by counting the colonies that formed on 10-fold serial dilutions of sample spread plated on agar-solidified tryptic-soy media, following visible growth after incubation at 25 °C for 48 h. Culturable refers to the ability of a cell to form a colony on agar-solidified media, designated here as the number of colony-forming units per ml (cfu ml^{-1}) of sample.

Sodium borate-buffered formalin and the SYBR Gold (Molecular Probes, Inc., cat. no. S-11494) nucleic acid-staining solutions were passed through 0.2 μm filters to remove extraneous particles and cells. A 10 ml sample was fixed in a final concentration of 5% buffered formalin and stained with SYBR Gold for 15 min. The sample was then filtered on a 5 mm spot of a 25 mm–0.2 μm black polycarbonate filter (Poretics, cat. No. K02BP02500) using a dot blot apparatus. Filters were mounted on glass microscope slides with the addition of 2 drops of an anti-fade solution, which consisted of 0.1% p-phenylenediamine (Sigma, cat. no. P-1519) in a 1:1 solution of phosphate-buffered saline and glycerin. All filtering and manipulations were conducted within a BioGard vertical laminar flow hood (Baker Company, model B6000-1) with a germicidal UV-C lamp. Cells on the filters were counted using a Nikon Optiphot epifluorescent microscope, equipped with B-2E (FITC), Y-2E (Texas Red) and EF4UV (DAPI) filter cubes, at a final magnification of 1000X. The total number of cocci and rods in 60 fields of view was determined, and the number of cells ml^{-1} was calculated by computing the cumulative average cell/

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field (field of view area at 1000X is 16741 μm^2). For samples from the ice core interior, the number of fields counted (60) yielded a counting error ranging from 10 to 69 cells ml^{-1}, depending upon the density of cells in the sample.

A.5. Fluorometry

The concentration of rhodamine 6G was quantitated using a model 112 Turner fluorometer, equipped with a 526 nm peak transmission filter and an emission detection filter that blocks all wavelengths <540 nm (peak rhodamine 6G emission is at 555 nm when excited at 526 nm). Standard curves ($R^2 > 0.99$) generated using stock rhodamine 6G concentrations ranging from 0.1 to 0.0005 mg l^{-1} were used to convert relative emission units into mg l^{-1} of rhodamine. The instrumental limit of detection is defined as 2 standard deviations of the y-intercept.

A.6. PCR amplification

Two μl of sample was used as the template for 30 cycles of PCR amplification (Eppendorf MasterTaq Kit; Brinkmann Instruments, Inc., cat. no. 954140091) in an Eppendorf Mastercycler Gradient Thermal Cycler using a 50 °C anneal temperature, a final $MgCl_2$ concentration of 1.5 mM, and the universal M13 forward and reverse nucleotide primers, for which annealing sites exist on the flanking regions of the pCRII-TOPO vector's multiple cloning site. A nested PCR was then performed with 2 μl of the product from the latter reaction and the same amplification conditions as above, except using the nucleotide primers ITS1 and ITS2 (Pélandakis et al., 2000), which specifically anneal to the flanking regions of the DNA insert and are internal to the PCR product generated during the first round of amplification. Samples of each PCR product were evaluated by electrophoresis through a 1% agarose gel followed by staining with ethidium bromide.

A.7. Determination of dissolved organic carbon (DOC)

Nonpurgeable dissolved organic carbon was determined with a Shimadzu TOC-5000A carbon analyzer. Standards were mixed from NIST traceable potassium hydrogen phthalate (Nacalais Tesque Inc. Lot # M4K9453). All sample glassware was prepared by soaking overnight in NoChromix acid solution followed by 6 nanopure deionized water rinses and 8 h of baking at 500 °C. Each sample was acidified with 2N HCl (ACROS 37% HCl and ultrapure water) and sparged with U.S.P. grade oxygen for 5 min to remove CO_2, followed by six replicate injections.

A.8. Mass spectral analysis of kerosene

Detection and quantification of the kerosene signature was determined with a HP 5890 GC column coupled to a HP 5970 series Mass Selective detector. The organic fraction of melted samples was extracted using 1 part methylene chloride and 3 parts melted ice core shavings in a glass conical vial with a Teflon lined screw cap. An internal standard of methyl stearate was included in the extraction process to monitor extraction efficiency. Samples were inverted and mixed for 10 min. After separation, the methylene chloride fraction was removed with a syringe. The aqueous phase was re-extracted to insure the removal of all organics. Two μl of the sample was injected into the detector. A sample of Jet-A kerosene was separated on a VG 70E GC column with a mass spectrometer detector to determine the hydrocarbon signature indicative of this mixture and for comparison with signatures obtained from ice core samples. Hydrocarbons typically fragment into ions of masses 57, 71, 85, and smaller (masses are denoted by m/z, the ratio of the mass to the number of ion charges). The ion with a mass of 71 (ranging between 70.70 and 71.70) represented the specific marker compound for hydrocarbons and was scanned for by setting the mass spectrometer to run in the selective ion mode. HP Chem Station software (version B.02.05) was used to calculate peak areas of the mass 71 ion. The most abundant ion of methyl stearate has a mass of 74 (73.70 to 74.40), which was used to calculate the relative response. The peak area of the hydrocarbon signature (mass 71) divided by the peak area of the internal standard (methyl stearate; mass 74) provided a relative response value. The relative response value was plotted against kerosene concentration to generate a standard curve for determining the concentration of kerosene in melted ice core samples.

A.9. Scanning electron microscopy

Samples for scanning electron microscopy were filtered onto clean 25 mm, 0.2 μm polycarbonate filters (Poretics, cat. No. K02BP02500) sputter coated with 10 nm Au–Pd. Cells were imaged using a cryogenic scanning electron microscope (JEOL-6100 SEM).

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