Annals of Glaciology 33 2001

A microscopic approach to investigate bacteria under in situ conditions in sea-ice samples

Karen Junge,[1] Christopher Krembs,[1] Jody Deming,[1] Aaron Stierle,[2] Hajo Eicken[2]
[1]*Astrobiology Program, School of Oceanography, University of Washington, Seattle, WA 98195, U.S.A.*
[2]*Geophysical Institute, University of Alaska Fairbanks, Fairbanks, AK 99775-7320, U.S.A.*

ABSTRACT. Microbial populations and activity within sea ice have been well described based on bulk measurements from melted sea-ice samples. However, melting destroys the micro-environments within the ice matrix and does not allow for examination of microbial populations at a spatial scale relevant to the organism. Here, we describe the development of a new method allowing for microscopic observations of bacteria localized within the three-dimensional network of brine inclusions in sea ice under in situ conditions. Conventional bacterial staining procedures, using the DNA-specific fluorescent stain DAPI, epifluorescence microscopy and image analysis, were adapted to examine bacteria and their associations with various surfaces within microtomed sections of sea ice at temperatures from −2° to −15°C. The utility and sensitivity of the method were demonstrated by analyzing artificial sea-ice preparations of decimal dilutions of a known bacterial culture. When applied to natural, particle-rich sea ice, the method allowed distinction between bacteria and particles at high magnification. At lower magnifications, observations of bacteria could be combined with those of other organisms and with morphology and particle content of the pore space. The method described here may ultimately aid in discerning constraints on microbial life at extremely low temperatures.

1. INTRODUCTION

Recently, interest has increased substantially in understanding the constraints on microbial abundance, activity and survival in extreme environments, since they present Earth analogs for possible extraterrestrial habitats in our solar system. Liquid- or frozen-water environments, such as Antarctic lakes (Karl and others, 1999; Priscu and others, 1999), supercooled cloud droplets or permafrost soils (Stone, 1999), where micro-organisms experience very low temperatures, are now being investigated as analogs of environments on Mars or the Jovian moon Europa.

Sea ice, an important component of the cryosphere and the global climate system, provides the coldest habitat on Earth for marine life, with temperatures ranging from 0° to −35°C (Maykut, 1986). Based on bulk measurements from melted sea-ice samples, extensive microbial communities are known to develop annually within the ice cover of the polar oceans in spite of such extreme temperatures and highly variable salinities (for reviews see Horner 1985; Palmisano and Garrison, 1993). Heterotrophic bacteria comprise one of the major groups of these communities (Sullivan and Palmisano, 1984), as evidenced by measures of bacterial production (Grossmann and Dieckmann, 1994; Helmke and Weyland, 1995) and the existence of a microbial loop (Kottmeier and Sullivan, 1990; Laurion and others, 1995). However, melting destroys the micro-environments within the ice matrix and precludes an examination of microbial populations at a spatial scale relevant to the organism.

Studies investigating bacteria in sea ice have relied on bulk analyses of melted ice samples or occasionally on extraction of the brine for separate bulk analysis. Data on the small-scale distribution of bacteria within the ice matrix have so far not been available, mainly due to the challenges of investigation at sub-zero temperatures. The need for development of an in situ bacterial detection technique is evident as theoretical models addressing spatial and other limits on bacteria in ice are being devised (Price, 2000).

Here, we describe the development of a method that allows for in situ microscopic observation of bacteria localized within the three-dimensional network of brine inclusions in sea ice. Such in situ microscopy provides a basis for relating characteristics of the bacterial community to its physical habitat (e.g. the morphology of brine inclusions or quantity and quality of particulate materials within the ice) on a spatial scale relevant to the micro-organisms. Bacterial abundance and distribution under in situ conditions, undisturbed in their spatial orientations, has only recently been investigated directly in other habitats, such as basaltic rock (Tobin and others, 1999), soil and biofilms (Barbara and Mitchell, 1996; DeLeo and others, 1997) and lake water (Krembs and others, 1998). For marine habitats, the study of microbial communities still relies largely on bulk analyses of homogenized samples on a macroscale. The method presented here can help to advance our understanding of marine microbial life, at a scale relevant to the organism, in a habitat covering 21×10^6 km^2 on this planet (Maykut, 1986).

2. METHODS

A summary of the in situ microscopic method developed for detecting micro-organisms within the ice matrix at relevant spatial and in situ temperatures is presented in Figure 1.

https://doi.org/10.3189/172756401781818275 Published online by Cambridge University Press

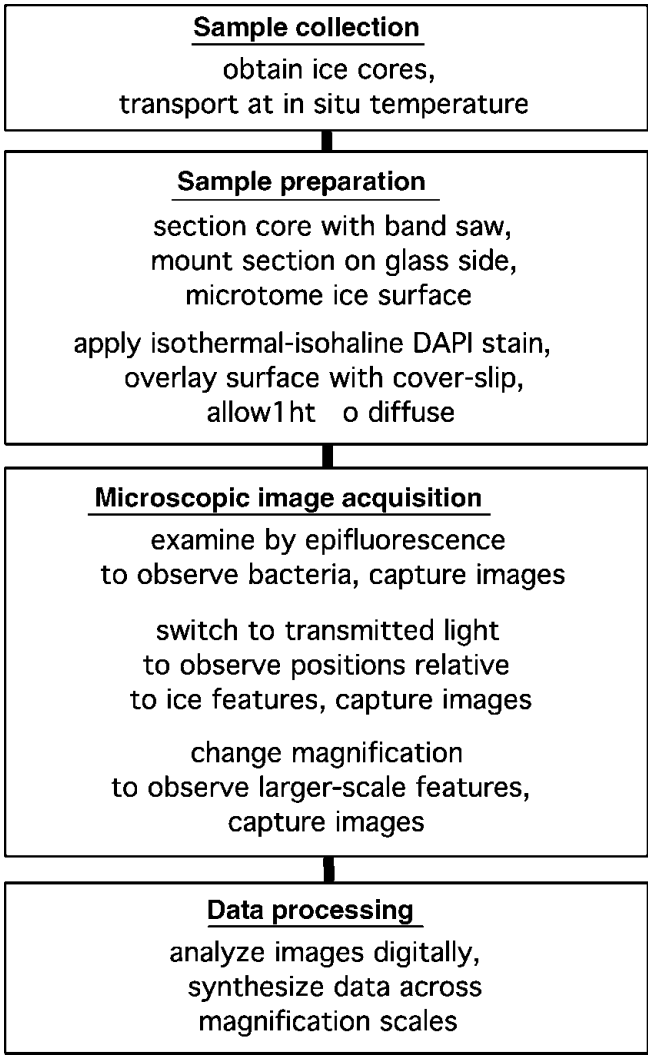

Fig. 1. Summary flow diagram of the developed method.

Below we describe individual steps taken in the development of the method.

2.1. Sample collection, storage and temperature control

Sea-ice samples were collected during the wintertime temperature minimum in March 1999 from two sites readily accessed from Barrow, Alaska, U.S.A.: one on the coastal fast ice of the Chukchi Sea and the other in nearby Elson Lagoon. Ice cores were taken using a 10 cm diameter ice auger. Samples were representative of first-year sea ice, with characteristic temperature and salinity profiles (Eicken and others, 2000). While most of the properties of the Elson Lagoon ice were comparable to those of the Chukchi Sea, the particulate content in the upper sediment-rich layers of the shallow lagoon ice was markedly higher, generally exceeding 10 mg L^{-1}. Ice samples with in situ temperatures of −15°C (at 25 and 35 cm below the ice surface of the Chukchi Sea and Elson Lagoon, respectively) and −2°C (from the bottom-most centimeters of the Chukchi Sea ice) were selected for microscopy. Replicate samples of the Elson Lagoon ice were used to test the method over a range of temperatures (−2°, −5°, −10° and −15°C).

The ice cores were placed in insulated containers that maintained the samples at or near the lower in situ temperature (−15°C) during sample transport (Eicken and others, 2000). The close proximity of Barrow and the University of Alaska Fairbanks (UAF) allowed for rapid sample transfer to a −15°C laboratory, where all samples were stored until processing. Sample processing was also performed in a temperature-controlled cold room at UAF, which accommodated the equipment required for preparation of ice sections, as well as the microscope and image-acquisition system. Placement of a Hobo Temperature logger (Onset Computers Corporation, Pocasset, MA) on the microscope stage verified that the examination unit held the desired temperature within 1°C during sample processing and analysis.

2.2. Sea-ice sample preparation

Three types of ice samples were prepared for analysis: (1) sections of artificial sea ice prepared from decimal dilutions of a known bacterial culture; (2) sections of the Barrow ice samples; and (3) melted Barrow ice samples.

Artificial sea-ice preparation

Three decimal dilutions (10^{-1}, 10^{-2} and 10^{-3}, corresponding to approximately 10^7, 10^6 and 10^5 bacteria mL^{-1}) of a turbid culture of the psychrotolerant sea-ice bacterium *Planococcus mcmeekenii* (Junge and others, 1998) were prepared in 0.2 μm filtered and autoclaved artificial sea water (ASW; 24 g NaCl, 0.7 g KCl, 5.3 g $MgCl_2$, 7.0 g $MgSO_4 7H_2O$, 1.3 g TAPSO [3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid] buffer in 1 L of distilled water, with pH adjusted to 7.5 using 0.2 N NaOH). The culture was diluted after reaching late-log growth phase, when the average cell diameter was approximately 1 μm. A volume of 10 mL of each dilution and of sterile ASW (negative control) was placed in an ice tray in the −15°C laboratory to induce artificial sea-ice formation. Sections of the ice cubes were prepared, processed and examined at −15°C, as outlined below for the natural sea-ice samples. Another 10 mL of each dilution was preserved with pre-filtered (0.2 μm) formaldehyde (2% final concentration) and processed for bacterial counts, as described below for melted-ice controls.

In order to determine the average depth to which bacteria (of typical 1 μm diameter) could be visualized within the ice, artificial sea ice was prepared from ASW containing cells of *P. mcmeekenii* that had been stained prior to ice formation. This use of pre-stained cells also allowed a test of whether or not all bacteria are excluded from the solid ice into liquid brine (along with salts) as ice forms from sea water. A 50 mL volume of turbid culture in late-log growth stage was stained with DAPI (final concentration 3 μg mL^{-1}; see section 2.3), diluted with 450 mL of ASW in a plastic beaker and placed into the temperature-controlled laboratory set at −2°C. Ice formation was induced by cooling the laboratory from −2°C to −15°C over the course of 10 days. Sections of these artificial ice preparations containing pre-stained cells were then prepared, processed and examined at −15°C, as outlined below.

Ice sections

Ice sections were prepared from both the artificial and natural sea-ice samples, essentially as described earlier (Eicken, 1993; specifics available from the authors). Briefly, ice sections 5–6 mm thick were prepared using a standard, steel-blade bandsaw. Each section was attached to a glass slide with fresh-water droplets frozen to the sample perimeter. The surface of the mounted sections was subsequently removed at approximately 15 μm intervals with a sled microtome, as described in Eicken (1993). Microtoming leaves the underlying ice intact and does

https://doi.org/10.3189/172756401781818275 Published online by Cambridge University Press

not induce artifacts on a macroscopic or microscopic scale. Samples were handled only with gloved hands, avoiding contact with the surface as well as introduction of extraneous fluorescent particles (threads, lint and dust particles, according to Cercone and Pedone, 1987). Ice sections were either stained (see section 2.3) or left unstained and examined as described below (see section 2.5).

Melted-ice controls

To compare results obtained from sections of natural ice with conventional bacterial counting procedures, three additional sections of similar size and core depth (and corresponding temperature) were melted in artificial brine (ratio of ice to brine was 1:1) at the corresponding examination temperature of the ice section. The artificial brine, used to reduce the possibility of cell lysis due to sudden changes in osmotic pressure, was prepared with distilled, deionized 0.2 μm filtered water (Nanopure ultrapure water system, Barnstead/Thermolyne, Dubuque, IA) and artificial sea salts (Instant Ocean, final concentration 250 ppt; Eicken and others, 1998). After ice formation and equilibration of the brine with the ice during a 24 h period in a freezer chest at −25°C, the solution was filtered through a 0.2 μm syringe filter (Corning Glass Works, New York, NY) for immediate use.

2.3. Bacterial staining procedures

The DNA-specific fluorescent stain 4',6'-diamidino-2-phenylindole 2HCl (DAPI), which fluoresces blue when bound to DNA and excited with light at a wavelength of 365 nm (Porter and Feig, 1980), was used to stain bacteria in all of the different sample preparations. When bound to non-DNA material or unbound, DAPI may fluoresce over a range of yellow colors (Kepner and Pratt, 1994; Mostajir and others, 1995).

Ice sections

To apply DAPI stain to sea-ice sections at in situ temperature without altering the ice surface, DAPI staining solutions (3 and 20 μg mL^{-1} final concentration) were prepared and equilibrated to the desired temperature and salinity. The 3 μg mL^{-1} DAPI solution was prepared from a stock solution of 20 μg mL^{-1} and an artificial brine, prepared as described above except to final concentration of 38, 87, 142 or 178 ppt, and placed in the cold room set to the desired examination temperature of −2°, −5°, −10° or −15°C, respectively. After ice formation and equilibration of the DAPI–brine staining solution with the ice at the desired temperature for at least 24 h, the final solution was filtered through a 0.2 μm syringe filter for immediate use. Care was taken at each stage to protect the solution from exposure to light. For two ice sections, isothermal–isohaline DAPI staining solutions (20 μg mL^{-1} final concentration) were prepared at −5° and −15°C by adding varying amounts of artificial sea salts corresponding to 87 and 178 ppt, respectively, to the DAPI stock solution. Both staining solutions were first tested on melted ice samples at room temperature to verify successful and differential staining of sea-ice bacteria (blue) and non-bacterial particulate materials (yellow) at the high salt concentrations used, as described below for melted-ice controls.

To stain ice sections, a drop of the appropriate DAPI–brine staining solution was applied to the microtomed surface of the sample and overlain with a glass cover-slip. The sample was allowed to stain for at least 1 h in the dark before microscopic observation. To check and account for autofluorescence, an unstained sample was examined microscopically prior to staining.

Melted-ice controls

Melted natural sea-ice samples, decimal dilutions of the artificial sea-ice preparations and ASW blanks were preserved with pre-filtered (0.2 μm) formaldehyde (2% final concentration) for subsequent analysis in the Seattle-based laboratory at the University of Washington. Where necessary, ASW was added to achieve a total volume of 10 mL, followed by 2 mL of the DAPI staining solution (20 μg mL^{-1}). After 7 min of staining, the sample was filtered onto a 0.2 μm polycarbonate track-etch black membrane filter (Micron Separation Inc., Westborough, MA). The filter was mounted on a microscopic slide, spotted with a drop of immersion oil, overlain with a cover-slip and examined by epifluorescence microscopy, according to standard procedures (Porter and Feig, 1980).

2.4. Microscope, filter sets and imaging system

A Zeiss Axioskop 2 microscope with a set of 2.5×, 5×, 20× and 63× (immersion) objectives and fitted with an epifluorescence illumination system and a 50 W mercury lamp was utilized for transmission and epifluorescence microscopy of the ice sections. It was modified in the factory for operation at sub-zero temperatures (−2° to −25°C) by exchanging all lubricants to maintain low viscosities at low temperatures. Non-fluorescent, filter-sterilized artificial brine prepared as described above was used as the immersion fluid for the 63× objective, which was a ceramic-tipped model. The combined requirement for sufficient working distances (i.e. 250 μm or more) to detect bacteria within the ice and for resilience against high-salinity solutions (ceramic tip) made it necessary to work with a 63× objective. Furthermore, the aperture of the 63× objective is large enough to allow for additional magnification with the image-processing set-up described below. The additional magnification more than compensates for the difference between 100 and 63×, with the resulting final magnification (3230×; see below) being approximately twice as high as that used for standard epifluorescence microscopy bacterial counts.

For epifluorescence work, the microscope was equipped with an optical filter set for DAPI (365 nm excitation, 395 nm beam splitter and long-pass 420 nm emission). Images of fluorescent cells and other features were captured using a MTI DC330E 3CCD color camera and Scion CG-7 RGB Color PCI Frame grabber. At the highest magnification, 1 μm in the sample slide corresponded to 10 linear pixel dimensions, with a total image size of 768 by 576 pixels. Pixels were digitized to 8 bits (512 gray levels) for each of the three RGB color channels. Image analysis, including segmentation, editing and cell counting, was done on the host computer (G3 Macintosh with 128 MB). All images were acquired, calibrated, analyzed and displayed using a variant of NIH Image v 1.62a (Rasband and Bright, 1995). The microscope features coupled with the video imaging system used facilitated visualization at a maximum magnification of 3230×.

2.5. Transmission and epifluorescence microscopy of ice samples

The following steps were taken to ensure that DAPI-stained

https://doi.org/10.3189/172756401781818275 Published online by Cambridge University Press

Table 1. Number of bacteria determined by in situ DAPI staining of ice sections compared to melted control samples

Sample examined	*Examination temperature*	*Number of bacteria visualized*	*Volume of ice section examined*	*Number of bacteria in ice section*	*Number of bacteria in melted-ice controls*[a]	*Percentage of total detected by in situ method*
	°C		mL	mL^{-1}	mL^{-1}	%
Artificial ice + culture						
10^{-1} dilution	−15	62	1.07×10^{-5}	5.79×10^{6}	$(1.03 \pm 0.87) \times 10^{7}$	56
10^{-2} dilution	−15	58	4.80×10^{-5}	1.21×10^{6}	$(1.88 \pm 0.85) \times 10^{6}$	64
10^{-3} dilution	−15	46	9.28×10^{-5}	2.48×10^{5}	$(3.00 \pm 1.93) \times 10^{5}$	83
Elson Lagoon ice	−2	21	2.03×10^{-4}	1.04×10^{5}	$(8.41 \pm 1.73) \times 10^{5}$	12
(35 cm below ice surface)	−5	33	2.06×10^{-4}	1.60×10^{5}	$(6.77 \pm 0.63) \times 10^{5}$	24
	−5	59[b]	2.06×10^{-4}	2.86×10^{5}	$(6.77 \pm 0.63) \times 10^{5}$	42
	−10	18	1.98×10^{-4}	9.08×10^{4}	$(8.26 \pm 2.36) \times 10^{5}$	11
	−15	16	2.04×10^{-4}	7.85×10^{4}	$(7.13 \pm 1.11) \times 10^{5}$	11
	−15	18[b]	7.93×10^{-5}	2.27×10^{5}	$(7.13 \pm 1.11) \times 10^{5}$	32
Chukchi Sea ice (25 cm below ice surface)	−15	10	2.00×10^{-4}	5.00×10^{4}	$(7.51 \pm 1.07) \times 10^{4}$	67

[a] Mean of triplicate subsamples ± standard error.

[b] Using a DAPI concentration of 20 μg mL^{-1} (all other samples, 3 μg mL^{-1}).

fluorescent cells were distinguished from either autofluorescent or amorphous fluorescent material. Unstained samples were examined extensively (500 microscopic fields per sample) in search of autofluorescing bacteria-sized and -shaped particles; none were observed. In stained samples, only fluorescent particles with morphologies, sizes and color (blue) consistent with DAPI-stained microbial cells were counted. Cell counting was initiated at least 10 μm into the ice to avoid recording possible contaminants on the surface of the section. Prolonged excitation of the sample was avoided, since photobleaching began to occur after several minutes.

Artificial sea-ice sections

For ice sections prepared from decimal dilutions of the bacterial culture, a minimum of 50 bacteria were enumerated while recording the total number of microscopic fields examined (as displayed on the monitor). For ice sections prepared from sterile ASW (negative controls), 500 microscopic fields were examined. The location of the ice surface was established by focusing on ice-grain boundaries reaching the underside of the cover-slip under transmitted light. The thickness of the ice layer examined in each sample was 100 μm (as this length was determined to be the average depth to which individual bacterial cells of 1 μm diameter were still discernible; see below). Given the total number of fields and the ice depth examined, the total number of bacteria per volume of ice was calculated and compared to the total number of bacteria counted in the initial dilution samples.

For ice sections prepared from ASW containing prestained cells of the bacterial culture, four separate brine inclusions, extending to >200 μm depth and containing many bacteria, were examined to determine the greatest depth at which individual bacteria of 1 μm diameter were still discernible. Ninety microscopic fields were examined to determine the fraction of bacteria found only in brine inclusions vs those frozen directly into the ice-crystal lattice.

Natural sea-ice sections

After staining an ice section, a minimum of 500 microscopic fields were examined by epifluorescence microscopy using the optical filter set for DAPI. When bacteria were encountered, images were recorded under DAPI excitation and transmitted light. These images were later analyzed to enumerate bacteria and determine their locations within the ice matrix and associations with ice features (e.g. brine channels, ice crystal walls, particles or other organisms). As for artificial sea-ice samples, examination began at least 10 μm below the ice surface for a total examination thickness of 100 μm. The total number of bacteria per volume of ice was calculated and compared to the concentration of bacteria determined in parallel melted samples. Microscopic observations were also performed at decreasing magnification using the 63×, 20×, 5× and 2.5× objectives to combine bacterial observations with those of other organisms and with morphology and particle content of the pore space.

Melted-ice controls

Bacterial abundance in melted samples was determined by standard DAPI-staining, sample filtration and epifluorescence microscopy, counting a minimum of 20 randomly selected fields on the filter or a minimum of 200 bacteria (Porter and Feig, 1980). These bacterial numbers were used to calculate the percentage of bacteria detectable within artificial and natural sea-ice sections.

3. RESULTS AND DISCUSSION

The fluorescent DNA-specific stain DAPI is commonly used in final concentrations of < 1 μg mL^{-1} for the enumeration of bacteria in many aquatic habitats (see review by Kepner and Pratt, 1994). We have adapted this conventional staining method and found that the use of higher concentrations of the stain (3 and 20 μg mL^{-1}) in temperature-equilibrated brine solutions allowed for successful detection and enumeration of bacteria within brine inclusions of the sea-ice matrix at extremely low temperatures (Table 1; Figs 2–6). Using high DAPI concentrations also allowed a clear distinction between bright blue-fluorescing DAPI-stained bacteria and stain bound to amorphous or particulate material which generally fluoresced yellow (Figs 4c and 5b; Porter and Feig, 1980; Mostajir and others, 1995).

Epifluorescent microscopic examination of the ice sections prior to staining revealed numerous inorganic particles, especially in Elson Lagoon ice samples, many of which fluoresced under ultraviolet illumination. However, no bacteria-sized and -shaped autofluorescing particles were detected in

https://doi.org/10.3189/172756401781818275 Published online by Cambridge University Press

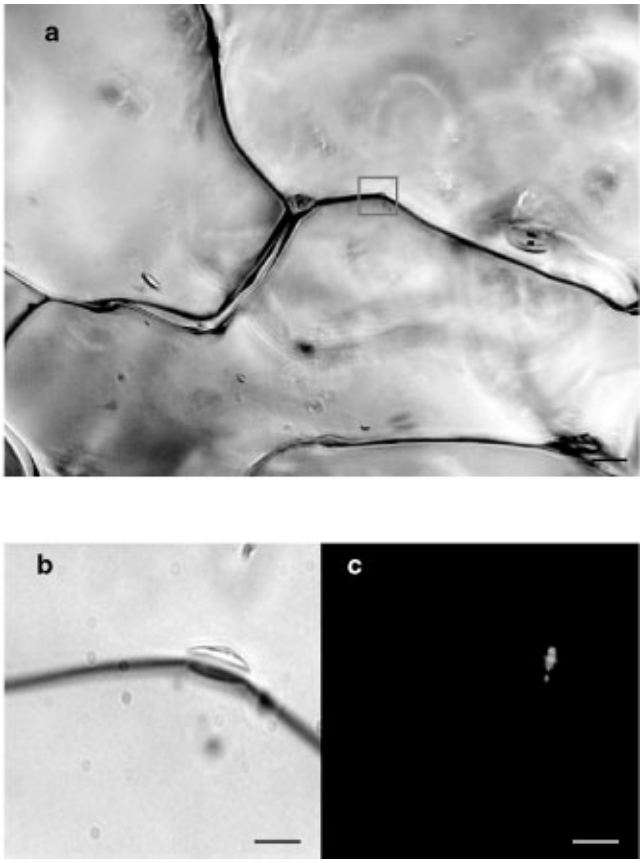

Fig. 2. (a) Transmitted light image of grain boundaries with fluid inclusions; bar = 100 μm; box refers to enlarged images in (b) and (c). (b) Transmitted light image of detail of grain boundaries in (a) with small brine pocket; bar = 10 μm. (c) Epifluorescent image of DAPI-stained (blue) bacteria at the wall of the brine pocket in (b); bar = 10 μm.

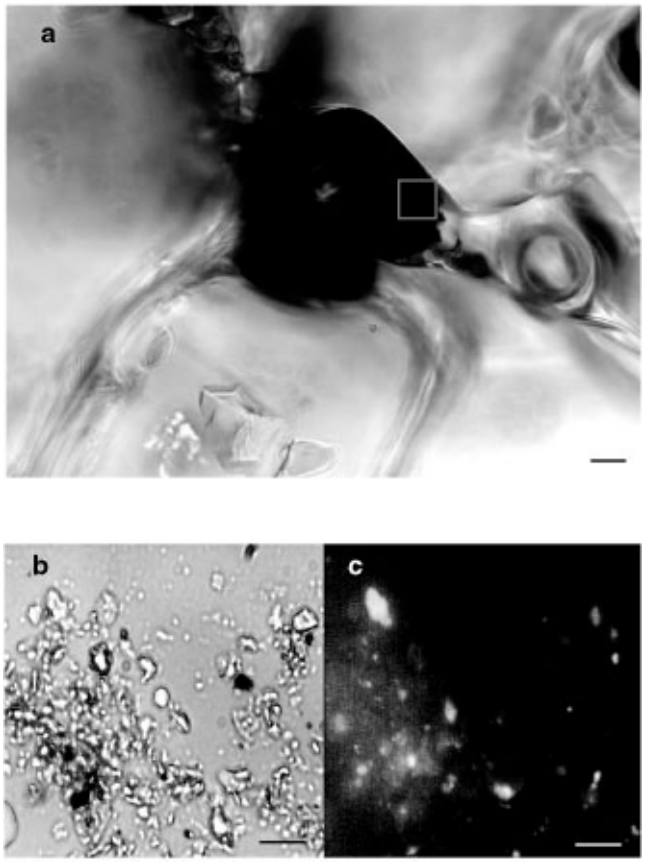

Fig. 4. (a) Transmitted light image of brine pocket filled with particulate material (dark area); bar = 100 μm; box refers to enlarged images in (b) and (c). (b) Transmitted light image of detail of particulate material filling the brine pocket in (a); bar = 10 μm. (c) Epifluorescent image of small, coccoid-shaped DAPI-stained (blue) bacteria, contrasting with more yellow-fluorescent (non-bacterial) particles; bar = 10 μm.

the 500 fields examined before staining. Although the presence of particles may complicate the enumeration of microbial cells (e.g. by obscuring them from view), bacteria were detected readily and unequivocally in stained samples even when rich in particles (Figs 4 and 5). Bacterial cell sizes were not rigorously quantified in this study, but a spectrum of bacterial sizes and morphologies was observed, ranging from the most abundant, small coccoid cells (≤1 μm; Figs 4–6) to a diverse mixture of rods (usually 1–3 μm long; Fig. 2), including a pair of very large rods (observed once, 10 μm long; Fig. 3).

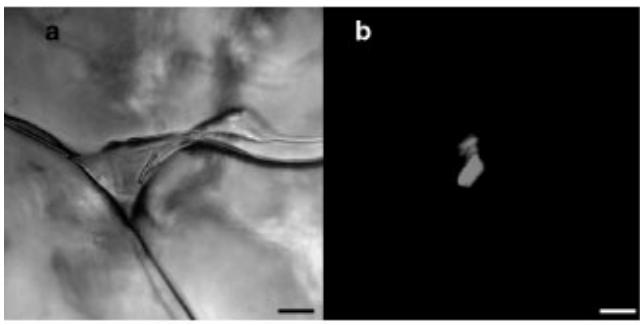

Fig. 3. (a) Transmitted light image of brine inclusion at triple point between three grains; bar = 20 μm; note two large rod-shaped bacteria along the wall of the inclusion (close inspection reveals apparent cell division). (b) Epifluorescent image of the same (DAPI-stained) bacteria as in (a); bar = 10 μm; note reflection of blue fluorescence from the organisms off the ice walls (an effect also mildly evident in Fig. 2c).

In artificial sea ice containing cultured sea-ice bacteria at known concentrations, 56–83% of the cells were detected using the in situ microscopic method (at highest magnification) at −15°C (Table 1). In artificial sea ice containing pre-stained cells of cultured sea-ice bacteria, the majority of cells (38 of 40, or 95%) were observed within brine inclusions (triple point junctures, brine veins, small brine pockets); only two were judged to be frozen into the ice crystal itself. In sections prepared from the natural wintertime sea-ice samples, the total numbers of bacteria stained and detected in the 500 fields examined were low, but when scaled to the volume of sample examined, they represented 12–67% of the total number in the melted-ice controls (Table 1).

The higher detection success with the artificial sea-ice preparations may be due to differences in the ice structure of artificial ice preparations and natural ice samples, with ice crystals of the latter observed to be significantly larger. In addition, many wintertime sea-ice bacteria were smaller than the 1 μm diameter cells of *P. mcmeekenii* and may have evaded visualization in this study. The lower detection success in Elson Lagoon samples may be due to the greater presence of particles possibly obscuring some cells or interfering with stain diffusion. The primary reason for an overall detection success of ≤67% in natural ice samples, however, relates to the fraction of pore spaces within the ice

https://doi.org/10.3189/172756401781818275 Published online by Cambridge University Press

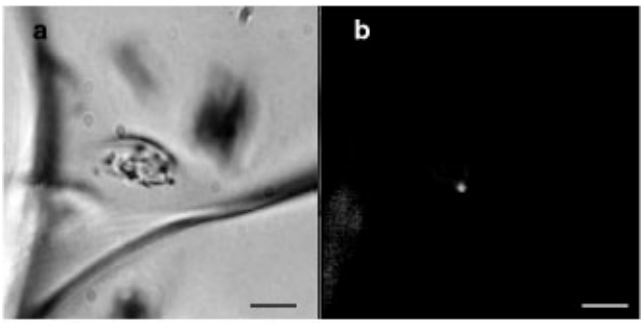

Fig. 5. (a) Transmitted light image of a cluster of particles in a brine inclusion; bar = 10 μm. (b) Epifluorescent image of a single, small, coccoid-shaped DAPI-stained (blue) bacterium, present among the more yellow-fluorescent particles; bar = 10 μm.

sections that actually received the DAPI–brine staining solution, as explained below.

By using a staining solution equilibrated to in situ temperature and salinity, we were able to introduce a dye specific for double-stranded DNA within bacterial cells with minimal disturbance of the sample and on a scale relevant to the bacteria. Staining the samples for 1 h allowed the dye to penetrate by diffusion far below our observation depth (110 μm into the ice), as verified by the faint blue background fluorescence of deep (>200 μm) brine pockets. However, as demonstrated with artificial sea-ice preparations containing pre-stained bacterial cells, 1 μm diameter cells could be detected on average only to a depth of 110 μm.

Furthermore, only those brine pores connected with the ice surface by veins or brine channels, whether shallow or deep in the ice section, could receive the dye solution. In a parallel study of diatoms present within the lower part of the ice core from the Chukchi Sea (readily visualized with or without staining, unlike bacteria; Fig. 6a and b), approximately 60% of the pores were inferred to have received the stain (personal communication from C. Krembs, 2000), in keeping with our bacterial detection success of ≤67% in field samples from the same site.

Although applying a drop of staining solution onto the surface of the ice could possibly result in fluid flow in the upper veins (and thus in altered positions of unattached bacteria therein), we did not observe any alterations of the brine-pocket size and shape or any movement of particles within brine channels when comparing observations of the same area of ice made before and after application of the staining solution. We further avoided fluid-induced artifacts (as well as any bacterial contaminants) by excluding the upper 10 μm layer of ice from the examination protocol.

Life at low temperature in sea ice very much depends on the evolution of the morphology and distribution of brine inclusions. The isothermal–isohaline staining technique described above represents an important step in the development of a comprehensive image-analysis and classification scheme that would ultimately allow us to quantitatively link the distribution and size of different classes of fluid and particulate inclusions to the characteristics of the microbial community at low temperatures. As evident in Figures 2 and 3, interconnected systems of narrow veins persist to temperatures of −15°C. The microscopic images indicate that both inorganic (Fig. 4) and biogenic inclusions (Figs 5 and 6) are largely confined to brine-filled pores. Here, triple points between adjacent grains (such as the example shown in Figure 3, with dimensions typically larger than about 20 μm) may play a particular role because of enhanced fluid flow (induced by local and large-scale temperature gradients) and subsequent physical concentration of inorganic and biogenic inclusions. On the other hand, liquid films on grain boundaries, such as between adjacent crystals of granular ice shown in Figure 2 (with typical widths on the order of 10 μm at −15°C), have also been found to harbor bacteria. The images presented in Figure 2b and c also raise the question to what extent individual bacteria interact with the ice matrix, such that enlargement of sheet-like inclusions (typically only a few micrometers wide for intergranular brine sheets) into actual brine pockets is a result of interaction between organisms or organic exudates and the surrounding ice. On a larger scale, this effect is apparent in Figure 6a and b, with diatom cells and bacteria surrounded by a residual brine volume.

4. CONCLUSIONS

With the development of this in situ microscopic method for

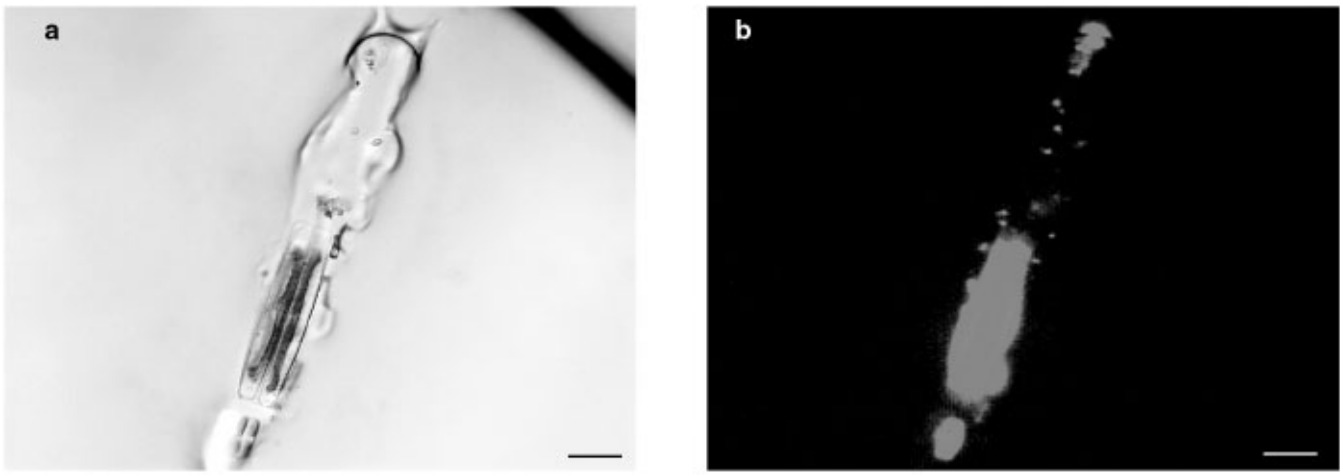

Fig. 6. (a) Transmitted light image of particulate material and a diatom in brine inclusion; bar = 20 μm; note green autofluorescence of chlorophyll inside the diatom. (b) Epifluorescent image of the same (DAPI-stained) diatom as shown in (a), along with many small, coccoid-shaped blue-fluorescing bacteria; bar = 20 μm.

https://doi.org/10.3189/172756401781818275 Published online by Cambridge University Press

detecting micro-organisms within the ice matrix at relevant spatial scales and in situ temperatures, many research questions now become tractable. The power of the described in situ microscopy method is in providing a basis for relating characteristics of bacteria to their physical habitat and thereby aiding in discerning physical and possibly chemical constraints on microbial communities within frozen matrices on a spatial scale relevant to the organisms. It is less useful for determining total abundances in sea ice, since only those brine pores open to the microtomed surface of the ice section can receive the stain. We are currently pursuing the possible linkage between particulate content of sea ice, microscale brine features and bacterial sustenance (and activity) at very low temperatures, as well as bacterial associations with other organisms and their organic exudates. The development of additional stains, chemical reagents or molecular probes amenable to isothermal equilibration in brine could lead to direct examination of extracellular enzymatic activities, bacterial motility and chemotaxis (Price, 2000), or the abundance and behavior of specific Bacteria, Archaea or multicellular micro-organisms (e.g. protists) within their native habitats at very low temperatures. The method can also be advanced towards in situ measurements of the microscale chemical conditions (e.g. pH, oxygen, nitrogen speciation or organic carbon content) that may enable microbial survival and activity even under the most extreme of wintertime conditions in sea ice.

In principle, one could use this in situ microscopic method to introduce dyes or other reagents into liquid veins present not only in sea ice, but also in other frozen habitats such as lake or glacial ice, permafrost, cloud droplets, and conceivably even ice cores returned from Mars or Europa. In advance of the return of extraterrestrial samples, exploration of specific physical and chemical constraints on microbial life at extremely low temperatures in Earth ice matrices should aid in evaluating and planning tests of the habitability of frozen systems elsewhere.

ACKNOWLEDGEMENTS

This research was supported by a U.S. National Science Foundation (NSF)–Office of Polar Programs–Life in Extreme Environments (LExEn) award to J. Deming and H. Eicken and the University of Washington Astrobiology Program. We are grateful for additional NSF support through the Barrow Arctic Science Consortium, especially the kind assistance of D. Ramey. We also thank S. Carpenter for technical support and O. Prange for help in generating the figures.

REFERENCES

Barbara, G. M. and J. G. Mitchell. 1996. Formation of 30- to 40-micrometer-thick laminations by high-speed marine bacteria in microbial mats. *Appl. Environ. Microbiol.*, **62**(11), 3985–3990.

Cercone, K. R. and V. A. Pedone. 1987. Fluorescence (photoluminescence) of carbonate rocks: instrumental and analytical sources of observational error. *J. Sediment. Petrol.*, **57**(4), 780–782.

DeLeo, P. C., P. Baveye and W. C. Ghiorse. 1997. Use of confocal laser scanning microscopy on soil thin-sections for improved characterization of microbial growth in unconsolidated soils and aquifer materials. *J. Microbiol. Methods*, **30**(3), 193–203.

Eicken, H. 1993. Automated image analysis of ice thin sections—instrumentation, methods and extraction of stereological and textural parameters. *J. Glaciol.*, **39**(132), 341–352; Erratum: **39**(133), p. 727.

Eicken, H. *and 20 others*. 1999. Ice tank studies of physical and biological sea-ice processes. *In* Shen, H. T., *ed. Ice in surface waters. Vol. 1.* Rotterdam, A.A. Balkema, 363–370.

Eicken, H., C. Bock, R. Wittig, H. Miller and H.-O. Poertner. 2000. Nuclear magnetic resonance imaging of sea-ice pore fluids: methods and thermal evolution of pore microstructure. *Cold Reg. Sci. Technol.*, **31**(3), 207–225.

Grossmann, S. and G. S. Dieckmann. 1994. Bacterial standing stock, activity, and carbon production during formation and growth of sea ice in the Weddell Sea, Antarctica. *Appl. Environ. Microbiol.*, **60**(8), 2746–2753.

Helmke, E. and H. Weyland. 1995. Bacteria in sea ice and underlying water of the eastern Weddell Sea in midwinter. *Mar. Ecol. Prog. Ser.*, **117**(1–3), 269–287.

Horner, R. A. 1985. *Sea ice biota.* Boca Raton, FL, CRC Press Inc.

Junge, K., J. J. Gosink, H.-G. Hoppe and J. T. Staley. 1998. *Arthrobacter*, *Brachybacterium* and *Planococcus* isolates identified from Antarctic sea ice brine. Description of *Planococcus mcmeekenii*, sp. nov. *Syst. Appl. Microbiol.*, **21**(2), 306–314.

Karl, D. M., D. F. Bird, K. Bjorkman, T. Houlihan, R. Shackelford and L. Tupas. 1999. Microorganisms in the accreted ice of Lake Vostok, Antarctica. *Science*, **286**(5447), 2144–2147.

Kepner, R. L. and J. R. Pratt. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiol. Rev.*, **58**(4), 603–615.

Kottmeier, S. T. and C. W. Sullivan. 1990. Bacterial biomass and production in pack ice of Antarctic marginal ice edge zones. *Deep-Sea Res.*, **37**(8), 1311–1330.

Krembs, C., A. R. Juhl, R. A. Long and F. Azam. 1998. Nanoscale patchiness of bacteria in lake water studied with the spatial information preservation method. *Limnol. Oceanogr.*, **43**(2), 307–314.

Laurion, I., S. Demers and A. F. Vézina. 1995. Microbial food web associated with the ice algal assemblage: biomass and bacterivory of nanoflagellate protozoans in Resolute Passage (High Canadian Arctic). *Mar. Ecol. Prog. Ser.*, **120**(1–3), 77–87.

Maykut, G. A. 1986. The surface heat and mass balance. *In* Untersteiner, N., *ed. Geophysics of sea ice.* London, etc., Plenum Press, 395–463. (NATO ASI Series B: Physics 146.)

Mostajir, B., J. R. Dolan and F. Rassoulzadegan. 1995. A simple method for the quantification of a class of labile marine pico- and nano-sized detritus: DAPI yellow particles (DYP). *Aquat. Microb. Ecol.*, **9**(3), 259–266.

Palmisano, A. C. and D. L. Garrison. 1993. Microorganisms in Antarctic sea ice. *In* Friedmann, E. I., *ed. Antarctic microbiology.* New York, etc., Wiley–Liss Inc., 167–218.

Porter, K. G. and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.*, **25**(5), 943–948.

Price, P. B. 2000. A habitat for psychrophiles in deep Antarctic ice. *Proc. Natl. Acad. Sci. U.S.A.*, **97**(3), 1247–1251.

Priscu, J. C. *and 11 others*. 1999. Geomicrobiology of subglacial ice above Lake Vostok, Antarctica. *Science*, **286**(5447), 2141–2144.

Rasband, W. S. and D. S. Bright. 1995. NIH Image: a public domain image processing program for the Macintosh. *Microbeam Analysis Soc. Jour.*, **1995**(4), 137–149.

Stone, R. 1999. Permafrost comes alive for Siberian researchers. *Science*, **286**(5437), 36–37.

Sullivan, C. W. and A. C. Palmisano. 1984. Sea ice microbial communities: distribution, abundance, and diversity of ice bacteria in McMurdo Sound, Antarctica, in 1980. *Appl. Environ. Microbiol.*, **47**(4), 788–795.

Tobin, K. J., T. C. Onstott, M. F. DeFlaun, F. S. Colwell and J. Fredrickson. 1999. In situ imaging of microorganisms in geologic material. *J. Microbiol. Methods*, **37**(3), 201–213.