Video Article

Removal of Exogenous Materials from the Outer Portion of Frozen Cores to Investigate the Ancient Biological Communities Harbored Inside

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URL: https://www.jove.com/video/54091
DOI: doi:10.3791/54091

Keywords: Molecular Biology, Issue 113, Permafrost, ice cores, microbial communities, decontamination, qPCR, soil, bacteria, archaea

Date Published: 7/3/2016

Citation: Barbato, R.A., Garcia-Reyero, N., Foley, K., Jones, R., Courville, Z., Douglas, T., Perkins, E., Reynolds, C.M. Removal of Exogenous Materials from the Outer Portion of Frozen Cores to Investigate the Ancient Biological Communities Harbored Inside. *J. Vis. Exp.* (113), e54091, doi:10.3791/54091 (2016).

Abstract

The cryosphere offers access to preserved organisms that persisted under past environmental conditions. In fact, these frozen materials could reflect conditions over vast time periods and investigation of biological materials harbored inside could provide insight of ancient environments. To appropriately analyze these ecosystems and extract meaningful biological information from frozen soils and ice, proper collection and processing of the frozen samples is necessary. This is especially critical for microbial and DNA analyses since the communities present may be so uniquely different from modern ones. Here, a protocol is presented to successfully collect and decontaminate frozen cores. Both the absence of the colonies used to dope the outer surface and exogenous DNA suggest that we successfully decontaminated the frozen cores and that the microorganisms detected were from the material, rather than contamination from drilling or processing the cores.

Video Link

The video component of this article can be found at https://www.jove.com/video/54091/

Introduction

The cryosphere (*e.g.*, permafrost soils, ice features, glacial snow, firn, and ice) offers a glimpse into what types of organisms persisted under past environmental conditions. Since these substrates can be tens to hundreds of thousands of years old, their microbial communities, when preserved frozen since deposition, reflect ancient environmental conditions. To appropriately analyze these ecosystems and extract meaningful biological information from frozen soils and ice, proper collection and processing of the frozen samples is necessary. This is of utmost importance as climate projections for the 21st century indicate the potential for a pronounced warming in Arctic and sub-Arctic regions[1]. Specifically, Interior Alaska and Greenland are expected to warm approximately 5 °C and 7 °C, respectively by 2100[2,3]. This is expected to significantly impact soil and aquatic microbial communities, and therefore, related biogeochemical processes. The warmer temperatures and altered precipitation regime are expected to initiate permafrost degradation in many areas[2-5] potentially leading to a thicker, seasonally thawed (active) layer[6,7], the thawing of frozen soils, and the melting of massive ice bodies such as ground ice, ice wedges, and segregation ice[8]. This would dramatically change the biogeochemical attributes in addition to the biodiversity of plants and animals in these ecosystems.

Glacial ice and syngenetic permafrost sediment and ice features have trapped chemical and biological evidence of an environment representing what lived there at the time the features formed. For example, in Interior Alaska, both Illinoisan and Wisconsin aged permafrost are present and this permafrost in particular provides unique locations dating from modern to 150,000 years before the present (YBP) that contain biological and geochemical evidence of the impact of past climatic changes on biodiversity. As a result, these sediments provide a record of the biogeochemistry and biodiversity over many thousands of years. Since the area has low sedimentation rates and has never been glaciated, undisturbed samples are accessible for collection and analysis, either drilling vertically into the soil profile or drilling horizontally in tunnels. More importantly, extensive records exist that especially highlight the unique biogeochemical features of permafrost in this region[9-14]. Specifically, the application of DNA analysis to estimate presence and extent of biodiversity in both extant and ancient ice and permafrost samples enables exploration of the linkage of ancient environmental conditions and habitat to occupation by specific organisms.

Previous studies have identified climatic impacts on mammals, plants and microorganisms from samples dating to 50k YBP[11, 15-19], though each study used a different methodology to collect and decontaminate the permafrost or ice cores. In some instances, the drilling cores were sterilized[16, 20-21], though the specific methodology did not clarify whether foreign nucleic acids were also eliminated from the samples. In other

studies, bacterial isolates[15] (*e.g.*, *Serratia marcescens*) as well as fluorescent microspheres[22] have been used to measure the efficacy of decontamination procedures.

This experiment was part of a larger study investigating microbial communities from permafrost samples dating back to approximately 40k YBP. The specific objective of this portion of the study was to successfully decontaminate ice and permafrost cores. To our knowledge, no methodology has integrated the use of solutions designed to eliminate foreign nucleic acids and associated nucleases from the outer portion of the frozen cores. This is despite the fact that these solutions are commonly used to decontaminate laboratory equipment for molecular experiments.

Once the cores were decontaminated, genomic DNA was extracted using the protocols developed by Griffiths *et al.*[23] and Töwe *et al.*[24], quantified using a spectrophotometer, and diluted with sterile, DNA-free water to achieve 20 ng per reaction. Bacterial 16S rRNA genes were amplified with primers 331F and 797R and probe BacTaq[25] and archaeal 16S rRNA genes were amplified with primers Arch 349F and Arch 806R and probe TM Arch 516F[26] under the following conditions: 95 °C for 600 sec followed by 45 cycles of 95 °C for 30 sec, 57 °C for 60 sec, and 72 °C for 25 sec with final extension at 40 °C for 30 sec. All qPCR reactions were conducted in duplicate. The 20 µl reaction volumes included 20 ng DNA, 10 µM of primers, 5 µM of the probe, and 10 µl of the qPCR reaction mix. Standards for bacterial and archaeal qPCR were prepared using genomic DNA from *Pseudomonas fluorescens* and *Halobacterium salinarum*, respectively. Both were grown to log phase. Plate counts were conducted and DNA was isolated from the cultures. Genomic DNA was quantified with a spectrophotometer with the assumption of one and six copies of the 16S rRNA gene per genome for *H. salinarum* and *P. fluorescens*, respectively[27-28]. Copy numbers of the bacterial and archaeal genes were calculated based on the standard curve, log-transformed to account for unequal variances between treatments, and assessed by ANOVA.

Community composition was determined by sequencing the 16S rRNA gene using flow cells and bridge amplification technologies and analyzing the communities with 'quantitative insights into microbial ecology' (QIIME)[29]. Forward and reverse reads were joined together and then sequences were filtered, indexed, and high quality representatives were selected for de novo operational taxonomic units (OTU) assignment through sequence alignment with a reference database. Aligned sequences were compared to a separate reference database for taxonomic assignment. A phylum level OTU table was created to determine general community composition.

Protocol

1. Equipment Preparation and Permafrost Core Collection

1. Equipment preparation and field sample collection and preservation gear
    1. Assemble auger for sample collection by inserting the drive adapter into the top of the barrel and rotating the lever to lock it in place. Pin the adapter tube onto the drive adapter and pin the motor onto the adapter tube. Insert the cutters on the barrel.
    2. Wear light duty suits, nitrile gloves, and masks to reduce any contamination to the samples. Wear ear protection and a hard hat for safety upon entering the Permafrost Tunnel (**Figure 1**).
    3. Enter the tunnel and select a location to collect the samples (**Figure 1B**).
    Note: For vertical or horizontal drilling location, select an area where there is known evidence that the material is frozen (*e.g.*, ice or permafrost), there is no known large root systems, and there is no known gravel. Remove all living plant material from the area before collecting a sample. If drilling vertically or horizontally, select an area that is relatively flat and accessible with the auger.
    4. Prepare a work station by layering the ground with a plastic material that has been sterilized with 70% isopropanol, DNA decontamination solution, and RNase decontamination solution.
    5. Place a 10 cm diameter polyvinyl chloride (PVC) pipe cut in half lengthwise on the workstation to provide a trough to hold the cores as they are removed from the auger. Decontaminate the PVC pipe with 70% isopropanol, DNA decontamination solution, and RNase decontamination solution.
    6. Near the work station, decontaminate the auger by spraying it with 70% isopropanol, DNA decontamination solution, and RNase decontamination solution. Remove solutions from the auger with a wipe.

2. Ice and permafrost core collection and storage
    1. Select an area of the wall to sample (see 1.2.1 Note).
    2. Decontaminate approximately 10 cm diameter of frozen wall area by wiping it with 70% isopropanol, DNA decontamination solution, and RNase decontamination solution.
    3. Elevate the decontaminated auger to the area of interest such that it is perpendicular to the sample area and begin drilling into the cleaned face of the wall (**Figure 1C, D**).
    4. Carefully remove the auger from sample collection area. Disconnect the auger from the motor and place the auger above the sterile PVC pipe in the clean work station. Carefully tilt auger such that the frozen cores slide out onto the sterile PVC pipe (**Figure 1E**).
    5. Decontaminate gloves with 70% isopropanol, DNA decontamination solution, and RNase decontamination solution.
    6. Pick up the ice and permafrost cores with sterile gloves and place them into sterile bags.
    7. Place the cores in a cooler or cold room until they are shipped or processed.
    8. Ship the frozen cores using dry ice to maintain them at a temperature below 0 °C.
    9. Immediately store the cores at -80 °C.

2. Permafrost and Ice Core Processing

1. Material Preparation
    1. Prepare sterile, nucleic acid free heavy duty aluminum foil, metal racks, glassware, metal forceps, and glass wool by baking in an oven at 450°C for 4 hr. Set aside these materials until used.

2. Sterilize 95% ethanol and ultrapure water by passing the solutions through a 0.22 µm filter.
3. Store ethanol solution at -20 °C and water at 4 °C.
4. Sterilize a plastic ruler by spraying it with 70% ethanol, DNA decontamination solution, and RNase decontamination solution and immediately wiping after each solution.

2. Bacterial Culture Preparation
    1. Prepare broth and plates to grow *Serratia marcescens*.
        1. For broth, combine 5 g tryptone, 1 g glucose, 5 g yeast extract, and 1 g potassium phosphate dibasic, and then add distilled water to reach 1 L. To make plates, add 15 g of agar to the above mix. For broth and plates, adjust pH to 7 and autoclave at 121 °C for 15 min.

    2. Prepare 1x phosphate buffed saline solution (PBS) by combining 8 g sodium chloride, 0.2 g of potassium chloride, 1.44 g of sodium phosphate dibasic, and 0.24 g potassium phosphate monobasic and add distilled water to reach 800 ml. Adjust pH to 7.4 and add distilled water to reach 1 L. Autoclave at 121 °C for 15 min.
    3. Inoculate broth with a sterile loop by dipping into the *S. marcescens* culture that was previously stored frozen at -80 °C. Under aseptic conditions, serial dilute the culture by adding 1 ml of culture to 9 ml of PBS and manually invert the solution. Add 1 ml of this dilution to 9 ml of PBS and manually invert the solution. Repeat eight more times until a 10^{-9} dilution is reached.
    4. Spread the 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} solutions onto agar plates by distributing 1 ml of broth onto the plate and spreading with a cell spreader. Do this in triplicate per dilution. Store the original culture at 4 °C.
    5. Incubate plates at 30 °C for 24 hr. Count the number of colonies to calculate the number of cells in the original culture. Note: The number of colonies in the original culture will depend on the growth rate of the bacteria.
    6. Pipet 250 µl of the original culture into a sterile 1.5 ml microcentrifuge tube. Dilute original culture by adding enough volume of the 1x PBS to obtain approximately 10^9 cells/ml as determined by colony count in the previous step. Pellet the cells in a microcentrifuge tube by centrifuging at 2,500 x g for 10 min.
    Note: The volume of 1x PBS will vary depending on the growth rate of the bacteria.
    7. In a sterile biohood, pipet off the broth and resuspend the cells in 1 ml 1x PBS buffer. Store at 4 °C until use or approximately two months.
    8. On the day of processing, dilute the *S. marcescens* culture from step 2.2.7 by adding 1 ml of the original *S. marcescens* culture to 39 ml 1x PBS buffer in a 50 ml centrifuge tube.

3. Prepare cold room space for core processing and preservation gear
    1. Prior to chilling the cold room, clean walls, floors, and metal table with a 1% bleach solution.
    2. Set temperature of cold room to approximately -11 °C.
    3. Once the cold room has reached the desired temperature, wear light duty suits, nitrile gloves, and masks to reduce any contamination to the cold room and the samples.
    Note: Two properly dressed individuals are required for this procedure.
    4. Bring the sterile materials (*e.g.,* aluminum foil, metal racks, glassware, tray, *S. marcescens* culture, and microtome blade) and samples into the cold room and place on the sterile table.

4. Permafrost and ice core processing in a cold room facility
    1. Place a permafrost core on a sterile, nucleic acid free sheet of aluminum foil.
    2. Lightly inoculate the outside of the core with the dilute culture of *S. marcescens* using a sterile foam plug (**Figure 2B**).
    3. Place the core on the sterile metal rack that sits above a tray.
    4. Sterilize the steel microtome blade with 70% ethanol, DNA decontamination solution, and RNase decontamination solution.
    5. Have Individual A clean nitrile gloves with 70% ethanol, DNA decontamination solution, and RNase decontamination solution and hold core at a 45° angle above the tray.
    6. Have Individual B gently scrape approximately 5 mm of the outside of the entire core, including the ends, using the sterile blade while Individual A turns the core after each scrape (**Figures 2C** and **3A, B**). Wipe the blade with 70% ethanol, DNA decontamination solution, and RNase decontamination solution as needed.
    7. Have Individual B pour filter-sterilized 95% ethanol over the core carefully and quickly, while Individual A turns the core as the solution is poured (**Figures 2D, 3C**).
    8. Have Individual B immediately rinse the core with filter-sterilized water.
    9. Replace used metal rack on tray with a new sterile metal rack.
    10. Have Individual A clean his or her nitrile gloves with 70% ethanol, DNA decontamination solution, and RNase decontamination solution and hold the core at a 45° angle above the tray.
    11. Have Individual B spray the entire core with DNA decontamination solution.
    12. Have Individual B immediately rinse the core with filter-sterilized water.
    13. Have Individual B spray the entire core with RNase decontamination solution.
    14. Have Individual B immediately rinse the core with filter-sterilized water.
    15. Place the core on a sterile sheet of aluminum foil and lightly wrap.

5. Thaw outer core
    1. Place a sterile metal rack over a sterile glass dish in a sterile biohood with laminar flow.
    2. Place two agar plates specific to *S. marcescens* in the glass dish below the sterile rack to collect liquid from the core (**Figure 2E**).
    3. Place the core on the sterile metal rack.
    4. Allow approximately 2-5 mm of the outer surface of core to thaw at 23 °C (on average, this will occur within approximately 10 min). Turn the core approximately 90° every 2 min.
    5. Swab entire surface of core using sterile forceps and sterile glass wool and inoculate two agar plates specific to *S. marcescens* by swabbing these materials onto the surface of the plates. Inoculate two new plates with the original culture used to dope the outside of the core, which was exposed to the low temperatures of the cold room.

6. Measure outer dimensions of thawed cylindrical core by placing a sterile ruler near, but not touching the core.
7. Place the core into large sterile bag and store it at -80 °C.

6. Check for growth
    1. Incubate agar plates at 23 °C for one week.
    2. Examine agar plates for growth of *S. marcescens* colonies.
        1. If there are no visible colonies on the plate, proceed to step 2.5.3.
        2. If colonies appear on plate, repeat from step 2.1.1 in section 2 "Permafrost and ice core processing".
    3. Obtain the core from freezer and aseptically transfer it to a sterile bag.
    4. Store the core in a sterile bag at 4 °C for approximately 24-48 hr to thaw the entire core.

3. Obtain Subsample for Nucleic Acid Extraction from Ice Cores and Permafrost

1. Nucleic acid extraction from ice cores
    1. Mix the thawed material from the ice core by gently agitating the bag (**Figure 2G**).
    2. Pour the thawed material into a sterile container with a 0.22 µm filter under vacuum to collect microorganisms.
    3. Aseptically remove the filter with sterile forceps and place it in a sterile 2 ml ceramic bead tube (1.4 mm).
    4. Store the filter at -80 °C.

2. Nucleic acid extraction from permafrost cores
    1. Mix the thawed material from the permafrost core by gently kneading the bag.
    2. After the permafrost has been well mixed, obtain a subsample of approximately 0.5 g bulk soil with a sterile scoopula and place it in a tared sterile 2 ml ceramic bead screw-cap tube (1.4 mm) that sits upright on a balance.
    3. Store subsamples at -80 °C.
    4. Obtain gravimetric water content of samples.
        1. Measure 10 g of the permafrost with a sterile scoopula and place in a tared tin on a balance. Measure wet mass of permafrost and tin.
        2. Incubate permafrost in an oven set at 105 °C for 24 hr. Measure dry mass of permafrost and tin.
        3. Calculate the gravimetric water content by subtracting the wet mass of permafrost by the dry mass of the permafrost and dividing by the dry mass of permafrost.
    5. Store permafrost at -80 °C.

4. Extract Nucleic Acids from Permafrost and Ice Cores

1. Material preparation
    1. Prepare amber vials to hold solutions by treating with 0.1% diethylpyrocarbonate (DEPC), incubating O/N at 37 °C, and autoclaving.
    2. Prepare 240 mM potassium phosphate buffer solution. Add 2. 5 g of potassium phosphate monobasic and 38.7 g of potassium phosphate dibasic. Adjust final volume to 500 ml by adding sterile water.
    3. Prepare hexadecyltrimethylammonium bromide (CTAB) extraction buffer by dissolving 4.1 g sodium chloride in 80 ml water and slowly adding 10 g CTAB while heating and stirring. Adjust final volume to 100 ml by adding sterile water.
    4. Add equal volumes of phosphate buffer solution and CTAB buffer and filter sterilize with a 0.22 µm filter. Cover bottle with aluminum foil and store at RT.
    5. Prepare 1.6 M sodium chloride solution (NaCl) by combining 9.35 g NaCl and 100 ml sterile water. Add 10 g polyethylene glycol 8000 to the 1.6 M NaCl solution and filter sterilize. Store at 4 °C.

2. Nucleic acid extraction according to a modified version of Griffiths *et al.*[22] and Töwe *et al.*[23]
    1. Wear light duty suits, nitrile gloves, and masks. Clean laboratory space and pipets with 70% ethanol, DNA decontamination solution, and RNase decontamination solution.
    2. Remove subsample (filter from ice core or permafrost soil sample) in 2 ml ceramic bead screw-cap tube from -80 °C freezer and thaw at RT.
    3. Add 0.5 ml of hexadecyltrimethylammonium bromide (CTAB) extraction buffer and vortex briefly.
    4. Add 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8) and shake tubes horizontally for 10 min using a flat panel adapter on a vortexer.
    5. Following bead-beating, centrifuge tubes at 16,100 x g for 5 min at 4 °C.
    6. Remove aqueous layer to a new sterile 1.5 ml tube and mix with an equal volume of chloroform-isoamyl alcohol (24:1). Centrifuge at 16,100 x g for 5 min at 4 °C.
    7. Remove aqueous layer to a new sterile 1.5 ml tube and add two volumes of 30% polyethylene glycol 8000 and 1.6 M NaCl. Incubate for 2 hr at 4 °C.
    8. Centrifuge at 16,100 x g for 10 min at 4 °C.
    9. Wash nucleic acid pellet with approximately 500 µl of ice-cold 70% ethanol and centrifuge at 16,100 x g for 10 min at 4 °C.
    10. Air dry pellet in a sterile biohood for 2 hr. Resuspend in 50 µl DNase/RNase-free TE buffer (pH 8.0). DNA is ready for downstream applications such as PCR and qPCR.
    11. Determine concentration of DNA with a spectrophotometer.
    12. Dilute DNA with sterile, DNA-free water to achieve 20 ng per reaction.

Representative Results

The presented method could be used to decontaminate frozen samples collected from various cryosphere environments, from glacial ice to permafrost. Here, we present data specifically collected from ice and permafrost samples collected from the Engineering Research and Development Center - Cold Regions Research and Engineering Laboratory (ERDC-CRREL) Permafrost Tunnel located in Fox, AK (**Figure 1A** and **1B**). The Permafrost Tunnel extends approximately 110 m into the side of Goldstream valley and provides access to ice rich silt and alluvium[30-31]. Samples from an ice wedge and frozen soil were carefully collected in triplicate from the walls of the tunnel on 24 October 2014. At the time of sampling, the temperature of the permafrost wall was -2.9 °C. Samples were collected from an ice wedge feature approximately 27 m from the portal of the tunnel, and frozen soil at 35 m and 60 m from the portal of the tunnel (**Figure 1C** and **1D**). Special care was taken during sample collection to limit contamination of the ice and permafrost cores (**Figure 1E**). The frozen cores were handled according to our decontamination protocol (**Figure 2**) and transported on dry ice to the CRREL soil microbiology laboratory in Hanover, NH for processing.

All cores were processed using the protocol described in Section 2 using sterile microtome blades and solutions (**Figure 3A, B,** and **C**). The goal of this study was to successfully extract endogenous nucleic acids from the frozen samples to determine the microbial communities present at the time that the material was deposited. The decontamination protocol was assessed by doping the cores with a dilute culture of *Serratia marcescens* and then examining the Petri plates for growth after the cores were treated[31]. The absence of *S. marcescens* colonies indicated that the exogenous microorganisms were properly removed from the outer portion of the core. However, the absence of colonies would not indicate whether exogenous DNA was removed from the outer portion of the core. Therefore, we sequenced samples from the ice core to check for DNA from *Serratia sp*. Because microbial abundance in the ice wedge was likely significantly lower than in the permafrost, we used the ice cores to determine if DNA from *S. marcescens* would be present following the decontamination protocol. If the cores were not properly decontaminated, then sequences related to *S. marcescens* would be expected to be present in the samples. **Figure 4** shows the low bacterial diversity within the ice core from 16S rRNA gene sequencing results. Sequences related to *Pseudomonas* sp., of the phylum Gammaproteobacteria, dominated the ice samples. Members related to Planctomycetia were also present in the ice, but to a lesser extent. Of particular note is the absence of *Serratia sp.* in the sequencing results, suggesting that the decontamination protocol sufficiently removed exogenous DNA.

Furthermore, there is an additional risk of contamination during the extraction of nucleic acids. Negative extraction blanks were used to reveal contamination from exogenous nucleic acids. The DNA from the samples and negative controls were amplified using qPCR. qPCR data showed that the permafrost harbored bacteria, but archaea were not detected in the permafrost (**Table 1**). Successful decontamination was further evidenced by the lack of bacterial or archaeal amplicons in extraction blanks, in conjunction with positive amplification of the controls, *Pseudomonas fluorescens* and *Halobacterium salinarum* (**Table 1**). The abundance measurements revealed that there were no significant differences in bacterial abundance between the cores collected at 35 m from the portal as compared to the 60 m cores (**Table 1**). Ongoing research is being conducted to determine whether microbial community composition was different between the cores.

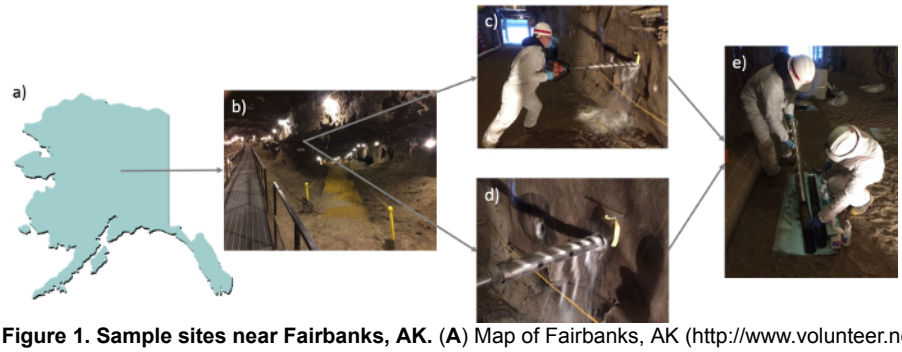

Figure 1. Sample sites near Fairbanks, AK. (**A**) Map of Fairbanks, AK (http://www.volunteer.noaa.gov/alaska.html), (**B**) ERDC-CRREL Permafrost Tunnel, (**C**) collection of permafrost cores using the SIPRE auger, (**D**) view of auger entering permafrost wall, and (**E**) preparing the core for archival. Please click here to view a larger version of this figure.

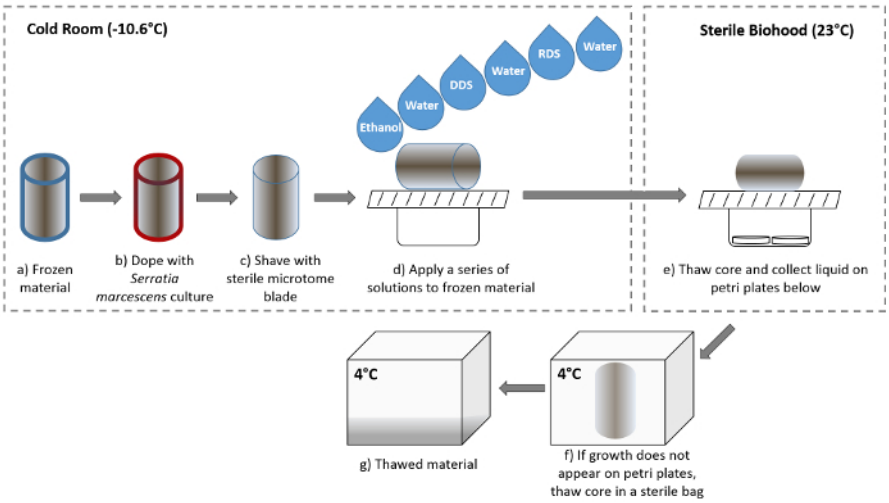

Figure 2. Schematic of decontaminating frozen material. (**A**) The frozen material (*e.g.,* ice core or permafrost core) is processed in a cold room. (**B**) It is purposefully contaminated with a bacterial culture. (**C**) The core is shaved with a sterile microtome blade to remove the outer 5 mm portion. (**D**) A series of solutions are applied to further decontaminate the outer portion of the sample. DDS indicates DNA decontamination solution and RDS indicates RNase decontamination solution. (**E**) By placing the core in a sterile biohood held at RT, the outer 2-5 mm of the material thaws. The liquid that drips from the ice or permafrost is collected in Petri plates and the core is swabbed and plated to check that the core was properly decontaminated. (**F** and **G**) If bacterial growth is not detected, then the core is thawed at 4 °C in a sterile container. Please click here to view a larger version of this figure.

Figure 3. Decontamination of permafrost cores in cold room under sterile conditions. (**A**) Remove the outer layer of the permafrost core with a metal microtome blade by scraping. (**B**) A closer view of a scrape. (**C**) Solution rinse to decontaminate the outer layer of the core. Please click here to view a larger version of this figure.

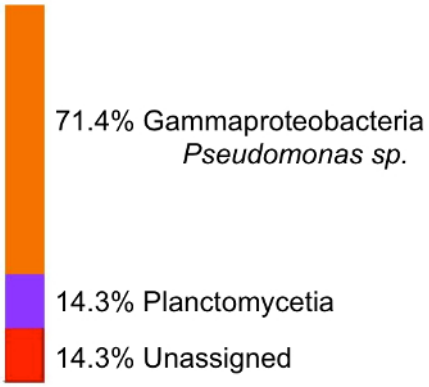

Figure 4. Microbial sequences from the ice wedge samples. Bar chart showing the average relative abundance of bacterial phyla present in the ice wedge samples by percentage. Please click here to view a larger version of this figure.

		Bacterial copy numbers * g^{-1} dry soil	
Sample	**n**	**Average * 10^6**	**Standard Error * 10^6**
35 m core	3	1.85	0.79
60 m core	3	3.58	0.75
Extraction Blank	1	ND	ND

Table 1. Bacterial abundance in permafrost. qPCR results showing the abundance of bacteria in the permafrost samples and associated extraction blanks. Values in table are reported as mean ± standard error. n is the number of samples. ND indicates not detected.

Discussion

The cryosphere offers access to preserved organisms that persisted under past environmental conditions. Though the recovered taxa may not represent the complete historic community, those recovered from analysis of glacial ice and permafrost samples can yield valuable historic information about select time periods[15-16]. For instance, meaningful biological information has been drawn from ice studies investigating anaerobic activity in the Greenland ice sheet[20] and permafrost studies investigating carbon cycling processes as a result of thaw[33] and have provided insight into fungal diversity from permafrost in Beringia[16]. Proper sample collection and decontamination must occur before conducting downstream analyses to investigate biological communities within these frozen materials. Even though these steps have significant implications on interpreting the data, many studies do not offer specific details on how the samples were collected and processed or images showing how to handle the frozen materials. For instance, previous studies have doped the outer portion of cores with fluorescent microspheres or known bacteria[15], though the exact concentrations of these materials were not described. Other studies have described decontamination protocols in detail, but have not applied high throughput analyses to test the efficacy of the methodology[15, 32]. Therefore, a detailed screening of intact bacteria and exogenous DNA through growth on Petri plates and qPCR was used to determine whether the sample collection, preservation, and analytical procedures were sterile enough to identify microbial communities present in the frozen materials.

This protocol has been modified to successfully isolate genetic material of interest from ancient cores. Care such as wearing specific gear and decontaminating supplies must be taken during the collection, processing, and extraction from the cores since contamination could occur at any step. Furthermore, shipping the cores with dry ice or a comparable cooling agent is imperative because if the cores thaw during transit, then there is a higher propensity that contaminants from the outer region of the core may migrate to the inner portion of the core. To further minimize the potential for contamination, consider collecting larger cores with a motorized auger rather than a push core. We found that the push cores were too small to properly decontaminate in the scraping step. Alternatively, a larger volume of sample could be extracted from the larger cores, which is especially important for samples with a low abundance of biological material. Additionally, the larger volume affords more room for error such that if *Serratia marcescens* colonies are detected on plates, then the core is large enough to be processed again.

Various supplies were tested to determine the most efficient method to decontaminate the cores. For instance, sterile aluminum foil, rather than glass dishes or plastic materials, allowed for the core to be placed on a sterile surface quickly and easily. Also, non-traditional supplies such as a metal rack and a tray proved to be beneficial to allow the scraped outer materials to accumulate away from the core, decreasing the risk of re-contaminating the cores. In earlier iterations of the protocol, the scraping occurred on a flat surface, which increased the risk of contamination. Finally, sterile bags, rather than glass dishes, were found to be conducive to mixing and storing the samples.

This protocol was targeted to eliminate microorganisms and nucleic acids on the outer portion of the frozen cores. While isopropanol and ethanol are effective disinfectants, they do not remove nucleic acids. Therefore, solutions that remove nucleic acids and associated enzymes from the outer portion of the cores were used. These solutions are commonly used to remove nucleic acids and associated nucleases from laboratory supplies and equipment. When testing their efficacy on laboratory surfaces, RNA decontamination solution removed more exogenous materials than the DNA decontamination solution[34]. Using nucleases to decontaminate the outer portion of the core might not always be the preferred method because genetic materials of interest may also be removed from samples with a low abundance of a particular organism. In particular, genetic material stored in permafrost and ice for extended periods of time undergo degradation. Because microorganisms are in a high abundance in these Alaskan samples, the concern that the solutions would remove a high proportion of endogenous genetic materials was

greatly reduced. However, caution should be taken when using solutions that contain nucleases to ensure that the nucleic acids that have been severely degraded or are from organisms in low abundance are not removed by the nucleases.

The absence of *S. marcescens* colonies on the Petri plates provided confidence that the intact cells were removed from the outer portion of the permafrost cores. Furthermore, analysis of ice cores that underwent the same protocol showed no detectable sequences related to *S. marcescens*. Sequence results revealed that only *Pseudomonas* were detected in these samples, even though both *Pseudomonas* sp. and *Serratia* sp. are within the class Gammaproteobacteria. Together, the absence of the *S. marcescens* colonies on Petri plates and the absence of DNA amplicons from qPCR suggest that the frozen cores were successfully decontaminated. Therefore, the microorganisms detected were likely embedded in the frozen material, rather than contamination from drilling or processing the cores. Other drilling techniques include hydraulic drilling to penetrate soil or ice at greater depths. Though this method would be beneficial to investigate low microbial abundance in these oligotrophic environments, it does not reveal whether drilling fluids would be successfully removed. Furthermore, if high throughput sequencing is not part of the downstream analysis, specific PCR reactions targeting *S. marcescens* should be used to amplify the extracted DNA.

The results from our example dataset showed that intact genomic DNA was successfully extracted from the frozen materials. Furthermore, both the permafrost and ice wedge harbored bacteria, as evidenced by qPCR and sequencing, respectively. The Alaskan discontinuous permafrost from this study contained similar bacterial numbers as permafrost in the Canadian high Arctic[35], though the Alaskan samples contained an order of magnitude fewer bacteria than permafrost from the Tibetan Plateau in Qinghai province, China[36] and active layer/permafrost soils from Nunavut, Canada[37]. Similar to an ice wedge collected in Nunavut, Canada[37], sequences related to Gammaproteobacteria, specifically *Pseudomonas*, which are common to soils and metabolically diverse, dominated the Alaskan ice wedge in this study. In fact, Katayama *et al.*[11] isolated bacteria within the phyla Actinobacteria, Bacilli, and Gammaproteobacteria from one of the ice wedges from the ERDC-CRREL Permafrost Tunnel. These studies corroborate the detection of Gammaproteobacteria in this study. Planctomycetia, which are common to aquatic sample, were also detected in the ice wedge. These organisms have been found in active layer soils above permafrost in northeastern Siberia[38], in addition to permafrost in the Tibetan Plateau in Qinghai, China[39].

Many studies have investigated the presence of archaea, particularly methanogens, in permafrost, with the notion that as permafrost thaws, methanogens will likely become more active, contributing to the efflux of methane to the atmosphere[21, 33-34]. Surprisingly, archaea were not detected in the Alaskan ice wedge or permafrost samples even though both Euryarchaeota and Crenarchaeota were found in permafrost from the Canadian high Arctic[17] and methanogens were found in permafrost samples from the Arctic tundra in Russia[21].

Frozen materials harbor ancient microorganisms, providing a record of biogeochemical processes that occurred many thousands of years ago. This biodiversity is of great interest under the current climate warming regime because microorganisms that were once restricted in the cryosphere environment may become liberated when the frozen materials thaw. In order to confidently identify the biodiversity harbored in these frozen materials, the frozen soils and ice samples must be properly handled and decontaminated. Here, we present a protocol to remove foreign cells and DNA from frozen samples to ensure that the microorganisms detected were from the material, rather than contamination from drilling or processing the cores.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was funded through the U.S. Army Engineer Research and Development Center, Basic Research Program Office. Permission for publishing this information has been granted by the Chief of Engineers.

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