Atmos. Chem. Phys. Discuss., 14, 32093–32131, 2014
www.atmos-chem-phys-discuss.net/14/32093/2014/
doi:10.5194/acpd-14-32093-2014

Atmospheric Chemistry and Physics Discussions

A next generation sequencing of Arctic bacteria in snow and frost flowers: identification, abundance and freezing nucleation

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Received: 5 November 2014 – Accepted: 6 November 2014 – Published: 19 December 2014

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Published by Copernicus Publications on behalf of the European Geosciences Union.

Abstract

During the spring of 2009, as part of the Ocean–Atmosphere–Sea Ice–Snowpack (OASIS) campaign in Barrow, Alaska, USA, we examined the identity, population, freezing nucleation ability of the microbial communities of five different snow types and frost flowers. In addition to the conventional culture-based PCR identification approach, we deployed a state-of-the-art genomic Next Generation Sequencing (NGS) technique to examine diverse bacterial communities in Arctic samples. 11–18 known phyla or candidate divisions were identified with the great majority of sequences (12.3–83.1 %) belonging to one of the five major phyla: *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Cyanobacteria*. At the genus level, 101–245 different genera were detected. The highest number of cultivable bacteria in cultured samples was observed in frost flowers (FF) and accumulated snow (AS) with 325 ± 35 and 314 ± 142 CFU mL^{-1}, respectively; and for cultivable fungi 5 ± 1 CFU mL^{-1} in windpack (WP) and blowing snow (BS). Complementary morphology and ice-nucleating abilities of the identified taxa were obtained using high resolution electron microscopy and ice nucleation cold-plate, respectively. Freezing point temperatures for bacterial isolate ranged from -20.3 ± 1.5 to -15.7 ± 5.6 °C, and for melted samples from 9.5 ± 1.0 to 18.4 ± 0.1 °C. An isolate belonging to the Bacillus species (96 % similarity) had ice nucleation activity of -6.8 ± 0.2 °C. Comparison with Montreal urban snow, revealed a seemingly diverse community of bacteria exists in the Arctic with many originating from distinct ecological environments, and we discuss the potential impact of microbial snow in the freezing and melting process of the snowpack in the Arctic.

1 Introduction

The snow pack has been shown to act as an important matrix for (photo) chemical and biological reactions of organic compounds (Ariya et al., 2011). Snow and ice provide large surface areas which consist of interstitial air, water and ice that may exchange

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chemical and biological matter with the atmospheric boundary layer. Previous reports described snow pack exchange and transformation processes that included trace gas exchange, scavenging, photolysis and adsorption (Kos et al., 2014), and more recently, biological transformations have been considered. Yet, the role of bio-organic molecules, including micro-organisms, in oxidation, freezing nucleation, gas-particle transfer and aerosol formation remains poorly understood,

Climate change has been linked to changes in snow and ice patterns in the Arctic, potentially impacting the Earth's albedo and atmospheric energy balance (Grenfell and Maykut, 1977; Grenfell and Perovich, 1984, 2004; Hanesiak, 2001). Atmospheric transport events such as dust storms initiated long distances away have been considered to influence the Arctic climate. Saharan dust, for instance, has been reported as a source of certain biological particles to reach the Arctic region (Barkan and Alpert, 2010). In 1976, an Asian dust storm was responsible for bringing as much as 4000 t of dust h^{-1} to the Arctic (Rahn et al., 1977). Dust has also been shown to transport microbial (Zhang et al., 2007, 2008a, b). Bacteria and fungi have been detected in Asian dust (Choi et al., 1977; Yeo and Kim, 2002; Wu et al., 2004; Ho et al., 2005) and in African desert winds (Griffin et al., 2001, 2003, 2006, 2007; Kellogg et al., 2004; Prospero et al., 2005) whereby some have been found to be viable and survived (Griffin et al., 2001; Prospero et al., 2005). Recently, the increase in the number of storms has been associated with the efficient long-range transport of dust, microbial and other chemicals to the Arctic regions (Clarke et al., 2001; Grousset et al., 2003; Uno et al., 2009). During long distance transportation, air masses may undergo chemical and physical transformation under extreme environmental conditions such as high levels of solar radiation, multiple freeze–thaw cycles, relatively acidic conditions, and predominantly inorganic salts (Jickells, 1999; Ariya et al., 2002, 2009; Cote et al., 2008). Little is known on the effects of the photochemical and aging processes of the chemical and biological composition of dust particles, or whether chemical properties and the genomic structure of microbial entities transported with dust, are altered, or mutated during long distance transport.

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Pure water droplets homogeneously freeze in the atmosphere at approximately −38 °C. Entities such as particles including mineral dust, soot, and biological materials (DeMott et al., 2003; Möhler et al., 2007) may serve as ice nuclei (IN) which enhance freezing at much higher temperatures in a process known as heterogeneous nucleation (Pruppacher and Klett, 1997). Depending on the nature of the impurities, heterogeneous nucleation can occur over a wide range in temperatures. Although, dust particles are generally assumed to be the most important global effect on ice nucleation, several strains of *Erwinia herbicola* (Lindow, 1978), *Pseudomonas fluorescens* (Maki and Willoughby, 1978), *Pseudomonas viridflava* (Paulin, 1978), and *Xant/zomonas campestris pathovar translucens* (Lim et al., 1978) are recognized amongst the most efficient IN in biological particles. Yet the global effect of the importance of biological ice nuclei is still a subject of debate. Some strains of *Pseudomonas syringae* can initiate water freezing at temperatures as high as −2 °C (Orser et al., 1985) which have also been detected in clouds and snow (Amato et al., 2005, 2007; Vaïtilingom et al., 2012; Lohmann and Feichter, 2005; Joly et al., 2013). Atmospheric microbial, besides being considered as an efficient ice nuclei (Constantinidou, 1990; Kieft and Ahmadjian, 1989; Möhler et al., 2007, 2008; Pouleur, 1992; Ariya et al., 2014; Mortazavi et al., 2008) have also been suggested to act as cloud condensation nuclei (Bauer et al., 2003; Möhler et al., 2007).

Both natural and anthropogenic sources can contribute to precipitation in Arctic regions (Hansson et al., 1993; Hinkley, 1994). There is some evidence for the observed increase in the number of storms in certain areas of the globe which can alter the transport and distribution of chemicals or biological entities (Wang et al., 2011; Zhang et al., 2007; Erel et al., 2006) with potential impacts on precipitation patterns (Sempere and Kawamura, 1994; Satsumabayashi et al., 2001). Although the pivotal role of dust in the atmospheric global circulation (Dunion and Velden, 2004; Wu, 2007), radiative budget (Sokolik and Toon, 1996; Kaufman et al., 2001), air pollution (Prospero, 1999; VanCuren, 2003) and cloud formation (Toon, 2003) has been documented, there is little known about how the newly introduced pool of transported microbial entities by dust to

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the Arctic impacts the change of the total Arctic microbial pool or affects the freezing and melting processes of snow and ice matrices in this region.

Several studies using standard microbiology techniques have shown that there is a diverse population of bacteria in the snow. Recent developments in high-throughput sequencing (HTS) techniques (Loman et al., 2012a, b), such as Next Generation Sequencing (NGS), also allow for metagenomic investigations of microbial populations in environmental samples. The present study was performed as part of the international Ocean Atmosphere Sea Ice Snowpack (OASIS) campaign (2009) in Barrow, Alaska. The aim of the study was to evaluate in five different snow types and frost flowers in the Arctic the: (i) identification and quantification of the number density of viable bacterial and fungal colonies, (ii) determination of the ice nucleation (IN) property of: (a) selected isolated bacteria and (b) melted samples, and (iii) identification of the total bacterial pool using Next Generation Sequencing. We herein provide further information on the biological composition of Arctic snow and frost flowers at genomic level, shed light on the potential influence of atmospheric transport on the change of microbial diversity, and discuss their potential roles in the freezing-melting processes of ice-snow in the Arctic.

2 Experimental methods

2.1 Study sites

Five different types of Arctic snow were studied: (i) accumulated snow, (ii) windpack, (iii) blowing snow, (iv) snow hoar, (v) fresh snow and frost flowers which were collected from 4 to 20 March 2009 during the OASIS campaign in Barrow, AK, USA. Detailed snow sampling procedures has been described elsewhere (Kos et al., 2014). Snow samples were collected from a field dedicated to snow research in the clean air sector at 71.31° N, 156.6° W, 400 m to the southeast of the Barrow Arctic Research Center (BARC) and frost flower samples were collected from sea ice at 71.36° N, 156.70° W.

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Vehicle access was restricted to the snow sampling area, and equipment was transported on foot with a hand pulled sled to limit local pollution. Snow sampling devices were sterile and single-use. A sterile HDPE spoon (Fisher) was used to collect the first 3 cm of the surface snow to fill the sterile high-density polyethylene (HDPE, Fisher) sample containers (220 mL). Similarly, frost flower samples were collected by carefully lifting the frost flower off the surface with a shovel. Snow temperature was measured using a long-stemmed thermometer (Fisher) and the meteorological conditions were recorded (air temperature, wind direction, and cloud cover). Samples were kept frozen (−20 °C) until shipped out by airfreight (transit time 41 h) in commercial coolers (Coleman). The maximum temperature upon arrival in the laboratory at McGill University in Montreal was −5 °C. Arctic samples were stored in a freezer at −20 °C (Viking) until analysis. Surface snow samples from heavy snowfall regions in the province of Quebec, Canada: Mont-Tremblant, the city of Montreal (urban snow) and its suburb Pierrefonds were also collected using similar techniques.

2.2 Isolation of viable microorganisms and Drop-freezing assays

Snow and frost flower samples were melted directly by transferring from freezer to refrigerator at 4 °C, under sterile conditions and these conditions were maintained at all times using sterile instruments and materials or certified sterile single-use supplies. Once melted, they were kept on ice for the IN experiment, or transferred to the laminar flow hood to culture the microorganisms. To grow the microorganisms, one millilitre of Arctic samples (snow and frost flower) were placed in standard 100 mm × 15 mm sterile plastic Petri dishes (Fisher). The media used for bacteria were tryptic soy agar (TSA), and R2A agar, a low nutrient medium used to improve the recovery of stressed bacteria. For fungi, mycological agar at neutral pH, and sabouraud dextrose agar (SDA) at a low pH of approximately 5.6 (all media by Becton, Dickson and Co) were used. Plates were incubated at 4 °C and were regularly checked for growth and the colonies were counted.

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Drop-freezing assays were done on: (i) viable isolated bacteria obtained from Arctic samples, and (ii) melted Arctic samples. Viable bacteria isolated from Arctic samples grown on Petri dishes were mixed with sterile ultrapure water (Millipore). The optical density of 1 at 600 nm was used to adjust the concentrations of bacteria to 10^8 cells mL^{-1}. IN experiments were performed using a homemade copper cooling plate (cooling rate of 1 °C min^{-1}), a technique first described by Vali (1971). The copper plate was coated evenly with commercial Vaseline™ petroleum jelly. The samples were kept on ice and were loaded as 10 µL droplets. Tap water was also used as a control that showed IN activity between positive (*Pseudomonas syringae*) and negative controls (ultrapure water). The temperature of each frozen droplet was recorded. IN temperatures are a simple average of the temperatures at which a sample group of drops freezes, i.e., the sum of the freezing temperature of each drop in the ensemble divided by the total number of drops.

2.3 Bacterial DNA isolation, amplification of 16S rDNA, sequencing and identification

Ready-Lyse Lysozyme was used to lyse the cell, and DNA was extracted and purified using either a DNAeasy kit (Qiagen) or a Master Pure DNA purification kit (Epicentre Technologies) according to the manufacturer's instructions. Extracted DNA was amplified by PCR in a final volume of 25 µL using 16S universal primers (Forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-ACGGCTACCTTGTTACGACTT-3′, Integrated DNA Technologies). An Eppendorf™ tube containing all the ingredients but DNA was used as control (blank) for every set of PCR. PCR included 35 cycles of denaturing at 94 °C for 1 min., annealing at 55 °C for 1 min., and extending at 72 °C for 2 min., followed by a 7 min. final extension at 72 °C. The PCR product was separated and analyzed in 1.2 % agarose gel electrophoresis stained with ethidium bromide.

The PCR products of 16S rDNA genes obtained from the cultured bacterial colonies were sequenced at McGill University and at the Génome Québec Innovation Centre,

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Montreal, Canada. The 16S rDNA sequences were aligned and compared with those available in the GenBank databases using the BLASTN (Basic Local Alignment Search Tool) through the NCBI (National Center for Biotechnology Information server) to identify sequences that share regions of homology with isolated sequences.

2.4 454 Pyrosequencing, and Electron microscopy analysis

The DNA from snow samples was extracted. A barcoded 16S rDNA tag was used to amplify three distinct regions (V1–3) of the bacterial 16S rDNA gene (~ 500 bp). The forward primer consisted of 454 Life Science adaptor A, a unique 10 base barcode rapid library MID, and the specific forward primer sequence: 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTATTACCGCGGCTG CTGG-3′ and the reverse primer consisted of 454 Life Science adaptor B fused to the specific reverse primer sequence: 5′-CCTATCCCCTGTGTGCCTTGGCAGT CTCAGAGAGTTTGATCCTGGCTCAG-3′. Amplification was done in triplicate and performed in a 20 µL reaction volume containing 13.85 µL of RNase and DNase free water, 2 µL of DNA template, 2 µL of 10x AccuPrime PCR buffer (Invitrogen), 1 µL of 2 µM of each primer, and 0.15 µL of AccuPrime Taq DNA polymerase Hifi (Invitrogen). Cycling conditions were performed at 95 °C for 2 min, followed by 30 cycles at 95 °C for 20 s, 56 °C (V1–3 primer set), 72 °C for 5 min and 4 °C forever. PCR products were purified with AMPure XP beads (Agencourt), and eluted in 20 µL of ultrapure water. The quality and size of the amplicons were assessed on a 2100 Bioanalyzer using a DNA 1000 kit (Agilent Technologies) and quantified with the PicoGreen assay (Invitrogen). The amplicons library was pooled in equimolar amounts. The pool was sequenced unidirectionally from adaptor A with the Genome Sequencer FLX Titanium (454/Roche) at McGill University and at the Génome Québec Innovation Centre, Montreal, Canada. The generated sequences from pyrosequencing was analyzed with the software MOTHUR for pre-processing (quality-adjustment, barcode split), identification of operational taxonomic units (OTUs), taxonomic assignment,

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community comparison, and statistical analysis (Schloss et al., 2009). Sequences were filtered to minimize the effects of poor sequence quality and sequencing errors.

Analysis Transmission electron microscopy (TEM) in conjunction with EDS analyses were used on Arctic snow samples and frost flowers to detect microbial and chemical compounds. Samples were freeze-dried. A sample solution of 7 µL was put onto a 200 mesh carbon-coated copper grid for 1 min. It was negative stained with 2 % uranyle acetate for 30 s. Imaging was done under TEM (Hitachi H7500 operated at 100 keV and spot size 5), a Philips CM200 200 kV TEM equipped with Gatan Ultrascan 1000 2 k × 2 k CCD Camera System (Model 895) and EDAX Genesis EDS Analysis System.

3 Results and discussion

Recent observations have indicated that snowpack is indeed a complex microhabitat that permits the growth of diverse microorganisms allowing for chemical and photobiological reactions to occur (Amoroso et al., 2009). A clear understanding of the bacterial population and their interactions will further reveal the role these play in altering the Arctic environment and its climate. In this study, the Next Generation Sequencing (NGS) technique in conjunction with a classical cultural method were used to identify and compare the bacterial community in different types of Arctic snow and frost flowers. Moreover, the Arctic microbial population was compared to urban snow from the cold North American City of Montreal. NGS analysis was performed using 1/8 of sequencing plate of GS FLX Titanium (454/Roche) for reading. Individual sequences were based on the V1–V3 primers. Using GS FLX Titanium (450/Roche), a total of 88 937 reads was made for all the samples with the average number of reads being 17 787. The lowest and highest number of reads was obtained for windpack, WP, (13 831) and urban snow, US, (24 968) respectively. The total number of bases was 32 284 312. The lowest and highest number of bases belonged to blowing snow, BS, (4 164 307), and urban snow, US, (7 966 589) respectively. The average raw read length varied among the samples and was obtained as: 542 ± 23 bases (urban snow, US), 422 ± 21 bases

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(blowing snow, BS), 542 ± 23 bases (snow hoar, SH), 533 ± 23 bases (windpack, WP), and 556 ± 24 bases (frost flower, FF). After trimming and passing through quality control, the final read length was recovered as: 319 ± 18 bases (urban snow, US), 299 ± 17 bases (blowing snow, BS), 401 ± 20 bases (snow hoar, SH), 385 ± 20 bases (windpack, WP), and 419 ± 20 bases (frost flower, FF) (Table A1).

The diversity of the bacterial communities for four different snow types and frost flowers was estimated using the Simpson Diversity Index, Rarefaction Metric, and species richness using the nonparametric Chao index (Chao, 1984). The Simpson Diversity Index which takes into account both species' richness, and an evenness of abundance among the species present reached a plateau after the sequencing of sampling of about 5000 for BS, 6000 for WP, 7000 for US, 8000 for SH, and 10 000 for FF (Fig. A1a). The Chao index gave values between 1500 and 7500 with BS exhibiting the lowest richness (Fig. A1b). The richness in total bacterial communities of Arctic samples was estimated by rarefaction analysis. The shapes of the rarefaction curves did not reach asymptote indicating that bacterial richness for most samples especially for urban snow, and windpack is not yet complete (Fig. A1c). Using the 3 % cut-off value in sequence differences for OTU, the estimates of the richness of total bacterial communities ranged from 1033 in BS, 1971 in WP, 1956 in SH, 1933 in US and 1605 in FF (Fig. A1c). Based on these analyses, the order of the highest diversity of bacteria to the lowest was observed in: windpack, snow hoar, urban snow, frost flowers, and blowing snow.

In the second-generation sequencing part of this study, we focused only on pyrosequencing for bacteria, which was feasible under our existing facilities; thus, the other biological species were not explored. Yet, analysis using high resolution electron microscopy (Fig. 1) revealed the appearance of the existence of several biological materials, remnants of biological activities, and not only biological entities in their entirety.

The individual sequences represented known phyla or candidate divisions as: 11 (urban snow), Arctic samples: 18 (WP), 16 (SH), 15 (BS), and 18 (FF) (see also the Table A2a). The majority of sequences (12.3–83.1 %) belonged to one of the five ma-

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jor phyla: *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Cyanobacteria*. The major phyla for urban snow and Arctic samples were as follows: (i) urban snow: *Proteobacteria* (49.04 %), *Bacteriodetes* (47.5 %), (ii) windpack: *Proteobacteria* (66.1 %), *Cyanobacteria* (12.3 %), (iii) snow hoar: *Proteobacteria* (67 %), *Firmicutes* (13.6 %), (iv) blowing snow: *Proteobacteria* (83.1 %), *Firmicutes* (6 %), *Actinobacteria* (5.09 %) and (v) frost flowers: *Proteobacteria* (50.2 %), and *Actinobacteria* (32.8 %) (Table A2a). *Proteobacteria* was the most widely expressed phylum among all the Arctic samples tested with the most abundance observed in blowing snow.

At the genus level, sequences represented 134 different genera for urban snow; Arctic samples: 245 for windpack, 139 for snow hoar, 101 for blowing snow, and 158 for frost flowers. The distribution of bacterial genus observed at greater than 1 % and the number of occurrence for each percentage observed for any genus in total bacteria is shown in Fig. 2. The top four genera with the highest percentage detected for each sample were as follows: urban snow, US: *Flavobacterium* (40 %), *Polaromonas* (11.2 %), *Variovorax* (6.6 %) and *Sandarakinorhabdus* (6 %); windpack, WP: *Methylobacterium* (9 %), *Sphingomonas* (4.9 %), *Lamprocystis* (4.5 %), and *Roseateles* (4.4 %); snow hoar, SH: *Roseateles* (18.6 %), *Methylobacterium* (14.1 %), *Bacillus* (5.7 %), and *Streptococcus* (5.7 %); blowing snow, BS: *Methylobacterium* (15.3 %), *Bradyrhizobium* (1.6 %), *Bacillus* (1 %), *Sphingomonas* (1 %); and for frost flowers, FF: *Propionibacterineae* (32.9 %), *Roseateles* (8 %), *Staphylococcus* (6.7 %), and *Candidates Pelagibacter* (6.1 %) (Table A2b).

NGS analysis clearly showed variation of distinct sets of microbial among different Arctic samples and urban snow. This observation suggests the importance of specific physical and chemical characteristics of each snow type that may serve as a predictor of microbial abundance and composition; specifically favoring growth conditions for microbial communities that originated from diverse sources.

Table 1 shows the analysis of NGS results encompassing bacteria at genus level that have been previously detected: (i) in Asian or African dust storms, (ii) with antifreeze and/or ice nucleation properties, and (iii) in cold oceanic water. Note that the

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existence of these bacteria does not ensure the expression of their property, and thus the existence of ice nucleating and freezing bacteria does not reflect their expression in environmental matrices. The percentage of bacteria (at genus level) that were previously observed in Asian or African dust samples were in the range of 36–47 % for all the snow categories and frost flower samples. Only a very small percentage of identified bacteria with previously demonstrated antifreeze property (Mills, 1999; Yamashita et al., 2002), were detected in: windpack: 1 %, surface snow hoar: 0.2 %, and frost flowers: 1 %. Urban Montreal snow samples had the highest number (7 %). 14–16 % of samples contained bacteria with ice nucleation properties (Table 1a). A very small percentage of identified bacteria showed both ice nucleation and antifreeze properties: the highest percentage was observed in urban snow samples (0.1 %) and the lowest was observed in blowing snow (0.03 %). Only 0.01 % of bacteria in windpack snow, surface hoar snow and frost flowers had both ice nucleation and antifreeze properties at different environmental conditions (Kawahara et al., 2004; Lorv et al., 2014). Similarly, Arctic samples showed a minute number of bacteria that have been previously detected in cold oceanic water (0.01–0.04 %); none were detected in urban snow samples, under the experimental conditions in this work.

Some of the bacteria in Arctic samples have previously been identified in Asian or African storms with ice nucleation (Kellogg et al., 2004; Griffin, 2007) or antifreeze properties (Smith et al., 2013). Within these bacterial genera pool, 2–3 % of Arctic snow samples and urban snow showed ice nucleation properties with only 0.2 % in frost flowers (Table 1b). Bacteria with antifreeze properties were observed for only 0.4 % in frost flowers and 1 % in both blowing snow and surface hoar snow. Higher numbers of such bacteria were observed for windpack (6 %). Interestingly, 13 % of bacteria originating from dust storms in urban snow had antifreeze properties. The possible introduction of antifreeze bacteria from the ocean into the air by different mechanisms such as the bursting of frost flowers by wind and fresh snowfall may further provide and facilitate infiltration into the snowpack (Rankin et al., 2002). The detection of a high number of bacteria with a vast genetic diversity pool, using NGS analysis, further illustrates that

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the snowpack is a heterogeneous soup of microbial entities. The chemical environment of the snowpack is constantly evolving by novel streams of chemicals through fresh precipitations, wind transportation and metabolic activity of microbial. With the increased incidence of dust storms, likely due to climate change, the detection of specific bacteria from hot areas into the Arctic environment may suggest a shift in the balance of "native bacterial populations" in the Arctic. One may speculate that it might be conceivable to consider interactions among the heterogeneous population of microbial in Arctic samples, including non-native taxa adaptation in the Arctic snow-ice genome. Though the Arctic does not provide a native habitat for non-native bacteria or biological species originating from elsewhere in the world, their entrance into the Arctic may affect certain bio-chemical reactions, or alter the nutrient pool for the other native microbial entities. In turn, it might affect the ratio and the survival rate of certain populations of microbial with freezing or anti-freezing properties, impacting the melting of ice or snowpack in the Arctic region.

Viable bacteria and fungi were quantified by incubating Arctic samples in a medium at cold temperatures (4 °C). Only a small fraction of a microbial community, especially from extreme environments such as the Arctic can be grown under laboratory conditions since many factors such as the composition of the medium that fully supports the basic needs of microorganisms for growth is not known. This notion was further confirmed as cultivable bacteria encompassing 0.1 to 3 % of the total bacteria, which was detected by NGS technique. Thus, the identified number of cultivated bacteria and fungi isolate from different snow categories and frost flowers does not reflect the actual number of microbial and should be considered as the lower limit, and therefore more metagenomic analysis such as NGS, is essential to decipher the complex pool of microorganisms in the Arctic. The cultivable bacteria might be representative of the active fraction of cultivable bacterial snow communities (Ellis et al., 2003; Frette et al., 2004), as was detected for bacteria living in different environmental samples such as soil, and marine samples (Pinhassi et al., 1997; Rehnstam et al., 1993).

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Figure 3a (bacteria) and b (fungi) show the variability in numbers of colony-forming units (CFU) within and between the different sample types using two different media (R2A and TSA) for bacteria and (SDA and mycological) for fungi. The average number of viable bacteria was higher than the number of fungi in Arctic samples. Overall, a higher number of CFU was observed in the R2A medium with a more limited nutrient content than TSA, wherein most aerobic bacteria are able to grow. In the R2A plates, the highest number of cultivable bacteria was observed in frost flowers (FF) and accumulated snow (AS) with 325 ± 35 and 314 ± 142 CFU mL^{-1}, respectively (Fig. 3a). However, the highest number of cultivable fungi grown in the mycological plate was observed in windpack (WP) and blowing snow (BS) with 5 ± 1 CFU mL^{-1} (Fig. 3b).

Both NGS and cultural method analysis showed a very high number of bacteria in frost flowers as compared to the other snow types that we tested. In recent years, special attention has been focused on the role of frost flowers as a contributing factor to changing the chemistry of the atmosphere in the Arctic. These are: (i) an important source of sea-salt aerosol (Rankin et al., 2002; Perovich and Richter-Menge, 1994; Martin et al., 1995), (ii) a contributing factor in releasing the ozone-depleting molecule, bromine monoxide (BrO), as was detected by satellite (Kaleschke et al., 2004), and (iii) as a source of sea ice bacteria (Collins et al., 2010).

Moreover, with their physical structure and chemical composition, frost flowers might provide a habitat for microbiological bodies such as bacteria, as well as protective and favorable conditions for metabolic and photochemical reactions (Bowman and Deming, 2010). The observed simple organic compounds and increased concentrations of both formaldehyde (Barret et al., 2009), hydrogen peroxide (Beine and Anastasio, 2009) within frost flower, may suggest that bacteria could act as a substrate for the photolytic production of oxidants (Bowman and Deming, 2010), and simple organic compounds (Ariya et al., 2002). The regular release mechanism of bacteria through frost flower, such as those with high ice nucleation activity, into the atmosphere, with potential transportation, may provide an additional impact on bioaerosol lower tropospheric mixing ratios (Jayaweera and Flanagan, 1982).

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As opposed to frost flowers, accumulated snow is characterized by several layers of snowfall which may have experienced repeated freeze–thaw cycles, and solar irradiation exposure. Analysis by cultural method showed that the highest number of bacteria is present in accumulated snow samples. Each fresh snowfall adds new nutrients and microorganisms to the old pool of accumulated snow. With the detection of more than 100 organic species in the aerosols at Alert in the Canadian high Arctic (February–June) (Fu et al., 2008), the snow layers could be further enriched with nutrients by the air/snow exchange (Xie et al., 2007; Cincinelli et al., 2005). Over time, bacterial populations in accumulated snow may increase by their sustainability and slow growth at very low temperature (−2 to −35 °C) (Junge et al., 2004; Gilichinsky et al., 1995; Panikov and Sizova, 2007; Bakermans et al., 2003).

Different types of Arctic snow and frost flower samples were tested for IN activities using obtained cultured bacterial colonies as well as whole melted Arctic samples. Ultrapure Milli-Q water (18 Ω resistance), tap water, and *P. syringae* mixed in ultrapure water were used as controls. Tap water contains organic impurities that allow ice to nucleate at warmer temperatures than ultrapure water. The individual average freezing temperature of bacterial isolates from different types of Arctic samples is shown in Fig. 4a. To explore the impact of undetected biological and chemical contents of snow samples on IN activity, the IN activity of each melted Arctic sample was directly measured and its IN activity was compared with the corresponding freezing temperature of the average sum of the total individual isolated bacterial colonies (Fig. 4b). The freezing temperatures of the average sum of the total individual (ASTI) isolated bacterial colonies fall at intermediate values between sterile ultrapure water (−24.3 ± 1.2 °C) and tap water (−15.3 ± 0.9 °C). The highest and lowest ice nucleation activity of bacteria were observed in fresh snow (ASTI: −15.7 ± 5.6 °C) and frost flowers (ASTI: −20.3 ± 1.5 °C) respectively. The ice nucleation activity of fresh snow was comparable to tap water (−15.3 ± 0.9 °C) (Fig. 4b).

Many of the bacterial isolates in different categories of Arctic samples showed a moderate IN activity at −15.9 ± 0.4 and −17.2 ± 0.8 °C in windpack (WP), −15.2 ± 1 and

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−16.1 ± 1.4 °C in blowing snow (BS), −15.2 ± 0.6, −12.9 ± 0.2, and −17.3 ± 0.3 °C in accumulated snow (AS), and −14 ± 0.4, −13.7 ± 0.2, and −6.8 ± 0.2 °C in fresh snow (FS) (Fig. 4a). Interestingly, bacteria with a type 2 ice nucleation ability at −6.8 ± 0.2 °C was isolated in fresh snow. This bacteria was identified with 96 % similarity to the Bacillus species (Table 2).

Among tested bacterial colonies, fresh snow showed the highest variation in ice nucleation activities; higher variation was observed for accumulated snow as compared to frost flowers. Different factors such as nutrient limitation and low temperature observed in the Arctic might have further shifted the ice nucleation activity of bacteria to the higher temperature (Nemecek-Marshall et al., 1993). Frost flower, a bridge between sea ice and the atmosphere and linking bio-abiogenic materials, had the lowest average ice nucleation activity. This might be related to its salinity and ability to accumulate different chemicals.

The observed range of IN activity in melted snow and frost flower samples was between −9.5 ± 1. °C (FS) and −18.4 ± 0.1 °C (FF) (Fig. 4.B). Interestingly, all the melted Arctic snow samples and frost flowers showed ice nucleation activity at the range very close to the lowest recorded ice nucleation activity of individual cultivable bacterial colonies. This observation may indicate an additional role of other uncultivable microbial and components in Arctic snow samples and frost flowers which are important in increasing the ice nucleation temperature. Culture-dependent methods selectively isolate a plate-growth-adapted subpopulation from the microbial communities which may represent the majority of the total bacterial numbers in samples (Pinhassi et al., 1997; Rehnstam et al., 1993), but not necessarily the total richness of the bacterial population (Amann et al., 1995; Onstott et al., 1998).

Table 3 contains the identified cultivable bacteria found in each category of Arctic samples (snow and frost flowers). Isolated bacteria were identified belonging to different genus, such as: *Afipia Genosp*, *Bacillus*, *Paenibacillus*, *Microbacterium*, and *Kocuria*. The highest IN activity corresponded to genus: *Bacillus* and *Paenibacillus* with −6.8 ± 0.2 °C and −15.2 ± 1 °C respectively (Table 2). At the 16S rDNA gene level,

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they exhibited about 96 and 95 % similarity to reported species of *Bacillus* sp., *Bacillus megaterium*, *Bacillus flexus*, and *Bacillus aryabhattai*; and *Paenibacillus amylolyticus*, *Paenibacillus xylanexedens*, and *Paenibacillus tylopili* respectively. Interestingly, Geobacter species, shown to oxidize radioactive materials such as uranium and precipitate uranium into the environment, were found in the windpack (Table 2).

It is noteworthy that although we focused on bio-organic materials, as we can see from HR-TEM/EDS analysis, there are inorganic matter in snow as well, which can contribute to ice nucleation. Hence, to evaluate the snow freezing properties, the complex chemical and bio-chemical pool of molecules and particles should be considered.

4 Conclusions

We herein examined the identity, population and ice nucleation ability of the microbial communities of five different snow types and frost flowers during the spring 2009 campaign of the Ocean–Atmosphere–Sea Ice–Snowpack (OASIS) program in Barrow, Alaska, USA. We used the Next Generation Sequencing (NGS) technique to examine the true bacterial communities in snow and frost flowers, in addition to conventional culture techniques. We observed much more insight into the wide range of taxa available in different types of snow and frost flowers. Arctic samples and reference urban snow represented 11–18 known phyla or candidate divisions. The great majority of sequences (12.3–83.1 %) belonged to one of the five major phyla: *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Cyanobacteria*. At the genus level, 101–245 different genera were detected. A largely diverse community of bacteria exists in the Arctic with many originating from remote ecological environments such as dust storms. Biological materials are shown to act as reactive sites for (photo-chemical) reactions and thus further studies are recommended to decipher further the complexity of the snow chemical pool due to the existence of such reactions. It is conceivable that the changes to the ratio of antifreeze bacteria on ice nucleation bacteria may have an impact on the melting and freezing processes of snowpack or frost flowers. It is

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thus conceivable that this shift in bacterial population could ultimately affect the snow melting-freezing processes. Further studies are required to evaluate whether change of nucleation patterns due to biological entities are indeed linked to climate change.

Acknowledgements. We thank Gregor Kos for providing us with snow and frost flowers samples from Barrow, AK. We are also grateful to Paul Shepson of Purdue University, Jan Bottenheim and Sandy Steffen from Environment Canada for logistical support, Florent Dominé and Didier Voisin from the Laboratoire Glaciologie et Géophysique Environnement and Harry Beine from UC Davies for their cooperation during sampling. We thank O. Cavaliere for proofreading the manuscipt. Funding from NSERC, CFI and FRQNT is kindly acknowledged.

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Table 1. Relative abundance of origin and physical properties of analyzed NGS bacteria. Bacteria in Arctic samples: blowing snow (BS), surface hoar snow (SH), windpack snow (WP), frost flowers (FF), and urban snow (US) were analyzed for their origin, and ice nucleation/melting properties as detected by Roche 454 GS-FLX Titanium; **(A)** total bacteria in Arctic samples: (i) previously observed in Asian/African (dust); (ii) cold oceanic water, (iii) with antifreeze (AF), (iv) ice nucleation (IN), and (v) IN and AF property; **(B)** in subtotal of total Arctic bacterial pool originated from Asian/African dust: (i) with AF, or (ii) IN property.

(A) Total bacteria in Arctic samples					
Snow Categories	Dust	Cold Oceanic Water	AF	IN	IN & AF
US	47 %	0 %	7 %	15 %	0.1 %
BS	36 %	0.01 %	0.10 %	15 %	0.03 %
SH	44 %	0.04 %	0.20 %	16 %	0.01 %
WP	39 %	0.01 %	1 %	16 %	0.01 %
FF	44 %	0.01 %	0.10 %	14 %	0.01 %

(B) Subtotal of Arctic bacteria in Asian/African dust origin		
Snow Categories	AF	IN
US	13 %	2 %
BS	1 %	3 %
SH	1 %	3 %
WP	6 %	2 %
FF	0.40 %	0.2 %

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Table 2. Ice nucleation of identified viable cultivable bacteria in Arctic sample. Ice nucleation temperature of identified bacterial isolates in each snow categories: blowing snow (BS), fresh snow (FS), windpack (WP), and frost flowers (FF); accession number, a unique identifier given to a DNA sequence; identified species; and % similarity, the ratio of identical query bases to known bases in the database.

Snow Category	Bacterial Colony	Ice Nucleation Temp. (°C)	Accession #	Species	% Similarity
WP	A	−18.9 ± 2.5	U87778.1	*Afipia genosp*	86
			JF799916.1	*Bradyrhizobium*	87
			FR691406.1	*Bosea*	87
BS	A	−18.9 ± 1.6	JN082256.1	*Bacillus* sp.	97
			JN092792.1	*Bacillus flexus*	97
			HQ143640.1	*Geobacillus stearothermophilus*	97
	B	−15.2 ± 1	JF343205.1	*Paenibacillus amylolyticus*	95
			NR_044524.1	*Paenibacillus xylanexedens*	95
			HQ202814.1	*Paenibacillus tylopili*	95
FS	A	−6.8 ± 0.2	JN208198.1	*Bacillus* sp.	96
			AB648987.1	*Bacillus megaterium*	96
			JN092792.1	*Bacillus flexus*	96
			HQ857752.1	*Bacillus aryabhattai*	96
	B	−21.6 ± 1	JN085952.1	*Microbacterium* sp.	98
			JF700471.1	*Microbacterium hydrocarbonoxydans*	98
			HQ113206.1	*Microbacterium oxydans*	98
FF	A	−20.0 ± 1.5	HQ425309.1	*Kocuria* sp.	98
			FR682683.1	*Kocuria rhizophila*	98

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Table 3. Identification of viable cultivable bacteria in Arctic samples. Some of the bacterial colonies were identified in each snow categories: accumulated snow (AS), blowing snow (BS), fresh snow (FS), windpack (WP), and frost flowers (FF); accession number, the nearest neighbor found in the data base, a unique identifier given to a DNA sequence; identified species; and % similarity, the ratio of identical query bases to known bases in the database.

Snow Catergory	Bacterial Colony #	Accession #	Species	% Similarity
FF	1	GU975796.1	*Curtobacterium* sp. D2.2	97
		JF798380.1	*Curtobacterium luteum*	97
		FN178369.1	*Curtobacterium citreum*	97
		HM045842.1	*Bacillus* sp. WJ18	97
	2	EU196527.1	*Paracoccus* sp. B10	97
		DQ195864.1	*Rhodobacteraceae bacterium*	97
	3	HQ425309.1	*Kocuria* sp. M1-36	98
		FR682683.1	*Kocuria rhizophila*	98
	4	JN084144.1	*Curtobacterium oceanosedimentum*	93
		EF592577.1	*Flavobacterium oceanosedimentum*	93
		EU373393.1	*Bacillus subtilis*	93
FS	1	JN208198.1	*Bacillus* sp. DG7	96
		AB648987.1	*Bacillus megaterium*	96
		JN092792.1	*Bacillus flexus*	96
		HQ857752.1	*Bacillus aryabhattai*	96
	2	JN085952.1	*Microbacterium* sp. ZL2	98
		JF700471.1	*Microbacterium hydrocarbonoxydans*	98
		HQ113206.1	*Microbacterium oxydans*	98
AS	1	EU379295.1	*Micrococcus luteus*	92
	2	HE578790.1	*Micrococcus luteus*	95
		HM209728.1	*Micrococcus yunnanensis*	95
	3	EU584512.1	*Frigoribacterium* sp. Everest-gws-26	97
		AY599739.1	*Actinobacterium* TB3-4-I	97
	4	JF969180.1	Bacterium REGD8	95
		JF778689.1	*Sporosarcina* sp. DRB20	94
WP	1	JF728909.1	*Leifsonia* sp. DAB_MOR27	96
		DQ172984.2	Bacterium TSBY-9	96
		NR_042669.1	*Leifsonia kafniensis*	96
		NR_041548.1	*Microterricola viridarii*	96
BS	1	EF540454.1	*Brevundimonas* sp. d1M	96
		JN020187.1	Uncultured alpha proteobacterium clone cher4_1B_11	96
		NR_037106.1	*Brevundimonas variabilis*	96
	2	FR691407.1	*Brevundimonas* sp. R-36741	96
		AB452982.1	*Alpha proteobacterium* HIBAF003	96
	3	JF778709.1	*Paenisporosarcina macmurdoensis*	99
		JF778708.1	*Sporosarcina* sp. GRT2	99
	4	JF969180.1	Bacterium REGD8	98
		HM224487.1	*Sporosarcina* sp. TPD39	98
		JF778709.1	*Paenisporosarcina macmurdoensis*	98
	5	JN082256.1	*Bacillus* sp. cf30	97
		JN092792.1	*Bacillus flexus*	97
		HQ143640.1	*Geobacillus stearothermophilus*	97
	6	FJ487574.1	*Paenibacillus amylolyticus*	95
		NR_044524.1	*Paenibacillus xylanexedens*	95
		HQ202814.1	*Paenibacillus tylopili*	95

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Table A1. Bioinformatic analysis for quality assessment/control of Roche 454 GS-FLX Titanium reads data. The number of reads and bases in NGS analysis for each Arctic sample: blowing snow (BS), surface hoar snow (SH), windpack snow (WP), frost flowers (FF); and urban snow (US) were compared. The quality of each read was assessed and improved by discarding and trimming low quality reads.

Snow Types	Number of Reads	Number of Bases	Raw read length (avg/median/SD)	Trim length (avg/median/SD)	Final read length (avg/median/SD)
US	24 968	7 966 589	611/532/25	292/108/17	319/419/18
BS	13 939	4 164 307	422/526/21	124/74/11	299/430/17
SH	18 002	7 214 113	542/535/23	141/75/12	401/452/20
WP	13 831	5 321 162	533/530/23	149/73/12	385/449/20
FF	18 197	7 618 141	556/556/24	138/83/12	419/470/20

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Table A2. Relative abundance of bacterial taxa detected by Roche 454 GS-FLX Titanium using 16S rDNA gene. Detected phyla **(a)** and the top four genera with the highest percentage **(b)** in each Arctic sample: blowing snow (BS), surface hoar snow (SH), windpack snow (WP), frost flowers (FF); and urban snow (US). The number in the parenthesis shows the sequence percentage obtained from the overall bacterial pool.

(a) Phylum US	BS	SH	WP	FF
Proteobacteria (49 %)	*Proteobacteria* (83.1 %)	*Proteobacteria* (67 %)	*Proteobacteria* (60.5 %)	*Proteobacteria* (50.2 %)
Bacteroidetes (47.5 %)	*Firmicutes* (5.9 %)	*Firmicutes* (13.6 %)	*Cyanobacteria* (12.3 %)	*Actinobacteria* (32.7 %)
Actinobacteria (2.5 %)	*Actinobacteria* (5.1 %)	*Actinobacteria* (8.8 %)	*Bacteroidetes* (8.5 %)	*Firmicutes* (7.8 %)
Cyanobacteria (0.3 %)	*Cyanobacteria* (2 %)	*Cyanobacteria* (3.1 %)	*Planctomycetes* (4.6 %)	*Bacteroidetes* (2.9 %)
Candidate_division_TM7 (0.2 %)	*Bacteroidetes* (1.8 %)	*Bacteroidetes* (2.2 %)	*Actinobacteria* (4.3 %)	*Verrucomicrobia* (2.2 %)
Firmicutes (0.2 %)	*Acidobacteria* (0.6 %)	*Verrucomicrobia* (1.8 %)	*Firmicutes* (3.4 %)	*Planctomycetes* (1.9 %)
Candidate_division_OP10 (0.1 %)	*Planctomycetes* (0.3 %)	Candidate_division_OD1 (1.3 %)	*Verrucomicrobia* (2.6 %)	*Cyanobacteria* (0.8 %)
Acidobacteria (0.08 %)	SM2F11 (0.3 %)	Candidate_division_TM7 (0.8 %)	*Gemmatimonadetes* (1.6 %)	*Deferribacteres* (0.4 %)
Planctomycetes (0.08 %)	*Verrucomicrobia* (0.2 %)	*Planctomycetes* (0.4 %)	*Chloroflexi* (0.9 %)	Candidate_division_OD1 (0.3 %)
Chloroflexi (0.03 %)	Candidate_division_OD1 (0.2 %)	SM2F11 (0.3 %)	*Acidobacteria* (0.6 %)	SM2F11 (0.2 %)
Verrucomicrobia (0.03 %)	*Gemmatimonadetes* (0.2 %)	*Fusobacteria* (0.2 %)	Candidate_division_TM7 (0.2 %)	BD1-5 (0.1 %)
	WCHB1-60 (0.2 %)	*Synergistetes* (0.2 %)	*Chlorobi* (0.2 %)	Candidate_division_OP3 (0.05 %)
	Candidate_division_TM7 (0.1 %)	Candidate_division_OP11 (0.1 %)	Candidate_division_OD1 (0.1 %)	Candidate_division_TM7 (0.05 %)
	Chloroflexi (0.1 %)	*Acidobacteria* (0.04 %)	Candidate_division_OP11 (0.07 %)	*Chloroflexi* (0.05 %)
	Deferribacteres (0.1 %)	Candidate_division_OP10 (0.04 %)	*Fibrobacteres* (0.07 %)	*Fusobacteria* (0.05 %)
		Lentisphaerae (0.04 %)	SM2F11 (0.07 %)	*Gemmatimonadetes* (0.05 %)
			Candidate_division_OP10 (0.04 %)	*Lentisphaerae* (0.05 %)
			Deinococcus-Thermus (0.04 %)	*Spirochaetes* (0.05 %)

(b) Genus US	BS	SH	WP	FF
Flavobacterium (40 %)	*Methylobacterium* (15.3 %)	*Roseateles* (18.6 %)	*Methylobacterium* (9 %)	*Propionibacterineae* (32.9 %)
Polaromonas (11.2 %)	*Bradyrhizobium* (1.6 %)	*Methylobacterium* (14.1 %)	*Sphingomonas* (4.9 %)	*Roseateles* (8 %)
Variovorax (6.6 %)	*Bacillus* (1 %)	*Bacillus* (5.7 %)	*Lamprocystis* (4.5 %)	*Staphylococcus* (6.7 %)
Sandarakinorhabdus (6 %)	*Sphingomonas* (1 %)	*Streptococcus* (5.7 %)	*Roseateles* (4.4 %)	*Candidatus Pelagibacter* (6.1 %)

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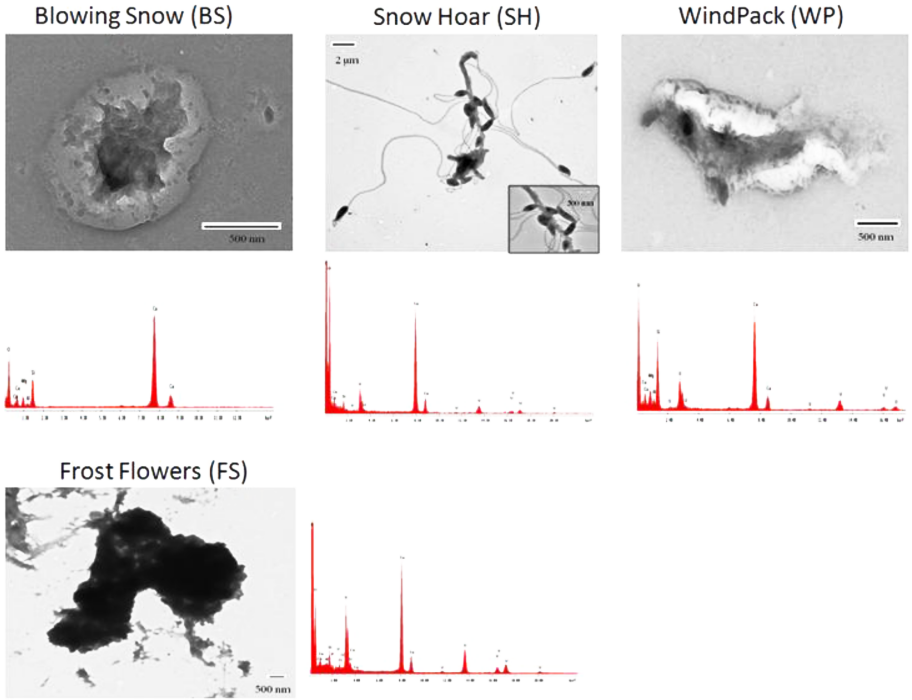

Figure 1. Analysis of snow-associated microorganisms by transmission electron microscopy (TEM) in conjunction with EDS. Microorganisms were detected in selected Arctic samples: blowing snow, BS; snow hoar, SH; windpack, WP, and frost flowers, FF.

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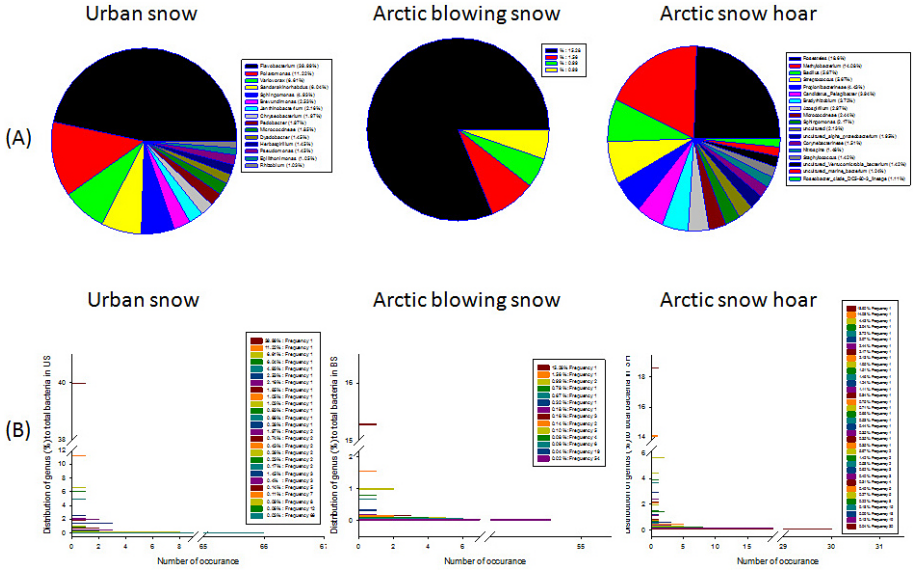

Figure 2.

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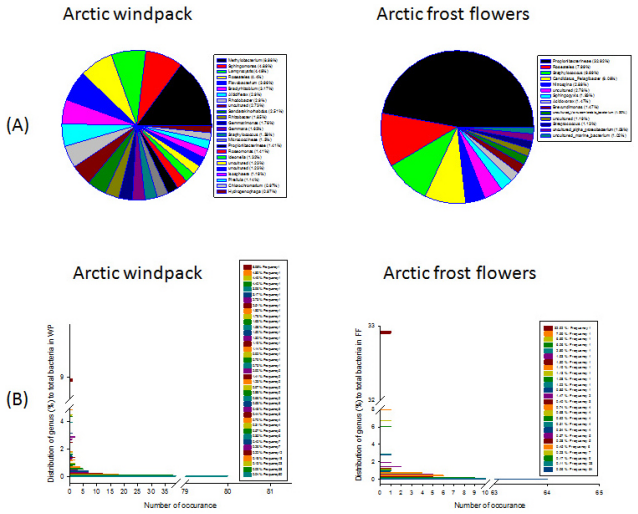

Figure 2. Bacterial community composition in Arctic samples and urban snow at genera level as detected by Roche 454 GS-FLX Titanium. **(a)** distribution of bacterial genus observed at greater than 1 %. **(b)** number of occurrence for each percentage observed for any genus in total bacteria.

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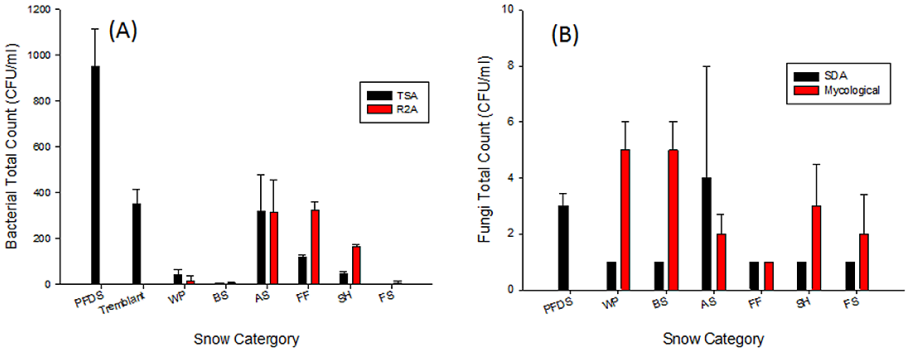

Figure 3. Concentration of viable cultivable bacteria and fungi in Arctic samples. Mean number of colony-forming units (CFU) mL^{-1} of snow on two media for: **(a)** bacteria (TSA and R2A) and **(b)** fungi (SDA and mycological agar) in each Arctic snow categories: accumulated snow (AS), blowing snow (BS), fresh snow (FS), surface hoar snow (SH), windpack (WP), and frost flowers (FF). Samples from several urban sites collected in Quebec, Canada: pierrefonds (suburb of Montreal; "PFDS"), and Mont-Tremblant (Laurentians; "Tremblant") are included for comparison. Error bars indicate SD of the mean for three experiments.

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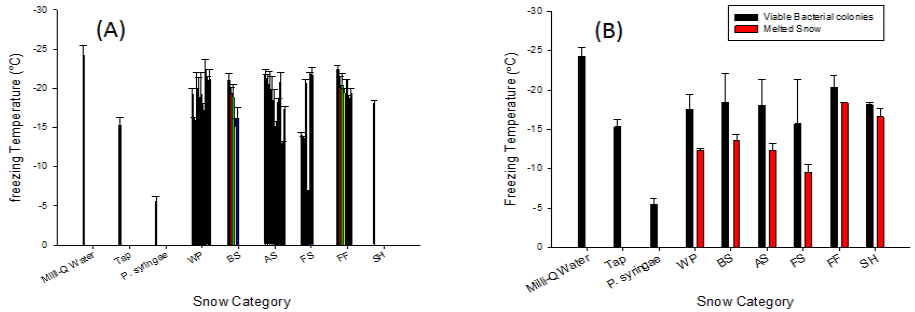

Figure 4. Ice nucleation activity of viable cultivable bacteria in Arctic samples. The average ice nucleation temperature of: **(a)** individual bacterial isolates and **(b)** total bacterial isolates and melted Arctic samples. Arctic snow categories: accumulated snow (AS), blowing snow (BS), fresh snow (FS), surface hoar snow (SH), windpack (WP), and frost flowers (FF). Controls: ultrapure water (milli-Q-water), tap water, and a suspension of laboratory-grown *Pseudomonas syringae* (*P. syringae*). Error bars indicate SD of the mean for three experiments.

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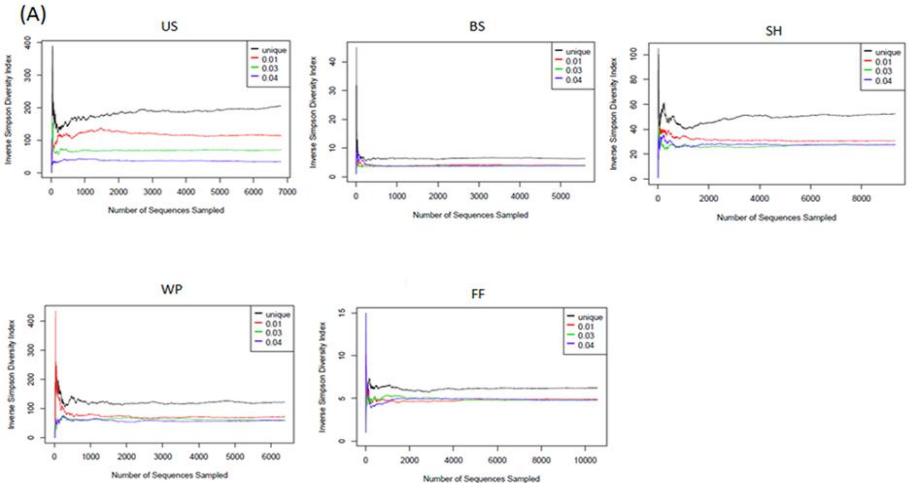

Figure A1.

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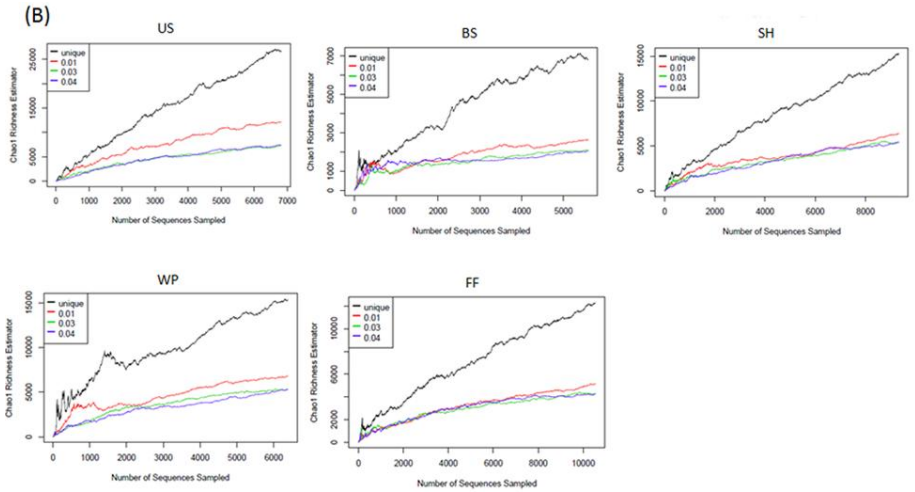

Figure A1.

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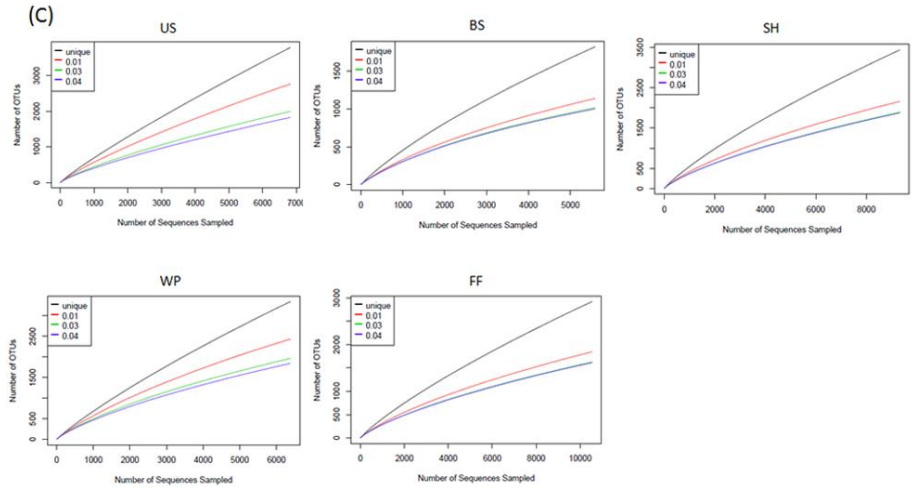

Figure A1. Bacterial diversity by 454 NGS analysis. The diversity of the bacterial communities for Arctic samples: blowing snow (BS), surface hoar snow (SH), windpack snow (WP), frost flowers (FF); and urban snow (US) was estimated using the Inverse Simpson Diversity Index **(a)**, species richness using the nonparametric Chao index **(b)**, and Rarefaction Metric (number of OTUs) **(c)** with 3 % cut-off value in sequence differences for OTU.

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