

Microbial sequences retrieved from environmental samples from seasonal Arctic snow and meltwater from Svalbard, Norway

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Abstract 16S rRNA gene (*rrs*) clone libraries were constructed from two snow samples (May 11, 2007 and June 7, 2007) and two meltwater samples collected during the spring of 2007 in Svalbard, Norway (79°N). The libraries covered 19 different microbial classes, including *Betaproteobacteria* (21.3%), *Sphingobacteria* (16.4%), *Flavobacteria* (9.0%), *Acidobacteria* (7.7%) and *Alphaproteobacteria* (6.5%). Significant differences were detected between the two sets of sample libraries. First, the meltwater libraries had the highest community richness (Chao1: 103.2 and 152.2) and Shannon biodiversity indices (between 3.38 and 3.59), when

compared with the snow libraries (Chao1: 14.8 and 59.7; Shannon index: 1.93 and 3.01). Second, β -LIBSHUFF analyses determined that the bacterial communities in the snow libraries were significantly different from those of the meltwater libraries. Despite these differences, our data also support the theory that a common core group of microbial populations exist within a variety of cryohabitats.

Keywords Arctic · Snow bacteria · Biodiversity · Environmental samples · Meltwater

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Introduction

A large portion of the Earth is cold: about 14% of the biosphere is polar and 90% (by volume) is cold ocean, with temperatures less than 5°C (Priscu and Christner 2004). Due to the strong feedback mechanisms between snow, ice and the atmosphere, the cryosphere influences the entire biosphere (Jones 1999). The seasonal snow cover is an important component of the cryosphere (Jones 1999), which can at times cover about 35% of the Earth's surface (Miteva 2007). Despite the importance of the cryosphere, the microbiological analysis of this environment is a relatively recent field of research. Microorganisms have been described in several extreme environments such as glacial ice (Christner et al. 2000; Skidmore et al. 2000; Christner et al. 2001), sea ice (Brinkmeyer et al. 2003) and polar snow (Carpenter et al. 2000; Amato et al. 2007), but their importance in cryosphere ecology has only begun to be recognized. Recent reports suggest that microorganisms impact the dynamics, composition and abundance of nutrients (Hodson et al. 2008), that they may shift surface albedo of snow and ice (Thomas and Duval 1995) and impact the hydrochemistry (Tranter et al. 2002). The

importance of bacteria in governing redox conditions and their role in Fe, S, N and P cycling is becoming apparent (Hodson et al. 2008). In addition, they are probably responsible for the metabolism and transformation of environmental contaminants such as mercury (Barkay and Poulain 2007; Poulain et al. 2007). While snow algae have been studied relatively extensively (Hoham 1975; Hoham and Duval 2001; Stibal et al. 2007), little is known about the bacteria inhabiting seasonal snow cover, especially polar snowpacks. Carpenter et al. (2000) reported low rates of DNA synthesis and the presence of *Thermus-Deinococcus*-like organisms in Antarctic snow. Amato et al. (2007) used culture-based methods to isolate 10 bacterial strains belonging to *Proteobacteria*, *Firmicutes* and *Actinobacteria* from a snowpit dug on a polythermal glacier in Svalbard (Norway). Unfortunately, culture-based techniques will not recover the full diversity of species present and might not even recover the dominant population (Ward et al. 1990). A metagenomic approach, which extracts and analyzes nearly all the microbial community DNA, should provide more detailed insight into polar snow microbial diversity. Such an approach was used to reveal relatively high microbial diversity in the snow of glaciers of the Tibetan Plateau around Mount Everest (Liu et al. 2009). Whether the snow in other environments is equally diverse has yet to be determined.

Here, we present the results of an exploratory study on microbial sequences retrieved from surface snow and meltwater samples from a high Arctic site, Svalbard, Norway (79°N), using culture-independent techniques. We hypothesize that the snow is a highly diverse ecosystem that might have some globally uniform characteristics in terms of microbial populations in cryohabitats.

Materials and methods

Samples for microbial analysis were taken during a 2007 springtime field campaign in Ny-Ålesund (Svalbard, Norway, 78°56'N, 11°52'E). A total of four samples were collected: an early spring (sample date May 11, 2007) snow sample (ESS), a late spring (sample date June 7, 2007) snow sample (LSS), a meltwater river (sample date June 11, 2007) sample (MWR) and a snow–soil interface meltwater (sample date June 10, 2007) sample (SSIM). Surface snow (3-cm depth) and meltwater samples were collected in 3 L sterile sampling bags using a Teflon shovel sterilized for microbial analyzes. To avoid contamination, Tyvex® body suits and latex gloves were worn during sampling and gloves were worn during all subsequent handling of samples. In the ESS sample, pH was slightly acidic (5.3) and the snowpack temperature was around freezing (<0°C), whereas in LSS, pH was circumneutral (6.4) and the

temperature was positive (around 1°C), indicating spring melt and the presence of liquid water within the snowpack. Meltwater samples were circumneutral, with a value of 6.8 for SSIM and 7.3 for MWR.

Microbial sample processing

Samples were processed immediately after collection in the field laboratory. Surface snow samples were left to melt at room temperature prior to being filtered onto sterile 0.22 µM 47-mm filters (Millipore) using a sterile filtration unit (Nalge Nunc International Corporation), while meltwater samples were filtered immediately. Filters were stored in sterile collection tubes at –20°C until further analysis. Procedural blanks were carried out by filtering Nanopure water (Siemens) using the same procedure.

DNA extraction

DNA was extracted using the protocol outlined in David et al. (2009). Briefly, filters were chopped and placed in a Fastprep® bead-beating tube (Lysing matrix E, MP Biomedicals) to which 1 mL of DNA extraction buffer (David et al. 2009) and 20 mg ml⁻¹ lysing enzyme (*Trichoderma harzianum*, Sigma L1412) were added. Tubes were left at room temperature for 1 h and then frozen at –20°C overnight. The frozen tubes were incubated at 65°C for 30 min and placed in a Fastprep® bead-beater (MP Biomedicals) set at speed 5.5 for 30 s. DNA was extracted from the water phase with an equal volume of chloroform:isoamyl alcohol (24:1) and precipitated with isopropanol.

DNA amplification

Since DNA yield was low (between 0.1 and 8 ng µl⁻¹), the DNA extracted from each sample was amplified using multiple displacement amplification with the illustra™ GenomiPhi™ V2 DNA Amplification Kit (GE Healthcare). Amplification was carried out according to the manufacturer's instructions, except that twice the amount of DNA was used.

Clone library construction

Using universal 16S rRNA gene (*rrs*) primers pA (5' AG AGTTTGATCCTGGCTGAG 3') and pH (5' AAGGAG GTGATCCAGCCGCA 3') and the illustra Hot Start Mix RTG (GE Healthcare) PCR kit, four bacterial clone libraries were constructed: ESS, LSS, MWR and SSIM. The 25 µL volume PCR reaction mix contained 0.6 µM of each primer, 5 µl GenomPhi-amplified DNA or 5 µl sterile water for the negative control. The PCR conditions used were 5 min at 95°C, followed by 45 cycles of 45 s of

denaturation at 95°C, 45 s of annealing at 56°C, and 45 s of elongation at 72°C. After a final 10-min extension at 72°C, PCR products were separated by 1%-agarose gel electrophoresis and purified using the NucleoSpin® Extract II kit (Clontech). Amplicons were cloned into pCR4-TOPO using a TOPO-TA cloning kit (Invitrogen) as per the manufacturer's instructions. Ninety-six clones from each library were screened for the correct-sized insert (1.5 kb) by PCR amplification using M13F (5' GTAAAA CGACGGCCAG 3') and M13R (5' CAGGAAACAGC TATGA 3') primers and the same protocol outlined above. Of these clones, a total of 192 PCR amplicons had the correct-sized insert and were sequenced in both directions (GATC Biotech). The sequencing run returned 155 sequences. Sequences were assembled using DNASTAR (DNASTAR®, Inc.) and average base pair coverage was 200. In the rare cases of base pair mismatch, chromatograms were consulted and the nucleotide was determined by two independent readers.

Clone library analysis

Sequences were aligned using the Clustal W alignment function in the BioEdit software package (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Distance matrices of the aligned sequences were generated for each library with the model F84 for nucleotide substitution using the DNADIST program in PHYLIP (version 3.6; Department of Genomic Sciences, University of Washington, Seattle, WA, USA; <http://evolution.genetics.washington.edu/phylip.html>). Biodiversity indices for clone libraries were generated by running the outfiles through the DOTUR 1.53 program (Schloss and Handelsman 2005). Rarefaction curves (Hughes et al. 2001) were drawn for all libraries and the Shannon biodiversity index (Magurran 1988), Chao1 species richness index (Chao 1984), as well as clone library coverage based on Good's coverage for an OTU definition (Good 1953) were determined using 97% identity level. The phylogenetic diversity of all four libraries was compared using the β -LIBSHUFF (<http://schloss.micro.umass.edu/software/slibshuff.html>) (Schloss et al. 2004). The program uses the Cramér-von Mises test (Pettitt 1982) to determine significant differences ($p < 0.05$ after applying the Bonferroni correction) among libraries independent of arbitrary OTU similarity percentage cut-off values and is designed to compare undersampled 16S rRNA gene libraries.

Phylogenetic analysis

Sequences were checked for chimera with the CHIMERA detection program (<http://rdp8.cme.msu.edu/cgis/chimera.cgi>) (Cole et al. 2003). Database sequences with the highest

degree of similarity were obtained with the NCBI BLAST-N program (<http://www.ncbi.nih.gov/blast>). The Ribosomal Database Project 10.0 Classifier tool (<http://rdp.cme.msu.edu>) was used to assign genus level groupings with 80% confidence (Cole et al. 2009). Phylogenetic trees were then generated using Clustal X (Thompson et al. 1997) to align sequences and run bootstrapping analysis (1,000 repetitions) with the Jukes–Cantor distance correction (Jukes and Cantor 1969) and the neighbour-joining method (Saitou and Nei 1987). The trees were visualized with NjPLOT (Perrière and Gouy 1996).

Results

16S rRNA gene (*rrs*) clone libraries

Four 16S rRNA gene (*rrs*) clone libraries were constructed from the ESS, LSS, SSIM and MWR samples from which a total of 155 independent sequences were obtained. All PCR amplification controls performed in parallel to the assays were negative. Based on the coverage estimates (Table 1), about 50 and 80% of the diversity in the clone libraries for snow samples was sampled (ESS and LSS, respectively), whereas meltwater clone libraries had lower values (around 30%). This is reflected in the rarefaction curves that begin to plateau for snow, but not for the meltwater libraries, which suggests that meltwater libraries were relatively undersampled (Fig. 1). The MWR library had the highest community richness (Chao1, Table 1), followed by SSIM, ESS and LSS. The 95% confidence interval of the Chao1 estimator overlapped between the ESS and meltwater samples. The meltwater libraries also had the highest Shannon biodiversity indices, followed by the two snow-derived libraries (ESS and LSS). Based on the β -LIBSHUFF analyses, the ESS clone library is significantly different from the LSS clone library ($p < 0.0001$). There was no significant difference between meltwater clone libraries ($p = 0.6826$), however, the snow libraries differed significantly from the meltwater libraries ($p < 0.0001$ for each pairwise comparison).

Sequence analysis

The combined four clone libraries covered 19 different microbial classes, with *Betaproteobacteria* (21.3%) and *Sphingobacteria* (16.4%) dominating. *Flavobacteria* (9.0%), *Acidobacteria* (7.7%) and *Alphaproteobacteria* (6.5%) were the next most-represented classes, with the remaining classes each representing less than 5% of the total. The genus for almost half of the sequences could not be determined with 80% confidence using the classifier tool, however, common genera existed for ESS and MWR

Table 1 Biodiversity indices and statistics among the snow and meltwater libraries

Library	Sample date	Individuals	OTUs	Chao1 (95% CI)	% coverage	H' (95% CI)
ESS	May 11, 2007	40	29	59.67 (38.40–124.45)	50	3.01 (2.73–3.29)
LSS	June 7, 2007	29	11	14.75 (11.64–33.02)	80	1.93 (1.54–2.30)
SSIM	June 10, 2007	40	32	103.20 (57.71–232.43)	33	3.38 (3.15–3.62)
MWR	June 11, 2007	46	39	152.20 (81.72–341.74)	26	3.59 (3.36–3.81)

Individuals number of clones sequenced, *OTUs* number of operational taxonomic units observed, *Chao1* Chao1 nonparametric richness estimate with 95% confidence interval in parentheses, *% coverage* percent library coverage based on Good's estimate, *H'* Shannon–Weaver diversity index with 95% confidence interval in parentheses

ESS, LSS, SSIM and MWR correspond to the early spring snow library, the late spring snow library, the snow–soil interface meltwater library and the meltwater river library, respectively

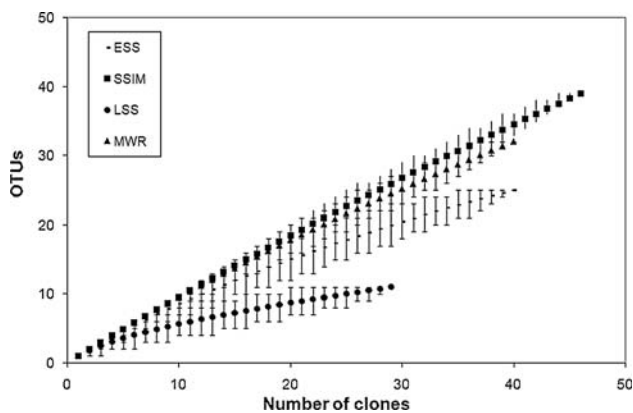


Fig. 1 Rarefaction curves for each clone library showing the number of clones sampled versus the number of operational taxonomic units (OTUs) within each library. Error bars represent the 95% CI. ESS, LSS, SSIM and MWR correspond to the early spring snow library, the late spring snow library, the snow–soil interface meltwater library and the meltwater river library, respectively

(*Curvibacter*), ESS and LSS (*Xiphinematobacteriaceae*_genera_incertae_sedis), MWR and SSIM (*Clostridium*, *Pedobacter*, *Niastella*, *Gp4*, *Gp6*) and for LSS and SSIM (*Flavobacterium*, *Polaromas*) (Supplementary Data, Fig. 1a, b, c).

The ESS library sequences contained 12 bacteria classes and one unclassified sequence. It was dominated by *Betaproteobacteria* (20.0%), *Alphaproteobacteria* (17.5%), *Sphingobacteria* (15.0%) and *Bacilli* (7.5%). *Flavobacteria* and *Cyanobacteria* comprised only a small percentage of the library (5.0% each) and some sequences related to eukaryote chloroplasts were also identified (Supplementary Data, Fig. 1). Most members of *Alphaproteobacteria*, *Bacilli* and *Sphingobacteria* are closely related to environmental clone sequences from alpine/polar environments. The LSS library was dominated by clones related to phototrophic eukaryotes (37.9%). A total of six different bacterial classes were represented, with *Betaproteobacteria* (27.6%) dominating, followed by *Cyanobacteria* (13.8%) and *Flavobacteria* (10.3%) (Supplementary Data, Fig. 1).

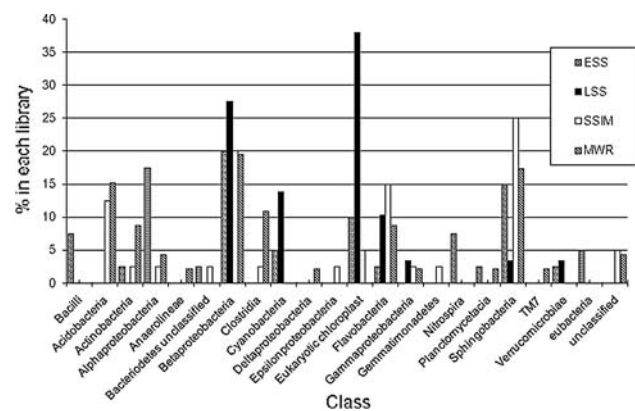


Fig. 2 Class distribution in each of the four clone libraries. The bars represent the percentage of each class in each library. ESS, LSS, SSIM and MWR correspond to the early spring snow library, the late spring snow library, the snow–soil interface meltwater library and the meltwater river library, respectively

A total of 12 classes were represented in the SSIM library, which was dominated by *Sphingobacteria* (25.0%), *Betaproteobacteria* (20.0%), *Flavobacteria* (15.0%) and *Acidobacteria* (12.5%). Half of the *Betaproteobacteria* had members with sequences nearest to environmental clone sequences retrieved from polar environments and snow (Supplementary Data, Fig. 1) and 60% of the *Flavobacteria* clustered with clone sequences from Arctic marine environments. The MWR library was dominated by *Betaproteobacteria* (19.6%), followed by *Sphingobacteria* (17.4%), *Acidobacteria* (15.2%) and *Clostridia* (10.9%). All *Flavobacteria* sequences had nearest neighbours that originated from Arctic marine environments, while 75% of *Clostridia* sequences were nearest to environmental clone sequences from alpine/polar environments (Supplementary Data, Fig. 1).

Common classes among all clone libraries were *Betaproteobacteria*, *Sphingobacteria* and *Flavobacteria*. Classes exclusive to snow libraries were *Verrucomicrobia* and *Cyanobacteria*, while *Acidobacteria* and *Clostridia* were limited to meltwater clone libraries (Fig. 2). *Bacilli*

and *Nitrospira* were only found in the ESS library, which also had the highest proportion of *Alphaproteobacteria* amongst all libraries. The LSS library contained no *Alphaproteobacteria* or *Actinobacteria*, unlike the other three libraries. The MWR library had a higher proportion of clones that were from the *Clostridia* class than the other libraries and no phototrophic organisms were present, while the SSIM library had the highest proportion of *Flavobacteria* (Fig. 2).

Clone sequences were also grouped based on the geographical origin of their nearest neighbour using the NCBI BLAST-N results. Sequences with nearest neighbours that were retrieved from alpine/polar environments (Antarctica/Arctic—soil, water, sediments and non-polar high altitude sites) represented 34.2% of all sequences, followed by sequences similar to isolates from soil (21.9%), aquatic (29.0%) and contaminated (11.0%) environments (Table 2). In the ESS library, sequences with nearest neighbours from aquatic sites accounted for 47.5% of the library, followed by alpine/polar sites (25.0%), soil (12.5%) and contaminated environments (10.0%). Almost all LSS sequences had nearest neighbours that were from alpine/polar (55.2%) or aquatic (41.4%) sites. The SSIM library was dominated by sequences with nearest neighbours from soil (32.5%) followed by alpine/polar and aquatic (25.0 and 22.5%, respectively) and contaminated environments (20.0%). The nearest neighbours of the sequences in the MWR library were mainly from alpine/polar regions (37.0%) and soil (32.6%) followed by aquatic environments (10.9%) (Table 2).

Discussion

Clone library analysis

Arctic snow and meltwater are highly diverse systems. The fact that the rarefaction curves fail to plateau for meltwater

Table 2 Distribution of clones in each of the four libraries based on nearest-neighbour isolation environment

Nearest-neighbour environment	Clones				
	Total	ESS	LSS	SSIM	MWR
Alpine/polar	34.2	25.0	55.2	25.0	37.0
Aquatic	29.0	47.5	41.4	22.5	10.9
Contaminated	11.0	10.0	3.4	20.0	8.7
Other	3.9	5.0	0.0	0.0	10.9
Soil	21.9	12.5	0.0	32.5	32.6

Values are in percent of total. ESS, LSS, SSIM and MWR correspond to the early spring snow library, the late spring snow library, the snow-soil interface meltwater library and the meltwater river library, respectively

libraries and only begin to plateau for early spring snow, suggests that further sampling or clone selection would reveal even higher diversity at the species level (Fig. 1) and that a large amount of the bacterial diversity still remains to be sampled from these environments. Based on the β -LIBSHUFF analyses, designed to analyze under-sampled libraries, snow bacterial communities (ESS and LSS) were significantly different ($p < 0.0001$) and snow and meltwater communities also differed significantly ($p < 0.0001$). Another estimator, the Chao1 nonparametric richness estimator, can be used as an alternative to the β -LIBSHUFF for comparing undersampled libraries since it approaches a stable maximum before rarefaction curves plateau (Kemp and Aller 2004). Although there is some overlap in the 95% confidence interval of the Chao1 estimator between the ESS and meltwater libraries (Table 1), no overlap is observed with the LSS library, further supporting the significance of the difference.

The observed differences in the retrieved sequences from the different libraries could be due to the seasonal changes in the respective microbial communities at the time of sampling, since the snow environment at the time of sampling differed in terms of temperature and pH. Another factor may also be atmospheric deposition. The relatively large percentage of cloned sequences with nearest neighbours isolated from the soil (12.5%) and contaminated environments (10.0%) in the ESS library might implicate atmospheric deposition of pollutants and/or microbes. Liu et al. (2006) also reported the presence of bacteria related to isolates from activated sludge in snow from glaciers in the Tibetan Plateau at altitudes above 5,000 m that they suggested originated from atmospheric deposition since bacteria attached to aeolian dust can be transported to glaciers (Yao et al. 2006) and microbial concentrations have been correlated to mineral particle density in the snow (Segawa et al. 2005). Whether these bacteria are then able to colonize and multiply in the snow depends on their ability to survive the harsh surface conditions, i.e. high UV radiation, temperature extremes, variable wind ventilation and variable (low) organic matter deposition (Jones 1999; Kuhn 2001; Hodson et al. 2008). The *Sphingobacteria* in the ESS library cluster with bacteria isolated from either Antarctica or tundra soils and are probably able to grow in the snow, since certain members are psychrotrophic and produce pigments that may protect them from solar radiation (Chauhan and Shivaji 1994).

We observed a decrease in biodiversity in the LSS library as compared to the ESS library and an increase in clones with nearest neighbours from polar/alpine environments and with DNA belonging to algae and *Cyanobacteria*. While it is possible that the proximity of the fjord to the sampling site may have influenced species composition in the LSS library due to sea spray, it is unlikely since most

of the sequences clustered with sequences retrieved from freshwater sources (lakes or glaciers, Supplementary Data, Fig. 1). The observed decrease in biodiversity in the LSS clone library may be due to meltwater washout. A decrease in microbial biomass and mineral particle concentration in mid-latitude mountain summer snow (June and July) due to meltwater washout was reported by Segawa et al. (2005), and meltwater has also been shown to washout nutrients (Kuhn 2001). Since meltwater integrates what is washed out of the snow, it would be expected to incorporate most species/classes found within the seasonal snowpack, with the exception of those able to attach themselves or form biofilms, thus reducing their mobility. The meltwater libraries sequenced contained all of the bacterial classes found in the ESS and LSS libraries with the exception of *Bacilli*, *Nitrospira*, *Verrucomicrobia* and *Cyanobacteria*.

Since meltwater also drains the soil, it would be expected that its community structure reflects this influence. For both meltwater libraries, over a third of the sequences had nearest neighbours isolated from the soil. Certain classes, such as *Acidobacteria* and *Clostridia*, were found in meltwater, but not snow, libraries. *Acidobacteria* are commonly isolated from soils (Jones et al. 2009), while *Clostridia* are anaerobic bacteria commonly isolated from Antarctic soils and anaerobic microbial mats (Miwa 1975; Spring et al. 2003; Stackebrandt et al. 2004).

Snow and meltwater diversity

Meltwater clone libraries were more diverse than snow libraries, with Shannon biodiversity indices between 3.38 and 3.59. These values are higher than those reported for meltwater libraries collected on the northern slope of Mount Everest: between 0.6199 and 2.398 (Liu et al. 2006). Bacteria in the Everest region were probably exposed to lower oxygen concentrations, stronger radiation and less nutrients than those from the Svalbard region, thus, the increased stress might have limited biodiversity. In the Everest meltwater clone libraries, the dominant groups were the *Bacteroidetes* group and *Betaproteobacteria*, similar to those found in this study. We also identified several *Actinobacteria* strains, which were absent in the Everest libraries. Cheng and Foght (2007) reported higher biodiversity indices (3.143–3.713) for libraries constructed from subglacial environments in the high Arctic, with *Bacteroidetes*, *Betaproteobacteria* and *Actinobacteria* dominating, and Skidmore et al. (2005) found mainly *Betaproteobacteria* and some *Bacteroidetes* in Canadian subglacial environments (Skidmore et al. 2005; Cheng and Foght 2007).

In contrast, clone libraries from snow samples from four different sites around Mount Everest had relatively high Shannon biodiversity indices, between 2.2 and 4 (Liu et al.

2009). Our snow libraries fell within this range (ESS: 3.01, LSS: 1.93). In Everest snow libraries, *Gammaproteobacteria* were dominant, followed by *Alphaproteobacteria* and *Actinobacteria*, unlike in our snow libraries where *Betaproteobacteria* dominated (Fig. 2). *Actinobacteria* and *Alphaproteobacteria* were only present in the ESS library, while only one *Gammaproteobacteria* clone was sequenced in the LSS library. In a study on the seasonal shifts in microbial populations in mid-altitude snowpacks, Segawa et al. (2005) reported that a large portion of their library was composed of *Betaproteobacteria* (Segawa et al. 2005). They also found a higher percentage of *Actinobacteria*, *Bacillus* and *Alphaproteobacteria* in the early spring (March) library than in the summer (June and August) libraries, similar to the shift we observed in our snow libraries. The biodiversity indices of our snow libraries are comprised between those of Arctic pack ice and Arctic microbial mats (0.812–1.291 and 3.50–4.41, respectively (Brinkmeyer et al. 2003).

Conclusion

The snowpack is a diverse habitat that remains largely unexplored as research efforts have focused on glacier systems and ice. Many studies, including this one, report the occurrence of related phylotypes from geographically diverse, but predominantly cold environments, and the community structure has common populations in different cryohabitats. Differences between the clone libraries from snow sampled during two different times demonstrate the need to further explore the seasonal evolution of the microbial community and the physiological state of the organisms. Our understanding of the microbial community needs to be expanded and functional analyses will determine both the effect of season on microbial activity as well as the extent that these communities influence snowpack dynamics such as nutrient cycling, gas transfer and environmental contaminant cycling and downstream ecosystem transfer.

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