Microbial Challenges and Solutions to Inhabiting the Dynamic Architecture of Saline Ice Formations

Marcela Ewert Sarmiento

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Reading Committee:

Jody W. Deming, Chair

John A. Baross

Rebecca Woodgate

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Abstract

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Marcela Ewert Sarmiento

Chair of the Supervisory Committee:

Walters Endowed Professor Jody W. Deming

Oceanography

Sea ice contains a microscopic network of brine inclusions effectively colonized by organisms from the three major clades of life. The architecture of this brine channel network is dynamic, with surface area, brine volume fraction, and brine salinity changing with temperature. This dynamic architecture may have also played a role in the origin and early evolution of life (Chapter 1). Sea-ice microorganisms experience multiple stressors, including low temperature, high salinity and fluctuations in those parameters. This dissertation discusses two bacterial adaptations to these challenges: the production of extracellular polysaccharide substances (EPS) and the accumulation of compatible solutes. Two Arctic bacteria were used as model organisms; the psychrophilic *Colwellia psychrerythraea* strain 34H (*Cp*34H), which grows at a comparatively narrow range of salinities, and the psychrotolerant *Psychrobacter sp.* strain 7E (*P*7E), which grows at a broad range of salinities. Chapter 2 presents experimental results evaluating the establishment of the sea-ice bacterial community by means of selective enrichment of EPS. Chapter 3 presents field measurements indicating that biological components of the sea-ice brines, including bacterial cells and

EPS from mixed sources (algal and bacterial), are expelled onto the ice surface and wicked upwards into snow, experiencing different degrees of cell loss depending on environmental conditions. Analysis of seasonal (Winter and Spring) *in situ* temperature and brine salinity data indicated that fluctuation regimes were significantly more energetic on the snow surface than in the ice column (Chapter 4), with implications for the microbial sea-ice population. Laboratory experiments exposing model organisms to freezing under constant and fluctuating regimes showed higher susceptibility to fluctuations by the stenohaline *Cp*34H than for the euryhaline *P*7E, with *P*7E undergoing fragmentation during the course of the freezing regime. The presence of compatible solutes significantly reduced cell loss in *Cp*34H. A synthesis of the microbial solutions to the challenges imposed by the dynamic structure of sea ice is discussed in Chapter 5, and testable hypotheses for future research were identified. Supporting information and the implementation of a science education and outreach activity based on this work is presented in the Appendices.

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DEDICATION

To my parents Yolanda and Michel, who taught me the love for nature and science,
my sister Ana, who taught me not to accept things as they are,
and my husband Don, who taught me patience.

Chapter 1

INTRODUCTION

The fundamental properties of water provide defining elements of both oceanography and astrobiology. Liquid water has been recognized as essential for life as we know it, even if not all environments with liquid water are expected to sustain life (*Jones and Lineweaver*, 2010). Liquid water, though, can only exist in its pure form at temperatures above –22 °C (independent of pressure) and pressures above 6×10^2 Pa (independent of temperature). Because most of the universe is characterized by low pressures and temperatures, liquid water is, thus, rare in the universe. Ice, referring hereafter to water ice unless indicated otherwise, can exist over a broader range of temperatures and pressures, acquiring a different crystalline structure under each set of conditions (Fig. 1.1). Under terrestrial (Earth) conditions, the vast majority of ice is in the form of hexagonal ice (Ih), with cubic ice (Ic) possibly forming in the upper atmosphere from droplets of water at very low temperatures (*Mayer and Hallbrucker*, 1987; *Salzmann et al.*, 2011). In the universe, other forms of ice exist at the very low pressures associated with the interstellar medium ($\approx 10^{-15}$ Pa) and the high pressures associated with the interior of icy exoplanets (*Ehrenreich et al.*, 2006; *Valencia et al.*, 2007) and icy moons (*Billings and Kattenhorn*, 2005). Ice is common in the galaxy, having being detected in planets, moons, comets and the interstellar medium. Most of the ice on Earth, which exists as glacial (freshwater) ice over Greenland and Antarctica, formed from atmospheric snow with limited mineral and chemical impurities (*Deming and Eicken*, 2007). Earth's sea ice, which is by definition formed from seawater (which is rich in impurities), represents a frozen habitat distinguished from other forms of ice Ih by its mushy (two phases) porous structure, having a relatively high content of liquid water and,

thus, greater habitability (*Deming and Eicken*, 2007).

Saline ice at temperatures below the eutectic of its salt components, as found in Earth's sea ice, consists of a scaffolding of solid ice penetrated by a network of pores and channels filled with a concentrated saline solution often called brine. Life in saline ice is thus confronted by the dual challenge of low temperature (–2 to –40 °C) and high salinity (40 to 200 in the practical salinity scale), while surrounded by a crystalline ice wall. This two-component structure (ice and brine) is dynamic, with changes in its architecture determined by temperature. Saline ice at temperatures below the eutectic could presumably exist in subsurface portions of the ice cover on Jupiter's moon Europa. The ocean salinity of Jupiter's moon Europa is still unknown, but even under the lower-end estimates ($<$ 15 g salt kg^{-1} water *Hand and Chyba*, 2007) the formation of saline ice could still be possible, as is seen on the Baltic Sea on Earth, where saline ice forms from brackish water (salinities $<$ 9 g salt kg^{-1} water; *Granskog et al.*, 2006). Saturn's moon Enceladus is also likely to harbor a saline ocean (*Zolotov*, 2007; *Postberg et al.*, 2011), having thus the potential to form saline ice.

This dissertation considers several types of ice, including artificially grown saline ice (Chapter 2) and saline snow and snow of limited impurities (Chapter 3). It relies mainly, though, on the properties of natural Arctic sea ice (all chapters) to guide the investigation of microbial challenges and solutions to inhabiting a frozen environment characterized by a dynamic architecture. Of primary interest are strategies for surviving, and even thriving, under the combined conditions of low temperature, high salinity and entrapment into a porous icy matrix. Such strategies include the use of extracellular polymeric substances (EPS, Chapters 2, 3 and 5) and intracellular compatible solutes (Chapter 4). The astrobiological relevance of ice is addressed in this chapter, including a review of its potential role in the origin and evolution of life, and several observations that I made in the course of this investigation. Three main topics are addressed: the role of interstellar ice in the formation of organic molecules and their delivery to rocky planets; sea ice as an environment participating in the origin and early evolution of life; and the possibility of detecting microbial life in extraterrestrial cryospheres by recognizing biosignatures similar to those generated

by current extremophiles inhabiting frozen environments on Earth.

1.1 The role of interstellar ice in the origin of prebiotic molecules

Stars may be the most conspicuous components of our galaxy, but far from a perfect vacuum, the space between them is filled with gas and microscopic dust forming what is known as the interstellar medium (ISM). The mass of the ISM corresponds to about 10 % of the mass of the Milky Way, and is comprised mostly of gas (99 %) and a small fraction of micron-sized dust (*Karttunen et al.*, 2003). The ISM plays a relevant role in the life cycle of stars by providing the raw material from which new stars (and their planetary systems) form, and receiving the material ejected by dying ones. In spiral galaxies, like the Milky Way, gas and dust concentrate on the galactic plane forming a thin disk. The disk itself has an uneven distribution of gas and dust, delimiting regions of various sizes and different physical and chemical properties (*Ehrenfreund et al.*, 2002). Regions with the highest density (average $\geq 10^3$ H_2 molecules cm^{-3}) are known as dense molecular clouds. These regions are highly heterogeneous, with clumps and cores of higher density.

Molecular clouds are important from an Astrobiology perspective since they are the birth place of stars and planetary systems. Their temperatures range from 10 to 20 K (–253 to –263 °C) in cold quiescent clouds to 100–300 K (–173 to 27 °C) in hot molecular cores, with both regions characterized by a rich chemistry, dependent on the dominant temperature (*Charnley et al.*, 2002). Interstellar dust grains in dense, cold molecular clouds are frequently covered with water-ice mantles, and to a lower extent CO, CO_2 and methanol ice (*Whittet et al.*, 1996; *Pontoppidan et al.*, 2004). Dust grains play a relevant role in the interstellar chemistry given their shielding role against incoming radiation and the catalytic properties of grain surfaces. Adsorbed molecules or atoms may migrate across the grain surface and react with other chemical species; however, at 10 K (–263 °C), the only atoms with enough mobility to interact are H, D, C, O, N (*Ehrenfreund et al.*, 2002), a fact reflected in the chemical composition of grain-surface molecules. Molecules formed on grain surfaces include molecular hydrogen (H_2), water (H_2O), ammonia (NH_3), methanol

(CH_3OH), formaldehyde (CH_2O), ethanol (CH_3CH_20H), ketene ($H_2C{=}C{=}O$), acetaldehyde (CH_3CHO), vinyl cyanide (CH_2CHCN) and ethyl cyanide (CH3CH2CN) (*Charnley et al.*, 2002). Among them, methanol (CH_3OH) and ammonia (NH_3) can lead to increasing chemical complexity (*Ehrenfreund et al.*, 2002). The warm, high-density regions of hot molecular cores (10^6 – 10^8 H_2 molecules cm^{-3}, T $\sim$ 100 – 300 K) are usually associated with embedded high-mass protostars. The regions in and surrounding the hot molecular cores host the majority of large, prebiotically-relevant molecules detected so far (*Remijan et al.*, 2004), with a high abundance of saturated molecules such as water (H_2O), ammonia (NH_3), hydrogen sulfide (H_2S), methanol (CH_3OH) and methyl cyanide (CH_3CN) among others (*Kim et al.*, 2002; *Schöier et al.*, 2002).

Most of the cloud-originated molecules are likely to be destroyed in the accretion phase of the star as they experience the high temperatures of the inner pre-solar nebulae. Nevertheless, pristine materials synthesized in the interstellar cloud can survive on the outskirts of the accretion disk; in fact, the cyano-radical (CN), hydrogen cyanide (HCN), hydrogen isocyanide (HNC), carbon monosulfide (CS), aldehyde group (HCO^+), ethynyl radical (C_2H) and formaldehyde (H_2CO) have been reported in two protoplanetary disks (DM Tau and GG Tau). These molecules, after some degree of modification, can be delivered later to already formed planets in the form of comets: agglomerates of frozen gases, ices, and rocky debris (*Charnley et al.*, 2002; *Ehrenfreund and Menten*, 2002). Cometary ices would be more processed than interstellar ices, because they are expected to come from interstellar material that evaporated, underwent a phase of hot-core chemistry, and finally recondensed onto precometary grains (*Charnley et al.*, 2002). Comets could act, nevertheless, as "chemistry messengers" (*Winnewisser*, 1997), connecting the chemical composition of the interstellar medium to the potential for life to arise on the surface of rocky planets from the inner region of the planetary system.

Inner planetary bodies of our Solar System were depleted of volatiles at the first stages of planetary formation. Water and volatiles currently forming Earth's atmosphere and hydrosphere were delivered, at least partially, by icy planetesimals formed at a distance of 4 –

7 AU from the sun and sent towards the inner Solar System by the gravitational effect of giant planets (*Delsemme*, 1998; *Owen*, 1998). Volatiles that accumulated on the planetary surface gave rise to an atmosphere and, eventually, a hydrosphere. This process is considered key to the development of a favorable environment for the origin of life, because the presence of a fluid envelope provides essential compounds (such as water), a place for the accumulation of organic material, regulation of temperature and slows down later cometary particles, thus preventing the destruction (by pyrolysis) of their organic molecules during their impact (*Chyba et al.*, 1990). To investigate potential molecules possibly brought by icy planetesimals, *Kobayashi et al.* (2004) irradiated interstellar-ice analogues composed of methanol (CH_3OH), ammonia (NH_3) and water (H_2O) with UV and gamma radiation. This treatment resulted in the production of amino-acid precursors which, after being subjected to hydrolysis (as might have occurred if accumulated in a primitive ocean), were transformed into glycine, alanine, aspartic acid, β-alanine, and α- and γ- aminobutiric acid. Of these amino acids, the first three are of biological relevance. When similar analogues were irradiated with circularly polarized UV light, the resulting mixture of alanine was non-racemic, with a slight enrichment of the L-enantiomer, the isomer mostly used by terrestrial organisms (*De Marcellus et al.*, 2011). Similar experiments with UV irradiation resulted in the production of N-heterocyclic molecules with the potential to perform as precursors of biological cofactors (*Meierhenrich et al.*, 2005). With evidence for the role of interstellar and cometary ice in the synthesis and delivery of prebiotic molecules to Earth, the stage is set for more complex developments leading to the origin of life, which we consider next from a bottom-up and a top-down approach.

1.2 Ice and the origin of life: a bottom-up approach

Multiple mechanisms have been proposed to explain the origin of life on Earth. The development of a unified model, however, has been hindered by conceptual problems including lack of a minimal definition of "life" (*Fry*, 1995) and the use of inductive generalizations from experimental data (*Wächtershäuser*, 1997). Hypotheses that explain the origin of

particular components (e.g., lipidic membranes, genetic material, etc.) or processes (e.g., replication, encapsulation, etc.) under defined environmental conditions, though, have lead to apparently disconnected models. A recent approach by *Stüeken et al.* (2013), to integrate available models and information recognizes that the Hadean Earth (4600 to 4000 million years ago) hosted multiple environments, with each environment providing unique chemical and physical settings for particular processes to take place. According to *Stüeken et al.* (2013), the origin of life could have taken place by the interaction of components developed in different settings within a "global chemical reactor." Frozen environments likely contributed to the origin of life by providing molecules of prebiotic interest formed in the interstellar medium and delivered by comets, as described in the previous section, and possibly played additional roles if frozen environments were present in the Hadean Earth.

Among the possible forms of ice on Earth, saline ice, formed from water with salt impurities, is of particular interest given the pockets of liquid water (brine) in its interior. A brine channel network provides a large number of compartments and an extensive interior surface area, estimated at 10^{14} - 10^{15} compartments with an area of 10^5 - 10^6 m^2 per cubic meter of ice, depending on the temperature (*Trinks et al.*, 2005). These compartments, from one or two millimiters to less than 10 μm in size (also depending on the temperature), would be subject to seasonal and daily fluctuations in temperature, resulting in seasonal and daily fluctuations in the concentration of solutes, as addressed for modern Earth in Chapter 4. The presence of sea ice in at least some geographical regions of the Hadean Earth is still debatable but has not been ruled out. Indeed, its potential role in the origin of life was considered by *Stüeken et al.* (2013) highlighting the ability of saline ice to provide encapsulation, cyclical dehydration, and chemical gradients. This hypothesis is supported by experimental work on the abiotic synthesis of nucleotides (*Menor-Salván et al.*, 2009), as well as nucleotide and amino-acid polymerization (*Vajda and Hollosi*, 2001; *Monnard and Ziock*, 2008), in laboratory-generated saline ice formations.

In experiments addressing the origin of RNA, urea solutions frozen for three weeks under a reduced atmosphere (nitrogen/hydrogen/methane, 30:30:40) resulted in the synthesis

of nucleotide bases after exposure to electrical discharges and fluctuating temperatures (*Menor-Salván et al.*, 2009). *Trinks et al.* (2005) demonstrated that activated nucleotides (5'-adenylic[2-methyl]imidazolides) in the presence of a poly(U) template were able to polymerize when subjected to freezing under fluctuating temperatures for over a year; the resulting polymers had a chain length of up to 400 nucleotides and high levels of linkage fidelity, both relevant properties for the non-enzymatic replication of prebiotic RNA molecules. Additional experiments with artificial sea ice also showed that RNA had affinity for surfaces inside the ice, including the ice walls of the brine channel network and the salt crystals precipitated at the lowest temperatures (*Trinks et al.*, 2005), suggesting the additional role of sea ice, through its microstructure and internal salt crystals, as a potential scaffolding for polymerization. Regarding the origin of proteins, current living systems contain proteins exclusively from the L-enantiomer of a defined set of amino acids (as opposed to a racemic mixture, as expected to occur in abiotic environments). Experiments by *Vajda and Hollosi* (2001) demonstrated a possible role for the interaction between amino acids and ice crystals in the origin of chirality. After subjecting razemic mixtures of activated amino acids to polymerization for 9 d, there was a higher selection of the L-enantiomer in samples frozen at –18 °C than in those kept at room temperature.

The next section elaborates on the interactions between biomolecules and the ice crystal lattice with regards to the origin of life following a top-down approach (e.g. based on information about current organisms; *Forterre and Gribaldo*, 2007). Experimental work and field observations (described in Chapters 2, 3 and 5) also address the interactions between biomolecules and the ice crystal lattice, but from the perspective of microbial survival and adaptation in modern sea ice, an extreme environment for Earth.

1.3 Ice and the origin of life: a top-down approach

Earth's cryosphere, the portions of the planet where water is frozen, has experienced changes in mass, volume and extent through geological time. The geological record indicates that Earth experienced multiple glaciations, periods with extensive ice covers. In some cases

ice may have covered the complete surface of the planet in what is known as "Snowball Earth" episodes (*Kirschvink*, 1992; *Hoffman et al.*, 1998). Earth's contemporary cryosphere is changing due to the ongoing increase in planetary temperature projected to continue into the next century as a result of anthropogenic activity. The present Earth cryosphere harbors different ice formations, all of which contain microbial life (e.g., *Deming and Eicken*, 2007). These formations include freshwater ice (glacial ice sheets, mountain glacial ice, and perennial and seasonal snow), saline ice (sea ice and marine ice shelves), and permafrost (frozen soil). The structural differences between freshwater and saline ice lead to intrinsically different microbial ecosystems, such that comparison between the two types of ice can be used to good effect, including when presenting the topic of life in the cryosphere to the general public (Appendixes F, G and H). Sea ice supports the most robust and dynamic of the cryospheric ecosystems (*Deming and Eicken*, 2007; *Deming*, 2010). Microorganisms that inhabit sea ice show unique adaptations to thrive within the fluids of the ice matrix, despite low temperatures, high concentration of salts and exposure to strong fluctuations in these parameters, a major theme of this dissertation (Chapters 3 and 4). If life appeared, evolved and presently thrives in frozen environments or settings closely associated with ice, biological systems will likely carry imprints demonstrating their current or past ability to interact with the ice lattice, throwing light upon the origin and early evolution of life. Furthermore, the ability to measure the interactions between terrestrial organisms and ice will advance the possibilities to recognize biosignatures in extraterrestrial icy environments.

Some of the protective mechanisms used by microbial life in saline ice are based on close interactions between biological molecules and the salt crystals that precipitate within the ice as temperature drops. A relevant example is the change in eutectic temperature effected by amino acids as studied by *Chen et al.* (2005). The presence of certain amino acids in NaCl solutions shifts the temperature of the eutectic crystallization of the salt, or even inhibits the ability of the solution to have an eutectic point. These effects are ascribed to an interaction of the amino acids with the salts such as to prevent the formation of $NaCl{\cdot}H_2O$ / ice crystals. The same amino acids that shift the eutectic temperature or inhibit the ability to have an eutectic point, have also been associated with protection of thylakoid membranes

subjected to freezing.

Other protective mechanisms are based on the interactions between biological components and the ice lattice. Different facets of the ice lattice offer points for hydrogen-bond formation where biological molecules can adhere with different degrees of affinity (*Tasaki and Okada*, 2009). Current living organisms have exploited this ice-affinity property by producing ice-active proteins and polysaccharides. Ice active molecules have the ability to affect the formation and structure of ice, either by promoting or inhibiting ice formation or by controlling size and shape of the resulting ice crystals (*Wharton et al.*, 2005).

Ice-active proteins are widespread in different taxonomic groups, including bacteria (*Raymond et al.*, 2007, 2008), fungi (*Raymond and Janech*, 2009; *Kondo et al.*, 2012), unicellular (*Raymond et al.*, 2009; *Bayer-Giraldi et al.*, 2011) and multicellular plants (*Griffith and Yaish*, 2004) and animals (*Graether et al.*, 2000). Many of the ice-active proteins derive their properties from careful spacing and orientation of specific amino-acid residues resulting in a structure that mimics one or more of the planes of growth of the ice crystal. The group of amino acids that participates in ice-activity balances amino acids of hydrophobic character with those that have the potential to create hydrogen bonds with the ice. Ice-active proteins can thus interact preferentially with ice (through hydrogen bonds) than with liquid water (due to the presence of hydrophobic amino acids; *Sharp*, 2011; *Kondo et al.*, 2012, and references within). Arrays of threonine, or of threonine and alanine, are common, with threonine providing the potential to make hydrogen bonds and alanine providing the hydrophobicity (*Graether et al.*, 2000; *Sharp*, 2011). Glycine clusters have also been associated with ice activity (*Graham and Davies*, 2005).

As reviewed above, the interaction between organic molecules and crystalline surfaces, such as ice or salt, can be relevant to the origin of biological polymers. Crystalline surfaces can act as a scaffolding, preferentially polymerizing those monomers able to adhere to them. Monomers likely available at the origin of life include those considered as consensus primordial amino acids, such as those listed by *Trifonov* (2000). Trifonov's consensus list is based on 40 parameters including the simplicity of the molecule, experiments of abiotic

synthesis and theories on the origin and evolution of the genetic code, and selects as earlier amino acids: Gly, Ala, Val, Asp, Pro, Ser, Glu, Leu and Thr. A similar analysis based only on likely prebiotic yields (under different prebiotic scenarios) was developed by *Higgs and Pudritz* (2009), resulting in a similar set of the 10 most likely amino acids used in primordial peptides: Gly, Ala, Asp, Glu, Val, Ser, Ile, Leu, Pro and Thr. This set of primordial amino acids is also consistent with a model for the origin of the genetic code based on doublet codons such as described by *Copley et al.* (2005).

In Table 1.1 I compare Trifonov's list of consensus primordial amino acids with those known to display ice affinity in current proteins and those known to interact with the salt crystals within saline ice. A contribution of this dissertation is my observation that amino acids involved in ice affinity in current proteins tend to correspond to the consensus primordial amino acids, which is not the case for amino acids that offer cryoprotection through affinity with the salts (Table 1.1). Because amino acids with ice activity have the ability to interact with the ice crystal lattice, this finding strengthens the hypothesis that interaction between prebiotic molecules and the ice lattice played a role in prebiotic chemistry.

Polysaccharides from different clades of organisms also present examples of ice activity. The polysaccharide fraction of extracellular polymeric substances (EPS) from the bacterium *Bacillus thuringiensis YY529* prevents the formation of ice nuclei (*Yamashita et al.*, 2002), and the polysaccharide fraction of EPS from the marine bacterium *Colwellia psychrerythraea* strain 34H (*Cp*34H) is selectively retained in saline ice, indicating ice activity (as described in Chapter 2 and Appendix A; *Ewert and Deming*, 2011). Furthermore, ice activity has been found in EPS from the arctic sea-ice alga *Melosira arctica* (*Krembs et al.*, 2011), and ice-active glycolipids have been found in diverse plants and animals from polar and temperate regions (*Walters et al.*, 2009, 2011). The precise mechanism by which these polysaccharides interact with the ice is not yet known, but in the case of EPS from *Melosira arctica*, the ice activity was eliminated by exposing EPS to a heat treatment or to the hydrolyzing action of an N-Glycosidase, suggesting the ice activity was associated with a glycoprotein (*Krembs et al.*, 2011). I suggest here that the attachment of the N-glycan to the protein fraction,

which occurs on the Asn residue of a sequon (sequence of three amino acids) consisting of Asn-X-Thr/Ser (with X representing any amino acid except Pro, *Stanley et al.*, 2009), could be a target area responsible for ice activity since, as seen in Table 1.1, Asn, Thr and Ser are common amino acids associated with ice-affinity proteins. I have also recently observed that the capsular (cell-held) polysaccharide of *Cp*34H, as described by *Carillo et al.* (2013), is decorated with appropriate amino acids to infer ice activity and thus to explain why this organism and its relatives with similar capsular polysaccharides can be found in sea ice.

The presence of ice-active molecules, proteins and polysaccharides in solutions subjected to freezing has measurable effects on the physical properties of the resulting ice. EPS from *Cp*34H produced macroscopic changes in saline ice which, as discussed in Chapter 2 and Appendix A, were associated with greater retention of brine in the ice. EPS from *Melosira arctica* resulted in changes of both the macroscopic properties and microscopic structure of the ice, including a higher concentration of salt in the ice, a brine-channel network with increased connectivity, and brine inclusions of different shape (as quantified by an increase in their fractal dimension) (*Krembs et al.*, 2011). Ice-active proteins from other organisms have also been shown to change ice structure by altering growth of the ice crystals and, consequently, the structure of the brine channels (*Raymond*, 2011, and references therein). For instance, the antifreeze protein of *Fragilariopsis cylindrus*, a polar diatom, changes the individual growth of ice crystals, promoting dendritic growth of the ice with a consequent increase in porosity (*Bayer-Giraldi et al.*, 2011). As discussed in Chapter 3, brines in newly formed sea ice are expelled to the ice surface and absorbed by frost flowers and snow. Because sea-ice brines are likely to contain these ice-active molecules and both snow and frost flowers are formed of ice crystals, the presence of ice-active substances may have a measurable effect on the physical properties of snow and frost flowers, including their temporal evolution, desalination, and capacity for wind dispersal, each of which represents an unexplored but testable hypothesis which I address on Chapter 3.

The modification of a physical environment by a biological molecule, such as the changes in ice structure attributable to ice-active proteins and polysaccharides, can be considered

a biosignature. The possibility to detect biosignatures based on biological interactions with the ice lattice depends on the development of suitable probes. *In situ* microscopical examination of extraterrestrial ice, aiming to evaluate its microstructure, could determine interconnectedness and fractal dimension of brine pores, which are associated with biologically altered liquid inclusions on Earth (*Krembs et al.*, 2011). Technology for collection and *in situ* microscopical evaluation of extraterrestrial ice, though, has not been developed. Another approach could be based in macroscopic changes associated with the changes in microstructure. For saline ice, in particular, the dielectric permittivity measured at MHz frequencies is related to the geometry and volume fraction of the brine inclusions, whereas the direct current (dc) conductivity is related to their geometry and connectivity (*Pringle and Ingham*, 2009). If the presence of ice-active biological molecules could be consistently associated with measurable changes in permittivity and/or dc conductivity, such parameters could be used to perform a preliminary evaluation of areas of astrobiological relevance, or perhaps used as biosignatures in regions dominated by ice Ih (the dominant form of ice on Earth). Surface landers could evaluate dc conductivity by deploying electrodes performing resistivity soundings, as is done on Earth's sea ice (*Pringle and Ingham*, 2009); dielectric permittivity could be measured by reflection of MHz pulses from the surface of the extraterrestrial ice, as described in *Pringle and Ingham* (2009) for Earth. Extrapolation of the use of this potential biosignature to extraterrestrial surfaces dominated with ice of a different crystalline structure (e.g. ice Ic – XV) would need further evaluation.

1.4 Conclusions

Water ice may have participated in different processes leading to the origin and early evolution of life on Earth as a result of interactions between prebiotic molecules and ice crystalline structure. Based on information available in the literature, key processes were recognized as follows. First, the surface of interstellar ice grains could participate in the formation of organic molecules of prebiotic relevance as determined by observations of organic molecules in star forming regions and models of surface chemistry. These molecules could have been

delivered to planetary surfaces by icy planetesimals. Second, if saline ice was present on the Hadean Earth, it could have provided a large number of compartments with a wide surface area of exposed crystal lattice that could have acted as scaffolding for the polymerization of primordial biological molecules. The ability of biological molecules to interact with the ice crystal lattice exists in extant ice-active proteins and polysaccharides, with amino acids involved in ice activity closely corresponding with consensus primordial amino acids. This observation strengthens the hypothesis that the ice lattice played a role in the origin of life. Finally, the modification of the ice microstructure resulting from the interaction between biological molecules and the ice lattice may provide a potential biosignature, if measurable in extraterrestrial frozen planets.

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Table 1.1: Amino acids in primordial peptides and extant ice-affinity proteins

Amino acid [a]	Primordial [b]	Ice activity [c]	Cryoprotection [d]
Gly	Yes	Yes (4)	In combination [e]
Ala	Yes	Yes (1, 6, 8)	In combination
Val	Yes	Yes (7, 9)	–
Asp	Yes	Yes (5)	NA [f]
Pro	Yes	–	Yes
Ser	Yes	Yes (7, 9)	In combination
Glu	Yes	–	NA
Leu	Yes	–	–
Thr	Yes	Yes (1, 2, 3, 5, 7, 8, 9)	Yes
Ile	Yes	–	–
Arg	–	–	Yes
Asn	–	Yes (1, 5)	–
Lys	–	–	Yes
Gln	–	–	NA
Cys	–	–	NA
His	–	–	Yes
Phe	–	–	–
Met	–	–	–
Tyr	–	–	NA
Trp	–	–	NA

[a] Listed by consensus chronological order of incorporation in the early genetic code according to *Trifonov* (2000). See details in the text.

[b] Listed amongst the 10 most likely primordial amino acids by *Higgs and Pudritz* (2009).

[c] Identified as responsible for conferring ice activity to selected proteins as described by (1) *Dalal and Sönnichsen* (2000), (2) *Graether et al.* (2000), (3) *Leinala et al.* (2002), (4) *Graham and Davies* (2005), (5) *Garnham et al.* (2008), (6) *Graham et al.* (2008), (7) *Middleton et al.* (2009), (8) *Sharp* (2011) and (9) *Middleton et al.* (2012).

[d] Confers cryoprotection by inhibiting or shifting the eutectic in saline ice as described by *Chen et al.* (2005).

[e] Cryoprotective effect evident only in combination with other substrates.

[f] Not assayed.

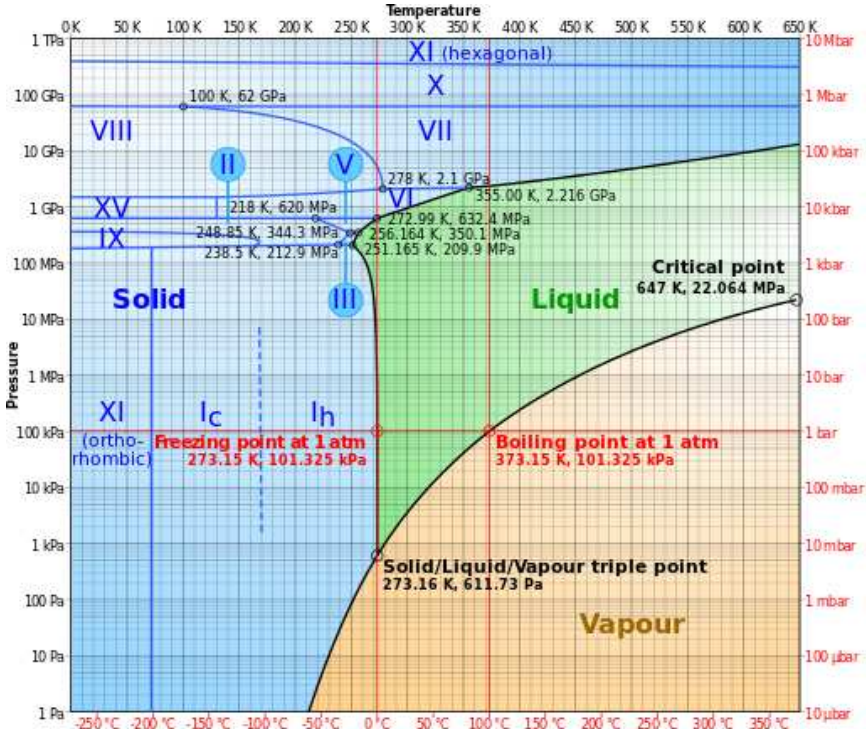

Figure 1.1: Phase diagram of water, retrieved from Wikimedia Commons (*User:Cmglee*, 2013). Roman numbers indicate different crystalline structures of ice, with ice Ih standing for hexagonal and Ic for cubic ice.

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Chapter 2

Selective retention in saline ice of extracellular polysaccharides produced by the cold-adapted marine bacterium *Colwellia psychrerythraea* strain 34H [1]

Abstract

The retention of salts in laboratory-grown ice was compared to the retention of extracellular polysaccharide substances (EPS) produced by the cold-adapted marine gammaproteobacterium, *Colwellia psychrerythraea* strain 34H. Saline ice was formed, by means of a cold-finger apparatus, from artificial seawater solutions containing either native dissolved EPS from *Cp*34H, the same EPS but heat-treated, or dissolved EPS from the uninoculated growth medium. Results indicated that only the native (unheated) EPS of *Cp*34H was retained preferentially in the ice. Temperature and volumetric measurements of the ice further suggested a link between the heat-labile fraction of this EPS of marine bacterial origin and potential habitat alteration. Bacterial EPS may join algal EPS in our understanding of how extracellular polymers help to establish and sustain the microbial community that inhabits sea ice.

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2.1 Introduction

Extracellular polysaccharide substances (EPS) [2] are complex polymers commonly produced by microbes (*Decho*, 2000) and composed mainly of neutral sugars with variable fractions of uronic acids, sulfates, amino sugars and proteins (*Mancuso Nichols et al.*, 2005). Some of these polymers are tightly bound to the microbial cell, whereas others are loosely attached, forming a more dispersed 'slime' (*Decho*, 1990). Generally, two fractions are recognized; particulate EPS (pEPS) corresponds to the EPS fraction > 0.4 μm and dissolved EPS (dEPS) to that < 0.4 μm. Among many roles, EPS act as ligands for metal cations, participate in the formation of marine aggregates and contribute to biogeochemical cycles (*Passow*, 2002; *Mancuso Nichols et al.*, 2005).

In sea ice, EPS produced by algae and bacteria have been considered to provide cryoprotection within the ice matrix (*Krembs et al.*, 2002; *Collins et al.*, 2008; *Marx et al.*, 2009; *Krembs et al.*, 2011) as well as a mechanism for organisms, particularly EPS-coated algae, to entrain selectively into the ice (*Meiners et al.*, 2003; *Riedel et al.*, 2007) and remain anchored within it (*Krembs and Deming*, 2008). Here we consider, via controlled laboratory tests, the entrainment of bacterial dEPS into growing sea ice. The entrainment of natural exopolysaccharides from Arctic surface seawater is discussed in Appendix B.

During the freezing process, sea ice retains a fraction of the microbial organisms, particles, salts and other solutes present in the source water; they are retained within liquid inclusions in the ice, brine-filled pores and channels (*Petrich and Eicken*, 2010) which constitute the habitable portion of the ice (*Junge et al.*, 2001). A larger fraction of solutes and particles, though, is expelled back into the water column. The proportion by which solutes are retained in sea ice can be described by 'effective segregation coefficients', the ratio between the concentration of solutes in the ice and the concentration of solutes in the source liquid from

[2] EPS refers to extracellular polysaccharide substances when the measuring technique quantifies only the sugar fraction, as in this chapter. The same abbreviation is also used for the more generic term: extracellular polymeric substances (as in Chapter 5), encompassing the greater chemical complexity of these polymers (*Wingender et al.*, 1999).

which the ice forms (*Eicken*, 2003). Effective segregation coefficients, k_{eff}, were initially defined for salts in seawater as:

$$k_{effs} = \frac{S_{ice}}{S_{source}}$$

where S_{ice} is the salinity of the melted ice (bulk salinity) and S_{source} the salinity of seawater away from the ice-water interface. Extending this definition to other solutes allows, for example, calculation of the segregation coefficient of exopolymers:

$$k_{effe} = \frac{[EPS]_{ice}}{[EPS]_{source}}$$

where $[EPS]_{ice}$ is the concentration of exopolymers in the melted ice, and $[EPS]_{source}$ is the concentration of exopolymers in the source solution.

Solutes in seawater will segregate proportionally to dissolved salts unless they interact with the ice crystals, in which case the segregation of the interacting solute will diverge from the segregation of the salts. To assess this divergence, segregation coefficients of different solutes can be normalized to generate an enrichment index (*Gradinger and Ikävalko*, 1998), defined as:

$$I_s = \frac{k_{effx}}{k_{effs}}$$

where k_{effx} is the segregation coefficient of the solute x. If $I_s = 1$, solute and salts are expelled in the same proportion and the solute is conservative. If $I_s > 1$, the solute is selectively retained (enriched) in the ice. In ocean environments that experience seasonal freezing, enrichment indices have been calculated from measurements of the concentrations of particles, cells and EPS in the ice and in underlying seawater (*Garrison et al.*, 1989;

Meiners et al., 2003; *Riedel et al.*, 2007). An alternative approach to compare concentrations of salts and solutes without considering concentrations in the source water is described in Appendix A.

The enrichment mechanism for organic 'impurities' (including microbes) present in seawater has usually been ascribed to a non-selective physical process, whereby suspended ice crystals, known as frazil ice, rise through the water column to consolidate an ice layer at the surface and collect particulate matter indiscriminately as they ascend by a filtering mechanism (Garrison and others, 1983, 1989). Evidence for a selective mechanism in the enrichment of particles and microorganisms, affected by cell size and possibly EPS coatings, comes from enrichment indices calculated for a variety of organisms in new Arctic sea ice (*Gradinger and Ikävalko*, 1998; *Riedel et al.*, 2007) and for bacteria and algae in artificial sea ice (*Grossmann and Gleitz*, 1993).

Algae have been reported early and often as enriched in sea ice (e.g., *Grossmann and Gleitz*, 1993; *Gradinger and Ikävalko*, 1998; *Riedel et al.*, 2007), with larger algal cells (>5 mm) presenting a higher average index ($I_s = 63.0$) than smaller algal cells ($I_s = 4.6$) or bacteria ($I_s = 5.1$) as reported in the most recent study (*Riedel et al.*, 2007). *Meiners et al.* (2003) reported an exception for pennate diatoms in nilas ice ($I_s = 0.5$).

Bacteria, on the other hand, were not reported as enriched in early studies of this subject (*Grossmann and Gleitz*, 1993; *Grossmann and Dieckmann*, 1994), and, when enrichment was reported later, the indices were always lower than those of algae (*Gradinger and Ikävalko*, 1998; *Riedel et al.*, 2007). As a result, the process of bacterial enrichment has been attributed to their pre-existing associations with larger cells (*Grossmann and Gleitz*, 1993; *Grossmann and Dieckmann*, 1994), with algal EPS (*Riedel et al.*, 2007) or with exopolymer particles (EPS > 0.4 μm) of indeterminate origin (*Meiners et al.*, 2003). In the cases where bacterial enrichment was not observed, the bacterial population of the ice had been inferred from the bacterial population measured in brines collected by centrifugation (as opposed to bulk sea-ice melting), an approach that would bias results towards a lower enrichment index if bacteria had remained within the ice matrix. The latter scenario is plausible given

that some bacteria produce ice-affine substances that remain attached to the ice fraction after brine removal by centrifugation (*Raymond and Fritsen*, 2000).

EPS have also been reported as enriched in sea ice, with the average index for particulate EPS (> 0.4 μm, of indeterminate origin) being of intermediate value and correlating positively with the enrichment of both algal cells and bacteria (*Meiners et al.*, 2003; *Riedel et al.*, 2007). These results have suggested that cellular coatings of EPS or EPS particles may play a role in the selective entrainment (or retention) of at least some microorganisms in the ice (*Meiners et al.*, 2003; *Riedel et al.*, 2007). The enrichment of dissolved organic materials, also observed in new sea ice (*Giannelli et al.*, 2001; *Thomas et al.*, 2001), suggests that dissolved EPS (< 0.4 μm) not yet examined in this way may have a similar fate.

The high enrichment indices for particulate EPS and (assumed) EPS-coated cells may be due to interactions between the extracellular polymers, or some component of them, and the ice crystals. Changes in ice-crystal morphology have been associated with the interaction between ice-affine molecules, particularly proteins, and ice crystals (*Raymond and Fritsen*, 2000). Sea-ice growth experiments in which the pore morphology was significantly altered in ice enriched with algal EPS relative to EPS-free ice (*Krembs and Deming*, 2008; *Krembs et al.*, 2011) thus provide support for linking ice affinity to ice enrichment. In these studies, the ice-altering property of algal EPS has been ascribed to a glycoprotein component, although ice activity has also been recently observed in polysaccharides with no detectable protein fraction (in xylomannans from insects; *Walters et al.*, 2009).

Perhaps because the majority of EPS found in polar sea ice is produced by algae (*Krembs et al.*, 2002; *Meiners et al.*, 2003; *Riedel et al.*, 2006), few studies have addressed the potential role of bacterially produced EPS in the entrainment of these smaller microorganisms into sea ice. Information from other (warmer) environments, however, shows that bacterial EPS can bind directly to mineral surfaces, altering their crystalline structures, or can develop strong complexes with inorganic ions, preventing mineral crystallization. In the former case, evidence suggests that a protein component of the EPS mediates the EPS-mineral interaction (*Kawaguchi and Decho*, 2002; *Perry et al.*, 2005). In the latter case, the presence

of bacterial EPS inhibited calcium carbonate precipitation (*Dupraz et al.*, 2009), an EPS-mineral interaction that, if translatable to subzero temperatures in brine, may be significant to the fate of CO_2 in sea ice (*Delille et al.*, 2007; *Rysgaard et al.*, 2007).

Moreover, some bacteria are known to produce proteins that interact at the molecular level with ice crystals (*Wilson et al.*, 2006; *Raymond et al.*, 2008) and, in non-saline laboratory systems, the selective partitioning of organic solutes into ice has been used to indicate ice affinity in proteins (*Marshall et al.*, 2004). Of particular relevance to this study is the recent isolation of an ice-binding protein from the spent culture medium of an Antarctic sea-ice bacterium of the genus *Colwellia* (*Raymond et al.*, 2007), suggesting that EPS produced by cold-adapted marine bacteria may include a protein fraction interactive with ice crystals that might contribute to the retention of bacterial cells in sea ice.

Here we propose that bacteria may be enriched in sea ice not only by their interaction with algae or algal EPS but also by the interaction of their own EPS with growing sea-ice crystals. To address this hypothesis, we examined the retention in artificial sea ice of EPS produced by the cold-adapted marine gammaproteobacterium, *Colwellia psychrerythraea* strain 34H (*Cp*34H), a genus and species often found in cold seawater and sea ice (*Deming*, 2010). In a departure from studies of particulate EPS (pEPS), we examined the dissolved (< 0.4 μm) fraction of EPS (dEPS). The goal was to determine whether or not these dEPS are selectively retained in the ice and, if so, whether that selective retention is attributable to a specific component of the EPS. We tested this hypothesis by determining and comparing the enrichment indices of three sources of EPS: EPS present in the spent culture media of *Cp*34H; a split of that same EPS subjected to heating to denature its heat-labile components; and EPS present in the uninoculated (also pre-heated) culture medium (derived from yeast extract). We also measured temperature and volume of artificial ice formed from these solutions, for an initial investigation of potential physical alteration of the ice by EPS of bacterial origin. Additional experiments comparing different freezing rates are described in Appendix A. Preliminary field experiments addressing retention of natural pEPS from Arctic winter surface seawater are described in Appendix B.

2.2 Methods

2.2.1 Source solutions

Ice was grown in triplicate experiments, completely randomized, from three source saline solutions containing high molecular weight polymers of different origin (Fig. 2.1). To obtain the first source solution, hereafter called native exopolymers or simply exopolymers, *C. psychrerythraea* strain 34H was grown at 2 °C, with shaking, in Marine Broth 2216 (Difco Laboratories) diluted to half organic strength with artificial seawater (ASW) as in *Marx et al.* (2009), until the culture reached maximum optical density (0.74 at A_{600}). The ASW contained the four major seawater salts (0.4 M NaCl, 9 mM KCl, 26 mM $MgCl_2$, 28 mM $MgSO_4$) and KH_2PO_4 buffer (salinity[3] 32, pH 7). For all experiments, a cell-free solution of exopolymers was obtained from the batch culture by low-speed centrifugation (20 min, 3000 g, 2 °C) to pellet most of the cells, and filtration of the recovered supernatant (0.45 μm filter, Millipore-HAWG 047 00) to remove cells remaining in suspension (according to *Huston et al.*, 2004) as well as pEPS. This gentle method for cell removal, validated microscopically during protocol development, reduces the risks of cell lysis, leakage of intracellular molecules, or disruption of macromolecules by collecting only the loosely attached exopolymers but not the capsular EPS tightly bound to the cells (*Nielsen and Jahn*, 1999). Furthermore, as the filtration step excludes what is typically considered the particulate fraction of EPS, the EPS examined here corresponds to the dissolved fraction of loosely attached 34H exopolymers.

The second source solution, called heat-treated (HT) exopolymers, was a split of the first solution subjected to 90 °C for 10 min (before storage at 2 °C). These heating conditions denature the secondary structure of proteins (even in the presence of hydrocolloids; *Ibanoglu*, 2005), but should not affect the concentration of polysaccharides given the short exposure time (according to rate constants of depolymerization for polysaccharides in *Lai et al.*, 2000.

[3]When referring to seawater, or solutions with seawater compositon, salinity values will be expressed in practical salinity units. When referring to NaCl saline solutions, concentration will be expressed in ppt.

The third source solution, called broth, was a split of the uninoculated medium used to culture *Cp*34H. This Marine Broth 2216, diluted to half organic strength, had been autoclaved (protein-denaturing conditions) prior to use.

All three source solutions were dialyzed with pleated dialysis tubing (3500 MWCO) in ASW for 2 days at 4 °C to ensure that all polymers tested were above a minimum size. After dialysis, 200 mL samples of the solutions were stored frozen at –15 °C until use.

Ice was generated using a cold-finger apparatus (Fig. 2.2a), first described by *Kuiper et al.* (2003) for recovering ice-affine proteins from freshwater solutions, modified for use with saline solutions (i.e., with corrodible metal parts being replaced with glass). The apparatus consisted of a glass tube connected to a circulating bath that kept antifreeze in the tube at the desired temperature, which in this study was –5 °C (Fig. 2.2a) as representative of sea-ice-forming conditions in the Arctic fall. Samples were placed in a 250 mL beaker immersed in a saline-water ice bath (salinity of 30) that kept the external temperature at –1.5 °C. For each experiment the cold finger was placed into the sample, and ice allowed to form, for a period of 6 h. The temperature of the unfrozen source solution was monitored during this period (Fig. 2.2b). Experiments were conducted under sterile conditions and using aseptic techniques to the extent possible in a 4 °C cold room, which helped to maintain reproducible experimental conditions.

At the end of each experiment, the cold finger was removed from the remaining liquid solution (Fig. 2.2c) and the ice was released from it. The ice was immediately photographed from three different angles (Fig. 2.2d): the top (Fig. 2.3a); and each of two sides (Fig. 2.3b and c). The top photography was used to verify circularity. The contours of the lateral photographs were extracted manually using an open-source vector graphics editor (Inkscape), and the volume was integrated using circular slabs of 0.125 cm thickness (Fig. 2.3d). To account for the small asymmetries in the ice hemispheres evidenced by the top photograph, the volumes calculated from the two lateral photographs were averaged. The volume occupied by the cold finger itself (2.4 cm^3), calculated from measured dimensions, was subtracted from the total.

The ice was then allowed to melt at room temperature. Bulk salinity, volume and EPS content were determined for both the melted ice and for the liquid solution that had remained unfrozen throughout the experiment. Salinity was measured at room temperature with a hand-held YSI-30 conductivity meter (accuracy of ± 0.1). EPS was measured as total concentration of hexoses, expressed as glucose equivalents, by the phenol sulphuric acid assay, as described by *Dubois et al.* (1956). To determine whether or not the liquid inclusions of the ice we grew contained exopolymers disproportionately to the salt also retained, we calculated the segregation coefficients, keff_s and keff_e, and the enrichment index, I_s, for EPS.

2.3 Results and discussion

Because growing ice rejects the salts present in a source solution (*Eicken*, 2003), k_{effs} should always be < 1.0. In our experiments, we observed an average value of k_{effs} of 0.39 ± 0.01 (± standard error, n = 3) in all three treatments (Table 2.1). There was no significant difference among treatments (p = 0.88, Kruskall-Wallis test, nonparametric test for three independent variables), indicating that salts were rejected similarly from the ice grown in all three cases.

As discussed above, if EPS in the source solution are expelled from the growing ice in the same proportion as the salts, then k_{effe} should be similar to k_{effs}; if EPS are retained preferentially in the ice, then k_{effe} should be greater than k_{effs}. Our experimental results show that k_{effe} for the HT exopolymers (0.38 ± 0.1) and the broth (0.40 ± 0.1) were similar to k_{effs} (0.39 ± 0.01), indicating no preferential retention of EPS. In contrast, the native (unheated) bacterial exopolymers had a higher k_{effe} of 0.63 ± 0.1 (Table 2.2), indicating their tendency to be retained in the ice. Using a Mann-Whitney U test (nonparametric test for small datasets, to compare two independent variables), we determined that the k_{effe} values for the HT exopolymers and the broth did not differ significantly (p = 0.83), suggesting that these two types of exopolymers segregated from the growing ice in a similar way. The k_{effe} of the native exopolymers, however, differed from the k_{effe} of the HT

exopolymers and the broth (p = 0.13), indicating with an 87 % confidence level that the native bacterial exopolymers segregated differently in the saline ice than in either the HT or the broth exopolymers.

The selective retention of EPS is quantified by I_s (Table 2.2), with $I_s > 1$ indicating selective retention. Neither the I_s values for EPS in the HT exopolymers ($I_s = 0.97 \pm 0.23$) nor the broth ($I_s = 1.02 \pm 0.14$) were significantly greater than 1.0 (one-tailed t test, 57 % level, p = 0.43). The only enrichment index that could be considered greater than 1 (with a confidence level of 89 %, p = 0.11) was for the native bacterial exopolymers ($I_s = 1.61 \pm 0.30$).

From these results we suggest that the loosely attached EPS produced by *Cp*34H can be retained preferentially in sea ice and that such retention is attributable to a heat-labile fraction of the EPS. If the selective retention of EPS from *Cp*34H can be generalized, this ice-adaptive characteristic could be exploited in the environment. Bacteria could use their EPS to associate directly with frazil ice crystals as they rise through the water column, or to be retained in the sea ice once formed, without depending on an association with algae, other larger particles or algal EPS. In a laboratory setting, selective retention of EPS may be exploited to develop physical isolation methods of such polymers (e.g. by freeze selection) without altering their chemical properties, as has been done for proteins (*Kuiper et al.*, 2003).

The heat-labile fraction of the exopolymers responsible for their selective retention in the ice may correspond to a protein fraction with ice-affinity properties. EPS from Antarctic bacterial isolates (near relatives of our test organism) have been shown to contain variable levels of protein, in some cases reaching 50 % of the total EPS composition (*Mancuso Nichols et al.*, 2005). Furthermore, ice binding proteins have been described for a number of cold-adapted microorganisms including sea-ice bacteria (*Raymond et al.*, 2007), glacial-ice bacteria (*Raymond et al.*, 2008), sea-ice diatoms (*Janech et al.*, 2006) and snow molds (*Hoshino et al.*, 2003); the ice-binding domain of the protein is similar in all cases. Because *Colwellia psychrerythraea* strain 34H does not have a gene encoding this ice binding protein

(*Raymond et al.*, 2007), the biochemical basis for the detected EPS enrichment is not yet clear. If the heat-labile fraction responsible for the selective retention of the native exopolymers from *Cp*34H is a protein with ice affinity, it may represent a different class of ice binding protein not yet identified genomically for *Cp*34H (*Methé et al.*, 2005). The ice-affinity property may also be dependent on the polysaccharide material itself, as happens for the thermal hysteresis-producing antifreeze from the freeze-tolerant beetle *Upis ceramboides* (*Walters et al.*, 2009) and suggested by the structure of *Cp*34H capsular polysaccharide (*Carillo et al.*, 2013).

An alternative explanation for the observed segregation coefficients is that exopolymers from the various sources were differentially expelled from the ice due to (1) differences in size or three-dimensional configuration of the polymers, or (2) changes in the structure of the ice matrix (e.g. more tortuous channels) due to interaction between the heat-labile component in the native exopolymers and the ice crystals. Our dialysis step, however, ensured that all solutions contained a similar concentration and size spectrum of polymers, all above 3500 Da (Table 2.2). Although we cannot rule out the possibility that differences in the three-dimensional configuration of the polymers influenced polymer expulsion from the ice, if changes in the physical microstructure of the ice contributed to a differential expulsion or retention of the polymers, those changes themselves would be attributable to the presence of ice-interactive polymers.

As a final point, note that molecules with surface affinity can modify the structure of the crystals to which they attach (*Kawaguchi and Decho*, 2002; *Perry et al.*, 2005). In particular, ice-binding proteins produced by several different microbes, including sea-ice bacteria, are known to modify the structure of ice crystals (*Raymond et al.*, 2007; *Kawahara*, 2008). A similar function for bacterial extracellular polysaccharides is not yet clear, but there is evidence to suggest that ice-algal EPS contain a glycoprotein fraction responsible for altering the pore morphology of sea ice (*Krembs et al.*, 2011). To address the potential alteration of sea-ice properties by native exopolymers from *Cp*34H, we examined two physical parameters in our experiments: the change in temperature of the source solution over the course of the

ice growing period, reflecting the temperature gradient in the ice, and the volume of ice that formed.

Although more detailed work is needed, both sets of results suggest that preferential retention of bacterial exopolymers may be associated with an increase in the liquid fraction of the ice. Specifically, the temperature change in the source solution over the 6 h experiment was similar ($p = 0.57$, Mann-Whitney U test) between the broth ($\delta T = 0.4 \pm 0.2$) and HT exopolymer solutions ($\delta T = 0.4 \pm 0.1$) but was lower for the native exopolymers ($\delta T = 0.1 \pm 0.1$; $p = 0.13$, Mann-Whitney U test) as seen in Table 2.3. The smaller change in the solution of native exopolymers suggests a stronger temperature gradient through the ice, which can be explained by a lower thermal conductivity due to an increased liquid fraction (liquid brine has lower thermal conductivity than frozen water; Pringle and Ingham, 2009). To evaluate alternative explanations (e.g., convection in the brine pores) would require detailed information on differences in ice-brine distribution.

Lower thermal conductivity is also associated with higher ice density (*Pringle and Ingham*, 2009). Although we did not measure the density of the ice directly, comparison of our ice volume measurements (bulk and unmelted) suggests a higher density in the ice formed from the source solution of native exopolymers. The bulk volume (pore water plus melted ice) of the melted ice (Table 2.3) agreed to within errors ($p = 0.49$, Kruskal-Wallis test) for the three test solutions. Because ice is less dense than water, we expected the volume of the unmelted ice, determined photographically, to be uniformly greater than the volume of melted ice. The photographic estimates, however, indicated differences between the native exopolymer experiment (smaller volume) and the other treatments ($p = 0.05$, Mann-Whitney U test), with no difference between the HT and broth exopolymers ($p = 0.83$, Mann-Whitney U test; Table 2.3). Although formation of higher-density ice from the solution of native exopolymers accounts for this difference, confirming that the results represent differences in the liquid fraction of the ice requires more detailed work.

Overall, the selective retention of native exopolymers produced by *Cp*34H and their apparent association with physical changes in the ice suggest that bacteria, like algae, have the

ability to modify the sea-ice environment and make it more habitable by increasing its liquid fraction. This finding agrees with previous evidence that algal EPS increases sea-ice habitability on the microscopic scale (*Krembs and Deming*, 2008; *Krembs et al.*, 2011) and that bacterial production of EPS in winter sea ice, in the absence of ice algae, serves a cryoprotectant function (*Collins et al.*, 2008). Although algal EPS production dominates during the biologically productive seasons in polar regions and thus may be expected to mediate physical alterations of sea ice on a larger scale, bacterial EPS production may nevertheless alter the physical properties of sea ice on a scale beneficial to the smallest of organisms that live throughout the ice in all seasons.

2.4 Conclusions

When artificial sea ice was formed from a solution containing dissolved native EPS from *Colwellia psychrerythraea* strain 34H, the EPS tended to be retained selectively in the ice compared to the salts also entrained ($I_s = 1.6 \pm 0.3$). The ability to be retained in the ice was lost if the exopolymers were first heated. This ability was also absent from other polysaccharides present in the culture medium used to grow *Cp*34H. We interpret these results as an ice-affinity property of the EPS likely due to a protein component, though the nature of that component is not yet identified and changes in the three-dimensional structure of the exopolymers cannot be ruled out. Our results also suggest differences in the liquid fraction of the ice formed from the solution of native bacterial exopolymers, but more work is needed to understand in detail the effects of bacterial EPS on the physical properties of ice and their specific roles as cryoprotectants and possibly osmoprotectants. The selective retention of bacterial EPS has implications for the segregation and retention of microorganisms in ice and for beneficial habitat alteration on the microscale. It may also contribute to developing methods for isolating chemically unaltered exopolymers for applications in cryopreservation or bioremediation.

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Table 2.1: Salinity of EPS-containing solutions at the start and end of an ice-growing experiment, with calculated segregation coefficients for the salt (k_{effs})

Source solution	Solution (start) [a]	Ice [b]	Solution (end) [c]	k_{effs}
Exopolymers	35.5 ± 0.4	14.0 ± 0.3	38.8 ± 0.5	0.39 ± 0.01
HT exopolymers	36.2 ± 0.3	14.2 ± 0.2	39.0 ± 0.1	0.39 ± 0.01
Broth only	35.7 ± 0.3	14.0 ± 0.3	38.5 ± 0.3	0.39 ± 0.01

[a] All salinity measurements are mean ± S.E. (n = 3).
[b] Ice formed after 6 h (melted for analysis).
[c] End refers to unfrozen solution remaining at 6 h.

Table 2.2: EPS concentration in source solutions at the start and end of an ice-growing experiment, with calculated segregation coefficients for exopolymers (k_{effe}) and enrichment indices for exopolymers relative to salt (I_s)

Source solution	Solution (start) [a]	Ice [b]	Solution (end) [c]	k_{effe}	I_s
Exopolymers	24.1 ± 0.5	15.3 ± 2.8	27.9 ± 0.7	0.63 ± 0.1	1.61 ± 0.30
HT exopolymers	22.6 ± 2.4	8.56 ± 1.9	25.5 ± 2.9	0.38 ± 0.1	0.97 ± 0.23
Broth only	25.8 ± 1.2	10.3 ± 1.3	36.0 ± 2.5	0.40 ± 0.1	1.02 ± 0.14

[a] All EPS measurements are glucose equivalents μg mL^{-1} (mean ± S.E., n = 3).
[b] Ice formed after 6 h (melted for analysis).
[c] End refers to unfrozen solution remaining at 6 h.

Table 2.3: Change in temperature of source solutions over 6 h (ΔT = |T at 6h – T initial |) and volume of ice grown by 6 h

Source solution	ΔT of solution over 6 h (°C)[b]	Volume of melted ice at 6 h (mL)[c]	Volume of unmelted ice at 6 h (cm^3)[d]
Exopolymers	0.1 ± 0.1	40 ± 2	45 ± 2
HT exopolymers	0.4 ± 0.1	43 ± 1	57 ± 4
Broth only	0.4 ± 0.2	40 ± 2	53 ± 3

[a] All values are mean ± S.E. (n = 3).
[b] Temperature was measured while the cold finger was in the solution.
[c] Volume of melted ice was measured at room temperature.
[d] Volume of unmelted ice was extrapolated from photographs taken immediately upon removal of the ice from the source solution.

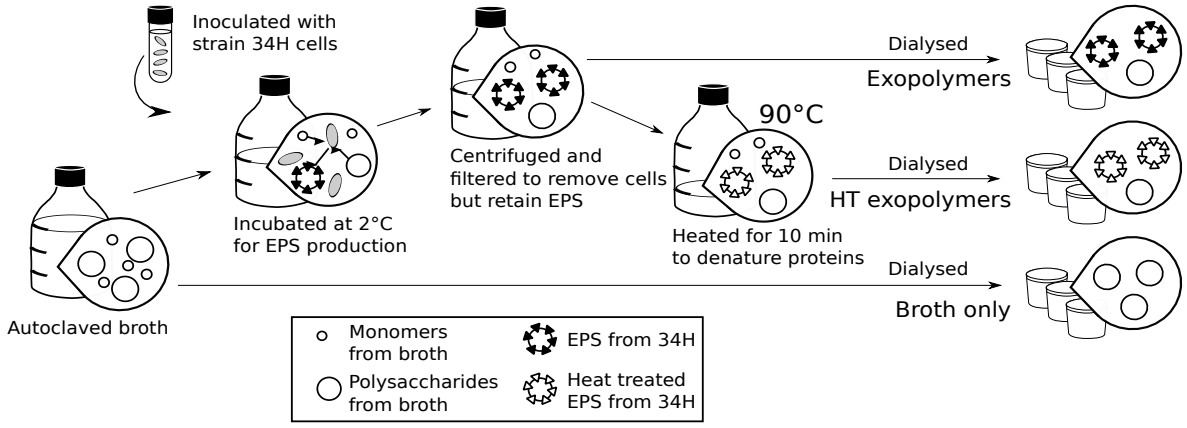

Figure 2.1: Preparation of source solutions: exopolymers: cell-free supernatant of the spent culture medium after growth of *Colwellia psychrerythraea* strain 34H; HT exopolymers: a split of the native exopolymer solution, heated to 90 °C for 10 min; and broth: uninoculated culture medium containing (autoclaved) polymers from yeast extract. Prior to use, all solutions were dialysed (3500 MWCO).

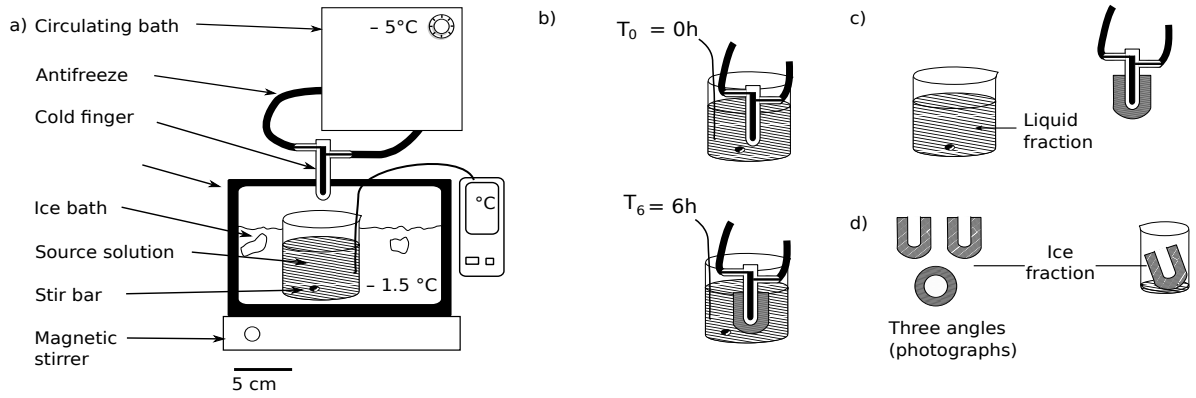

Figure 2.2: Operation of the cold finger apparatus: (a) the apparatus, as modified from *Kuiper et al.* (2003) for use with saline solutions, monitor sample temperature and keep a constant external temperature; (b) cold finger placed into source solution for 6 h; (c) formed ice removed; and (d) ice immediately photographed from the angles, then allowed to melt at room temperature.

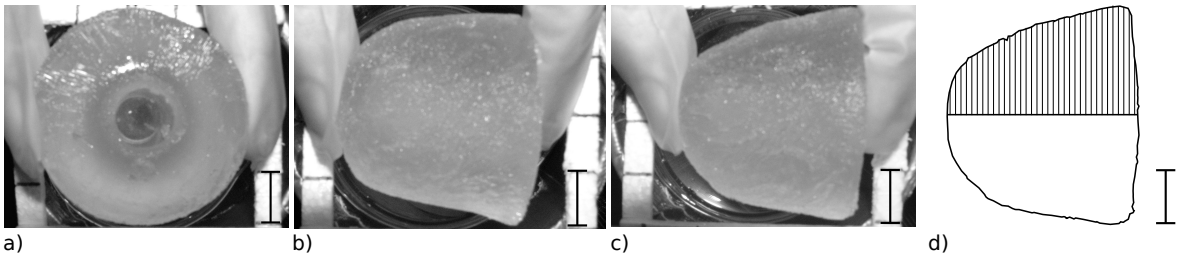

Figure 2.3: Example of calculation of ice volume by photographic approach: (a) the formed ice hemisphere photographed from the top, with axis perpendicular to underlying surface; (b) the ice photographed from the side, with axis parallel to underlying surface; (c) the ice photographed from the side after a 90 °C rotation; and (d) extrapolation of the ice volume from the contour of the hemisphere, integrating the volumes of slabs 0.125 cm wide (and subtracting the volume occupied by the cold finger itself). Bars indicate 1.25 cm.

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Chapter 3

Bacterial and extracellular polysaccharide content of brine-wetted snow over Arctic winter first-year sea ice[1]

Abstract

During freeze-up and consolidation, sea ice rejects to its surface brine of marine origin that is incorporated into overlying snow. To evaluate transport of biological components in brines from ice to snow, vertical profiles of temperature, salinity, bacterial abundance, and extracellular polysaccharide substances (EPS) were obtained through snow and first-year sea ice (Barrow, AK) in consecutive winters (2010, 2011). Snow profiles showed strong interannual variation, with 2010 presenting higher values and wider ranges in salinity (0.3 – 30.9, all salinity measurements are in practical salinity units), bacterial abundance (2.8×10^2 – 1.5×10^4 cells mL^{-1}), and particulate EPS (pEPS, 0.04 – 0.23 glucose equivalents (glu-eq) mg L^{-1}) than 2011 (0 – 11.9, 2.7×10^3 – 4.2×10^3 cells mL^{-1} and 0.04 – 0.09 glu-eq mg L^{-1}, respectively). Upper 10 cm of sea ice also differed interannually, with 2010 again presenting higher salinity (19.4, n = 1), bacterial abundance (5.4×10^4 – 9.6×10^4 cells mL^{-1}) and pEPS (0.13 – 0.51 glu-eq mg L^{-1}) than 2011 (7.7 – 11.9, 1.7×10^4 – 2.2×10^4 cells mL^{-1}, and 0.01 – 0.09 glu-eq mg L^{-1}, respectively). Transport of bacteria and pEPS from sea-ice brines into snow was evident in 2010 but not 2011, a year with more extreme winter conditions of colder temperature, thinner snow and stronger wind. By size fraction, the smallest EPS (< 0.1 μm) dominated (> 80 %) total EPS in both ice and snow; the > 3 μm fraction of EPS in snow appeared to have an atmospheric source. Evaluation of membrane integrity by

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Live/Dead stain revealed a high percentage (85 %) of live bacteria in saline snow, identifying this vast environment as a previously unrecognized microbial habitat.

3.1 Introduction

During its consolidation and cooling, Arctic first-year sea ice (FYI) expels to its surface a thin layer of brine (*Perovich and Richter-Menge*, 1994; *Ehn et al.*, 2007). This surface brine can be transported by capillary action into frost flowers (*Drinkwater and Crocker*, 1988) or snow (*Barber et al.*, 1995). Bacteria (referring collectively to Bacteria and Archaea) and organic material present in sea-ice brines (*Junge et al.*, 2001, 2004; *Krembs et al.*, 2002, 2011) are also available for transport. The incorporation of sea-ice bacteria and extracellular polysaccharide substances (EPS) into short-lived saline frost flowers has been shown in both field and laboratory settings (*Bowman and Deming*, 2010; *Aslam et al.*, 2012), but their transport and long-term (seasonal) persistence have not been demonstrated for snow over sea ice.

The majority of the Arctic sea-ice surface, with an estimated extent of 7×10^6 km^2 for seasonal ice in winter (*Nghiem et al.*, 2007), is covered with snow, mostly accumulated in early fall with some additional accumulation in late winter and early spring (*Warren et al.*, 1999; *Sturm et al.*, 2002). Snow deposited over new ice incorporates sea-ice brines from the ice surface or from collapsed frost flowers formed prior to snowfall (*Drinkwater and Crocker*, 1988; *Barber et al.*, 1995; *Massom et al.*, 2001). The brine-wetted basal snow, referred to as saline snow, has bulk salinities reported as > 10 ppt and up to 40 ppt in the fall, with salinity decreasing through winter due to gravity drainage (*Langlois et al.*, 2007). Salinity decreases upward from the basal stratum, reaching < 5 ppt at 10 cm above the ice surface (*Langlois et al.*, 2007; *Barber et al.*, 1995).

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[1]EPS refers here to extracellular polysaccharide substances because the measuring technique quantifies only the sugar fraction. The same abbreviation is also used for the more generic term: extracellular polymeric substances (as in Chapter 5), encompassing the greater chemical complexity of these polymers (*Wingender et al.*, 1999).

Although the physical and chemical properties of saline snow have been examined in some detail (*Langlois et al.*, 2007; *Domine et al.*, 2004; *Poulain et al.*, 2007; *Geldsetzer et al.*, 2009; *Barber et al.*, 2003), microbial studies targeting brine-wetted snow are not available. The few reported measurements of bacterial abundance in snow over Arctic sea ice range from 9 $\times 10^2$ to 5.2×10^5 cells mL^{-1} melted snow, with lowest values detected in upper spring layers (*Møller et al.*, 2011) and highest values in lower summer layers (*Poulain et al.*, 2007). Bacterial deposition with snow grains or atmospheric particles has been documented (e.g. *Segawa et al.*, 2005), but the potential of sea-ice brines as a source of bacteria (or EPS) to overlying snow has not been considered. Yet, substantial numbers of bacteria are present in the brines of upper sea ice and available for upward transport. A typical bacterial abundance (scaled to brine volume) in upper sea-ice brines of late fall FYI is 7×10^5 cells mL^{-1} (*Collins and Deming*, 2011), with midwinter values as high as 2×10^8 cells mL^{-1} (*Collins et al.*, 2008).

Upper FYI also contains high levels of EPS, with bulk winter concentrations of 1.5 mg C L^{-1} for particulate EPS (pEPS; >0.45 μm) (*Krembs et al.*, 2002). Even higher values of pEPS (2 – 570 mg C L^{-1}) were measured in brines expelled onto the surface of new ice formed in spring (*Bowman and Deming*, 2010). According to mesocosm work, the proportion of EPS in dissolved form (dEPS) increases in surface ice as the ice matures (*Aslam et al.*, 2012). We can find no reports of EPS concentration in snow over sea ice (or over ground).

The objective of this study was to examine the potential upward transport of bacteria and EPS in sea-ice brines, based on vertical profiles of biological and physical parameters for snow and underlying FYI. Results, interpreted in the context of salinity, ion composition, and meteorological information, underscore the influence of atmospheric conditions on the transport and persistence of brine, sea-ice bacteria, and EPS in snow throughout winter. Because the saline snow layer represents an areally vast habitat that persists until spring melt (*Barber et al.*, 1995), it offers a much longer timeframe and greater spatial scale than the frost-flower habitat for interaction between marine and atmospheric components. This interaction is of particular importance in view of current changes in the Arctic sea-ice cover.

The expected increase in the seasonal formation of FYI, which expels brine to its surface during consolidation, will thus also lead to an increase in the extent of saline snow.

3.2 Methods

3.2.1 Study Site and Sampling Approach

Samples of snow and underlying ice were collected from landfast FYI near Barrow, AK, during 10 – 15 February 2010 (BW'10) and 7 – 11 March 2011 (BW'11). Sampling sites were selected by their proximity (300 m) to the UAF Barrow Sea Ice Mass Balance Observatory Site (MBS; 156.5 °W, 71.4 °N, *Druckenmiller et al.*, 2009), which is 13 km from the NOAA weather station (156.8 °W, 71.3 °N; *NOHRSC*, 2011; Fig. 3.1). Sites chosen were flat areas of undeformed ice, although the BW'11 site was surrounded by rubbled ice. Samples were collected from random, undisturbed snow patches 20 m apart. BW'10 snow patches (n = 4) had different depths of 6, 9, 10 and 19 cm. BW'11 snow patches (n = 5) were uniform in depth at 8 – 9 cm, the average snow depth in a 100 m snow-depth transect (see Fig. C.1 in Appendix C). Additional samples (n = 17) were collected during BW'11 from two snow patches 3 cm deep.

3.2.2 Sample Collection and Physical Measurements

Snow layers, hardness, and vertical profiles of temperature and salinity (3-cm intervals) were measured in snow pits 60-cm wide according to *Sturm* (2009) with zero-depth defined at the ice-snow interface. Snow density and grain size were also determined during BW'11. Temperature was measured by hand-held temperature probe (precision 0.1°C); density, with a 100-cm^3 cutter and spring scale (Taylor-LaChapelle snow density kit, Model ST-2, Hydro-Tech). Snow grain size was visually estimated using a handheld magnifying glass and a gridded card. Samples of 0.1 L were collected for salinity.

For biological profiles, 3 – 6 L of snow were collected from a new wall, following either layers of different hardness (BW'10, 0 – 2 cm, 2 – 3 cm, 3 – 6 cm, 6 cm – surface) or uniform 3-cm intervals (BW'11). Samples were collected starting with the uppermost interval, removing snow until the required volume was achieved. To ensure that similar volumes of snow were collected from intervals of different thickness (BW'10), snow from the upper layers was carefully removed and set aside until enough material from the lower layers was exposed. Horizontal variability in salinity was measured in a 3-cm-deep snow patch by selecting random quadrats (n = 13) from a 61×61 cm square frame divided into 10×10 cm quadrats (BW'11). Samples were collected by removing the full depth of snow from each quadrat. Another 3-cm deep snow patch was sampled to determine bacterial viability in thin snow fully exposed to the atmosphere (2 L snow, n = 4; 1 L surface ice, n = 4). All volumes collected refer to unmelted samples.

The ice surface was cleaned of snow and, immediately upon exposure, punctured with a chisel, opening a 1-cm-deep slot for the temperature probe measurement. Adjacent ice surface was then fragmented with a chisel or ice pick to a depth of 2 – 3 cm. Ice fragments were collected for salinity (0.1 L) and biological (1 L) measurements. The full thickness of sea ice (1.1 m) was sampled with a CRREL-style core barrel of 8.5 cm inner diameter (BW'10). Following *Pringle and Ingham* (2009), temperature was measured in two ice cores at 10-cm intervals, and salinity on melted 10-cm sections. Four additional ice cores were cut into 10-cm sections for pEPS and bacterial abundance. All biological samples from snow and ice were collected using ethanol-rinsed tools, placed in sterile Whirl-Pack bags at *in situ* air temperature (–16 to –21 °C) and transported in an insulated cooler for processing at the Barrow Arctic Research Center (BARC). All samples were processed within 5 h after sampling.

3.2.3 Salinity and Ion Measurements

Bulk salinity, measured on melted samples using a handheld YSI-30 conductivity meter (accuracy ± 0.1), was reported as Practical Salinity (S_p) according to the Practical Salinity Scale (PSS-78; *UNESCO*, 1981). Samples may have composition anomalies, hence salinity values serve only as a reference of brine content (*Millero et al.*, 2008). Brine salinity and volume fraction were calculated using the phase equations for ice, as applied to snow by *Drinkwater and Crocker* (1988) and others (e.g. *Barber et al.*, 2003; *Langlois et al.*, 2007). The phase equations from *Cox and Weeks* (1983) were used, and brine volume fraction was expressed as fraction of total volume of ice plus brine (i.e., not including volume of air in the sample). Aliquots of one snow sample (BW'10) were stored at 2°C for later determination of the concentration of major ions (Ca^{2+}, K^+, Mg^{2+}, Na^+, Cl^-, SO_4^{2-}) at the University of Washington (UW) with an ion chromatography Dionex 120 and inductively coupled plasma-atomic spectrometry (ICAP 61E Model, Thermo Jarrell Ash Co.).

Enrichment factors (Ef) were calculated according to *Toom-Sauntry and Barrie* (2002) (Eq. 1) and using molar concentrations from *Pilson* (1998):

$$Ef(\mathrm{X}) = \left(\frac{[\mathrm{X}]}{[\mathrm{Mg}^{2+}]}\right)_{sample} \times \left(\frac{[\mathrm{X}]}{[\mathrm{Mg}^{2+}]}\right)^{-1}_{seawater} \tag{3.1}$$

where [X] and [Mg^{2+}] are the molar concentrations of the ions X and Mg^{2+} (the latter used as reference ion because Na^+ precipitates at low temperature and Cl^- is scavenged by snow).

3.2.4 Biological Measurements

Melting Protocols

To determine bacterial losses due to osmotic shock during sample melting, two types of melts (direct and saline) were compared for all samples, except BW'10 snow (direct only),

BW'10 surface ice (saline), and BW'11 viability assay (saline). In direct melts, samples were melted directly, controlling temperature but not salinity of the final melt; in saline melts, a saline solution was added to the sample to achieve a final salinity of the melt similar to the expected *in situ* brine salinity of the sample. Concentrations were scaled to original volume of sample (bulk-scaling). Saline solutions (milli-Q water and Sigma Sea Salts or NaCl) were prepared to a final concentration of 265 – 270 ppt and filtered (0.22 μm) at BARC (BW'10) or filtered and autoclaved in advance at UW (BW'11). Chilled saline solution was added to the sample to obtain a final meltwater salinity of 100 ppt (BW'11) or similar to *in situ* brine salinity (BW'10) as estimated by the phase equations of sea ice by *Cox and Weeks* (1983).

The choice of 100 ppt was informed by laboratory experiments, where bacterial isolates from frost flowers did not experience significant cell loss when exposed to a salinity change from 220 to 100 ppt (data not shown). Samples were melted at room temperature (shaking often, processing immediately) or in a 0°C water bath. Because bacteria and EPS partition into the brine phase of sea ice (*Junge et al.*, 2001; *Krembs et al.*, 2011), bulk concentrations were also scaled to brine volume (brine-scaling), first subtracting background levels from non-saline snow (if background exceeded sample level, no brine-scaling was made).

Bacterial Abundance

Total bacterial abundance was determined by epifluorescence microscopy as in *Collins et al.* (2008), fixing 15 – 45 mL of sample with 0.22 μm filtered formaldehyde (final concentration of 2 %) and processing at UW (BW'10). During BW'11, slides were prepared at BARC by filtering 70 – 300 mL onto 0.2 μm polycarbonate filters, then fixing with formaldehyde and staining with DAPI. Samples were kept in the dark at 4°C until counting at UW within 1 month of collection.

Extracellular Polysaccharide Substances

To determine particulate EPS (pEPS), 150 – 300 mL were gently filtered (< 350 mm Hg) onto 0.4 μm pore-size polycarbonate filters. Additional BW'11 samples were passed through consecutive polycarbonate filters: 800 mL onto a 3 μm pore-size filter (pEPS > 3 μm), 400 mL of filtrate onto a 0.4 μm pore-size filter (pEPS < 3 μm) and 50 mL of the resulting filtrate onto a 0.1 μm pore-size filter (dissolved EPS (dEPS) > 0.1 μm). The 0.1 μm filtrate was stored in acid-washed plastic bottles (dEPS < 0.1 μm). Samples were kept frozen at –20 °C until processed at UW within 1 (pEPS) and 5 (dEPS) months. Filters were resuspended in 0.25 mL of artificial seawater. Filtrates for dEPS < 0.1 μm were first dialyzed (SnakeSkin dialysis tubing, 3500 MWCO) for 24 h at 2 °C until reaching a salinity of < 2; dialysates were held at 80 °C for 3 – 4 days until volume evaporated to 1 – 2 mL. The phenol-sulfuric assay (*Dubois et al.*, 1956) was performed on the resuspensions and concentrated dialysates to quantify EPS as glucose equivalents (glu-eq) as in *Krembs et al.* (2011).

Bacterial viability

The Live/Dead assay (*Bac*Light kit L-13152, Invitrogen) was applied to saline snow and surface ice samples kept frozen at –20 °C and processed at UW within 2 months. The protocol of *Tam et al.* (2003) was modified to avoid thermal or osmotic shock of sea-ice bacteria as follows: melted samples were filtered onto a 0.22 μm polycarbonate filter at 2 °C; the filter was resuspended into 0.5 mL of chilled, filtered (0.22 μm), 100 ppt NaCl brine and incubated (30 min, 2 °C) with 0.5 mL 2:1 mixture of SYTO 9 and propidium iodide. A minimum of 20 fields and 200 "live" cells was counted with a Zeiss Universal epifluorescence microscope. The modified protocol was tested against cultures (2 °C, 35 ppt) of the marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H, confirming detection of "live" cells (preserved membrane) in exponential growth phase and "dead" cells in death phase.

3.2.5 Graphical and Statistical Analysis

To evaluate if sea-ice bacteria, EPS, and salts were incorporated in the same proportion in snow, the expected contents of bacteria (B_e) and pEPS ($pEPS_e$) were calculated assuming a 1:1 transport with the salts: $B_e = B_a + B_b$, where B_a is bacteria from the atmosphere (average bacterial number in snow samples with the lowest salinity, < 0.5) and B_b is bacteria from the brine (calculated by multiplying bulk snow salinity with the ratio of bacteria-to-salts in surface sea ice). Similar calculations were made for pEPS. Movement of bacteria or pEPS was assumed not to exceed that of salts. Statistical analyses were performed with R v.2.13.1 (*R Development Core Team*, 2011). Values are mean ± standard deviation. Statistical difference between groups was determined using independent two-sample t-test, paired t-test and analysis of variance with repeated measures. A Mann-Whitney test was used in cases where the normality assumption did not hold. Non-parametric Spearman's correlation analysis was used to evaluate relationships between variables.

3.3 Results

3.3.1 Meteorological and Environmental Conditions

Interannual comparison of parameters expected to influence the sampled microbial communities revealed more extreme conditions for BW'11 relative to BW'10 (Table 3.1): thinner snow depth, higher wind speed and, for comparable days in January, lower temperature and higher brine salinity at the ice surface. Seasonal and daily fluctuations in temperature, brine salinity and brine volume fraction during the 40 days leading to BW'11 were calculated for four environments: air (indicating conditions experienced by snow directly exposed to the atmosphere), ice-snow interface, and 10-cm and 20-cm depths into the sea-ice column. Daily fluctuations in all parameters were strongest, on average, for snow exposed to the atmosphere (Fig. 3.2). Compared to the ice column, temperature fluctuations were 16 times greater at the ice surface and 80 times greater in snow exposed to the atmosphere.

3.3.2 Physical Characterization of the Snow and Ice

Average snow density (BW'11) ranged from 286 ± 11 kg m^{-3} in the basal stratum to 320 ± 29 in the upper stratum (n = 5). Many profiles had an icy layer at 2 cm above the ice surface, with a hardness value of *blade* (see additional measurements of snow layers, hardness and grain size in Appendix C, Table C.1). Melted samples of saline snow contained a white mineral crystal metastable at room temperature, possibly a polymorph of $CaCO_3$ as found in *Fischer et al.* (2012).

For snow profiles of comparable depth, both years showed similar temperature and brine salinity, but bulk salinities and brine volume fractions were lower during BW'11 (Fig. 3.3). Temperature decreased upwards at –0.41 to –0.51 °C cm^{-1} in the snow and –0.12 °C cm^{-1} in the ice column (Fig. 3.3a; see full ice-core data in Appendix C, Fig. C.3). Since brine salinity is a function of temperature, estimated values show an opposing gradient, reaching highest values in the snow (Fig. 3.3b). Bulk salinity and brine volume fraction were highest at the ice-snow interface and lowest in the upper layers of snow (Fig. 3.3c, d). Saline snow showed limited horizontal variability in bulk salinity (11.3 ± 0.67; mean ± SD, n = 13; Fig. 3.3c).

Enrichment of major ions relative to Mg^{2+} (Fig. 3.4; see bulk concentrations in Appendix C, Table C.2) revealed strong sulfate depletion in the snow. Na^+ showed moderate depletion in the bottom 6 cm, whereas Ca^{2+} and K^+ showed slight depletion at all depths. Cl^- was enriched up to 6 cm above the ice.

3.3.3 Bacterial Measurements

For both years, bacterial abundance in snow (2.8×10^2 – 1.5×10^4 cells mL^{-1}) was always lower than in the uppermost 3 cm of ice ("surface ice," 1.3×10^4 – 9.6×10^4 cells mL^{-1}) which, in turn, was lower than in the upper 10-cm section of the BW'10 ice cores ("ice column," Fig. 3.5). During BW'10, bacterial abundance in snow declined upwards from the

ice surface, correlating significantly with depth and salinity (Fig. 3.5a, Table 3.2), with the minimum value of the study recorded at 4.5 cm. In contrast, all snow depths during BW'11 had similar bacterial abundances, with a higher minimum value (5.8×10^3 cells mL^{-1}) and no correlation with depth or salinity (Fig. 3.5b, Table 3.2).

Bacterial abundance in snow was lower than expected for a 1:1 transport with the salts (Fig. 3.5), with ratios of bacterial abundance to salts decreasing 7 to 10-fold from ice column to surface ice, and surface ice to saline snow (Fig. 3.5). A high percentage of "live" bacteria, determined by membrane integrity (Live/Dead stain), was observed for both saline snow (85 % ± 5 %, n = 4) and surface ice (78 % ± 7 %, n = 4) during BW'11.

Abundances in the BW'10 sea-ice column were distributed in a C-shaped profile (Appendix C, Fig. C.5). No differences due to melting procedure were observed, except for the upper section where a 55 % cell loss was observed for direct melts (n = 1, Fig. 3.5a). Significant cell loss was observed during BW'11 in direct melts of surface ice (30 % lower, p = 0.009, t-test) and the medium (3 – 6 cm) snow horizon (20 % lower, p = 0.041, t-test).

3.3.4 Extracellular Polysaccharide Substances Measurements

The sea-ice column had a relatively constant concentration of pEPS (but for the maximum value in the upper section, Appendix C, Fig. C.5), with a mean pEPS value (0.740 ± 0.73 mg glu-eq L^{-1}, n = 22) significantly higher than in snow (0.098 ± 0.07 mg glu-eq L^{-1}, n = 11; $p < 0.001$, Mann-Whitney). During BW'10, pEPS concentration in the snow decreased upwards from the surface ice, with a significant negative correlation with depth (Table 3.2); pEPS appeared to follow a 1:1 transport with salts (Fig. 3.6a). Although the correlation was not significant (p = 0.096), the low p-value provides evidence against the null hypothesis that pEPS does not correlate with salinity. During BW'11, pEPS correlated significantly with salinity but not with snow depth (Table 3.2). Unlike ratios of bacterial abundance to salts, the ratio of pEPS to salts decreased only by half between surface ice and saline snow during BW'10 and doubled during BW'11 (Fig. 3.6).

The mean total EPS (carbon equivalents) in BW'11 snow was 0.27 mg C L^{-1} ± 0.05 (n = 15). The smallest dEPS size fraction (< 0.1 μm) accounted for 85 – 90 % of the total EPS in both surface ice and snow (Fig. 3.7). Concentrations of the remaining EPS size fractions (dEPS > 0.1μm, pEPS < 3 μm and pEPS > 3 μm) differed significantly from each other at each snow depth ($p < 0.01$, analysis of variance with repeated measures), with the largest pEPS fraction having the highest concentrations at all snow depths ($p < 0.001$, paired t-test, Fig. 3.7). Concentrations of these same three EPS size fractions in the underlying ice did not differ significantly from each other.

3.3.5 Additional Scalings for Bacteria and EPS

Brine-scaling of bacterial abundance and EPS concentration (Appendix C, Table C.3) indicated similar patterns to bulk-scaling, with both parameters lower in saline snow than in surface sea ice, except during BW'11 when pEPS was slightly higher in the saline snow layer. Scaling to brine, which reflects the in situ conditions experienced by bacteria, also allowed comparisons of the amount of presumably protective pEPS available on a per cell basis. Highest values characterized saline snow (15 and 100 pg glu-eq $cell^{-1}$ during BW'10 and BW'11, respectively), and lower values, surface sea ice (4.3 and 2.3 pg glu-eq $cell^{-1}$ during BW'10 and BW'11, respectively). Likewise, total values of EPS per cell (BW'11) were two orders of magnitude higher in saline snow (3360 pg glu-eq $cell^{-1}$) than in surface ice (30 pg glu-eq $cell^{-1}$).

3.4 Discussion

3.4.1 Physical Properties

Ionic composition (Fig. 3.4) confirmed that the source of salt in the snow we sampled was sea-ice brine and not seawater flooding (sulfate and sodium depletion is associated with mirabilite precipitation in brines exposed to temperatures < –8 °C *Rankin et al.*, 2000).

Furthermore, brine content decreased away from the ice-snow interface, as expected for fluids incorporated by capillary rise (Fig. 3.3c, *Coléou et al.*, 1999). Low salinity snow samples had higher ion concentrations by 1 – 3 orders of magnitude, and a stronger SO_4^{2-} depletion signal, than reported for snowfall in the high Arctic (*Toom-Sauntry and Barrie*, 2002). Background salinity may include salt from blown saline snow or other atmospheric depositions (e.g. *Barrie et al.*, 1985; *Kumai*, 1985). Cl^- enrichment was likely due to the scavenging of surface reactive chloride compounds by the snow (*Toom-Sauntry and Barrie*, 2002).

Compared to literature values, bulk snow salinities were generally high in BW'10 and low in BW'11 (*Barber et al.*, 2003; *Langlois et al.*, 2007). BW'11 had a longer lead time for brine gravity drainage (*Langlois et al.*, 2007), but other factors may have contributed to the interannual differences. Air temperature during freeezup, lower in BW'10 (–18.6 °C) than BW'11 (–9.9 °C), may have resulted in faster freezing rates and a greater expulsion of brine then available for incorporation into snow. Snow porosity and grain size (*Coléou et al.*, 1999) also affect fluid incorporation into snow, but measurements for the time of snow deposition are lacking.

Snow had lower bulk salinity (0 – 39) than reported for frost flowers (FF, 10 – 120 ppt) (*Perovich and Richter-Menge*, 1994; *Bowman and Deming*, 2010), yet held a greater amount of salt per unit area of sea ice covered. The volume of snow covering 1 cm^2 would hold 37 – 64 mg salt in BW'10 (assuming snow density of 300 kg m^{-3}) and 9 – 12 mg in BW'11. FF would hold up to 6 mg salt per cm^{-2} (assuming weight per unit area of 25 – 50 mg cm^{-2} and occupation of one third of the available surface; *Perovich and Richter-Menge*, 1994). The higher salt content in snow per unit area supports the hypothesis that saline snow is a more important source of bromide salts and other salt aerosols to the atmosphere than FF (*Simpson et al.*, 2007; *Yang et al.*, 2008; *Roscoe et al.*, 2011).

3.4.2 Bacterial and EPS measurements

Total bacterial abundances in the snow were low to intermediate compared to the few reported values for snow on sea ice from spring and summer (*Poulain et al.*, 2007; *Møller et al.*, 2011). Influence of melting method on bacterial loss for the upper section of the ice was consistent with initial work by *Deming* (2010). Previous work by *Helmke and Weyland* (1995) showed no effect of melting protocols on the culturability of bacteria from winter sea-ice cores, but culturable bacteria are only a fraction of the total population in sea ice (*Junge et al.*, 2002).

Although estimates of EPS by the phenol-sulphuric acid method (assayed at absorbance wavelength of 490 nm) can underestimate acidic sugars (*Dubois et al.*, 1956) known to be present in EPS (*Underwood et al.*, 2010), EPS concentrations in the sea-ice column were consistent with the winter pEPS profile determined by *Collins et al.* (2008) using the Alcian Blue staining method. We found no measurements of EPS content in saline snow for direct comparison, but total values of dissolved organic carbon (DOC) reported for snow above the saline layer (1.3 - 4 mg C L^{-1}) were 5 to 15 times higher (*Møller et al.*, 2011). Atmospheric sources of DOC in snow, including EPS from aerosols, have been considered previously (*Leck and Bigg*, 2008). On average (mean $\pm$ SD), total dEPS accounted for 87 % $\pm$ 6 in upper low-salinity snow, with saline snow at 94 % $\pm$ 2 and surface ice at 93 % $\pm$ 2. These percentages agree with dominance of the dEPS fraction in new surface sea ice and frost flowers grown in mesocosms by *Aslam et al.* (2012), and observed in late spring sea ice (bottom 10 cm) in Barrow (72 % $\pm$ 9.5) by *Krembs et al.* (2011). Differences in the relative abundance of the two evaluated pEPS size fractions, with the largest fraction (> 3 μm) being dominant in the snow but not in the ice, suggest atmospheric contributions of pEPS to the snow. Correlation with salinity, however, which was negative in previous studies of sea ice (*Collins et al.*, 2008) and sea-ice brines (*Aslam et al.*, 2012), was significantly positive in BW'11 snow, indicating possible marine origin of the pEPS.

3.4.3 Bacterial and pEPS Transport in the Saline Snow Layer

In the first year of this study, bacteria and pEPS (> 0.45 μm) in the snow followed a gradient similar to salinity and had significant relationships with snow depth (Table 3.2) suggesting upward transport and incorporation with sea-ice brines. Upward transport of marine bacteria and pEPS was not clear in the second year, when higher background levels of bacteria and pEPS may have masked a comparatively smaller contribution from the brines. Higher pEPS values in surface ice during BW'10 (Fig. 3.6) and higher background levels in snow during BW'11 (0.09 mg glu-eq L^{-1} vs 0.04 mg glu-eq L^{-1}) likely contributed to these differences in gradients.

In both years, fewer bacteria were detected in the snow than expected from the concentration of salts (Fig. 3.5). One cause could be their selective retention in the ice due to ice affinity of the bacterial coating (implied by the work in Chapter 2, *Ewert and Deming*, 2011) or by physical blockage of the brine veins by pEPS (*Krembs et al.*, 2011), affecting the passive transport of bacteria and salts in different ways. Ratios of bacteria to salts in frost flowers (data from *Bowman and Deming*, 2010) are lower than in surface ice, in agreement with selective retention occurring before brine leaves the ice. Retention in the basal layer of snow (by ice affinity of cells to snow crystals) could also contribute to higher bacterial abundance in the lowermost snow layer.

3.4.4 Loss of Marine Bacteria and EPS in the Snow

Selective loss of bacteria in the surface environment would also account for the lower bacterial numbers observed in the surface environment. Bacterial loss over the course of winter (up to 49 %) has been observed in upper sea-ice horizons (upper 25 cm, *Collins et al.*, 2008), where it was attributed to osmotic lysis, viral lysis and/or cell impingement by ice or salt crystals. Viral lysis in particular may be triggered by fluctuations in salinity and temperature (*Ghosh et al.*, 2009; *Shkilnyj and Koudelka*, 2007) as occur in sea ice.

Fluctuations in temperature, brine volume and brine salinity were progressively less strong the deeper into the ice. This dampening effect of sea ice (*Collins et al.*, 2008; *Petrich and Eicken*, 2010) may explain higher bacterial abundance in the 10-cm ice horizon (BW'10) compared to surface ice, as well as the reduction in bacteria-to-salt ratios in samples near or above the sea ice surface (Fig. 3.5). The strongest fluctuations were registered at the air interface, with daily changes in brine salinity up to 180 (Fig. 3.2). Actual fluctuations in brine salinity may depend on available water content of the environment and time of exposure; for instance, smaller fluctuations should occur in the more ephemeral frost flowers, which have a freshwater (pure ice) content lower than saline snow.

Brine skim and frost flowers are young environments that, even if directly exposed to the air, still contain high numbers of bacteria and EPS per unit area (0.3×10^4 – 4.8×10^4 cells cm^{-2} and 0.2 – 36.2 μg glu-eq cm^{-2}, respectively; data from *Bowman and Deming*, 2010). Within hours to days, however, brine from both environments will be incorporated into deposited snow. The snow sampled in BW'10 had incorporated high amounts of salt per unit area (see above), but the numbers of bacteria (2.1×10^3 – 9.6×10^3 cells cm^{-2}) and pEPS (0.03 – 0.3 μg glu-eq cm^{-2}) per unit area were low. This apparent loss of bacteria in the snow may be due to a longer time of exposure, weeks to months, to more extreme environmental conditions of temperature and salinity (despite the higher relative proportion of protective EPS per cell in snow compared to sea ice). As we did not observe a higher proportion of "dead" cells in saline snow compared to surface ice, the mechanism accounting for the lower cell numbers in the snow must involve complete cell lysis (see Appendix D).

Another microbial stressor at the ice surface is UV-B irradiation. pEPS absorption spectra peaked in the UV range (data not shown), a characteristic of polysaccharides susceptible to UV-B photolysis (*Ortega-Retuerta et al.*, 2009). Penetration of UV light into snow, however, is limited by absorption and scattering, with attenuation of 1 order of magnitude in 4-cm deep snow patches (*King and Simpson*, 2001; *Cockell and Córdoba-Jabonero*, 2004). Also, UV-B driven photolysis may be irrelevant during periods of reduced daylight, but could occur in the uppermost layers of snow and thinner ($<$ 4 cm) snow packs during daylight

periods which, by the time of sampling, were 7 h in BW'10 and 10 h in BW'11.

Annual differences in the snow cover may have contributed to the observed differences in bacterial and EPS content. The deeper BW'10 snow cover provided milder conditions in the saline snow layer, with warmer temperatures (by 18.2 °C) and fresher salinities (by 72) than exposed brines. BW'11 had a thinner snow pack, with average snow depth of 4.7 cm (Mass Balance Site, 40 days leading to sampling), 28 days showing minimum snow depth $<$ 4 cm and only one day having maximum snow depth $>$ 20 cm. The thinner snow cover and lower air temperatures in BW'11 led to more extreme conditions in the saline snow layer, consistent with a greater loss of bacteria and pEPS in the snow. Furthermore, increasing daylight during BW'11 (up to 10 hours of light per day) augmented the potential for UV-B photolysis.

Testable hypotheses emerge from this work. The insulating role of the snow may translate to milder daily and seasonal fluctuations for thick snow packs, making them more habitable than thinner snow. Changing snow-depth differences could thus generate heterogeneity in the bacterial community of the ice-snow interface. Likewise, because snow accumulated over collapsed frost flowers shows higher salinity than snow accumulated directly over the ice (*Massom et al.*, 2001), it may also contain higher bacterial and pEPS abundances.

3.4.5 Potential Aerial Transport of Saline Snow

Wind speeds capable of initiating blowing snow events ($>$ 7 m s^{-1}; *Savelyev et al.*, 2006) were common during the weeks leading to sampling, and more frequent prior to BW'11 (68 days) than BW'10 (40 days). Bare ice was not evident, but snow at the Mass Balance Site was thinner than 3 cm on 10 out of 40 days leading to sampling, indicating blowing of saline snow in BW'11. Blowing snow could redistribute marine components and contribute to higher background levels of bacteria and EPS, masking existing gradients. Wind dispersal of the bottom 2 cm of snow, however, would have been limited by observed hard icy layers. Aerosol production and aerial dispersal from frost flowers remain open questions (*Obbard*

et al., 2009; *Roscoe et al.*, 2011), but frost flowers may play an indirect role by contributing brine, bacteria and EPS to the snow that collapses them.

3.5 Conclusions

Bacteria and EPS of marine origin are present in saline snow overlying Arctic first-year sea ice where they are susceptible to dispersal by snow-blowing events. The observed bacteria and EPS did not occur in the same proportion as the salts in this newly identified and potentially vast habitat, suggesting either selective retention in the source ice or loss after transport into snow. Losses may be due to the more extreme conditions encountered near the atmospheric interface, documented as wide daily fluctuations in temperature, brine salinity and brine volume fraction.

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Table 3.1: Meteorological parameters

Station	Parameter	BW'10	BW'11
NOAA[a]	Snow depth (cm)	30.4 (20.3 to 43.2)	20.1 (10.2 to 63.5)
	Air temperature (°C)	–22.4 (–41.1 to –1.1)	–20.5 (1.1 to –42.8)
	Wind speed (m s^{-1})	4.8 (0 to 16)	5.5 (0 to 21.6)
MBS[b]	Snow depth (cm)	30	4.1
	Air temperature (°C)	–26.3	–32.4
	Ice-surface temperature (°C)	–12.1	–25.4
	Ice-surface brine salinity	160	232

[a] Values are the average (and minimum to maximum) from NOAA-PABR Station for the period from ice formation (mid-November) to sampling week (10 – 15 Feb 2010; 7 – 11 Mar 2011).
[b] Daily averages are from the UAF Mass Balance Site for 23 Jan 2010 and 26 Jan 2011.

Table 3.2: Correlation coefficients (Spearman's) for parameters in snow

	Year[b]	Bacterial abundance	pEPS
Temperature	2010	0.08	–0.09
	2011	0.22	0.45
Salinity	2010	**0.84****	0.53
	2011	–0.27	**0.55***
Depth	2010	**–0.80****	**–0.69***
	2011	0.30	–0.43
pEPS	2010	**0.64***	
	2011	0.09	

[a] Bold highlights significant relationships at $p < 0.05$ (*) and $p < 0.005$ (**).
[b] Direct melts apply to 2010; saline melts to 2011.

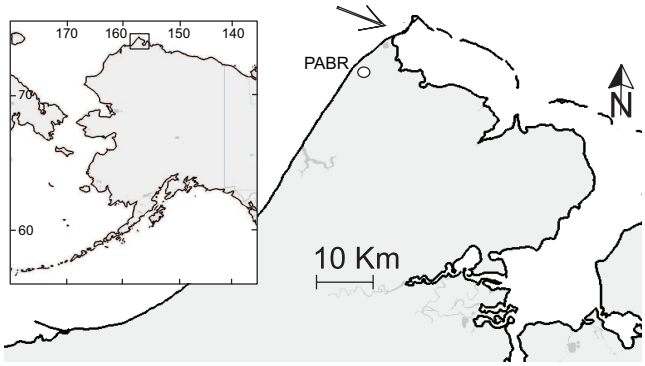

Figure 3.1: Location of sampling area (arrow) relative to the state of Alaska (insert; latitude in °N, longitude in °W) and the NOAA weather station at the Post Rogers Memorial Airport (circle, PABR) in Barrow.

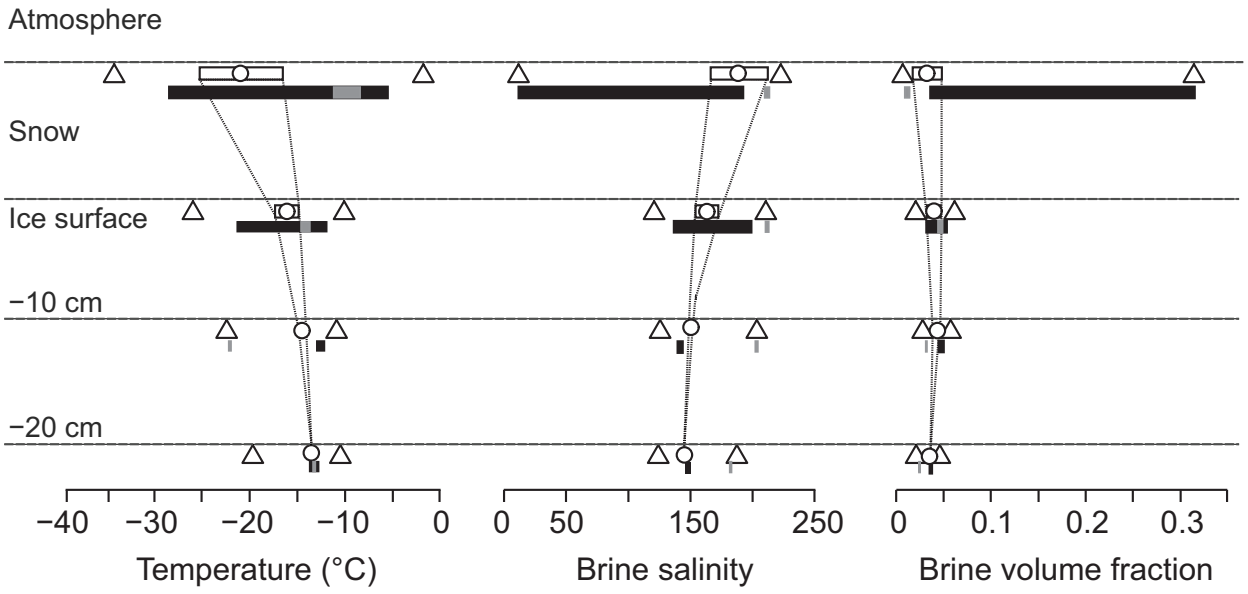

Figure 3.2: Daily and seasonal fluctuations in temperature, brine salinity and brine volume fraction at different depths in the snow and ice column. Symbols represent average (circles) and maximum and minimum (triangles) daily values for the 40 days leading to BW'11. Daily values correspond to MBS daily averages of temperature (measured at 15-min intervals, accurate to 0.2 °C; *Druckenmiller et al.*, 2009). Open bars represent average daily fluctuation, calculated as maximum minus minimum during a given day. Black bars represent the maximum daily fluctuation and gray bars the minimum daily fluctuation during the 40-day period. Brine salinity and volume fraction were calculated using equations from *Cox and Weeks* (1983), temperatures from MBS and average salinities at the study site.

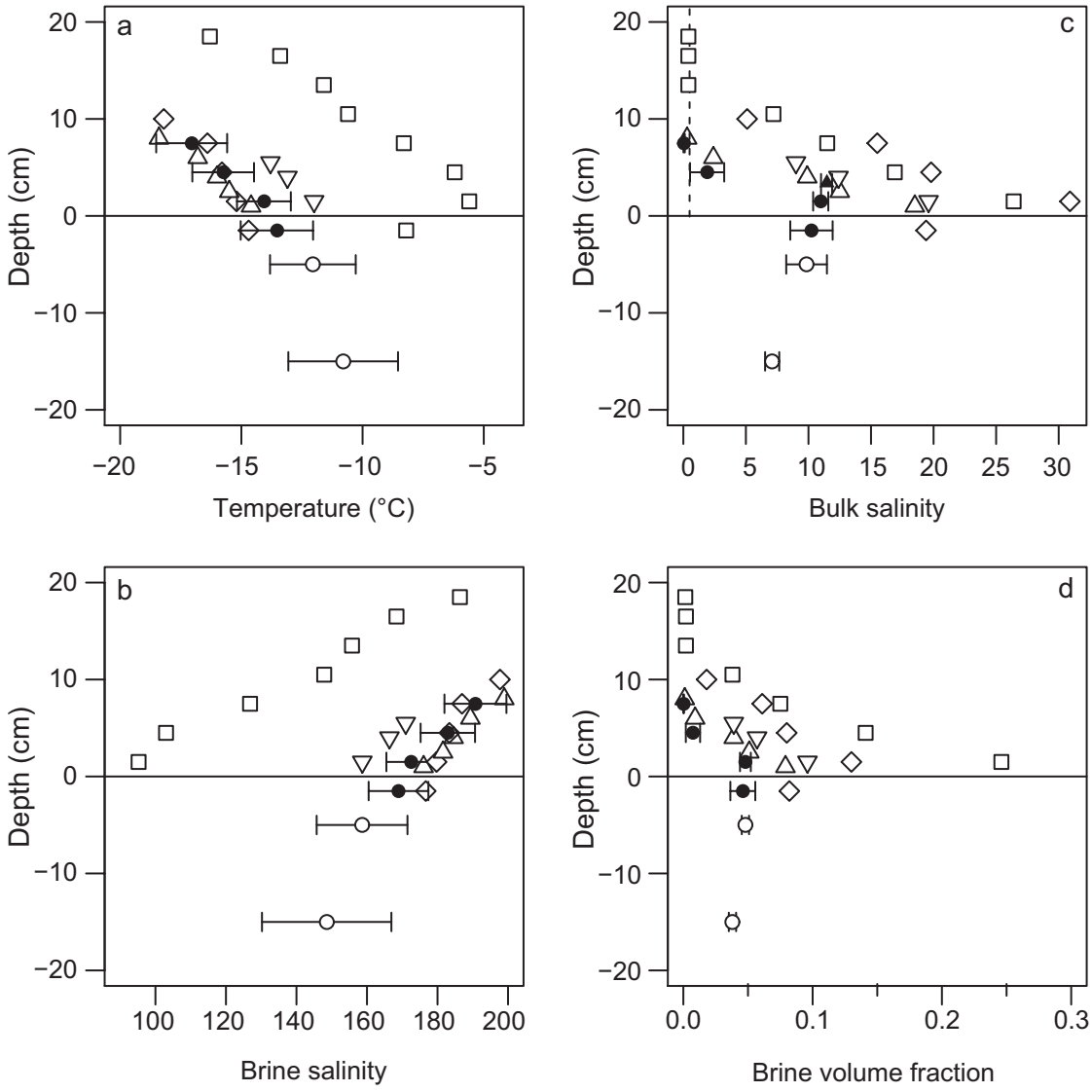

Figure 3.3: Depth profiles of temperature (a), brine salinity (b), bulk salinity (c) and brine volume fraction (d) in snow (positive depths) and sea ice (negative depths) during BW'10 (open symbols) and BW'11 (solid symbols). Open squares, triangles and diamonds indicate different snow pits of different depth. Symbols with error bars indicate mean ± SD, where n = 2 for open symbols and n = 5 for solid symbols (except the triangle in (c)). The solid triangle (c) indicates horizontal variability in a snow patch 3-cm deep (mean ± SD, n = 13). Solid lines indicate snow-ice interface; dashed line (c) indicates maximum background bulk snow salinity, defined as 0.5. Lowest bulk salinities in the snow were 0.3 (BW'10) and 0.1 (BW'11).

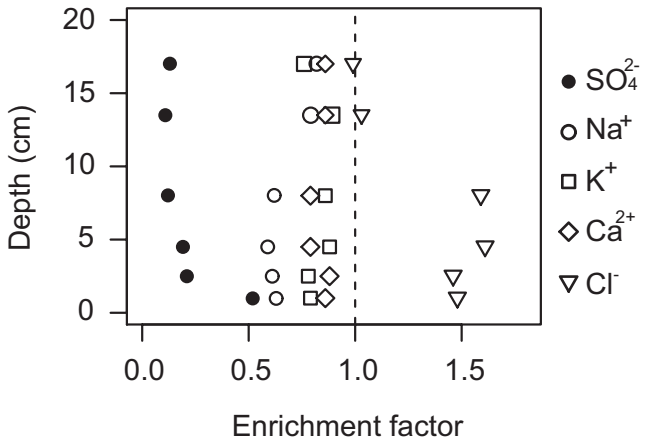

Figure 3.4: Depth profiles of enrichment factors (Ef, as defined in the text) for each of the major ions in seawater relative to Mg^{2+} (molar) in snow over sea ice (BW'10). Snow depth is distance from ice-snow interface.

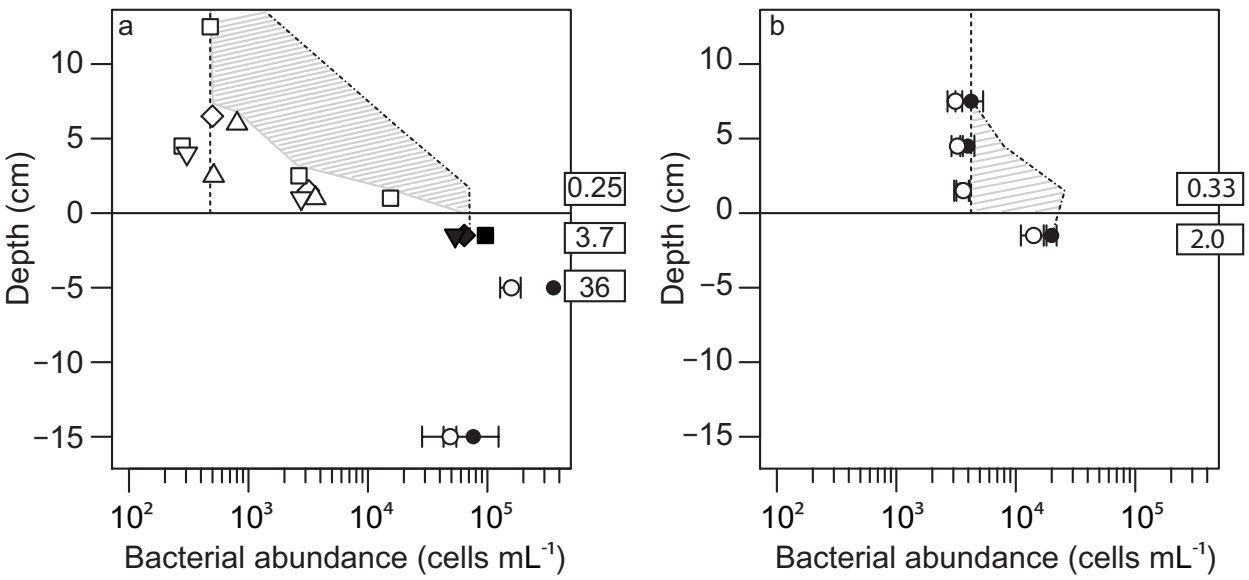

Figure 3.5: Depth profiles of total bacterial abundance in snow (positive depths) and sea ice (negative depths) during BW'10 (a) and BW'11 (b). Symbols with error bars indicate mean ± SD, where (a) n = 2 for means in the ice column and (b) n = 5 for means in both the snow and the ice column. Open symbols indicate direct melts; solid symbols indicate saline melts; symbols for different BW'10 snow pits as in Fig. 3.3. Solid lines indicate snow-ice interface; dashed lines indicate average bacterial abundance in background low-salinity (< 0.5) samples; hatched areas indicate difference between measured and expected (dot-dashed lines) bacterial abundance assuming 1:1 transport of cells with salts (calculated for saline melts). Numbers in boxes indicate ratios of cells to salts (10^3 cells per salinity unit) at given depths.

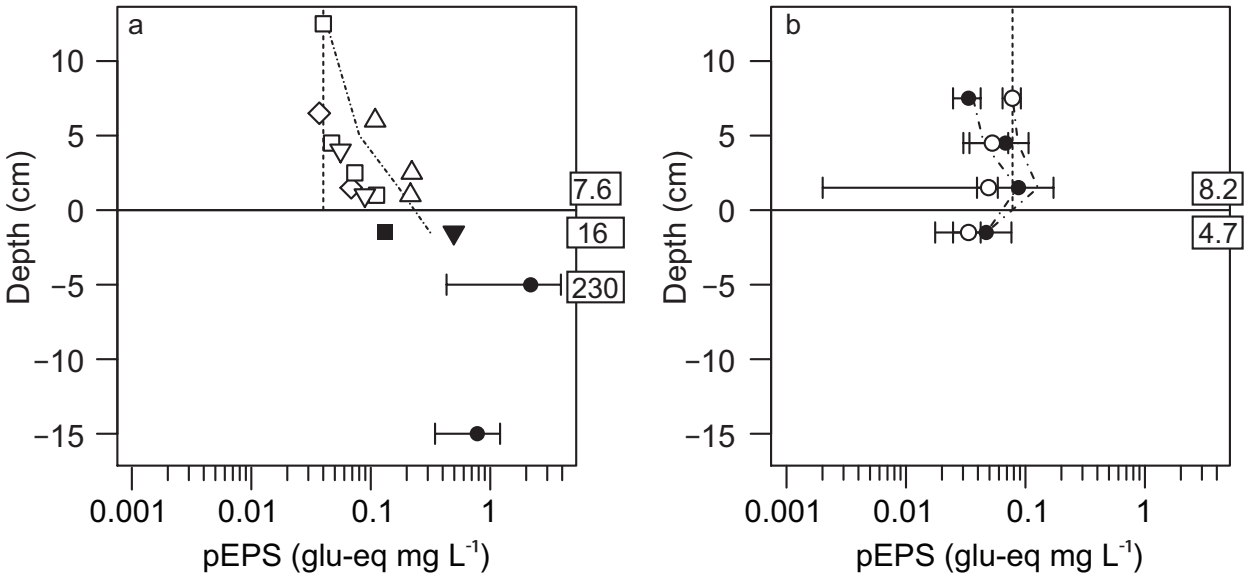

Figure 3.6: Depth profiles of bulk pEPS (> 0.4 μm) concentration in snow (positive depths) and sea ice (negative depths) during BW'10 (a) and BW'11 (b). Symbols, error bars and lines as in Fig. 3.5. Numbers in boxes indicate ratios of pEPS to salts (μg pEPS per salinity unit) at given depths.

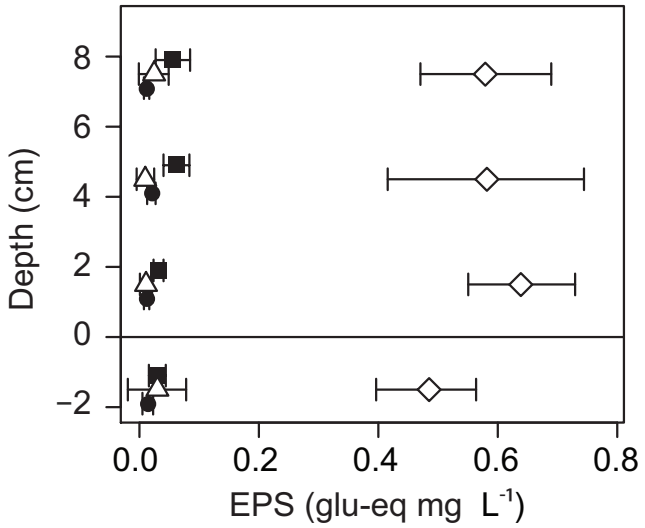

Figure 3.7: Depth profiles of bulk EPS size fractions (BW'11). Horizontal line indicates snow-ice interface. Open symbols are dEPS (< 0.4 μm), filled symbols are pEPS (> 0.4 μm). Diamonds indicate dEPS < 0.1 μm; triangles indicate dEPS > 0.1 μm. Circles indicate pEPS < 3 μm; squares indicate pEPS > 3 μm. All values are mean $\pm$ SD (n = 5).

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Chapter 4

Environmental fluctuations and the role of compatible solutes in the survival of sea-ice bacteria

Abstract

Bacteria inhabiting the upper sea ice column, and its associated environments, are subject to multiple stressors, including extremes of temperature and salinity, and fluctuations in these parameters. Due to the insulating properties of the snow and ice, sea-ice brines experience different ranges of temperature and salinity depending on the degree of exposure to the atmosphere. Examining winter and spring records of temperature and inferred brine salinity from first-year coastal sea ice (Barrow, Alaska) confirmed narrower ranges for the ice (that is, 10 cm below the ice surface) than the snow-atmosphere interface (10 cm above the ice surface). Short-period ($<$ 1 h) fluctuations of temperature and salinity were mild compared with diurnal and semi-diurnal fluctuations. Fluctuations with periods of 1 – 24 h were significantly more energetic at the snow-atmosphere interface than in the ice, and a clear diurnal regime was evident in the spring. In this chapter, laboratory experiments were performed to evaluate how well bacteria may survive freezing for short (40 min) and long (15 d) periods, under constant and fluctuating temperature regimes. Melting procedures (direct and brine melts), which subject organisms to a different degree of osmotic shock, were also evaluated. Experiments involved two Arctic isolates: obligate psychrophile *Colwellia psychrerythraea* strain 34H (*Cp*34H) and a new psychrotolerant isolate, *Psychrobacter sp.* strain 7E (*P*7E), which has a broader growth range of temperature and salinity. After freezing for 40 min, samples subjected to a direct melt procedure involving a relatively rapid (minutes) drop in salt concentration of 108 – 174 ppt yielded significantly lower cell numbers than samples melted into brine. Isolate *P*7E presented higher survival rates in

both treatments. Using brine melts, *Cp*34H yielded cell losses of $\sim$ 50 % when subjected to long term freezing (15 d) under fluctuating conditions, with significantly lower cell numbers than the constant freezing treatment at –20 ° C ($p < 0.01$, analysis of variance; ANOVA). The presence of either one of the compatible solutes, choline (a precursor), glycine betaine or proline, had a protective effect on *Cp*34H, significantly reducing cell loss under fluctuating conditions ($p < 0.01$, ANOVA). In contrast, freezing under either constant or fluctuating conditions, promoted miniaturization and fragmentation in *P*7E, with significant increase in total cell numbers in six of nine experiments ($p < 0.01$, ANOVA). Together, the environmental data and the laboratory results suggest that depth of the ice-snow cover, in determining the different fluctuation regimes of temperature (and salinity) throughout the system, influences the nature of the bacterial communities that survive the winter. Organisms able to tolerate wide changes in temperature and salinity would be favored in surface environments with stronger fluctuation regimes, while those with more restrictive tolerances would survive best in the ice column. The overall composition of the community may depend on the availability, in the inhabitable brine phase, of compatible solutes for osmoprotection.

4.1 Introduction

The environmental conditions that characterize the upper sea-ice column and associated environments, including the bare sea-ice surface, brine-skim layer, frost flowers and saline snow layer, impose several types of stress on the resident microbial communities. Lower temperatures and higher brine salinities characterize winter sea ice, whereas a marked seasonal change towards warmer temperatures and lower brine salinities occur in spring. The winter stresses can be considered in terms of transient (< 1 d) exposure to extremes in temperature and brine salinity, long-term (several days) exposure to such extremes, and fluctuations, mild or strong, in these parameters. Temperature fluctuations generate freeze-thaw events that affect the brine-channel network in sea ice, causing corresponding fluctuations in brine-volume fraction and brine salinity. Changes in temperature, osmolarity and liquid volume are particularly relevant to sea-ice microorganisms because the brine

phase defines their habitable space (*Junge et al.*, 2004). In relatively warm (subarctic) sea ice, changes in salinity help to shape microbial response and community structure (*Kaartokallio et al.*, 2005). A similar, yet possibly stronger response is expected from the resident microorganisms of environments directly exposed to a colder atmosphere, such as the environments of surface brine skim and frost flowers, which are subject to stronger extremes and fluctuations than the underlying ice column.

Exposure of the natural bacterial community of sea ice to long-term (70 – 440 d) winter freezing conditions results in cell losses of 24 – 49 % of the original population (*Krembs et al.*, 2002; *Collins et al.*, 2008). Mortality by long-term freezing may be caused by the up-shift in osmolarity associated with freezing, the formation of intracellular ice crystals, the reduction in habitable space, or the puncturing of the cell by ice crystals (*Mazur*, 1984). Of these potential causes, only the first has been demonstrated for bacterial cells, and only in laboratory studies of organisms from other environments. To cope with an up-shift in osmolarity, many bacteria respond by a transient accumulation of K^+ to equilibrate the osmotic pressure and limit water loss (*Heermann and Jung*, 2012). K^+ accumulation is accompanied by quick synthesis of glutamate to counterbalance ionic charges (*Dinnbier et al.*, 1988). K^+ and glutamate, which limit cell functioning at high concentrations, are then replaced by compatible solutes, a variety of organic molecules with the ability to increase the internal osmolarity of the cell without interfering with cellular functions (*Roberts*, 2005). Some of these solutes can have a protective effect against other conditions of stress such as desiccation, heat shock and low –including freezing– temperatures (*Welsh*, 2000; *Hoffmann and Bremer*, 2011).

Compatible solutes can be synthesized *de novo* by the organism, imported from the environment or converted from precursors, with direct uptake from the environment being the most energetically favorable (*Oren*, 1999). Osmolites used as compatible solutes by Bacteria and Archaea include sugars, phosphodiesters, glyceric acid derivatives, polyols and amino acids, of which glycine betaine and proline are of widespread use (*Roberts*, 2005; *Empadinhas and da Costa*, 2008; *Wargo*, 2013). Choline, even if not a compatible solute *per se*, can also serve

as osmoprotectant in organisms that have the ability to transform it into glycine betaine by means of a choline dehydrogenase and a glycine betaine aldehyde dehydrogenase (*Styrvold et al.*, 1986). Environmental sources of choline include the enzymatic degradation of phosphatidylcholine and sphingomyelin, abundant phospholipids of the eukaryotic membrane (*Wargo*, 2013); related betaine lipids have also been detected in marine algae (*Kato et al.*, 1996), although their role as potential sources of compatible solutes for bacteria has not been studied. The uptake of compatible solutes and their precursors in the estuarine environment is rapid (on the scale of nM h^{-1}) (*Kiene*, 1998), explaining very low environmental concentrations of their soluble forms (nanomolar in the case of choline; *Wargo*, 2013).

Membrane transporters allowing the uptake of compatible solutes are widespread in Bacteria. In particular, glycine betaine, choline and proline can be imported by primary transporters of the ATP-binding cassette (ABC) type (ATP-driven), or by secondary transporters of the betaine/choline/carnitine transporter (BCCT) family (driven by proton or sodium motive force; *Ziegler et al.*, 2010). BCCT transporters are transmembrane proteins with N- and C-terminal extensions on the cytoplasmic side, with the length of these extensions playing a critical role in the osmoregulation of the transporter (*Ziegler et al.*, 2010). The whole genome sequence of *Colwellia psychrerythraea* strain 34H has one putative gene assignment for a primary ABC transporter (CPS_4933 – CPS_4935) and five assignments for transporters of the BCCT family, of which two (CPS_4009 and CPS_1335) are expected to import choline and three (CPS_4027, CPS_3860 and CPS_2003) do not have a clear predicted substrate (*Collins and Deming*, 2013).

In addition to the stress of long-term freezing conditions, freeze-thaw cycles associated with short-term temperature fluctuations can also expose sea-ice bacteria to a down-shift in osmolarity. Osmolarity down-shifts result in a rapid water influx into the cell that can result in osmolysis. To prevent osmolysis, bacterial cells have mechanosensitive channels that open when intracellular pressure reaches a critical value, thus acting as "emergency valves." The opening of these channels leads to a rapid and non-specific, a release of solutes that does not require energy to be activated (*Morbach and Krämer*, 2002). This non-specific release

of solutes may include significant amounts of small organic molecules from the intracellular pool of the organism, including amino acids and low molecular weight sugars (*Halverson et al.*, 2000). After the osmotic shock, some of the released solutes are quickly recovered from the surrounding media by the cells (*Schleyer et al.*, 1993). The re-accumulation of compatible solutes may imply energy consumption for the cell, depending on the membrane transporter used for compatible solute uptake, or in those cases where *de novo* synthesis is required. Cell death resulting from environmental freeze-thaw stress has been considered as a possible source of nutrients and cryoprotectants to the surviving community, especially upon the arrival of spring (in soil communities; *Morley et al.*, 1983).

This chapter presents a series of three experiments designed to assess the impact of various freezing conditions on cold-adapted Arctic isolates with different growth ranges of temperature and salinity. Our working hypothesis was that bacteria with wider tolerances of temperature and salinity in their growth patterns could also better survive the more extreme and fluctuating conditions that characterize upper regions of the winter snow-sea-ice system, than their counterparts with narrower growth ranges.

The first isolate examined, *Colwellia psychrerythraea* strain 34H (*Cp*34H), is a psychrophilic gamma-proteobacterium enriched from cold (< 0 °C) marine sediments (*Huston*, 2003), with relatives of the same genus and species also found in sea ice (*Bowman et al.*, 1997; *Bowman et al.*, 1998; *Methé et al.*, 2005). *Cp*34H is characterized by comparatively narrow temperature (–12 to 13 °C) and salinity growth ranges (20 to 50). The second isolate is a psychrotolerant species of the genus *Psychrobacter*, not yet characterized at the species level, but designated as strain 7E in our culture collection (*P*7E). *P*7E was isolated as part of this dissertation from upper sea-ice brine (salinity of 128) collected from a first-year ice floe in the southern Beaufort Sea during winter. Like other species in this genus (*Romanenko et al.*, 2004), *P*7E can grow over a broad range of conditions of temperature (–1 [possibly lower] to 25 °C) and salinity (32 to 125).

Experiment 1 was performed to identify relevant responses of *Cp*34H to the various freezing regimes with and without an added compatible solute. Experiment 2 included both organ-

isms, increased the statistical power, the range of test temperatures (–10 to –25 °C), and tested for cell loss due to post-treatment sample processing. Experiment 3 focused on the effect of the more extreme conditions and the presence/absence of several different compatible solutes or compatible-solute precursors on both organisms. We chose as compatible solutes proline and glycine betaine, as well as the glycine betaine precursor choline. The choice of compatible solutes was based on physiological information available for *Cp*34H (*Collins and Deming*, 2013) and on genomic information available for both *Cp*34H and the permafrost isolate *Psychrobacter arcticus*, the closest relative of *P*7E for which a whole genome sequence is available (*Bakermans et al.*, 2006).

4.2 Methods

4.2.1 Seasonal fluctuations

In situ temperature data were obtained from the Mass Balance Observatory Site (MBS) established by the University of Alaska Fairbanks (*Druckenmiller et al.*, 2009) in coastal first-year sea ice near Barrow, Alaska (156.5 °W, 71.4 °N). Data sets were downloaded from the MBS website (*UAF*, 2011). Temperature measurements (accurate to ± 0.2 °C) were available for discrete depths in the ice and snow at 15-min intervals from January to June 2011. We focused our analysis on three environments: the ice column (i.e., 10 cm below the ice surface); the ice-snow interface (0 cm); and the snow-atmosphere interface (i.e., 10 cm above the ice surface). Brine salinity was estimated from temperature following phase equations for sea ice (*Cox and Weeks*, 1983; see also Chapter 5). Winter was defined as day of the year 25 – 78, with day 25 (Jan 25) being the first available measurement and day 79 (March 20) corresponding to both the spring equinox and the first inflexion point in the temperature data series. Spring was defined as days 79 – 156 (March 20 to June 5), with day 156 corresponding to the second inflexion point, just a few days before the summer solstice on day 171. Range of temperatures and brine salinities most likely to be experienced for each season and environment were determined using interquartile ranges and presented as boxplots.

Temperature and brine salinity fluctuate temporally at any given location in the ice or snow. In order to compare the intensity (energy) and frequency distribution of the fluctuations, power spectral densities were estimated with Welch's method according to *Emery and Thomson* (2001). Power spectral density was calculated for each of the three targeted locations in the ice-snow system, with winter and spring time series analyzed separately. The time series was divided into eight segments with 50 % overlap and a Hamming window function. Each segment was treated as an independent time series.

4.2.2 Isolates and growth ranges

We used subcultures of *Colwellia psychrerythraea* strain 34H, derived from the original isolate maintained at –80 °C. *Psychrobacter sp.* strain 7E was isolated from winter sea-ice brine collected from a first-year ice floe as part of the International Polar Year 2007–2008 Circumpolar Flaw Lead system study (*Barber et al.*, 2010) as follows. Using ethanol-rinsed equipment, several bore holes were drilled with an ice corer at 15-cm depth in first-year sea ice, and left covered overnight with Styrofoam plugs. Brines were collected the next day and pooled. Using aseptic technique, aliquots of brine were inoculated onto pre-chilled agar plates (based on Marine Broth 2216, Difco Laboratories, half organic strength but full seawater salinity of 32; $\frac{1}{2}\times$MB 2216) and incubated at –1 °C for two weeks. Colony isolation was achieved by sequential plating, during which a close association of this strain with a species of the genera *Psychroflexus* was observed (Appendix E). DNA was extracted from a single colony using the MoBio Ultraclean Microbial DNA isolation kit. The 16S rRNA gene from extracted DNA was amplified using primers 27F and 1492R. Amplicons were purified using the Fermentas GeneJet PCR purification kit and eluted in 35 μL of molecular grade H_2O. Purified products were centrivapped to dryness and re-suspended in 10 μL of molecular grade H_2O for sequencing on an Applied Biosystems capillary sequencer using primers 357F and 926R. Sequencing was carried out at the DNA Sequencing and Gene Analysis Centre at the University of Washington's Medicinal Chemistry Department. Resulting sequence was aligned using the Basic Local Alignment Search Tool (BLAST) of

the National Center for Biotechnology Information (NCBI).

Stock cultures of both organisms were grown and maintained in the rich medium $\frac{1}{2}\times$MB 2216. This medium, which provides optimal nutrient conditions, was also used to evaluate growth at different temperatures (–1, 2, 8, 13 and 21 °C, all at salt concentration of 35) and salt concentrations (35, 65, 80 and 125 ppt, all at a temperature of 8 °C). Salt concentration was increased by adding NaCl to the media before autoclaving. For Experiments 1 – 3, we used the more defined medium Lactate-Yeast Extract (LYE), prepared with 0.5 % lactate and 0.0014 % yeast extract in artificial seawater (ASW, 0.4 M NaCl, 9 mM KCl, 26 mM $MgCl_2$, 28 mM $MgSO_4$ and TAPSO buffer), as in (*Marx et al.*, 2009). Use of defined media was advantageous to evaluate the effect of specific compatible solutes. LYE was supplemented with glycine betaine (LYE-GB) by amending 500 mL of LYE with 0.25 mL of a 2M solution of betaine hydrochloride (SIGMA) in ASW, for a final concentration of 1 mM; NaOH was used to neutralize the LYE-GB medium to a pH of 7.3 before autoclaving.

The complete genome sequence for *Cp*34H is available (*Methé et al.*, 2005), but the genome of *P*7E has not yet been sequenced. The hypervariable V3–V5 region of the 16S RNA of *P*7E shares 97 % similarity with *Psychrobacter arcticus*, a species of the same genus isolated from Arctic permafrost, a frozen environment likely subject to similar constraints as sea ice. The available genome sequence of *P. arcticus* was thus evaluated as a proxy for *P*7E in comparative analysis with *Cp*34H. Genes were accessed online at MicrobesOnline (*Dehal et al.*, 2010; http://www.microbesonline.org) to check for homology, operons and putative function. Prediction of N- and C-terminal length was performed using the TMHMM method (http://www.cbs.dtu.dk/services/TMHMM/), which predicts transmembrane helices in proteins.

4.2.3 Bacterial measurements

Bacterial survival was measured in terms of cell abundance before and after an experimental treatment. Cell abundance was measured on samples fixed immediately after melting with

0.2 μm filtered 2 % formaldehyde and refrigerated in the dark until processing within 1 – 3 weeks, and determined by direct counts as follows. An aliquot of 80 μL (*Cp*34H) or 130 μL (*P*7E) of the fixed sample was resuspended in 3 mL of 0.2 μm filtered artificial sea water (ASW) amended with 2 – 3 drops of surfactant (Triton-X) and vortexed for 10 s to disperse cells. Resuspended samples were filtered onto 0.2 μm pore-size polycarbonate filters (Poretics) and stained for 10 min with the DNA-specific stain 4'-6'-diamidino-2-phenylindole (DAPI) as in *Marx et al.* (2009). Filters were mounted onto slides and examined with a Zeiss Universal epifluorescence microscope. A minimum of 20 fields or 200 bacteria were counted for each sample. One of the treatments from Experiment 3 (LYE-GB subjected to constant freezing at –20 °C) consistently showed cell clumping despite use of Triton-X. New aliquots were thus processed with more (5 drops) Triton-X and then sonicated for 30 s, 1 min or 5 min using a XL 2015 Sonicator (Misonix) with a 20 kHz intensity to disrupt clumps.

Daily mortality rates were calculated from average cell counts as $k = ln(\frac{X_0}{X_t})/t$, where k is the mortality rate, X_0 the bacterial abundance at the start of the experiment, X_t the bacterial abundance at the end of the experiment, and t the duration of the experiment (3 d or 15 d). Aiming to assess if daily mortality rate was constant through time, we calculated the rate from day 3 to 15 in Experiment 1 with $t = 12$ d, X_0 being the bacterial abundance at the end of the short-term experiment (3 d), and X_t the bacterial abundance at the end of the long-term experiment. This rate was compared with that of days 0 to 3.

4.2.4 Experiment 1

A comparative summary of conditions used for each of the three experiments is provided in Table 4.1. Experiment 1 was designed to explore relevant variables potentially affecting bacterial survival as measured by cell loss. Tested conditions included freezing temperatures that were constant (–13 or –20 °C) or fluctuating (between –2 and –17°C). Samples were kept frozen over short-term (3 d) or long-term (15 d) periods. Short-term treatments included

replicated temperatures (and associated brine salinities) recorded at the MBS during a weather warming event (Fig. 4.4a, b and Fig. 4.1) at 10 cm into the ice column (–13 °C) and near the atmosphere (fluctuating). Fluctuating conditions represent an extreme situation where brines at the ice-atmosphere interface are in thermal equilibrium with the air. Long-term treatments included a series of random warming (and cooling) events and their corresponding changes in brine salinity (Fig. 4.4b, d). Experiment 1 was performed with *C. psychrerythraea* strain 34H grown on media with and without compatible solutes. *Cp*34H was grown in 500 mL of LYE or LYE-GB at 8 °C to mid-exponential phase (5 d), reaching an optical density of 0.068. Aliquots of 5 mL were then transferred to sterile 15-mL test tubes held in an ice bath and then frozen for either 3 or 15 days under stable or fluctuating temperatures (n = 3 for each treatment). Additional replicated controls (n = 2) were placed at constant temperatures of 8 °C (to ensure the culture was healthy at time zero) and –20 °C (for an extreme comparison). One-mL aliquots were subsampled to measure initial cell abundance (n = 1). At the end of each treatment, samples were melted by placing test tubes in a 0 °C water bath (direct melt) and then immediately processed for cell abundance.

4.2.5 Experiment 2

Experiment 2 was designed to compare the effects of freezing temperature, constant and fluctuating, on *C. psychrerythraea* strain 34H and *Psychrobacter sp.* strain *P*7E, when frozen for short (40 min) or long (15 d) periods. Based on a power analysis of results of Experiment 1, the number of replicas for Experiment 2 was increased to six; replicated measurements of the initial bacterial abundance were included to extend the range of statistical analyses possible. To determine if the post-treatment melting method (direct or brine-melted) affected bacterial abundance, an additional comparative test was performed for samples subjected to short-term freezing.

The test organisms were grown in LYE at 8 °C to mid exponential phase (5 d). One-mL

aliquots were taken to measure initial cell abundance (n = 6). Additional 1-mL aliquots were transferred to sterile 1.5-mL microcentrifugue tubes kept in an ice bath and then frozen for 40 min or 15 d, under stable or fluctuating temperatures (n = 6). A second set of replicas was included for treatments in the short-term experiment to compare melting protocol during sample processing.

In the short-term tests, replicas were placed at –10, –15 or –20 °C with checks every 30 min to monitor freezing. Samples at warmer temperatures (< –20 °C) took longer to freeze (1 h) compared to the –20 °C samples (30 min); light shaking was required to promote nucleation. Estimated cooling rates were 0.2 – 0.7 °C min^{-1}. Once samples were frozen, they were left at the constant freezing temperature for 40 min. Estimated brine salinities corresponding to these temperatures were 143, 179 and 209. After incubation, the first set of replicas was then melted directly (direct melt), with the tube held in a water bath at 0 °C. The second set of replicas, also held in an ice bath, received 0.35 mL of pre-chilled 265 ppt sterile NaCl brine per microtube, achieving a final melt concentration of approximately 100 ppt (brine melt).

In the long-term tests, samples were held frozen at constant (–10, –15 or –20 °C) or fluctuating (between –25 °C and –7 °C) temperature. In the fluctuating treatment, temperature was shifted every 12 h simulating a diurnal fluctuation in temperature (24 h period). On one occasion, samples were left at –25 °C for 36 h. The estimated brine salinities were 143, 179 and 209 for the constant treatments and 232 and 109 for the fluctuating treatment. After 15 d under these freezing conditions, all samples were processed by brine melt as described above.

4.2.6 Experiment 3

Experiment 3 focused on the effects of the availability of compatible solutes. Both organisms were frozen for 15 d under either constant (–20 °C) or fluctuating (–7 to –25 °C) temperatures. The fluctuating environment was as described for Experiment 2, except that

the warmer temperature oscillated between –5.5 and –9 °C (–6.8 °C ±1.1, mean ± SD, n = 15), corresponding to brine salinities of 232 and 94 to 134 (mean 109 ± 12, n = 15). Both organisms were grown in LYE at 8 °C as in Experiment 2. Aliquots of 0.9 mL of culture were transferred to sterile 1.5 mL microcentrifugue tubes, held in an ice bath and amended with either 0.1 mL of LYE or 0.1 mL of a 10 mM solution (in LYE) of the compatible solute precursor choline or the compatible solutes glycine betaine and proline, for a final concentration of 1 mM. Amended media were designated as LYE-Cho (choline), LYE-GB (glycine betaine) and LYE-Pro (proline). After 15 days under freezing conditions, all samples were processed by brine melt as described above.

4.2.7 Graphical and statistical analyses

Means, standard deviation (SD), and 95 % confidence intervals (CI, shown in all figures) were calculated for all experimental data. Percentage of change in cell abundance was calculated as: $1 - \frac{C_i}{C_f}$, where C_i and C_f are the initial and final mean cell abundance. Mortality rates were calculated assuming constant mortality, with the equation

$$Kt = ln\frac{C_i}{C_f},$$

where K is the mortality rate; t, the time; and C_i and C_f as above. Statistical analyses for experimental data were performed using R v.2.13.1 (*R Development Core Team*, 2011). Environmental data from MBS were processed with Matlab as described in section 4.2.1.

The statistical tests of Shapiro and Bartlett were used to check assumptions of normality and equal variance. Experiment 1 was analyzed with a two-way analysis of variance (ANOVA) for unbalanced, proportional design, with each type of medium analyzed separately. Experiments 2 and 3 were analyzed separately for each test organism. Initial and final cell abundances (independently measured) were compared using t-tests. For Experiment 2, samples subjected to short-term freezing were analyzed with a two-way ANOVA to determine the effect of melting procedure at each temperature. The effect of freezing time (short-term vs.

long-term) was tested with a two-way analysis of variance (ANOVA) performed on samples subjected to brine melt and constant temperature (–10 °C, –15 °C or –20 °C). Fluctuating conditions were not testable under short-term freezing, and thus were compared with constant temperatures using a one-way ANOVA. For Experiment 3, a two-way ANOVA was used to compare the effect of freezing conditions (fluctuating vs. constant) using media supplemented with compatible solutes. When the medium had a significant effect, levels were analyzed with a post-hoc pairwise comparison using Tukey's Honest Significant Differences test. LYE-GB treatment was not included in this comparison because cell abundance under constant (–20 °C) conditions could not be determined due to cell clumping, but cell abundance for glycine betaine medium under fluctuating conditions was directly compared with LYE medium with a t-test.

4.3 Results

4.3.1 Temperature and salinity regimes

For all three environmental locations (ice column, ice/snow interface and snow/atmosphere interface) median temperatures were lower in winter than spring (Fig. 4.1). The difference between the winter and spring median temperatures, and the estimated brine salinities, was greatest for the snow-atmosphere interface ($\Delta T \sim 10.3$ °C; $\Delta S > 71$) and lowest for the ice column ($\Delta T \sim 5.3$ °C; $\Delta S \sim 40$). Interquartile ranges of temperature and brine salinity, representing the range of most frequent conditions in a given season, were narrower in the winter than in the spring, and narrower in the ice column than at the snow-atmosphere interface (Table 4.2). Total ranges, indicating the maximum temperature range experienced regardless of absolute temperature, were similar in both seasons for a given location, but they were, again, wider at the snow-atmosphere interface than in the ice column (Table 4.2).

This coarse measure of differences in the temperature and brine-salinity regimes was confirmed by power spectrum analysis. Power spectral density for temperature (Fig. 4.2) and for

calculated brine salinity (Fig. 4.3) showed similar behavior for the three environmental locations and both seasons, with the strength of the fluctuation decreasing, and noise level increasing, towards the high frequencies in all spectra. Fluctuations in the ice column were significantly smaller (95 % confidence interval) than at the snow-atmosphere interface for frequencies between 10^{-2} cycles per hour (cph, 4-d period) and 1 cph (1-h period). Spectral plots for spring (Fig. 4.2b, 4.3b) indicated a significant diurnal spectral peak at 0.04 cph (1-d period) consistently present in the three environments of interest. A small diurnal temperature fluctuation was noticeable in winter.

4.3.2 Isolates

Psychrobacter sp. strain 7E grew over a wider range of salinities (35 to 125) and temperatures (–1 to 25 °C) than *C. psychrerythraea* strain 34H (salinity 35 to 65, temperatures –1 to 13 °C) when cultured in $\frac{1}{2}\times$MB 2216 medium. *Cp*34H has been reported to grow at temperatures as low as –12 °C (*Wells and Deming*, 2006), with an extrapolated minimum of –14.5 °C (*Huston*, 2003), but temperatures below –1 °C were not tested in this thesis and have not been tested for *P*7E in general. For *Cp*34H, end-point (15-d) measurements of the 8 °C control culture were greater in LYE-GB medium ($2.4 \pm 0.06 \times 10^7$ cells mL^{-1}) than in LYE ($2.0 \pm 0.04 \times 10^7$ cells mL^{-1}, n = 2), a result also observed in optical-density growth curves (Appendix A). The presence of glycine betaine in the culture media, however, did not increase the salinity tolerance of *Cp*34H.

Genes related to compatible solute import and metabolism listed in Table 4.3 indicate that both *Cp*34H and *Psychrobacter arcticus* have putative transporters for compatible solutes or their precursors (betaine, choline and carnitine in both organisms; proline in *Cp*34H). The potential to transform choline to betaine is also evident by the presence of putative choline dehydrogenase and putative betaine aldehyde dehydrogenase.

The N- and C-terminal lengths of the BCCT family transporters differ greatly between *Cp*34H and *Psychrobacter arcticus*. Whereas *P. arcticus* has long C-terminal tails, putative

betT transporters from *Cp*34H have short N- and C-termini, with the N-terminus predicted to extend into the periplasmic space, not intracellularly as in other organisms (Table 4.3). The other three BCCT transporters from *Cp*34H have relatively long N-terminal tails and relatively short C-terminal tails.

4.3.3 Experiment 1

Initial cell numbers, similar for cultures grown on LYE (1.7×10^7 cells mL^{-1}) and LYE-GB (1.5×10^7 cells mL^{-1} for LYE-GB, n = 3, average ± SD) are compared to cell abundance at the end of the experiment in Fig. 4.5. Samples subjected to long-term freezing had significantly ($p < 0.05$) lower cell abundance than those subjected to short-term freezing (Fig. 4.5). A comparison of the 95 % confidence intervals in Fig. 4.5 shows lower cell abundance in the coldest temperature (–20 °C), even though the effect of temperature on cell abundance was only significant at $p < 0.1$. An analysis of power indicated that the statistical power $1–\beta$ was ~ 0.8 at $\alpha < 0.05$ for this experiment (compared to $1–\beta$ of > 0.95 at $\alpha < 0.05$ in Experiments 2 and 3, where n = 6). The majority of the cell loss occurred between days 3 and 15 for the milder (–13 °C) and fluctuating (–2 to –17 °C) temperatures (Table 4.4). At the coldest temperature (–20 °C), on the contrary, most of the cell loss occurred in the first three days of the experiment.

4.3.4 Experiment 2

Initial cell numbers for *Cp*34H ($2.8 \pm 0.3 \times10^7$ cells mL^{-1}) and *P*7E ($7.2 \pm 1.8 \times10^6$ cells mL^{-1}, n = 6, average ± SD) are compared to cell abundance at the end of the experiment in Fig. 4.6. None of the *P*7E replicas subjected to long-term freezing at –10 or –15 °C was frozen at the end of the experiment. Samples from the direct melts of *P*7E subjected to –10 °C were lost due to procedural error. Despite these caveats, after the short-term (40 min) freezing treatments, both organisms showed significantly lower bacterial abundance in samples subjected to the direct-melt protocol when compared to the brine melt ($p < 0.05$;

Fig. 4.6a, b). Brine melts did not show significant loss in cell abundance compared to the initial values for either *Cp*34H or *P*7E.

Samples subjected to long-term freezing (15 d) were melted only with the brine-melt approach; thus they were only compared with the brine-melt treatments of the short-term freezing. *Cp*34H had a significant difference in cell abundance due to length and temperature of freezing ($p < 0.05$). *P*7E, on the other hand, had a significant difference in cell abundance due to the interaction of temperature and freezing time ($p \leq 0.01$), but no difference could be explained statistically by freezing time or by freezing temperature only.

Even using the protective brine-melt protocol for sample processing, *Cp*34H had significant cell loss after 15 d of freezing under both temperature regimes (Fig. 4.6c, $p < 0.05$), with the percentage of cell loss increasing two- to three-fold compared to the short-term treatment. The greatest cell loss (Fig. 4.6c) and highest daily mortality rates (Table 4.5) occurred under the constant –10 °C and the fluctuating treatment. In contrast, cell numbers for *P*7E did not change significantly in samples kept at –10 or –15 °C for 15 d (these samples did not freeze), and actually increased ≥ 30 % under the fluctuating ($p < 0.01$) and constant –20 °C ($p = 0.06$) treatments. Both organisms presented significant ($p < 0.001$) changes in cell abundance depending on the temperature regime at which they were frozen for 15 d. The greater changes were seen in the fluctuating treatment, which resulted in a decrease in cell numbers for *Cp*34H and an increase for *P*7E.

4.3.5 Experiment 3

Initial cell numbers for *Cp*34H ($1.9 \pm 0.2 \times 10^7$ cells mL^{-1}) and *P*7E ($4.2 \pm 0.3 \times 10^6$ cells mL^{-1}, $n = 6$, average ± SD), lower than for Experiment 2, are compared to cell abundance at the end of the experiment in Fig. 4.7. Freezing *Cp*34H at constant –20 °C for 15 d in unamended medium LYE, or amended media LYE-Pro and LYE-Cho did not result in significant difference from initial cell numbers (Fig. 4.7a). In contrast, cell numbers for *P*7E significantly increased in this treatment for all media ($p < 0.01$, Fig. 4.7b). Cell

clumping was observed for both organisms when frozen in LYE-GB medium at constant –20 °C, preventing accurate cell counts. This treatment is thus not included in the statistical analysis.

Freezing under fluctuating temperatures (Fig. 4.7c) resulted in significant cell loss for *Cp*34H in LYE medium ($p < 0.001$) and the amended media LYE-Pro and LYE-GB ($p < 0.05$). The greatest percentage of cell loss was observed for LYE (Fig. 4.7c), which also had the highest daily mortality rate (Table 4.6). No significant cell loss was detected in LYE-Cho medium. *P*7E showed a significant increase in cell numbers in LYE and LYE-GB media ($p < 0.05$), but no difference in LYE-Cho and LYE-Pro (Fig. 4.7d). The increase in cell numbers observed for *P*7E when frozen in LYE medium under fluctuating conditions was higher in Experiment 2 than in Experiment 3, where initial cell numbers were also lower (Tables 4.5, 4.6).

Cell abundance for *Cp*34H was significantly ($p < 0.001$) different at the end of the experiment depending on media (excluding the GB treatment), temperature regime (constant vs. fluctuating) and the interaction of these two factors. Post-hoc tests showed higher numbers in media with compatible solute (LYE-Cho, LYE-Pro) compared to unamended LYE medium ($p < 0.001$), and no significant difference between LYE-Cho and LYE-Pro. For *P*7E, only the temperature regime showed a significant effect ($p < 0.05$). Cell numbers in LYE-GB, analyzed separately, were higher than those in LYE for both organisms ($p < 0.001$).

4.4 Discussion

4.4.1 Environmental parameters and the sea-ice microbial community

The median winter temperatures of –14, –16 and –20 °C observed at different depths in the ice-snow system we studied (Fig. 4.1), although considered limiting for growth in the majority of bacteria, may still be permissive for selected sea-ice organisms. *Psychromonas*

ingrahamii and *C. psychrerythraea* strain 34H can grow at –12 °C (*Breezee et al.*, 2004; *Wells and Deming*, 2006), with theoretical growth for *Cp*34H at –14.5 °C (*Huston*, 2003). The theoretical temperature limit for *Cp*34H growth, though, may not be as low as expected if high salinity (associated with very cold sea ice) is the primary limiting factor as suggested by experimental results discussed below. Organisms similar to *Planococcus halocryophilus* Or1 (from permafrost), which can grow at –15 °C (*Mykytczuk et al.*, 2013), could also be present in sea ice. Perhaps more importantly to survival, the limits for bacterial activity other than growth are less restrictive than this observed environmental range in sea ice and associated environments. Different types of metabolic activity are reported at temperatures from –5 to –32 °C (*Bakermans and Skidmore*, 2011 and references within).

The snow-atmosphere interface had the broadest temperature interquartile range (Fig. 4.1, Table 4.2) and higher energies in the power spectral density plots (Fig. 4.2, 4.3), reflecting atmospheric changes in temperature. The insulating properties of ice and snow were evident by milder fluctuations in the ice-snow interface and the ice column. Differences in cell abundance in saline snow under thin or thick snow covers have been attributed to different fluctuating regimes (Chapter 3; *Ewert et al.*, 2013).

Long-term freezing in sea ice has the potential to select for different bacterial populations, as has been proposed for soil ecosystems (*Morley et al.*, 1983). In the case of sea ice, differential selection could be particularly marked in environments characterized by the strongest fluctuations, namely brines exposed directly to the atmosphere (Fig. 4.2). This effect may also help to explain observed bacterial selectivity in saline frost flowers on the surface of new ice (*Bowman et al.*, 2013). Supporting this hypothesis, the two species evaluated in this study responded differently to long term freezing under constant and fluctuating temperatures, with different susceptibility to osmotic down-shift and different requirements of compatible solutes (Fig. 4.5 to 4.7).

Different growth ranges of the two species studied could also have played a role in their differential survival. During the warmer fraction of a fluctuation cycle, organisms may be able to perform functions otherwise limited by temperature, such as reproduction or repair.

Despite cold adaptation, however, growth of *Cp*34H at even moderate subzero temperatures may be limited by the corresponding brine salinity. *P*7E, on the other hand, even though less cold-adapted, has the advantage of tolerating a wider range of salinities, and thus may be active during the warming periods of the fluctuation. This suggestion is in keeping with the recognition that simultaneous adaptation to multiple stressors and a broad range of environmental conditions is characteristic of species inhabiting frozen environments such as permafrost (*Mykytczuk et al.*, 2013).

4.4.2 Effects of short-term freezing and melting on cell abundance

Throughout winter and spring, sea-ice microorganisms experience short-term temperature changes on timescales of hours to days (Fig. 4.1). Temperature fluctuations cause freeze-thaw events that alter the brine volume fraction of the ice, the brine salinity and the concentration of other solutes and particles. These events possibly impact the bacterial community by the formation of ice crystals during freezing and the exposure to osmotic shock during thaw.

To evaluate if mortality could occur in a limited number of freeze-thaw events imposed over a timescale of days, Experiment 1 (Fig. 4.4a, b) exposed *Cp*34H to two mild freeze-thaw events (Δ T = – 17 °C; Δ S = 155), which resulted in lower (though not significantly lower) cell numbers than one mild freeze-thaw event (Δ T = – 13 °C ; Δ S = 130) as experienced in the – 13 °C constant treatment. The highest mortality was observed for organisms subjected to the strongest freeze-thaw event (Δ T = – 20 °C; Δ S = 175), experienced at the – 20 °C treatment. Since the melting protocol included a rapid osmotic downshift for all samples, Experiment 1 could not distinguish if cell loss was due to the freezing or the melting only.

Experiment 2 exposed cells to similar short-term freeze-thaw events of both, lesser and greater magnitude, and differentiated cell mortality due to a freezing episode (formation of ice crystals, osmotic up-shift and reduction in habitable space) from the sum of freezing and melting (with the additional exposure to osmotic shock). The use of brine melts, protecting

against osmotic shock during melting, resulted in minimal (statistical insignificant) cell loss for both species whereas direct melts, exposing organisms to $\Delta S > 108$, increased by two-fold the percentage of cell loss (Fig. 4.6). Transient decreases in temperature, with concomitant changes associated with ice formation, are thus not strong contributors to cell mortality, compared to a down-shift in salinity. These single freeze-thaw events, developed over a timescale of hours, did not show difference due to the magnitude of the temperature change.

The increase in mortality associated with osmotic down-shift, even if observed for both organisms, was only significant for *C. psychrerythraea* strain 34H, presumably reflecting the more euryhaline character of *Psychrobacter sp.* strain 7E. *In situ* osmotic shock experienced by sea-ice communities during short-term warming events may favor species better adapted to osmolarity changes. For instance, the higher tolerance of osmotic down-shifts of *Psychrobacter* species, which have been detected in Summer sea ice (e.g. *Bowman et al.*, 1997), might help them better persist through winter and spring. The stronger short-term temperature fluctuations at the ice- or snow-atmosphere interface may impose stronger constraints than those at the upper-ice column (Fig. 4.2) where conditions remain relatively constant. In fact, no changes in the dominant members of the bacterial or archaeal community have been observed for the upper ice column through the winter (*Collins et al.*, 2010), though changes in surface communities of the ice-snow system remain to be examined.

The results of this study also highlight the need to use brine melts when processing sea-ice samples from very cold environments, as discussed in (*Miller et al.*, 2013), in keeping with observations by Ewert *et al.* (2013, Chapter 3) that winter surface sea-ice samples subjected to direct melting suffered significant cell loss.

4.4.3 Effects of long-term freezing on cell abundance

Long-term constant freezing at –10, –15 and –20 °C, in unamended medium, had a deleterious effect on *Cp*34H, with cell losses up to 45 % and daily mortality rates between 1.6

and 3.9 cells d^{-1} (Experiments 2 and 3). Whether or not the effect was due only to the prolonged exposure to low temperatures or only to the corresponding exposure to high salinities, cannot be deduced from these experiments. The highly deleterious effect of long-term freezing in Experiment 1, on the other hand, must have been due to the use of the direct melt approach for sample processing, for the effect was milder in Experiments 2 and 3 when similarly treated samples were melted into brine (Fig. 4.6c, 4.7a; LYE-medium at –20 °C).

Mortality of *Cp*34H during long-term freezing in a saline medium could have occurred by irreversible plasmolysis, the shrinking of the cytoplasm and consequent separation of the membrane from the cell wall. Upon freezing, cells are exposed to high external osmolarities, which cause them to loose water and dehydrate. Unless retained by a hydratable shell of EPS, water lost to the extracellular media may be incorporated into the growing ice lattice and thus be effectively removed from the vicinity of the organism. Cellular dehydration can cause plasmolysis, usually a reversible condition, except in extreme situations where it causes cellular damage and compromises cell survival (*Scheie*, 1969). Alternatively, when freezing rates are relatively rapid (as in these experiments), intracellular water cannot leave the cell fast enough, resulting in the formation of intracellular crystals associated with cell damage, at least for the case of eukaryotic cells (*Mazur*, 1984). Intracellular crystals prevent cell maintenance tasks, explaining the higher mortality rates observed at –10 °C in this study. Another possible mechanism to explain *Cp*34H mortality is viral activity. Changes in intracellular ionic content by up-shifts in salinity are known to induce lysogenic phages in other bacteria and result in cell lysis (*Shkilnyj and Koudelka*, 2007). *Cp*34H carries in its genome two putative lysogenic phages (*Methé et al.*, 2005), although attempts to induce them have not been successful (*Wells*, 2006). The more extreme conditions of the experiments in this study may have induced the expression of one or both viruses, with viral activity being higher at the warmer temperatures (–10 °C). However, neither cell plasmolysis nor viral presence in the media were evaluated in these experiments.

Daily mortality rates for *Cp*34H subjected to long term freezing at constant –20 °C (Tables 4.5, 4.6) were similar to those reported for natural bacteria communities in winter upper sea

ice (1 %) as calculated from data in *Collins et al.* (2008). Direct comparison between these rates, though, must acknowledge that cell loss in the environment is a complex process, involving a diverse sea-ice population, the presence of cryoprotective substances of algal origin (e.g. *Krembs et al.*, 2011), the presence of osmoprotective substances released by various organisms (*Kapfhammer et al.*, 2005), substantial physical losses (up to 50 %) due to brine drainage or expulsion, and variable mortality rates over the lifetime of the ice. Furthermore, our experimental setup had two properties usually associated with high mortality in frozen samples: rapid freezing rates and cells obtained from cultures in exponential growth phase (*Morley et al.*, 1983; *Mazur*, 1984).

Mortality rates are not expected to be uniform throughout the season. Daily mortality rates measured soon after freezing in Experiment 1 (Table 4.4, day 0 – 3) were 6 times higher than those measured a few days after freezing (days 3 – 15). This change may indicate acclimation to the freezing conditions, or an intrinsic phenotypic heterogeneity where some cells in the population die soon after entrapment in the ice and others are better fit to resist a particular set of extreme conditions (*Fraser and Kærn*, 2009). The reduction in mortality rate found in this experiment is also consistent with measurements by *Krembs et al.* (2002), who measured cell abundances before and after incubation of ice cores for 118 and 441 days at three temperatures (–5, –15 and –25 °C). In all cases, daily mortality rates between days 0 and 118 were higher than between days 118 and 441.

*P*7E was less susceptible to long-term freezing, consistently showing no change or even a significant increase in cell numbers after 15 d under constant –20 °C. Results from warmer temperatures are inconclusive because cultures did not freeze at temperatures above –15 °C, so no corresponding increase in salinity was experienced. Lack of freezing could be possibly due to the presence of extracellular antifreeze substances (not determined). Bacterial cultures remaining unfrozen at temperatures below the freezing point of seawater to –12 °C have been reported for *Cp*34H (*Wells and Deming*, 2006). In this study, all samples of *Cp*34H froze, but those at warmer temperatures had to be shaken to promote ice nucleation.

The increase in *P*7E cell numbers was associated with a decrease in cell size, indicating that

*P*7E may have gone through a process of fragmentation prompted by the extreme conditions experienced during freezing. Increase in cell numbers accompanied by a reduction in cell size has been observed in a species of marine *Vibrio* subjected to starvation, with increases in cell numbers from 100 to 800 % in the first two weeks (*Novitsky and Morita*, 1977). In independent studies, reduction in bacterial cell size associated with freezing temperatures was reported for permafrost isolates grown at –10 °C (*Bakermans et al.*, 2003) and increase in cell numbers was reported for soil bacteria frozen at constant –9 °C for 9 days (*Morley et al.*, 1983). Smaller cells have faster rates of water equilibration to external osmolarity, which may accelerate dehydration when cells are exposed to the high salinity of brines, but at the same time prevent the formation of intracellular ice crystals (*Mazur*, 1984).

4.4.4 Effects of a fluctuating environment

Temperature fluctuations produce freeze-thaw events that can increase bacterial mortality by exposing cells to down-shifts in osmolarity leading to osmotic lysis, as observed in the direct-melt samples of Experiment 2. As studied in other frozen environments, mortality depends on the number of repeated freeze–thaw events and the range of temperatures experienced (*Morley et al.*, 1983; *Liu et al.*, 2013), which in turn determine the extent of the osmotic down-shift. In this study, the two examined strains responded differently to freezing for 15 d under fluctuating conditions without compatible solutes. The high cell losses observed for *Cp*34H (Fig. 4.6c, 4.7c) fall within the range of 40 – 60 % reported for soil samples subject to repeated freeze-thaw cycles (*Morley et al.*, 1983). *P*7E, on the other hand, had significant increases in cell numbers in the range of 15 – 66 %, likely the result of cell fragmentation.

Fluctuations may also increase the energy requirements of the cell, beyond the already high energy demands associated with survival at low temperatures (*Bakermans and Nealson*, 2004) and at high salinities (*Oren*, 1999). For instance, up-shifts in salinity prompt a genetic response involving a likely transient up-regulation of multiple genes (*Bergholz et al.*,

2012; *Marin et al.*, 2004; *Mykytczuk et al.*, 2013), with genes involved in osmolyte transportation returning to their basal states once the organism is acclimated. The repeated update in gene-expression level associated with a fluctuating environment would involve an energetic cost for organisms. Another energetic cost will be associated with down-shifts in salinity. During osmotic down-shift, intracellular solutes are quickly released by non-specific mechanosensitive membrane channels to avoid cell lysis (Appendix E). After a few minutes, some of the solutes are recovered via specific transporters (*Schleyer et al.*, 1993), a process that requires energy (*Oren*, 1999). Repeated down-shifts are expected thus to increase the energy demands of the organism, even to a point where recovery of essential solutes is compromised.

Viral lysis can also contribute to mortality for sea-ice organisms experiencing freeze-thaw cycles. Cells plasmolysed as a result of osmotic up-shift are more susceptible to lysozymes, which can lead to cell lysis if the affected cell experiences a subsequent down-shift in osmolarity (*Scheie*, 1973), as would occur in a fluctuating environment. Lysozymes, or muramidases, are commonly expressed in bacteriophage as an entry/release mechanism to/from the cell (*Fastrez*, 1996). In particular, *Colwelliaphage* 9A, which infects *Cp*34H, contains three such muramidases, one of which has an amino-acid signature that classifies it as strongly psychrophilic (*Colangelo-Lillis and Deming*, 2013). Our experimental design did not include the observation of viral infection.

4.4.5 Protective role of compatible solutes

As predicted by the presence of proline, glycine-betaine and choline transporters (Table 4.3), compatible solutes present at the time of freezing decreased cell loss in *Colwellia psychrerythraea* strain 34H under fluctuating conditions (Fig. 4.7c). Glycine betaine had observable effects for both organisms under fluctuating conditions, improving *Cp*34H survival and significantly increasing cell numbers in *P*7E. Under constant –20 °C, glycine betaine promoted cellular aggregation in all samples for both species, a phenomenon reported

for *Vibrio cholerae*, whereby exogenous glycine betaine can induce biofilm formation under high-salinity conditions by promoting the synthesis of a biofilm polysaccharide (*Kapfhammer et al.*, 2005). In this study, biofilm formation was not induced in the fluctuating treatment, perhaps because glycine betaine accumulation was likely transient, released back to the media with every osmotic down-shift. In contrast, a constant –20 °C temperature would maintain a relatively stable interior ice-surface area, and also allow the precipitation of salt crystals within the brine inclusions, offering additional stable surfaces for attachment. Under fluctuating conditions the interior architecture and surface area of the brine network would be altered periodically, and salt crystals could re-dissolve during the warmer, lower-salinity periods. The availability of choline, a compatible solute precursor, also reduced mortality in *Cp*34H under fluctuating conditions (Fig. 4.7), confirming that one or both of its betT genes and betIBA operons (Table 4.3) are able to transport choline and transform it to betaine to act as compatible solute, as proposed by *Collins and Deming* (2013). A minor anti-viral protective feature of compatible solutes may also be at work in our experiments, as observed in experiments with *E. coli* where glycine betaine reduced by 10 % the induction of lysogenic viruses associated with salinity up-shifts (*Shkilnyj and Koudelka*, 2007). In the case of *Cp*34H, which can completely metabolize glycine betaine (*Collins and Deming*, 2013), this compatible solute could also provide a protective effect by acting as an additional energy source compensating the higher energy requirements described above.

Contrary to Experiment 3, where the addition of glycine betaine at the time of freezing increased *Cp*34H survival under fluctuating conditions (compared to unamended medium, Fig. 4.7), adding glycine betaine during *Cp*34H growth (days before the freezing event) did not improve survival (Fig. 4.5). This result is explained by the ability of *Cp*34H to metabolize glycine betaine, with cells consuming the bulk of available glycine betaine while growing for 5 d at 8 °C under permissive salinities, making it unavailable at the time of freezing. The consumption of glycine betaine in our cultures was confirmed by growth curves, where *Cp*34H produced a higher yield in LYE-GB than in LYE (Appendix A). Natural estuarine populations are known to metabolize glycine betaine derived from environmental choline in < 3 d at 27 °C, either by respiring it to CO_2 or incorporating it

as a source of carbon (*Kiene*, 1998). In particular, the betT transporter has been shown to participate in the incorporation of glycine betaine for catabolic functions, even when organisms are not subjected to high-salinity conditions (*Chen and Beattie*, 2008). On a related note, the minimal medium LYE used in this study contains yeast extract, which is known to contain glycine betaine (*Empadinhas and da Costa*, 2008). This glycine betaine from the media, though, was likely consumed before the start of freezing, thus reducing its availability as osmoprotectant.

The activation mechanism determining the expression of the BCCT transporters, and thus the uptake of glycine betaine, may have differed according to the length of the transporter C-terminus in each isolate. Long C-terminus tail are required for osmotic regulation of the betT transporter in *E. coli* and other bacteria (*Tøndervik and Strøm*, 2007; *Ziegler et al.*, 2010). The long (> 100 residues) C-terminal tails of the *Psychrobacter arcticus* BCCT transporters suggest their activity can be upregulated by osmolarity, possibly being the case in our *Psychrobacter sp.* isolate as well. All betT transporters from *Cp*34H, however, have a short C-terminus, implying that they are not activated as a response to salinity but perhaps as a response to temperature, as in the betP BCCT transporter of *Corynebacterium glutamicum* (*Özcan et al.*, 2005). This temperature trigger could explain why the presence of glycine betaine, in spite of increasing the survival of *Cp*34H under fluctuating conditions (Experiment 3; Fig. 4.7c), did not extend its salinity growth range. When *Cp*34H was grown in LYE and LYE-GB at 8 °C, the maximum salt concentration at which growth was detected was 60 ppt for both types of media (data not shown).

Upon osmotic down-shift, bacteria can release compatible solutes accumulated or synthesized in the cell, which then become available to other organisms. For example, glycine betaine present in the spent media of related *Vibrio* species effectively acted as an osmoprotectant (*Kapfhammer et al.*, 2005), offering a compelling perspective for studying the protective role of compatible solutes in mixed communities. In a mixed community, organisms without the ability to synthesize compatible solutes, but with appropriate transporters, could incorporate them for osmoprotection or consumption depending on the fluctuation

regime. The maximum effectiveness of compatible solutes in the sea-ice ecosystem could thus depend on the timing of their production and location in the ice column.

4.5 Conclusions

The effects of exposure to freezing under constant and fluctuating conditions, as determined by cell losses, was different for the two species evaluated in this study. *P*7E, tolerant to a wide range of temperatures and salinities, generally did not experience cell loss after freezing, but underwent a process of miniaturization and fractionation. *Cp*34H, with narrower growth ranges of temperature and salinity, had higher cell loss when exposed to freezing. More work could be done to determine if organisms with broader growth ranges are selectively selected in frozen environments subjected to fluctuating temperatures.

Cell loss in *Cp*34H was similar in samples frozen at constant temperatures of –20 °C and –15 °C, with a higher mortality rate occurring at the constant warmer temperature of –10 °C. Winter environmental data depict the snow-atmosphere interface as a more extreme environment than the upper ice column, with median temperatures up to 6 degrees colder and brine salinities up to 40 units higher. Differences in median values are insufficient, though, to account for the observed mortality, leaving the differences in fluctuation regime as the more important condition determining cell loss in sea ice. In fact, fluctuating regimes, significantly stronger at the snow-atmosphere interface than in the upper ice column, were consistently associated with elevated mortality in *Cp*34H. Higher mortality rates at the warmer temperature of –10 °C, possibly due to more rapid decay processes or increased viral activity, could translate into seasonal differences in the dynamics of the bacterial population with higher mortality rates by spring.

The presence of compatible solutes at the time of freezing improved the survival of *Cp*34H. Glycine betaine, in particular, induced biofilm formation in both, *Cp*34H and *P*7E when frozen for 15 d at constant –20 °C, highlighting the role of compatible solutes in stress-associated responses for organisms frozen under conditions similar to those experienced

during winter in the upper sea-ice column.

Future work could explore the role of temperature and salinity in the incorporation of compatible solutes by sea-ice bacteria, in particular *Cp*34H, which, we have hypothesized may incorporate glycine betaine as a response to down-shifts in temperature. Another profitable avenue of work would examine if selective mortality takes place in sea ice and associated environments characterized by different fluctuation regimes depending on the salinity and temperature growth ranges of the bacterial community.

4.6 Acknowledgments

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Table 4.1: Summary of performed experiments

Exp.	Organism	Melt protocol[b]	Compatible solutes[c]	> –20 °C		–20 °C		Fluctuation	
				Short	Long	Short	Long	Short	Long
1	*Cp*34H	Direct	GB	✓	✓	✓	✓	✓	✓
2	*Cp*34H-*P*7E	Direct, Brine	—	✓	✓	✓	✓	—	✓
3	*Cp*34H, *P*7E	Brine	Pro-Cho-GB	—	—	—	✓	—	✓

[a] Samples were exposed to given temperature conditions during a short (3 d in Experiment 1, 40 min in Experiment 2) or a long period of time (15 d in all experiments).
[b] Samples were melted either directly (Direct melt), in a pre-chilled NaCl brine solution achieving a final melt concentration of approximately 100 ppt (Brine melt), or using both approaches as in Experiment 2.
[c] Each treatment included either one of the compatible solutes proline (Pro), glycine betaine (GB) or its precursor choline (Cho).

Table 4.2: Total and interquantile ranges [a] of temperature (T) and salinity (S)[b] recorded at different depths of the ice-snow system at MSB (2011)

Environment	Season	Total range		Interquantile range	
		T (°C)	S	T (°C)	S
Ice column	Winter	12.4	83	3.6	23
	Spring	15.9	179	7.8	87
Ice-snow interface	Winter	16.5	90	4.6	28
	Spring	20.0	191	9.6	105
Snow-atmosphere interface	Winter	30.1	136	6.7	42
	Spring	28.5	232	13	136

[a] Ranges are independent of absolute values.
[b] Brine salinity, expressed according to practical salinity scale, and calculated according to (*Cox and Weeks*, 1983).

Table 4.3: Genes annotated with putative functions in compatible solute metabolism and transport

Organism	Gene	Gene (N-/C-terminus) [a]	Function
Colwellia psychrerythraea strain 34H	betT	CPS_4009 (3/27) CPS_1335 (3/27)	Putative BCCT transporter
	betI	CPS_4012 CPS_1332	Transcriptional regulator
	betB	CPS_4011 CPS_1333	Betaine aldehyde dehydrogenase
	betA	CPS_4010 CPS_1334 CPS_0670 CPS_3434	Choline dehydrogenase
	MaoC	CPS_0671 CPS_3435	Acyl dehydratase
	betP (?)	CPS_2003 (42/9) CPS_4027 (42/22) CPS_3860 (19/16)	Putative BCCT transporter
	ProX	CPS_4933	Amino-acid ABC active transporter
	ProV	CPS_4935	ATP-binding protein
	ProW	CPS_4934	Proline /glycine betaine ABC transporter
	PutP	CPS_3347 CPS_0068 CPS_3463 CPS_0939 CPS_3419	Na^+/proline symporter
	CirA	CPS_3344	Outer membrane receptor protein
	CaiC	CPS_3345	Acyl-CoA synthetases (AMP-forming) / AMP-acid ligases II
	CaiD	CPS_3346	Enoyl-CoA hydratase / carnithine racemase
Psychrobacter arcticus	betU	Psyc_1301 (8/185)	Putative BCCT transporter
	betT	Psyc_0727 (18/177)	Putative BCCT transporter
	betT	Psyc_0826 (18/44)	
	betI	Psyc_0730	Transcriptional regulator
	betA	Psyc_0728	Choline dehydrogenase
	betB	Psyc_0729	Betaine aldehyde dehydrogenase
	PutP	Psyc_1415	Na^+/proline symporter

[a] Numbers in parantheses indicate length in number of amino acids of N- and C-terminal tails for BCCT-family transporters as predicted by the TMHMM method following *Chen and Beattie* (2008) (http://www.cbs.dtu.dk/services/TMHMM/). MaoC genes CPS_0671 and CPS_3435 are included in this list, as they are predicted to belong to the same operon as betA genes CPS_0670 and CPS_3434, respectively. CaiC, CaiD and CirA genes are included, as they are predicted to belong to the same operon as PutP gene CPS_3347.

Table 4.4: Daily mortality rates [a] for Experiment 1 (3 and 15 d of freezing)

Time (d)	Media	–13 °C		–20 °C		Fluctuating	
		% d^{-1}	Cells d^{-1}	% d^{-1}	Cells d^{-1}	% d^{-1}	Cells d^{-1}
1 - 3	LYE	— [b]	—	12	18×10^5	1	1.9×10^5
	LYE-GB	—	—	11	15×10^5	—	—
3 - 15	LYE	4	6.4×10^5	2	2.3×10^5	6	6.9×10^5
	LYE-GB	6	8.1×10^5	2	2.2×10^5	4	5.6×10^5

[a] Daily mortality rate expressed as percentage (%) of the population or total number of cells lost per day.
[b] Indicates no cell loss.

Table 4.5: Daily mortality rates [a] for Experiment 2 (15 d of freezing)

Isolate	–10 °C		–15°C		–20 °C		Fluctuating	
	% d^{-1}	Cells d^{-1}	% d^{-1}	Cells d^{-1}	% d^{-1}	Cells d^{-1}	% d^{-1}	Cells d^{-1}
*Cp*34H	4	8.0×10^5	2	3.8×10^5	2	4.0×10^5	5	9.7×10^5
*P*7E	2	1.0×10^5	1	0.4×10^5	— [b]	—	—	—

[a] Daily mortality rate expressed as percentage (%) of the population or total number of cells lost per day.
[b] Indicates no cell loss.

Table 4.6: Daily mortality rates [a] for Experiment 3 [b] (15 d of freezing)

Temperature	LYE		LYE-Pro		LYE-Cho		LYE-GB	
	% d^{-1}	Cells d^{-1}	% d^{-1}	Cells d^{-1}	% d^{-1}	Cells d^{-1}	% d^{-1}	Cells d^{-1}
–20 °C	0.3	0.5×10^5	— [c]	—	0.4	7.6×10^5	— [d]	—
Fluctuating	5	6.3×10^5	1	1.3×10^5	0.1	0.2×10^5	1	1.5×10^5

[a] Daily mortality rate expressed as percentage (%) of the population or total number of cells lost per day.
[b] *Psychrobacter sp.* did not present cell loss in any of the treatments.
[c] Indicates no cell loss.
[d] Not measured due to cell aggregation.

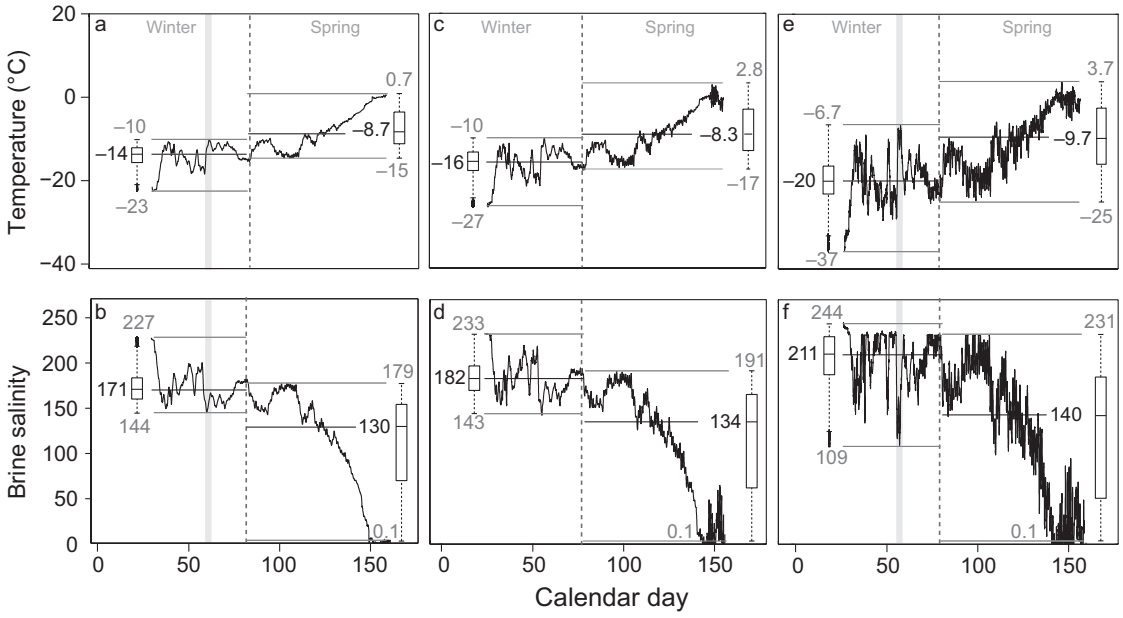

Figure 4.1: Seasonal temperatures (a, b, c) recorded at MBS during winter and spring 2011 and corresponding estimated brine salinity (c, d, e) for the upper ice column (a, d), ice-snow interface (b, e) and snow-atmosphere interface (c, f). For each season and environment, boxplots illustrate conditions most frequently experienced; black lines and numbers indicate the median and gray lines and numbers indicate maximum and minimum. The shaded vertical line in panels a, c, d and f indicates the warming event replicated in Experiment 1. The vertical dashed line marks the spring equinox. Modified from Fig. 3 and 4 in *Ewert and Deming* (2013); see Fig. 5.3 and 5.4 in Chapter 5

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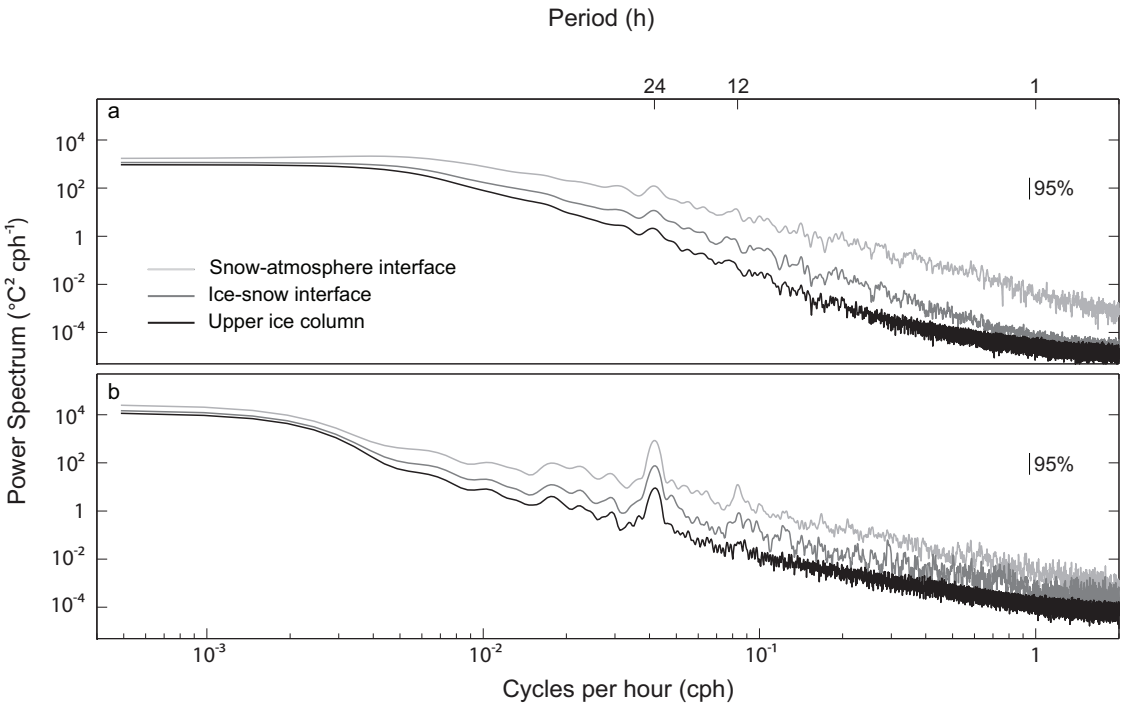

Figure 4.2: Power spectral density for temperature variation (°C), calculated from data in Fig. 4.1 for winter (a) and spring (b) 2011. Lines of different darkness indicate fluctuations at the upper ice column (light gray), ice-snow interface (dark gray) and snow-atmosphere interface (black). Vertical dash indicates 95 % confidence interval. Diurnal, semi-diurnal and hourly periods are indicated on the secondary (upper) X-axis.

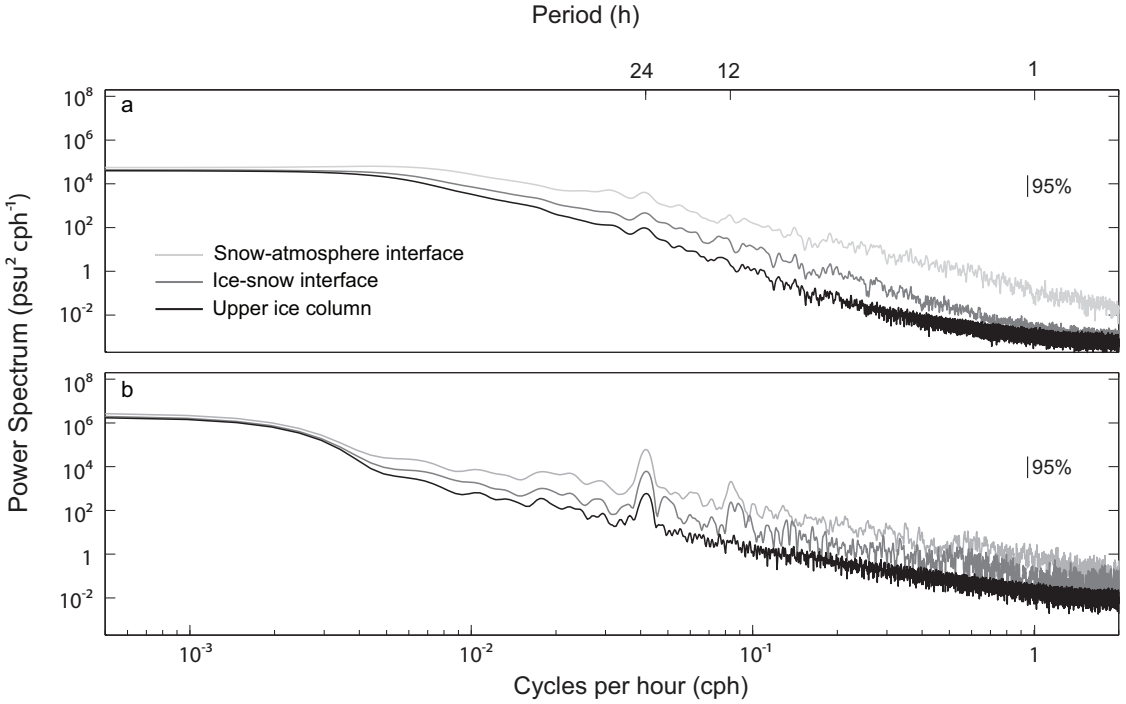

Figure 4.3: Power spectral density for variations in brine salinity, calculated using phase equations for sea ice (*Cox and Weeks*, 1983) and temperature measurements at MBS during winter (a) and spring (b) 2011. Lines, confidence interval and secondary axis as in Fig. 4.2.

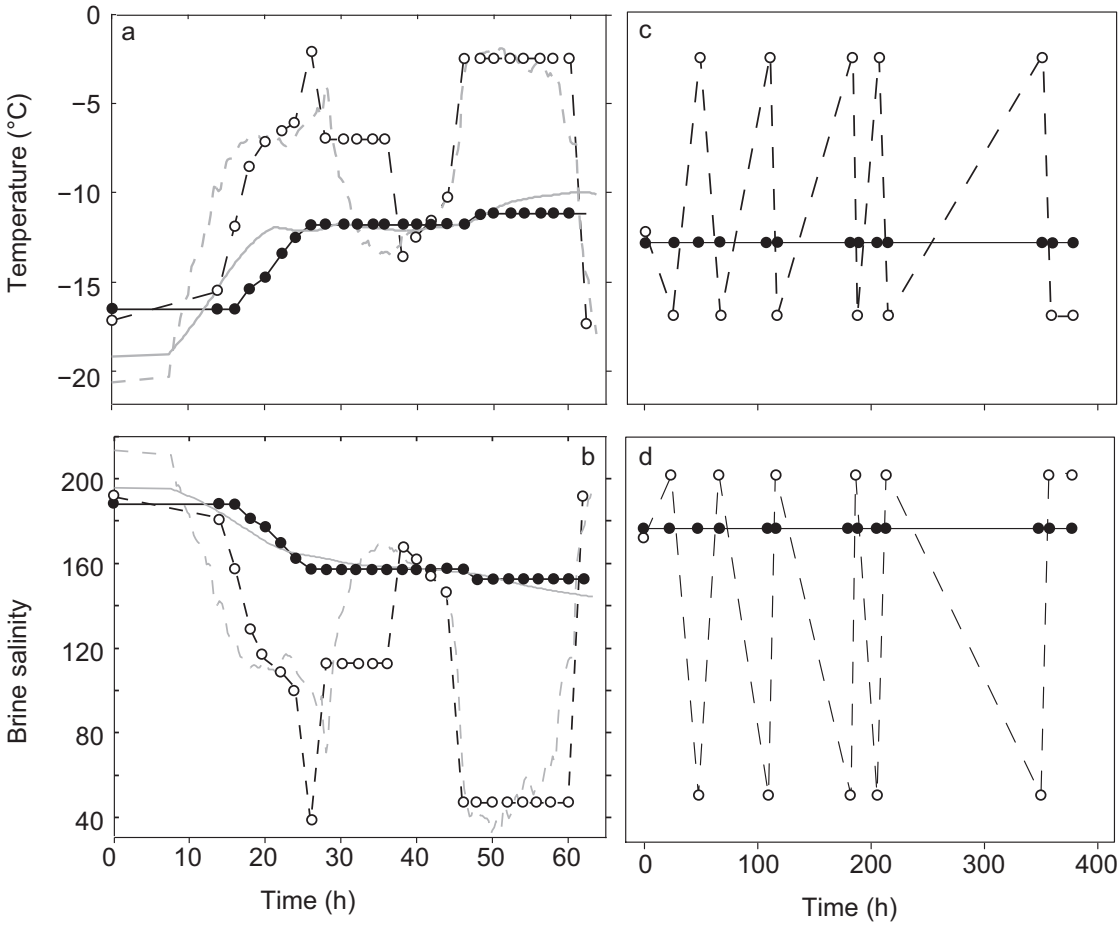

Figure 4.4: Temperature and calculated brine salinity for short-term (a, b) and long-term (c, d) freezing (Experiment 1). Circles indicate actual experimental temperatures for the fluctuating (open) and stable (filled) treatments. For short-term freezing, experimental temperatures were selected to follow closely the environmental values represented by the shaded area in Fig. 4.1. Gray lines (a, b) indicate environmental values recorded at MBS during days 54 to 58 (2011) at 10 cm below the ice-snow interface (continuous line) or 10 cm above the snow-atmosphere interface (dashed line). The snow-atmosphere conditions were selected to correspond to a more extreme case than the snow surface data presented in Fig. 4.1, where brines would be in thermal equilibrium with the air.

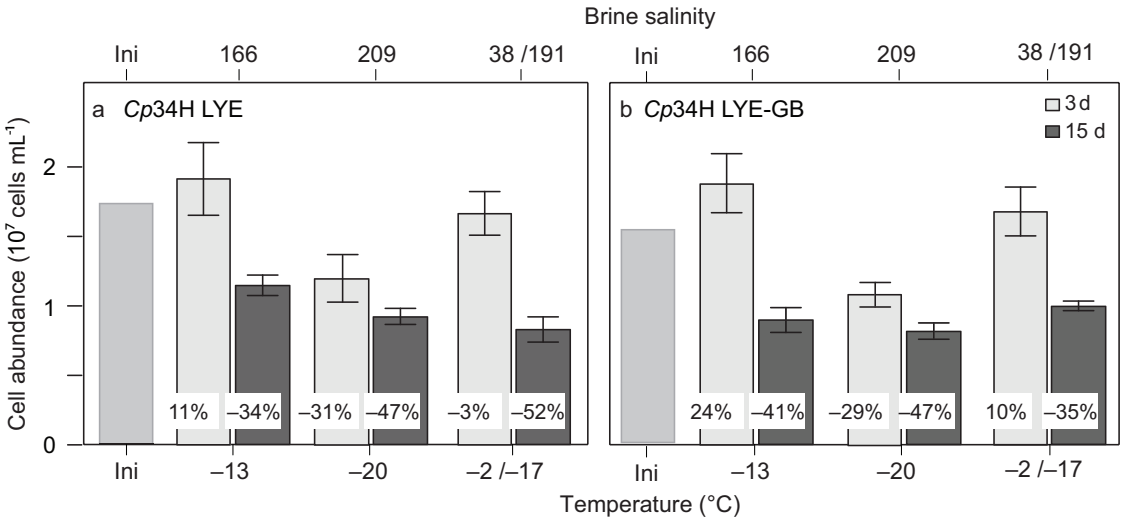

Figure 4.5: Comparison of initial (ini) and endpoint cell abundances for *Cp*34H grown in LYE medium (a) or LYE-GB medium (b), held frozen for 3 d (light bars) or 15 d (dark grey bars) under stable (–13 °C, –20 °C) or fluctuating (–2 to –17 °C) conditions, then melted directly for sample processing (Experiment 1). Corresponding brine salinities in secondary (upper) labels. Error bars represent mean with confidence interval (n = 3; n = 2 for –20 °C). Numbers in white boxes indicate percentage of change compared to initial cell abundance.

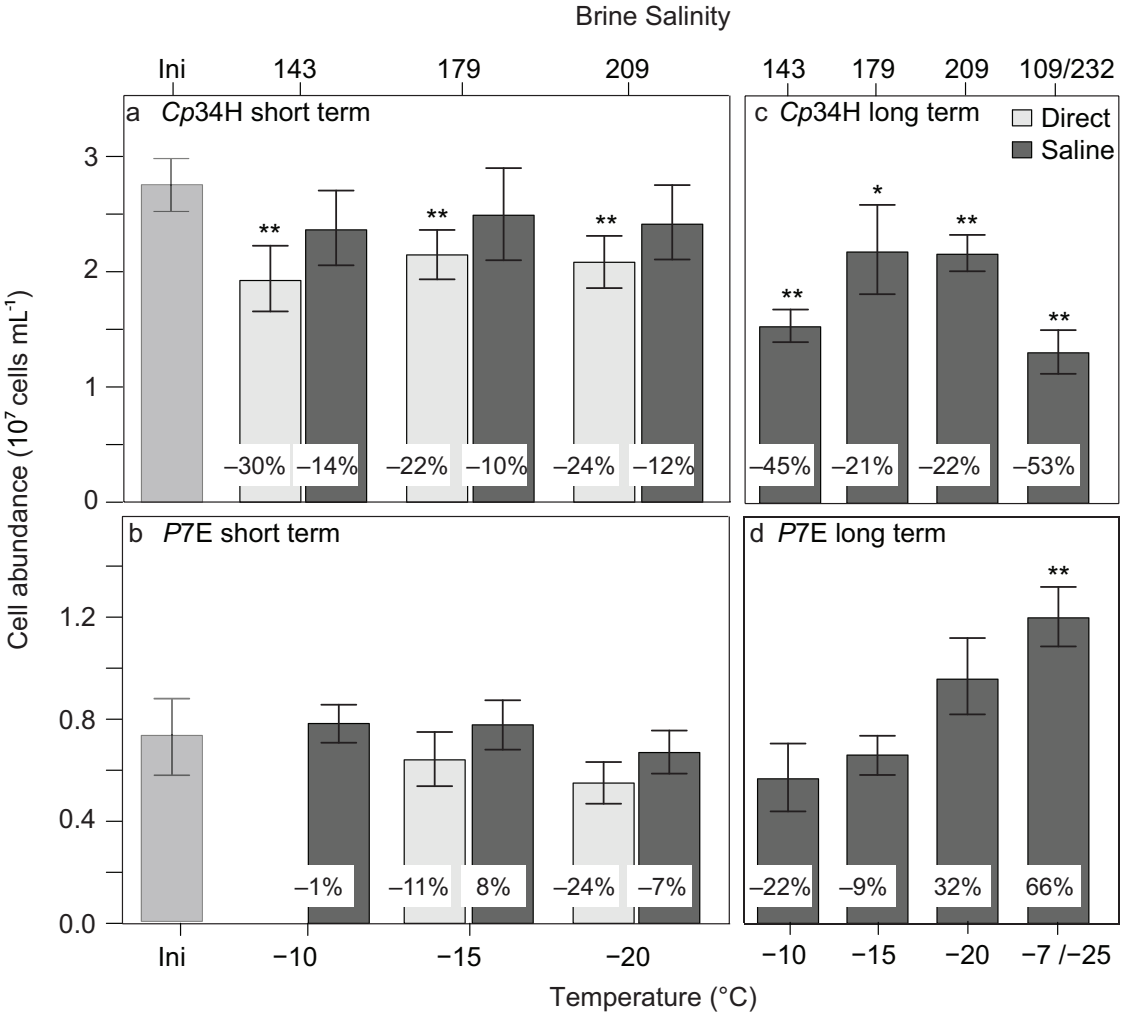

Figure 4.6: Comparison of initial (ini) and endpoint cell abundance for *Cp*34H (a, b) and *P*7E (c, d) grown in LYE medium, and hold frozen for 40 min (a, c) or 15 d (b, d) under constant (–10 °C, –15 °C and –20 °C) or fluctuating (–7 to –25 °C) temperatures; corresponding brine salinities in secondary (upper) labels (Experiment 2). Two melting methods, direct (light bars) and brine melt (dark bars), were assayed in the short-term treatment. Error bars represent mean with 95 % confidence interval (n = 6). Asterisks indicate bacterial abundance has changed significantly (* $p < 0.05$, ** $p < 0.01$, t-test) from initial abundance. Numbers in white boxes indicate percentage of change compared to initial cell abundance. See text for additional description of statistical comparisons among treatments. Missing bar on panel b (*P*7E, direct melt, –10 °C) corresponds to samples lost during processing. Bars in panel d for treatments at –10 °C did not freeze during the 15-d incubation (see text for details).

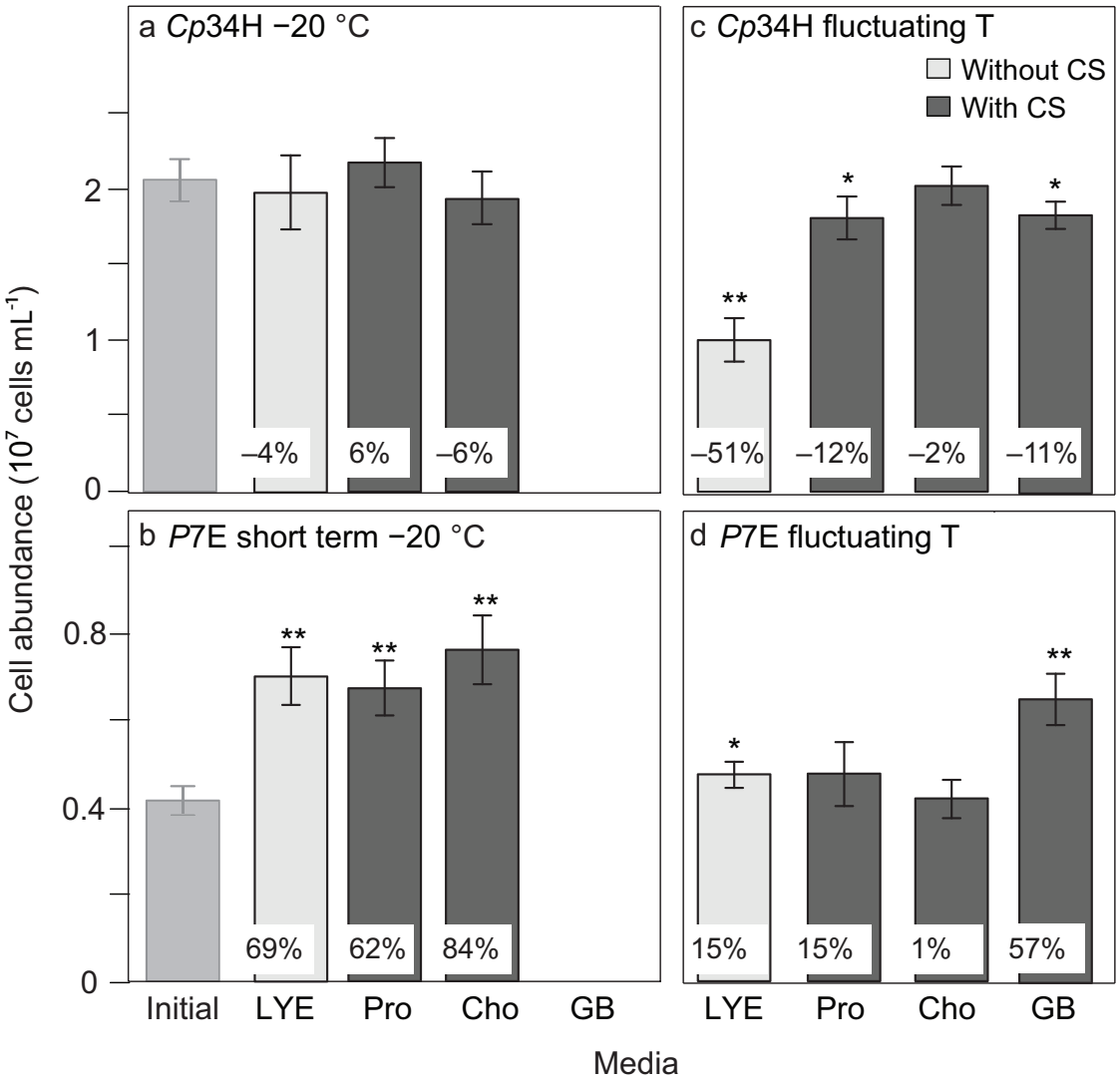

Figure 4.7: Comparison of initial (ini) and endpoint cell abundance for *Cp*34H (a, b) and *P*7E (c, d) exposed to freezing for 15 d under stable (–20 °C and salinity 209; a, b) or fluctuating conditions (–6 to – 25 °C and salinity 101 to 232; c, d) (Experiment 3). Treatments correspond to LYE medium amended with compatible solutes to a final concentration of 1 mM as follows: LYE (no amend, light bars); and Cho (choline), GB (glycine betaine), and Pro (proline) (dark bars). Missing bars in panels a, b correspond to samples where cell abundance could not be determined due to cell aggregation (see text for details). All samples were processed by brine melt. Error bars, asterisks and numbers in white boxes are as in Fig. 4.6 (n = 6). In panel c, all amended treatments differ significantly from the unamended LYE treatment; in panel d, only LYE-GB differs. See text for statistical details.

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Chapter 5

Sea ice microorganisms: environmental constraints and extracellular responses[1]

Abstract

Inherent to sea ice, like other high latitude environments, is the strong seasonality driven by changes in insolation throughout the year. Sea-ice organisms are exposed to shifting, sometimes limiting, conditions of temperature and salinity. An array of adaptations to survive these and other challenges has been acquired by those organisms that inhabit the ice. One key adaptive response is the production of extracellular polymeric substances (EPS), which play multiple roles in the entrapment, retention and survival of microorganisms in sea ice. In this concept paper we consider two main areas of sea-ice microbiology: the physico-chemical properties that define sea ice as a microbial habitat, imparting particular advantages and limits; and extracellular responses elicited in microbial inhabitants as they exploit or survive these conditions. Emphasis is placed on protective strategies used in the face of fluctuating and extreme environmental conditions in sea ice. Gaps in knowledge and testable hypotheses are identified for future research.

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[1]This chapter was originally published as Ewert, M., and J.W. Deming (2013). Sea Ice Microorganisms: Environmental Constraints and Extracellular Responses. *Biology*, 2, 603–628 . It represents a summary understanding of this dissertation subject, after the conclusion of the experimental work reported in Chapters 2 to 4 and the publication of Chapters 2 and 3.

5.1 Introduction

Sea ice is a dynamic, porous matrix that harbors within its interior network of brine pores and channels an active (e.g., *Junge et al.*, 2004; *Søgaard et al.*, 2010) and diverse (*Brown and Bowman*, 2001; *Brinkmeyer et al.*, 2003; *Bowman et al.*, 2012; *Maas et al.*, 2012) community. The sympagic (ice-associated) community has multiple trophic levels including photosynthetic bacteria and algae, chemoautotrophic bacteria and archaea, and heterotrophic bacteria, archaea, flagellates, fungi and small metazoans (*Horner et al.*, 1992; *Gradinger and Ikävalko*, 1998; *Krembs et al.*, 2000; *Lizotte*, 2003; *Collins et al.*, 2010; *Bowman et al.*, 2012). Members of this community, particularly the bacteria and algae, play important roles in cycling carbon (*Miller et al.*, 2011; *Lee et al.*, 2012) and nitrogen (*Kaartokallio*, 2001; *Rysgaard and Glud*, 2004) in polar regions; selected bacteria also respond to pollutants such as crude oil (*Delille et al.*, 1997; *Brakstad et al.*, 2008) and mercury (*Barkay and Poulain*, 2006; *Møller et al.*, 2011).

The seasonal (autumnal) decrease in temperature that leads to the formation of sea ice in polar waters progressively reduces the liquid phase of the ice—the brine volume fraction—and consequently increases the concentration of solutes and particles in the brine. Phase equations of sea ice (*Assur*, 1958; *Cox and Weeks*, 1983; *Leppäranta and Manninen*, 1988) are frequently used to estimate brine salinity and brine volume fraction based on the temperature of the ice and its bulk salinity (salinity after melting). Temperature determines solute concentration such that when the ice reaches a temperature of -5 °C, just $\sim$3 °C below the freezing point of seawater, the estimated brine volume fraction has decreased below 0.3 (even as low as 0.05 for ice with low bulk salinity; Fig. 5.1a) and the estimated brine salinity has increased to nearly 100 (Fig. 5.1b). At extreme winter temperatures, salt precipitation within the brine phase adds complexity to these constraints (see deflection points at -22.9 °C, the eutectic for hydrohalite, in Fig. 5.1b). Organisms, previously at seawater temperature and salinity, are thus exposed to much lower temperatures, higher salinities, and reduced habitable space soon after entrapment in sea ice.

Other properties of sea-ice brines also show temperature-dependent changes. For instance, the solubility of biologically relevant gases, including CO_2 and O_2, decreases as the salinity of brines increases, translating to a general degassing effect throughout the winter season (*Thomas et al.*, 2010). Subsequently, biological O_2 consumption can further reduce O_2 concentration in some sections of the sea-ice column leading to the development of microbial communities and processes that are favored by low oxygen conditions (*Kaartokallio*, 2001; *Petri and Imhoff*, 2001; *Rysgaard and Glud*, 2004).

Summer brings a new set of changes. Melting of snow and then surface ice, and the consequent formation of melt ponds, exposes sea-ice microorganisms to salinities close to that of freshwater, or else flushes them back to the ocean through brine drainage (*Meiners et al.*, 2008; *Lee et al.*, 2012). Although disadvantageous to halophiles, this surface melting generates an environment suitable for the growth of a distinctive community of freshwater microorganisms (*Brinkmeyer et al.*, 2004; *Gradinger et al.*, 2005). Increased solar radiation in the spring and summer also promotes growth of photosynthetic organisms, further influencing sea-ice brine composition and prompting the secretion of protective screening and quenching compounds (*Mundy et al.*, 2011). The onset of significant algal photosynthesis imposes additional challenges to the resident microbial community in the form of oxidative stress (by the increase of O_2) or decrease in pH (by consumption of CO_2 and consequent shifts in the carbonate chemistry of brine).

Some microorganisms have constitutive adaptations (expressed constantly) that allow them to thrive or survive under specific conditions of high salinity or low temperature. In a variable environment such as sea ice, however, acclimation mechanisms that allow a microorganism to function across a range of conditions may have an advantage over constitutive mechanisms finely tuned to an extreme, but relatively constant, environment. Microbial adaptations to sea ice may involve intracellular processes, membrane proteins or the secretion of extracellular polymeric substances (EPS). The latter can modify the immediate surroundings of the organism and its neighbors. The protective role of EPS, which tends to be gelatinous in nature, has been recognized since the earliest stages of sea-ice microbiology,

as this account from *McLean* (1918) relates:

> "It is a curious fact, and yet a well-known experience, to find that bacteria may live dormant in ice for prolonged periods, and that infection may be carried through ice, but it is not so generally recognized that some bacteria prefer to grow on ice. Microorganisms, as a rule, are capable of resisting a low temperature when their ordinary activities cease, and they tend, either as single units or in clusters, to throw out a mucilaginous protein substance for their protection."

This review addresses some of the physico-chemical parameters that define the sea-ice environment on a seasonal basis and the protective responses they elicit in microorganisms, particularly bacteria, the category of microorganisms known to inhabit all depths, forms and seasons of sea ice (Fig. 5.2). It diverges from prior reviews on sea-ice microorganisms and their environment (e.g., *Horner et al.*, 1992; *Legendre et al.*, 1992; *Ackley and Sullivan*, 1994; *Staley and Gosink*, 1999; *Thomas and Dieckmann*, 2002; *Mock and Thomas*, 2005; *Deming*, 2009) by giving particular attention to the protective role of EPS in the face of extreme fluctuations in key environmental parameters characteristic of the Arctic. In doing so, gaps in knowledge and new directions for research are identified.

5.2 Sea ice as a microbial environment

5.2.1 Incorporation into sea ice

Sea ice formation starts in the fall. When water reaches its freezing point, ice crystals, known as frazil ice, form throughout the upper water column and rise to the surface accumulating in a slush. This surface slush continues to freeze, consolidating into an upper layer of ice. Ensuing sea-ice growth depends on the conditions of the ocean. Calm conditions promote the formation of congelation ice, formed by platelets adhering at the bottom of an already established ice layer. Turbulent conditions lead to frazil ice growth, where ice crystals form throughout the mixed water column and rise to the surface where they coalesce

(*Petrich and Eicken*, 2010). The growth of sea ice produces a porous structure that, unlike glacial ice, which is formed from fresh water, retains abundant impurities that were present in the source water. Salts and other solutes, organic and inorganic particles, and microorganisms are rejected by the growing ice lattice into interconnected liquid inclusions within the ice. The liquid inclusions, brine channels and pores, form an interconnected network that accumulates high concentrations of solutes and constitutes the inhabited fraction of sea ice (*Junge et al.*, 2001).

Different processes can contribute to the retention of some of the microorganisms, particles and organic substances entrained into the ice from the source water (Fig. 5.2). Physical concentration can enrich algal cells, either through scavenging by frazil ice or by water circulation through the newly established ice layer (*Weissenberger and Grossmann*, 1998). Scavenging occurs when frazil ice crystals drift towards the surface, dragging in their path particles and algal cells from the water column that will later concentrate in the ice (*Garrison et al.*, 1983, 1989). The physical concentration of algae has been observed in the field, with algal cells >10 μm preferentially enriched in young Arctic sea ice (*Gradinger and Ikävalko*, 1998). The ability of marine algae to nucleate ice crystals (*Knopf et al.*, 2011) could also contribute to the enrichment process of sea-ice algae by favoring the formation of ice crystals in their immediate environment that can lift them towards the consolidating ice layer.

In contrast, enrichment of smaller bacterial (and archaeal) cells in sea ice does not appear to occur directly by physical processes. Bacterial incorporation can be facilitated by the presence of algae, through bacterial association with algal cells or aggregated algal EPS, that are then concentrated by physical processes (*Grossmann and Dieckmann*, 1994; *Riedel et al.*, 2007; *Weissenberger and Grossmann*, 1998). Attachment of bacteria to algae in young Arctic sea-ice samples has been observed but, because insufficient data are available to consider it a widespread phenomenon, the potential role of EPS is highlighted instead (*Riedel et al.*, 2007). Indeed, particulate EPS (pEPS; > 0.4 μm) of likely algal origin are rapidly enriched in newly forming sea ice (*Meiners et al.*, 2003; *Riedel et al.*, 2007). Bacterial EPS also have the potential to play a role in the entrainment and retention of bacteria in ice either

directly or by promoting attachment to algal cells or detrital particles amenable to physical entrainment. Dissolved EPS (dEPS; < 0.4 μm) produced by *Colwellia psychrerythraea* strain 34H (*Cp*34H), a model marine psychrophilic bacterium (*Deming*, 2010), was found to be selectively retained in saline ice under experimental conditions (Chapter 2; *Ewert and Deming*, 2011). Marine bacteria with the ability to produce an EPS coating with similar properties could be retained in the ice by this means alone, independently of association with algal cells or their byproducts, an hypothesis that remains to be tested.

As sea ice consolidates, brines in the upper layers of the ice are expelled upwards to the ice surface forming a surface skim layer (Fig. 5.2). Sea-ice microorganisms (primarily bacteria), EPS and dissolved organic compounds are carried with the brine to this even colder habitat at the ice-atmosphere interface. A fraction of the bacteria and EPS may be selectively retained in the ice, however, following the arguments and potential mechanisms outlined for initial entrainment into the ice (EPS coatings and attachment to larger particles or ice crystals). Brines in this skim layer, and the bacteria and organic substances within it, can subsequently be incorporated into frost flowers that form on the new ice surface (*Bowman and Deming*, 2010) or into the saline snow layer, which represents a vast bacterial habitat in its own right (Chapter 3; *Ewert et al.*, 2013). Frost flowers and saline snow will have brine inclusions with properties similar to those of the ice, but exposed to more extreme environmental parameters.

5.2.2 The low temperature constraint

A defining characteristic of the sea-ice environment is temperatures below, and sometimes well below, 0 °C. Meltponds, the accumulated meltwater from snow and surface ice during the summer season, are the only ice-associated environments with temperatures above 0 °C (between 0.4 and 1.5 °C, according to *Lee et al.*, 2012). Sea-ice temperatures range typically between -2 °C and -30 °C with the coldest temperatures recorded in the upper 10 cm of sea ice during Arctic winter (*Collins et al.*, 2008) (winter lows for Antarctic sea ice are less

extreme). Environments associated with the surface of new ice in winter, such as the brine skim layer and frost flowers, can be exposed to air temperatures below –30 °C.

As a result of the insulating properties of ice and snow, environments experiencing the most severe fluctuations in temperature (and thus brine salinity) are those directly exposed to the winter atmosphere: the brine skim layer and frost flowers on the surface of new ice and, to a lesser degree, the saline snow layer when snow is blown to minimal thickness (as discussed in Chapter 3; *Ewert et al.*, 2013). To illustrate a typical range of temperatures and fluctuations seasonally experienced in Arctic sea ice and associated environments, Fig. 5.3 presents detailed temperature measurements from the Mass Balance Observatory Site from the University of Alaska Fairbanks (*Druckenmiller et al.*, 2009). This observatory, located in landfast coastal sea ice in the coast of Barrow, Alaska (156.5° W, 71.4° N), measures air-snow-ice-water temperature profiles throughout the winter and spring seasons.

Low temperatures impose constraints at different levels in the single-cell microorganism and, consequently, elicit responses spanning different aspects of its physiology. Reaction and transport rates decrease with temperature, slowing most physiological processes. Protein folding is affected by a decrease in hydrophobic forces and changes in hydration (*Georlette et al.*, 2004). Membranes become rigid. Nucleic acids become more stable, which hinders replication, transcription and translation processes. Microbial solutions to these constraints also span the gamut of possibilities. Cold-active enzymes remain functional at low temperatures by favoring amino acids that allow higher flexibility and structural modifications that provide ligands better access to the catalytic site (*Georlette et al.*, 2004).

Bakermans et al. (2007) found that *Psychrobacter cryohalolentis* K5, a psychrotolerant bacterium isolated from permafrost, showed important changes in its proteome with up to 30% of the proteins having significantly different levels of expression when exposed to low temperatures. Among these proteins were cold-shock chaperones to facilitate translation, cold-adapted alleles that would allow a same function be performed at two different temperatures, and an increase in the expression of certain transporters. Bacteria also respond to low temperatures by changing the type of fatty acids and carotenoids present in their membranes

and altering the membrane protein content (*Bakermans et al.*, 2007; *Deming*, 2009; *Shivaji and Prakash*, 2010). In fact, some bacteria can perceive changes in temperature through a membrane-bound sensor that triggers the expression of cold-activated genes (*Shivaji and Prakash*, 2010).

Given the prevailing low temperatures, organisms inhabiting sea ice and associated environments can be expected to use many of these strategies to cope with low-temperature constraints. When compared with the underlying water, the sea-ice bacterial community is enriched in culturable taxa considered psychrophilic, *i.e.*, uniquely adapted to low temperatures (*Bowman et al.*, 1997). Also, psychrophilic organisms tend to be more abundant in the upper layers of the ice, which have been exposed to the coldest temperatures during the winter (*Bowman et al.*, 1997). Extracellular enzymes from sea ice have also been recognized for their unusually low optimal temperatures (*Huston et al.*, 2001), especially those assayed in winter ice (*Deming*, 2007).

5.2.3 The brine channel network

The brine channel network containing the liquid fraction of the ice has a complex three-dimensional structure (*Weissenberger et al.*, 1992). For relatively warm ice near the ice-water interface, brine channels range in diameter from a few to hundreds of micrometers; the network is dominated by the smallest channels (<40 μm) that account for about 50 % of the surface area (*Krembs et al.*, 2000). Brine inclusions are characterized by their volume fraction and connectivity, temperature-dependent properties that determine the permeability of the ice. For ice with a given bulk salinity, the size and connectivity of the brine inclusions will decrease with temperature until the ice reaches a critical porosity where it is no longer permeable (*Golden et al.*, 2007). According to phase equations of sea ice, the threshold for fluid permeability in sea ice occurs when the porosity approaches 5%, which occurs at a temperature of about -5 °C for ice with a salt concentration of 5 ppt (*Golden et al.*, 1998). Once the ice reaches the permeability threshold, pockets of brine become isolated

from the underlying seawater and from each other (though micrometer-scale connections remain possible; *Deming*, 2007). Shrinking of brine inclusions leads to an increase in the concentration of salts and other solutes, and of organisms and other particles present in the brines. This temperature-dependent concentration of solutes exposes sea-ice organisms to seasonal changes in salinity. The uppermost section of the ice experiences the most drastic changes, where, as temperature decreases in the winter, the concentration of salts in the brine can reach a salinity of 220, or 6 – 7 times higher than that experienced by microorganisms in seawater before their entrapment in the ice. As salts in the brine concentrate above their saturation points, ice experiences the successive precipitation of ikaite ($CaCO_3{\cdot}6H_2O$) at -2.2 °C, mirabilite ($Na_2SO_4{\cdot}10H_2O$) at -8.2 °C, and hydrohalite ($NaCl{\cdot}2H_2O$) at -22.9 °C (*Assur*, 1958; *Rysgaard et al.*, 2011). Salt precipitation changes the ionic composition of sea-ice brines with respect to the source seawater and generates additional solid surfaces (salt crystals) with which microorganisms can interact. Such interactions have not been explored, except conceptually (see Section 5.3.3).

Temperature-dependent reduction of brine volume also increases the percentage of brine channel area covered by organisms (*Krembs et al.*, 2000), as well as the concentration of bacteria, viruses and free DNA within the brine (*Collins and Deming*, 2011a,b) simultaneously increasing their contact rates in exponential fashion (*Wells and Deming*, 2006). High concentrations and contact rates with viruses and nucleic acids have been hypothesized to promote lateral gene transfer in sea ice (*Deming*, 2007; *Collins*, 2009). Properties of the brine channel network, mainly its connectivity and volume fraction, also affect the type of predators and the predator-prey dynamics of the sea-ice community. In general, larger predators only access the lowermost sections of the ice (*Gradinger et al.*, 1999), with pores of less than 200 μm considered a refuge for smaller organisms (microalgae, ciliates and bacteria; *Krembs et al.*, 2000). Some metazoan predators such as rotifers and turbellaria, though, are flexible enough to squeeze into brine channels with diameters much smaller than their bodies, and also adjust their body size according to changes in ambient salinity (*Krembs et al.*, 2000). In the smallest sea-ice brine inclusions, viruses take the role as main predators (*Collins et al.*, 2010; *Collins and Deming*, 2011a).

Observations by *Krembs et al.* (2011) confirmed the role of EPS-producing microorganisms in directly influencing the properties of the brine channel network. The presence of EPS increased the abundance of pores in the ice by 15% (over EPS-free ice) and led to the formation of pores with convoluted irregular shapes (*Krembs et al.*, 2011). The effects of EPS were also evident in the larger pores (> 250 μm), where perimeter-to-length ratios corresponded to a fractal geometry, as opposed to the Euclidean geometry characteristic of pores in artificially grown sea ice lacking EPS (*Krembs et al.*, 2011). The presence of EPS thus affects the habitability of sea ice by increasing the volume of the habitable liquid phase and the interior ice-liquid surface area available for "colonization." These effects result from interactions between EPS and the ice, whether by clogging the brine channel network, changing the viscosity of the brine or directly associating with the ice crystals (*Krembs et al.*, 2011).

Sea-ice microorganisms can also modify the brine channel network through their antifreeze proteins, another type of extracellular substance produced by both sea-ice diatoms (*Janech et al.*, 2006) and bacteria (*Raymond et al.*, 2007). Extracellular antifreeze proteins secreted by the sea-ice diatom *Fragilariopsis cylindrus* can alter the microscopic and macroscopic structure of saline ice, opening the possibility for this protein and similar ones to play an important role in shaping the sea-ice microbial environment if produced in sufficient quantities (*Raymond*, 2011; *Bayer-Giraldi et al.*, 2011). The presence of extracellular organic substances with the ability to change macroscopic and microscopic structure of sea ice suggests a possible need to re-evaluate the applicability of *Cox and Weeks* (1983) equations to describe brine salinity and brine volume fraction in natural, EPS-rich sea ice (Fig. 5.1a). The issue is of particular relevance since the phase equations of sea ice are a common tool for estimating the brine salinities experienced by sea-ice organisms *in situ*.

5.2.4 The brine salinity constraint

The high salinity characteristic of sea-ice brine imposes at least two types of constraints on resident microorganisms. First, high concentrations of salts tend to affect the functioning of proteins, including precipitating them. Bacteria and archaea inhabiting high salinity environments tend to have, as a response, acidic proteins that, given their abundant negative charges, remain soluble and functional at higher salinities than basic proteins. Second, high environmental salinity exposes organisms to high osmotic pressure that drives water out of the cell, resulting in potential dehydration, loss of turgor pressure and reduction of cell volume. To counteract this water efflux, microorganisms of all types compensate for excessive concentrations of external solutes by accumulating compatible solutes in the interior of the cell. The general microbial ability to tolerate and even thrive in sea-ice brines comes with the added benefit of refuge against metazoan predators more susceptible to increases in salinity, such as those reported by *Krembs et al.* (2000).

The osmotic up-shift that occurs with ice formation happens quickly as the temperature of the ice drops (Fig. 5.1b). Sea-ice brines, though, are distinguished from other high-salinity environments not only by subzero temperature but also by extreme fluctuations in salinity (Fig. 5.4). A common bacterial response to osmotic up-shift starts with the transient accumulation of K^+ and glutamate, accompanied by a release of putrescine to balance intracellular charges (*Dinnbier et al.*, 1988; *Wood*, 1999). Avoiding growth limitations inherent to an intracellular accumulation of salts, microorganisms replace the accumulated K^+ with compatible solutes, which are either imported or synthesized directly in the cell (*Dinnbier et al.*, 1988). Compatible solutes are small, water-soluble organic molecules that increase the osmolarity of the cytoplasm without the disruptive effect of salt ions (*Roberts*, 2005). Dozens of compatible solutes have been described for bacteria and archaea, including free amino acids and their derivatives, sugars and their derivatives, and polyols and their derivatives. Among the most common compatible solutes are betaine, ectoine, trehalose, α-glucosylglycerol and glutamate (*Roberts*, 2005). A suite of genes allows for the transport of compatible solutes from the environment and/or their synthesis in the cell (*Mao et al.*,

2010).

Not all organisms accumulate compatible solutes. Some extremely halophilic microorganisms, such as *Halobacterium salinarum*, which grows optimally at 26% NaCl, compensate high external concentration of solutes by incorporating salts in their cytoplasm. To keep cytoplasmic proteins functioning after the accumulation of salts, *H. salinarum* expresses an unusually high ratio of acidic to basic proteins (4.9 for the complete proteome; *Kennedy et al.*, 2001). Membrane proteins, adapted to function when directly exposed to the high-salinity environment, present a similar tendency to be acidic independently of the intracellular accumulation of organic solutes or K^+ salts in the cytoplasm (*Saum et al.*, 2012).

Partial proteomes available to examine for bacteria known from sea ice, when compared with bacteria and archaea from other saline and fresh-water environments (following *Saum et al.*, 2012), also present the signature of a high acid-to-basic ratio in their membrane proteins (Table 5.1), consistent with the high salinities seasonally experienced in sea-ice brines. Their cytoplasmic ratios, however, do not compare with the extremely halophilic reference strain. Although the difference may be domain-specific (*Halobacterium*, contrary to its name, is an archaeal genus), we hypothesize that the salting-in strategy used by *H. salinarum*, with long term accumulation of K^+ ions in the cytoplasm and the majority of cytoplasmic proteins being acidic, has not been adopted by microorganisms from the sea-ice environment despite exposure to high brine salinities. Greater physiological flexibility will be provided by use of the compatible solutes strategy in the face of strong seasonal fluctuations in salinity inherent to sea-ice brines. Note that *Psychromonas ingrahamii*, which had the lowest ratio of acidic to basic proteins compared with other marine isolates considered (Table 5.1), was isolated from the sea ice/water interface, an environment with lower salinities and species not necessarily adapted to the higher salinities of the brine channel network (*Aslam et al.*, 2012a).

Extracellular polymeric substances are also used by sea-ice microorganisms as a response to elevated salinities. The sea-ice diatom *Fragilariopsis cylindrus* has been shown to increase the production of all types of EPS (soluble, insoluble and frustule-associated) when frozen

under high-salinity conditions (*Aslam et al.*, 2012a). Likewise, high concentrations of EPS from a sea-ice isolate of the bacterial genus *Pseudoalteromonas* were shown to extend the range of salinities at which this strain could grow, while also providing protection against freeze-thaw cycles (*Liu et al.*, 2013).

Osmotic down-shift will be experienced in the late spring and the summer as the ice warms and melts. The extent of the osmotic down-shift depends on summer brine drainage. If melting prompts the brine to be flushed back into the ocean, microorganisms may not likely experience salinities much lower than seawater. If melting results in the formation of surface meltponds, then one of two conditions will follow: meltponds connected to seawater will have salinities close to 29, similar to those in nearby surface water; unconnected meltponds will have salinities below 5, reaching values as low as 0.1 (*Lee et al.*, 2012). In the latter case, microorganisms will be exposed to a drastic down-shift in salinity, which could result in the lysis of a significant fraction of the population. For bacteria, even if not directly lysed, a down-shift may prompt lysogenic viruses (already carried by the cell) to enter the lytic stage in those cells with an active metabolism (*Ghosh et al.*, 2009; *Shkilnyj and Koudelka*, 2007) and lead to bacterial loss by that mechanism. The possibility that an EPS coating may protect against a drastic down-shift in salinity or viral lysis has not been tested (see Section 5.3.2).

5.2.5 Insolation

Solar radiation drives numerous reactions, biotic and abiotic, including the alteration and destruction of biologically relevant molecules. Processes driven by solar radiation, and the responses they trigger in microorganisms, are of particular relevance in polar regions where strong seasonal changes in insolation occur. At the organism level, UV radiation (UVR) can damage DNA and other nucleic acids by the formation of thymine dimers. UVR is known to decrease viability in bacteria from aquatic ecosystems (*Thomas et al.*, 2009) and damage the photosynthetic potential of benthic algae (*Underwood et al.*, 1999).

Given the potential detrimental effects of high irradiation, microorganisms have developed multiple protective responses, including the production of shading pigments, antioxidant compounds, and the performance of rapid DNA repair. For instance, in sediment-associated diatoms, exposure to UV-B prompts motility (away from radiation) and the production of carotenoid pigments able to function as quenching agents (*Underwood et al.*, 1999). The synthesis of mycosporine-like amino acids (MAA), a type of UVR-screening compound, is widespread in marine microscopic algae, especially those associated with surface blooms (*Jeffrey et al.*, 1999). Similar responses to UVR are found in the sea-ice microbial community. Uusikivi and collaborators measured relatively high concentrations of MAA in Baltic sea ice, particularly in the surface layers (*Uusikivi et al.*, 2010). Likewise, Mundy and collaborators reported the production of carotenoid pigments and mycosporine-like amino acids by algal communities associated with sea ice during the melting season of Arctic coastal first year ice, under high levels of UVR (*Mundy et al.*, 2011). Motility by sea-ice algae in response to changing irradiance has been suggested (*Horner and Schrader*, 1982) but, to the best of our knowledge, has not been confirmed as a mechanism of photoadaptation in sea ice. The general effects of UVR on EPS are less clear, with some (non-sea-ice) studies finding an increase (*Thomas et al.*, 2009) and others a decrease (*Underwood et al.*, 1999) in EPS content of the UV-exposed community.

Solar radiation further influences the sea-ice ecosystem by driving reactions that modify the dissolved organic carbon (DOC) pool. The potential of organic compounds to participate in photochemical reactions can be inferred from their absorption of visible radiation and UVR. DOC from spring sea ice, known to absorb UVR, has been shown to undergo varied photochemical reactions including changes in bioavailability and photooxidation to CO_2 (*Thomas et al.*, 2010; *Norman et al.*, 2011). EPS are also affected by solar radiation. Ortega-Retuerta and collaborators demonstrated that transparent exopolymer particles (an alternative descriptor for pEPS) from natural North Sea water and from cultures of the marine diatom, *Chaetoceros affinis*, can be photolysed by UV-B (290 – 315 nm), and to some extent by UV-A (315 – 400 nm) and photosynthetically active radiation (400 – 700 nm) (*Ortega-Retuerta et al.*, 2009).

Possible effects of solar radiation on EPS specific to sea-ice environments have been considered (*Bowman and Deming*, 2010) but not tested to our knowledge. As part of a larger study (Chapter 3; *Ewert et al.*, 2013), we collected pEPS samples from Arctic upper sea ice and saline snow. The susceptibility of these EPS to photochemical reactions was then examined by measuring absorption spectra. Absorption, converted to Napierian absorption coefficients (m^{-1}), was higher in the UV-B range (Fig. 5.6), suggesting that EPS associated with the winter sea-ice bacterial community may be susceptible to photochemical changes during the spring and summer when the radiation level increases. Similar profiles to the one in Fig. 5.6 have been observed for particulate organic matter from late-winter surface Baltic sea ice (*Uusikivi et al.*, 2010), except for a peak in the 320 – 345 range associated with MAA that was absent from our samples. Samples from Baltic sea ice (*Uusikivi et al.*, 2010) were collected after the snow melt and contained a community of microscopic algae likely responsible for the production of MAA; in contrast, our samples were collected before snow melt and dominated by a bacterial community, explaining the absence of a MAA signature. In fact, Cockell and collaborators found that a snow cover of 5 – 15 cm thickness could reduce the transmittance of UV radiation by an order of magnitude and reduce the impact of radiation in bacterial spores (*Cockell et al.*, 2003). The snow cover over sea ice may thus act as a seasonal shading agent, protecting surface sea-ice microorganisms against UV radiation. This protective cover, though, is highly heterogeneous in thickness and melts early in the season (*Sturm and Massom*, 2010).

5.3 Extracellular responses to sea-ice environmental constraints

5.3.1 Extracellular polymeric substances

Extracellular polymeric substances (EPS), composed primarily of polysaccharides, are commonly produced by a wide range of microorganisms from both terrestrial and marine environments. EPS differ amongst organisms and producing conditions in sugar chain length and branching, sugar composition, type of sugar linkages, and the presence of additional chem-

ical groups such as sulfates, proteins, lipids and even nucleic acids (*Ruas-Madiedo and de los Reyes-Gavilán*, 2005; *Mancuso Nichols et al.*, 2005a). Different bacterial strains can produce EPS of different chemical composition and structure (*Lemoine et al.*, 1997; *Mancuso Nichols et al.*, 2005a), but a single strain can also produce more than one kind of EPS (*Schiano Moriello et al.*, 2003). Likewise, the type and amount of EPS produced by a bacterial strain can be modified by exposure to certain environmental conditions such as salinity (*Vyrides and Stuckey*, 2009), temperature (*Mancuso Nichols et al.*, 2005b) or presence of heavy metals (*Guibaud et al.*, 2005). Hence, the term EPS does not refer to a single chemically defined molecule but a complex mixture of diverse polysaccharides and ancillary compounds. Because EPS measurements typically quantify only the polysaccharide fraction of these components, the term EPS has also been used to refer specifically to extracellular polysaccharide substances. We use EPS throughout this review in its broadest meaning, unless otherwise specified.

EPS can be either tightly bound to the cell surface, loosely attached, or cell free (*Decho*, 1990). Cell-free hydrophobic EPS from mesophilic bacteria have been shown to self-assemble into polymer microgels and to accelerate the self-assembly of microgels in seawater, with implications for concentrating organic-rich substrates for bacterial degradation (*Ding et al.*, 2008). These properties, however, were inhibited at low temperature for the particular polysaccharides studied (*Ding et al.*, 2008). The extent to which such self-assembly may occur in the sea-ice environment, where solute concentrations are high and EPS may be derived from psychrophilic microorganisms, has not been fully explored. An initial analysis in winter ice indicated minimal self-assembly (*Krembs et al.*, 2002), yet EPS aggregates with spherical diameters between 2 and 50 μm have been observed in sea ice (*Meiners et al.*, 2003).

5.3.2 EPS in sea ice

Most of the EPS in sea ice can be attributed to production by ice algae, either before or after entrainment into the ice. Even in regions of the ice dominated by bacteria, the trail of algal EPS is expected to overwhelm the amount produced by sea-ice bacteria (*Collins et al.*, 2010; *Krembs et al.*, 2011). Diatoms may produce distinctive EPS depending on their particular sea-ice habitat. Two diatoms isolated from the sea-ice brine network, *Fragilariopsis curta* and *F. cylindrus*, produced complex polysaccharides of higher molecular mass, with low relative abundance of glucose but high relative content of galactose, xylose and fucose. In contrast, a species of *Synedropsis* from the ice-water interface produced EPS dominated by low-molecular weight polysaccharides with low complexity and high relative content of glucose (*Aslam et al.*, 2012a).

EPS abundance in sea ice and associated environments has been quantified in numerous studies beginning with those by *Krembs et al.* (2002) and by *Meiners et al.* (2003). During fall, the number of EPS particles in sea ice can be an order of magnitude higher than in underlying water and often correlates with the presence of sea-ice algae (*Meiners et al.*, 2003). The dissolved EPS fraction is consistently more abundant in sea ice (*Krembs et al.*, 2011) and sea-ice associated environments such as frost flowers (*Aslam et al.*, 2012b) and saline snow (*Ewert et al.*, 2013).

The EPS pool in sea ice is established during ice formation (Chapter 2; *Riedel et al.*, 2007; *Ewert and Deming*, 2011; *Aslam et al.*, 2012b) but can be modified subsequently by the entrained microorganisms. For instance, sea-ice microorganisms can add EPS to the existing pool by producing it *in situ* as a stress response, a process inferred from the increase in EPS concentration in winter sea ice (*Collins et al.*, 2008; *Krembs et al.*, 2002). On the other hand, bacteria may selectively degrade and consume certain fractions of the EPS pool, changing its overall chemical composition and size fractionation, as suggested by the detailed analyses of *Underwood et al.* (2010).

The widespread, yet heterogeneous (e.g., *Underwood et al.*, 2010), presence of EPS in sea

ice and associated environments may reflect the varied functions these polymers perform at different ecosystem levels (*Decho*, 1990; *Wolfaardt et al.*, 1999). At the microorganism level EPS have been associated with cell adhesion and aggregation (*Mora et al.*, 2008), motility (*Lind et al.*, 1997), affinity for metals (*Baker et al.*, 2010), and with providing a sticky framework to keep extracellular enzymes in the immediate vicinity of the cell (*Decho*, 1990). EPS can also provide protection against toxic heavy metals (*Bitton and Freihofer*, 1977) and desiccation (*Knowes and Castenholz*, 2008). All of these functions have relevance in sea-ice environments. In particular, recent experimental data have shown that EPS can play a role in protecting sea-ice bacteria (*Liu et al.*, 2013) and diatoms (*Aslam et al.*, 2012a) against the challenges of high-salinity brines. These results are in agreement with data from other environments where high-salinity stress triggered changes in the type and amount of EPS produced by microorganisms from anaerobic sludge (*Vyrides and Stuckey*, 2009) and by freshwater cyanobacteria (*Ozturk and Aslim*, 2010). Likewise, EPS could have a role in protection against low salinity shocks. The marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H, whose immediate relatives are found in sea ice, increased the amount of EPS produced per cell when exposed to low salinities not permissive of growth (*Marx et al.*, 2009). The survival benefit was implied but not directly tested.

5.3.3 Influence of EPS on physical-chemical properties of sea ice

Further insight into the protective role of EPS comes from experiments by *Krembs et al.* (2011), who observed that artificial ice formations containing algal EPS had higher bulk salinities than EPS-free counterparts. This result has been related to the potential of EPS to form "plugs" in the brine channels, increasing the amount of salts that are retained (Fig. 5.2). Following the phase equations of sea ice, higher bulk salinities result in higher brine volume fractions under similar temperature regimes (Fig. 5.1b), effectively increasing the available habitable space for microorganisms.

The salinity of the brine pockets, however, is conventionally described as a function of

temperature only and does not depend on the bulk salinity of ice. Following earlier work (*Krembs et al.*, 2011; *Krembs and Deming*, 2008), we suggest in Fig. 5.1b that the presence of EPS may have an effect on the validity of traditional phase equations when applied to natural sea ice. Some possible mechanisms may involve extracellular polymers (whether EPS or proteins; Chapter 2; *Ewert and Deming*, 2011) with ice activity interacting with the ice surface of the brine pores and channels. If an important fraction of the surface area is covered, the growth of ice crystals might be restricted, resulting in local areas with lower salinity than predicted. EPS may also partition the brine within an ice pore creating microscale salinity gradients that affect ice crystal growth in currently unpredictable ways (*Krembs and Deming*, 2008). Another option could be antifreeze proteins, whereby more water in the liquid state would mean lower salinities. An EPS plug physically decreasing the minimal size of the brine pocket would have a similar effect.

Divalent cations present in sea-ice brines can also interact with charged groups in the backbone of EPS. In the marine environment, this interaction has been suggested to play a role in the binding of key nutrients for the cell such as iron (*Mancuso Nichols et al.*, 2005c). Likewise, the interaction of EPS with Ca^{2+} determines self-assembly of marine gels, which can in turn increase the availability of nutrients for the microbial population (*Verdugo*, 2012). In the case of sea ice, the relationship between EPS and Ca^{2+} may figure in the fate of carbonates in sea ice (*Rysgaard et al.*, 2012). Relationships between bulk measures of dissolved organic matter and $CaCO_3$ precipitation were not evident in Antarctic sea ice (*Fischer et al.*, 2012), but experiments specifically using EPS under ice-brine conditions have not been reported. If the dissolved organic matter measured in seawater by *Chave and Suess* (1970) included EPS, then evidence exists for a role in delaying the onset of $CaCO_3$ precipitation. Data on the interactions between EPS and calcium in other environments (*Braissant et al.*, 2003) may inform first tests of this hypothesis for sea ice.

Bergmann and collaborators used conductometric titrations to estimate the amount of binding sites for divalent cations present in ionic and nonionic bacterial extracellular polysaccharides (*Bergmann et al.*, 2008). Conductometric titrations (*Farris et al.*, 2011) measure

changes in conductivity resulting from the addition of a saline solution to a solution of interest, and provide information on the charge density of polyelectrolytes such as ionic polysaccharides. A non-ionic polysaccharide such as dextran has a titration curve where no interaction with Ca^{2+} ions is evident. Xanthan, being an ionic polysaccharide, has a titration curve with a clear offset due to its conductive properties and the presence of associated counter-ions. Its curve also shows two segments with distinctive slopes, indicating that Ca^{2+} ions interacted with the polysaccharide until all binding sites were occupied (*Bergmann et al.*, 2008).

Following this approach, conductometric titrations were performed on solutions (in deionized water) of dextran, xanthan, and EPS obtained from a culture of *C. psychrerythraea*. A blank with no polysaccharide added was also included (see Fig. 5.5 for details). The resulting titration curves and the slopes of their respective linear regressions (Fig. 5.5) agree with results from Bergmann and collaborators (*Bergmann et al.*, 2008). The titration curve of dextran and the blank closely resemble each other, whereas the titration curve of xanthan has an offset and two segments with distinctive slopes. The slope of the 34H EPS curve is the same as the slope of the first segment for xanthan, the segment where interaction with Ca^{2+} is expected; there is no change in the slope, however, and the offset is 6 times higher. EPS from strain 34H thus likely contains charged polysaccharides with abundant backbone charges and associated counter-ions (high curve offset) and multiple binding sites for Ca^{2+} (no change in slope over the tested range of $CaCl_2$ concentrations). The presence of charged EPS from this Arctic marine psychrophile has implications for the dynamics of carbonates in the sea-ice environment given that charged polysaccharides, unlike non-ionic polysaccharides, have known effects on the precipitation of carbonates (*Hardikar and Matijević*, 2001).

Interactions between cations and polysaccharides also confer both algal and bacterial EPS with the potential to adsorb heavy metal contaminants such as Cd^{2+} (*Guibaud et al.*, 2005), Pb^{2+} (*Comte et al.*, 2008) and Hg^{2+} (*Zhang et al.*, 2013), which can then be incorporated into the food chain (*Bhaskar and Bhosle*, 2006; *Schlekat et al.*, 2000). High concentrations of

these heavy metals have been found in the Arctic marine food web, in levels comparable to those from temperate regions with high anthropogenic activity, and exceeding in some cases the guidelines for human consumption by the World Health Organization (*Campbell et al.*, 2005); EPS from sea-ice organisms may be playing a role in the fate of these contaminants. Of special interest is the dependence of heavy metal adsorption to EPS on properties such as salinity, pH and Ca^{2+} concentrations (*Bhaskar and Bhosle*, 2006; *Comte et al.*, 2008; *Zhang et al.*, 2013), properties that undergo seasonal changes in sea-ice brines.

EPS can also interact with other extracellular macromolecules, most relevantly with proteins. Non-covalent interactions between EPS and proteins allow the formation of complexes, coacervates and aggregates, increasing the range of pH in which a protein is soluble (*Turgeon et al.*, 2007). In the case of cold-adapted marine organisms, EPS have been shown to increase the stability and half-life of a cold-active extracellular aminopeptidase from *C. psychrerythraea* 34H (*Huston et al.*, 2004). The interaction of EPS with antifreeze proteins is of particular interest for it could allow the accumulation of such proteins in the immediate vicinity of the cell, concentrating the antifreeze effects of the protein (*Bayer-Giraldi et al.*, 2011) to the benefit of sea-ice inhabitants as temperatures drop seasonally. Interactions between EPS and proteins under *in situ* conditions relevant to microbial life in sea ice remain largely unexplored.

5.4 Prospectus for future research

In focusing on the extracellular responses of sea-ice microorganisms to the sometimes severe and fluctuating environmental conditions of their habitat, our goal has been to highlight a number of features, particularly regarding EPS, that have not been fully explored or that raise new research directions. The extracellular products of microorganisms entrapped in sea ice are known to influence the microstructure of the ice, and thus its habitability, but how they may influence the effectiveness of traditional equations for calculating key parameters of sea ice or function at the micrometer scale within an ice pore is not clear. Unanswered questions involve the potential role of EPS in mitigating the salt concentration directly

experienced by the cell, contributing to the adaptive strategy of compatible solutes, and blocking viral attack. EPS interactions with other exudates, including ice-active proteins and hydrolytic enzymes that serve a substrate-acquisition function for the cell, are poorly known. If the unexplored interactions of EPS with inorganic ions, particularly Ca^{2+}, were to be as significant in sea ice as they are in other environments, then the implications for carbon transport through the sea-ice cover could be quantitatively important. As much as has been learned over the past decades about sea-ice microorganisms and their self-protective responses to the constraints of their habitat, more awaits discovery.

5.5 Acknowledgments

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Table 5.1: Ratio of acidic to basic proteins in partial proteomes of selected microorganisms.

Organism	**Membrane**	**Cytoplasmic**	**Environment**
Extremely halophilic			
Halobacterium salinarum	3.88	16.8	Highly saline lakes
Halophilic			
Psychrobacter cryohalolentis	2.22	3.27	Cryopeg
Roseobacter denitrificans	2.22	3.00	Marine
Psychrobacter arcticus	2.14	3.34	Permafrost
Sphingopyxis alaskensis	1.90	2.23	Marine
Shewanella frigidimarina	1.52	2.90	Marine, sea ice
Colwellia psychrerythraea strain 34H	1.47	3.17	Marine sediments, sea ice
Shewanella oneidensis	1.47	3.26	Anaerobic sediments
Marinobacter aquaeolei	1.42	3.13	Marine
Oceanobacillus iheyensis	1.15	3.78	Marine sediments
Psychromonas ingrahamii	1.10	2.00	Sea ice / water interface
Non-halophilic			
Flavobacterium psychrophilum	0.75	1.44	Freshwater fish
Lactococcus lactis subsp. lactis	0.74	4.03	Gut flora
Sphingomonas wittichii	0.69	2.28	River

[a] Ratio of proteins with isoelectric point (pI) < 7 to proteins with pI > 7; pI calculated with the *Compute pI/Mw* tool from the ExPasy Bioinformatics Resource Portal (*ExPASy*, 2012).

[b] All reviewed protein entries for each organism retrieved from the UniProtKB data base on November 2012 (*Uniprot*, 2011), annotated for location as either "membrane" or "cytoplasmic."

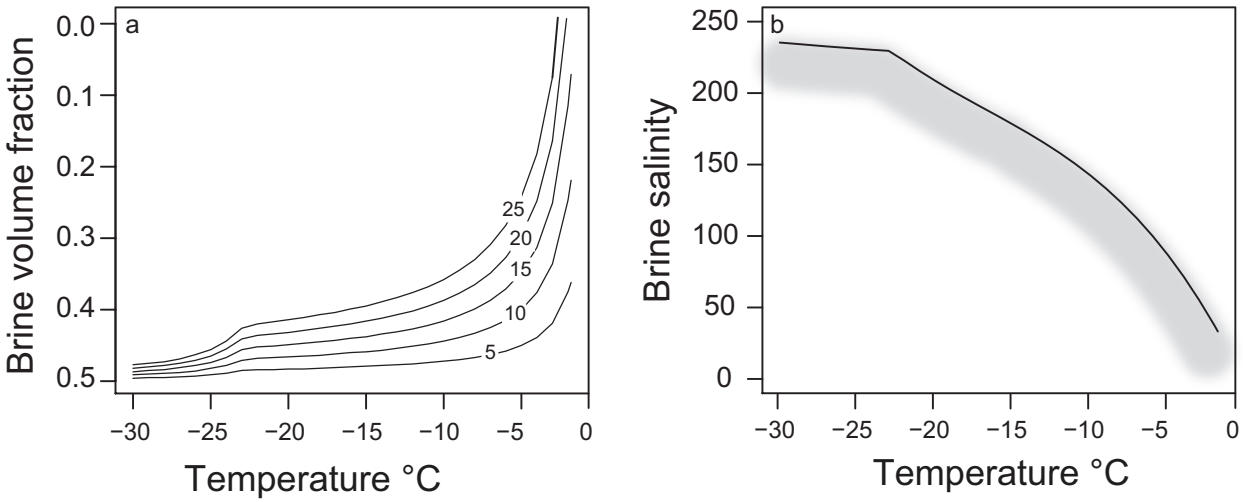

Figure 5.1: Dependence of brine volume fraction (**a**) and brine salinity (**b**) on sea-ice temperature, according to phase equations from *Cox and Weeks* (1983). Contour lines in (**a**) indicate the effect of different bulk salinities on brine volume fraction. Brine salinity (**b**) is independent of bulk ice salinity, conventionally determined only by temperature; we suggest, by the shadowing of the line, that the presence of extracellular polymeric substances (EPS) produced by sea-ice organisms may influence brine salinity in as yet unpredictable ways (see Section 5.3.3).

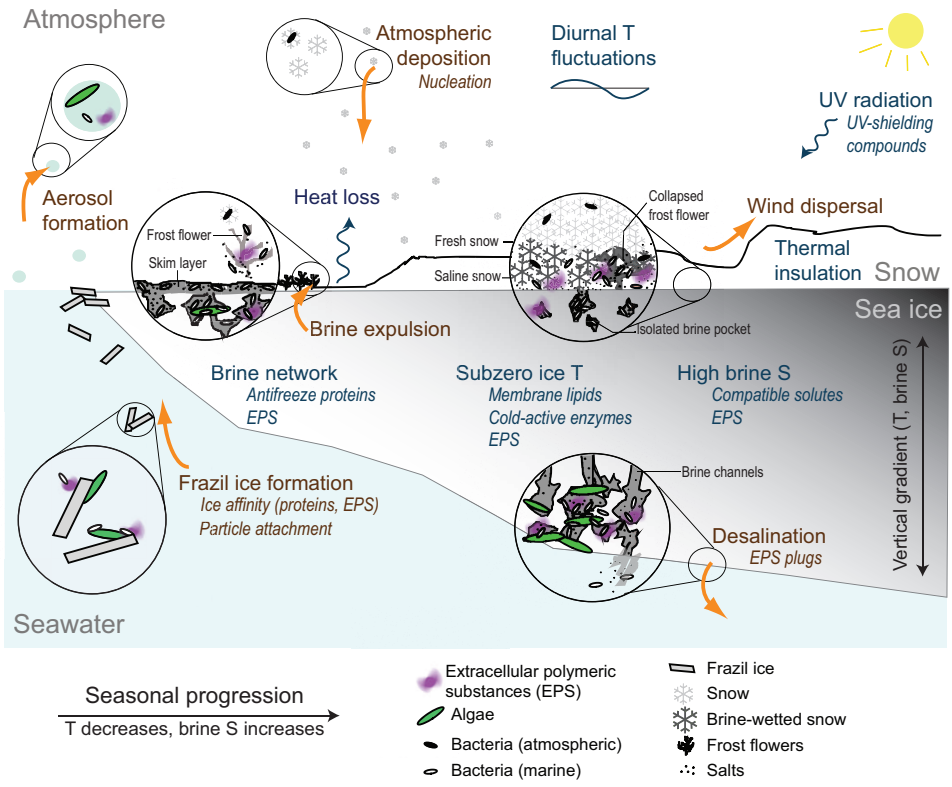

Figure 5.2: Schematic diagram of seasonal (fall through winter) processes influencing microorganisms in sea ice, including transport mechanisms (orange arrows) and some of the microbial adaptive responses (italics). During sea-ice formation, larger organisms ascend with rising frazil ice crystals; smaller bacteria and archaea likely attach to algae, particles or ice crystals. Once entrained in the ice, microorganisms inhabit a network of brine channels where they experience low temperature (T), high brine salinity (S) and reduced living space, but are protected from fluctuations in air temperature by the insulating properties of snow and ice. As sea ice consolidates, brines are expelled into the ocean (desalination) and onto the surface; a fraction of the microorganisms, EPS and other components of the brine are expelled, too. Surface-expelled brines and their contents form a skim layer that can be incorporated into frost flowers and snow, prone to wind dispersal. The skim layer and frost flowers, directly exposed to the atmosphere, experience more extreme fluctuations in temperature and brine salinity and, as the sun rises in late winter, greater UV exposure. From remaining areas of open water, including leads, wind can transport marine microorganisms in aerosols. Airborne microorganisms (including terrestrial bacteria) can nucleate snow and return to the ice/snow surface.

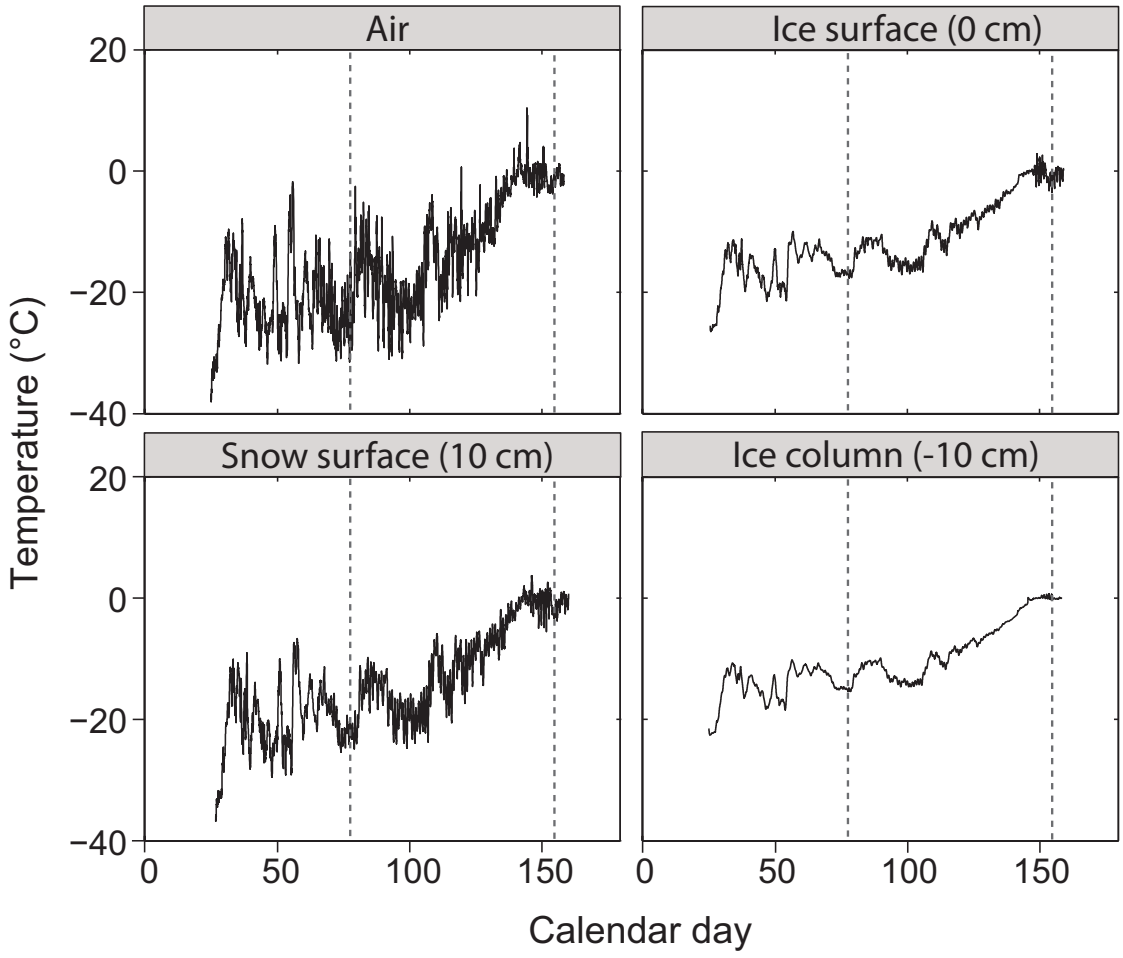

Figure 5.3: Temperature recorded at the Mass Balance Observatory Site (Barrow, AK, USA) during 2011 (days of year 25 – 158) at different depths above and below the ice surface. Dashed lines mark seasonal transitions. Spring equinox was on day 79, summer solstice on day 171.

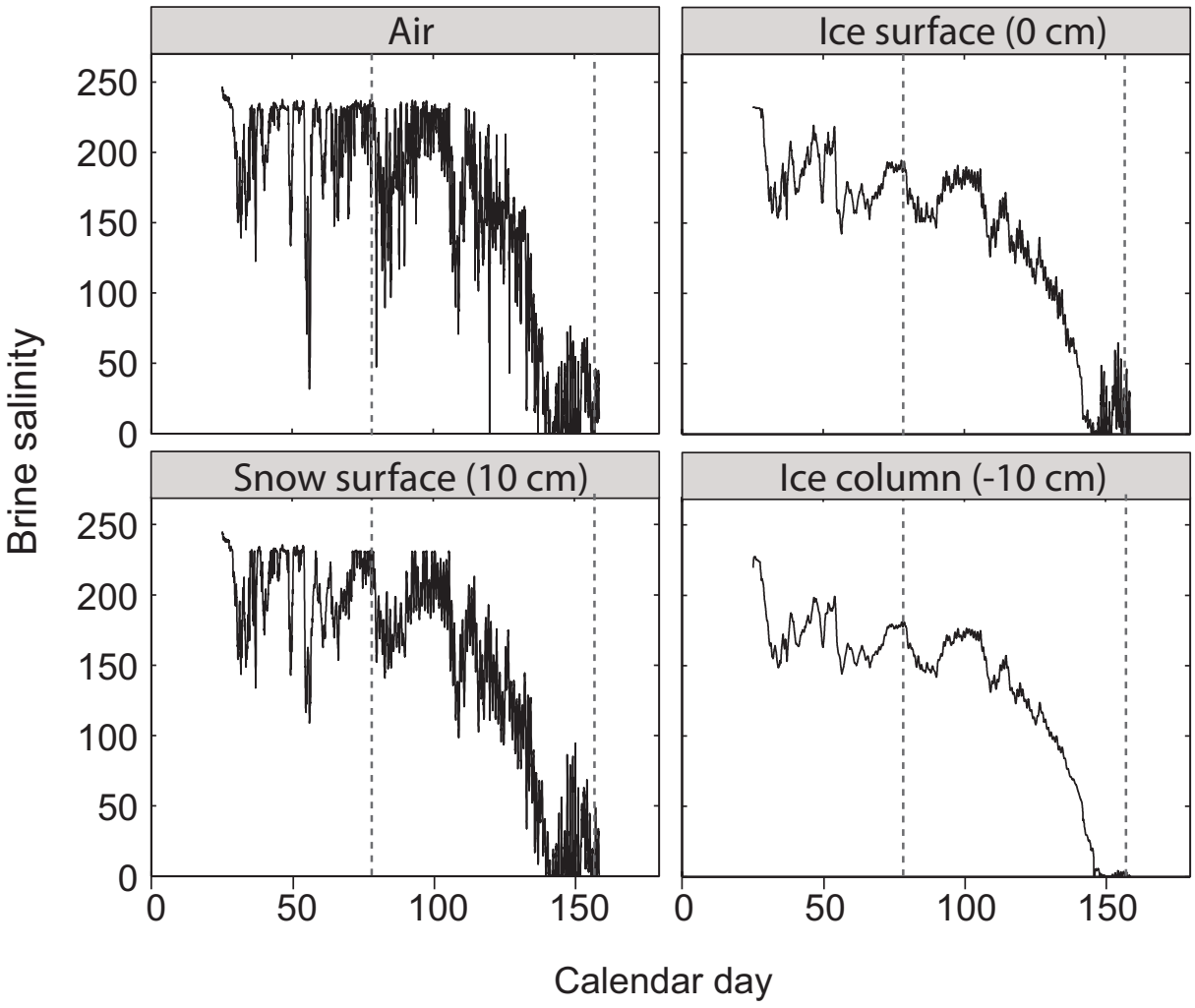

Figure 5.4: Brine salinity calculated from temperature data in Fig. 5.3. Depths and dashed lines as in Fig. 5.3. Brine salinity calculated using air temperature represents the extreme situation in which expelled sea-ice brines are directly exposed to the atmosphere and in thermal equilibrium with it.

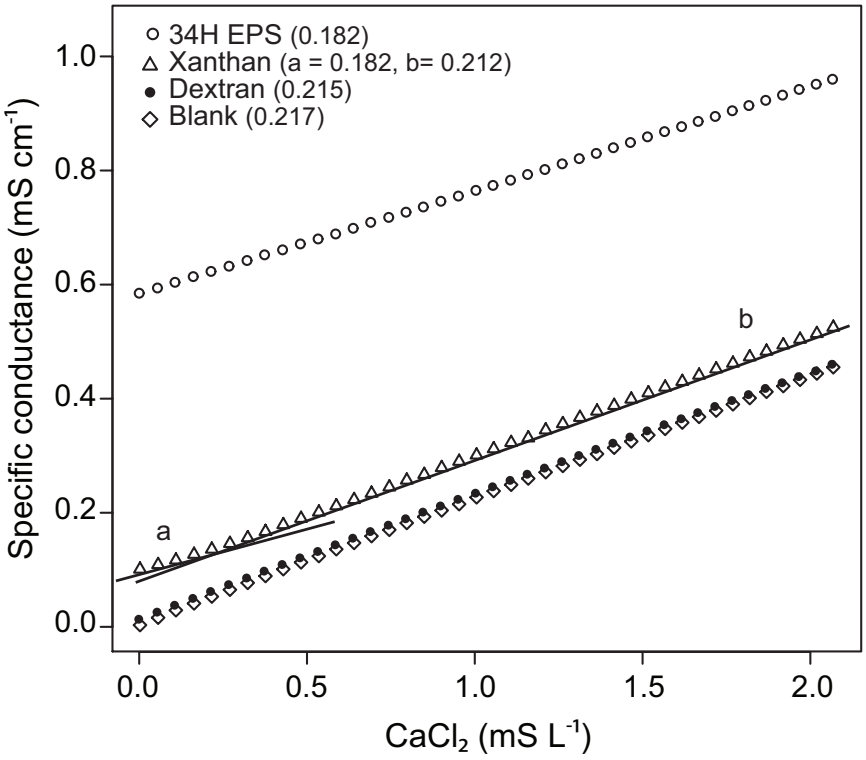

Figure 5.5: Conductometric titration of polysaccharide solutions with $CaCl_2$ (0.05 M). Each data point shows the effect of increasing concentration of $CaCl_2$ on preexisting solutions of polysaccharide (0.5 g L^{-1}). Value in parentheses is the slope of the titration curve. Slopes were calculated using linear regressions, all of which have $R > 0.99$ and p value < 0.001. Experiments were performed at room temperature, with less than 1 degree difference among treatments (blank, 22.0 °C ± 0.1; dextran, 22.1 °C ± 0.1; xanthan 22.0 °C ± 0.1; 34H EPS, 22.9 °C ± 0.1). Cell-free EPS from *Cp*34H was extracted by centrifugation and precipitation with ethanol as in *Marx et al.* (2009), followed by freeze-drying.

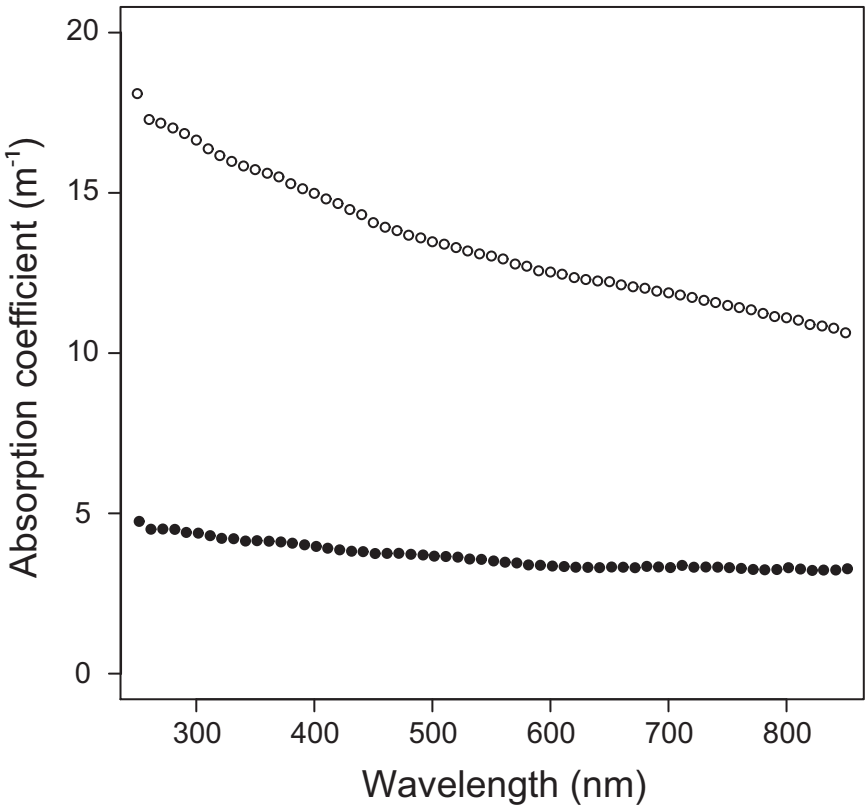

Figure 5.6: Absorption spectra for pEPS solution concentrated from surface samples of winter first year ice (open circles, 13 mg glu-eq mL^{-1}) and saline snow (filled circles, 9.3 mg glu-eq mL^{-1}). Samples were collected offshore Barrow, Alaska, in February 2010, filtered onto 0.4 μm polycarbonate filters as described by Ewert and collaborators (*Ewert et al.*, 2013), kept frozen in the dark at -20 °C for 20 months, and resuspended in 1.5 mL of distilled water for analysis.

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Appendix A

Extracellular polysaccharide substances (EPS) from *Colwellia psychrerythraea* strain 34H

A.1 Introduction

Extracellular polysaccharide substances (EPS) are complex macromolecules primarily comprised of polysaccharides [1] , but also containing variably sized fractions of protein, (phospho)lipid, humic substances or nucleic acid. EPS are commonly produced by bacteria and serve a variety of functions from the cellular to the ecosystem level, including the formation of bacterial aggregates and attachment to surfaces (*Wingender et al.*, 1999). Bacteria can produce different types of EPS when exposed to various environmental conditions; e.g., changes in the protein fraction, polysaccharide size or composition have been observed with changes in temperature (*Mancuso Nichols et al.*, 2005), pH (*Sánchez et al.*, 2006), culture media (*McKellar et al.*, 2003), inorganic nutrients (*Zhan et al.*, 1991) and starvation (*Myszka and Czaczyk*, 2009). Changes in the quantity of EPS produced by *Colwellia psychrerythraea* strain 34H (*Cp*34H), our model psychrophilic bacterium, have been associated with responses to environmental stress (*Marx et al.*, 2009). Although changes in the composition are not yet known, the dissolved fraction of *Cp*34H EPS (dEPS) carries a component that provides for selective entrainment of the dEPS in saline ice (Chapter 2). Recent work shows that the amino acid composition of the capsular (cell-held) polysaccharide of *Cp*34H may account for selective entrainment of the organism itself (*Carillo et al.*, 2013). To contribute further to the existing body of information on *Cp*34H EPS and prepare

[1] EPS refers to extracellular polysaccharide substances when the measuring technique quantifies only the sugar fraction, as in this appendix. The same abbreviation is also used for the more generic term: extracellular polymeric substances (as in Chapter 5), encompassing the chemical complexity of these polymers (*Wingender et al.*, 1999).

for experiments described in Chapter 4, changes in the production of total EPS and dEPS were examined according to growth phase, particularly in minimal media. This appendix describes the results from that work, as well as from experiments complementary to those described in Chapter 2 on the ice-affinity properties of *Cp*34H dEPS from cultures grown in rich media.

A.2 Methods

A.2.1 Growth curves and EPS production in minimal media

Colwellia psychrerythraea strain 34 H (*Cp*34H) was cultured at different temperatures (2, 8 and 13 °C) in three types of minimal media: sarcosine-lactate-vitamins (SLV), lactate-yeast-extract (LYE) and LYE supplemented with glycine betaine (LYE-GB). LYE was prepared in artificial sea water (ASW) as described in *Marx et al.* (2009), using 5 g L^{-1} lactic acid (Sigma-Aldrich) and 0.014 g L^{-1} yeast extract. LYE-GB was prepared by adding glycine betaine to a final concentration of 1 μM as described in Chapter 4. SLV was prepared in ASW as described in *Collins and Deming* (2013), using 2 g L^{-1} sarcosine, 0.5 g L^{-1} calcium L-lactate and 0.5 mL L^{-1} 100× RPMI-1640 vitamin solution. Comparisons were made with cultures in one type of rich media, Marine Broth 2216 (Difco Laboratories) diluted to half organic strength ($\frac{1}{2}$×MB 2216) with ASW as also described in Chapter 2. ASW contained the four major seawater salts (0.4 M NaCl, 9 mM KCl, 26 mM $MgCl_2$ and buffer, either KH_2PO_4 or TAPSO (3-N-[Tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid]), for a final salinity of 32 (practical salinity) and pH of 7. Bacterial growth was determined by optical density measured at 600 nm (OD-600); number of cells was determined by microscopic observation as described in Chapter 4. Measurements of dEPS were made by centrifuging 50 – 200 mL of culture for 20 min (3000 g at 2 °C) and filtering the resulting exudate on either a 0.4 μm polycarbonate filter (dEPS) or a 0.2 μm filter (dEPS $< 2\mu$m). For SLV cultures, total EPS was measured by centrifuging 1 mL of culture for 45 s ($\sim$ 15000 g), pelleting the cells and performing the phenol-sulphuric

assay on the supernatant as described in *Marx et al.* (2009).

A.2.2 Additional cold-finger experiments on the selective retention of dEPS

Cell-free exudate from *Cp*34H was obtained as described in Chapter 2. Cultures were grown in $\frac{1}{2}\times$MB 2216 at 2 °C until mid-exponential phase, centrifuged for 20 min (3000 g at 2 °C) and filtered through a 0.4 μm polycarbonate filter (dEPS). Cold-finger experiments were performed as described in Chapter 2, except that the source solutions (dEPS, heat-treated [HT] dEPS and $\frac{1}{2}\times$MB 2216) were not dialyzed and the temperature settings of the cold-finger were –10, –15 and –20 °C, as representative of new ice forming in open leads during the colder conditions of Winter. Due to the resulting faster freezing rates, ice growth was allowed only for 1 h.

To determine if consecutive cycles of freezing would change the proportion of dEPS retained in the ice, a second experiment employed consecutive freezing (re-freezing) of solutions already fractionated by the cold-finger apparatus in the experiments described in Chapter 2. The starting solution was the melted ice fraction from each treatment (dEPS, HT dEPS and $\frac{1}{2}\times$MB 2216). Because the volume available from the melted ice fraction was low, replicas were combined to have enough volume for the next experiment. The initial salinities and EPS concentrations differed between treatments and the volume of the initial solution (125 mL) was less than the volume used in the previous experiments. As in Chapter 2, the cold finger was set at a temperature of –5 °C and the duration of the experiment was 6 h.

A.2.3 Statistics and additional calculations

Segregation coefficients, k_{effs} and k_{effe} and enrichment index, I_s, were calculated as described in Chapter 2. In those cases where salinity and EPS concentration for the source solution were missing, the ratios R_s and R_e were calculated as follows:

$$R_s = \frac{S_{ice}}{S_{sol}}$$

$$R_e = \frac{[EPS]_{ice}}{[EPS]_{sol}}$$

where S_{ice} and S_{sol} are the salinities of the ice and the remaining solution, and $[EPS]_{ice}$ and $[EPS]_{sol}$ are the concentrations of EPS in μg glu-eq mL^{-1} in the ice and remaining solution, respectively. These ratios can also be normalized to determine enrichment ($R_e/R_s > 1$).

Brine volume fraction and brine salinity were calculated for the artificial saline ice, following *Cox and Weeks* (1983). All salinity values are expressed in the practical salinity scale. These parameters were also calculated for experiments described in Chapter 2. Given the setup of the cold finger apparatus, the resulting saline ice had a temperature gradient. Brine volume fraction and salinity were thus calculated using a temperature average between the cold finger itself (in equilibrium with the innermost part of the ice) and the temperature of the solution (in equilibrium with the outermost part of the ice).

Means and standard deviation (SD) were calculated for all experimental data. Graphical and statistical analyses for experimental data were performed using R v.2.13.1 (*R Development Core Team*, 2011). A two-way analysis of variance (2-way ANOVA) was used to evaluate differences among treatments.

A.3 Results and discussion

A.3.1 Cell growth and EPS production in rich and minimal media

Total EPS per cell changed during the growth phases of *C. psychrerythraea* strain 34H when grown in SLV, with high values in the first days of growth that equilibrated at about

1 pg glu-eq cell^{-1} after 5 d (Fig. A.1). *Cp*34H had a dEPS yield of 30.7 ± 3.9 μg glu-eq mL^{-1} (mean ± SE, n = 10) for cultures growing at 2 °C in rich media ($\frac{1}{2}$×MB 2216) and harvested in late exponential phase (OD = 0.7). Short-term cultures of *Cp*34H at 2 and 13 °C presented lower yields of the smaller size fraction of dEPS (< 0.2 μm) in minimal media SLV (6 d) than in rich media $\frac{1}{2}$×MB 2216 (3 d; Table A.1). End-point values of dEPS for *Cp*34H growing in SLV and $\frac{1}{2}$×MB 2216 at 2 °C were 670 ± 140 and 20.5 ± 4.8 fg glu-eq cell^{-1}, respectively. Higher EPS per cell has been associated with a response to environmental stress (*Marx et al.*, 2009) which when considered next to the lower cell yields of this study, suggests that minimal media provide less than optimal growing conditions for *Cp*34H than organically complex media.

Some bacteria have been reported to produce, under starvation conditions, a different type of EPS that facilitates biofilm formation (*Myszka and Czaczyk*, 2009). Attachment has been observed for *Cp*34H when cultured in the minimal media LYE and SLV. Preliminary experiments showed that *Cp*34H can attach to biofilm chambers when cultured in LYE for 15 d at 8 °C but not in $\frac{1}{2}$×MB 2216 (S. Carpenter, personal communication). Cultures of *Cp*34H growing in SLV also attached to the bottom of a glass flask when kept for 1 month at 8 °C (Fig. A.2). These results were confirmed with cultures of *Cp*34H in LYE-GB at 8 °C which, when subjected to freezing for 15 d at –20 °C, formed aggregations as described in Chapter 4. Whether or not *Cp*34H produces a different type of EPS when growing in minimal media remains an open question.

The growth curves of *Cp*34H in SLV (250 mL, shaking, 2 °C) resulted in a generation time of 175 h and maximum yield of 2.4 ×10^7 cells mL^{-1} (OD-600 = 0.11; Fig. A.3). Growth curves in LYE and LYE-GB (250 mL, no shaking, 8 °C) presented a comparable maximum OD-600 for LYE-GB (0.12), but a lower yield for LYE (0.06; Fig. A.3). The presence of glycine betaine, or its derivative, sarcosine, increased cell yield for *Cp*34H, confirming its ability to metabolize compatible solutes, as described in *Collins and Deming* (2013).

A.3.2 Selective retention of dEPS in artificial sea-ice

Cold-finger experiments performed at faster freezing rates (e.g. at lower temperatures) resulted in higher salt retention in the ice (Table A.2), as expected; the faster freezing rate, the greater the quantity of entrapped brine given less time for brine expulsion or desalination (1 h vs. 6 h). Values for R_e were significantly higher (p = 0.001, 2-way ANOVA) for the unheated *Cp*34H dEPS at –5 °C (Chapter 2) than for dEPS present in $\frac{1}{2}\times$MB 2216 for all fast freezing rates (at –10, –15, –20 °C; Table A.3), confirming and extending conditions for the selective retention of *Cp*34H dEPS, reported for slower freezing rates in Chapter 2. Likewise, the ratio R_e/R_s for the faster freezing rates was significantly higher (p = 0.002, 2-way ANOVA) for the (unheated) *Cp*34H dEPS compared to the media control, although no significant difference was detected between freezing temperatures (Table A.4). These results suggest that the selective retention of dEPS could take place not only during the Fall freeze-up, as discussed in Chapter 2, but also at colder temperatures. This result is relevant for new ice formed during the winter in open leads or cracks in the ice. Preliminary experiments with natural EPS from surface seawater, sea-ice brines and first-year sea ice show k_{effe} values considerably higher than 1 (Table B.1 in Appendix B), supporting this hypothesis.

The values for brine volume fraction presented in table A.5 support the hypothesis in Chapter 2 that the presence of *Cp*34H dEPS increases the liquid fraction of saline ice, not only its salt content, which may have implications for the habitability of sea ice (*Krembs et al.*, 2011).

Table A.1: Production of dEPS < 2 μm (glu-eq μg mL^{-1})[a] by *Cp*34H during early exponential phase

Media	2 °C	13 °C
$\frac{1}{2}\times$MB 2216	0.81 ± 0.11	1.06 ± 0.01
SLV	0.186 ± 0.07	0.193 ± 0.01

[a] All values are mean ± S.D.; n = 3.

Table A.2: Salinity of EPS-containing source solutions at the start of an ice-growing experiment, of the ice formed after 6 h (melted for analysis), and of the unfrozen solution remaining at 6 h, with calculated segregation coefficients for the salt (k_{effs}) and ratio of salt retention. Data presented for the initial experiments at –5 °C (mean ± S.E., n = 3), refreezing experiments at –5 °C (Refr, n = 1) and experiments at lower temperatures (–10, –15, –20 °C, n = 3).

Experiment (°C)	Source solution	Salinity solution (0 h)	Salinity ice (6 h)	Salinity solution (6 h)	k_{effs}	R_s
–5	*Cp*34H dEPS	35.5 ± 0.4	14.0 ± 0.3	38.8 ± 0.5	0.39 ± 0.01	0.36 ± 0.01
	HT dEPS	36.2 ± 0.3	14.2 ± 0.2	39.0 ± 0.1	0.39 ± 0.01	0.36 ± 0.01
	$\frac{1}{2}$×MB 2216	35.7 ± 0.3	14.0 ± 0.3	38.5 ± 0.3	0.39 ± 0.01	0.36 ± 0.01
–5 (Refr.)	*Cp*34H dEPS	2.7	2.2	6.6	0.85	0.31
	HT dEPS	3.4	2.5	9.0	0.72	0.26
	$\frac{1}{2}$×MB 2216	1.7	2.2	6.0	0.56	0.33
–10	*Cp*34H dEPS		21.1 ± 0.2	34.6 ± 0.2	—	0.61 ± 0.01
	$\frac{1}{2}$×MB 2216	—	20.4 ± 0.1	34.2 ± 0.3	—	0.60 ± 0.01
–15	*Cp*34H dEPS	—	22.5 ± 0.1	37.1 ± 0.6	—	0.61 ± 0.01
	$\frac{1}{2}$×MB 2216	—	22.1 ± 0.2	38.8 ± 0.8	—	0.57 ± 0.01
–20	*Cp*34H dEPS	—	23.6 ± 0.2	41.4 ± 0.3	—	0.57 ± 0.01
	$\frac{1}{2}$×MB 2216	—	22.6 ± 0.3	39.4 ± 1.3	—	0.57 ± 0.02

Table A.3: dEPS concentration in source solutions at the start of an ice-growing experiment, in ice formed after 6 h, and in solution remaining at 6 h, with calculated (mean ± S.E., n = 3) segregation coefficients for dEPS (k_{effe}) and ratios of dEPS retention for initial experiments at – 5 °C, refreezing experiments at – 5 °C (Refr, n = 1), and experiments at lower temperatures (–10, –15, –20 °C, n = 3)

Experiment (°C)	Source solution	EPS solution (0 h)	EPS ice (6 h)	EPS solution (6 h)	k_{effe}	R_e
–5	*Cp*34H dEPS [a]	24.1 ± 0.5	15.3 ± 2.8	27.9 ± 0.7	0.63 ± 0.1	0.55 ± 0.1
	HT dEPS [b]	22.6 ± 2.4	8.56 ± 1.9	25.5 ± 2.9	0.38 ± 0.1	0.29 ± 0.04
	$\frac{1}{2}$×MB 2216	25.8 ± 1.2	10.3 ± 1.3	36.0 ± 2.5	0.40 ± 0.1	0.34 ± 0.1
–5 (Refr.)	*Cp*34H dEPS	12.0	17.0	32.8	1.4	0.52
	HT dEPS	18.5	10.9	34.8	0.59	0.31
	$\frac{1}{2}$×MB 2216	22.3	13.2	37.1	0.59	0.36
–10	*Cp*34H dEPS	—	41.7 ± 2.7	37.7 ± 2.5	—	1.1 ± 0.1
	$\frac{1}{2}$×MB 2216	—	11.7 ± 1.8	60.1 ± 4.9	—	0.22 ± 0.04
–15	*Cp*34H dEPS	—	44.9 ± 4.1	39.7 ± 7.2	—	1.19 ± 0.2
	$\frac{1}{2}$×MB 2216	—	50.8 ± 18	73.3 ± 3.4	—	0.68 ± 0.2
–20	*Cp*34H dEPS	—	28.7 ± 4.6	36.8 ± 3.3	—	0.78 ± 0.1
	$\frac{1}{2}$×MB 2216	—	23.2 ± 7.16	60.0 ± 8.5	—	0.45 ± 0.2

[a] dEPS were measured as glucose equivalents (glu-eq μg mL^{-1}).

[b] HT refers to heat-treated.

Table A.4: Enrichment indices and ratio of dEPS[a] to salt retention

Experiment (°C)	Source solution	I_s [b]	R_e/R_s [b]
–5	*Cp*34H dEPS	1.61 ± 0.30	1.52 ± 0.3
	HT dEPS	0.97 ± 0.23	0.79 ± 0.1
	$\frac{1}{2}$×MB 2216	1.02 ± 0.14	0.92 ± 0.2
–5 (Refr.)[c]	*Cp*34H dEPS	1.7	1.7
	HT dEPS	0.8	1.2
	$\frac{1}{2}$×MB 2216	1.1	1.1
–10	*Cp*34H dEPS	—	1.82 ± 0.2
	$\frac{1}{2}$×MB 2216 [d]	—	0.38 ± 0.1
–15	*Cp*34H dEPS	—	1.95 ± 0.4
	$\frac{1}{2}$×MB 2216	—	1.2 ± 0.4
–20	*Cp*34H dEPS	—	1.37 ± 0.3
	$\frac{1}{2}$×MB 2216	—	0.79 ± 0.3

[a] dEPS were measured as glucose equivalents (glu-eq μg mL^{-1}).
[b] All values are mean ± S.E.; n = 3 unless noted otherwise.
[c] Refreezing experiments; n = 1.
[d] Mean ± SD; n = 2.

Table A.5: Volume of ice, brine volume fraction and brine salinity for initial experiments at – 5 °C, refreezing experiments at – 5 °C (Refr), and experiments at lower temperatures (–10, –15, –20 °C) [a]

Experiment (°C)	Source solution	Ice volume (melted)[b]	Brine salinity[c]	Brine volume fraction[c]
–5	*Cp*34H dEPS	40 ± 2	36 ± 1	0.38 ± 0.02
	HT dEPS	43 ± 1	38 ± 1	0.36 ± 0.01
	$\frac{1}{2}$×MB 2216	40 ± 2	41 ± 2	0.33 ± 0.02
–5 (Refr.)[a]	*Cp*34H dEPS	39	—	—
	HT dEPS	50	—	—
	$\frac{1}{2}$×MB 2216	31	—	—
–10	*Cp*34H dEPS	26 ± 1	—	—
	$\frac{1}{2}$×MB 2216	31 ± 1	—	—
–15	*Cp*34H dEPS	42 ± 3	—	—
	$\frac{1}{2}$×MB 2216	51 ± 3	—	—
–20	*Cp*34H dEPS	61 ± 3	—	—
	$\frac{1}{2}$×MB 2216	68 ± 2	—	—

[a] All values are mean ± S.E.; n = 3 except refreezing experiments where n = 1.
[b] Volume of melted ice was measured at room temperature.
[c] Brine parameters were derived using equations from *Cox and Weeks* (1983).

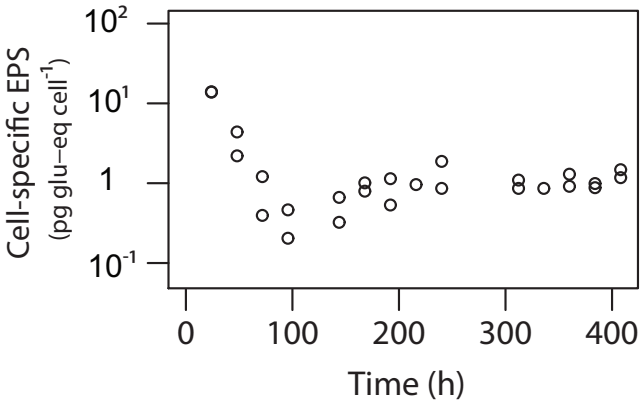

Figure A.1: Yield of EPS per cell for *Cp*34H cultured at 2 °C in SLV minimal media

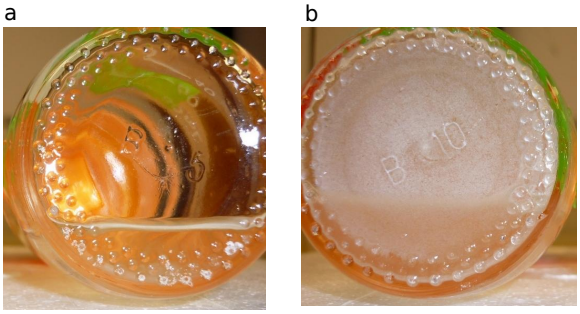

Figure A.2: Qualitative observation (visible by the naked eye) of no *Cp*34H attachment to glass in SLV (a) and strong cell attachment to glass in $\frac{1}{2}\times$MB 2216 (b). Pictures show the bottom of a glass flask (tilted on its side) holding stationary phase cultures grown at 2 °C without shaking for 1 month.

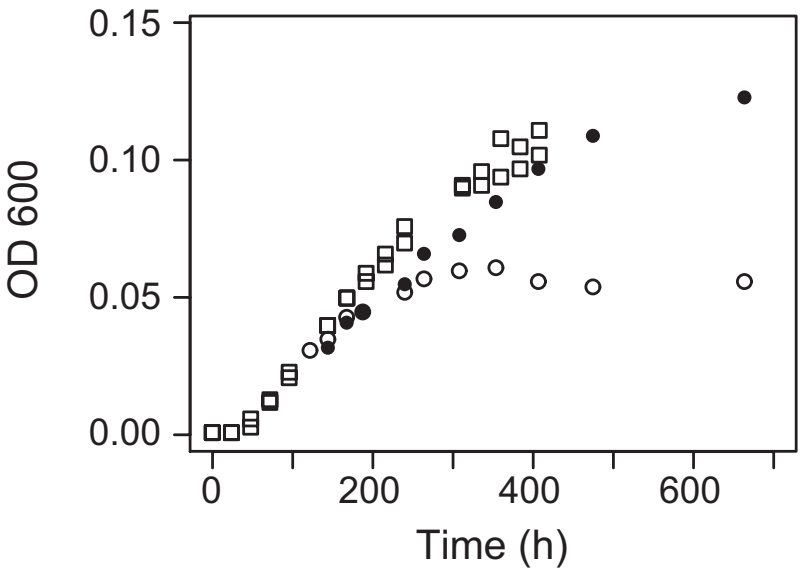

Figure A.3: Growth curves of *Cp*34H in SLV (squares), LYE (open circles) and LYE-GB (filled circles) media.

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Appendix B

Selective retention in artificial sea ice of extracellular polysaccharides from arctic surface seawater, sea ice and sea-ice brine

B.1 Introduction

Extracellular polysaccharide substances (EPS) from *Colwellia psychrerythraea* strain 34H were selectively retained in artificial saline ice when exudates from bacterial cultures are frozen by means of a cold-finger apparatus (Chapter 2). This appendix describes preliminary field experiments designed to evaluate whether or not if environmental EPS present in sea ice and associated environments also have a similar capacity for selective retention.

B.2 Methods

B.2.1 Sample collection

Experiments were performed on three types of environmental samples associated with Arctic winter sea ice: surface seawater (SSW, collected from under the ice), first year sea ice (FYI) and sea-ice brine. All samples were collected from the CCG icebreaker *Amundsen* during January 2008, as part of the Circumpolar Flaw Lead Systems Study (CFL) in the Beaufort Sea. Samples were collected from an ice floe near to the flaw lead, in which the ship was stationed. Surface water was collected within 1 m of the bottom of the ice, with an ethanol-rinsed hand-held Niskin bottle deployed through a hole in the ice. The bottom 15-cm section of a FYI core was cleanly collected with a 9-cm diameter Mark II coring system (Kovacs Enterprises). Brine was collected by "sack-hole" sampling, following *Thomas et al.* (2001),

by removing a 30-cm short core from the surface of the FYI and covering the resulting hole for overnight drainage and accumulation of brine. SSW and and brine were placed in sterile 1-L bottles, FYI in a sterile whirlpack bag, and kept close to ambient temperature until processing shortly after collection. Once aboard the ship, FYI was mechanically crushed within the whirlpack bag, aseptically transferred to a sterile melt jar, and melted in brine solution to avoid osmotic shock, as in (*Collins et al.*, 2008). Brine and SSW were processed directly.

B.2.2 Metal rods

Principles involved in the cold-finger method described in Chapter 2 were applied in the field by using a series of 12-cm long, stainless steel rods, each attached to tubing for safe handling (Fig. B.1a). The handles were wrapped in electrical tape to create a close fit to the tube mouth of 15-mL polypropylene test tubes. An acrylic disc was constructed to serve as a support base for the assembled metal rods, by opening four perforations of 1-cm diameter each. Before use, the metal rods were cleaned with ethanol, rinsed with distilled water and placed in sterile polypropylene 15-mL test tubes (Fig. B.1b). The test tubes containing the metal rods were moved to a –80 °C freezer for 7 – 8 h. Preliminary tests using an IR thermometer showed that the metal rods left at –80 °C whether for 1 or 24 h, had a surface temperature of –20 °C after a short exposure to room temperature comparable to the timeframe of the experiments.

B.2.3 Ice formation

Soon after collection, 1 L of sample was placed in a sterile 2 L plastic container held in a saline ice bath. The ethanol-cleaned acrylic disc was placed on top of the container and secured by metal hooks. The metal rods were removed from the freezer, quickly introduced into the sample (through the disc) and held in place for 1 min, during which time ice grew on the surface of the rods according to the temperature differential between rod and

sample. The disc holding the rods was then carefully removed and the rods were placed simultaneously into empty, sterile, 50-mL polypropylene tubes to collect the ice which soon slid off the rod (Fig. B.1b). The entire procedure was repeated twice to collect enough ice volume for analysis. The ice was left at room temperature and processed as soon as it melted for particulate EPS (pEPS) analysis as described in Chapter 3. Subsamples of the initial and remaining sample solution were also taken for pEPS analysis. Segregation coefficients (k_{effe}) and pEPS ratios (R_e) comparing pEPS in the ice with pEPS in the initial or remaining solution, respectively, were calculated as described in Chapter 2 and Appendix A:

$$k_{effe} = \frac{[EPS]_{ice}}{[EPS]_{source}}$$

$$R_e = \frac{[EPS]_{ice}}{[EPS]_{sol}}$$

B.3 Results and discussion

Concentrations of pEPS in the initial sample, and in the resulting ice and remaining sample solution, are presented in Table B.1, with the corresponding segregation coefficients and pEPS ratios. The starting range of concentrations of pEPS was narrow for these sample types, though the highest value was observed in SSW and the lowest in the brine. The low values in the brine may be due to retention of pEPS in the brine channels, even as the brine itself drained into the sack hole used for collection.

All samples showed enrichment coefficients and pEPS ratios > 1, indicating that pEPS from these sources segregated preferentially into the ice. These values were also higher than those determined for dEPS from *Colwellia psychrerythraea* strain 34H (Chapter 2 and Appendix A) using the cold-finger apparatus, even for experiments performed at the same estimated temperature of –20 °C. Environmental pEPS from sea ice and associated environments may thus have a higher potential for selective retention in the ice. SSW showed the highest k_{effe}

and R_e values, whereas FYI and brine presented similar values, both lower than in SSW. This comparative finding may indicate different types of pEPS, with different affinities for ice, in these sample types. Because salinity values were not collected systematically, enrichment indices, which would confirm the selective retention of pEPS over salts, could not be calculated.

B.4 Conclusions

Although the described metal-rod approach to testing for selective entrainment of organic entities into sea ice can benefit from optimization and repeated deployment, initial results suggest its potential as a new tool for this type of investigation. Winter Arctic sea ice and associated environments contained pEPS with the capacity to be selectively retained in the ice. This potential appeared to be higher in surface sea water samples, fostering speculation that the composition of entrained EPS may change over time as sea ice ages and encased organisms produce EPS for purposes of cryoprotection or osmoprotection (as discussed in Chapter 5 and references therein).

Table B.1: Retention of pEPS and enrichment index for metal-rod freezing experiments with natural samples [a]

Source solution	pEPS solution (0 min)	pEPS ice (1 min)	pEPS solution (1 min)	k_{effe}	R_e
Surface seawater	0.15	1.24	0.05	8.3	23.0
Sackhole Brine	0.09	0.38	0.13	4.3	2.9
First-year ice	0.12	0.49	0.15	4.0	3.2

[a] All concentrations in μg gluc-eq mL^{-1}; values represent measurements (n = 1) performed on combined samples of ice produced by multiple metal rods.

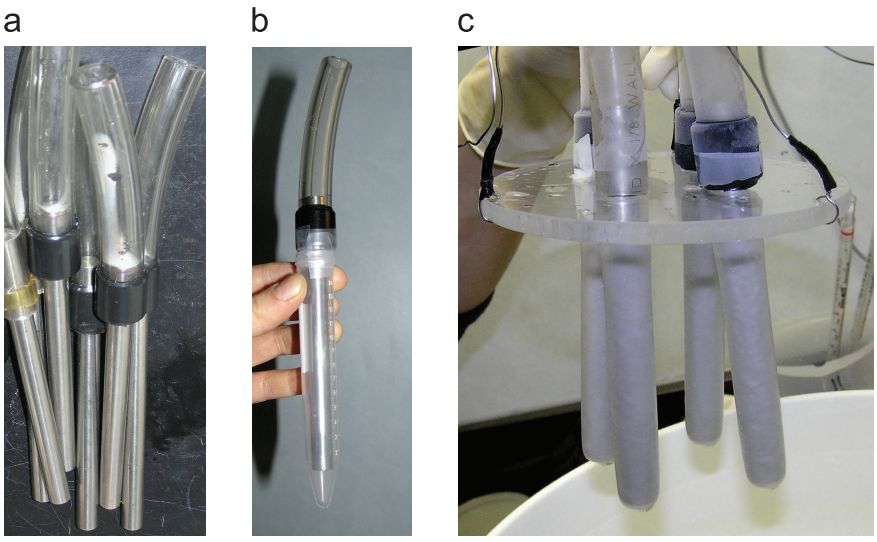

Figure B.1: Configuration of the metal rods used to test for selective retention of environmental pEPS. Metal rods consisted of 12-cm long, stainless steel rods with a tubing handle (a). After ethanol sterilization, metal rods were placed in sterile 15-mL polypropylene tubes and chilled to –80 °C before the experiment (b). Metal rods, held by an acrylic disc, were immersed in the solution of interest for one minute and then removed with the ice that had grown on their surfaces (c). Ice was melted in separate sterile polypropylene tubes for processing.

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Appendix C

Complementary measurements of saline snow[1]

This data set contains additional measurements and scalings of snow properties, ion composition, pH, bacterial abundance and extracellular polysaccharide substances (EPS) for sea ice, and snow over sea ice, complementary to information presented on Chapter 3. Samples were collected during the winters of 2010 (BW'10) and 2011 (BW'11) off the coast of Barrow, Alaska, as described in Chapter 3. Snow depth cross section (100-m transect), average snow depth and sampling locations for BW'11 sampling site are presented in Fig. C.1. Table C.1 presents detailed information on layers, hardness, and visual estimates of snow grain size and shape (estimated with a hand-held magnifying glass and a gridded card) for all snow samples.

Melted samples of saline snow contained a white mineral crystal metastable at room temperature. When observed with light microscopy, crystals had two representative shapes, club-like and trapezoidal (Fig. C.2). Aliquots of one snow sample (BW'10) were stored at 2°C and processed at the University of Washington (UW) within 5 months of collection to determine pH (digital ionalyzer, Orion Research 501, ROSS 81-55 pH electrode) and concentration of major ions, Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}, Cl^{-}, SO_4^{2-} (as described in Chapter 3). In BW'11, pH was directly measured in the field with pH strips, with two samples kept frozen at –20 °C to measure pH after five months by digital ionalyzer; an offset of 2.4 units was found between the two measuring methods. Bulk ion concentrations are presented in Table C.2 and were used in Chapter 3 to calculate BW'10 enrichment factors (Fig. 3.4). Bulk

[1]A version of this appendix was originally published as supplementary material in: M. Ewert, S. D. Carpenter, J. Colangelo-Lillis, and J. W. Deming (2013), Bacterial and extracellular polysaccharide content of brine-wetted snow over Arctic winter first-year sea ice. *J. Geophys. Res.*, 118, 726–735, doi:10.1002/jgrc.20055.

pH presents a clear gradient, with high pH at the high-salinity samples from the ice-snow interface, and low pH at the low-salinity snow surface (Fig. C.4). Bulk pH in the uppermost layer of sea ice is in the range reported for marine brines (8.3 – 8.5, *Miller et al.*, 2011; 8.5 – 8.8, *Papadimitriou et al.*, 2007) and similar to pH values of 8.1 – 8.7 reported by *Kalnajs and Avallone* (2006) for Antarctic frost flowers during the Austral winter. Likewise, pH in saline snow is similar to that observed by *Poulain et al.* (2007) for snow over sea ice in the Canadian High Arctic (6 – 9) but it is higher than the 5.4 – 7.3 range measured for snow in Antarctica (*Kalnajs and Avallone*, 2006), which may be due to differences in salinity among the two sets of snow samples. The upper, non-saline snow has lower pH values, as expected by the presence of acidic sulfates and nitrates of atmospheric origin (*Barrie et al.*, 1985). The pH values for non-saline snow are slightly higher than values reported for snow over land in Barrow (4.7 – 5.5), although such values were taken in a year with above average concentration of atmospheric haze, which is a source of sulfate to the snow (*Douglas and Sturm*, 2004). The buffering effect of marine brines on the snow, evidenced by the relationship between pH and salinity (Fig. C.4b), has implications for the environmental conditions experienced by the microbial community in the snow.

Full depth-ice core measurements are presented in Fig. C.3 (physical parameters) and Fig. C.5 (cell abundance and particulate EPS); these figures complement partial ice-core data presented in Fig. 3.3, 3.5 and 3.6 of Chapter 3. Bulk bacterial abundance and EPS were scaled to brine volume (Table C.3) by dividing the bulk value by the brine volume fraction of the corresponding sample.

Table C.1: Physical parameters of snow (hardness, grain size, grain shape) by layer, on individual samples.

Year	Sample	Layer (cm)[a]	Hardness[b]	Grain Size (mm)[c]	Grain shape [d]
	1	0–2	Blade	(nm)	(nm)
		2–11	Pencil	(nm)	(nm)
	2	0–2	1-finger	(nm)	(nm)
		2–3	Blade	(nm)	(nm)
		3–9	Pencil	(nm)	(nm)
BW'10	3	0–2	1-finger	(nm)	(nm)
		2–3	Blade	(nm)	(nm)
		3–6	1-finger	(nm)	(nm)
		6-19	Pencil	(nm)	(nm)
	4	0–2	Pencil	(nm)	(nm)
		2–6	Pencil	(nm)	(nm)
	1	0–4	Pencil	3	ws/dh
		4–8.5	1-finger	1	ws
		8.5–9	Pencil	1	ws
	2	0–2	Pencil	2	ic/dh
		2–3	Blade	1–2	ic/ws
		3–6	1-finger	1–2	ws
		6–8	1-finger	1	ws
	3	0–3	Pencil	1–2	ic/dh
		3–6	1-finger	1	ws
BW'11		6–9	Pencil	0.5–1	ws
	4	0–3	Pencil	3	ws/dh
		3–4	Blade	(nm)	(nm)
		4–6	1-finger	1	ws
		6–9	Pencil	1	ic/ws
	5	0–3	Pencil	3	ic/dh
		3–4	Blade	2	ws
		4–6	1-finger	2	ws
		6–9	Pencil	1	ic/ws
	6	0–3	Pencil	(nm)	(nm)

[a] Height interval at which each layer occurred, with 0 being the ice surface.
[b] Hardness measured in hand values according to *Colbeck et al.* (1990).
[c] Intervals indicate range of dominating values.
[d] ws = wind slab, dh = depth hoar (small plates), ic = ice crust, (nm) = not registered; grain shape identified following *Sturm* (2009).

Table C.2: Depth profile of major ions in snow during BW'10[a]. Values for Standard Sea Water (*Pilson*, 1998) included for comparison.

Depth (cm) [b]	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	SO_4^{2-}
15	5.008	0.102	0.694	0.116	7.108	0.048
12	5.381	0.124	0.740	0.124	7.898	0.042
6	97.43	1.708	11.15	1.708	182.9	0.610
3	147.0	2.674	17.33	2.674	289.2	1.790
2	217.4	4.715	27.41	4.715	412.8	3.029
0	354.6	7.134	42.77	7.137	655.5	11.85
-100	480.57	10.46	54.14	10.53	559.4	28.93

[a] All concentrations in mmol L^{-1}.

[b] Snow depth measured as distance from ice-snow interface with 0 being the basal layer of snow. Depth –100 represents values for Standard Sea Water.

Table C.3: Biological parameters scaled to brine for surface sea ice and saline snow.

Year	Sample[b]	Melt[c]	Bacterial [d] abundance	pEPS[e]	pEPS > 3 μm	pEPS < 3 μm	dEPS > 0.1 μm	dEPS < 0.1 μm
2010	Ice	s	8.4 ± 1.8	3.7 ± 3.3	(nm)[f]	(nm)	(nm)	(nm)
2010	Snow	d	0.3 ± 0.2	0.4 ± 0.2	(nm)	(nm)	(nm)	(nm)
2011	Ice	d	3.2 ± 1.1	0.8 ± 0.4	(nm)	(nm)	(nm)	(nm)
2011	Ice	s	4.5 ± 1.0	1.0 ± 0.7	0.34 ± 0.25	0.69 ± 0.35	0.66 ± 1.16	10.8 ± 2.7
2011	Snow	d	0.1±0.1	(nm)	(nm)	(nm)	(nm)	(nm)
2011	Snow	s	(nm)	1.2 ± 1.6	(nm)	0.03 ± 0.07	(nm)	1.6 ± 1.2

[a] Means and standard deviations (SD) were calculated for n = 5, except for BW'10 ice (n = 3 for bacterial abundance, n = 2 for pEPS) and BW'10 snow (n = 4).

[b] "Ice" corresponds to surface ice and "snow" to saline snow layer.

[c] See methods in Chapter 3 for description of direct (d) and saline (s) melts.

[d] Bacterial abundance as cells$\times 10^5$ mL^{-1} brine.

[e] All EPS concentrations in mg glu-eq L^{-1} brine.

[f] Not measured (nm) indicate that no sample was collected (2010, 2011 direct melt) or that the background value was higher than the value at the surface layer of snow (2011 saline melt; see Chapter 3 for discussion).

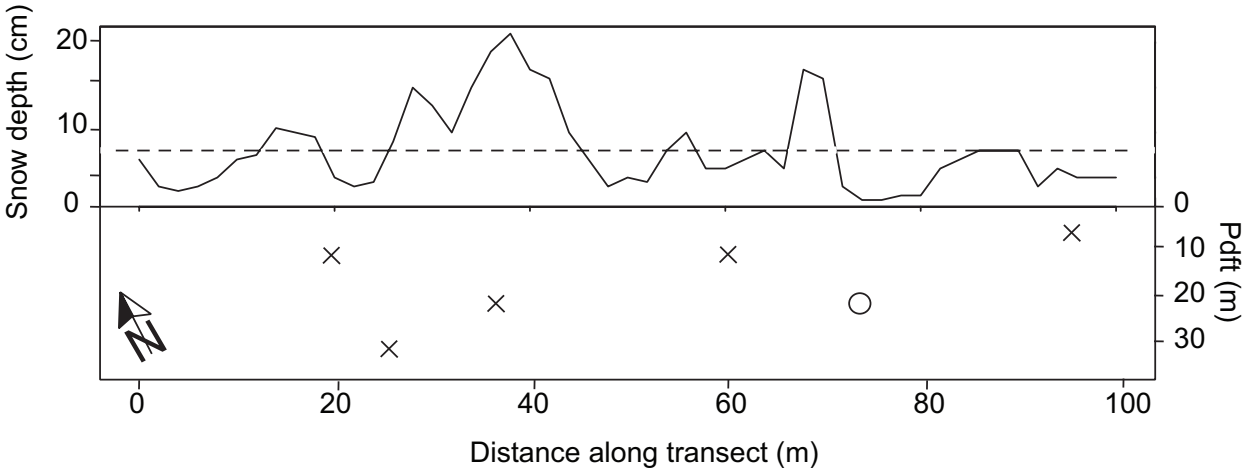

Figure C.1: Snow depth cross section for the BW'11 sampling site, with dashed line indicating average snow depth of 8 cm in a 100-m transect. Symbols indicate sampling locations at perpendicular distance from the transect (pdft). Snow depth was heterogeneous in all directions; depths on the transect do not represent depths at the sampling locations. Samples for depth profiles (X) were collected from snow patches with a depth close to average; samples for spatial variability in salinity and Live/Dead analysis (o) were collected from a patch 3-cm deep.

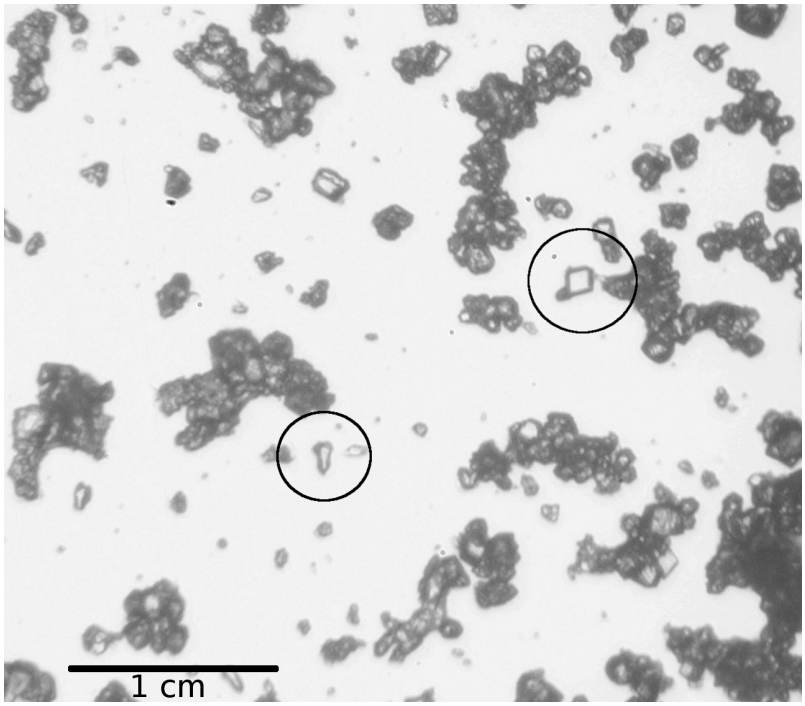

Figure C.2: Ligth microscopy observations of white mineral crystals precipitated from saline snow samples. Circles highlight two characteristic shapes. One of these species may be a polymorph of $CaCO_3$, as discussed in Chapter 3.

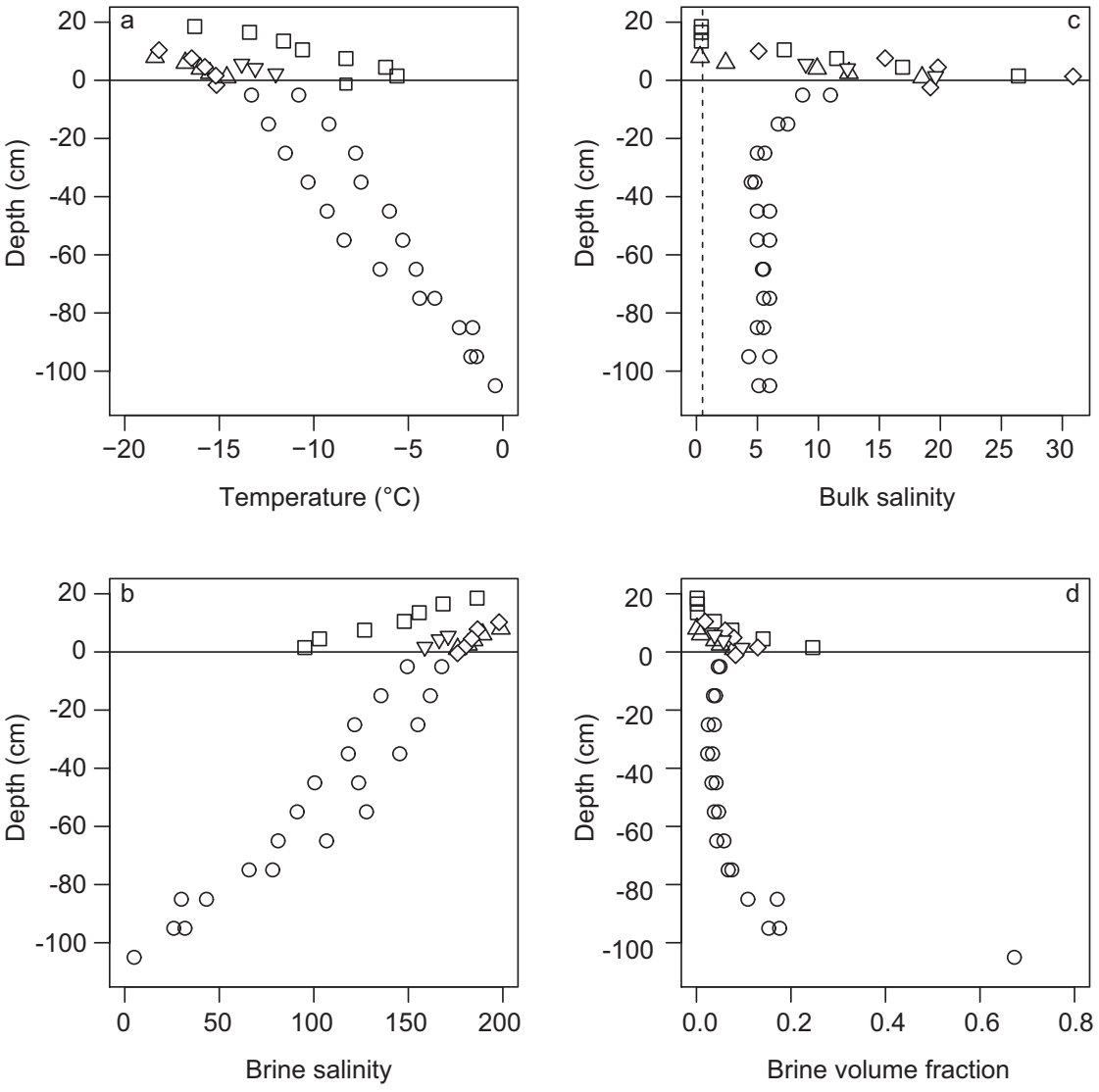

Figure C.3: Depth profiles from BW'10 including complete ice core data for temperature (°C) (a), brine salinity (b), bulk salinity (c) and brine volume fraction (d) in snow (positive depths) and sea ice (negative depths). Solid lines indicate snow-ice interface; dashed line (c) indicates maximum background bulk snow salinity of 0.5.

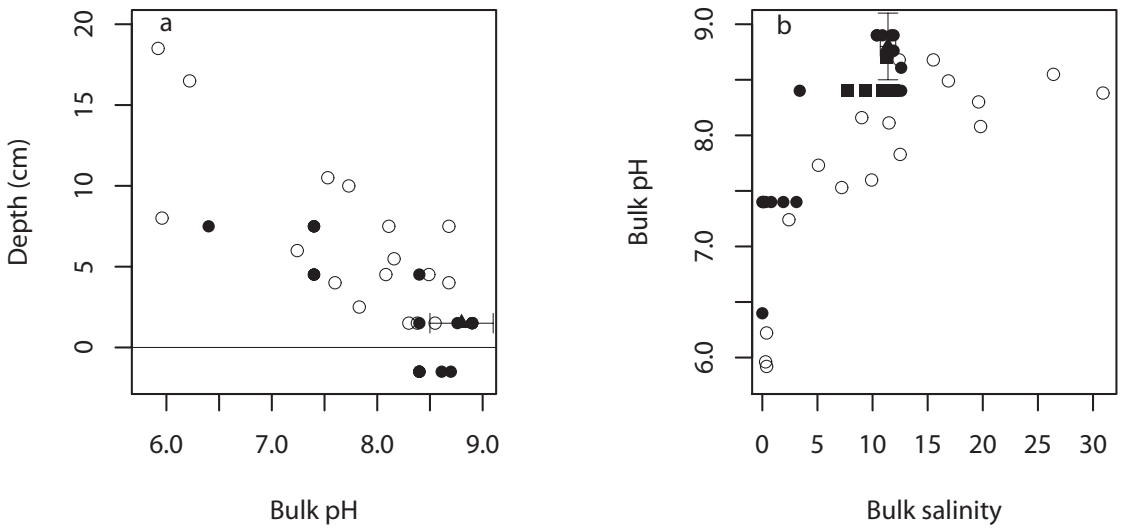

Figure C.4: (a) Bulk pH depth profiles and (b) relationship between pH and bulk salinity in snow and surface sea ice. Open symbols indicate BW'10 data; filled symbols indicate BW'11 data. Filled squares (b) indicate the sea-ice data.

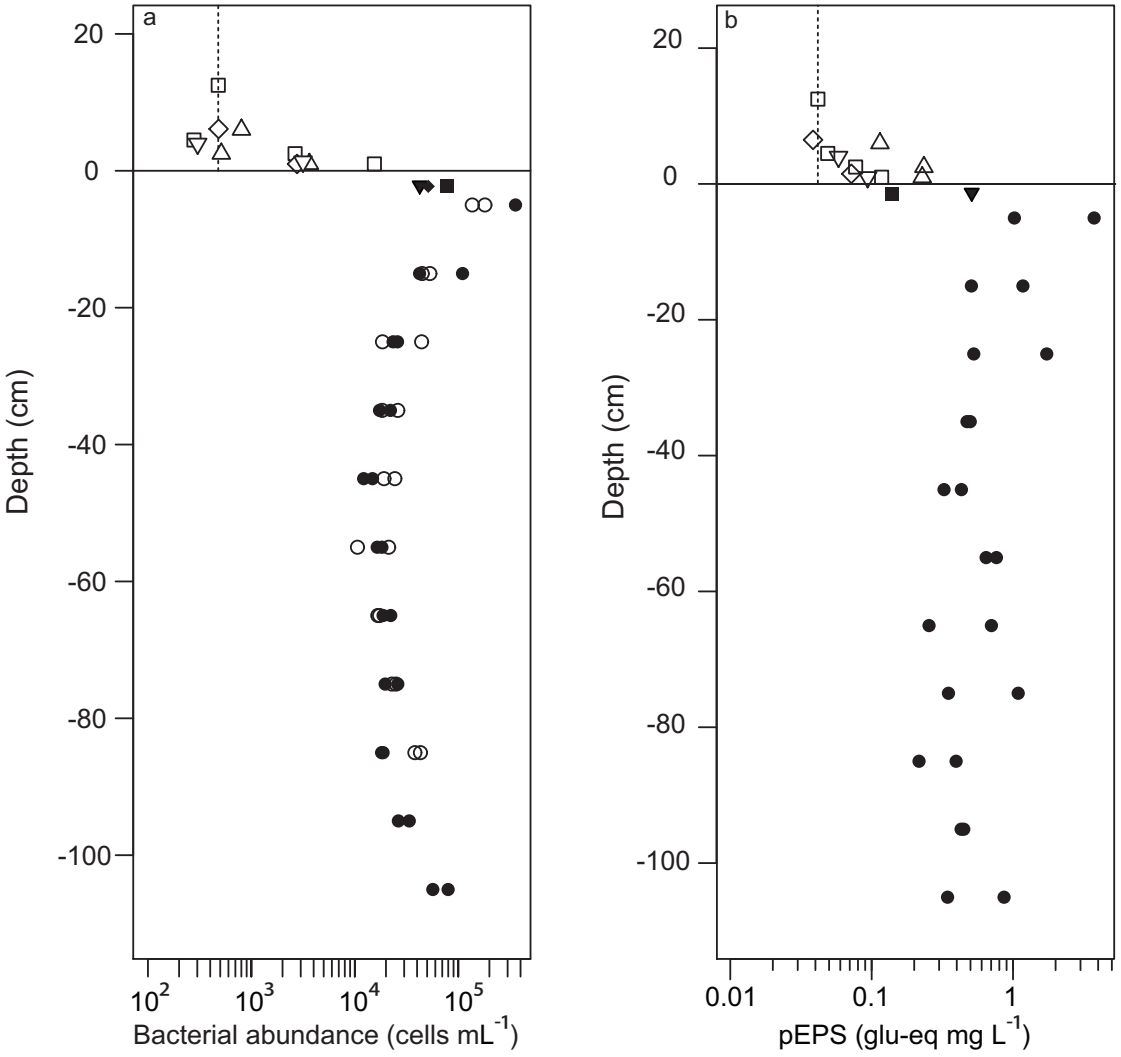

Figure C.5: Depth profiles from BW'10 including complete ice core data for total bacterial abundance (a) and bulk pEPS (b) in snow (positive depths) and sea ice (negative depths). Open symbols indicate direct melts; solid symbols indicate saline melts. Solid lines indicate snow-ice interface; dashed lines indicate average bacterial abundances in the background low-salinity samples (< 0.5).

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Appendix D

Cell loss induced by osmotic down-shock in an extremely halophilic bacterial culture enriched from Arctic frost flowers

Due to the high salinity of winter sea-ice brines, microorganisms inhabiting them are prone to osmotic down-shifts associated with daily fluctuations and seasonal changes in temperature (Chapter 5). An osmotic down-shift can trigger osmolysis in microbial cells. Understanding the extent of cell loss due to osmotic down-shifts is relevant to explaining changes in sea-ice microbial abundance and community structure throughout the winter, as discussed in Chapter 4, but it also has implications for the practical aspect of processing sea-ice samples.

Available protocols for melting sea ice include: melting sea ice directly (direct melt); melting samples in filtered seawater (seawater melt); or melting samples in artificial brine to achieve a final salinity similar to the expected in situ salinity of sea-ice brines (isohaline melt). Direct melts showed losses of 13 to 97 % of eukaryotic cells when compared with seawater melts; losses depended on the taxa (with ciliates and flagellates being the more susceptible) and had an effect on the species composition (*Garrison and Buck*, 1986; *Gradinger*, 1999). Direct melt, however, showed no difference from seawater melt for most taxa of sea-ice algae (except chrysophytes), if performed slowly at 4 °C (*Mikkelsen and Witkowski*, 2010). The effect of melting protocols on sea-ice bacteria has been limited to the culturable fraction, showing no difference by melting protocol in winter sea-ice cores (*Helmke and Weyland*, 1995). Since culturable bacteria represent only a fraction of the total number in sea ice, the effect of melting method on total bacterial numbers needs to be considered.

Results from upper winter sea-ice presented in Chapter 3, as well as unpublished work

reported by *Deming* (2010), indicated that neither direct nor seawater melt offer enough protection for bacteria inhabiting the upper regions of winter sea ice, where salinities can reach values above 200. To further investigate the osmotic sensitivity of bacteria inhabiting highly saline ice formations, an extremely halophilic culture (frost flower culture 3E) was obtained from winter Arctic frost flowers (FF). This appendix presents the isolation process of 3E, and experimental work where 3E was grown at a salt concentration of 200 ppt and then subjected to different degrees of osmotic down-shock.

D.1 Methods

D.1.1 Enrichment procedure

Bacterial enrichment cultures were obtained from frost flowers growing on newly formed winter sea-ice in the Southern Beaufort Sea (during the Circumpolar Flaw Lead system study 2007 – 2008; 71.05 °N, 125.53 °W). A small lead that had formed in a first-year ice floe, 870 m from where the ship was stationed, was closely monitored for three days, with new iced forming on the second day and frost flowers fully developed by the third day. The new ice was 10 cm thick by the time frost flowers were collected; air temperature was –28.2 °C above the frost flowers and –18.5 °C at the bare ice surface. Frost flowers were collected by carefully scraping the surface of the ice with an ethanol-cleaned spatula, placing the material in sterile bags and melting directly at 10 °C, shaking often to prevent warming. As soon as the frost flowers were melted, aseptic technique was used to inoculate aliquots into pre-chilled liquid media (Marine Broth 2216 half organic strength, or $\frac{1}{2} \times 2216$) in test tubes that were then incubated at –1 °C for two weeks. An additional aliquot was used to measure bulk salinity by refractometer. Turbid cultures were sequentially transferred into $\frac{1}{2} \times 2216$ media adjusted to higher salinities with NaCl, until growth was observed at 200 ppt and –1 °C. From these final enrichment tubes, sequential streak-plating was performed using pre-chilled agar plates of $\frac{1}{2} \times 2216$ at seawater salinity and –1 °C, until homogeneous colonies of the same size and morphology were obtained. DNA was extracted and purified

as described in Chapter 4. The Bacterial 16S rRNA gene was amplified and sequenced as described in Chapter 4, but the results indicated the possible presence of more than one organism. In the absence at this time of more information to confirm or reject culture purity, this extremely halophilic culture enriched from frost flowers will be designated hereafter as FF culture 3E.

D.1.2 Salinity and temperature growth range

To determine the salinity range of 3E, a stock culture was inoculated into $\frac{1}{2}\times$MB 2216 media of different salt concentrations. $\frac{1}{2}\times$MB 2216 prepared as described in Chapter 2 and amended with NaCl to concentrations of 35, 80, 164, 200 or 220 ppt. All tubes were incubated at –1 °C without shaking for at least 2 weeks. Temperature growth range was determined in similar fashion but only for salinity of 35 ppt.

D.1.3 Osmotic shock experiments

FF culture 3E was grown at 2 °C in $\frac{1}{2}\times$MB 2216 media amended with NaCl to 200 ppt. Once the culture reached late exponential phase, 0.5 mL aliquots were transferred to sterile microcentrifugue or borosilicate tubes, in replicate for each treatment. Sterile, pre-chilled distilled water was added in different quantities for each treatment to reach a final salt concentration of 2, 35 or 100 ppt. One treatment received instead an aliquot of sterile NaCl solution with a concentration of 200 ppt. Each of these treatments was immediately mixed by vortexing the tubes for 1 s. All tubes were held in an ice bath to avoid shock due to warming. Samples were incubated for either 10 min (n = 3) or 12 h (n = 2), fixed with 0.2 μm filtered 37 % formaldehyde for a final concentration of 2 % and refrigerated in the dark until processing within 1 week. Cell abundance was determined by direct counts performed by microscopic observation on fixed samples as described in Chapter 4. All counts were corrected for the dilution treatment to enable cross comparison; starting cell abundance was not determined but the 200-ppt treatment represents the no-shock control (presumably

no loss of cells) against which the other treatment results can be compared. Graphical and statistical analyses were performed using R v.2.13.1 (*R Development Core Team*, 2011). Shapiro and Bartlett tests were used for assumptions of normality and equal variance; data were analyzed with an one-way ANOVA.

D.2 Results and discussion

Frost flower samples yielding FF culture 3E had a bulk salinity of 65. FF culture 3E was able to grow at all tested salt concentrations, from 35 to 220 ppt, and temperatures from –1 to 21 °C at 35 ppt. After being subjected to osmotic down-shift, cell abundance was similar in all treatments except for the strongest shift tested (from 200 to 2 ppt), where cell numbers were 40 – 50 % lower (Fig. D.1). Using an ANOVA test, differences among treatments were significant at 93 % for the 10-min experiment ($p = 0.07$) and 96 % for the 12-h experiment ($p = 0.06$). Microscopic observations showed that samples subjected to the strongest down-shift in salt concentration presented compromised cells, with poorly defined edges and an abundance of residual DNA indicating osmotic lysis in this treatment (Fig. D.2). FF culture 3E, with the capacity to grow across a wide range of salinities, could be expected to tolerate strong osmotic shifts within that range. This expectation was supported by three experiments were no difference in cell numbers was observed following exposure of the culture to changes in salt concentratio up to > 165 ppt. The more drastic changes (~ 200 ppt) did impact the integrity of the cells, resulting in lower cell counts.

D.3 Conclusions

Osmotic downshift associated with processing of sea-ice samples is known to cause cell losses in eukaryotic organisms, but its effect on bacteria is not well determined. A bacterial culture obtained from winter frost flowers, FF culture 3E, showed cell loss (significant at 93%) when exposed to an osmotic shock of ~ 200 ppt. This change in salt concentration could be similar to changes experienced during direct melt of winter samples of upper sea

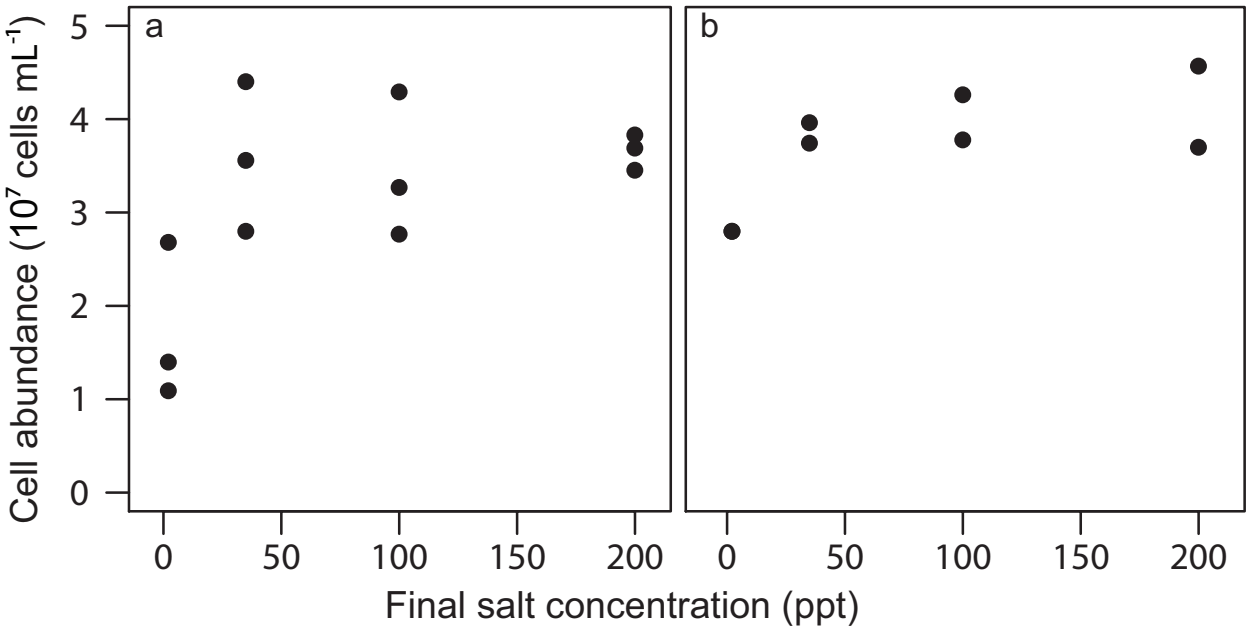

Figure D.1: Cell abundance after osmotic down-shift from 200 ppt to 2 (most extreme change), 35, 100 and 200 ppt (no change in salt concentration). Samples were exposed to the new conditions for 12 min (a; n = 3) or 12 h (b; n = 2).

ice. Because the sea-ice bacterial community is a complex one, where some organisms may be better adapted to down-shifts in salinity than others, the use of saline or isohaline melts for sea-ice samples where the *in situ* brine salinity is close to or above 200 is recommended.

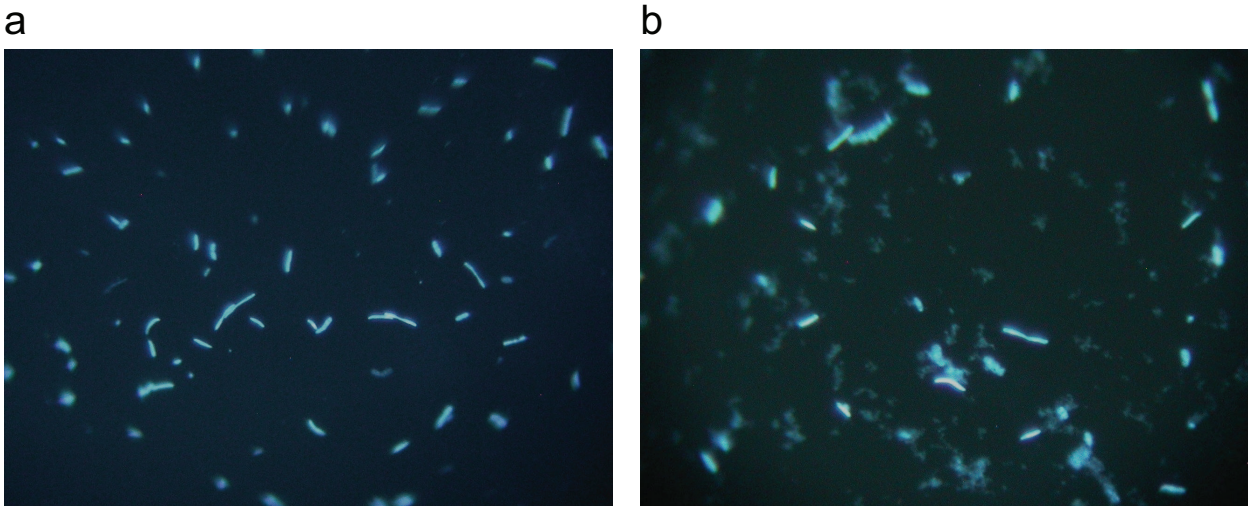

Figure D.2: Microscopic observations of DAPI-stained cells growing at 200 ppt, when kept at the same salt concentration (a) or exposed to a sudden osmotic down-shift to 2 ppt (b). Note compromised cells and presence of residual genetic material in (b).

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Appendix E

Strategies for osmolarity protection in Arctic sea-ice bacterial isolates as predicted by genomic analysis of related species

E.1 Introduction

Organisms inhabiting sea-ice brines are subject to strong changes in osmolarity associated with freezing and thawing of the ice (Chapter 5). Due to a reduction in the volume of the brine inclusions in the ice, freezing is associated with an increase in brine salinity; thawing, on the other hand, results in a salinity down-shift. Up-shifts in salinity affect bacteria by causing plasmolysis, a shrinkage of the cytoplasm that results in separation of the membrane from the cell wall. Down-shifts in salinity cause osmolysis, the breaking of the cell membrane due to increasing extracellular pressure (Appendix D). These changes in brine salinity can occur with the seasonal freezing in the Fall and thawing in the Spring. Daily salinity and temperature fluctuations are also frequent for sea-ice brines exposed to the atmosphere (e.g. Fig. 5.4), with long term exposure to these fluctuations causing significant cell loss for some bacteria (Chapter 4). To cope with these short- and long-term changes in osmolarity, bacteria have developed strategies to control the concentration and type of solutes present in the cell.

This appendix describes the isolation and identification of two sea-ice bacteria from high salinity brines (125, practical salinity) collected from upper winter sea ice, an environment expected to have moderate to high fluctuations in salinity, and thus, be inhabited by organisms likely able to respond to such changes. The first isolate is from the class of Flavobacteria, possibly a new species of the genus *Psychroflexus*; the second isolate is a gammaproteobacterium of the genus *Psychrobacter*. Close relatives to these isolates,

that also have full genome sequences available, were identified by V5–V6 16S rRNA gene regions. The selected organisms were *Psychroflexus torquis*, a psychrophilic Flavobacterium isolated from Antarctic sea ice (*Bowman et al.*, 1998), with 92 % similarity to our isolate *Psychroflexus sp.* strain 1E (*Pf*1E); and *Psychrobacter arcticus*, a gammaproteobacterium isolated from Arctic permafrost (*Bakermans et al.*, 2006), with 97 % similarity to our isolate *Psychrobacter sp.* strain 7E (*P*7E). The genomes of *Psychroflexus torquis* and *Psychrobacter arcticus* were analyzed in depth to determine potential strategies for osmolarity protection. The genome of *Colwellia psychrerythraea* strain 34H (*Cp*34H), a model psychrophilic organism isolated from Arctic sediments (*Methé et al.*, 2005) but susceptible to cell loss with long-term freezing (Chapter 4), was analyzed for comparative purposes.

E.2 Methods

Psychroflexus sp. strain 1E (*Pf*1E) and *Psychrobacter sp.* 7E (*P*7E) were isolated from upper first-year sea-ice brines during the Winter of 2008. Brine samples were collected with aseptic tools, after overnight accumulation in bore holes as described in Chapter 4. Using aseptic technique, aliquots of brine were inoculated onto pre-chilled agar plates (Marine Broth 2216, half organic strength and full seawater salinity, $\frac{1}{2}\times$ MB 2216) and incubated at –1 °C. The V5-V6 16S rRNA gene regions sequenced and aligned as described in Chapter 4. Because standard phenol-chloroform DNA extraction was ineffective for *Pf*1E due to the unusually hard consistency of its colonies, a MoBio Power Biofilm DNA isolation kit was used, with positive results. Genome analysis of *Psychroflexus torquis* and *Psychrobacter arcticus* was performed using the tools available at MicrobesOnline (*MicrobesOnline*, 2013; *Dehal et al.*, 2010). At this time, the genome of *P. torquis* is incomplete, such that operon prediction was not always possible.

E.3 Results and discussion

E.3.1 Isolates

Enrichment cultures leading to the isolation of *Psychrobacter sp.* strain 7E and *Psychroflexus sp.* strain 1E, yielded colonies 3 – 6 mm in diameter, light-pink in color, and with a highly elastic consistency; the consistency of the colonies coincided with the definition of a "ropy" EPS-producing colony by (*Ruas-Madiedo and de los Reyes-Gavilán*, 2005), where the colonies are able to produce strands when extended with an inoculation loop. During sequential plating for purification, the presence of two distinctive colony types, each notably different from the initial types, was revealed. Because considerable effort was required to purify each type, a close consortial relationship between the two resulting isolates may pertain. After purification, the first isolate produced 3 – 10 mm bright orange colonies with a very hard consistency that could not be disturbed with a standard inoculation loop; this colony type maintained its integrity even after transfer to liquid media and vortexing for 1 min. The isolate was characterized as a strictly psychrophilic organism (growth in $\frac{1}{2} \times MB2216$ from –1 to 13 °C, where –1 °C was the lowest temperature tested) and identified as *Psychroflexus sp.* strain 1E (*Pf*1E) with 92 % similarity to *Psychroflexus torquis* strain ATCC 70075, the closest relative with a whole genome sequence available. When left to grow in $\frac{1}{2}\times$MB 2216 liquid media at 8 °C for 1 month, *Pf*1E resulted in a highly gelatinous culture that generated a plug in the upper section of the test tube (Fig. E.1). The bright orange colonies of *Pf*1E are characteristic of bacteria from the Bacteroidetes phylum (*O'Sullivan et al.*, 2006); *Pf*1E may contain proteorhodopsin pigments as do other sea-ice bacteria in the Flavobacteriaceae family (*Koh et al.*, 2010), including *P. torquis*, (its genes P700755_00947 and P700755_31809 encode putative proteorhodopsins). The second isolate purified from the initial consortium formed small (1 – 2 mm) white colonies with a very soft consistency. It was characterized as a psychrotolerant organism (growth in $\frac{1}{2}\times$ MB 2216 –1 to 21 °C) and identified as *Psychrobacter sp.* strain 7E (*P*7E), with 99 % similarity to both, *Psychrobacter nivimaris* strain 88/2-7 (*Heuchert et al.*, 2004) and *Psychrobacter*

proteolyticus strain 116 (*Denner et al.*, 2001), and a 97% similarity with *Psychrobacter arcticus*, the closest relative with a whole genome sequence available.

E.3.2 Response to fluctuations in osmolarity

At 8 °C, both *Psychrobacter sp.* strain 7E and *Psychroflexus sp.* can grow at salt concentrations of 35 to 125 ppt. For *P*7E, a higher salt concentration of 180 ppt was also tested, but no growth was observed. Growth rates at the salt concentration of 125 ppt were low for both organisms (with turbidity reached in 1 month for *P*7E and $>$ 1 month for *Pf*1E). Growth at salt concentrations below 35 ppt was not explored for either isolate. In contrast, *Colwellia psychrerythraea* strain 34H (*Cp*34H) grows at salt concentrations of 20 – 50 ppt (*Wells and Deming*, 2006; *Marx et al.*, 2009).

For *Cp*34H, genes identified as likely involved in the response to an osmolarity up-shift are depicted in Fig. E.2; for a down-shift in Fig. E.3. For *P. arcticus* (as representative of *P*7E) up-shift genes are shown in Fig. E.4 and down-shift genes in Fig. E.5. For *P. torquis* (as representative of *Pf*1E) up-shift and down-shift genes are shown in Fig. E.6 and E.7, respectively. Although not evident in these comparative figures, some of the genes included in them may play a role in the response to both an up-shift and a down-shift of salinity. All of the genes are categorized and compiled by function and organism in Tables E.1 - E.7, to indicate distinctive and overlapping genes. These tables also contain information pertaining to genes in the same operon as the target genes of interest, even if their role in osmolarity regulation is not clear. Because the genome of *P.torquis* is not complete, its genomic analysis must be considered preliminary.

Immediately after an osmotic up-shift, cells maintain their turgor by transiently accumulating K^+ ions which are imported into the cytoplasm by means of K^+-uptake proteins (*Dinnbier et al.*, 1988; Table E.1). Because K^+ is not a compatible solute, its long term storage may affect cellular functions. If the high-osmolarity conditions continue, bacteria import compatible solutes into the cell by means of membrane transporters (Table E.2).

Many types of small organic molecules serve as compatible solutes, with betaine, ectoine and proline amongst the more commonly used (*Roberts*, 2005). Choline, precursor to betaine, is not a compatible solute *per se*, but many bacteria have the ability to oxidize it into betaine for osmolarity protection (Table E.3). Another strategy against fluctuating salinities is the export of excess Na^+ ions by Na^+ / H^+ antiporters which exchange Na^+ with H^+ protons from the environment (*Krulwich et al.*, 2009). Since Na^+ ions are a major constituent of sea-ice brines, Na^+ / H^+ antiporters (Table E.4) may be playing a protective role in sea-ice bacteria.

Bacterial cells exposed to a drastic down-shift in salinity immediately release intracellular ions to relieve extreme intracellular turgor and prevent cell lysis (*Halverson et al.*, 2000). The release of intracellular ions occurs through mechanosensitive ion channels (Msc) which act as "emergency valves" by being mechanically activated when the intracellular pressure reaches a target threshold (Table E.5). There are at least three major types, mini (MscM), small (MscS) and large (MscL) conductance mechanosensitive ion channels (*Naismith and Booth*, 2012). Each type of channel is activated at different membrane tensions, opens to different diameters and has different ion specificities (*Kung et al.*, 2010). MscL, in particular, are wide channels up to 30 Å that open at very high tensions (just before the lytic limit of the cell) and with very low specificity, allowing the passage of molecules as large as 1000 Da (*Kung et al.*, 2010). Minutes after the release of intracellular solutes via Msc, as prompted by an osmotic down-shift, organisms transport some of the solutes back to the cell by means of transport proteins, including the K^+-uptake proteins discussed before (Table E.1, *Schleyer et al.*, 1993). Another strategy to maintain cell functions at low osmolarity is the accumulation of polyamines via spermidine/putrescine transporters (Table E.6). Polyamines play a role in compensating the protein-DNA interactions at low osmolarity, the condition when intracellular concentrations of putrescine increase (*Capp et al.*, 1996).

Changes in osmolarity have also been associated with the expression levels of porins OmpC and OmpF. In the model organism *Escherichia coli*, porins OmpC and OmpF are differentially expressed depending on the osmolarity of the media, with OmpC preferentially

expressed at high osmolarity and OmpF at low osmolarity (*Csonka and Hanson*, 1991). The expression of these porins is regulated by the osmolarity sensor EnvZ/OmpR (*Wood*, 1999) but also by temperature (*Csonka and Hanson*, 1991). Putative genes associated with the long term osmolarity sensing and regulation are listed in Table E.7.

As observed in Tables E.1 - E.7, the selected model organisms have different sets of genes related to osmolarity response, as well as different numbers of copies for each gene. The differences cover both the responses for up-shifts and down-shifts in salinity. *P. torquis* seems to rely heavily on the import of K^+ given its 11 K^+ transporters, whereas *P. arcticus* and *Cp*34H each carry only two. All organisms have the gene-based potential to import compatible solutes from the media, but the number of available transporters differs by organism. *Cp*34H has numerous genes for the import of compatible solutes, with 5 putative BCCT transporters and 6 putative proline transporters. In contrast, *P. arcticus* and *P. torquis* have only 3 putative BCCT transporters and 1 putative proline transporter. All organisms have the putative ability to use choline as a source of the compatible solute betaine. MscL genes were only present in *P. arcticus* and *P. torquis*, not in *Cp*34H, suggesting a greater susceptibility of *Cp*34H to highly fluctuating environments, as demonstrated by laboratory experiments with live cultures (Chapter 4).

E.4 Conclusions

Two bacterial species possibly related by a close consortial relationship, *Psychrobacter sp.* strain 7E and *Psychroflexus sp.* strain 1E, were isolated from high salinity brines collected from Arctic upper sea-ice in the Winter. Both isolates can grow at salt concentrations of 125 ppt and temperatures of –1 °C. Potential genes involved in the response to up-shifts and down-shifts in salinity were listed for closely related species *Psychrobacter arcticus* and *Psychroflexus torquis*, as well as for the psychrophilic bacterium *Colwellia psychrerythraea* strain 34H. Some putative genes are shared by all organisms, indicating widespread strategies to respond to osmolarity changes. These genes include trKA for potassium uptake, betT for betaine / choline or carnitine transport, the operon betIBA for choline conversion

into glycine betaine, and the MscS genes for small conductance mechanosensitive channels. The number of copies of PutP and ProX, proline transporters, was higher in *Cp*34H than in the other organisms, possibly indicating differences in the use of proline as compatible solute. A potentially important difference is the presence of large conductance mechanosensitive channels in *P. arcticus* and *P. torquis* but not in *Cp*34H, which may be indicative of differences in the ability to resist fluctuations in salinity.

E.5 Acknowledgments

J.S. Bowman extracted and sequenced the DNA of the isolates.

Table E.1: Candidate genes involved in K^+ transport as a response to up-shifts in osmolarity

Gene	Function	*Cp*34H	*P. arcticus*	*P. torquis*
trkA	K^+ uptake protein	CPS_0017	Psyc_1606	P700755_00587
				P700755_02512
				P700755_03032
				P700755_15596
				P700755_21076
				P700755_24511
				P700755_25011
				P700755_28795
				P700755_31739
*sun	SSU rRNA methyltransferase B	CPS_0018		
*fmt	Methionyl-tRNA formyltransferase	CPS_0019		
*def1	Polypeptide deformylase	CPS_0020		
ktrB	K^+ uptake transporter		Psyc_1607	
trkH	K^+ uptake protein	CPS_0015		P700755_00582
* pepQ	Xaa-Pro dipeptidase	CPS_0013		

* In the same operon as the gene immediately above.

Table E.2: Candidate genes involved in the transport of compatible solutes as a response to up-shifts in osmolarity

Gene	Function	*Cp*34H	*P. arcticus*	*P. torquis*
betT	Putative BCCT transporter	CPS_4009	Psyc_1301	P700755_17029
		CPS_1335	Psyc_0727	P700755_18079
			Psyc_0826	P700755_19282
*MarR [a]	Transcriptional regulator	CPS_1337		
betL [b]	Secondary BCCT transporter	CPS_4027		
		CPS_3860		
		CPS_2003		
ProX	Amino acid ABC active transporter	CPS_4933		P700755_32574
*ProV	Amino acid ABC transporter, ATP-binding protein	CPS_4935		P700755_26982
*ProW	Permease protein	CPS_4934		P700755_21471
PutP	Na^+/proline symporter	CPS_0068	Psyc_1415	
		CPS_0939		
		CPS_3347		
		CPS_3463		
		CPS_3419		
*CirA	Outer membrane receptor protein	CPS_3344		
*CaiC	Acyl-CoA synthetases (AMP-forming) / AMP-acid ligases II	CPS_3345		
*CaiD	Enoyl-CoA hydratase/carnithine racemase	CPS_3346		

[a] marR has an homologue in *Vibrio cholera* named cosR (VC_1278), whose functions, including the repression of compatible solute transporters, have been described in *Shikuma et al.* (2012). The cosR gene also upregulates the porine ompT protein, which is homologous (40 %) to the putative porine CPS_1206 in *Cp*34H.

[b] When blasted against *Cp*34H genome, gene ISM_11110 (rbtB) from *Roseovarius nubinhibens* (a gene known to transport DMS, *Sun et al.*, 2012) showed 55 % similarity with CPS_2003 (betL transporter).

Table E.3: Candidate genes involved in choline catabolism

Gene	Function	*Cp*34H	*P. arcticus*	*P. torquis*
betI	Transcriptional regulator	CPS_4012	Psyc_0730	P700755_32579
		CPS_1332		
*betB	Betaine aldehyde dehydrogenase	CPS_4011	Psyc_0729	P700755_33650
		CPS_1333		
*betA	Choline dehydrogenase	CPS_4010	Psyc_0728	P700755_14335
		CPS_1334	Psyc_1614	P700755_20851
				P700755_33069
betA	Choline dehydrogenase	CPS_0670		
		CPS_3434		
*MaoC		CPS_0671		
		CPS_3435		

* In the same operon as the gene immediately above.

Table E.4: Candidate genes involved in Na^+ export as a response to up-shifts in osmolarity

Gene	Function	*Cp*34H	*P. arcticus*	*P. torquis*
nhaC	Na^+/H^+ antiporter	CPS_2476		P700755_16864
		CPS_3465		
		CPS_4723		
		CPS_2809		
*pepN	aminopeptidase N	CPS_2808		
nhaD	Na^+/H^+ antiporter	CPS_3512		
nhaP	Na^+/H^+ antiporter	CPS_3408	Psyc_1305	P700755_05749
Smf	DNA processing chain A			P700755_05744

* In the same operon as the gene immediately above.

Table E.5: Candidate mechanosensitive ion channels

Gene	Function	*Cp*34H	*P. arcticus*	*P. torquis*
MscS	Small mechanosensitive ion channel	CPS_0104	Psyc_0970	P700755_28720
		CPS_1419	Psyc_0625	P700755_15106
		CPS_1746		
		CPS_4603		
		CPS_1934		
		CPS_0969		
		CPS_4829		
*PlsC	Acyltransferase family protein	CPS_4830		
MscS	Small mechanosensitive ion channel	CPS_0961		
*RpoE	RNA polymerase sigma-70 factor	CPS_0962		
MscS	Small mechanosensitive ion channel			P700755_09141
MetG	Methionyl-tRNA synthetase			P700755_09146
MscS	Small mechanosensitive ion channel			P700755_19267
NarK	Nitrate/nitrite transporter			P700755_19272
MscL	Large conductance mechanosensitive ion channel		Psyc_0340	P700755_20866

* In the same operon as the gene immediately above.

Table E.6: Candidate genes involved in the transport of polyamines as a response to a downwhift in osmolarity

Gene	Function	*Cp*34H	*P. arcticus*	*P. torquis*
potA	Spermidine/putrescine ATP-binding protein	CPS_2039	Psyc_1545	P700755_10895
				P700755_14500
*potB	Permease protein	CPS_2038		P700755_10905
				P700755_22959
*potC	Permease protein	CPS_2037		P700755_10900
				P700755_26567
*potD	Periplasmic spermidine/putrescine binding protein	CPS_2036		P700755_26347
potG	Putrescine ABC transporter, ATP-binding protein	CPS_4672		
*potL	Putrescine ABC transporter, permease protein	CPS_4674		
*potH	Putrescine ABC transporter, permease protein	CPS_4673		
*potF2	Periplasmic putrescine-binding protein	CPS_4671		

* In the same operon as the gene immediately above.

Table E.7: Candidate genes involved in sensing and regulatory responses to osmolarity

Gene	Function	*Cp*34H	*P. arcticus*	*P. torquis*
EnvZ	Osmolarity sensor protein	CPS_4596	Psyc_2021	
*OmpR	Transcriptional regulatory protein	CPS_4597	Psyc_2022	
BaeS	Osmolarity sensor protein		Psyc_2020	
OmpC	Outer membrane protein (porin)	CPS_1206		
		CPS_2010		
		CPS_2472		
		CPS_1206		
OmpN	Outer membrane protein (porin)		Psyc_1642	

* In the same operon as the gene immediately above.

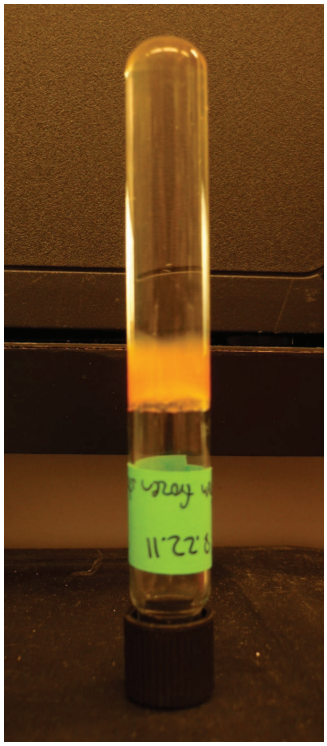

Figure E.1: *Psychroflexus sp.* strain 1E grown in $\frac{1}{2}\times$MB 2216 liquid media at 8 °C after one month. Test tube was photographed upside-down to illustrate the effect of the (likely) EPS plug on the surface of the culture holding the remaining fluid in place.

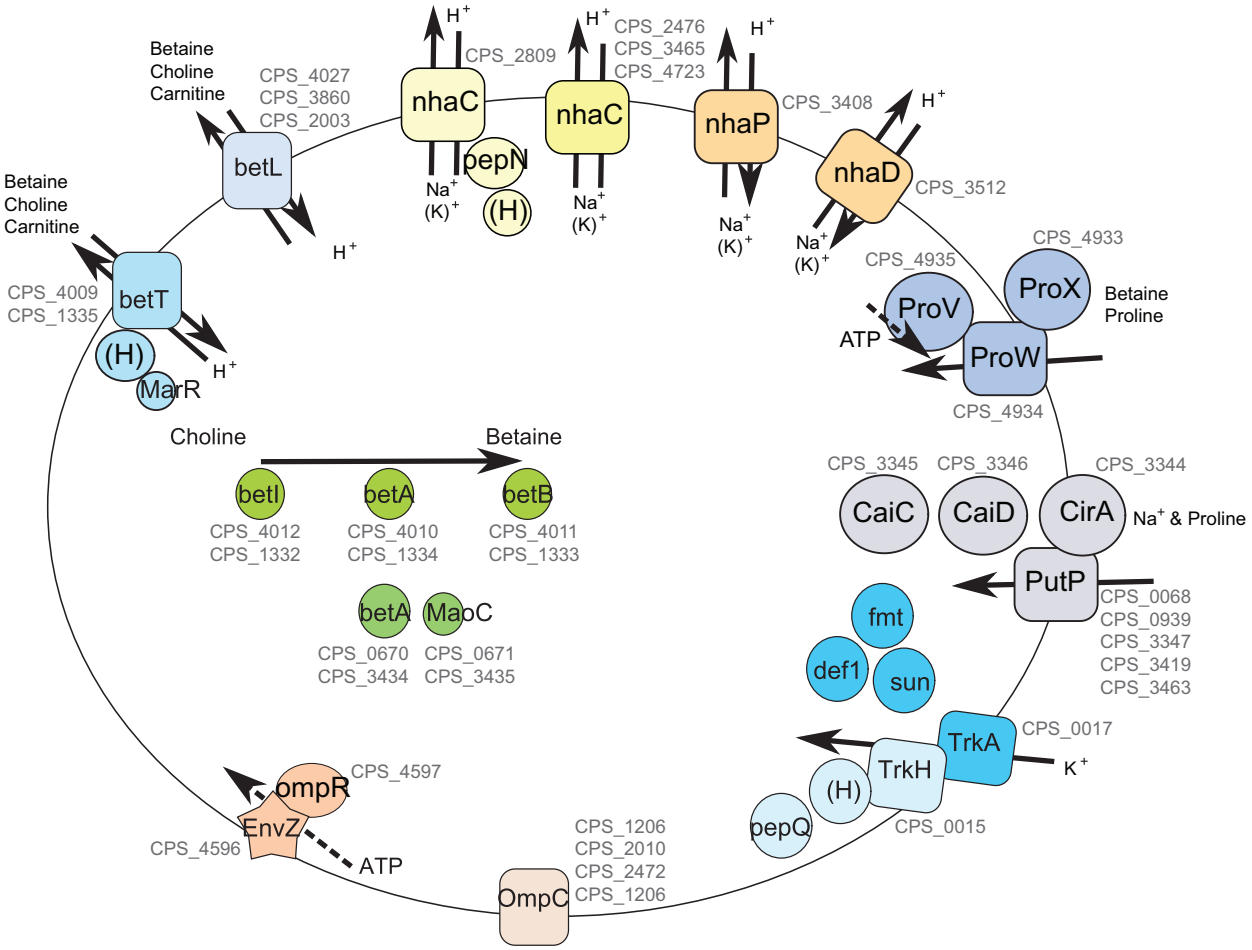

Figure E.2: Representation of a bacterial cell with candidate proteins involved in response to osmolarity up-shifts, based on candidate genes present in *Colwellia psychrerythraea* strain 34H genome. Gene loci from *Cp*34H listed adjacent to the proteins of interest. Squares indicate membrane proteins, circles indicate cytoplasmic (non-membrane) proteins, stars indicate proteins involved in gene regulation. Black arrows indicate transport of solutes and dashed arrows, chemical transformation between molecules. Genes predicted under the same operon have the same color.

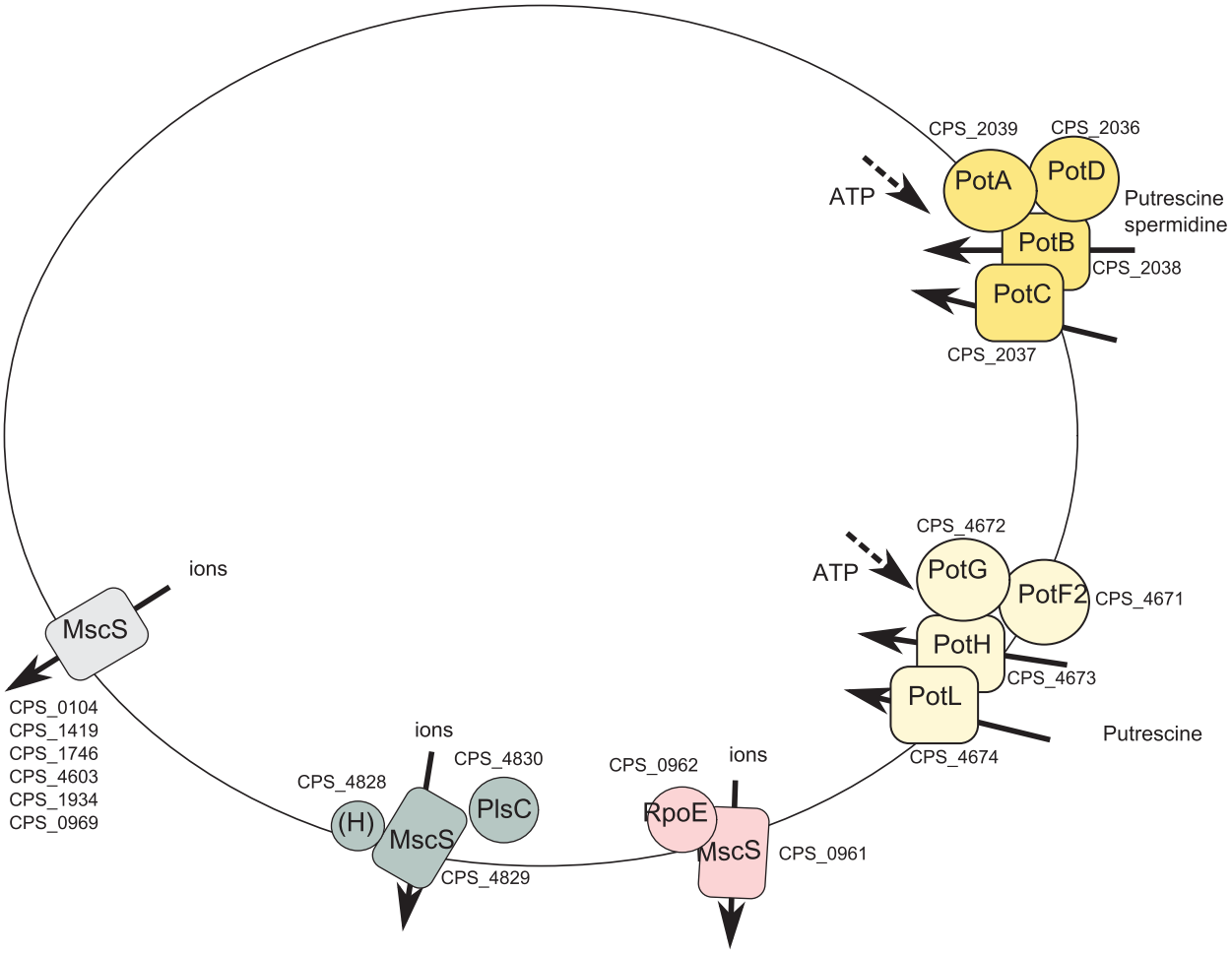

Figure E.3: Representation of a bacterial cell with candidate proteins involved in response to osmolarity down-shifts, based on candidate genes present in *Colwellia psychrerythraea* 34H genome. Symbols as in Fig. E.2.

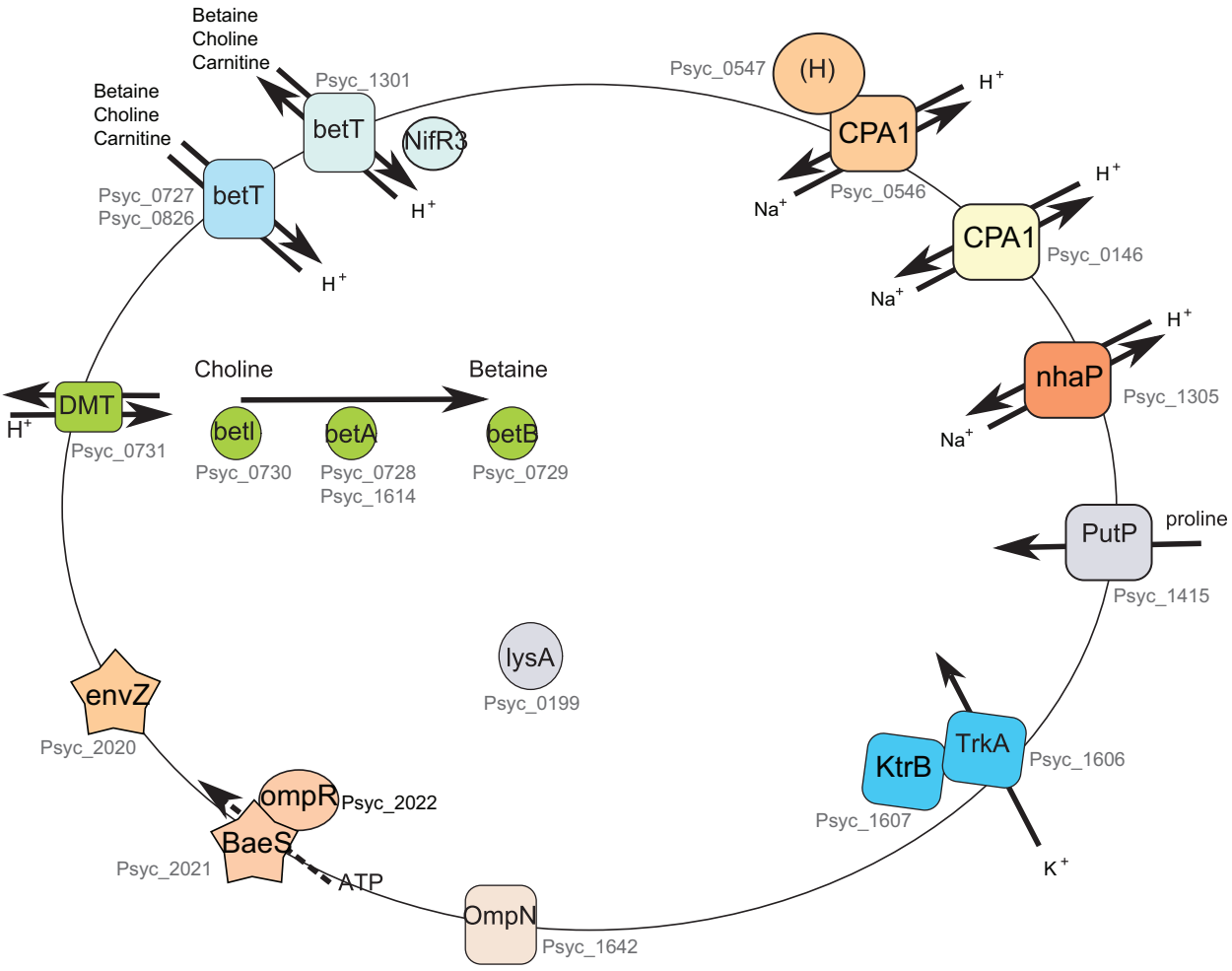

Figure E.4: Representation of a bacterial cell with candidate proteins involved in response to osmolarity up-shifts, based on candidate genes present in *Psychrobacter arcticus* genome. Symbols as in Fig. E.2.

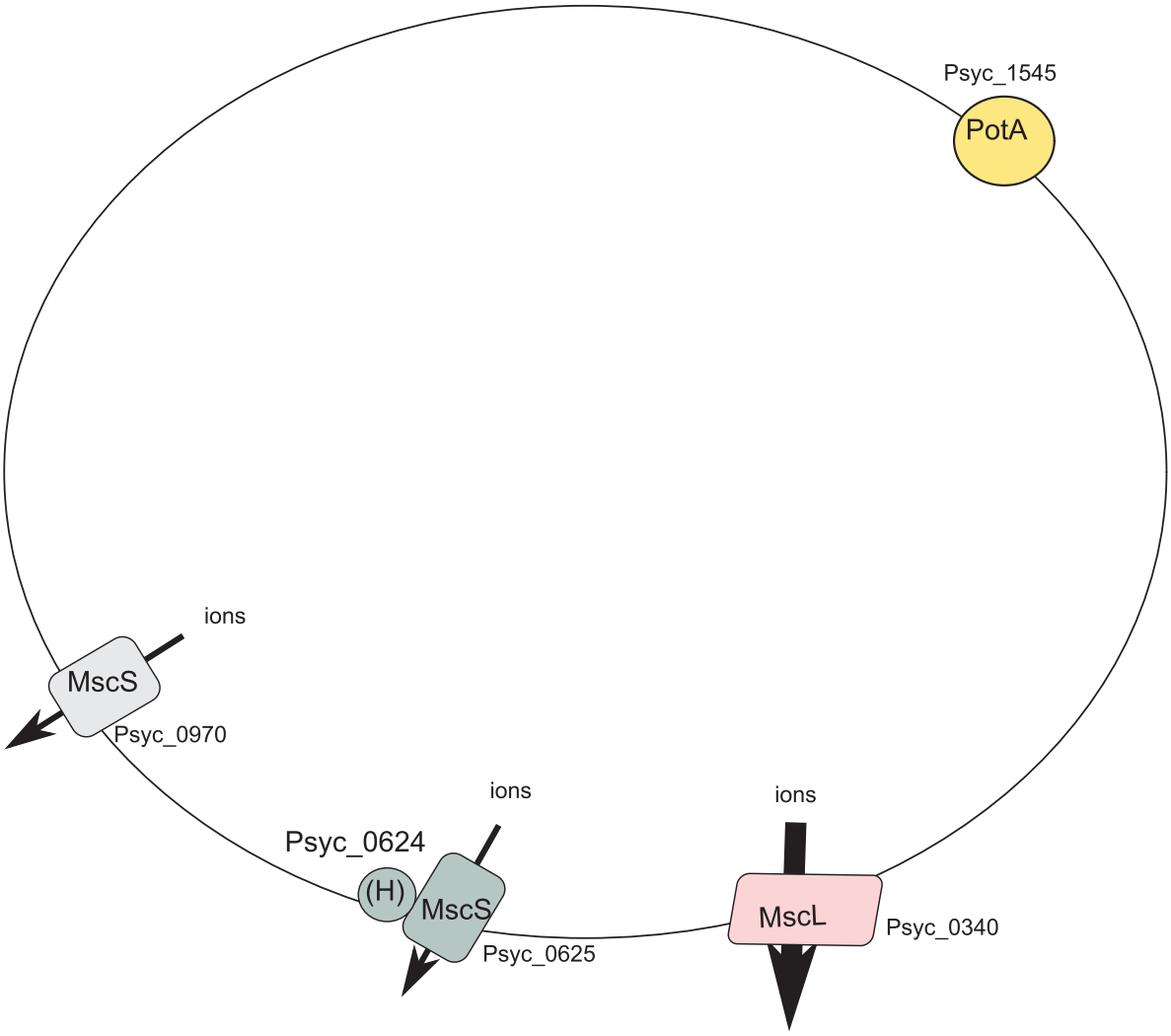

Figure E.5: Representation of a bacterial cell with candidate proteins involved in response to osmolarity down-shifts, based on candidate genes present in *Psychrobacter arcticus* genome. Symbols as in Fig. E.2.

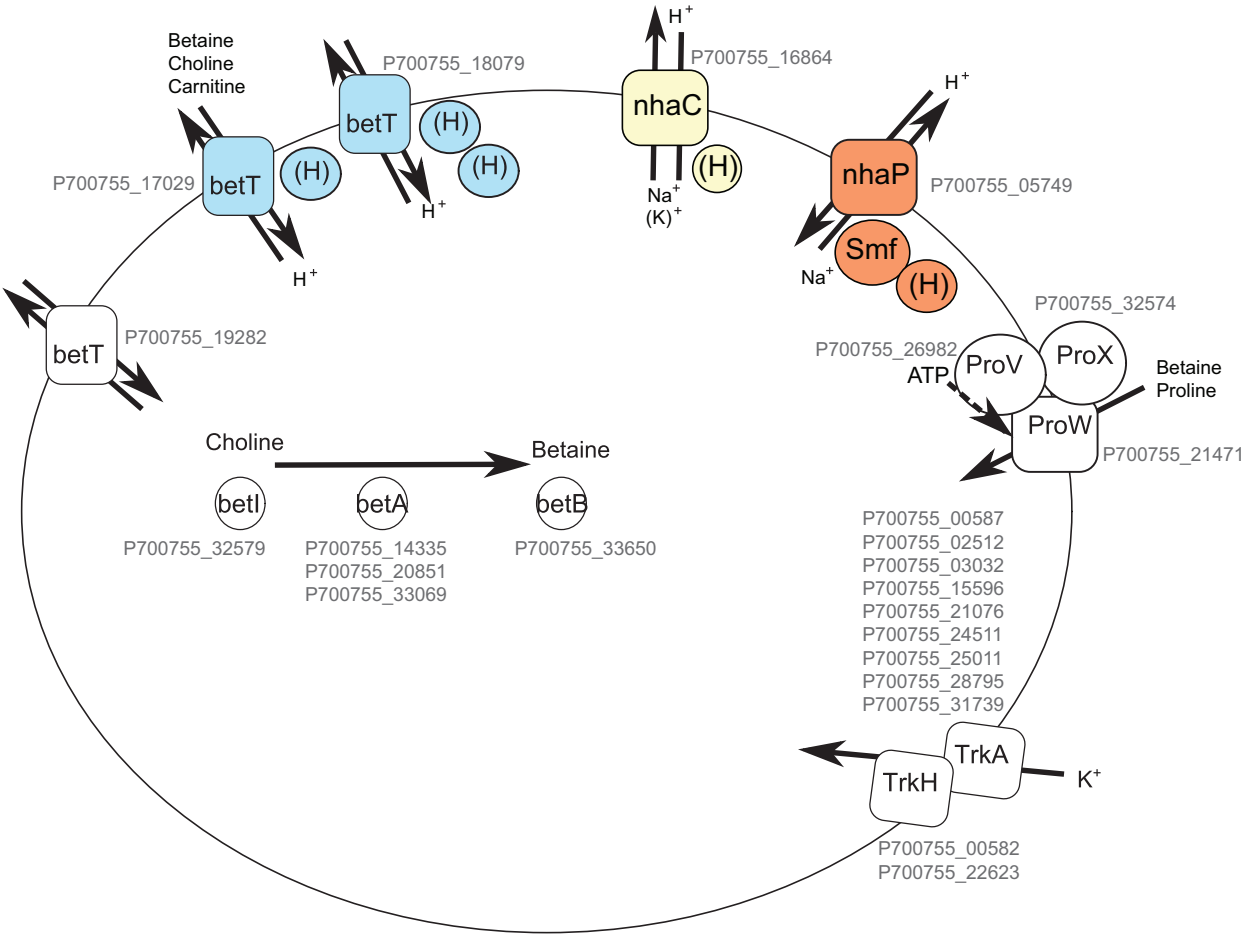

Figure E.6: Representation of a bacterial cell with candidate proteins involved in response to osmolarity up-shifts, based on candidate genes present in *Psychroflexus torquis* genome. Symbols as in Fig. E.2.

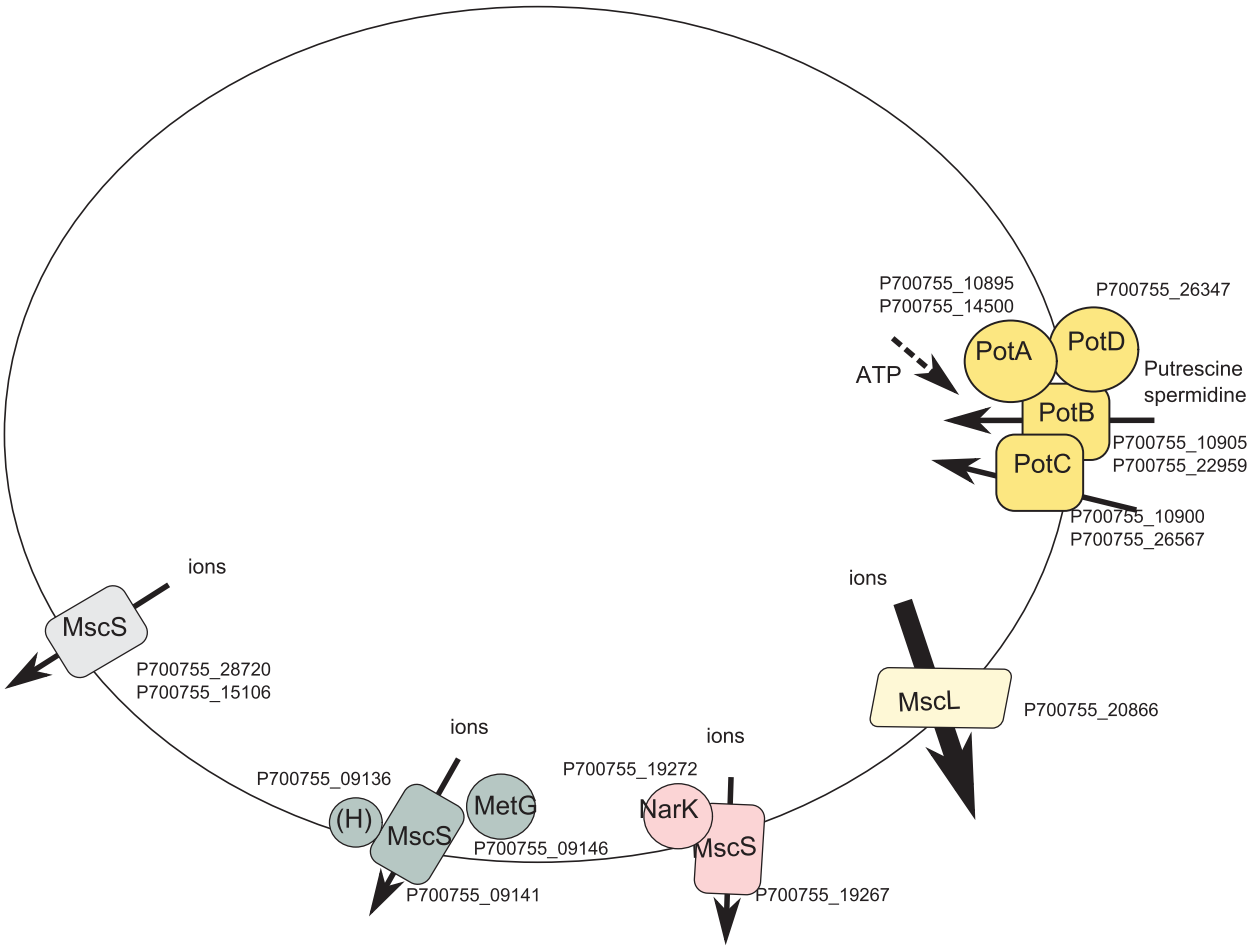

Figure E.7: Representation of a bacterial cell with candidate proteins involved in response to osmolarity down-shifts, based on candidate genes present in *Psychroflexus torquis* genome. Symbols as in Fig. E.2.

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Appendix F

Development of a public education and outreach activity linking Earth's cryosphere and icy planets in the search for life elsewhere

F.1 Introduction

Astrobiology topics, of interdisciplinary character, are effective tools for science education (e.g. *Oliveira and Barufaldi*, 2009; *Wells et al.*, 2007). The public education and outreach (EPO) activity described here links two Astrobiology topics: sea-ice ecosystems with the study of cryospheres (frozen regions of planets and moons). Two components, a web site and a table-top activity, were designed to encourage exploration, through astrobiology-relevant questions, of disciplines such as Microbiology, Polar Sciences, Geology, Astronomy and Planetary Sciences. Both components presented up-to-date scientific information, also illustrating aspects of the practice of scientific research, and the need of multidisciplinary efforts to answer astrobiological questions. The overall project was designed to be used by other scientists looking to reach public audiences in similar ways. Components were translated to Spanish to reach broader audiences. This appendix describes the development of the table-top activity, which was presented to general and K-12 audiences.

F.2 Methods

F.2.1 Table-top activity

The table-top activity was designed and implemented as part of the Portal to the Public program from the Pacific Science Center (PSC) (*Portal to the Public*, 2011). The process

involved in-depth exploration of the EPO-relevant aspects of the topic, as well as training in the delivery of scientific content to different audiences. The activity prototype was evaluated during one-on-one meetings with PSC staff, informal presentations to VPL members and a formal presentation to the Discovery Corps youth (a group of experienced PSC volunteers). Appendixes G (in English) and H (in Spanish) include a step-by-step description of the construction of the activity.

F.3 Results

F.3.1 Below freezing: a table-top activity

The design and visual aid content for the activity (one full-size poster and two additional small posters) were successfully completed and translated to Spanish. The activity was presented at four consecutive Polar Science Weekends (2010 – 2013) for a total of 80 h on the museum floor, bringing together the topics of Astrobiology and Polar Sciences in a public venue. In spite of not having precise estimates of the number of people actually participating in the activity, statistics from PSC show that Polar Science Weekends are well attended events, with a final attendance (4 d) of 6,918 participants in 2011 and 9,156 in 2012. Knowledge required to perform the activity is easily transferable to other scientists; the activity was used by other two Astrobiology students, Jesse Colangelo-Lillis and Evan Firth, during three of the four Polar Science Weekends.

PSC also offered the opportunity to present the activity at their Scientist Spotlight and Life Sciences Research Weekend. The portability of the activity allowed to transport it to rural areas and successfully present it to broader audiences at Toppenish (WA) in an event coordinated by Washington NASA Space Grant and the Yakama Nation. A case summary of this activity was sent for inclusion in the "Public Engagement with Science Catalog" compiled by the Museum of Science, Boston (*Ewert*, 2012).

Qualitative assessment of the activity indicated public interest and engagement, with fre-

quent questions and willingness to explore different topics ranging from microbiology to astronomy. Given that the starting point of the activity was sea ice and the degree of participation was voluntary, not all participants engaged in discussions of planetary sciences; many of them, however, inquired about topics related to climate change.

F.4 Conclusions

The development and implementation of a table-top activity addressing the study of cryospheres was successful, presenting to the public a variety of Astrobiology topics. The website (not currently active) provided a good working ground to evaluate relationships among Astrobiology topics. The support network was a key factor in the completion of the projects. Completion of the table-top activity was fully achieved due to the strong support network of PSC personnel, in particular Lauren Burman, Lauren Russell, Dana Vukajlovich y Stephanie Fitzwater provided extensive training. The PSC gave multiple opportunities to host the activity.

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Appendix G

BELOW ZERO: LOOKING FOR MICROBES IN THE COLDEST PLACES [1]

Abstract

The construction of hands-on activities is a valuable tool for scientists interested in public education and outreach. This manuscript describes my experience designing, building and presenting a hands-on activity for small groups of k-9 children. The activity, focused in my research on polar environments, presents key elements of polar microbiology, evokes expeditions to remote regions and questions the possibility of extraterrestrial life, bringing up characteristic elements of scientific endeavor. Below are described the objectives of this hands-on activity, a framework to introduce the topic, detailed instructions to build the activity, and a description of my experiences during the implementation phase, which took place in collaboration with Pacific Science Center (Seattle, U.S.A).

G.1 Objectives

- Present, in a playful and approachable way, concepts related to life in extreme environments polar microbiology and astrobiology.
- Convey a key result of polar microbiology: sea ice contains more organisms than glacial ice on land.
- Allow children to experience aspects of the scientific endeavor, including hypothesis

[1] A Spanish version of this Appendix is available in Appendix H.

formulation, comparison of results, use of replicas and negative results.

- Eliminate communication barriers between scientists and public, sharing mutual concerns, questions and answers.

- Offer to the public an interactive and memorable experience during their visit to the museum by means of one-to-one conversations.

- Stimulate children imagination by encouraging them to take the role of field explorers and lab researchers.

- Offer a conceptual framework to discuss the environmental aspects related to climate change.

G.2 Theoretical framework

Air temperature above the Arctic Ocean is so low that seawater freezes. During winter time, when temperatures reach lows of 50 celcius degrees below zero (–50 °C, –58 °F), sea ice extends from coast to coast in this ocean. Sea ice, unlike fresh-water ice like the one on mountain tops, is porous, permeated by minute channels filled with brine. Brine is an aqueous solution with high concentrations of salt, which remains liquid at temperatures as low as –40 °C and hosts sea salts, organic matter and microbes. Microbes inhabiting this brine channels are adapted to survive extreme conditions of low temperature and high salinity. Fresh-water ice, on the contrary, does not have brine channels, containing, thus, negligible amounts of liquid water, organic matter and microorganisms. This type of ice is characteristic of mountain glaciers.

The difference in microbial abundance between sea ice and fresh-water ice is a key concept to understand the role of each type of ice in the ecosystem. It is also a gateway to discuss topics related to microbial life, life in extreme environments, the Arctic ecosystem, and even the possibility of extraterrestrial life. The activity described below was developed through

the "Portal to the Public" program (*Portal to the Public*, 2011) from Pacific Science Center (PSC), aiming to involve participants in discovering a concept, imagining places never visited and facing aspects of scientific labor such as select appropriate experiments, making replicas and obtaining conclusions.

G.3 The activity

The facilitator starts the activity by looking for personal connections between the participants and the topic, asking questions such as: "which is the coldest place you have visited? What types of ice have you seen"? Likewise, questions like "Do you know what a microbe is? Do you think there are microbes in the ice"? are pertinent to determine if participants are familiar with basic concepts central to the activity. (Table G.1).

Children are then invited to play a game, where they are asked to take the role of polar scientists exploring the Arctic. The goals of the game are to collect ice from two environments: the Arctic Ocean (sea ice - saline) and a glacier in Greenland (glacial ice - fresh water), and then determine which one has more bacteria. Sampling sites are color coded in a map (Fig. G.1a). As the goals of the activity are explained, the following questions can be asked to the participants: "What types of clothes would you take to the Arctic? How would you get there"? If the activity takes place in an appropriate context, the use of costumes can also be implemented. Costumes can include field clothing such as gloves, boots, coats, parkas and goggles; likewise, laboratory coats can be made available to highlight the differences between laboratory and field work. In each place two samples are taken, corresponding to "replicas." Each sample is represented by a wooden stick painted with the same color coding as the map. To further increase the complexity of the activity it is possible to add one additional comparison, having available sea ice samples collected in two seasons, Winter and Summer.

After imagining what is appropriate clothing and transportation for a Polar expedition, participants choose a sample and wait for their imaginary return to the laboratory to analyze

it. This is a good moment to point out that the scientific endeavor is a long-term process, where many times it is not possible to know the properties of a sample until it has been returned to a laboratory for in-depth analysis. While samples are transported back to the laboratory, participants are invited to propose hypothesis through questions such as "Which sample do you think will have more microbes? Do you think all samples are going to be similar or different"? If there is no time constraint, an option is to keep a log book, either in a blackboard or notebook, with the development of the activity and the proposed hypothesis are written down. Upon their return to the laboratory, participants enact the process of sample analysis by inserting the wooding stick into a container with small steel balls or pellets (Fig. G.1b). Each wooden stick hides a given number of magnets (Fig. G.2a, b), thus the "sample analysis" will attach a different number of steel pellets representing different microbial abundances for each sampling site (Fig. G.1c). Once all samples have been analyzed, the results are compared with each other and with the proposed hypothesis. Participants are asked to explain the results, and if a log book is being kept, this is a good moment to stress the steps taken so far: asking a question, proposing a hypothesis, collecting samples, analyzing results and generating conclusions.

The activity is designed for sea ice samples to have more microbes than those from fresh-water ice. Higher microbial abundance in saline ice is due to the presence of brine pockets and channels, which offer a space available for microbial colonization. Furthermore, brine channels have liquid water which, in spite of high salinities, sustains these small organisms. Glacial ice, on the other hand, does not have enough space nor liquid water to sustain a high number of microbes.

If a freezer is available, differences between saline and fresh-water ice can be demonstrated by using home made ice cubes, prepared from water with and without salt, on top of which a few drops of food coloring are applied. Food coloring will make evident the higher porosity of the saline ice. This point of the activity is ideal to discuss the implications from the results, including the amazing adaptations from extremophile microbes and, depending on the available time, invite participants to consider a broader range of topics including polar

sciences, climate change and astrobiology (Table G.2).

This activity is designed in such a way that, during sample processing and discussion of results, participants will also face challenges commonly found in the process of scientific discovery (Table G.3). For instance, replicate samples are never identical, and one of the samples from glacial ice does not have any microbe (e.g. Fig. G.1c).

G.4 Instructions for building the activity

G.4.1 Materials

- Printed map
- Wooden sticks for the "samples" (at least two for each sampling site)
- Acrylic paint of different colors (a different color for each sampling site)
- Small, flat magnets
- Silicon (or similar adhesive)
- Cardboard
- Fabric tape
- Printed map
- Jar (wide mouth)
- Small steel balls or pellets (bb's)
- Tray for placing the samples

G.4.2 Building the activity

A map of the Arctic is printed with color coded points highlighting the geographical location of the sampling sites, as well as a well known reference point: the North Pole. Colors must be easily distinguishable and accessible for color-blind participants (color blind simulators are available online to evaluate the accessibility of digital images).

Samples are built with wooden sticks painted with acrylic paints according to the color coding of the sampling sites in the map. Each sampling site has two samples, represented replicate measurements. Microbial abundance is defined by transforming, in a scale of one to five, real data on microbial abundance from corresponding sampling sites (Table G.4). Based in this transformation, one to five magnets are glued to the lower part of the wooden stick (Fig. G.2.a). In the upper part the wooden stick is labeled with the information of the sampling site (name and number) as well as additional information about the sample type (salinity, season and temperature).

To represent the concept of heterogeneity in nature, magnets are distributed in such a way that replicas are not identical. Once the magnets are in place, the lower section is covered by a piece of cardboard and fabric tape to homogenize the appearance of all samples independent of the number of magnets present (Fig. G.2.b). This homogeneization contributes to simulate the expectation generated in scientific research when possible results from an experiment are unknown until all data are gathered and analyzed. At this point in the construction process, it should be verified that the magnets can effectively pick up the steel pellets.

Once samples are built, steel pellets are placed in a wide-mouth jar, lid-covered for transport. The jar is filled such that, after introducing a sample, the steel pellets completely cover the area with the magnets. As the activity is developed samples are placed in a tray and, at the end, pellets are returned to the jar (Fig. G.1b). To illustrate additional concepts it is possible to prepare a poster on the Arctic region and the sea-ice ecosystem.

G.5 Experiences at Pacific Science Center

This activity has been presented in multiple opportunities at Pacific Science Center (PSC), for general public and school visits, for a total of 76 h of museum floor (Fig. G.3). The activity has also been presented at special events in rural areas of Washington state, which was possible due to its easy transport.

G.5.1 Preliminary conversation: connecting with the topic

Many visitors, when asked about their experiences in cold places, remembered trips to the mountain or winters with snow. Other participants, though, expressed doubts on the relevance of the activity for their group. For instance, would the activity provide new knowledge to visitors originally from places close to the Arctic? Or in the opposite case, would they be able to understand the activity in spite of never having seen snow before? In this cases it was necessary to confirm that the activity is flexible and can be accommodated to personal interest and background.

Some visitors, specially the younger ones or those in rural regions, did not know the meaning of the word "microbes." Other visitors were more likely to recognize alternative names such as "germ" or "bacteria." Differences in background knowledge highlight the relevance of a constant interaction with the public. For the goals of this activity it was enough to use the general concept of a microbe as a very small living being. It was useful to complement this practical definition by mentioning that there are many types of microbes: some cause disease, others help producing food (e.g. cheese), whereas others play important roles in the ecosystem. Likewise, it was relevant to mention the widespread planetary presence of microbes. These topics offer ample possibilities to connect with the every-day experiences of the participants (Table G.1).

G.5.2 Public reception of the activity

The activity was positively received, specially by k-9 children. Participants between 7 - 11 years old took the greatest advantage, enjoying the playful aspect of the activity, while at the same time proposing hypothesis, performing the experiment and analyzing the results. When children where asked to describe what clothes should they were in a polar expedition, or what transport should they use, laughter was frequent as their own expectations were compared with actual requirements. Children in this age group discussed concepts related to the need of replication (e.g. why not all samples from the same place are equal?) as well as negative results (why is there a sample with no microbes in it?). The activity was more successful when developed in small groups with one to eight participants. With some modifications, though, it could be adapted to present to bigger groups, for instance in classroom visits. Younger children focused in the playful aspect, trying to fish pellets out in a consistent way, surprising themselves with unexpected results (e.g. samples with too few or too many pellets) and worked with the concepts of "more" and "less."

Results were less consistent when the activity was presented to teenagers, showing interest in some occasions but not always. In cases where participants showed interest, it was possible to develop in depth concepts related with the topic of Astrobiology, as well as the need of replication in scientific experiments and the use of averages to present scientific results. Adults showed interest in the general topic, but rarely on the activity itself. Images in the poster caught their attention, as well as the opportunity to talk about polar microbiology and climate change.

G.5.3 Final thoughts

This activity achieved the goals of designing and constructing a low-cost, hands-on activity, aiming to illustrate a key concept of polar sciences incorporating at the same time characteristics of the scientific endeavor. The resulting activity is easy to transport and has been

presented at PSC and in school visits to rural areas. With the current design it is necessary to have a facilitator in charge of the activity, but it has the potential to be transformed into a stand-alone activity for a museum setting.

Flexibility during the presentation of the activity was key to maintain participants interested, answering to particular needs and maximizing the learning experience. Even though there were common elements, each interaction was different. The poster, with images of the Arctic, was useful to guide conversations toward polar sciences and astrobiology, increasing the time participants spent interacting at the activity.

This activity can be easily adapted to other contexts sharing a similar goal of ilustrate scientific contexts based in quantitative differences among factors. For instance, microbial abundance in tropical lakes, air or soil; contaminant levels in aquatic environments; number of copies of a gene in different species, etc. Likewise, if materials are available, students could be assigned as a science project to construct a replicate of this activity to illustrate a concept of interest.

G.6 Acknowledgments

I want to acknowledge staff from the Portal to the Public program of Pacific Science Center: Lauren Burman, Lauren Russell, Dana Vukajlovich and Stephanie Fitzwater. The Virtual Planetary Laboratory and the Applied Physics Laboratory provided funding for this project. My advisor, Deming, reviewed the activity proposal and supported its presentation at PSC. Ángela Perez reviewed the manuscript and made valuable suggestions to improve it.

Table G.1: Concepts related to the sea-ice microbial life and example questions for different age groups. The selection of topics from this and the following tables should fit the group age, personal interest and previous knowledge of participants.

Topic	Possible questions / comments
What are microbes	Have you ever heard the word "microbe"? The word "germ"? The word "bacteria"? How big is it a microbe? Is it smaller or bigger than your finger? Can you see microbes directly with your eyes? When was it the first time that you heard the word "microbe" or "bacteria"? Have you ever had to explain your son/daughter what a microbe is?
Microbial distribution	Where do you think that microbes can be found? Tell me a place and I will tell you if it has microbes. Do you think there are places on Earth withouth microbes?
Functions	Do you know what do microbes do? Can microbes cause disease? How can a microbe in the soil be beneficial for a plant? Can you imagine a microbe recycling nutrients? Do you like yogurt? or cheese? both are made with the help of microbes! Do you know the name of a microbe? Have you heard any news related to microbes?

Table G.2: Additional concepts in polar sciences, climate change and astrobiology.

Topic	Possible questions / comments
Polar sciences	Where can you find ice? What is it ice made of? Do all water bodies on the planet contain the same type of water? Have you ever gone to the ocean? How can you get to the Arctic? Have you heard recently about the Arctic on the news? Do you know what do polar bears eat?
Climate change	Have you heard about climate change? Sea ice cover is quickly changing in the Arctic.
Astrobiology	If you wanted to go to a place colder than the Arctic or Antarctica, where would you go? Can there be life outside planet Earth? Where? How can extraterrestrial life be detected? If a space ship left today for Europa (Jupiter's moon), how old would you be when the spaceship arrives to its destiny? Have you ever thought about the problem of interplanetary contamination?

Table G.3: Topics related to the practice of scientific research.

Topic	Possible questions / comments
Exploration and field work	Have you heard about the North Pole? If you were on an Arctic expedition... what clothes would you wear? How can you get from here to the Arctic? What transportation mean would you use? How could you collect sea-ice samples?
Laboratory work	Microbes are so small, that you can't know how many of them are until you analyze the samples in the laboratory! To answer interesting questions, you can compare samples from different places or taken in different seasons.
	Why do samples from the same sampling site differ? Why do you need replicas? What does "average" mean?
Negative results	Why there are no microbes in some samples? Perhaps the method does not work if there are too few microbes! (detection limits) Could the experiment have failed? What can you do to prove that a place has no microbes at all?

Table G.4: Estimates of microbial abundance in different ice types and its equivalent in number of magnets per sample.

Sample type	Estimated microbial abundance (cells mL^{-1}	Equivalent in number of magnets per sample
Sea ice (winter)	$1 \times 10^4 - 1 \times 10^5$	2 – 3
Sea ice (summer)	$1 \times 10^6 - 1 \times 10^7$	4 – 5
Glacial ice	$1 - 1 \times 10^3$	0 – 1

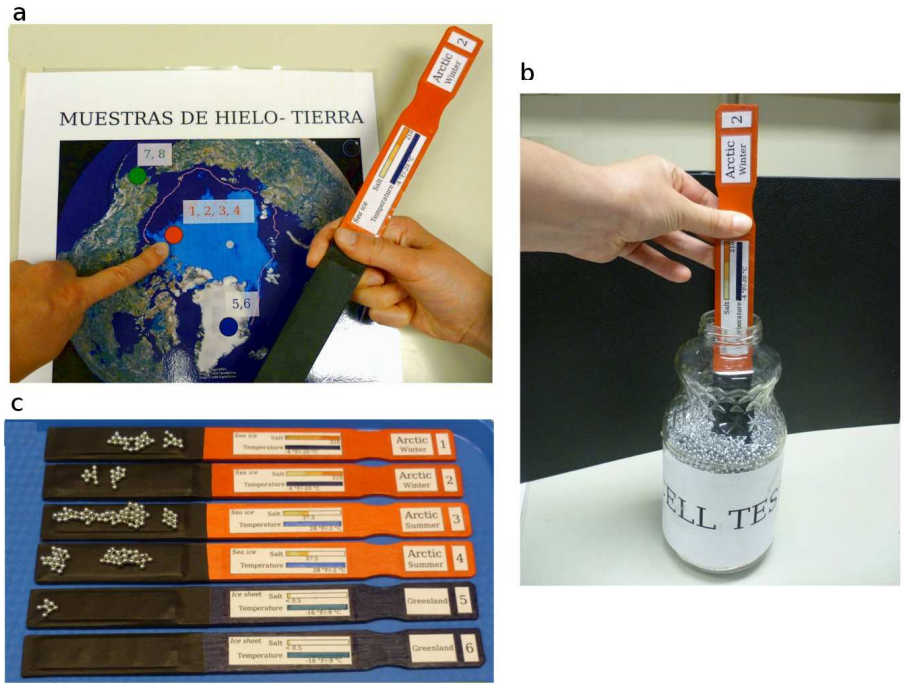

Figure G.1: Activity development. a) Sampling site selection; b) determining bacterial abundance; c) results according to sample type (from top to bottom): 1, 2 sea ice (Winter); 3, 4 sea ice (Summer); 5, 6 glacial ice.

Figure G.2: Materials for the activity. a) Placing the magnets. b) Fabric tape finish.

Figure G.3: Activity demonstration at Pacific Science Center

Bibliography

Portal to the Public (2011), *Implementation Manual*, Pacific Science Center, Explora, North Museum of Natural History, Institute for Learning Innovation.

Appendix H

Bajo Cero; buscando microbios en los lugares más fríos: construcción de una actividad lúdica para la presentación en museos de ciencias[1]

Resumen

La construcción de actividades interactivas es una herramienta valiosa para científicos interesados en la divulgación científica. En este artículo describo mi experiencia diseñando, construyendo y presentando una actividad interactiva para grupos pequeños de niños y niñas en edad escolar. La actividad, centrada en mi trabajo sobre microbiología de ambientes polares, presenta aspectos claves de la microbiología polar, evoca expediciones a regiones remotas, cuestiona las posibilidades de vida extraterrestre y enfrenta aspectos característicos del quehacer científico. A continuación se presentan los objetivos de la actividad, un marco teórico sobre el tema, instrucciones detalladas para construir la misma, y finalmente, una descripción de las experiencias durante la implementación de la actividad, que fue llevada a cabo en colaboración con el Centro de Ciencias del Pacífico (Seattle, E.E.U.U.).

H.1 Objetivos

- Presentar, de manera lúdica, conceptos relacionados con la vida en ambientes extremos, la microbiología polar y la astrobiología.

- Transmitir un resultado clave de la microbiología polar: el hielo marino contiene más organismos que el hielo glacial en los continentes.

[1]This Appendix describes the construction and implementation of an outreach and public education activity as described in Appendix F. An English translation is found on Appendix G

- Permitir que niños y niñas experimenten el quehacer científico a través de la formulación de hipótesis, la comparación de resultados, la utilización de réplicas y la ocurrencia de resultados negativos.

- Eliminar barreras de comunicación entre el científico y el público, compartiendo inquietudes, preguntas y respuestas.

- A través de la conversación directa, ofrecer un aspecto interactivo y memorable de la visita al museo.

- Estimular la imaginación de los niños y niñas, animándolos a representar el rol de exploradores en el campo e investigadores en el laboratorio.

- Ofrecer un soporte conceptual para discutir aspectos ambientales relacionados con el cambio climático.

H.2 Marco teórico

El Océano Ártico es un lugar tan frío que el agua salada del mar se congela. En invierno, cuando las temperaturas alcanzan los 50 grados centígrados bajo cero (–50 °C), el hielo marino cubre este océano de costa a costa. A diferencia del hielo de agua dulce, como el que se encuentra en los picos de las montañas, el hielo marino es poroso y está atravesado por diminutos canales llenos de salmuera. La salmuera es una solución acuosa con altas concentraciones de sal, que permanece líquida hasta los – 40 °C y alberga sales marinas, materia orgánica y microbios. Estos microbios que habitan los canales de salmuera están adaptados para sobrevivir condiciones extremas de baja temperatura y alta salinidad.

El hielo de agua dulce, al contrario, no posee bolsillos o canales de salmuera. Como consecuencia, contiene una cantidad ínfima de agua líquida en su interior, tiene muy poca materia orgánica y alberga un número mínimo de organismos. Este tipo de hielo es característico de los glaciales en las montañas.

Esta diferencia entre la abundancia de microbios en el hielo marino y el de agua dulce es un concepto esencial para comprender el papel de cada tipo de hielo en el ecosistema. Es, a la vez, un punto de entrada para discutir temas relacionados con la vida microbiana, los organismos extremos, el ecosistema del Ártico e, incluso, la posibilidad de vida extraterrestre. La actividad que se presenta a continuación fue desarrollada a través del programa "Portal al Público" (*Portal to the Public*, 2011) del Centro de Ciencias del Pacífico, buscando involucrar al participante en el descubrimiento de un concepto; imaginar sitios no visitados y enfrentar aspectos de la labor científica como seleccionar experimentos, hacer replicas y obtener conclusiones.

H.3 La actividad

El primer paso consiste en generar conexiones personales de los participantes con el tema, por ejemplo a través de preguntas como ¿Cuál es el lugar más frío en el que has estado? ¿qué tipos de hielo haz visto? Igualmente es oportuno indagar si los participantes están familiarizados con conceptos básicos que se usarán en la actividad tales como el de "microbios" a través de preguntas como ¿Sabes qué es un microbio? ¿Crees que en el hielo puede haber microbios? ¿Si es así, por qué? (Tabla H.1).

El juego como tal inicia invitando a los participantes a imaginar que son científicos polares en un viaje de exploración en el Ártico. Se explica entonces los objetivos del juego: colectar hielo en dos ambientes: el Océano Ártico (hielo salado) y un glaciar en Groenlandia (hielo de agua dulce o glacial), y determinar cuál tiene más bacterias. Los sitios de muestreo se indican en un mapa siguiendo un código de color (Fig. H.1a). A medida que se explica la actividad, se pregunta a los participantes ¿Qué clase de ropa llevarías al Ártico? ¿Cómo llegarías hasta allá? Si el contexto de la actividad lo permite, se puede implementar el uso de disfraces, como guantes, botas, abrigos, parkas, gafas de esquí y otro equipo de campo usado en lugares de baja temperatura; igualmente, se pueden tener disponibles batas blancas de laboratorio, resaltando las diferencias entre el trabajo de campo y el de laboratorio. En cada sitio se toman dos muestras, lo que en términos científicos corresponde a "réplicas". Cada una de las

cuatro muestras disponibles está representada por un palo de balso pintado según el código de color del mapa. En caso de querer aumentar la complejidad de la actividad, se puede añadir un objetivo adicional: comparar el hielo marino que se colecta en dos estaciones: invierno y verano.

Después de imaginar qué clase de ropa y medio de transporte se debe usar en una expedición Polar, los participantes escogen una muestra, y esperan su regreso al laboratorio para analizarla. Este es un buen momento para señalar que la actividad científica es un proceso laborioso, donde muchas veces es imposible conocer las características de una muestra hasta que ésta ha sido transportada y analizada en el laboratorio. Mientras se transportan las muestras de regreso se invita a los participantes a crear hipótesis por medio de las preguntas ¿Cuál muestra tendrá más microbios? ¿Será que las muestras son iguales o distintas? De haber suficiente tiempo, se puede llevar una bitácora, anotando las hipótesis formuladas en un tablero o en una hoja de papel. Una vez de regreso en el laboratorio, los participantes simulan el análisis de la muestra colocando el palo de balso en un recipiente con balines (Fig. H.1b). Dado que los palos de balso esconden un número específico de imanes (Fig. H.2a, b), el "análisis" revelará una cantidad de balines que representa la abundancia microbiana en el sitio de muestreo (Fig. H.1c). Cuando se han analizado todas las muestras, se pasa a comparar las muestras entre sí y con las hipótesis planteadas. A continuación se fomenta la generación de explicaciones de los resultados obtenidos. Si se está llevando una bitácora, se pueden escribir los resultados y enfatizar los pasos realizados: pregunta, hipótesis, muestreo, análisis de resultados y conclusiones.

La actividad está diseñada para que las muestras de hielo salado tengan más microbios que las de hielo de agua dulce. Esta diferencia se debe a que los poros y canales de salmuera del hielo salado ofrecen un amplio espacio para ser colonizado por los microbios. Al mismo tiempo, los canales de salmuera ofrecen agua líquida, la cual, a pesar de ser muy salada, abastece las necesidades de estos diminutos organismos. El hielo glacial, por el contrario, no ofrece suficiente espacio y tiene muy poca agua líquida para mantener vida microbiana en su interior. Si se dispone de una nevera, las diferencias entre hielo salado y de agua dulce

se pueden demostrar con cubos de hielo de fabricación casera (con y sin sal) a través de la aplicación de unas gotas de colorante de comida en la parte superior del hielo. Este punto es ideal para entablar conversaciones acerca de las implicaciones de los resultados, incluyendo las increíbles adaptaciones de los microbios y, dependiendo del tiempo disponible, invitar a los participantes a discutir un rango más amplio de temas, incluyendo, Ciencias Polares, el Cambio Climático Global y la Astrobiología (Tabla H.2).

Esta actividad está diseñada para que, en el proceso de analizar las muestras y los resultados, los participantes también enfrenten dificultades características del descubrimiento científico (Tabla H.3). Por ejemplo, las réplicas nunca son idénticas y una de las muestras de hielo glacial no tiene ningún microbio (ej. Fig. H.1c).

H.4 Instrucciones para la construcción de la actividad

H.4.1 Materiales

- Mapa impreso
- Palos de balso para las "muestras" (al menos dos por cada sitio de muestreo)
- Pintura acrílica de diferentes colores (un color diferente para cada sitio de muestreo)
- Imanes pequeños planos
- Silicona (o adhesivo similar)
- Cartón
- Cinta de tela
- Mapa impreso

- Frasco de boca ancha
- Balines
- Bandeja para colocar las muestras

H.4.2 Construcción de la actividad

Se imprime un mapa del Ártico donde se ha señalado con colores la ubicación geográfica de los sitios de muestreo y un punto de referencia conocido: el Polo Norte. Los colores deben ser fáciles de distinguir y accesibles para personas con daltonismo (en la red se encuentran programas de simulación "color blind simulators" para evaluar la accesibilidad de imágenes digitales).

Las muestras se construyen con palos de balso pintados con vinilo según el código de color del mapa. Para cada sitio se designan dos muestras, representando "réplicas" de una misma medida. La abundancia microbiana se define transformando, a una escala de 1 a 5, datos reales de abundancia microbiana en cada sitio de muestreo (Tabla H.4). Con base en esta transformación, se pegan con silicona de 1 a 5 imanes en la parte inferior del palo de balso (Fig. H.2.a). En la parte superior se coloca una etiqueta con la información del "sitio de muestreo" (nombre y número) e información adicional sobre las muestras (salinidad, estación y temperatura).

Para representar el concepto de heterogeneidad en la naturaleza, los imanes se distribuyen de forma que las réplicas no sean idénticas. Una vez colocados los imanes, la sección se cubre con un rectángulo de cartón y cinta de tela para disminuir las diferencias visuales entre muestras (Fig. H.2.b). Esto también simula la expectativa que se genera en la investigación científica al desconocer los posibles resultados de un experimento. En este punto se verifica que los imanes puedan atraer los balines efectivamente.

Una vez terminadas las muestras, se colocan los balines en una jarra de boca ancha que se

pueda tapar para el transporte. La jarra se llena hasta una altura tal que al introducir una muestra los imanes queden totalmente cubiertos. Las muestras se colocan en una bandeja y, al terminar, los balines se regresan a la jarra (Fig. H.1b). Para ilustrar conceptos adicionales, se puede preparar un póster con fotos del Ártico, hielo polar y los organismos que lo habitan.

H.5 Experiencias en el Centro de Ciencias del Pacífico (PSC)

La actividad ha sido presentada en múltiple oportunidades en el Centro de Ciencias del Pacífico (PSC por sus siglas en inglés), atendiendo visitas tanto del público general como de colegios, para un total de 76 horas de presentación al público (Fig. H.3). También ha sido presentada en eventos especiales en zonas rurales del estado de Washington, lo cual fue posible dada su facilidad de transporte.

H.5.1 Conversación preliminar: generando nexos con el tema

Muchos visitantes, al preguntar sobre sus experiencias en lugares fríos, recordaron paseos a la montaña o su experiencia con la nieve. Otros participantes, sin embargo, expresaron dudas sobre la pertinencia de la actividad para su grupo. Por ejemplo, podría la actividad aportar algo nuevo para ellos, que crecieron en un sitio muy cerca al Ártico? O en el caso contrario, podrían entender la actividad a pesar de nunca haber visto nieve? En estos casos fue importante confirmar que la actividad es flexible y se puede acomodar a distintas experiencias personales.

En cuanto al tema de los "microbios", algunos visitantes, en especial los más pequeños o aquellos en regiones rurales, nunca habían escuchado la palabra. Otros visitantes reconocían más fácilmente palabras como "germen" o "bacteria". Esto resalta la importancia de una constante interacción con el público. Para los objetivos de esta actividad fue suficiente utilizar el concepto general de microbio como un ser vivo muy pequeño. Resultó también útil mencionar que hay muchos tipos de microbios: algunos causan enfermedades, otros ayudan

a producir alimentos como el queso, mientras que otros cumplen funciones importantes en el ecosistema. Igualmente, es clave mencionar que los microbios tienen una amplia distribución en el planeta. En este punto hay muchas posibilidades para conectar dichos conceptos con la experiencia cotidiana del participante (Tabla H.1).

H.5.2 Recepción de la actividad

La actividad fue bien recibida, en especial por niños y niñas en edad escolar. Los participantes que más aprovecharon la actividad fueron aquellos entre 7 y 11 años quienes, a la vez que disfrutaron el aspecto lúdico, se plantearon hipótesis, realizaron el experimento y analizaron los datos. Al pedir a los niños y niñas que describieran qué ropa usarían para llegar al Polo, o qué medios de transporte, las risas eran frecuentes cuando se comparaban sus expectativas con las experiencias de científicos reales. Los niños y niñas en este grupo de edad discutieron conceptos relacionados con la necesidad de tomar réplicas (¿por qué no todas las muestras de un mismo sitio son iguales?) y los resultados negativos (¿por qué una de las muestras no tiene ningún microbio?). La actividad resultó más exitosa al trabajar en grupos pequeños, entre 1 y 8 participantes, pero, con ciertas modificaciones, podría adaptarse a grupos más grandes para la presentación en colegios. Los niños más pequeños se enfocaron en el aspecto lúdico, intentando sacar balines de manera consistente, sorprendiéndose con resultados inesperados (e.j. resultados negativos o las muestras con mayor cantidad de balines) y trabajaron los conceptos de “más” y “menos”.

Los resultados fueron menos consistentes al presentar la actividad a adolescentes, despertando interés en ciertas ocasiones, pero no en todas. En casos dónde se despertó interés, fue posible desarrollar con mayor profundidad los conceptos relacionados con la necesidad de replicas en los experimentos científicos, el uso de promedios para presentar resultados científicos y el tema de la Astrobiología. La mayoría de adultos mostraba interés en el tema general, aunque no siempre en la actividad, siendo más atraídos por las imágenes visuales del póster, o por la simple oportunidad de conversar conceptos relacionados con microbiología

y cambio climático.

H.5.3 Reflexiones finales

Con esta actividad se lograron los objetivos de crear una actividad lúdica, de bajo costo, que permitiera ilustrar un concepto clave de las ciencias polares e incorporar, al mismo tiempo, aspectos relacionados con el quehacer científico. La actividad es fácil de transportar, y ha sido presentada en el PSC y en visitas a zonas rurales. Con el diseño actual es necesaria la presencia de un facilitador, pero en dado caso se podría convertir en una actividad autónoma para dejar de manera permanente en un museo.

La flexibilidad durante la presentación de la actividad fue clave para mantener el interés de los participantes, responder a sus necesidades particulares y maximizar el aprendizaje. Aunque hubo elementos comunes, todas las interacciones fueron diferentes. El póster resultó útil para mostrar imágenes del Ártico, y dirigir la conversación hacia temas de las ciencias polares y la Astrobiología, incrementando el tiempo de interacción de los participantes.

Esta actividad se puede adaptar fácilmente a otros contextos, donde el objetivo principal sea ilustrar conceptos científicos basados en diferencias cuantitativas entre factores. Por ejemplo, abundancia microbiana en ecosistemas tropicales, cantidad de contaminantes en un lago prístino vs. aguas de desecho, microbios en el aire vs. microbios en el suelo, número de copias de un gen entre una especie y otra, etc. Igualmente, en caso de tener acceso a los materiales, se puede asignar a estudiantes como proyecto de ciencias, bien sea en el salón de clase o en un club de ciencias, la construcción de una réplica de la actividad ilustrando algún concepto de interés.

H.6 Agradecimientos

Quiero agradecer al personal del Centro de Ciencias del Pacífico asociado programa Portal al Público: Lauren Burman, Lauren Russell, Dana Vukajlovich y Stephanie Fitzwater. Los

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Tabla H.1: Conceptos relacionados con la vida microscópica del hielo marino y ejemplos de preguntas para distintos grupos de edad, que permitan relacionar los conceptos preliminares con experiencias cotidianas del participante.

Tema	Posibles preguntas/ comentarios
Qué son los microbios	¿Has oído alguna vez la palabra "microbio"? La palabra "germen"? La palabra "bacteria"? ¿Qué tan grande es un microbio? Más grande o más pequeño que tu dedo? ¿Puedes verlos a simple vista? ¿Cuándo fue la primera vez que escuchaste la palabra microbio (o bacteria)? ¿Alguna vez has tenido que explicarle a tus hijos qué es un microbio?
Distribución microbiana	¿Dónde crees que hay microbios? Dime un lugar y yo te digo si tiene o no microbios. ¿Piensas que puede haber lugares en el planeta Tierra sin microbios?
Funciones	¿Sabes qué hacen los microbios? ¿Los microbios pueden causar enfermedades? ¿Cómo puede un microbio en el suelo ayudar a una planta? ¿Alguna vez te imaginaste a un microbio que recicla nutrientes? ¿Te gusta el yogur? ¿O el queso? ¡ambos necesitan la ayuda de microbios para su producción! ¿Te sabes el nombre de algún microbio? ¿Has escuchado alguna noticia relacionada con microbios?

Tabla H.2: Conceptos adicionales sobre Ciencias Polares, Cambio Climático y Astrobiología.

Tema	Posibles preguntas/ comentarios
Ciencias polares	¿Dónde puedes encontrar hielo? ¿De qué está formado el hielo? ¿Toda el agua que encuentras en el planeta es igual? ¿Has viajado al mar? ¿Como se puede llegar al Ártico? ¿Has escuchado hablar del Ártico en las noticias? ¿Sabes qué comen los osos polares?
Cambio climático	¿Has oído hablar sobre el cambio climático? La cobertura de hielo está cambiando rápidamente en el Ártico.
Astrobiología	Si quisieras ir a un lugar más frío que el Ártico o la Antártida, ¿a dónde irías? ¿Puede haber microbios fuera del planeta Tierra? ¿En qué lugares? ¿Cómo se puede detectar vida extraterrestre? Si hoy sale una nave espacial para Europa (la luna de Júpiter), ¿qué edad tendrías cuando la nave llegue a su destino? Has considerado alguna vez el problema de la contaminación interplanetaria?

Tabla H.3: Temas de conversación relacionados con la investigación científica. La selección de los temas de discusión sugeridos en esta y otras tablas, debe acomodarse al grupo de edad, interés y conocimiento previo de los participantes.

Tema	Posibles preguntas/ comentarios
Exploración y trabajo de campo	¿Has oído hablar del Polo Norte? Si fueras en una expedición al Ártico... ¿Qué clase de ropa utilizarías? ¿Cómo puedes llegar desde aquí hasta el Ártico? ¿Qué medio de transporte utilizarías? ¿Cómo se podrían colectar las muestras del hielo?
Experimentación	Los microbios son tan pequeños, que cuando estás tomando las muestras no puedes saber cuántos colectaste! Para obtener una respuesta más interesante, se pueden comparar muestras tomadas en distintos lugares o en distintas estaciones.
Replicas	¿Por qué las muestras de un mismo sitio no son idénticas? ¿Por qué se necesitan réplicas? ¿Qué es un "promedio"?
Resultados negativos	¿Por qué no aparecen microbios al analizar esta muestra? ¡Tal vez el método no funciona cuando hay muy pocos microbios! (límites de detección) ¿Tal vez el experimento falló? ¿Qué se puede hacer para comprobar que en realidad no hay microbios en ese lugar?

Tabla H.4: Estimativos de abundancia microbiana en distintos tipos de hielo y su equivalente en número de imanes por muestra.

Tipo de muestra	Abundancia microbiana estimada (células mL^{-1}	Equivalente en número de imanes por muestra
Hielo marino (invierno)	$1 \times 10^4 - 1 \times 10^5$	2 – 3
Hielo marino (verano)	$1 \times 10^6 - 1 \times 10^7$	4 – 5
Hielo glacial	$1 - 1 \times 10^3$	0 – 1

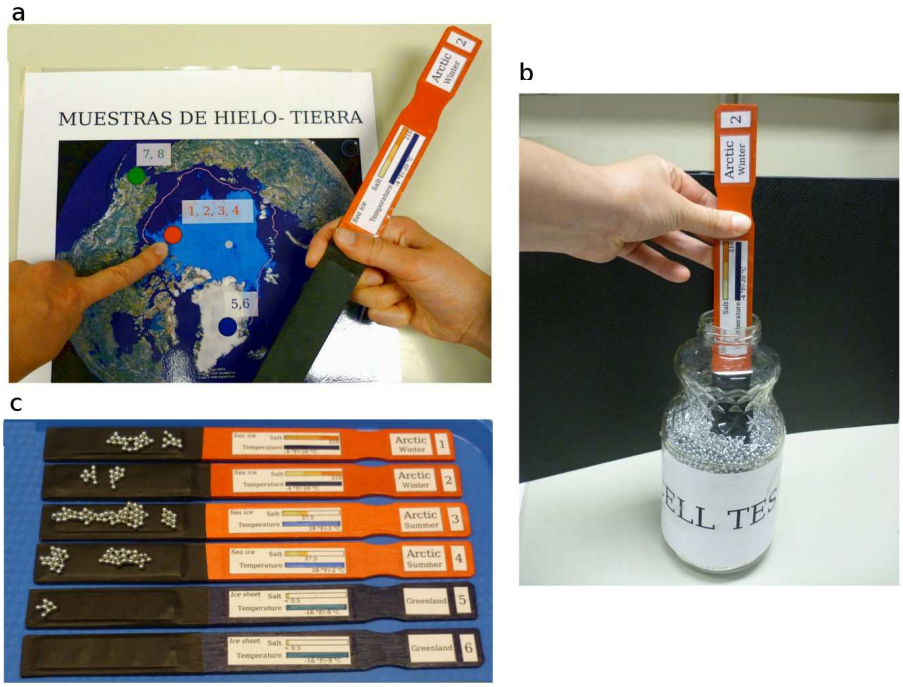

Figura H.1: Desarrollo de la actividad. a) Selección del sitio de muestreo; b) determinación de la abundancia de bacterias; c) resultados según muestra (en orden de arriba a abajo): 1, 2 hielo marino (invierno); 3, 4 hielo marino (verano); 5, 6 hielo glacial.

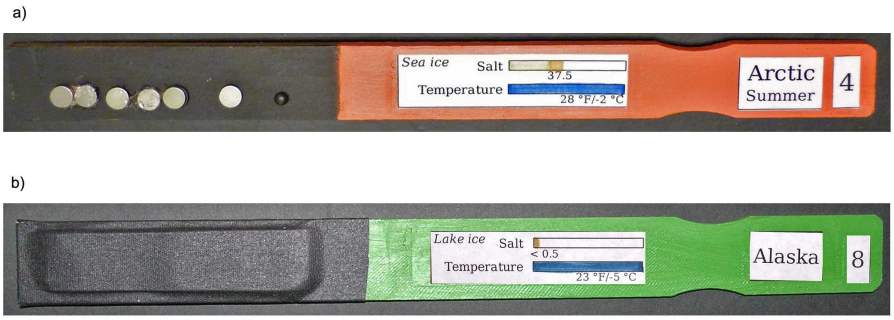

Figura H.2: Palitos en construcción. a) Colocación de los imanes. b) Acabado con cinta de tela.

Figura H.3: Ejemplos de la presentación de la actividad en el Centro de Ciencias del Pacífico

Bibliografía

Portal to the Public (2011), *Implementation Manual*, Pacific Science Center, Explora, North Museum of Natural History, Institute for Learning Innovation.

MARCELA EWERT SARMIENTO

UW-Oceanography, Box 357940, Seattle, WA 98195 • (206) 484-7646 • mewerts@uw.edu

EDUCATION

Ph.D. Biological Oceanography and Astrobiology, University of Washington, Summer 2013 (expected)
Microbial challenges and solutions to inhabiting the dynamic architecture of saline ice formations. Adviser: Dr. Jody W. Deming.

M. S. Biological Oceanography, University of Washington, 2009
Ice affinity of extracellular polysaccharides produced by Colwellia psychrerythraea *strain 34H.* Adviser: Dr. Jody W. Deming.

M. S. Astronomy, National University of Colombia, 2006
Approaching habitability in star forming regions: a study of their chemical properties. Adviser: Dr. Juan Manuel Tejeiro.

B. S. Biology, with Honors, National University of Colombia, 2003
The notion of life within the theories of life's origin. Adviser: M.S. Eugenio Andrade

TEACHING EXPERIENCE

Teaching Assistant, University of Washington
· **Deep Sea Exploration: Submarine Volcanoes and Novel Life Forms**. Winter 2013. Instructor: Dr. Deborah Kelley. Advised students on scientific-abstract writing and graded assignments. Science and non-science majors (100-level class).
· **Arctic Change**. Spring 2011. Instructor: Dr. Rebecca Woodgate. Gave selected lectures, designed and led activities and round-table discussions for weekly quiz session, and graded assignments. Science and non-science majors (100-level class).
· **Energy and the Environment: Life Under the Pale Sun**. Spring 2009. Instructor: Dr. Peter Rhines. Science and non-science majors. Gave lectures on Biology and Chemistry, advised on weekly journal and essay writing (200-level Honors class).
· **Lecturer** in UW-Bothell undergraduate (Ecology, BES 312) and UW-Oceanography graduate programs (Biological Oceanography, OCN 535).

Facilitator, University of Washington Annual TA/RA conference, 2010, 2011.
· Panelist on **International TA panel**. Prepared and led the workshops **Motivating your students** and **Activities to engage your students**.
· Facilitator at the School of Oceanography & Aquatic and Fishery Sciences TA orientation.

Mentor, University of Washington, 2009, 2011.

· Mentored junior graduate students from Applied Math, Microbiology and Oceanography. · Mentored a Discovery Corps high school student participating as a science intern in the laboratory.

Developer of evaluation instruments, National University of Colombia, 2005.

· Developed standardized evaluation instruments for natural sciences in 5^{th} and 9^{th} grades.

SCIENCE EDUCATION (INFORMAL SETTINGS)

Pacific Science Center Fellow, 2009 – 2013.

· Developed **hands-on activities** on Polar Sciences to present in Scientist Spotlight (2010-2012), Life Sciences Research Weekend (2009) and Polar Sciences Weekend (2009 – 2013) with more than **80 hours** on the museum floor.

· Contributed to fellows prototyping event.

· Panelist in the "Portal to the Public Synthesis Meeting: Innovations to Advance Public Engagement with Scientists in Informal Science Education (ISE) Institutions (2010").

· Featured in the Aug. 4^{th} 2011 PSC Newsletter (www.pacificsciencecenter.org/Articles/coolscientist.html).

Science Communicator, UW-School of Oceanography, 2006 – 2013

· Tens of hours in K-12 class visits, lab tours, job shadowing and science nights in Seattle (WA), Toppenish (WA), Barrow (AK) and Bogotá D.C. (Colombia).

· General public talks for FAMELAB (Denver, 2012); Saturday School Yard talk (Barrow, 2011); Queen Anne Science Cafe (Seattle, 2010); Diálogos KYEF (Bogotá D.C., 2009)

Astronomy Club Manager, Planetarium of Bogotá, 2005 – 2006

· Creation, development and management of Astronomy Clubs for children and teenagers.

· Developed hands-on activities and presented them in weekly meetings with K-12 students.

PROFESSIONAL SERVICE

· Assistant - local organizing committee, **Surface Ocean Lower Atmosphere Study, Open Science Conference (2012)**, Cle Elum, WA..

· Co-convener **European Geosciences Union 2012**, Session CR9.10: Sea ice physical and biological processes and interactions with climate.

· Organizing committee, Astrobiology Graduate Student Conference, **AbGradCon 2009**, an international conference hosting 67 Astrobiology students from 8 countries and 34 universities.

AWARDS AND FELLOWSHIPS

· Student Poster Awards

Surface Ocean Lower Atmosphere Study, SOLAS Conference (2012), Cle Elum, WA.

International Symposium on Sea Ice in the Physical and Biogeochemical System (2010; 2^{nd} prize), Trømso, Norway

Inuit Partnership of Excellence - Circumpolar Flaw Lead, All Hands Meeting (2009; special mention for effective science communication to Inuit laymen), Winnipeg, Canada

· Acceptance to the IPY International Sea-Ice Summer School (2007), Svalbard, Norway.
· Vatican Observatory Fellowship in Astrobiology (2006 – 2008).
· Acceptance to the Vatican Observatory Summer School (VOSS) in Observational Astronomy and Astrophysics (2005), Castel Gandolfo, Italy.
· Graduate School Scholarship for Science Students, National University of Colombia (2004).

FIELD EXPERIENCE

· **Winter 2010, 2011**. Two 9-day expeditions on coastal sea ice near **Barrow**, Alaska, from the Barrow Arctic Research Center.
Snow and surface ice sample collection and processing for EPS content, EPS size fractionation, bacterial abundance and Live/Dead analysis.

· **Winter 2008**. Three-week research cruise in the **Beaufort Sea** aboard the Canadian Coast Guard research icebreaker *Amundsen* (Circumpolar Flaw Lead expedition, during the International Polar Year).
Collected sea ice, brine, frost flowers and water samples for isolation of extremophile bacteria.

· **Fall 2006, 2008, 2010** Four-day UW-Astrobiology workshops at Yellowstone National Park (WY, MT, ID), the Mojave Desert Research Station (CA), and Easton Glacier on Mt. Baker (WA).

PUBLICATIONS

Miller, L.A., M. Gosselin, L.L. Sørensen, G.S. Dieckmann, A. Fransson, F. Fripiat, C.S. Garbe, C. Hughes, D. Lannuzel, J.-L. Tison, M.A. van Leeuwe, E.W. Wolff, **M. Ewert** and J. Bowman (2013) Methods for Biogeochemical Studies of Sea Ice: The State of the Art. *Elementa* (in preparation)

Carillo, S., G. Pieretti, E. Parrilli, A. Casillo, F. Sannino, **M. Ewert**, J.W. Deming, R. Lanzetta, M.A. Parrilli, M.L. Tutino and M.M. Corsaro (2013) The capsular polysaccharide of the obligate psychrophile marine bacterium *Colwellia psychrerythraea* strain 34H: Isolation and structural Characterization (in preparation)

Ewert, M. and J. W. Deming (2013). Sea ice microorganisms: Environmental constraints and extracellular responses. *Biology*, 2:603-628

Ewert, M., J. W. Deming, J. Collangelo-Lillis and S.D. Carpenter (2013). Marine microbial content of the brine-wetted snow cover over Arctic first-year sea ice. *J. Geophys. Res.*, doi:10.1002/jgrc.20055

Ewert, M. and J. W. Deming (2011). Selective retention in saline ice of extracellular polysaccharides produced by the cold-adapted marine bacterium *Colwellia psychrerythraea* strain 34H.*Ann. Glaciol.*, 52:111-117

Balbin, A., C. Chica and **M. Ewert** (2000) Building a setting for the origin and evolution of primordial proteic sequences (in Spanish) Acta Biológica Colombiana, Oct. pp. 49–50, Memorias II Encuentro Científico de Estudiantes de Biología

SELECTED PRESENTATIONS

Ewert, M. & J. W. Deming (2013) Compatible solutes promote survival of sea-ice bacteria under fluctuating-temperature regimes. Poster, Polar and Alpine Microbiology Conference, Big Sky, MN, 8–12 September.

Deming, J. W., J. S. Bowman, **M. Ewert** and R.E. Collins (2012) Microbial life at the boundary between sea ice and atmosphere. Invited presentation given by J. W. D., IPY 2012 Conference: From Knowledge to Action, Montreal, Quebec, 22–27 April.

Ewert, M., J. Colangelo-Lillis, S. D. Carpenter and Deming, J. W. (2012) Transport of marine microbes and polysaccharides from first-year sea ice into snow and implications for marine-atmospheric exchange. Poster, SOLAS Open Science Conference, Cle Elum, WA, 7–10 May.

Deming, J. W., **M. Ewert**, J. S. Bowman, J. Colangelo-Lillis and S. D. Carpenter (2010)

Brine-wetted snow on the surface of sea ice: a potentially vast and overlooked microbial habitat. Poster, AGU- Fall Meeting, San Francisco, CA, 13–17 December.

Deming, J. W., J. S. Bowman, **M. Ewert.** and R.E. Collins (2010) Airing the implications of finding microbes in frost flowers. Oral presentation by J. W. D., IPY Oslo Science Conference 2010, Oslo, Norway, 8–12 June.

Ewert, M. and J. W. Deming (2010) Selective retention of extracellular polysaccharides, produced by the cold-adapted marine bacterium *Colwellia psychrerythraea* strain 34H. Poster, International Symposium on Sea Ice in the Physical and Biogeochemical System, Trømso, Norway, 31 May–4 June.

Ewert, M. and J. W. Deming (2009) Cultivation of microbial extremophiles from winter sea ice and frost flowers. Poster, IPY - Circumpolar Flaw Lead System Workshop, Winnipeg, Canada, 1–4 November.

Anderson, R., **M. Ewert**, E. Stueeken and OoL Seminar 2009 (2009) The World-Wide-Web Origin of Life: using a collaborative website as a tool to develop an integrative model for the origin of life. Poster, AbGradCon 2009, Seattle, WA, USA.

Ewert, M. & Deming, J. W. (2008) Natural exopolymers: ice affinity and modification of the environment as strategies for survival in sea ice. Poster, AbSciCon 2008, Santa Clara, CA, 14–17 April.

Ewert, M. (2007) Life in sea-ice: production of extracellular polymers as an adaptive strategy. Oral presentation (in Spanish), I Seminar on the Origin of Life, National University of Colombia. Bogotá, Colombia. 2 October.

Ewert, M. (2006) Inquiries on habitability: the chemical environment of star forming regions. Poster, Pale Blue Dot III: Searching for Life on Distant Worlds, Chicago, USA. 17–20 September.

Ewert, M. (2004) The notion of life within theories of life's origin. Poster, Bioastronomy 2004, Reykjavík, Iceland. 12–16 July.

ADDITIONAL PROFICIENCIES

Computational. Platforms: Windows, Linux. Languages: R, LaTeX, C++

Languages. Spanish, English, French (written and spoken fluency).