Microbial Evolution In Sea Ice: Communities To Genes

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Abstract

Microbial Evolution In Sea Ice: Communities To Genes

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Microbial communities encased in growing sea ice must contend with the combined stresses of low temperature and high salinity, environmental pressures that only intensify over the course of the winter. This harsh physical environment was expected to negatively impact both the abundance and diversity of the microbial community entrained within the ice, hypotheses which were tested in Chapters 1 and 2, respectively. While the overall abundance of microorganisms decreased in the coldest ice, extracellular polymeric substances were produced throughout the winter in all measured horizons. Microbial communities entrained from seawater into sea ice were preserved in the ice, with communities dominated by SAR11 Alphaproteobacteria (Bacteria) and Marine Group I Crenarchaeota (Archaea) found essentially unchanged throughout the winter. These results informed further hypotheses on the potential for increased lateral gene transfer by conjugation, transduction, or natural transformation in sea ice, addressed in Chapter 3 by measurement of the concentrations of bacteria, viruses, and extracellular free DNA in natural sea ice. These hypotheses were supported by the measurement of up to 100× more extracellular free DNA in sea ice brine than in the underlying seawater and extremely high virus-to-bacteria ratios (up to 2820), with predicted virus-to-bacteria contact rates up to 844× those expected in the underlying seawater. In Chapter 4 a comparative analysis of the genome of a model psychrophilic γ-proteobacterium, *Colwellia psychrerythraea* strain 34H, was used to examine the potential for the exchange of genes of particular utility in permanently cold habitats. Phylogenetic analysis and G+C content were used to identify a genomic island in *C. psychrerythraea* strain 34H containing a number of genes encoding proteins involved in the degradation

of abundant compatible solutes like glycine betaine. Furthermore, the positive growth of *C. psychrerythraea* strain 34H on sarcosine (a derivative of glycine betaine) as a sole carbon and nitrogen source suggested that the laterally transferred genes were expressed in vitro.

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ACKNOWLEDGMENTS

"If it was easy, everyone would do it," I would sometimes tell myself when it seemed like it was just too much for one person... and then I did it. And sure enough, up there on the Title Page is just one name. But that's a lie. It's like claiming that I grew a tree just because I planted a nut and sat there for 7 years, checking in on it every once in a while, without mentioning The Sun, The Air, The Rain, The Soil, or The Diazotrophs, each of which helped me grow the tree without my even having to ask, because that's just what they do. If you are reading this, you are one of those ingredients, even if you don't realize it, and even if your name isn't listed below.

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Just because I'm carving my name into this here tree, don't git to thinking that I growed it all by myself. I thank you all.

DEDICATION

To

leaving it better

than you found it

INTRODUCTION

> "On the theory of natural selection we can clearly understand the full meaning of that old canon in natural history, '*Natura non facit saltum.*' This canon, if we look to the present inhabitants alone of the world, is not strictly correct; but if we include all those of past times, whether known or unknown, it must on this theory be strictly true." Charles Darwin, On the Origin of Species

Despite the underlying influence of microorganisms in every known habitable environment on Earth, the processes by which they adapt to invade and colonize new environments are not well understood. Like all Terrestrial life, microorganisms adapt by gradual change through mutation and natural selection; however, another mode of evolution, saltation via lateral (or horizontal) gene transfer (LGT) is now known to influence the evolution of microorganisms—in short, making genetic leaps where none were predicted by theory. While mechanisms for lateral gene transfer have been known for decades from laboratory investigations, the relative importance of these mechanisms in different environments is virtually unknown. Sea ice is an environment which has appeared repeatedly (but not continuously) through Earth's history and is present today in both polar regions. Microorganisms and their biomolecules can be concentrated in the physical matrix of the ice, where the environmental conditions can be extremely challenging, combining multiple conditions known separately to favor the occurrence of gene exchange. Recent evidence indicates that other planets in our solar system, prominently Mars and Europa, also appear to harbor extensive cold, salty habitats where microorganisms might conceivably survive and exchange genes. Driven by a desire to understand the mechanisms by which the microbial inhabitants of extreme environments—on Earth or possibly elsewhere—adapt to thrive in those environments, I pursued the following underlying hypothesis: sea ice acts as a hotspot for gene exchange in the marine environment.

This dissertation is the culmination of a series of studies undertaken to characterize the microbiological habitat (Chapter 1) and community ecology (Chapter 2) of Arctic winter

sea ice, a microbiological environment about which little is known. The findings from these chapters were instrumental in the formulation and initial evaluation of the hypothesis stated above. Further efforts provided first estimates of the abundance of the raw materials necessary for LGT in sea ice (Chapter 3), as well as evidence supporting the influence of LGT on the genome and lifestyle of *C. psychrerythraea* 34H, a psychrophilic polar marine microorganism that has been isolated from sea ice (Chapter 4).

Sea ice habitat

Arctic sea ice covers an area of about 15×10^6 km^2 at its maximal extent during the winter (Antarctic 18×10^6 km^2; Fetterer and Knowles, 2002), providing an extensive (albeit shrinking: Stroeve et al., 2005; Serreze et al., 2007) habitat for microorganisms. Sea ice is also a dynamic habitat, changing dramatically over the course of days, weeks, and seasons. First-year sea ice forms in the autumn as the air temperature begins to drop below the freezing point of seawater, about −1.8℃ (for detailed description of ice growth see Eicken, 2003, and references therein). Minute ice crystals form at the surface and can be mixed by wind and waves into the upper water column. As the crystals collect at the ocean's surface they scavenge particles, including phytoplankton like diatoms, and concentrate them at the surface. Bacteria attached to the particles are entrained into the growing ice (Garrison et al., 1983, 1989; Gradinger and Ikavalko, 1998); others are likely to be passively incorporated from the seawater (Weissenberger and Grossmann, 1998). As the temperature continues to drop heading into winter, more of the interstitial water freezes and the ice compacts, depressing the turbulent mixing in the water column. At this point the ice begins to grow in a slower, more orderly fashion, by freezing seawater to the bottom of the ice sheet. Crystals grow at the base of the ice sheet and exclude contaminating molecules and particles, including salt ions and most bacteria, into pore spaces. Salts, cells, inorganic particles, dissolved nutrients and (presumably) viruses all become enriched in these pore spaces, also called brine pockets, channels, or more generally, inclusions. The salinity of the brine inclusions is inversely proportional to the temperature of the ice, leading to an unstable density gradient (i.e. colder, higher salinity brines overlay warmer, lower salinity inclusions) that drives convective overturn within the ice and expulsion of brine from the base of the ice sheet as it is forming. Unattached cells and viruses in the brine are probably expelled from the ice as well. As the temperature continues to decrease, the connections

between brine channels constrict and the movement of brine essentially ceases near a bulk salinity of 5‰ and a temperature of −5°C (Golden et al., 1998; Eicken, 2003). At this point any microorganisms inside the ice become 'trapped' and subject to the whims of the harsh winter environment. The temperature continues to drop through the winter and the ice can reach temperatures below −25°C for days or weeks, with corresponding brine salinities above 225‰, almost 7× the salinity of the underlying seawater. At the approach of spring, sea ice microbial communities centered around primary production by sea ice algae form, usually near the base of the ice sheet. These communities can be extraordinarily productive, particularly at the base, partly because within the ice sheet they are able to maintain themselves in a high-light environment relative to phytoplankton in the underlying seawater, and receive replenishing nutrients from seawater flushing the ice. Unique and highly-culturable bacterial communities can be isolated from these environments (Junge et al., 2002), which may persist until the breakup of the ice sheet due to infiltration and flushing by melt pond formation from overlying snow.

Hypothesis 1: Winter sea ice acts as a selective barrier to the survival of microorganisms (Chapters 1 & 2).

Potential adaptations to the extreme conditions faced by microorganisms in winter sea ice brines include the production of extracellular polymeric substances (EPS; Krembs and Engel, 2001; Krembs et al., 2002; Meiners et al., 2003; Mancuso Nichols et al., 2005) and compatible solutes like proline, ectoine, and glycine betaine (Bremer and Krämer, 2000; Roberts, 2005). Because EPS is extracellular and compatible solutes may escape from cells through lysis or leakage and be transported into the cell, these adaptations may afford protection to the unadapted fraction of the community as well (Welsh, 2000; Krembs et al., 2002). The mechanism of action of EPS is not clear but it may depress the freezing point of the brine and alter the microstructure of the brine channels to prevent impingement of ice crystals onto cell surfaces (Krembs et al., 2000, 2002; Krembs and Deming, 2008). Compatible solutes are generally small neutral or zwitterionic molecules highly concentrated inside the cell to maintain turgor pressure in the presence of low temperatures or high salt concentrations (Welsh, 2000; Sleator and Hill, 2002; Roberts, 2005). They can be synthesized de novo or imported from the medium; some stabilize proteins and DNA, others protect DNA from cleavage by endonucleases (Welsh, 2000). EPS and compatible solutes may also

potentially be utilized as energy sources by microorganisms in the ice.

Extreme conditions in sea ice may result in increased microbial mortality, but the mechanisms of cell death are not known. Two likely sources of microbial death in the ice are physical disruption of cells by changes in osmolarity or the impingement of ice crystals (Krembs et al., 2002), leading to cell damage and lysis; and, biological disruption, either lysis by phage or the rare surviving protistan grazer in the winter ice, or due to biochemical injury. Although increased salinity is likely to cause cell shrinkage (dessication) and inhibit activity and growth, it is less likely to rupture the cell membrane and lead to lysis than is hypotonic osmotic shock. No published studies report the isolation of extremely halophilic microorganisms from sea ice, implying that the majority of the surviving cells are either simply preserved in the ice or utilize some specific adaptation, like the production of EPS or compatible solutes, to tolerate salts. Dessication probably inhibits internal ice crystals from growing and perforating the cell wall, while EPS production could be a strategy to avoid physical disruption from external ice crystals. Lysis by protistan grazers in the ice is well documented in warmer ice (Garrison and Close, 1993; Stoecker et al., 1997, 1998; Gradinger and Ikavalko, 1998) and is probably a viable mechanism for mortality down to −10°C or so. Viruses are known from sea ice (Maranger et al., 1994; Borriss et al., 2003; Gowing et al., 2002, 2004) and viral production has been documented in incubations of natural communities under sea ice brine conditions of −12°C and 160‰ salt (Wells and Deming, 2006c), but only the active fraction of the microbial population would likely be susceptible to lysis by phage infection. Consequences of microbial lysis in sea ice include the release of nutrients, enzymes, and cryo- and osmoprotectants into the brine that might be utilized by other microorganisms, the release of intracellular signalling molecules that could influence the behavior of neighboring microorganisms, the release of phage (potentially including transducing phage), and the release of nucleic acids that could be utilized as an energy source or for lateral gene transfer via transformation.

Of the little known about possible community dynamics over time and increasingly severe conditions in sea ice, most data derive from Antarctic sea ice (Delille and Rosiers, 1996; Fiala et al., 2006), which generally experiences less severe conditions than Arctic sea ice. Most frequently, microbial studies of sea ice have focused on the summer season, with some attention paid to (warm) ice near the seawater interface during colder seasons (e.g., see reviews by Lizotte, 2003; Mock and Thomas, 2005; Deming, 2009). Evidence

that biological processes may be occurring in the coldest parts of sea ice during the winter includes detection of increases in high molecular weight organic material in Arctic ice held at temperatures of −5 to −20°C (Krembs et al., 2002) and regeneration or utilization of nutrients at subzero temperatures (Dieckmann et al., 1991; Garrison and Close, 1993; Gleitz et al., 1995; Thomas et al., 1995; Kaartokallio, 2001). Although always working with melted samples at temperatures above −2°C, early investigators of potential microbial processes in Antarctic winter sea ice also demonstrated bacterial production (Kottmeier and Sullivan, 1987) and enzyme activity (Helmke and Weyland, 1995).

Microbial community succession is known to occur in the spring and summer (Garrison and Close, 1993; Gleitz et al., 1996; Delille and Rosiers, 1996; Stoecker et al., 1998; Delille et al., 2002; Lizotte, 2003; Fiala et al., 2006), and during the short relatively warm winter-spring sea ice season in the Baltic Sea (Kaartokallio et al., 2008), but little is known about community dynamics in the polar winter (Delille, 1993; Helmke and Weyland, 1995; Fiala et al., 2006). A selective effect has been observed in Antarctic sea ice as an increase in the ratio of psychrophilic to psychrotolerant colony-forming bacteria in the upper ice during the months between autumn freeze-up and spring (Delille and Rosiers, 1996). Unlike winter ice microbial communities, the spring and summer communities are well described from both Antarctic and Arctic sea ice (Bowman et al., 1997a,b; Junge et al., 2002; Brinkmeyer et al., 2003), and are primarily associated with ice algae in the lower tens of centimeters of the ice. These communities tend to be dominated by easily culturable heterotrophic bacteria. No Archaea have been described from these warm ice communities, though Archaea were found in newly formed frazil ice in the Southern Ocean (Murray et al., 1998). In the only prior study of the phylogenetic community structure of Bacteria and Archaea in winter sea ice, the community was dominated by attached Bacteria, though Archaea made up to 3.4% of the cells (Junge et al., 2004).

Most of the microorganisms entrained into sea ice derive from seawater (as opposed to atmospheric sources). Terrestrial input in coastal environments may also contribute to the community in Arctic ice (Wells and Deming, 2003; Garneau et al., 2006; Wells and Deming, 2006a), an input not as likely for Antarctic ice due to the small influx of terrestrial meltwater there. Although some fraction of the 10^5–10^6 cells ml^{-1} found in polar seawater may be adapted for growth in sea ice and simply persist until the next freeze-up, the majority are more likely to be adapted for pelagic growth (although this

concept has not been tested quantitatively). Unless there is a strong preferential selection for ice-active microorganisms in the initial stages of sea ice growth (unknown at this point, but a potential mechanism is found in gas vacuolate bacteria Gosink et al., 1998), the expectation is that most of the microbial community in first year sea ice is derived from ubiquitous seawater microorganisms that are not specially adapted for growth in sea ice. If the extreme environmental (as opposed to biological) conditions in winter sea ice act as a selective pressure then those seawater microorganisms should preferentially lyse, enriching the community for those microorganisms capable of surviving and multiplying in the ice, e.g. those with adaptations to the winter sea ice environment. If instead the conditions do not impose a selective pressure then even those microorganisms not adapted to the winter conditions should survive, even if they are unlikely to be active and so perhaps best described as 'preserved.'

In Chapters 1 and 2 I investigated the community ecology of Bacteria and Archaea in Arctic winter sea ice, including physical-chemical (i.e. temperature, salinity) characterizations of the brine environment within which these microorganisms persist. The abundance and diversity of microorganisms within the ice were asessed over the course of a winter season, in addition to measurements of extracellular polymeric substances and other particulates in the ice. The hypothesis that winter sea ice acts as a selective barrier to the survival of microorganisms was not upheld; instead, conditions within winter sea ice were found to preserve bacterial and archaeal communities similar to those originally entrained into the ice despite significant decreases in microbial abundance, indicating that species-specific mortality was rare.

Hypothesis 2: Multiple conditions favoring lateral gene transfer coexist in sea ice, making it a 'hot spot' for microbial gene exchange in the marine environment (Chapters 3 & 4).

The movement of genetic material from one organism to another *sans* reproduction is called lateral (or horizontal) gene transfer (LGT). Recent complete genome sequencing of hundreds of microorganisms has confirmed the pivotal role that LGT has played in the evolution of existing microbial genomes (Syvanen, 1994; Doolittle, 1999a,b; Brown, 2001; Gogarten et al., 2002; Lawrence and Ochman, 2002; Lawrence, 2002; Syvanen, 2002; Brown, 2003; Lawrence and Hendrickson, 2003; Beiko et al., 2005; Beiko and Hamilton, 2006). This genomic evidence has led to conclusions that LGT is widespread among all three Domains

of life and intensive, with perhaps 15% (and perhaps much more) of the genes in any microbial genome likely obtained via LGT, depending on life history characteristics (Garcia-Vallve et al., 2000) and measurement technique. However, the hypothesis that LGT is an active mechanism in microbial evolution today—and not simply the relic of an earlier mode (Woese, 2002; Kurland et al., 2003)—is more difficult to address with genomics alone. Most commentators on the importance of lateral gene transfer have focused on the past, asking questions about how reliable the universal phylogenetic tree is and how it has been shaped by LGT. There is an unfilled need to amend gene sequence-based investigations with in situ experiments using environmentally relevant microorganisms to address the potential for and relative rates of LGT in the natural environment today and in the future.

Unknown to date is the relative importance of the three known mechanisms of LGT on the evolution of microbial genomes in any environment, much less in perennially cold marine systems, which make up >90% of the volume of the world's oceans. The first of these mechanisms to be discovered was transformation, the direct uptake and integration of extracellular donor DNA by a 'competent' host cell (Griffith, 1928; Avery et al., 1944). Next came the discovery of conjugation, a biochemically complex system (often plasmid-borne) requiring direct contact between donor and host cells (Lederberg and Tatum, 1946), and the discovery of phage as agents of LGT, called transduction (Zinder and Lederberg, 1952). Although these mechanisms have been long utilized in the laboratory setting (e.g. see examples in Sambrook and Russell, 2001) and genome sequencing data indicate they must have occurred in the natural environment, to date relatively few studies have investigated even the potential for LGT in situ.

The potential for lateral gene transfer in an environment depends on the presence of the necessary raw materials Transformation requires free DNA, either dissolved (Paul et al., 1987, 1991b) or bound (Lorenz et al., 1981; Lorenz and Wackernagel, 1987, 1990; Stewart et al., 1991), and transformation frequency is generally proportional to DNA concentration (Frischer et al., 1993; Sikorski et al., 1998). Measurements of dissolved DNA concentration in seawater vary greatly, from 0.1~50 μg L^{-1} (compiled in Lorenz and Wackernagel, 1994; Dröge et al., 1999) with mean values <10 μg L^{-1}. Free DNA could accumulate in sea ice brines via cell lysis by phage or grazers (Proctor and Fuhrman, 1990; Turk et al., 1992), the release of DNA-containing EPS (Steinberger and Holden, 2005; Allesen-Holm et al., 2006; Bockelmann et al., 2006), or simply concentration from the source seawater. Naturally

competent cells (genetically and phenotypically able to take up DNA) are also required for transformation. A pair of studies found that about 15% of a marine microbial community was naturally competent (Frischer et al., 1990, 1994) to take up plasmid DNA from a marine *Vibrio* strain.

Transduction requires the presence of phage, which have been observed and enumerated in melted sea ice samples (Maranger et al., 1994; Gowing et al., 2002, 2004; Wells and Deming, 2006c), and more specifically transducing phage, which have not yet been identified from sea ice, though transducing phage from the marine environment are known (Baross, 1972; Wommack and Colwell, 2000). Wells and Deming (2006c) calculated contact rates between cells and viruses that were 13–600× higher in sea ice at temperatures below −24°C than in the underlying seawater, due to the brine concentrating effect.

Conjugation requires a donor cell containing a mobile genetic element (usually a conjugative plasmid) and an adjacent recipient cell (usually lacking the mobile genetic element). Numerous plasmids were identified from cultured isolates of Antarctic sea ice bacteria (Kobori et al., 1984), but conjugative capacity was not determined.

The biomedical literature is replete with reports of rapidly-spreading antibiotic resistant microorganisms in hospitals and biofilms (e.g. see Neu, 1992, references therein, and many of the hundreds of papers citing it), but existing reports of LGT in the environmental literature focus primarily on the terrestrial environment, in the contexts of genetically modified agricultural products and bioremediation. Studies of LGT in the marine environment are sparse (Gauthier and Briettmayer, 1990; Hermansson and Linberg, 1994; Lorenz and Wackernagel, 1994; Dröge et al., 1999), however, several studies (mostly in microcosms) have demonstrated that marine microbial communities can and do undergo gene exchange under permissible conditions (Baross, 1972; Stewart and Sinigalliano, 1990; Paul et al., 1991a; Dahlberg et al., 1998a,b; Jiang and Paul, 1998), although the mechanism was not always known.

The presence of biotic or abiotic surfaces often increases the frequency of observed gene transfer in microcosm and laboratory studies (Hermansson and Linberg, 1994) by stabilizing extracellular DNA and attracting high cell densities, but sea ice as a source of surfaces for gene transfer (e.g. the ice matrix or entrained aggregates) has never been studied. Stressful conditions also tend to induce the pathways responsible for LGT, including: chemical stressors (e.g. mutagens, heavy metals, reactive oxygen species, divalent cations), radiation

(UV light, radioactivity), pH and temperature (heat or cold shock). In addition to the physical environment, the biological-chemical environment can affect the frequency of LGT in bacterial cells. Biological factors that have been shown to induce pathways of LGT include antibiotics, viral infection, starvation, attachment, quorum sensing, and high cell density.

If sea ice were found to be a hotspot for LGT, the distribution of transformants from sea ice has potential evolutionary significance. Arctic sea ice can entrain, transport and distribute particles and possibly pollutants (Nurnberg et al., 1994; Eicken et al., 1997; Pfirman et al., 1995; Stierle and Eicken, 2002; Eicken et al., 2005) throughout the Arctic Ocean faster than ocean currents alone and can be expected to do the same for recombinant microorganisms. This rapid and widespread distribution of a new lineage may promote the fixation of the new recombinant genome into the metapopulation, for example by spreading genes during a selective sweep that might be detectable by metagenomics (Cohan, 2001; Nesbø et al., 2005).

Although transduction and conjugation are both likely important means of LGT in certain environments, transformation might be particularly interesting to study in sea ice for a number of reasons. Sea ice brines may harbor large amounts of extracellular DNA– some concentrated from seawater and some released by cells lysing in the ice. More free DNA leads to more encounters between extracellular DNA and naturally competent cells if present and thus higher frequencies of transformation. Transformation might also be important in the adaptation of microorganisms to sea ice because while transformation is often more frequent between closely related species, the source DNA can originate from any organism in the environment, whether Bacteria, Archaea or Eukarya or from viruses. In contrast, while some marine phage may be generalists capable of infecting a wide range of hosts, most are probably fairly specific in their host requirements (Wommack and Colwell, 2000). Transformation is also of interest because the potential for natural competence is encoded by the host genome (rather than by phage or plasmid) and so the mechanism could be studied in silico with genomics, proteomics, or transcriptomics. Finally, of the known mechanisms for gene transfer in the environment, transformation is the most straightforward to study in the laboratory, requiring only a simple genetic system and purified DNA, not the discovery and isolation of a cold-active transducing phage or conjugative plasmid.

If LGT occurs in sea ice, it should be evident in the genomes of microorganisms

that live there. Genomic methods to identify LGT include analyzing %G+C nucleotide composition, codon usage patterns, and most convincingly, phylogenetic analysis to identify genes having inconsistent phylogeny with a conserved marker like the 16S ribosomal RNA gene (Ragan, 2001; Gogarten et al., 2002). If LGT is an important mechanism for microbial adaptation to new environments, then genes acquired by LGT need to provide some selective advantage there. One recently identified example of a laterally transferred gene conferring a selective advantage in the ocean is the case of proteorhodopsin, a light driven proton pump, transferred between Archaea and Bacteria in the photic zone (Frigaard et al., 2006). Further important cases of LGT in the marine and estuarine environments include cell surface-associated proteins, among others lying in 'genomic islands', that may play a role in niche differentiation in marine cyanobacteria (Rocap et al., 2003; Coleman et al., 2006), and the case of the estuarine *Vibrio cholerae*, an opportunistic human pathogen causing thousands of deaths per year. The pathogenicity of *V. cholerae* is dependent on at least one lysogenic phage and a genomic (or 'pathogenicity') island (Faruque and Mekalanos, 2003).

The genome of a model psychrophilic γ-proteobacterium isolated from Arctic marine sediments, *Colwellia psychrerythraea* strain 34H, was recently published (Methé et al., 2005). Most cultured representatives of the genus *Colwellia* are cold-adapted and some have been isolated from sea ice (including *C. psychrerythraea* strain 34H) and surfaces, including marine sediments. The genome of *C. psychrerythraea* strain 34H shows plentiful evidence of adaptation to cold environments, including genes involved in maintaining membrane fluidity, forming, transporting, and degrading compatible solutes, and synthesizing extracellular polysaccharides and enzymes (Methé et al., 2005). Other interesting abilities may include degradation of a variety of simple (e.g. C1) and complex (e.g. aromatic) compounds, and the mitigation of reactive oxygen species (Methé et al., 2005). If some of these genes were transferred laterally into the genome, they should be detectable by comparative genomic approaches. The genomes of other cold-adapted bacteria are now available, including *Pseudoalteromonas haloplanktis* (Médigue et al., 2005) and *Psychrobacter arcticus* (RefSeq: NC_007204), and the future availability of more genomes will enable more extensive analyses of LGT in these low temperature environments.

Using genomics it is possible to identify sets of genes with conserved function, allowing the prediction of that function in other genomes. For example, several marine *Vibrio* spp. have a conserved set of genes important in natural competence for transformation, including

those for a Type IV pilus complex. The Type IV pilus is required for twitching motility and often important in pathogen virulence, but the gene products are also required for natural transformation in a number of bacteria (Graupner et al., 2000; Averhoff and Friedrich, 2003; Meibom et al., 2005). Besides analysis of gene sequences, analysis of gene expression is also a powerful method for interrogating genomes. One recent study used gene chip microarrays to identify genes from *Vibrio cholerae* that were up-regulated in the presence of chitin hexamers (Meibom et al., 2004), including those involved in natural competence. Chitin seems to be an important site of attachment and gene exchange in the marine environment, perhaps not surprisingly since it is one of the most abundant biopolymers on earth. Recent studies have demonstrated that *V. cholerae* (Meibom et al., 2005) and *V. fischeri* (Pollack et al., 2007) are both naturally competent for transformation when attached to chitin, a common starvation-induced stress response in marine *Vibrio* spp. Chitin and other surfaces for attachment (including entrained inorganic particles and the ice walls of the brine channels) are prevalent in sea ice, as are stressors that are known to induce pathways for lateral gene transfer, like osmotic shock, temperature shock, and high cell density.

In Chapter 3 I investigated various physical, chemical, and biological parameters of sea ice as they pertained to lateral gene transfer by conjugation, transduction, and transformation. In Chapter 4 I used the genome of a model psychrophile, *C. psychrerythraea* 34H, to identify a case of inter-Order LGT that would be expected to increase the fitness of the bacterium in sea ice.

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Chapter 1

SPATIAL HETEROGENEITY AND TEMPORAL DYNAMICS OF PARTICLES, BACTERIA, AND PEPS IN ARCTIC WINTER SEA ICE

1.1 Abstract

Abundances of particles, total bacteria, and particulate extracellular polymeric substances (pEPS) in Arctic sea ice were tracked through a winter season to examine the impact of combined extremes of low temperature and high salinity on the prokaryotic microbial community. Three horizons, centered at depths of 25, 45, and 65 cm from the ice surface, with mean seasonal temperatures of −20, −17, and −14℃, respectively, were sampled 16 times over the course of 12 weeks. Microscopic counts of bacteria (stained with DAPI) and particles (stained with acridine orange) reflected the dynamic conditions of the growing ice sheet, with greater abundances and variability in the upper ice horizons compared to the lower. The trend of higher particle and bacterial abundances in the upper ice was corroborated by several full-depth profiles taken during the expedition, which also displayed significantly decreasing cell abundance with depth. Bacterial abundance declined slowly and significantly with time in the upper and middle ice horizons, but not in the lowest, suggesting that much of the prokaryotic microbial community is resilient to extreme environmental conditions. We found that pEPS concentrations increased significantly with time and with decreasing temperatures in all depth horizons, which may lend support to the argument that sea ice bacteria produce EPS in situ as a cryoprotectant.

1.2 Introduction

Arctic sea ice covers an area of about 15×10^6 km^2 at its maximal extent during the winter (Antarctic 18×10^6 km^2; Fetterer and Knowles, 2002), providing an extensive (albeit shrinking: Stroeve et al., 2005) habitat for microorganisms. Winter sea ice is an extreme environment, characterized by limited light, subzero temperature (−2 to −35℃) and high salinity (37 to 237‰) in the brine inclusions where most of the organisms have been

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observed to reside (Junge et al., 2001). Little is known about these microbial inhabitants; the assumption that they are largely inactive during the winter has been tested infrequently (Junge et al., 2004; Wells and Deming, 2006). Even less is known about possible community dynamics over time and increasingly severe conditions, and then only from studies of Antarctic sea ice (Delille and Rosiers, 1996; Delille et al., 2002; Fiala et al., 2006), which generally experiences less severe conditions than Arctic sea ice. Instead, microbial studies of sea ice have focused on the summer season, with some attention paid to (warm) ice near the seawater interface during colder seasons (e.g., see reviews by Lizotte, 2003; Mock and Thomas, 2005).

Evidence that biological processes may be occurring in sea ice during the coldest months of winter includes detection of increases in high molecular weight organic material in Arctic ice held at temperatures of −5 to −20°C (Krembs et al., 2002) and regeneration or utilization of nutrients at subzero temperatures (Dieckmann et al., 1991; Garrison et al., 1993; Gleitz et al., 1995; Thomas et al., 1995; Kaartokallio, 2001). Although always working with melted samples at temperatures above −2°C, early investigators of potential microbial processes in Antarctic winter sea ice also demonstrated bacterial production (Kottmeier et al., 1987), enzyme activity (Helmke and Weyland, 1995), and an increase in the ratio of psychrophilic to psychrotolerant colony-forming bacteria in the upper ice during the months between autumn freeze-up and spring (Delille and Rosiers, 1996). A recent study of Arctic sea ice by Junge et al. (2004) utilized fluorescence in situ hybridization to demonstrate the presence of metabolically active cells (virtually all were attached) and relatively more members of the Cytophaga-Flavobacter-Bacteroides group (up to 50%) and Archaea (up to 3%) in the coldest sections of the ice at −15 to −20°C. Although microbial succession, particularly in the ice-algal community, is understood to occur during the warmer months (detailed in Lizotte, 2003), the results of Junge et al. (2004) are suggestive of Bacterial and Archaeal succession during winter. Because the gradients they observed were spatial (vertical profiles in single cores) and not over seasonal time, the concept of succession for these microbial domains remains elusive.

The purpose of this study was to build upon the "snapshot" work done previously during Arctic winter and test hypotheses concerning the effects of increasingly severe environmental conditions on natural microbial communities over time. A unique opportunity to overwinter in the Arctic allowed us to access the same ice field repeatedly and take advantage of

the expected impermeability of sea ice at winter temperatures well below −5°C (Golden et al., 1998). This impermeability means that sea ice impurities, including entrained microorganisms, should be conveniently trapped for study for the duration of the winter season. In addition to basic physical-chemical parameters and total bacterial (prokaryotic) abundance, we measured the concentration of inanimate particles and of particulate extracellular polymeric substances (pEPS), the latter believed to serve as cryoprotectants in winter sea ice (Krembs et al., 2002). A parallel study of Bacterial and Archaeal microbial diversity and succession in the ice, using 16S rDNA-based fingerprinting techniques, is reported in Chapter 2.

1.3 Materials and Methods

1.3.1 Ice-core and seawater sampling

All samples were collected as part of the international Canadian Arctic Shelf Exchange Study (CASES) overwintering expedition while the CCGS *Amundsen* was immobilized in the first-year sea ice of Franklin Bay, Northwest Territories, Canada (at 70.0°N, 126.3°W; Fig. 1.1), where the water depth was 232 m. Air temperature was measured on the bridge of the ship by the Meteorological Observations team before day 22 and after that by an in situ instrument array stationed within 100 m of the coring field (data made available by T. Papakyriakou). The same instrument array measured ice temperatures in situ at depths of 1–100 cm at hourly intervals from day 22. Average daily temperatures were computed and extrapolated to the thickness of the ice sheet using the linear part of the temperature-depth curve. Ice temperatures reported prior to day 22 were measured by inserting a hand-held thermocouple into a hole drilled into the center of each 10-cm section of the physical core immediately after collection (see below).

Ice cores were collected at least weekly during the period of 10 January–28 March 2004 (CASES Legs 4 and 5), using a manually-driven MARK II ice auger (9-cm internal diameter, Kovacs Enterprise), from an undeformed undisturbed field of the first-year ice designated for biological sampling and located 1600 m east of the ship. On day 41 we also sampled at the mouth of the Horton River (Station 'Angaguk', 70.0°N, 126.7°W; Fig. 1.1), 18 km from the main station. On each sampling day one full-length core was taken from a previously unvisited portion of the ice field for standard measurements of ice thickness, temperature, and bulk salinity. The core was then cut into 10-cm sections (from the top) and later melted

aboard ship to determine bulk salinity using a temperature-corrected conductivity probe. In situ brine salinities were calculated from the in situ temperature and bulk salinity of a given ice section, using the equations of Cox and Weeks (1983).

Three sea ice cores for particulate parameters (particles, bacteria, and pEPS) were collected in a square pattern within 1 m of the full-length standard core, which lay at one corner, as above but without coring down to seawater. These shorter cores were cut in the field, using an ethanol-sterilized saw, into 10-cm sections centered at each of three depths from the ice surface: 25, 45, and 65 cm, designated horizons I, II, and III, respectively. Each section was also labeled by calendar day of sampling; e.g., 10-I, 10-II, etc. On days 35 and 38 we sampled an additional horizon centered at 15 cm, labeled horizon IV, in addition to those samples taken at this depth on days 27 and 40 (see below). The ice sections were placed into separate sterile Whirl-Pak bags and transported in an insulated cooler back to a shipboard cold room set at 0°C, where they were processed as soon as possible. Each was mechanically crushed (still within its sterile bag) and then allowed to melt into a prefiltered (0.22 µm) artificial brine solution (prepared from Instant Ocean salts; Marineland) at 0°C, using an ice:brine volume ratio of 1:2. In order to protect against osmotic shock and possible cell lysis, we used the isothermal-isohaline melting approach of Junge et al. (2004) for each ice section at >−15°C: i.e. the salinity of the artificial brine solution was adjusted to obtain a final meltwater salinity near the in situ salinity of the original brine inclusions in the ice. This melting approach also kept the temperature of the meltwater from rising above the in situ ice temperature before melting was completed within 12 hours. Resource constraints impelled that we melt ice horizons below −15°C into a brine of salinity 275‰, the salinity appropriate for −15°C sections.

On days 27, 40, and 88, single full-length cores were taken as above to determine vertical profiles of total bacterial abundance; on days 27 and 40 all depths were sampled, on day 88 alternating 10 cm sections were sampled, starting from the top. In some cases, the abundance of particles (day 88) and concentration of pEPS (day 27) in the full-length cores was also determined. Under-ice seawater samples were collected from the CTD rosette lowered through the ship's moon pool (at 5–20 m depth) during CASES Leg 3 on days 343, 348, and 355 (of the year 2003). On days 28, 35, 74, and 88 (of 2004; CASES Legs 4 and 5) under-ice seawater samples were collected with a hand-held 2-L Niskin bottle lowered through a hole in the ice 400 m from the designated ice-sampling field. These water samples

were processed similarly to the melted sea ice samples, except that particle content was not evaluated for the 2003 samples.

1.3.2 Abundance of particles, total bacteria, and pEPS

Immediately following completion of the melting process, a 40-ml subsample from each melted section was fixed with 0.2-µm-filtered 2% formaldehyde (final concentration) and stored at 4℃ in the dark for later enumeration by epifluorescence microscopy. All samples were processed in the home laboratory within 4 months of sampling. Total bacteria were counted within that period, while inanimate particles were counted within two years. Although a subset of the samples were examined after staining with 4',6'-diamidino-2-phenylindole (DAPI; Porter and Feig, 1980), most were subjected to a dual-staining procedure, simplified from Schmidt et al. (1998). Samples were stained with 3,6-bis[dimethylamino]acridinium chloride (acridine orange (AO); Hobbie et al., 1977) for 3 min, filtered (<350 mm Hg) onto 0.2-µm pore size polycarbonate membrane filters (Poretics), then stained for 10 min with DAPI. Total bacteria were counted on all filters using DAPI optics (365 nm excitation, 395 nm splitter and 420 nm long pass barrier) while particles were counted on all dual-stained filters using AO optics (450–490 nm excitation, 510 nm splitter and 520 nm long pass barrier). A minimum of 20 fields or 200 particles or bacteria were counted from each sample at a magnification of 625× or 1562.5×, respectively. Inanimate particles were counted (limited to maximum dimensions of 2–150µm) and defined as those that stained red with AO but not blue by DAPI (if they appeared under DAPI optics they were usually yellow); they were generally angular and appeared lithogenic (obvious organic detritus like the occasionally observed diatom frustule did not stain red). Although size distributions were not quantified in detail, their maximum dimensions ranged from 2µm to 100µm (aggregates were counted as single particles) with most being <10 µm. For analysis the total number of particles and cells were scaled to volume of unmelted sea ice (calculated from length and diameter of sea ice section using a density of 0.92 g cm^{-3}), as well as to brine volume, calculated from temperature and bulk salinity using the equations of Cox and Weeks (1983).

Another subsample of 150 ml was filtered (<350 mm Hg) onto a 24-mm diameter 0.4-µm pore size membrane filter to quantify particulate extracellular polymeric substances (pEPS) by the Alcian blue method (Passow and Alldredge, 1995). Filters were stained with Alcian

blue solution for 1 min, rinsed with distilled water, and stored at −20°C until analysis within one year. Stain was dissolved for 12 h in 2 or 6 ml of 80% sulfuric acid and centrifuged. The pEPS concentration was measured spectrophotometrically at 787 nm and reported in xanthan gum equivalents (μg XGEQV), as calculated from a standard curve of xanthan gum in 180‰ artificial brine. Standard curves of xanthan gum prepared in artificial brines of different salinities, up to 250‰, did not differ significantly from the curve based on 180‰ brine. As for particles and cells, measurements of pEPS were scaled to volume of unmelted sea ice, as well as to calculated brine volume. Efforts to measure dissolved EPS in Franklin Bay sea ice yielded results below the detection limits of the assay.

1.3.3 Statistical and graphical analyses

All statistical analyses were performed with R v.2.5.0 (R Development Core Team, 2008), an open-source statistical software package. Because distributions of particulate parameters were predominantly not normally distributed (using the D'Agostino-Pearson test for normality; Zar, 1999), they were analyzed with nonparametric statistics, including the Spearman rank correlation (r_S), unless otherwise noted. Particulate parameters were also analyzed for outliers, defined as points > 1.5× the interquartile range (IQR) from the third quartile (Q3), and extreme outliers, defined as points > 3× the IQR from Q3. Values described as 'outlier-adjusted means' were calculated by trimming the single highest extreme outlier and taking the mean of the remaining values. Robust linear regression models that include but minimize the effect of outliers were used to calculate slopes for particulate parameters over the course of the ice-sampling period. Specifically, the R function *rlm* performs iterated re-weighted least squares with an M-estimator; the default settings (Huber's method with tuning constant k = 1.345) were used. Confidence intervals for the slopes were calculated but the significance of the models was not evaluated; significance was determined only by the Spearman rank correlations. In all cases, significant correlations were defined by $p < 0.05$. Figure 1.1 was drawn using the open-source Generic Mapping Tools accessed via "Online Map Creation" (https://www.aquarius.geomar.de/omc/). Other figures were generated with Matlab (R13.0.1, The Mathworks, Inc.).

1.4 Results

1.4.1 Physical conditions of the ice field

Full depth contours

Over the course of the 12-week study the air temperature fluctuated between −18°C and −38°C (Fig. 1.2) as the thickness of the ice increased from 91 cm on day 10 (January 10) to 165 cm on day 88 (March 21). Snow cover was typically ≤ 5 cm. Maximum and minimum ice temperatures measured near the surface (at 5 cm depth) were −18 and −32°C respectively, while the overall temperature maximum at the ice-water interface was near the freezing point of the seawater, −1.8°C (Fig. 1.3(a)). As expected, average daily ice temperature increased monotonically and mostly linearly with depth (Fig. 1.3(a)). In situ brine salinity at 5 cm depth, calculated from ice temperature (Cox and Weeks, 1986), ranged from 195 to 238‰, with a daily brine salinity minimum of ~35‰ at the ice-seawater interface (Fig. 1.3(b)). Vertical profiles of bulk salinity generally conformed to the C-shaped curve typical of first-year sea ice (Cox and Weeks, 1988; Nakawo and Sinha, 1981), with higher salinities at the upper and lower boundaries (Fig. 1.3(c)), and included additional salinity maxima in the interior of the ice, as also observed by Nakawo and Sinha (1981). The fraction of the ice volume occupied by brine, calculated from ice temperature and bulk salinity (Cox and Weeks, 1983), ranged from 0.4 to 18% throughout the ice (Fig. 1.3(d)). Excluding the ice-water interface (bottom 10 cm), the mean brine volume fraction in the ice was 2.5%, within the bottom 10 cm it was 10%.

Selected ice horizons

Temperatures in the selected ice horizons (centered at depths of 25, 45 and 65 cm) varied by 10–12°C, reflecting dampened atmospheric temperatures which fluctuated over a 20°C range (Table 1.1, Fig. 1.3(a)). Calculated brine salinities in these horizons varied by factors of 1.3–1.5 (Table 1.1, Fig. 1.3(b)), while bulk salinities varied more, by factors of 1.5–2.1 (Table 1.1, Fig. 1.3(c)). The brine volume fraction, calculated from the temperature and bulk salinity (Cox and Weeks, 1986), varied by factors of 1.8–3. (Table 1.1, Fig. 1.3(d)), with the largest change occurring in the upper horizon. In all horizons, temperature and bulk salinity each correlated significantly and negatively with time (Table 1.2). Temperature (and thus brine salinity) varied significantly with depth among the three depth horizons (F

= 225, p < 0.001, ANOVA), as did brine volume fraction (F = 15, p < 0.001, ANOVA), although bulk salinity did not (F = 0.5, p > 0.1, ANOVA).

1.4.2 Abundance of particles, bacteria and EPS

Full depth snapshots

Particle abundance measured in a full-length core from day 88 decreased significantly with depth ($r_S = -0.9$, p < 0.001, n = 9) over two orders of magnitude, from 2.6×10^5 to 1.8×10^3 particles ml^{-1} ice, at 5 and 165 cm, respectively (Fig. 1.4A). Total bacterial abundance (Fig. 1.4B), normally distributed in the full-length cores, also decreased significantly with depth in two cores, from day 27 (r = −0.73, p < 0.01, n = 12) and day 40 (r = −0.77, p < 0.01, n = 12). In a third core from day 88, an inflection point was observed at 105 cm; counts decreased to this point (r = −0.98, p < 0.001, n = 6), below which they increased (though not significantly) to the bottom at 165 cm depth (r = 0.92, p < 0.1, n = 4). Mean values ± standard error of the mean (σ_M) on days 27, 40, and 88 were $5.4 \pm 0.5\times10^4$, $4.4 \pm 0.5\times10^4$, and $3.2 \pm 0.6\times10^4$ cells ml^{-1} ice, respectively. A significant difference among these means was detected (F = 8.5, p < 0.01, ANOVA). Follow-up one-sided t tests, based on the hypothesis that bacterial abundance decreased over time in the ice, indicated no significant decrease in mean bacterial counts between days 27 and 40 (t = 1.5, p < 0.1) or days 40 and 88 (t = 1.6, p < 0.1), but a significant decrease between days 27 and 88 (t = 2.9, p < 0.01) was detected.

The mean ($\pm \sigma_M$) bacterial abundance in under-ice seawater before ice sampling began was $2.9 \pm 0.2\times10^5$ bacteria ml^{-1}, while the mean during the course of ice sampling was $2.2 \pm 0.1\times10^5$ bacteria ml^{-1}. These means were significantly different from each other (t = 4.7, p < 0.01, t test). They were also 1.4–8.1× higher than the mean counts per volume of ice, as expected given the brine (and presumably bacterial) expulsion inherent to the initial ice formation stage (which we were not present shipboard to measure).

Concentrations of pEPS, measured in the full length core from day 27 (Fig. 1.4C) were not significantly correlated with depth in the ice ($r_S = -0.13$, p > 0.1, n = 12). The core had a mean concentration ($\pm \sigma_M$) of 35 ± 3 μg XGEQV L^{-1} ice, excluding a single outlying point of 200 μg XGEQV L^{-1} ice at a depth of 15 cm. Chlorophyll a and phaeopigment concentrations measured from two full-length cores collected by others on days 20 and 38

were very low, although isolated small peaks in phaeopigment were observed in the upper ice at 5 cm on day 20 and at 15 cm on day 38 (S. Brugel, personal communication). Ratios of phaeopigment to chlorophyll a were inversely related to depth in the ice (S. Brugel, personal communication).

Selected ice horizons

Particle abundance

The abundance of inanimate particles in Franklin Bay sea ice horizons I, II, and III (Fig. 1.5), when scaled to ice volume, ranged over more than two orders of magnitude from 5.7×10^3 to 1.3×10^6 particles ml^{-1} ice, and when scaled to brine volume, from 3.6×10^5 to 1.5×10^8 ml^{-1} brine (Table 1.3). The particle counts from horizons I and II were significantly positively skewed, with the highest outlier in horizon I (day 60; Fig 1.5A) alone more than doubling the mean from 3.7 to 8.3×10^4 particles ml^{-1} ice. Extreme outliers in horizon II (at 9× and 6× the IQR from Q3 on days 10 and 35, respectively, Fig. 1.5B) caused the mean value of 2.5×10^4 particles ml^{-1} ice to be 1.4× the median.

Particle abundance, whether scaled to ice or brine volume, did not change significantly over the ice-sampling period except in horizon II, where it decreased with time (by ~ 47%), and also correlated positively with bulk salinity ($p < 0.01$ in both cases, Table 1.4), implying a loss of particles with brine expulsion (Fig. 1.5B). Lacking measurements of particle abundance at the start of ice formation, we cannot estimate the fraction of particle loss that could be attributable to brine expulsion. When scaled to brine volume, particle abundance correlated inversely with bulk salinity in horizons I and III ($p < 0.05$, Table 1.5).

Significant differences in median particle abundance were detected among the selected ice horizons, whether scaled to ice volume ($H = 11$, $p < 0.01$, Kruskal-Wallis test, Table 1.4) or brine volume ($H = 12$, $p < 0.01$, Kruskal-Wallis test, Table 1.5). Follow-up Wilcoxon rank sum tests indicated that horizons I and II each contained significantly more particles (scaled to ice volume) than horizon III ($W = 354$, $p < 0.01$, and $W = 404$, $p < 0.05$, respectively), while horizon I did not contain more particles than horizon II ($W = 569$, $p > 0.05$) except when scaled to brine volume ($W = 618$, $p < 0.05$).

The mean ($\pm\ \sigma_M$) particle abundance in Franklin Bay sea ice horizon IV was $4.7 \pm 1.1\times10^5$ ml^{-1} ice ($n = 6$), a factor of 5.7× greater than the concentration in horizon I. In

the near-shore Angaguk sea ice (horizons I–IV, n = 12), the mean particle abundance was 2.2 ± 0.6×10^5 particles ml^{-1} ice, significantly different from the equivalent mean of 0.7 ± 0.2×10^5 particles ml^{-1} ice in Franklin Bay horizons (t = 2.5, p < 0.05, t test).

Total bacterial abundance

Bacterial abundance in Franklin Bay sea ice horizons I, II, and III (Fig. 1.6) ranged from 3.6×10^3 to 3.4×10^6 cells ml^{-1} ice and 2.3×10^5 to 2.0×10^8 ml^{-1} brine (Table 1.3). The bacterial distribution in horizon I (only) was significantly positively skewed (with a mean value 3.3× the median when scaled to ice volume), due to the presence of a number of outliers, including extreme outliers at 5, 7, 8, 25, and 87 times the IQR from Q3. The highest outlier alone caused a doubling of the mean from 7.5 to 16×10^4 bacteria ml^{-1} ice.

Bacterial abundance decreased significantly with time in horizons I and II (Figs. 1.6A and 1.6B) when scaled to ice volume (Table 1.4), but not to brine volume (Table 1.5). Cell loss rates (± 95% confidence intervals) of 500 (± 290) and 250 (± 170) cells ml^{-1} ice d^{-1} were calculated for horizons I and II, respectively, for overall losses of ~49% and 38% during the ice-sampling period. Cells were retained in the ice in similar proportions to salt in horizons II (0.94 ± 0.38) and III (1.01 ± 0.47), but in horizon I the mean retention for cells was greater (4.4 ± 14.9, median = 1.2) due to the outliers described above. Assuming cells acted conservatively with respect to bulk salinity (as found above for horizons II and III), then 50% and 57% of the cell loss over time could be attributable to brine expulsion from horizons I and II, respectively. Considering the measured retention of cells in horizon I, then 11% or 42% of the cell loss could be attributable to brine expulsion, using the mean or the median as the estimate, respectively. Given these calculations, it is surprising that bacterial abundance correlated positively with bulk salinity only in horizon III (Table 1.4).

Significant differences in median cell abundance with depth in the ice were detected whether scaled to ice volume (H = 12, p < 0.01, Kruskal-Wallis test) or to brine volume (H = 19, p < 0.001, Kruskal-Wallis test). Follow-up Wilcoxon rank sum tests indicated that horizon I contained more cells than both horizon II (W = 1066, p < 0.01) and horizon III (W = 1110, p < 0.01), but horizon II did not contain more cells than horizon III (W = 684, p > 0.1) even when scaled to brine volume (W = 895, p > 0.1).

The mean (± σ_M) bacterial abundance in the uppermost horizon (IV) of Franklin Bay sea ice was 12 ± 3.5×10^4 ml^{-1} ice (n = 8), not significantly different from the outlier-adjusted

mean of horizon I (7.5 ± 1.7×10^4 bacteria ml^{-1}; t = 1.1, p > 0.1, t test). In Angaguk sea ice, the mean bacterial abundance for all four horizons (I–IV, n = 12) sampled was 7.6 ± 0.7×10^4 ml^{-1} ice, which did not differ from the mean of 8.0 ± 2.7×10^4 bacteria ml^{-1} ice for the equivalent Franklin Bay horizons (t = 1.6, p > 0.1, t test).

pEPS content

Concentrations of particulate EPS (pEPS) in Franklin Bay sea ice horizons I, II, and III ranged from 13 to 860 μg XGEQV L^{-1} ice (Fig. 1.7), or 0.7 to 99 μg XGEQV ml^{-1} brine (Table 1.3). The pEPS distribution was positively skewed in horizons I and II with mean values of 2× and 1.3× the median, respectively, when scaled to brine volume, and extreme outliers at 5 and 8 times the IQR from Q3 in horizon I and a single extreme outlier at 5 times the IQR from Q3 in horizon II.

When scaled to either ice (Table 1.4) or brine volume (Table 1.5), pEPS concentrations in all three horizons increased significantly with time and with decreasing temperature. Accumulation rates (± 95% CI) of 1.6 (± 1.1), 1.9 (± 0.7), and 1.4 (± 0.5) μg XGEQV L^{-1} ice d^{-1} were calculated for horizons I, II, and III, respectively (Fig. 1.7A–C). Additionally, pEPS in the upper horizons, scaled to ice volume (Table 1.4), and in all horizons scaled brine volume (Table 1.5) increased significantly with decreasing bulk salinity.

Differences in median pEPS concentration with depth were significant only when scaled to brine volume (H = 6.9, p < 0.05, Kruskal-Wallis test), not ice volume (H = 5.2, p < 0.1, Kruskal-Wallis test). Follow-up Wilcoxon rank sum tests indicated that horizon I contained a significantly higher median pEPS concentration (scaled to brine volume) than horizon III (W = 853, p < 0.01), but no differences were detected between horizons I and II (W = 693, p > 0.1) or horizons II and III (W = 739, p < 0.1).

The mean (± σ_M) pEPS abundance in the upper horizon (IV) of Franklin Bay sea ice was 260 ± 120 μg XGEQV L^{-1} ice (n = 6), which did not differ from the mean of horizon I (160 ± 30 μg XGEQV L^{-1} ice; t = −0.90, p > 0.1, t test). In Angaguk sea ice (horizons I–IV, n = 4), the mean pEPS abundance was 440 ± 290 μg XGEQV L^{-1} ice, also not different from the mean of 120 ± 25 μg XGEQV L^{-1} ice for the equivalent Franklin Bay horizons (t = −1.3, p > 0.1, t test).

Overall correlations

Other correlations, particularly those among the particulate variables (particles, bacteria, and pEPS) were scattered and generally weak (Tables 1.4 and 1.5). The large number of correlations calculated here warrants careful scrutiny of perceived significance because the results have not been explicitly corrected for multiple comparisons. If the generally overly-conservative Bonferroni correction (Zar, 1999) were applied in this study, correlations with $p < 0.001$ (38% and 58% of the significant correlations in Tables 1.4 and 1.5, respectively) would be upheld. This analysis reiterates the finding by Junge et al. (2004) that correlations among microbially relevant variables scaled to brine volume are stronger than those when scaled to ice volume.

1.5 Discussion

In this study, the unique overwintering expedition we joined allowed us to test whether the abundance of microbial communities encased in first-year sea ice changes significantly as winter progresses or whether the increasingly severe conditions in their interior brine habitats instead act to preserve the microorganisms. Measured and calculated profiles of biologically-relevant physical parameters including air (Fig. 1.2) and ice temperature (Fig. 1.3(a)), brine salinity (Fig. 1.3(b)), bulk salinity (Fig. 1.3(c)), and brine volume fraction (Fig. 1.3(d)) documented the extreme conditions confronted by the biotic component of winter sea ice. They also demonstrated the presence of spatial variability on the order of decimeters and temporal dynamics on the order of days to weeks.

1.5.1 Spatial variability

Full depth profiles of winter ice showed that the concentrations of inanimate particles (Fig. 1.4A) and total bacteria (Fig. 1.4B) were highest in the surface ice and decreased significantly with depth in the core. This trend was corroborated by the differences in mean concentrations of both parameters among the selected horizons sampled during the study: particle and bacterial content of the upper ice horizons (IV and I) were significantly higher, by 3–34× and 4–9×, respectively, than of the lower ice horizons II and III. Other studies of the vertical distribution of bacteria in sea ice have typically focused on the warmer seasons, when bacterial maxima are associated with ice-algal populations in lower portions of the ice (Gosink et al., 1993; Gradinger and Zhang, 1997). In a year-round study of Antarctic sea

ice, however, distributions more similar to those reported here were observed in the winter months (biomass maxima in upper ice), even if the vertical gradients were only coarsely resolved and no accompanying physical parameters were reported (Delille et al., 2002).

Observations of particle and bacterial maxima in surface ice and numbers decreasing with depth can be explained by a combination of two factors: 1) the disproportionate incorporation of particles and bacterioplankton by frazil ice scavenging during early sea ice formation, as has been observed or discussed by others (Garrison et al., 1989; Grossmann and Gleitz, 1993; Grossmann and Dieckmann, 1994; Grossmann, 1994; Gradinger and Ikavalko, 1998; Eicken et al., 2005); and 2) higher concentrations of particles and bacterioplankton in the source waters for ice formed in autumn (horizons IV and I), for example by particles originating from the Horton River or the Smoking Hills, with diminishing numbers as winter progressed (and the ice sheet grew to include horizons II and III and lower). The second factor is supported by comparison of mean bacterioplankton abundance before (when it was higher) and after the start of the winter ice-sampling in this study. Winter reductions in bacterioplankton abundance have also been reported for surface waters north of our study site (Sherr et al., 2003). Additionally, higher resolution sampling by others (Alonso-Sáez et al., 2008) detected a trend of increasing bacterioplankton abundance towards the end of winter that we were not able to detect in our limited set of seawater samples. This increase might explain the (statistically weak, $p < 0.1$) increase in bacterial (though not particle) abundance in the most recently formed bottom layers of the longest of the three full-depth cores, taken on day 88. An alternative explanation in light of the continuing decrease of inanimate particle counts in the same ice (Fig. 1.4A) is in situ bacterial growth, perhaps benefiting from fresher pigment content in the bottom ice. Without multiple cores to assess variability in these bottom ice parameters, however, we cannot resolve the issue.

In contrast to the particle and bacterial trends, the full depth profile of pEPS content failed to indicate a vertical gradient in the ice (Fig. 1.4C); only a weak gradient was observed when scaled to brine volume (data not shown), which may imply differential accumulation rates with depth. The maximum pEPS concentration appeared in the upper ice (above the horizons selected for time-series analyses), in keeping with disproportionate particle entrainment during early ice formation, but the remaining pEPS concentrations indicated relative constancy with depth. Although nearly corresponding peaks in pEPS and pigments may suggest an isolated algal source at 15 cm in the upper ice, the overall scarcity of diatoms

in microscopic analyses and very low pigment values argue against pEPS production by sea-ice algae in the cores we examined, as has been invoked for other winter sea ice samples of considerably higher pEPS content (Krembs et al., 2002).

Besides variability with depth in the ice, spatial heterogeneity in the horizontal dimension was indicated by the presence of extreme outliers in the particulate data sets from the upper ice horizons sampled throughout the study (Figs. 1.5, 1.6, 1.7). Although the highest particle, bacterial, and pEPS loads were not always concurrent in the ice (and did not drive correlations with time), their disproportionate occurrence within the upper ice horizon can again be explained as the combined result of rapid freezing and scavenging of a patchy distribution of particle-rich seawater into young ice followed by a transition to slower, more orderly growth of congelation ice (in the lower depth horizons) from relatively particle-poor seawater. High spatial variability has been observed previously on spatial scales relevant to our sampling scheme in both Arctic (Gosselin et al., 1986; Rysgaard et al., 2001; Granskog et al., 2005) and Antarctic sea ice (Eicken et al., 1991), but not for either a bacterial or winter data set that highlights upper very cold ice. A specific example of marked spatial heterogeneity in our study is found on day 60, when the overall maximum particle abundance and pEPS concentration were recorded, as were high bacterial abundance and unusually low bulk salinity. The detection of outliers of bacterially relevant parameters in this study suggests the presence of infrequent but distinctive anomalies in the ice that may offer unusual overwintering habitats to the entrained microorganisms. Testing this idea would require simultaneous analysis of multiple bacterial and microstructural features of unmelted winter sea ice, a conceivable prospect given the work of Junge et al. (2001), Krembs et al. (2002), and Stierle and Eicken (2002).

1.5.2 Temporal dynamics

The standard scaling of material abundance to bulk ice parameters (whether ice melt, area, or volume Horner et al., 1992; Junge et al., 2004) serves the purpose of evaluating large-scale patterns, as in this and many other studies. Individual bacteria, however, operate on a vastly smaller scale in winter sea ice brines than can be represented by bulk analyses. Absent microscopic analyses of unmelted ice (as in Junge et al., 2001), we relied upon comparative results of different scalings, to total volume of ice versus the volume of brine in the ice (as in Junge et al., 2004) for insight on temporal changes at the bacterial scale.

We found that bacterial abundance scaled to ice volume decreased significantly (Table 1.4) over time in the upper colder ice horizons sampled (Fig. 1.6A and B), but not in the lower warmer one (Fig. 1.6C). Krembs et al. (2002) estimated losses of similar magnitude at −15°C from a much smaller dataset of laboratory ice incubations. Further calculations, based on the assumption that our 2003 bacterioplankton measurements accurately reflected source waters, indicated that sufficient brine expulsion may have occurred during the course of the study to account for up to 57% of these losses, depending on the ice horizon. We had expected any advective losses from the very cold ice horizons of this study to be negligible or undetectable based on evidence that the bulk permeability of sea ice is very low to nonexistent at brine volume fractions of less than 5%, which is the case at temperatures below −5°C and bulk salinities of 5‰ and higher. Whether or not brine (and bacterial) expulsion actually occurred during the winter, a portion of the cell losses must still be attributable to mortality within the ice. In contrast, when bacterial abundance was scaled to brine volume, the concentration of cells did not change over time (Table 1.5). From the perspective of the individual bacterium, its "neighborhood" population remained constant through the winter. The suggestion of a steady-state concentration of cells in the brine is intriguing from a number of perspectives, including the possibility of viral lysis or quorum sensing at very low temperature and high salinity, but the mechanism(s), physical or biological, responsible for steady-state populations in these cold brines are not known.

As a biological explanation for within-ice bacterial losses, top-down control by protozoan grazers during our study is not likely, given spatial restrictions on grazers in the pores of very cold winter sea ice (Eicken, 1992; Krembs et al., 2000) and the absence of any observations to our knowledge of active heterotrophic eukaryotes in winter sea ice. Delille et al. (2002) reported the presence of significant protozoan biomass in Antarctic sea ice surface layers during early austral winter but without corresponding measures of ice conditions (temperature or salinity) or grazing activity. Dinoflagellate cysts have sometimes been observed in upper horizons of Antarctic sea ice, but excystment has only been reported to take place at temperatures warmer than −9°C and corresponding brine salinities <140‰ (Stoecker et al., 1997, 2000), conditions not met in the upper horizons of the ice that we studied.

A more likely biological mechanism for explaining bacterial loss is the lysis of cells by viruses, as suggested by Wells and Deming (2006) in a study parallel to ours. They reported

the presence of abundant (2–82×10^6 ml^{-1} brine) viruses in winter sea ice collected from the same designated ice field in March 2004. From their week-long experimental incubations of isothermal-isohaline ice melts (limited logistically to a temperature of −12℃), periodic decreases in bacterial abundance at approximate rates of 10^4–10^5 cells ml^{-1} ice d^{-1} can be estimated. If these decreases were due to cell lysis by viruses, as the authors implied, then our much lower estimated decreases of <500 cells ml^{-1} ice d^{-1} could be explained readily by viral activity. Although we did not measure viral abundance over time in the ice to address this hypothesis directly, we used their diffusion-based model from which the effects of decreasing temperature and brine volume fraction on the encounter rate between viruses and bacteria in winter sea ice brines could be calculated. The resulting potential contact rates for conditions in the upper ice we sampled (horizon I) averaged 2400× the rate for underlying seawater at −1℃.

Another set of mechanisms to account for cell loss includes physical stressors in the ice. Low temperature and high salinity are frequently used to preserve foods and other perishable goods given their inhibitory effects on microbial activity and growth, but they are not generally useful for lysing microbial cells. On the contrary, we might expect melting and freshening of sea ice to be more stressful for the microbial community than freezing because lower salinity and higher temperature (as characterize surface melt ponds on Arctic sea ice, which eventually drain and flush salt from the system) both cause cell lysis in cold-adapted marine bacteria (Morita, 1975). Additional causes of cell lysis in winter sea ice may be impingement of ice or salt crystals, causing physical disruption of cell walls. Although we were not able to evaluate such effects directly, we did measure the concentration of pEPS, in part due to earlier indications that pEPS protect ice-algal cells against the impingement of ice crystals under winter conditions (Krembs et al., 2002; Meiners et al., 2003).

We found a strong positive correlation between pEPS concentration and time in Arctic winter sea ice (Fig. 1.7, Tables 1.4 and 1.5), with an estimated net production of a few percent per day. The mean pEPS concentration in our ice horizons (117 μg XGEQV L^{-1}) was lower than the mean measured in the bottom 4 cm of the ice (185 μg XGEQV L^{-1}) by Riedel et al. (2006) in March during the same cruise, and much lower than that measured by the same authors later in the season (maximum of ~10^4 μg XGEQV L^{-1}) during high ice-algal production and by Krembs et al. (2002) in coastal Chukchi Sea ice in February (>10^3 μg XGEQV L^{-1}). Based on cell abundance and a cell-specific production factor of 4 amol C

cell^{-1} h^{-1} (Stoderegger and Herndl, 1999) we estimated bacterial pEPS production in upper ice horizons of ~3 µg C L^{-1} ice d^{-1}. We are unable to confidently compare these rates to others in the literature (Meiners et al., 2004; Riedel et al., 2006) due to a misprint in the conversion factor used in the other studies, but we note that their conclusion that bacterial pEPS production is negligible in sea ice may need to be reconsidered after multiplication of published production rates by a factor of 24. Also, although we cannot exclude the possibility that dissolved EPS released by ruptured cells aggregated and contributed to the pEPS we measured, evidence against the extracellular conversion of DOC to pEPS in winter sea ice has been reported (Krembs et al., 2002) and dissolved EPS was undetectable in our samples.

This is the only study of its kind in Arctic winter sea ice, but several Antarctic studies have provided information on the microbial content of sea ice at shallow stations (10–20 m water depth) throughout an annual cycle (Delille and Rosiers, 1996; Delille et al., 2002; Fiala et al., 2006). Generally expected swings in bacterial abundance were reported by Delille and Rosiers (1996), especially for lower portions of the ice, including greater abundances in newly formed ice, winter minima, and spring maxima. Because these authors binned their data, however, reporting only means and standard deviations on a monthly basis, distinguishing spatial from temporal variability in the upper ice horizons relevant to our study is not possible. More problematic for comparative analysis is that none of these Antarctic studies reported temperature in the ice or its bulk salinity. In the case of Delille and Rosiers (1996), the relatively thick snow cover and thin ice (maximum of ~ 1.2 m) often found in the Antarctic would imply warmer temperatures than at our Arctic site (with ice up to 1.8 m thick, <5 cm snow, and air temperature minima of <−33°C). In the case of Delille et al. (2002), they reported thin snow cover (<2 cm, but up to 50 cm) and air temperatures down to −30°C at the near-shore Antarctic study site where they found enriched protozoan and microalgal populations in the upper ice during ice formation and decreasing abundances starting in July and August, when conditions would likely have been most similar to those during our study. In the case of Fiala et al. (2006), a winter air temperature minimum of −30°C with little snow cover was reported in another near-shore Antarctic study site. These authors, who measured aerobic heterotrophic bacterial colony forming units (CFU; growth at 4°C) and various eukaryotes, found an association between CFU and algae in the ice, attributing a decrease in CFUs with decreasing algal biomass to

a nutrient-limitation response. For their station (D) most similar to our Franklin Bay site, based on its distance from shore and low chlorophyll a biomass (<1 µg L^{-1} in the upper half of the ice), they reported CFUs that may have decreased somewhat over the winter before increasing to their maximum values in the spring, along with low or undetectable abundances of protozoa and ciliates (<5 ml^{-1} in either case) with higher concentrations lower in the ice. Given the relative infrequency of sampling, lack of variability estimates and other statistical considerations, unconstrained physical conditions, and methodological differences, systematic comparisons among these studies are not possible but appear to converge on the general phenomenon that initial elevations in bacterial abundance in young ice decrease over the course of the winter.

1.6 Conclusions

Our results have shown the particular importance of acquiring and reporting physical parameters, particularly temperature and bulk salinity, for the subsequent analysis of microbiological data from sea ice. In winter sea ice, more so than in summer ice, understanding the microbial ecology requires a parallel appreciation of the physical context of the ice and its strong gradients. When scaled to ice volume we found significant gradients of bacterial concentrations in the ice with depth and time, with both the highest mean concentrations and greatest losses occurring in the upper, colder and more saline ice. When scaled to brine volume, however, no apparent change was detected in bacterial concentration over time at any depth we studied, though the change in concentration with depth was magnified. In spite of bulk cell losses, individual organisms do not appear to experience a drop in neighboring numbers and a sizable fraction of the encased population persists through winter. In possible aid of this persistence was the observed in situ production of particulate EPS, presumably by bacteria responding to the combined extremes of low temperature and high salinity that challenge them each Arctic winter season, at least at the start of this century.

1.7 Acknowledgments

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Table 1.1: Physical parameters for the three ice horizons centered at depths of 25 (I), 45 (II) and 65 (III) cm over sampling days 10–88.

		Ice parameters		Brine parameters	
Section		Temperature (℃)	Bulk salinity (‰)	Salinity (‰)	Volume (% of ice)
I	range	−26 to −15	2.8–5.9	180–230	0.6–2.1
	mean (median)	−20 (−20)	4.6 (4.6)	210 (210)	1.5 (1.6)
	n	71	16	16	16
II	range	−22 to −15	3.3–5.4	170–220	1.2–2.2
	mean (median)	−17 (−16)	4.6 (4.6)	190 (180)	1.8 (1.9)
	n	71	16	16	16
III	range	−18 to −9	3.4–5.2	140–200	1.4–3.0
	mean (median)	−13 (−12)	4.4 (4.3)	160 (160)	2.1 (2.1)
	n	71	16	16	16

Table 1.2: Correlation coefficients (Pearson's r) among physical parameters for the three ice horizons. Not significant (ns) indicates p ≥0.05. Bold indicates most significant relationships.

		Day	p	Temperature	p
Bulk salinity	I	−0.53	<0.05	0.31	ns
(n = 16)	II	−0.67	<0.01	0.38	ns
	III	−0.71	<0.01	0.51	<0.05
Temperature	I	−0.46	<**0.001**		
(n = 71)	II	−0.73	<**0.001**		
	III	−0.84	<**0.001**		

Table 1.3: Particulate parameters for the three ice horizons over sampling days 10–88.

Section		Particles		Bacteria		pEPS	
		$\times 10^3$ ml^{-1} ice	$\times 10^5$ ml^{-1} brine	$\times 10^3$ ml^{-1} ice	$\times 10^5$ ml^{-1} brine	μg XGEQV L^{-1} ice	μg XGEQV ml^{-1} brine
I	range	5.7–1300	27–1500	3.6–3400	42–2000	20–860	1.1–99
	mean (median)	83 (22)	85 (19)	160 (48)	110 (35)	160 (110)	14.7 (7.5)
	n	27	27	40	40	35	35
II	range	6.4–150	3.6–66	5.7–72	3.0–42	13–540	0.7–30
	mean (median)	25 (18)	14 (10)	36 (33)	21 (18)	110 (80)	7.1 (5.4)
	n	34	34	38	38	34	34
III	range	8.0–21	4.6–11	4.2–94	2.3–45	19–190	0.8–13
	mean (median)	14 (13)	8 (8)	37 (39)	18 (18)	77 (70)	4.3 (4.8)
	n	17	17	41	41	36	36

Table 1.4: Correlation coefficients (Spearman's ρ) among particulate parameters scaled to ice volume for the three ice horizons. Not significant (ns) indicates p ≥0.05. Bold indicates most significant relationships.

Parameter		Particles	p	Bacteria	p	pEPS	p
Day	I	−0.18	ns	−0.41	<0.01	0.45	**<0.001**
	II	−0.50	<0.01	−0.45	<0.01	0.71	**<0.001**
	III	−0.35	ns	−0.27	ns	0.62	**<0.001**
Temperature	I	0.07	ns	0.22	ns	−0.42	**<0.001**
	II	0.30	ns	0.28	ns	−0.73	**<0.001**
	III	0.50	<0.05	0.20	ns	−0.61	**<0.001**
Bulk salinity	I	−0.16	ns	0.23	ns	−0.48	<0.01
	II	0.48	<0.01	0.23	ns	−0.39	<0.05
	III	−0.08	ns	0.42	<0.01	−0.30	ns
Particles	I			0.06	ns	0.14	ns
	II			0.11	ns	−0.41	<0.05
	III			−0.21	ns	−0.11	ns
Bacteria	I					−0.24	ns
	II					−0.42	<0.05
	III					−0.16	ns

Table 1.5: Correlation coefficients (Spearman's ρ) among particulate parameters scaled to brine volume for the three ice horizons. Not significant (ns) indicates p ≥0.05. Bold indicates most significant relationships.

Parameter		Particles	p	Bacteria	p	pEPS	p
Day	I	0.07	ns	−0.02	ns	0.60	**<0.001**
	II	−0.15	ns	0.00	ns	0.75	**<0.001**
	III	−0.20	ns	0.23	ns	0.76	**<0.001**
Temperature	I	−0.35	ns	−0.20	ns	−0.63	**<0.001**
	II	0.09	ns	−0.09	ns	−0.76	**<0.001**
	III	−0.01	ns	−0.30	ns	−0.74	**<0.001**
Bulk salinity	I	−0.45	<0.05	−0.23	ns	−0.65	**<0.001**
	II	0.14	ns	−0.22	ns	−0.48	<0.01
	III	−0.55	<0.05	−0.09	ns	−0.48	<0.01
Particles	I			0.17	ns	0.46	<0.05
	II			0.05	ns	−0.08	ns
	III			−0.14	ns	0.43	ns
Bacteria	I					0.20	ns
	II					0.02	ns
	III					0.18	ns

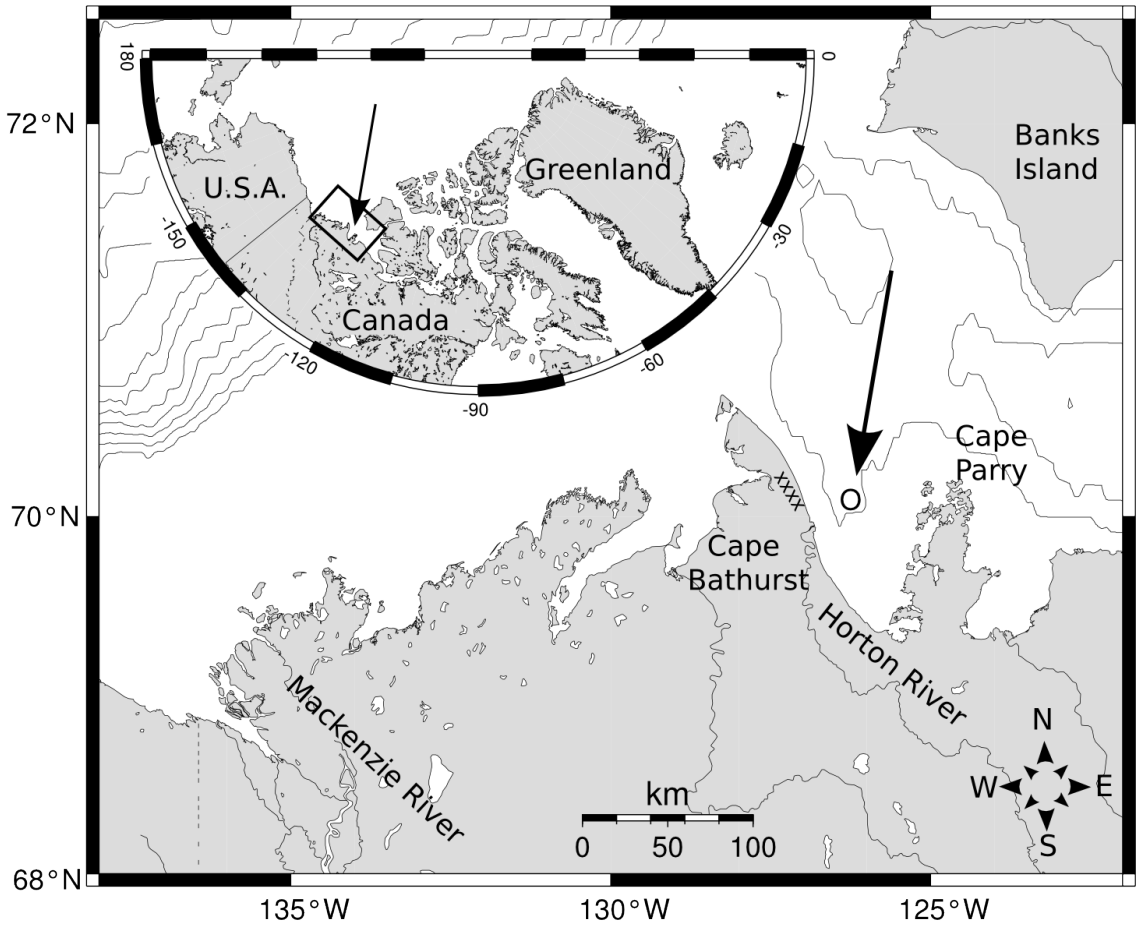


Figure 1.1: Map of Franklin Bay, bounded on the west by Cape Bathurst and on the east by Cape Parry, showing the overwintering site of the CCGS Amundsen (o) during CASES 2003–2004, the Horton River outflow, and riverine station Angaguk (A).

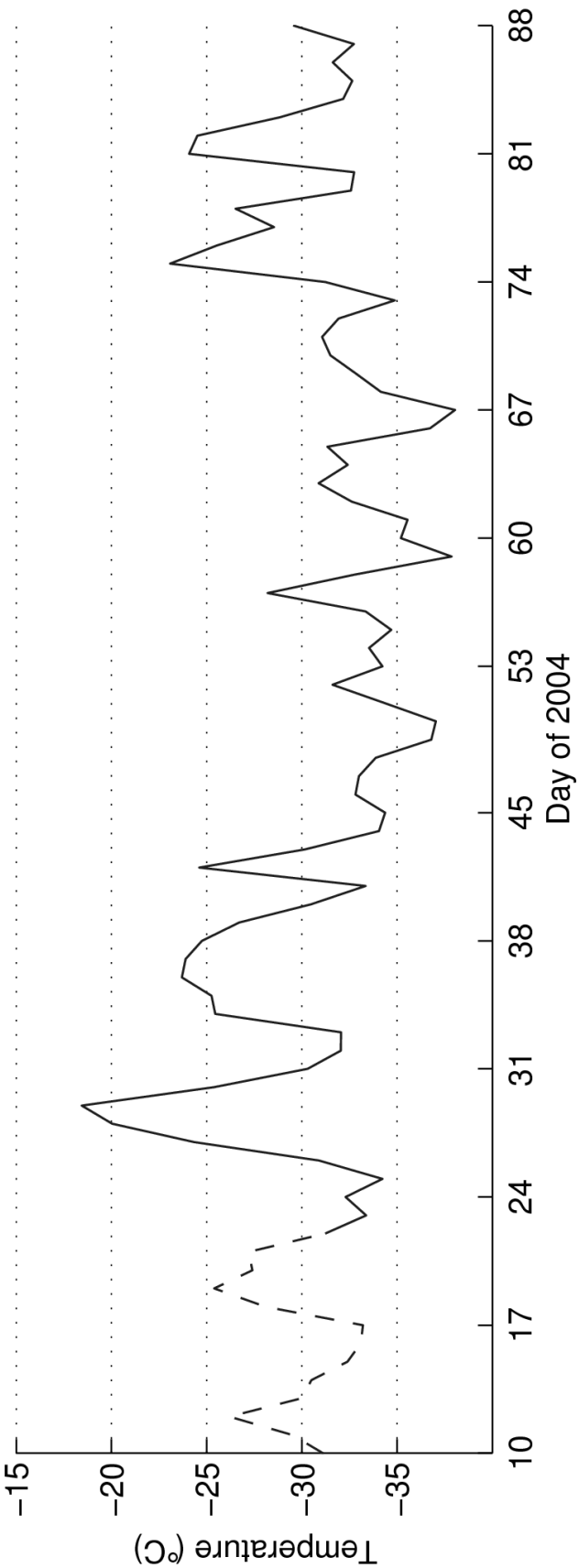
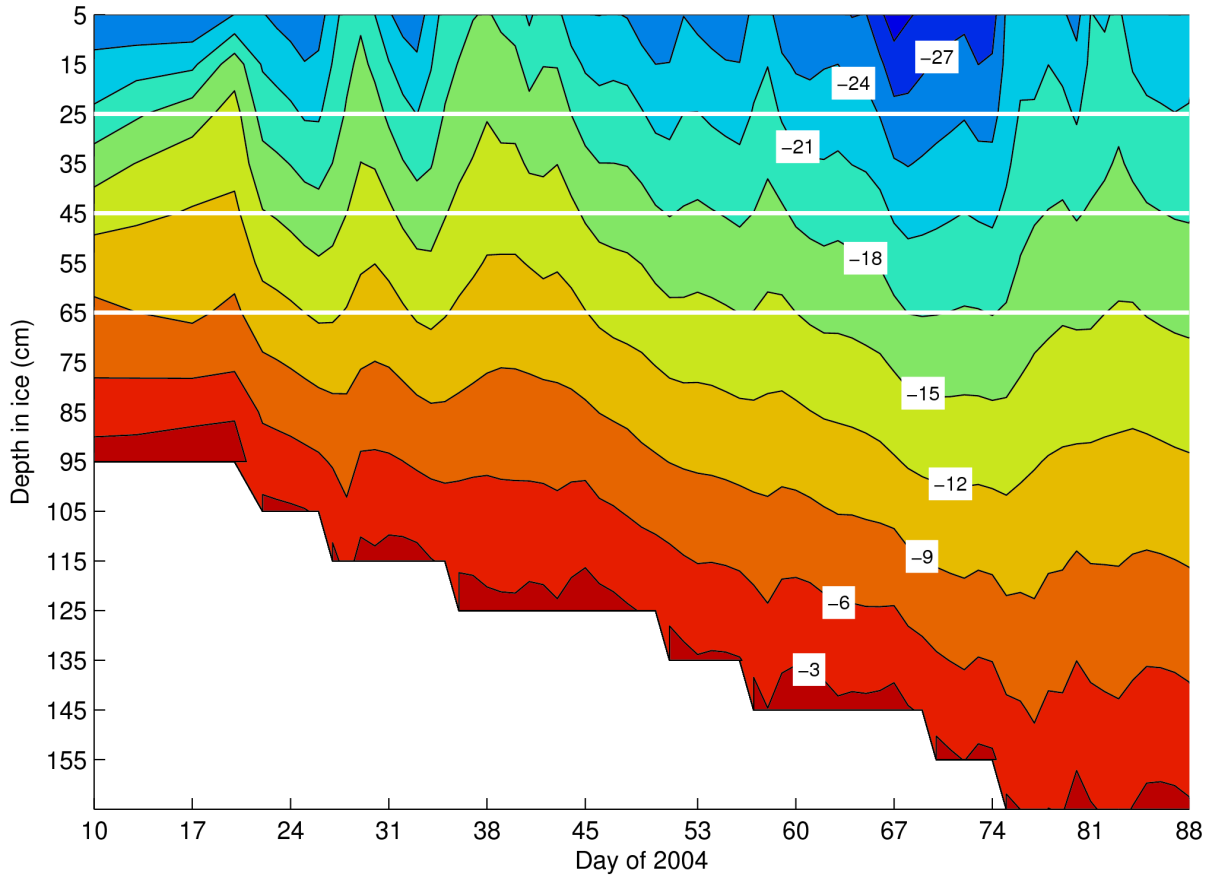
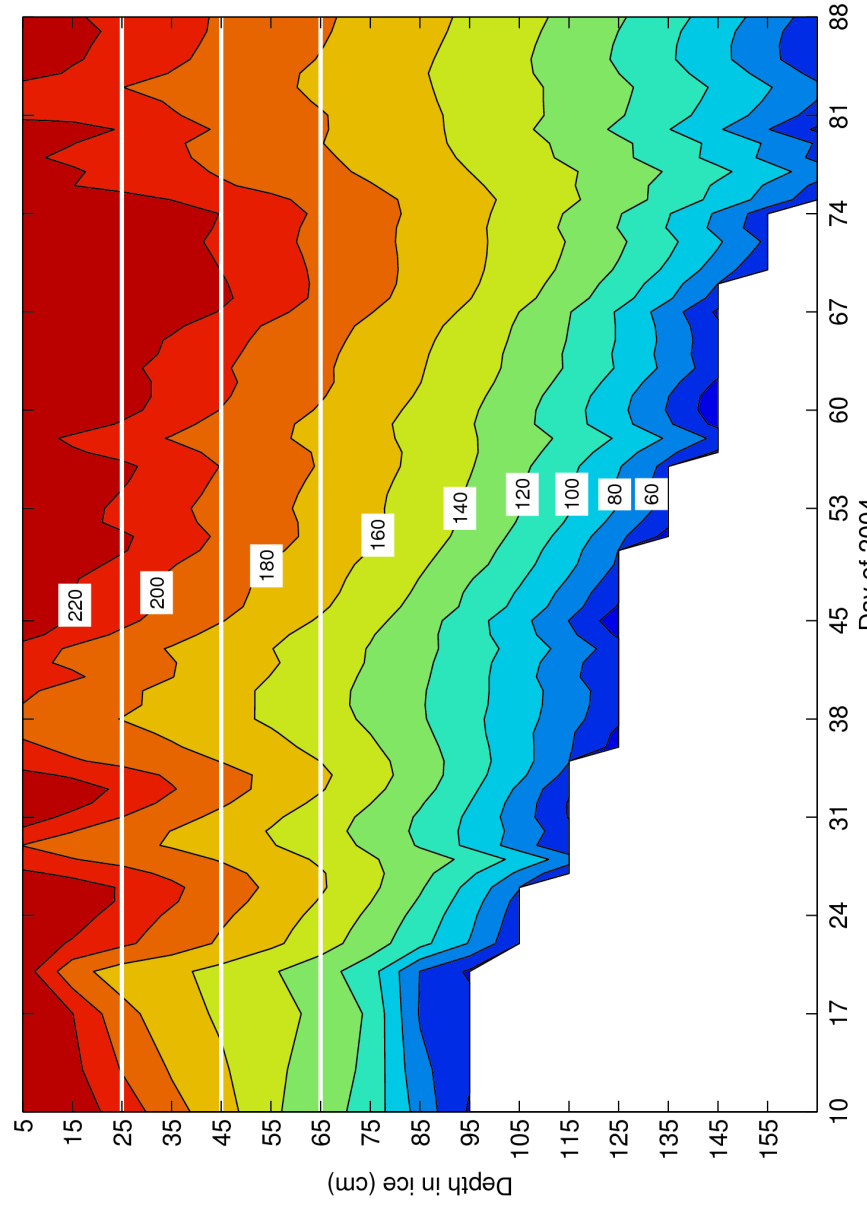


Figure 1.2: Average daily air temperature (℃) over the course of the study. Dotted line indicates air temperature as measured from bridge of ship; solid line indicates air temperature as measured by the in situ weather station.



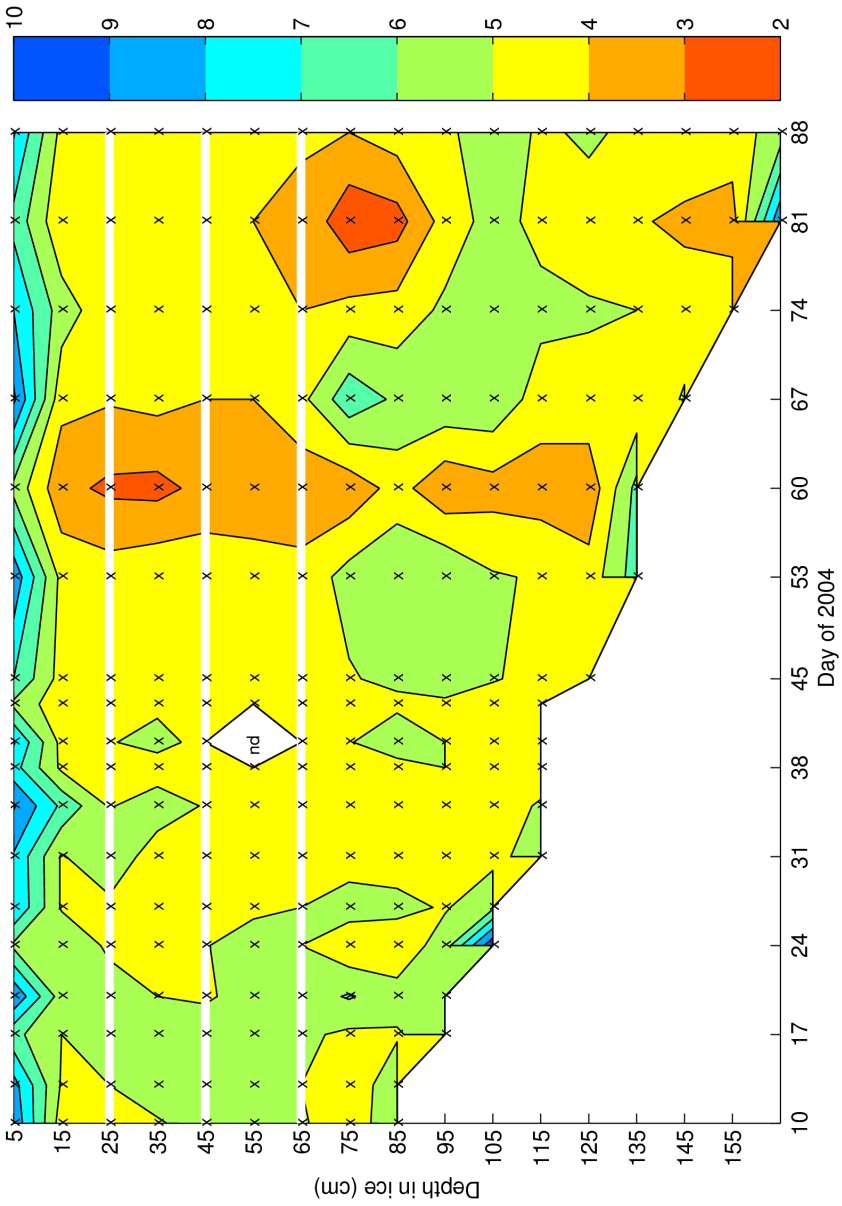
(A)

Figure 1.3: Physical conditions of the ice field over the course of the study: (A) average daily ice temperature (℃) over the course of the study; (B) brine salinity (‰; calculated from ice temperature); (C) bulk salinity (‰); (D) brine volume fraction (%; calculated from ice temperature and bulk salinity, values range up to 18% near the ice-water interface). White horizontal lines mark the center of each ice horizon (I, II, and III) selected for particulate analyses.



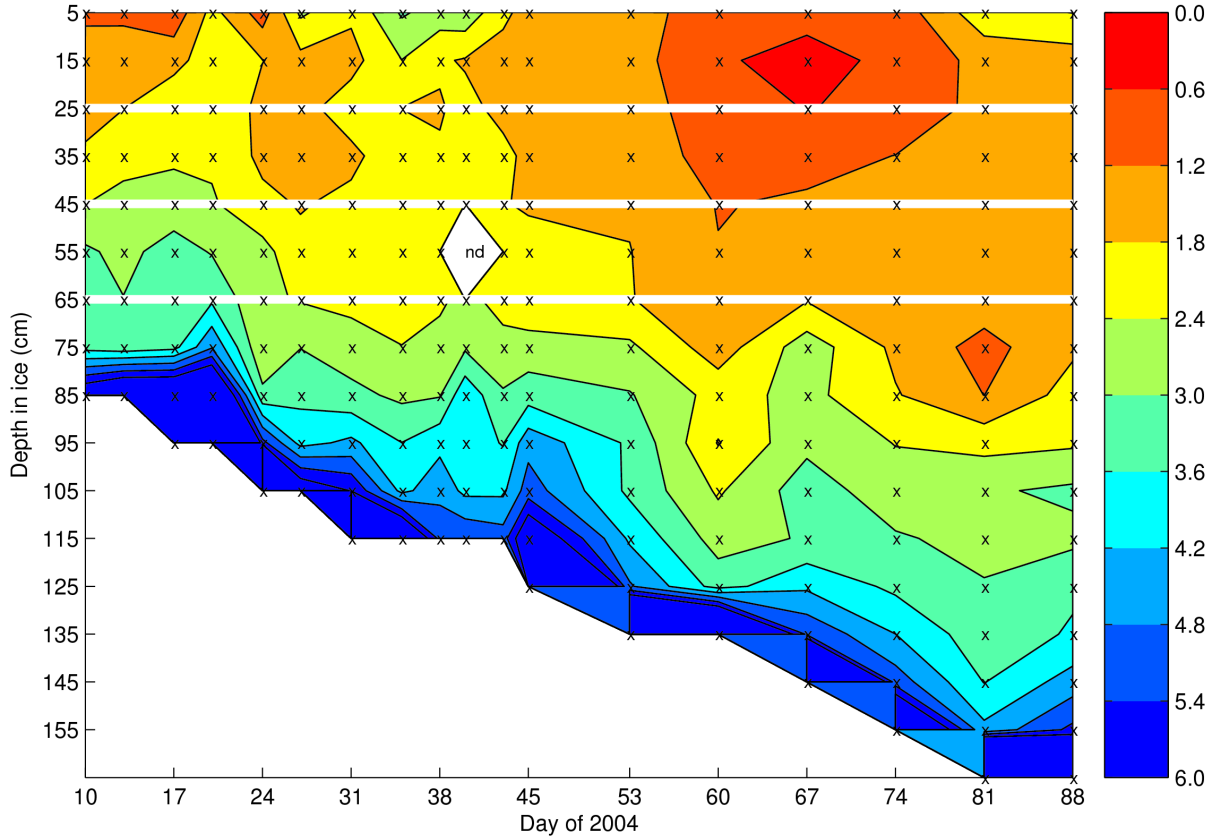
(B)

Figure 1.3: (cont.)



(c)

Figure 1.3: (cont.)



(D)

Figure 1.3: (cont.)

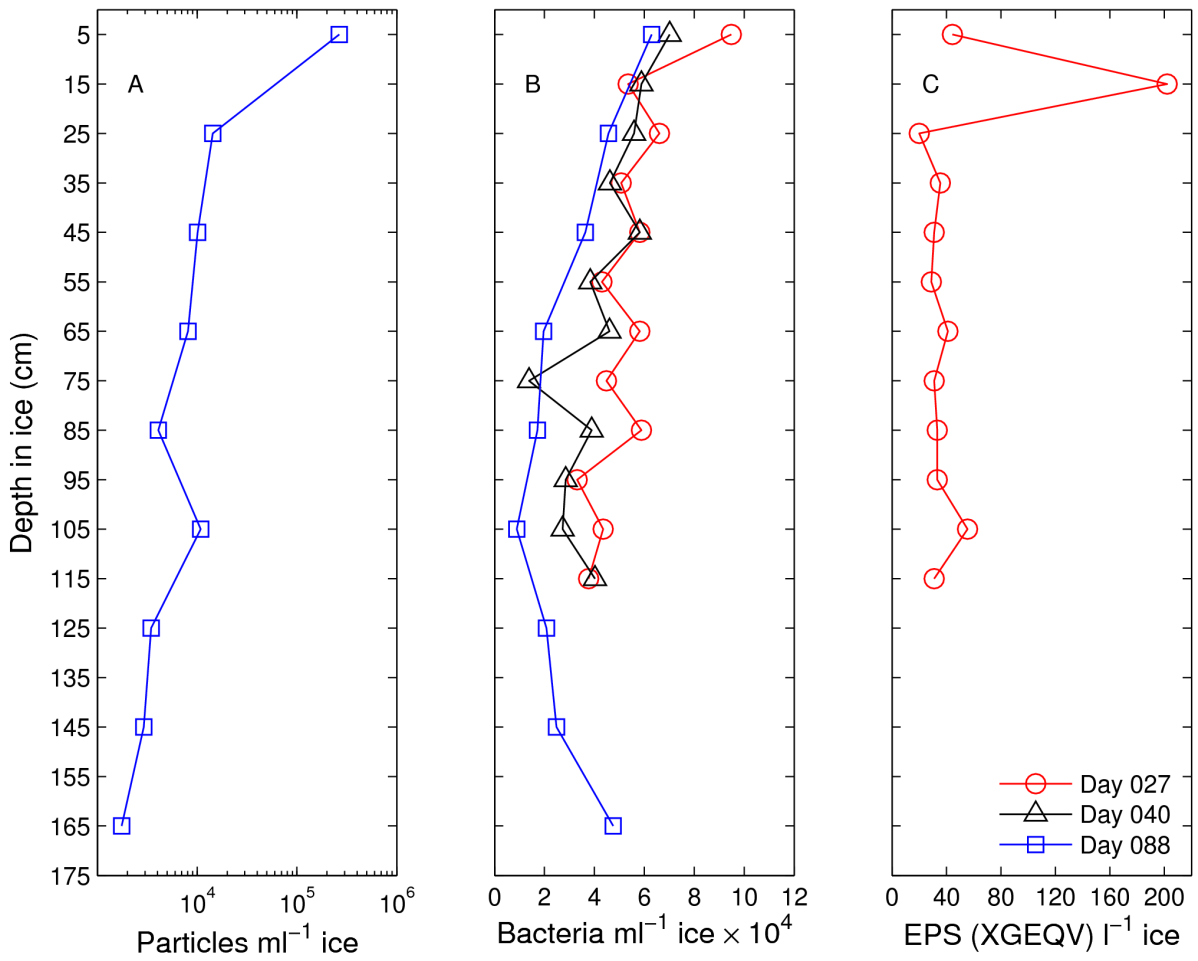


Figure 1.4: Full depth profiles as snapshots of (A) particles ml^{-1} ice on day 88, (B) bacteria ml^{-1} ice on days 27, 40, and 88, and (C) pEPS in xanthan gum equivalents (μg XGEQV) L^{-1} ice on day 27.

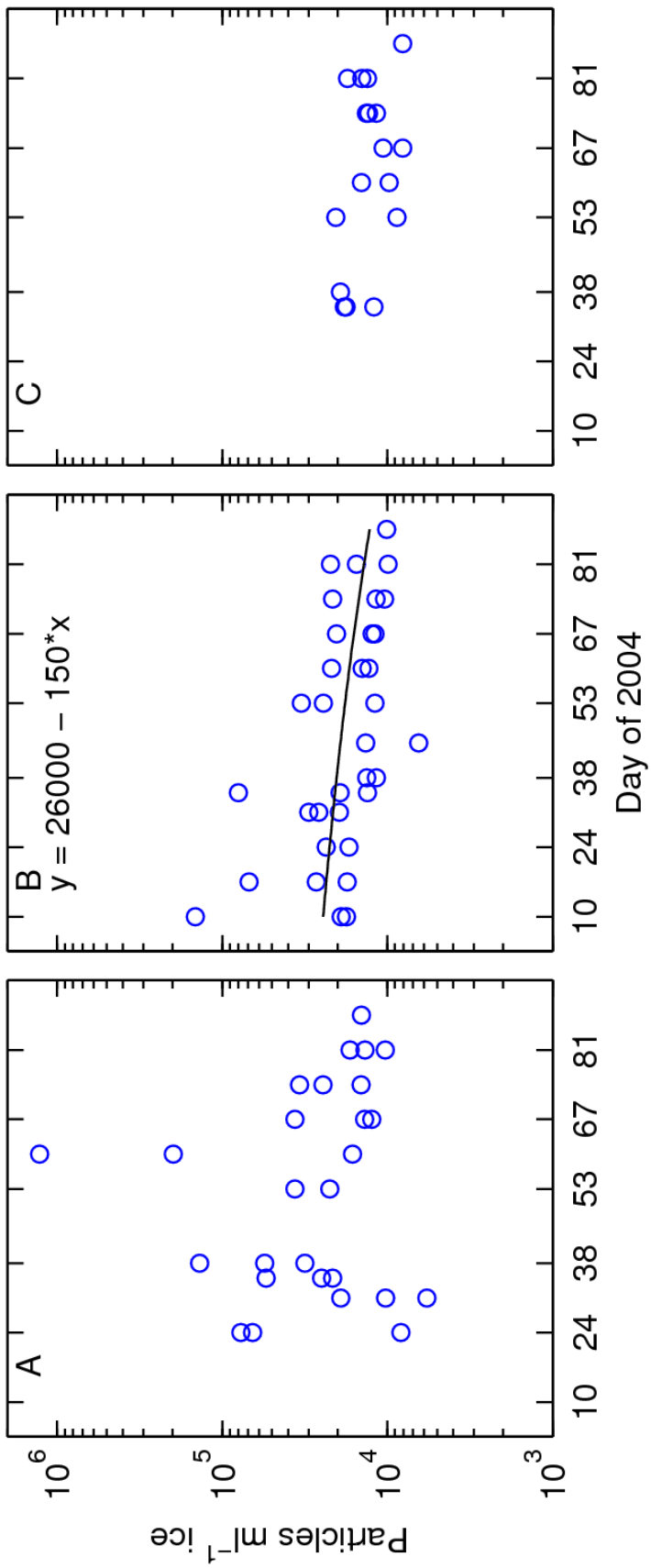


Figure 1.5: Microscopic counts of particles (ml^{-1} ice) in (A) horizon I, 25 cm, (B) horizon II, 45 cm, and (C) horizon III, 65 cm over the course of the study. A robust linear model was fitted to the data in B (solid line, equation shown above).

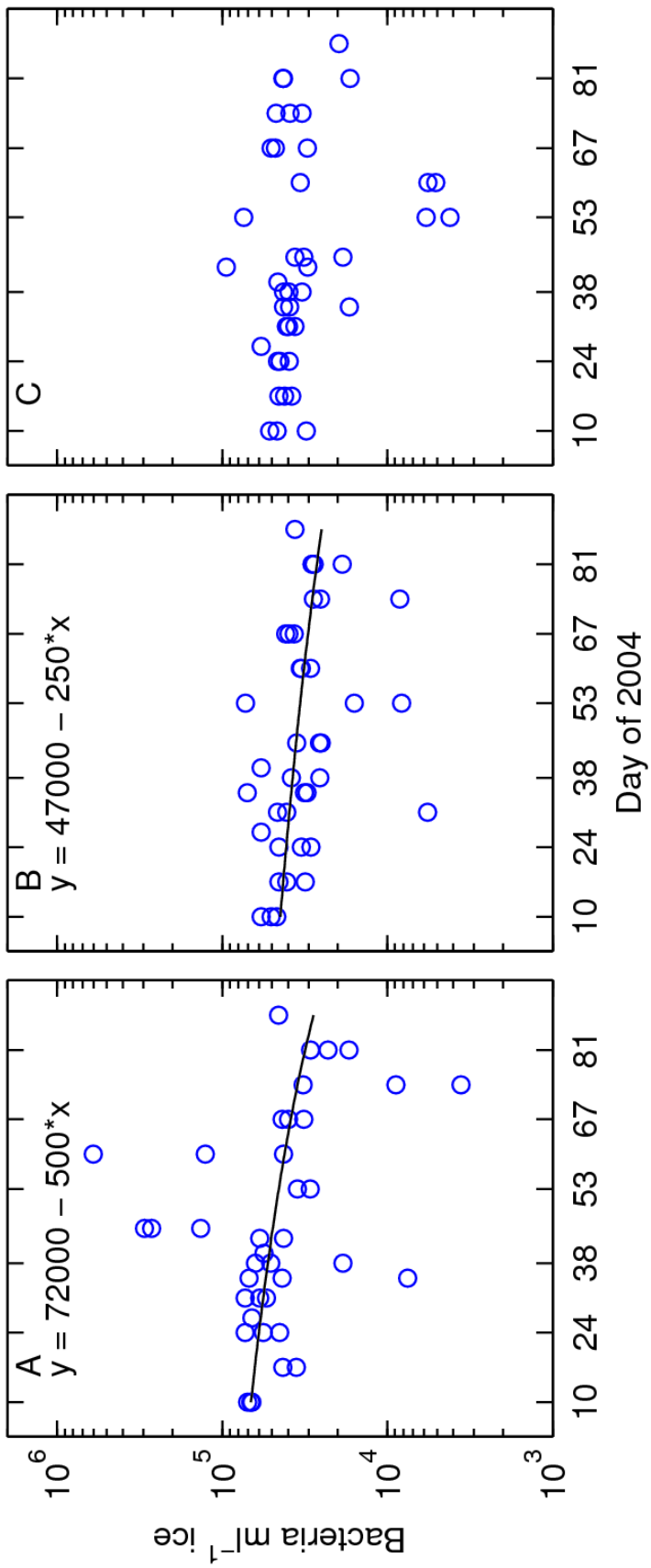


Figure 1.6: Microscopic counts of bacteria (ml^{-1} ice) in (A) horizon I, 25 cm, (B) horizon II, 45 cm, and (C) horizon III, 65 cm over the course of the study. Robust linear models were fitted to A and B (solid lines, equations shown above).

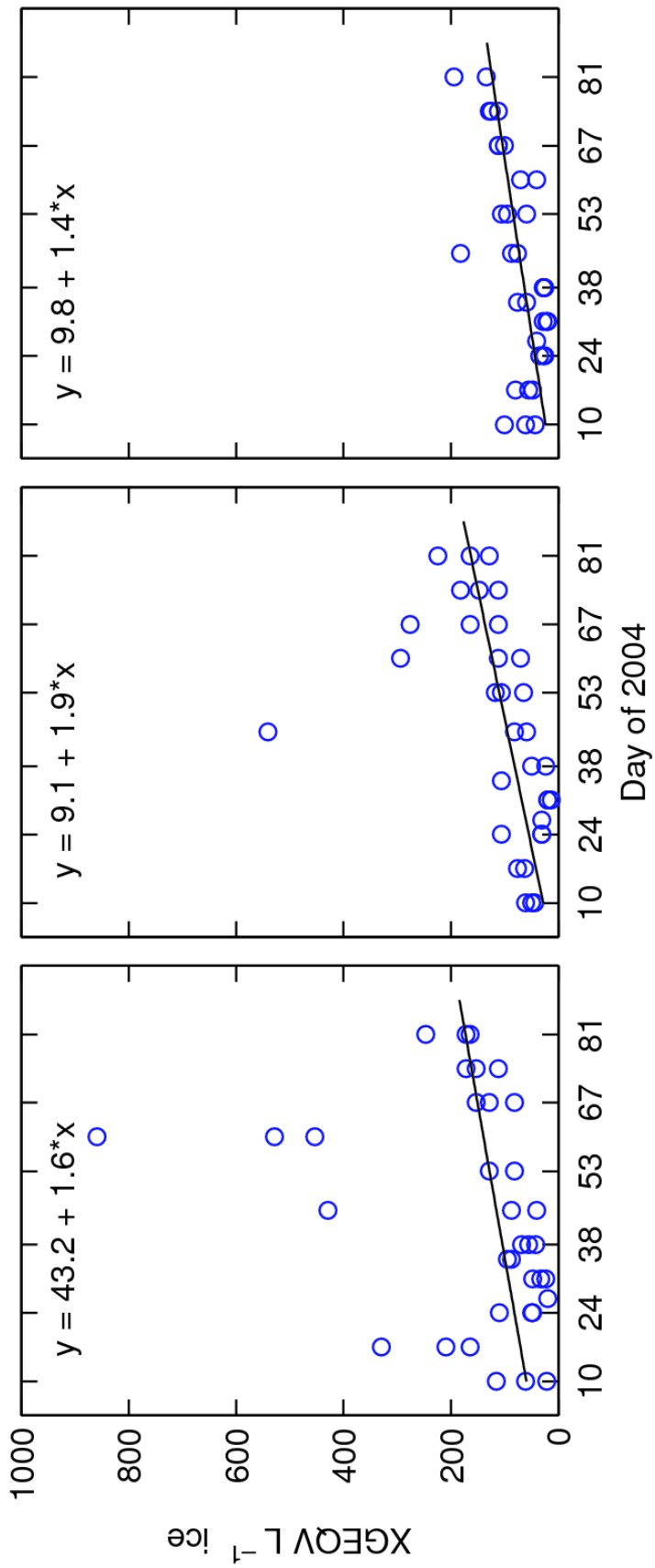


Figure 1.7: Measurements of pEPS in xanthan gum equivalents (μg XGEQV) L^{-1} ice in (A) horizon I, 25 cm; (B) horizon II, 45 cm; and (C) horizon III, 65 cm over the course of the study. Robust linear models were fitted to A, B and C (solid lines, equations shown above).

Chapter 2

PERSISTENCE OF BACTERIAL AND ARCHAEAL COMMUNITIES IN SEA ICE THROUGH AN ARCTIC WINTER

2.1 Abstract

The structure of bacteria communities in spring and summer sea ice differs from that in seawater prior to fall freeze-up, suggesting selection during ice formation or taxon-specific mortality of the ice-entrapped microorganisms during winter. We tested this hypothesis by weekly sampling (January–March 2004) of the upper horizons of first-year sea ice (Franklin Bay, Western Arctic) that experienced temperatures from −9°C to −26°C, generating community fingerprints and clone libraries for Bacteria and Archaea from this ice and underlying seawater. Despite severe conditions and significant decreases in microbial abundance, no significant changes in richness or community structure were detected in the ice. Furthermore, communities of Bacteria and Archaea in the ice resembled those in underlying seawater, consisting primarily of SAR11-clade Alphaproteobacteria and Marine Group I Crenarchaeota, neither of which is known from spring or summer sea ice. The bacterial ice library contained clones of Gammaproteobacteria from oligotrophic seawater clades (e.g. OM60, OM182) but no clones from gammaproteobacterial genera commonly detected in spring and summer sea ice by similar methods (e.g. *Colwellia*, *Psychrobacter*). Selection during ice formation and mortality during winter appear to play minor roles in the process of microbial succession that leads to distinctive spring and summer sea ice communities.

2.2 Introduction

Arctic sea ice covers an area of about 15×10^6 km^2 at its maximal extent during winter (Antarctic 18×10^6 km^2; Fetterer and Knowles, 2002), providing an extensive (albeit shrinking; Serreze et al., 2007) habitat for microorganisms. During the annual lifetime of polar sea ice, it experiences wide ranges in environmental conditions, yet changes in its microbial (bacterial and archaeal) communities through the seasons, especially winter, are not well known (as reviewed by Mock and Thomas, 2005; Deming, 2009). Winter sea

ice, particularly in the Arctic, is an extreme environment characterized by limited light, very cold temperatures in its upper horizons (to −35°C) and correspondingly high salinity (37 to 237‰) in its brine inclusions, where most of the organisms have been observed to reside (Junge et al., 2001). The assumption that these microbial inhabitants are largely inactive during the winter has been tested infrequently. Working with first-year sea ice north of Barrow (Alaska) during the coldest month of the year (March), Junge et al. (2004) observed that a small percentage of cells (0.5–4.0% by CTC staining) were metabolically active to −20°C, the coldest ice-horizon examined. More recently, Wells and Deming (2006b), working with landfast sea ice in Franklin Bay (Western Arctic, also in March), detected an increase in bacterial numbers (doubling time > 4 days) in one of the three ice-brine samples they incubated at −12°C (salinity of 160‰).

Relative to other marine environments, bacteria in sea ice have proven highly amenable to cultivation; e.g. up to 62% of total counts from Arctic summer ice (Junge et al., 2002), and 50% from Antarctic winter ice (Helmke and Weyland, 1995). The bacterial groups that have been cultured from polar sea ice in general consist primarily of Gammaproteobacteria of the orders Oceanospirillales and Alteromonadales, the marine *Roseobacter* clade of Alphaproteobacteria (Bowman et al., 1997; Brown and Bowman, 2001; Brinkmeyer et al., 2003; Buchan et al., 2005), and the Bacteroidetes (also known as Cytophaga-Flavobacter-Bacteroides). Less frequently, Betaproteobacteria and high G+C Gram positives (Actinobacteria) have also been obtained in culture from Antarctic sea ice (Junge et al., 1998) and Baltic Sea ice (Kaartokallio et al., 2005). Results from culture-independent methods, including cloning and sequencing of 16S rRNA genes and fluorescent in situ hybridization (FISH), overlap remarkably well with culture-based results, confirming the prevalence of these bacterial groups in spring and summer sea ice (Brown and Bowman, 2001; Brinkmeyer et al., 2003). In contrast, Archaea have not been known to exist in sea ice until recently, even though they are prevalent members of Arctic pelagic communities (Wells et al., 2006; Galand et al., 2006; Galand et al., 2008b) and comprise a sizable fraction of the biomass in Antarctic frazil ice in late winter (DeLong et al., 1994). To date, Archaea have only been detected in Arctic winter sea ice (using FISH), where they comprised a small percentage (≤ 3.4%) of the total microbial community (Junge et al., 2004), appearing to be absent in sea ice of later seasons by both culture-based and culture-independent methods (Brown and Bowman, 2001; Brinkmeyer et al., 2003).

Possible shifts in community composition and richness in Arctic winter ice over seasonal time and increasingly severe conditions are not known. Based on cultivation studies of Antarctic sea ice, psychrophiles are thought to selectively outlive or outcompete psychrotolerant species during winter (Helmke and Weyland, 1995; Delille and Rosiers, 1996; Helmke and Weyland, 2004; Fiala et al., 2006), leaving them in a position to dominate during the biologically productive spring and summer months. Alternatively, these common heterotrophic sea ice microorganisms may be enriched into newly forming sea ice if attached to larger eukaryotic phytoplankton, which are selectively entrained into sea ice by frazil ice scavenging (Grossmann, 1994). The potential dynamics of microbial communities during entrainment or after entrapment in polar sea ice have not previously been examined using phylogenetic approaches (for any season), though some researchers have investigated the winter succession of bacterial and archaeal communities in the polar water column (Murray et al., 1998; Murray and Grzymski, 2007; Alonso-Sáez et al., 2008). The use of PCR-based techniques for high-throughput analysis of microbial community structure, including terminal restriction fragment length polymorphism (T-RFLP) analysis and automated ribosomal intergenic spacer analysis (ARISA), have proven useful in revealing spatial and temporal patterns in other marine environments at higher resolution than achieved with cloning and sequencing (Osborn et al., 2000; Hewson et al., 2007; Fuhrman et al., 2008). One of these 'fingerprinting' techniques, temperature gradient gel electrophoresis, was applied to investigate bacterial community dynamics and succession within the relatively warm (> -5°C) and thin (< 30 cm) winter ice cover of the brackish (5–6‰) Baltic Sea (Kaartokallio et al., 2008). Differences in the spatial and temporal distributions of common sea ice Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes over the two-month lifetime of the ice cover were linked to exchange processes at the ice-water interface and progression of the ice-algal bloom, reflecting community dynamics unlikely to pertain to much colder, thicker and more light-limited Arctic winter sea ice.

During the Canadian Arctic Shelf Exchange Study (CASES), when the CCGS *Amundsen* was immobilized in the landfast sea ice of Franklin Bay (Western Arctic) through the winter, we investigated the spatial heterogeneity and temporal dynamics of particulate matter, including microorganisms and particulate extracellular polymeric substances (pEPS), within the ice (Collins et al., 2008). The same ice field was cored repeatedly each week from January through March, a period when the ice thickness increased from 90 to 200 cm and upper ice

temperatures were well below −5℃, leaving the ice effectively impermeable (Golden et al., 1998) and microbial communities conveniently entrapped. We focused on the upper 70 cm of the ice sheet, analyzing the contents of three 10-cm horizons centered at 25, 45, and 65 cm below the ice surface (reserving subsamples for later analyses of community structure and richness). The results of this study indicated winter losses of significant fractions (38 and 49%) of the total number of bacteria in the two coldest ice horizons sampled, where in situ temperatures had ranged from −15 to −26℃ (25 cm) and −12 to −22℃ (45 cm; the range at 65 cm was −9 to −18℃; Collins et al., 2008); at these temperatures brine salinities are also extreme, ranging from 130 to 230‰. We attributed some of the bacterial losses to virally mediated mortality, given measurements of viral production in selected ice-brines from the same ice field (Wells and Deming, 2006b). We attributed the persistence of the majority of bacteria throughout the ice to the generic cryoprotective effects of pEPS (Krembs and Deming, 2008), the amount of which had increased significantly in all three horizons during winter (Collins et al., 2008).

Here, we investigate effects of the increasingly severe conditions in the aforementioned sea ice on the structure and richness of the natural microbial communities found within it, beginning several weeks after ice formation. Because bacterial communities that thrive within spring and summer sea ice differ substantially from free-living pelagic communities prior to freeze-up, we hypothesized that the extreme conditions presented by winter sea ice would exert a selective pressure on the microbial community, favoring the survival and subsequent dominance of the easily-cultured psychrophilic Bacteria already known from spring and summer sea ice. To test this hypothesis we performed community fingerprinting of Bacteria (by ARISA) and Archaea (by T-RFLP) on the entire sample set collected previously during the CASES overwintering expedition (Collins et al., 2008), also generating complementary clone libraries of bacterial and archaeal 16S rRNA genes from selected samples of both winter sea ice and under-ice seawater. Although we were unable to test directly for selection during the fall freezing process, our results also bear upon this concept.

2.3 Results

Community dynamics

Bacterial ARISA

Contrary to expectation, no changes in the community structure (Fig. 2.2) or richness (Fig. 2.2) of the sea ice bacterial community were detected through the winter. A large majority (77.5%) of the total ARISA signal intensity (i.e. global cumulative peak height) derived from 14 operational taxonomic units (OTUs) present in all 11 successfully analyzed sea ice samples (Table 2.2). Clone libraries of the 16S-ITS-23S region identified some of these common OTUs, including SAR11 clade Alphaproteobacteria, which made up 50% of the total signal intensity (Table 2.2), in agreement with the dominance of this clade in those clone libraries; OTUs best matching *Polaribacter*, a genus of Bacteroidetes, made up a further 12%. Several more OTUs found to persist through the winter had best matches to various Alphaproteobacteria, Gammaproteobacteria, and Flavobacteria (Table 2.2). Correlation analysis revealed no significant change ($p < 0.05$) in community structure similarity (Sørensen's index; 84 ± 7%, Fig. 2.2) or richness (range 19–29, Fig. 2.2) over time in any horizon. Clustering analysis also indicated no differences among communities by depth horizon but the limited number and nonuniform distribution of successfully amplified samples precluded further statistical assessments of richness or community structure as a function of depth in the ice. The under-ice seawater sample (88-SW), though lacking about 30% of the OTUs found in every ice sample, was nevertheless dominated by many of the same OTUs detected in the sea ice library (Table 2.2), and had a Sørensen's similarity index of 64.3% relative to ice sample 24-II.

Several OTUs found to persist in ice through the winter had no representatives in the bacterial clone library; these OTUs summed to 15% of the total signal, indicating that potentially important groups were missed by the clone library approach. Nevertheless, overlap between clone library sequences and ARISA OTUs (Table 2.2, Table 2.3) was substantial. Of the 50 identifiable bacterial subtypes (members of the same phylotype, as identified by 16S rRNA gene sequence, with variable ARISA lengths) detected in the clone libraries, 32 subtypes had predicted ARISA lengths ± 1 bp of an ARISA OTU (Table 2.2, Table 2.3); 10 more subtypes had putative matches ± 2.5 bp.

Archaeal T-RFLP

Archaeal 16S rRNA genes were readily amplified from these winter sea ice samples, yielding a larger data set than for Bacteria yet still no changes in community structure (Fig. 2.2) or richness (Fig. 2.2) of the entrapped archaeal communities were detected through the winter. A large majority (88%) of the total T-RFLP signal intensity derived from 17 OTUs present in all 21 successfully analyzed sea ice samples (Table 2.4). Those OTUs included several matching the Marine Group I.1a Crenarchaeota, which comprised 45% of the total signal intensity (Table 2.4), in agreement with the dominance of this clade in the clone libraries (Table 2.6). OTUs best matching the Marine Group II.b Euryarchaeota made up a further 25% of the total signal intensity. Correlation analysis revealed no significant change ($p < 0.05$) over time in community structure similarity (81 ± 6%, Fig. 2.2) or richness (range 18–33, Fig. 2.2) in any horizon.

No trend in spatial distribution was observed in the dominant members of the community, but several minor unidentified OTUs exhibited such patterns: best matches to Marine Group I.3a Crenarchaeota (515 bp) and *Thermoplasma* (228 bp) were found primarily in upper ice horizons; OTUs with best matches to Marine Group I.1c Crenarchaeota (499 bp), Methanobacteria (477 bp), RC-V Euryarchaeota (531 bp), and unknown groups (529 bp, 322 bp) were found primarily in lower ice horizons (Table 2.4, Table 2.5). The under-ice seawater samples, 35-SW and 88-SW, contained most of the same OTUs dominant in the sea ice library (Table 2.4, Table 2.5) and did not cluster separately from the ice samples, though some minor OTUs were present only in the seawater samples: 317 bp, 428 bp, and 602 bp. Relative to ice sample 17-II, seawater samples 35-SW and 88-SW had Sørensen's similarity indices of 70.8% and 67.8%, respectively.

All of the phylotypes detected in the archaeal clone libraries were also detected as T-RFLP OTUs, though many OTUs found to persist through the winter had no representatives in our clone libraries, possibly as a result of the different primers used for cloning and fingerprinting. Several of these OTUs were putatively identified using a database of sequences from a recent study (Galand et al., 2006) conducted near the outflow of the Mackenzie River (due west of our study site; Fig. 2.1), including Methanomicrobiales, uncultured methanogen-associated groups LDS and RC-V, and uncultured members of the Marine Group I.3a and Marine Group I.3c Crenarchaeota. Other OTUs were putatively identified as halophiles (Halobacteriales, 152 bp) or thermophiles (e.g. *Methanothermus*,

615 bp and *Thermoplasmata*, 228 bp), based on a database of predicted terminal restriction fragment lengths of archaeal sequences from GenBank.

Community composition

Bacterial community

The dominant phylotypes in both the sea ice and seawater libraries (70% and 48% of sequences, respectively; Table 2.6) were associated with the common seawater clade of SAR11 Alphaproteobacteria (Fig. 2.3), consistent with their dominance in the ARISA analysis (Table 2.2). Two major SAR11 subtypes were detected; neither showed a differential distribution between the sea ice and seawater libraries (Table 2.5).

Overlap between the sea ice and seawater libraries was limited: only 6 of 44 phylotypes were shared between ice (28 total phylotypes) and water (22 total phylotypes), but due to the common dominance of SAR11, no statistical difference was detected between the libraries using WebLIBSHUFF ($p_{ice-water} = 0.143$; $p_{water-ice} = 0.183$). Differences were evident in the relative occurrence of Gammaproteobacteria, which appeared primarily in the sea ice library, and of Bacteroidetes, found primarily in the seawater library (Table 2.5). The most abundant gammaproteobacterial phylotypes clustered with cultured oligotrophic bacterioplankton clades OM182 and OM60 (Fig. 2.4, Table 2.5). The Bacteroidetes sequences were dominated by polar marine Cryomorphaceae phylotypes, present only in the seawater library. Despite being represented by only 5 sequences in the libraries, the single *Polaribacter* phylotype consisted of 4 subtypes, none of which was shared between the sea ice and seawater libraries. Together, two of these subtypes accounted for 11.2% of the total signal intensity in the ARISA analysis (Table 2.2). Each library included a different OM43-clade *Methylophilales* phylotype, as well as several distinct marine *Roseobacter* phylotypes (Fig 2.3, Table 2.5). Other unshared phylotypes belonged to the high G+C Gram positive Actinobacteria (sea ice and seawater), Verrucomicrobia and Deltaproteobacteria (sea ice only), and the uncultured 'Marine Group A' division (seawater only). The seawater library had a greater Chao1 index of richness (174 for seawater, 39 for ice) which was likely underestimated due to lesser coverage (61% for seawater, 88% for ice), but the libraries had similar Shannon diversity indices (2.25 for seawater, 2.43 for ice).

Additionally, eukaryotic 18S rRNA gene sequences were detected in the seawater library,

though not the sea ice library. These 13 sequences were closely related to uncultured marine stramenopile group MAST-1, clade NS1A, a group with worldwide distribution including Arctic and Antarctic surface waters (Lovejoy et al., 2006; Massana et al., 2006). The predicted ARISA fragment lengths for these sequences were less than 100 bp so their detection by ARISA was not likely.

Archaeal community

Archaeal sequences were present in both the sea ice and seawater clone libraries, from which a total of 7 phylotypes were detected with high similarities to existing Arctic archaeal clone sequences. The great majority (91%) of sequences from the Archaeal libraries belonged to Crenarchaeota of the Marine Group I clade, most of which fell into a single phylotype of the Marine Group I.1a (Fig. 2.5, Table 2.8). The remaining crenarchaeal sequences were scattered among 4 more phylotypes within the Marine Group I.1a and Marine Group I.1c clades (Fig. 2.5). Two phylotypes belonged to uncultured Euryarchaeota of the Marine Group II.b clade clusters 5 and 7. The libraries had high coverage (> 95% each), similar richness (5 for ice, 6 for seawater) and similar indices of Shannon diversity (0.61 for ice, 0.76 for seawater).

2.4 Discussion

The expectation that selective losses of dominant bacterial and archaeal community members would occur under the extreme conditions of winter sea ice was not realized in this study. Although both bacterial growth and mortality had previously been inferred from changes in total bacterial counts (Wells and Deming, 2006b; Collins et al., 2008), microbial community succession was not detectable in the upper ice horizons we sampled. We found no significant change in bacterial or archaeal richness or community similarity over the 3-month period of our investigation (Fig. 2.2, Fig. 2.2). Persistence of the dominant members of the sea ice microbial community over the course of the winter is also evidenced by the high proportion of total signal associated with members that were present in every sea ice sample analyzed by ARISA (Table 2.2) and T-RFLP (Table 2.4).

Based on documented cell loss in the coldest ice horizons we studied (Collins et al., 2008) and high rates of culturability but low bacterial diversity in spring and summer sea ice (Junge et al., 2002), we had hypothesized a decrease in bacterial and archaeal richness over

the course of the winter as common seawater microorganisms perished under the pressures of extreme environmental conditions. Indeed, previous studies of cultivated bacteria have reported a higher proportion of psychrophilic to psychrotolerant strains present in sea ice relative to seawater (Kaneko et al., 1977; Delille and Rosiers, 1996; Delille et al., 1997; Fiala et al., 2006), suggesting selective forces at work during ice formation. Another recent study used molecular methods to track changes in microbial community structure towards higher proportions of cultivable Bacteroidetes and Gammaproteobacteria in Baltic Sea ice (Kaartokallio et al., 2008), indicating succession within relatively warm and thin sea ice. In contrast to these studies, we observed that the survival of microorganisms in Arctic winter sea ice was essentially independent of taxonomy, indicating that the extreme conditions presented by winter sea ice exerted limited selective pressure on the entrained microbial community. Quantitative measurements of differences in relative abundance of specific groups of microorganisms, using techniques like FISH (Alonso-Sáez et al., 2008), quantitative PCR of 16S rRNA and functional genes like amoA (Galand et al., 2009b), or massively parallel hypervariable tag sequencing (Galand et al., 2009a; Huse et al., 2008) are presently being used to investigate the responses of bacterial and archaeal communities to selective pressures in the water column, but these techniques have not yet been applied to investigate selection in sea ice. Their use might reveal subtle patterns of succession that escaped the sensitivity limits of our methods.

Many Bacteria and Archaea may lie dormant in winter sea ice, surviving without reproducing and thus limiting the possible mechanisms of selection (Kaneko et al., 1977; Helmke and Weyland, 1995). Although Bacteria and Archaea were presumed to 'survive' if their DNA was present on our membrane filters, we have no data demonstrating their continued viability in situ. Protistan bacterivores, which can selectively graze bacteria based on size and biochemical cues, are essentially absent from very cold Arctic winter ice (Krembs et al., 2002) and so are unlikely to have contributed any selective effect, although they may play an important role in shaping the microbial community in warmer ice. The persistence of the ice microbial communities that we measured over 3 months of winter is consistent with the view that cells are relatively inactive in cold winter sea ice but survive in the absence of bacterivores, whereas relatively active under-ice seawater communities are subject to predation and do change over the course of winter (Murray and Grzymski, 2007; Alonso-Sáez et al., 2008). Complete preservation in the ice, however, conflicts with

reports of actively respiring cells in Arctic winter sea ice (Junge et al., 2004) and, from the same ice we sampled, examples of bacterial mortality (Collins et al., 2008) and viral and bacterial production in experimental ice-brines (Wells and Deming, 2006b). To reconcile the general absence of species-specific mortality reported here with the presumed presence of a mixture of active and inactive populations (whether taxonomically similar or not), both populations must share similar mortality rates. This assumption can be fulfilled if the primary mechanisms of mortality in the ice are taxonomically non-selective, despite the possibility that they may each affect active and inactive cells differently.

Two prime mechanisms to consider for non-selective mortality are virally-mediated lysis and cell damage from co-occurring extremes in temperature and salinity. Modeled contact rates between viruses and bacteria in sea ice brines are extremely high (up to 600 times that in seawater; Wells and Deming, 2006b) and, under environmental stress, host specificity may give way to a broad range of infectable hosts (Wells and Deming, 2006a), linking both mechanisms under consideration. Viral production (whether by species-specific or generalist phages) may play a role in mortality of any taxonomic group that remains active in the ice, whereas dormant populations might be affected if lysis is achieved not by viral reproduction within an active host but via 'lysis from without' due to a large number of attached viruses (Delbrück, 1940).

While the dominant sources of mortality for microorganisms in winter sea ice remain unknown, a generalized protective mechanism has been proposed for cells within sea ice: coatings of hydrated, extracellular polymeric substances (EPS) that simultaneously buffer against external ice-crystal damage, osmotic shock and viral attack (Krembs et al., 2002; Krembs and Deming, 2008). EPS are produced by many sea ice bacteria in culture (Mancuso Nichols et al., 2005; Marx et al., 2009), are colonized by bacteria in sea ice (Meiners et al., 2004), and were found to increase in abundance in the ice we studied over the course of the winter (Collins et al., 2008). Production of EPS by a subset of microorganisms entrained into sea ice might serve all entrapped within it, including common seawater species that are not themselves adapted to life in sea ice, thereby limiting species-specific mortality.

The structure of these persistent winter sea ice communities more closely resembled that of communities in fall and winter seawater (whether measured in this study or reported by others) than previously observed communities in spring and summer sea ice. The winter ice bacterial community was dominated by the common pelagic SAR11-clade Alphaproteobac-

teria, with only much smaller complements of Alphaproteobacteria and Flavobacteriales well known from spring and summer sea ice. While sea ice in those later seasons has appeared devoid of Archaea, all of our winter sea ice (and seawater) samples contained them, with the dominant archaeon belonged to the Marine Group I.1a Crenarchaeota. Beyond the notable resemblance to polar seawater communities, our results also indicate that microorganisms from a variety of habitats, including terrestrial soil, riverine waters, and marine sediment, entrain into sea ice and persist through the winter. Likely sources of the non-marine microorganisms include freshwater from the nearby Horton River, eroded soils from the Smoking Hills, and terrestrial organic matter from the Mackenzie River, which is the largest source of suspended particulates to the Beaufort Shelf (Macdonald et al., 1998).

The dominant bacterial and archaeal phylotypes we observed in our winter ice samples have not previously been detected in sea ice of later seasons. We identified SAR11-clade Alphaproteobacteria and Marine Group I Crenarchaeota in winter sea ice by both clone library sequencing and molecular fingerprinting, indicating that these microorganisms were incorporated into the ice we sampled during its growth and that they persisted through winter.

With regards to the dominant SAR11 phylotypes detected in our winter ice samples, all were highly similar to sequences from the coastal Beaufort Sea (Galand et al., 2008a), the central Arctic Ocean (Bano and Hollibaugh, 2002; Malmstrom et al., 2007), and Antarctic surface waters (Murray and Grzymski, 2007), but were absent both from summer sea ice (Brown and Bowman, 2001; Brinkmeyer et al., 2003), and autumn sea ice near Antarctica (Brinkmeyer et al., 2003). To date the only cultured representative of the SAR11 clade is '*Candidatus* Pelagibacter ubique', an obligately oligotrophic alphaproteobacterium distributed widely throughout the waters of the world's oceans (Morris et al., 2002). Although we detected two clades of SAR11 ITS sequences, no evidence for differentiation between sea ice and seawater was found that might indicate ecotype differentiation between environments (as suggested by García-Martínez and Rodríguez-Valera, 2000). Because sequences within the SAR11 clade made up a larger fraction of the late winter sea ice library (70%) than the under-ice seawater library (48%), however, SAR11 may overwinter more successfully in ice than in seawater. This inference is consistent with quantitative FISH studies by Alonso-Sáez et al. (2008), who detected a seasonal decrease in the relative abundance of SAR11 in Franklin Bay surface waters from a high of 36% of total DAPI counts

at the time of freeze-up in December to a low of 18% by late winter in March. Likewise, using FISH in the western Arctic Ocean, Malmstrom et al. (2007) noted a seasonal increase in the relative abundance of SAR11 in surface waters during summer compared to spring.

The dominant archaeal phylotypes in our sea ice and seawater libraries, Marine Group I Crenarchaeota (Fig. 2.5, Table 2.8) have been previously identified from central Arctic seawater (Bano et al., 2004), Beaufort shelf nepheloid layers and riverine particles (Galand et al., 2006; Galand et al., 2008b), and Antarctic seawater growing frazil ice (DeLong et al., 1994), but were absent from summer and autumn sea ice (Brown and Bowman, 2001; Brinkmeyer et al., 2003). These phylotypes clustered with the 'Marine' Group I Crenarchaeota rather than the 'Freshwater' Group I which are prominent in the Mackenzie River (Galand et al., 2008b,a). The seasonally high relative abundance of the Marine Group I Crenarchaeota that we detected both by T-RFLP (45% of total signal intensity) and clone library sequencing (91% of sequences in both libraries) in Franklin Bay is consistent with FISH counts in surface waters at the same site, showing a winter high for this group (to 16% of DAPI counts) that decreased to undetectable levels by late summer (Alonso-Sáez et al., 2008). This trend is also consistent with results from the Southern Ocean showing that the relative abundance of Marine Group I Crenarchaeota was highest during winter and inversely correlated with algal biomass (chlorophyll a) on seasonal time scales (Murray et al., 1998; Church et al., 2003). The rarer Marine Group II Euryarcheota we detected were also closely related to phylotypes found prevalently in archaeal communities from the coastal Beaufort Sea (Galand et al., 2006; Galand et al., 2008b) and central Arctic Ocean (Bano et al., 2004). Though Archaea were identified by Junge et al. (2004) in winter sea ice, their FISH probes were generic for the domain Archaea so no further taxonomic assignment was possible.

Bacterial sequences from several frequently-cultured groups of copiotrophic sea ice bacteria were absent from our winter sea ice clone libraries, replaced instead by groups whose cultured members prefer low nutrient concentrations. Neither the bacterial sea ice nor seawater library harbored sequences from cold-adapted genera of the Oceanospirillales, Alteromonadales, or Flavobacteriales that are commonly cultured and cloned from sea ice, including *Colwellia*, *Glaciecola*, *Halomonas*, *Marinobacter*, *Pseudoalteromonas*, *Psychrobacter*, *Psychromonas*, or *Shewanella* (Fig. 2.4, Table 2.5). Instead, the majority of gammaproteobacterial phylotypes, derived mostly from the sea ice library, were related

to recently-cultured oligotrophic seawater clades OM60 and OM182 (Cho and Giovannoni, 2004). The OM60 clade is prominent in Arctic seawater (Bano and Hollibaugh, 2002; Kellogg and Deming, 2009), as is the seasonally-abundant OM182 clade (Grzymski et al., 2006; Murray and Grzymski, 2007), which is also prevalent in Antarctic surface waters (Murray et al., 1998; Murray and Grzymski, 2007). A representative from the OM60 clade was reported in Arctic pack ice (Brinkmeyer et al., 2003), but no members of the OM182 clade have previously been identified from sea ice. Cultured isolates of sea ice Alphaproteobacteria generally cluster with marine *Roseobacter*, but only one phylotype detected in our libraries was most similar to a cultured representative (*Sulfitobacter sp.*). Three phylotypes falling into the uncultured *Roseobacter* RCA cluster were detected infrequently in our libraries, consistent with observations of their low abundance in seawater at the Franklin Bay overwintering station (< 5%; Alonso-Sáez et al., 2008). Bacteroidetes are well-known from sea ice and often make up a sizable fraction of the community in polar seawater (Bano and Hollibaugh, 2002; Wells and Deming, 2003; Malmstrom et al., 2007), including at the Franklin Bay overwintering site (Alonso-Sáez et al., 2008), but made up only a small fraction of the bacterial clones in our winter sea ice library.

Polaribacter, a genus of Bacteroidetes appearing in the ARISA analysis, provided a notable exception to the lack of representation by cultivated isolates in the winter sea ice we sampled (Table 2.2 and Table 2.3). Because *Polaribacter spp.* have been identified as abundant Bacteroidetes in Arctic and Antarctic sea ice (Brown and Bowman, 2001; Junge et al., 2002; Brinkmeyer et al., 2003), the recent genome sequence of Antarctic seawater isolate *Polaribacter irgensii* strain 23-P (GenBank accession: AAOG00000000) may help elucidate their adaptations to sea ice (e.g. their frequent ability to form gas vacuoles; Auman et al., 2006). Despite their documented association with sea ice (Gosink et al., 1998; Junge et al., 2002) we were not able to detect a preferential entrainment of *Polaribacter spp.* into the ice relative to other taxonomic groups that persisted through the winter because we also detected them in the underlying seawater by both cloning and ARISA. However, the high intra-specific ITS variability we noted within this phylotype may provide useful markers for testing future hypotheses regarding ecotype differentiation due to selective entrainment into sea ice by these successful sea ice colonizing bacteria.

Overall, the dominant presence of common seawater microorganisms and the absence of many known sea ice microorganisms in the Arctic winter ice we studied indicate that species-

specific mortality during the winter was rare. The degree of similarity between winter ice and seawater communities also implies that selection during the freezing process must have been relatively minor. The distinctive nature of well known microbial communities in sea ice of the warmer biologically productive seasons must not be predetermined by selective survival of community members exposed to freezing and severe winter conditions, but rather as a result of competitive outgrowth by copiotrophs that overwinter below detection limit or arrive as immigrants once the warming ice becomes permeable.

2.5 Experimental Procedures

Ice core and seawater sampling

Sampling location and procedures have been reported in detail by Collins et al. (2008), along with measurements of air and ice temperature, bulk ice and brine salinity, brine volume fraction, and content of total particulate matter, bacterial abundance and pEPS in the ice. Briefly, each week from 10 January (calendar day 10) to 28 March 2004 (day 88), three ice cores were drilled from a designated field of landfast first-year sea ice in Franklin Bay, Northwest Territories, Canada (at 70.0°N, 126.3°W; 16 km from the mouth of the Horton River) without reaching seawater to capture 10-cm depth horizons centered at 25, 45, and 65 cm from the ice surface and designated horizons I, II, and III, respectively. Freezing dates for horizons I and II were predicted to be 9–18 November and 26 November–5 December, respectively, while horizon III was observed to freeze from 14–20 December 2003.

The ice sections were cut aseptically in the field, placed into sterile Whirl-Pak bags and transported in an insulated cooler to a shipboard cold room set at 0℃, where they were processed within 24 h. To protect against osmotic shock and possible cell lysis, each section (after mechanical crushing) was melted into 0.22 μm-filtered artificial brine solution (prepared as in Collins et al., 2008) at 0℃, using an ice:brine volume ratio of 1:2. After subsampling for other variables (Collins et al., 2008), the remainder of each melted sample was gently filtered onto a 47-mm diameter 0.22-μm nitrocellulose filter (Millipore) and stored at −80℃ for later DNA extraction.

Under-ice seawater samples were collected on calendar days 35 and 88 by lowering a hand-held 2-L Niskin bottle through a hole in the ice to the base of the ice sheet. Designated 35-SW and 88-SW, the seawater samples, each with a salinity of 30, were returned to the ship, filtered immediately, and the filters stored at −80℃, as for fully melted sea ice samples.

DNA extraction

Within 2 years of collection, each filter was removed from storage at −80℃ and cut into small fragments with sterilized scissors. For horizons II and III, all three filters (one from each of the three ice cores) from each sampling day were combined in a single tube. In horizon I, due to low DNA yields, six filters—three from each of two sampling days in adjacent weeks—were combined in a single tube; i.e. from the following pairs of calendar sampling days: 10 and 17, 24 and 31, 60 and 67, and 74 and 81. Each tube then received, per filter, 800 μl STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 40 μl 20% SDS. After incubation at 65℃ for 20 min, each tube was vortexed, then centrifuged at 1400× g for 15 min. The supernate was transferred to a Centricon YM-100 centrifugal filtration device (Millipore) to concentrate and de-salt the genomic DNA, according to the manufacturer's recommendations. The recovered volume was increased to 600 μl with TE buffer before three rounds of phenol/chloroform extraction, followed by ethanol precipitation and re-suspension in 50 μl TE buffer. Total DNA concentration was measured in a SpectraMaxM2 plate reader (Molecular Devices) using PicoGreen fluorescence (Invitrogen) according to the manufacturer's recommendations. Recovery of genomic DNA was < 1–33% based on cell counts (Collins et al., 2008), assuming 2.5 fg DNA per bacterium (Button and Robertson, 2001). No attempt was made to separate the DNA of viable cells from that of dead cells.

Community fingerprinting

Bacterial DNA fragments were PCR-amplified for ARISA using fluorescently labeled (6-HEX) forward primer Uni1392F, and unlabeled reverse primer R23S-125R (sequences located in Table 2.1). Amplified bacterial DNA was pooled from two PCR amplifications, each containing 3–117 ng total DNA. Partial archaeal 16S rRNA genes were PCR-amplified for T-RFLP using fluorescently labeled (6-FAM) forward primer Arch109F, and unlabeled reverse primer Arch915R. Amplified archaeal DNA from four PCR amplifications was pooled, then digested with restriction enzyme HpyCH4III at 37℃ for 6h, which was determined empirically to enable complete digestion without overdigestion. PCR amplifications using archaeal primers were generally more robust than with bacterial primers, even though archaeal abundance was likely only a few percent of the bacterial abundance (Junge et al., 2004), a phenomenon which has also been observed in a highly saline Arctic spring system

(T. Niederberger, personal communication). All DNA fragments were analyzed on a MegaBACE1000 capillary gel electrophoresis instrument (Molecular Dynamics Inc.).

Electropherograms were analyzed using DAx analysis software (v8.0, Van Mierlo Software Consultancy). A low-pass Fourier transform was applied to ARISA electropherograms to reduce noise and increase peak calling efficiency. Heights of saturated peaks in several T-RFLP electropherograms were estimated by fitting a Gaussian function to the non-saturated points, using the open-source statistical package R (R Development Core Team, 2008). For both ARISA and T-RFLP, a peak was called if its height was > 5× the baseline root-mean-square noise level (< 1.0% of the cumulative peak height for each profile). Profiles with cumulative peak heights less than 1×10^4 RFUs (ARISA) or 8×10^4 RFUs (T-RFLP) were removed from the analysis. The peaks in the remaining samples were binned using in-house software (`http://rocaplab.ocean.washington.edu/cgi/dakster/index.html`) and distance cutoffs of 1 bp for fragment lengths of 70–700 bp, 2 bp for 700–1200 bp, and 4 bp for > 1200 bp. Each bin, representing a 16S-ITS-23S ribosomal DNA fragment (ARISA) or terminal restriction fragment (T-RFLP), was designated an operational taxonomic unit (OTU). The cumulative peaks heights of each OTU were used as gross measures of relative abundance to compare fingerprinting with clone libraries. A presence/absence matrix containing OTUs with at least one peak height greater than 1.0% (ARISA) or 0.25% (T-RFLP) of the sample's cumulative peak height was used for calculation of richness and pairwise similarity, and non-parametric multivariate analyses, performed with PRIMER v6 (Clarke and Gorley, 2006). Pairwise distances were calculated as Sørensen's similarity coefficient ($C_S = \frac{2C}{A+B}$, where A and B are the number of OTUs in each of two samples, and C is the number of shared OTUs between the two samples; Hughes et al., 2001); samples were then clustered by Group Average and the significance of each cluster was calculated by SIMPROF at the 95% confidence level. Pearson correlation coefficients for Sørensen's similarity with date were calculated in R, as were partial Pearson correlation coefficients for richness with date controlling for the cumulative peak height of each profile. A correlation function in the open-source plotting program Qtiplot was used to calculate the point of maximum covariance between ARISA RFUs and clone library phylotype frequencies to correct for different running rates of fluorescent labels 6-HEX and ROX (used for the ARISA ladder), resulting in an adjustment of ARISA OTU lengths by −7 bp, near the −5.5 bp difference determined by Hahn et al. (2001).

Clone library construction and sequencing

Four clone libraries were constructed for phylogenetic analysis, using the ribosomal RNA operon, from two samples: under-ice seawater from day 35, used to create libraries FB04bw and FB04aw; and winter sea ice from horizon I (combined days 74 and 81), used to create libraries FB04bi and FB04ai, where FB04 = Franklin Bay 2004, b = Bacteria, a = Archaea, w = water, and i = ice. Bacterial 16S-ITS-23S ribosomal DNA was amplified using primer pair Uni515F/R23S-125R (Table 2.1). Archaeal 16S ribosomal DNA was amplified using primer pair Arch21F/Arch958R. Four separate amplifications were performed for each sample and each was reconditioned using one-tenth of the PCR product as template for an additional 4 cycles with fresh reaction mix (Thompson et al., 2002). The pooled reconditioned PCR product was cloned using the TOPO-TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's recommendations. Bacterial clone libraries were subjected to dye-termination sequencing at the High-Throughput Genomics Unit (HTGU), Department of Genome Sciences, University of Washington. Archaeal clone libraries were sequenced on a MegaBACE1000 capillary gel electrophoresis instrument. Bi-directional double-stranded sequences were obtained for > 900 bp of the bacterial and archaeal 16S rRNA genes and the complete intergenic transcribed spacer region (ITS) for Bacteria. Sequencher software (v4.6, Gene Codes Corp.) was used to call bases and construct contigs which were checked and edited manually as necessary. Most of the bacterial sequences included the 16S–23S intergenic transcribed spacer (ITS) region, which we excluded from phylogenetic analyses but from which we calculated predicted ARISA fragment lengths and defined subtypes of 16S rRNA gene phylotypes. Seventeen sequences related to *Stenotrophomonas maltophila* were removed from the bacterial sea ice library as probable contaminants. ARISA fragment lengths predicted from these sequences (777 and 779 bp) overlapped with those predicted from other clone library sequences, but these fragments were observed only rarely in the ARISA dataset. Bacterial sequences were deposited in Genbank with accession numbers EU836892–EU837057 and FJ753995–FB754002; archaeal sequences: EU486859–EU486955; and eukaryotic sequences: FJ753982–FJ753994.

Phylogenetic analysis

Small subunit ribosomal rRNA gene sequences were aligned using the NAST aligner (DeSantis et al., 2006) at Greengenes (`http://greengenes.lbl.gov`). All sequences were checked for chimeras with Bellerophon (Huber et al., 2004) and Mallard (Ashelford et al., 2006); none were detected. Sequence alignments were imported into ARB (Ludwig et al., 2004) and edited manually as necessary. Bootstrapped phylogenetic trees were constructed in PAUP* v4.10beta (Swofford, 2003). ModelTest was used to determine the optimal nucleotide substitution model for maximum likelihood tree construction (Posada and Crandall, 1998). In ARB, partial sequences were added to the tree by parsimony and assigned phylotypes based on their location within the tree. Distance matrices calculated in PAUP* using the Tamura and Nei (1993) model of nucleotide substitution were used with WebLIBSHUFF (Schloss et al., 2004) to compare clone libraries statistically, and with DOTUR (Schloss and Handelsman, 2005) to define phylotypes and calculate estimates of species richness and diversity using the Chao1 and Shannon indices, respectively. Phylotypes were defined by > 98% similarity for bacterial sequences and > 99% for archaeal sequences. Subtypes were designated if multiple fingerprinting fragments were predicted within any single phylotype. The archaeal sequences were subsequently used to aid the choice of restriction enzymes for community profiling by T-RFLP (Collins and Rocap, 2007).

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Table 2.1: Primers used in this study. Universal name as described by Alm et al. (1996)

Common name	Universal name	Sequence (5'–3')	Usage	Reference
Arch21F	S-D-Arch-0002-a-S-20	TTCCGGTTGATCCYGCCGGA	Archaeal cloning	DeLong (1992)
Arch109F	S-D-Arch-0109-a-S-17	ACKGCTCAGTAACACGT	T-RFLP	Grosskopf et al. (1998)
Arch915R	S-D-Arch-0915-a-A-20	GTGCTCCCCCGCCAATTCCT	T-RFLP	Stahl and Amann (1991)
Arch958R	S-D-Arch-0958-a-A-19	YCCGGCGTTGAMTCCAATT	Archaeal cloning	DeLong (1992)
Uni515F	S-*-Uni-0515-a-S-19	GTGCCAGCMGCCGCGGTAA	Archaeal sequencing, Bacterial cloning	Lane (1991)
Uni515R19	S-*-Uni-0515-a-A-19	TTACCGCGGCKGCTGGCAC	Archaeal sequencing	Lane (1991)
Uni1392F	S-*-Uni-1392-a-S-15	GYACACACCGCCCGT	Bacterial sequencing, ARISA	Hewson and Fuhrman (2004)
Uni1392R	S-*-Uni-1392-a-A-15	ACGGGCGGTGTGTRC	Bacterial sequencing	Field et al. (1988)
R23S-125R	L-D-Bact-0125-a-A-15	GGTTBYCCCATTCRG	Bacterial cloning, ARISA	modification of Hunt et al. (2006)
M13F	—	GTAAAACGACGGCCAG	Archaeal and Bacterial sequencing	Invitrogen
M13R	—	CAGGAAACAGCTATGAC	Archaeal and Bacterial sequencing	Invitrogen

Table 2.2: Bacterial ARISA OTUs in Franklin Bay (FB) sea ice horizons I–III (representing depths of 25, 45, and 65 cm below the ice surface) and under-ice seawater (SW). Each horizon is ordered by calendar day (left to right). Shown are OTUs present in every sea ice sample; additional OTUs are presented in Table 2.3. A black dot indicates the presence of a peak; alternating gray and white rows are used for clarity. '% height' indicates the percentage of the total global peak height in each OTU.

	FB I			FB II							FB III	FB SW	%	Phylotype of best clone
	24	60	74	24	31	38	53	60	67	74	31	88	height	library match
683	•	•	•	•	•	•	•	•	•	•	•	•	37.3	SAR11: B1-a, B2, B3-a, B17-a
786	•	•	•	•	•	•	•	•	•	•	•		7.1	Polaribacter: B28-a
666	•	•	•	•	•	•	•	•	•	•	•	•	6.5	SAR11: B1-b
702	•	•	•	•	•	•	•	•	•	•	•	•	6.3	Alphaproteobacteria: B13-b; OM182: B20
237	•	•	•	•	•	•	•	•	•	•	•		3.1	—
738	•	•	•	•	•	•	•	•	•	•	•	•	2.8	—
860	•	•	•	•	•	•	•	•	•	•	•	•	2.6	Polaribacter: B28-d
391	•	•	•	•	•	•	•	•	•	•	•		2.3	—
704	•	•	•	•	•	•	•	•	•	•	•	•	2.3	Alphaproteobacteria: B13-b; OM182: B20
661	•	•	•	•	•	•	•	•	•	•	•		1.7	SAR11: B1-b, B9, B10; Cryobacterium: B42
688	•	•	•	•	•	•	•	•	•	•	•	•	1.7	—
856	•	•	•	•	•	•	•	•	•	•	•	•	1.5	Polaribacter: B28-d
539	•	•	•	•	•	•	•	•	•	•	•	•	1.2	—
756	•	•	•	•	•	•	•	•	•	•	•	•	1.1	—

Table 2.3: Bacterial ARISA OTUs in Franklin Bay (FB) sea ice horizons I–III (representing depths of 25, 45, and 65 cm below the ice surface) and under-ice seawater (SW). Each horizon is ordered by calendar day (left to right). Shown are OTUs that matched the predicted fragment length from a clone library sequence and OTUs present in at least 70% of the sea ice samples, excepting OTUs present in Table 2.2. A black dot indicates the presence of a peak; alternating gray and white rows are used for clarity. '% height' indicates the percentage of the total global peak height in each OTU.

	FB I			FB II							FB III	FB SW	%	Phylotype of best clone
	24	60	74	24	31	38	53	60	67	74	31	88	height	library match
672	•	•	•	•	•		•	•	•	•	•	•	3.5	—
680	•	•	•	•		•	•	•	•	•	•	•	3.3	SAR11: B1-a, B2, B4, B5-b, B7, B8; OM182: B17-a
816	•	•	•	•		•	•	•	•	•	•		1.4	Alphaproteobacteria: B12; Methylophilus: B24; Polaribacter: B28-b
839	•	•	•		•	•		•	•	•	•	•	1.4	Methylophilus: B23
820	•	•	•	•	•			•	•	•	•		1.0	Chromatiales: B22
734	•	•	•		•			•	•	•	•		0.8	—
595	•	•	•	•	•	•		•		•	•	•	0.7	Cryomorphaceae: B32
784	•							•		•			0.7	Polaribacter: B28-a
1100	•	•	•		•			•	•	•			0.6	OM182: B18-b
781			•							•			0.6	Janthinobacterium: B25
544	•	•	•		•					•		•	0.5	Cryomorphaceae: B29
777	•		•					•		•	•		0.5	OM182: B19
664	•						•		•			•	0.5	SAR11: B1-b, B3-b, B5-a, B6
801			•							•			0.5	Proteobacteria: B27; Actinobacteria: B43
551	•		•					•			•	•	0.4	Cryomorphaceae: B29, B31
600	•	•	•		•	•				•			0.4	Cryomorphaceae: B32
531		•	•					•	•	•			0.2	Cryomorphaceae: B34
548												•	0.2	Cryomorphaceae: B29, B31
779	•		•										0.1	Janthinobacterium: B25
513		•											0.1	Saprospira: B40
775	•												0.1	Formosa: B38
623								•	•		•		0.1	Marine Group A: B44
850								•			•		0.1	Polaribacter: B28-c
697			•										0.1	Alphaproteobacteria: B13-a; OM182: B17-b
825								•					0.1	Chromatiales: B22
847								•				•	0.1	Deltaproteobacteria: B26; Polaribacter: B28-c
629												•	0.1	Marine Group A: B44
676			•										< 0.1	SAR11: B1-a
769										•			< 0.1	Flavobacteriaceae: B35
1016	•												< 0.1	Verrucomicrobia: B41

Table 2.4: Archaeal T-RFLP OTUs in Franklin Bay (FB) sea ice horizons I-III (representing depths of 25, 45, and 65 cm below the ice surface), and under-ice seawater (SW). Each horizon is ordered by calendar day (left to right). Shown are OTUs present in every sea ice sample; additional OTUs are presented in Table 2.5. A black dot indicates the presence of a peak; alternating gray and white rows are used for clarity. '% height' indicates the percentage of the total global peak height in each OTU. Best sequence database matches are demarcated by origin: †, this study; ‡, Galand et al. (2006); and §, GenBank. MGI, Marine Group I Crenarchaeota; MGII, Marine Group II Euryarchaeota; LDS and RC-V refer to clades of uncultured Euryarchaeota.

	FB I			FB II									FB III									FB SW		%	Taxonomic groups of
	24	60	74	17	24	31	38	53	60	67	74	81	10	17	24	31	53	60	67	74	81	35	88	height	best database matches
528	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	23.9	MGI.1a†‡, LDS‡
92	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	23.8	MGII.b†‡, MGII.a†‡, RC-V‡
326	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	20.8	MGI.1a‡
615	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	5.1	*Methanothermus*§
143	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	3.2	—
280	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	2.6	Methanomicrobiales‡
402	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	2.2	—
612	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	1.4	—
368	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	1.1	—
447	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	0.7	—
201	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	0.7	—
511	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	0.6	—
798	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	0.5	—
152	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	0.4	Halobacteriales§
360	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	0.4	—
183	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	0.3	—
217	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•			0.3	—

Table 2.5: Archaeal T-RFLP OTUs in Franklin Bay (FB) sea ice horizons I-III (representing depths of 25, 45, and 65 cm below the ice surface), and under-ice seawater (SW). Each horizon is ordered by calendar day (left to right). Shown are OTUs present in at least 50% of the sea ice samples, and those with at least one peak height greater than 0.25% of the sample's cumulative peak height, excepting OTUs present in Table 2.4. A black dot indicates the presence of a peak; alternating gray and white rows are used for clarity. '% height' indicates the percentage of the total global peak height in each OTU. Best sequence database matches are demarcated by origin: †, this study; ‡, Galand et al. (2006); and §, GenBank. MGI, Marine Group I Crenarchaeota; MGII, Marine Group II Euryarchaeota; LDS and RC-V refer to clades of uncultured Euryarchaeota.

	FB I			FB II										FB III									FB SW		%	Taxonomic groups of
	24	60	74	17	24	31	38	53	60	67	74	81	10	17	24	31	53	60	67	74	81	35	88	height	best database matches
219																	•					•		1.0	—
94									•		•	•							•			•	•	0.8	—
91					•														•					0.8	MGII.b†‡, MGII.a†‡, RC-V‡
513					•														•					0.5	MGI.3a‡
524			•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	0.4	Freshwater Group I.1a‡
87		•				•		•	•	•	•	•	•			•	•	•			•		•	0.4	RC-V‡
531				•					•	•			•		•	•	•	•		•	•	•		0.4	—
529				•			•		•						•	•	•	•	•					0.4	—
222			•		•	•		•	•			•	•			•		•	•		•	•	•	0.4	RC-V‡, LDS‡
321											•								•			•	•	0.3	—
761	•	•	•	•	•	•	•	•	•	•	•	•				•	•	•	•		•	•	•	0.3	—
282		•	•				•	•	•	•	•					•			•			•	•	0.3	Methanomicrobiales‡§
461											•	•									•		•	0.3	—
380		•	•	•	•		•		•	•	•	•	•			•	•	•	•	•	•	•	•	0.2	—
325					•														•			•		0.2	—
516					•														•				•	0.2	—
458																	•					•		0.2	—
301																	•					•		0.2	Desulfurococcales§
272		•	•	•	•		•		•	•	•	•	•		•	•	•	•	•		•	•	•	0.2	—
322					•		•					•					•	•	•		•			0.2	—
515			•	•	•		•		•															0.2	MGI.3a‡
498		•	•	•	•		•	•	•	•	•	•	•		•	•	•	•	•	•	•	•		0.1	MGI.1c†‡
455		•			•	•	•	•	•	•	•	•	•			•	•	•	•	•	•	•	•	0.1	—
609			•	•	•	•	•					•		•	•	•	•	•	•		•	•		0.1	*Methanococcus*§, *Picrophilus*§
477				•	•			•	•				•		•	•	•	•	•	•	•	•		0.1	Methanobacteria§
567																				•				0.1	—
287		•	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	•	•	•	0.1	MGI.3c‡
157		•		•	•	•			•	•		•	•		•	•	•	•	•	•	•	•		0.1	—
499				•	•	•	•	•	•	•	•	•	•			•	•	•			•		•	0.1	MGI.1c†‡
132		•			•			•	•	•		•	•			•	•	•	•		•	•	•	0.1	MGI.1a†
137		•	•		•	•	•	•	•	•	•	•				•		•				•		0.1	—
228		•						•	•													•		0.1	*Thermoplasma*§
247																	•					•		0.1	—
231													•											0.1	—
197																				•				< 0.1	—

Table 2.6: A summary of the bacterial and archaeal clone libraries from sea ice horizon I (25-cm depth) and under-ice seawater, including the abundance of major taxonomic groups.

	Sea ice	Seawater
Day of year collected	74+81	35
Temperature at collection	−22℃	−1.7℃
Bacterial clone libraries	FB04bi	FB04bw
All Bacteria	109	46
Proteobacteria		
Alphaproteobacteria		
SAR11 clade	76	22
Other	5	3
Gammaproteobacteria	12	2
Betaproteobacteria	2	1
Deltaproteobacteria	1	0
Unclassified Proteobacteria	1	0
Bacteroidetes		
Flavobacteria	6	13
Sphingobacteria	2	3
Other		
Actinobacteria	3	1
Verrucomicrobia	1	0
'Marine Group A'	0	1
Archaeal clone libraries	FB04ai	FB04aw
All Archaea	52	45
Crenarchaeota		
Marine Group I	46	41
Euryarchaeota		
Marine Group II	6	4

Table 2.7: Bacterial phylotypes (defined by >98% 16S rRNA gene similarity) in clone libraries from late winter sea ice (FB04bi) and under-ice seawater (FB04bw). Subtypes were designated by predicted ARISA lengths, calculated for primer pair Uni1392F and R23S-125R. 'Unknown' indicates partial sequence for which ARISA length could not be calculated.

Taxonomy	Phylotype	Sea ice clones	Seawater clones	ARISA length
	Proteobacteria: Alphaproteobacteria			
SAR11	B1-a	41	16	678–683
	B1-b	3	3	661–663, 665–666
	B1-unknown	1	—	—
SAR11	B2	14	2	680–682
SAR11	B3-a	2	—	682
	B3-b	3	—	664
SAR11	B4	3	—	680
SAR11	B5-a	1	—	663
	B5-b	2	—	679
SAR11	B6	2	—	663
SAR11	B7	2	—	681
SAR11	B8	1	—	681
SAR11	B9	1	—	662
SAR11	B10	—	1	662
Sulfitobacter	B11-unknown	1	—	—
Roseobacter cluster	B12	2	—	813
Unclassified	B13-a	1	—	699
	B13-b	1	—	702
Unclassified polar	B14	—	1	1051
Roseobacter cluster	B15-unknown	—	1	—
Roseobacter cluster	B16-unknown	—	1	—
	Proteobacteria: Gammaproteobacteria			
OM182	B17-a	2	1	681–682
	B17-b	1	—	695
OM182	B18-a	2	—	916–917
	B18-b	2	—	1096
OM182	B19	2	—	777
OM182	B20	1	—	702
OM60	B21-a	1	—	979
	B21-unknown	—	1	—
Chromatiales	B22	1	—	820
	Proteobacteria: Betaproteobacteria			
Methylophilus	B23	—	1	837
Methylophilus	B24	1	—	816
Janthinobacterium	B25	1	—	779
	Proteobacteria: Deltaproteobacteria			
Unclassified	B26	1	—	844
	Proteobacteria: Unclassified			

(cont.)

Taxonomy	Phylotype	Sea ice clones	Seawater clones	ARISA length
Unclassified	B27	1	—	796
		Bacteroidetes: Flavobacteria		
Polaribacter	B28-a	2	—	786
	B28-b	—	1	817
	B28-c	1	—	845
	B28-d	1	—	856
Cryomorphacaea	B29	—	4	548–549
Cryomorphaceae	B30	—	1	709
Cryomorphaceae	B31	—	1	549
Cryomorphaceae	B32	—	1	596
Cryomorphaceae	B33	—	1	719
Cryomorphaceae	B34	—	1	532
Flavobacteriaceae	B35	1	1	770–771
Flavobacteriaceae	B36	1	—	—
Cellulophaga	B37	—	1	1085
Formosa	B38	—	1	776
		Bacteroidetes: Sphingobacteria		
Unclassified	B39	2	—	895
Saprospira	B40	—	3	514
		Verrucomicrobia		
Verrucomicrobaceae	B41	1	—	1012
		High G+C Gram Positive: Actinobacteria		
Cryobacterium	B42	3	—	661–662
Unclassified	B43	—	1	796
		'Marine Group A'		
Unclassified	B44	—	1	624

Table 2.8: Archaeal phylotypes (defined by >99% 16S rRNA gene similarity) in clone libraries from late winter sea ice (FB04ai) and under-ice seawater (FB04aw). Subtypes were designated by predicted TRF lengths, calculated for primer/enzyme combination Arch109F/HpyCH4III. 'Unknown' indicates partial sequence for which TRF length could not be calculated.

Taxonomy	Phylotype	Sea ice clones	Seawater clones	TRF length
		Crenarchaeota: Marine Group I		
Cluster 1a	A3-a	34	31	527–528
	A3-b	1	—	132
	A3-unknown	8	4	—
Cluster 1a	A4-a	1	1	528
	A4-unknown	1	2	—
Cluster 1a	A5	1	1	528
Cluster 1a	A6	—	1	528
Cluster 1c	A7	—	1	499
		Euryarchaeota: Group II.b		
Cluster 7	A1-a	4	2	93
	A1-unknown	—	1	—
Cluster 5	A2	2	1	93

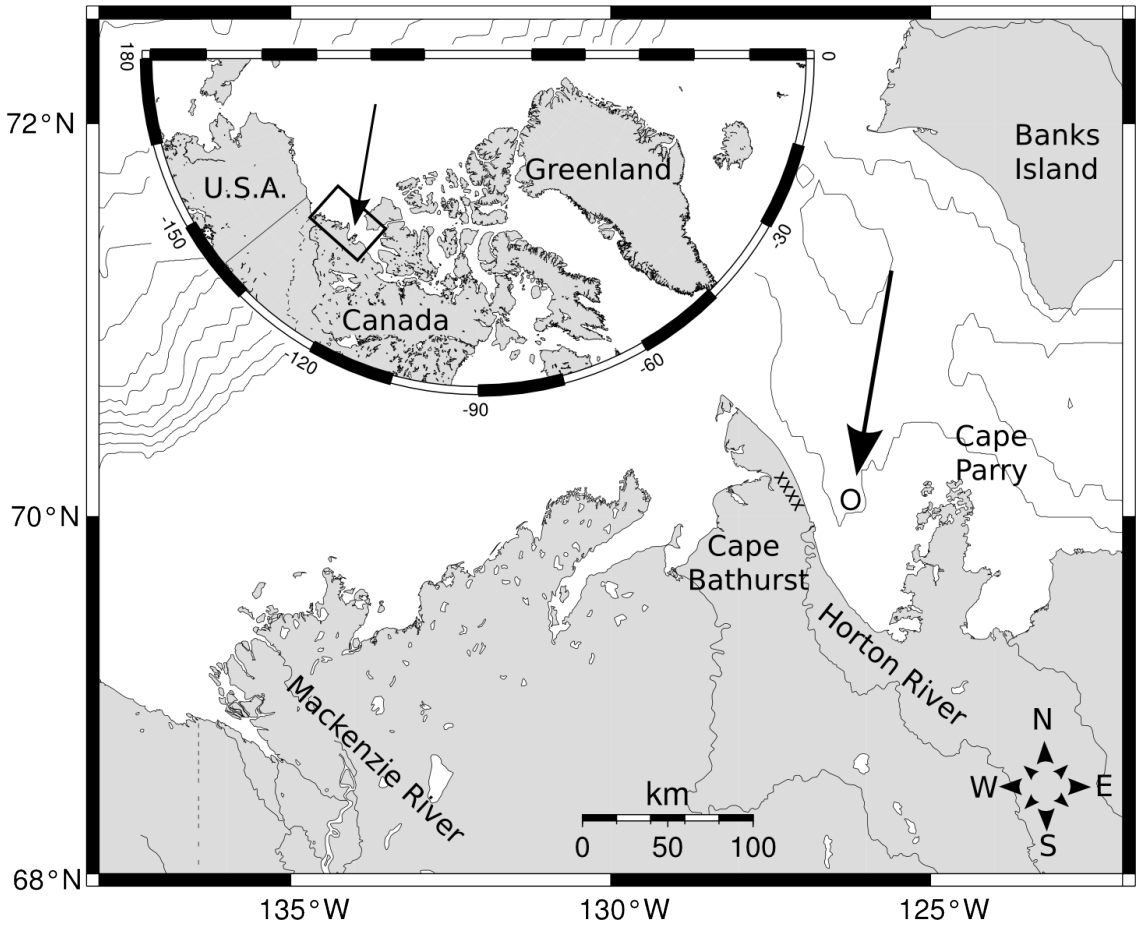

Figure 2.1: Map of the vicinity of Franklin Bay, Northwest Territories, Canada, indicating Franklin Bay (arrows), the primary sampling site at the CCGS *Amundsen* overwintering station (∘) and nearby Smoking Hills (x).

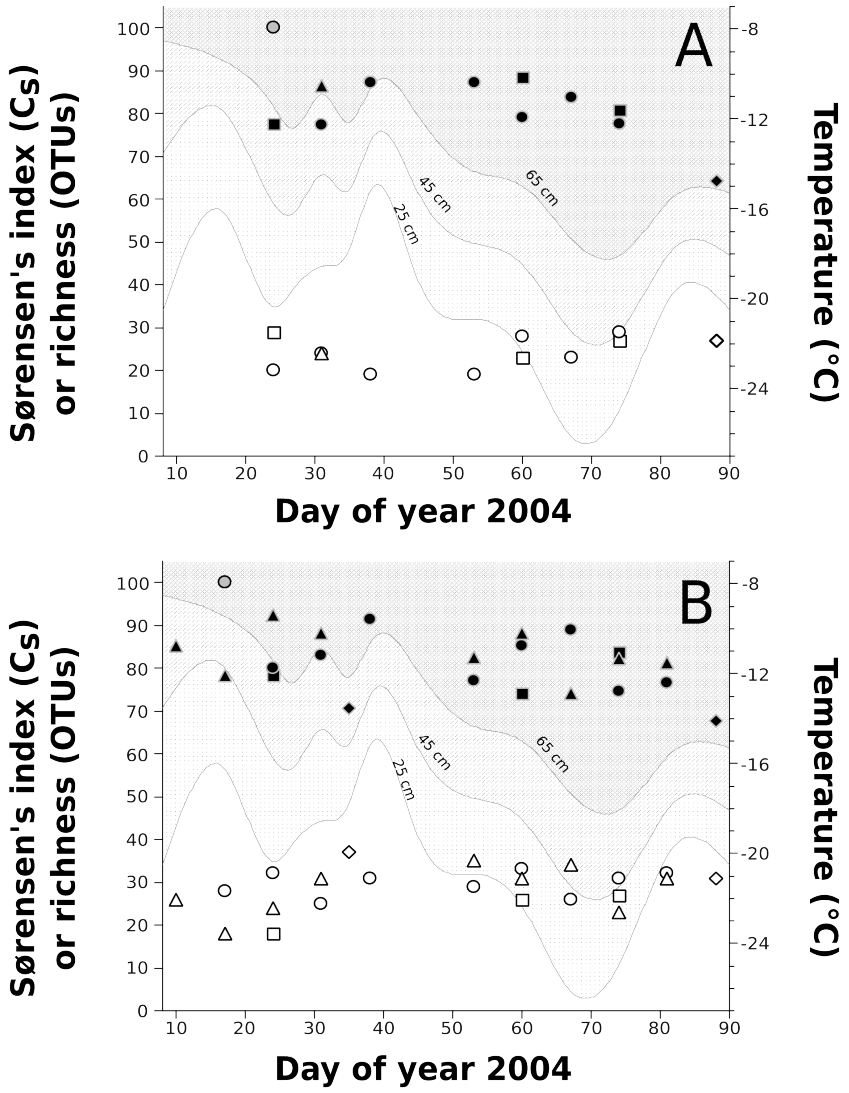

Figure 2.2: Pairwise similarities (black symbols) and richness (white symbols) of (A) bacterial and (B) archaeal communities in Arctic winter sea ice horizons I–III, representing depths of 25, 45, and 65 cm below the ice surface. Temperature contours for each horizon are labeled by depth (from Collins et al., 2008). Sørensen's similarity index, C_s (%), was calculated for each sample relative to the first sample in horizon II (filled gray circle). Richness was defined as the number of OTUs with at least one peak height greater than 1.0% (Bacteria) or 0.25% (Archaea) of the sample's cumulative peak height. Pearson correlation coefficients of pairwise similarity or richness with date were not significant (at $p<0.05$) for any horizon in either community. Shape of symbol demarcates origin: horizon I (□), horizon II (○), horizon III (△), seawater (◇).

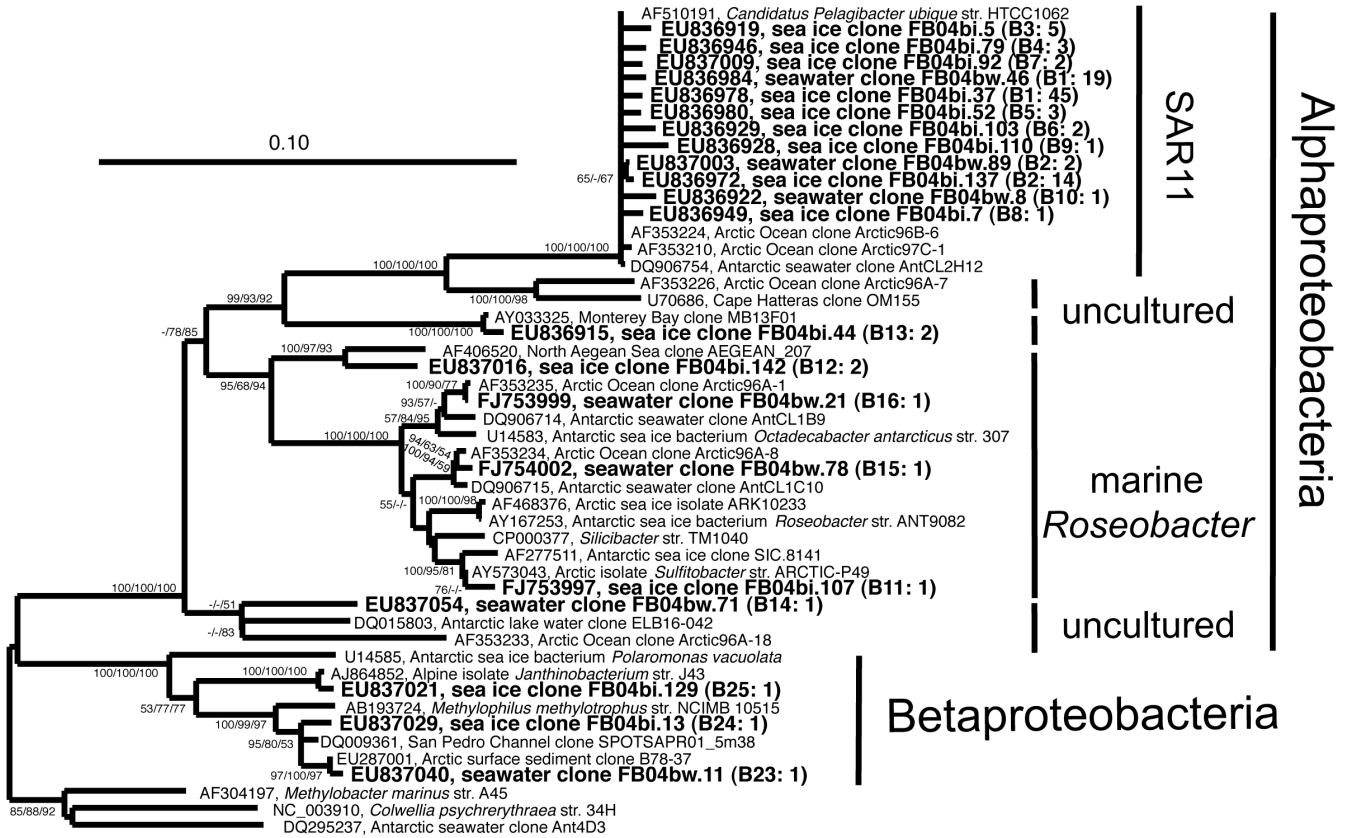

Figure 2.3: Phylogenetic tree of alphaproteobacterial and betaproteobacterial 16S rRNA gene sequences. Tree topology was defined by the consensus of 1000 maximum parsimony bootstrap replications utilizing 291 parsimony-informative nucleotides. Branch lengths were defined by Tamura-Nei distances calculated from 558 hypervariable-masked nucleotides. Node values indicate percentage of 10000 distance, 1000 maximum parsimony, and 100 maximum likelihood bootstrap replications, respectively; only bootstrap values greater than 50% are shown. One sequence from each phylotype (defined by >98% similarity) from each library in this study is shown in bold, followed in parentheses by the phylotype and number of sequences from each library within that phylotype.

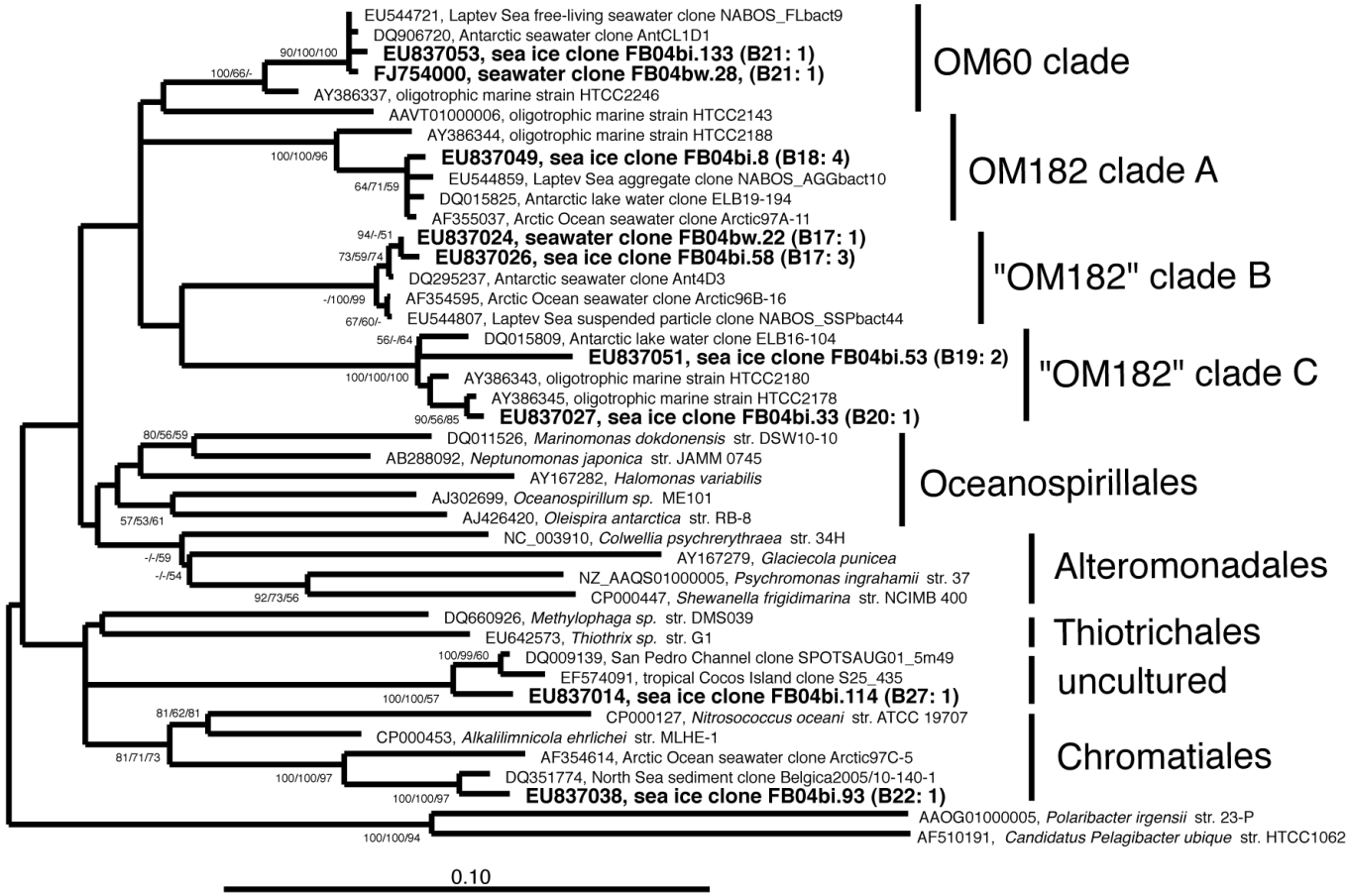

Figure 2.4: Phylogenetic tree of gammaproteobacterial 16S rRNA gene sequences. Tree topology was defined by the consensus of 1000 maximum parsimony bootstrap replications utilizing 240 parsimony-informative nucleotides. Branch lengths were defined by Tamura-Nei distances calculated from 570 hypervariable-masked nucleotides. Other features as in Fig. 2.3.

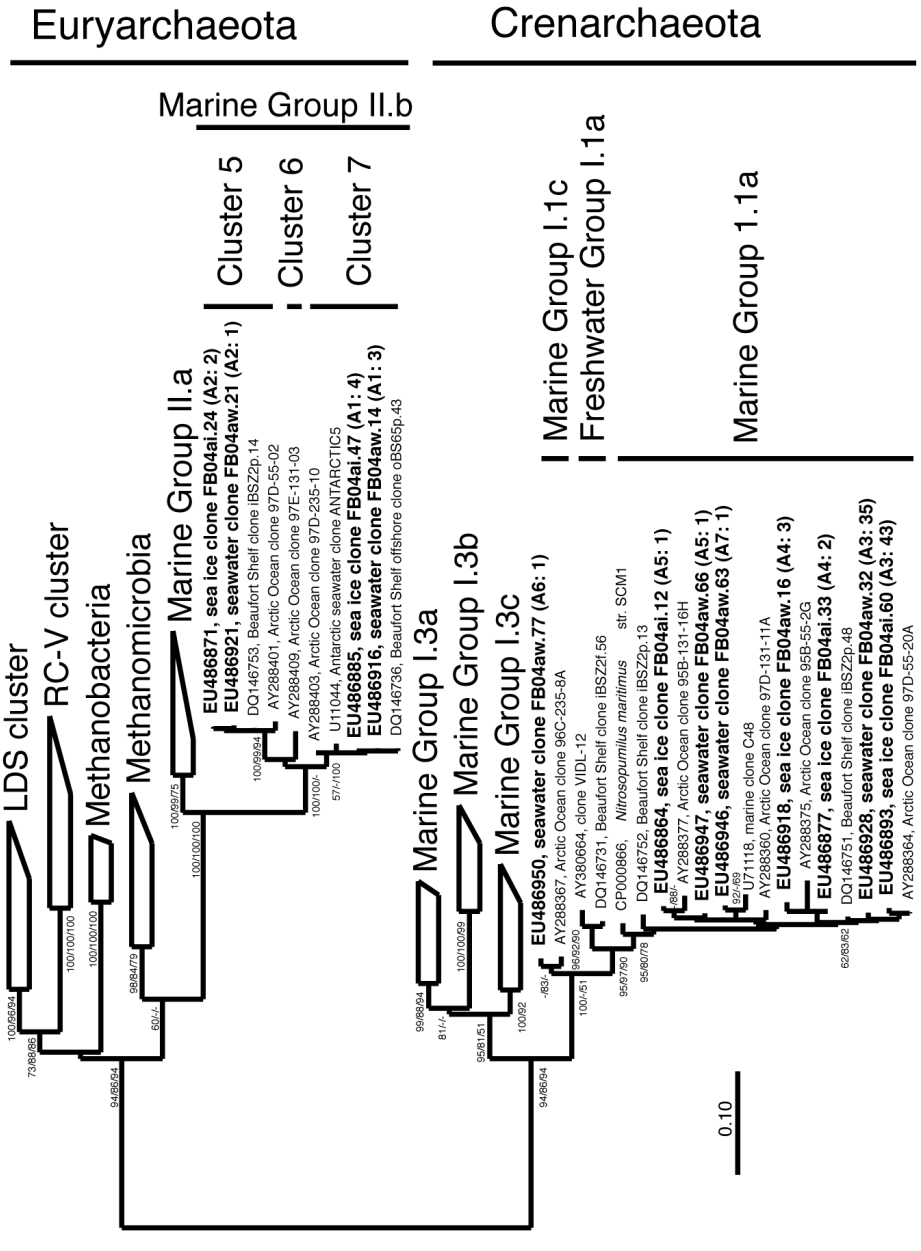

Figure 2.5: Phylogenetic tree of archaeal 16S rRNA gene sequences. Tree topology was defined by the consensus of 1000 maximum parsimony bootstrap replications utilizing 511 parsimony-informative nucleotides. Branch lengths were defined by Tamura-Nei distances calculated from 807 hypervariable-masked nucleotides. Phylotypes were defined by >99% similarity. Other features as in Fig. 2.3.

Chapter 3

ABUNDANT DISSOLVED GENETIC MATERIAL IN SEA ICE: A HOT SPOT FOR LATERAL GENE TRANSFER?

3.1 Abstract

Lateral gene transfer (LGT) is a an integral component of microbial evolution, but the relative importance of each identified mechanism of gene exchange (transduction, conjugation, and transformation) is not known in the environment. Sea ice is an environment characterized by extreme conditions of temperature and salinity, among other stress-inducing factors that may increase the susceptibility of bacterial hosts to exogenous DNA. We measured concentrations of dissolved DNA, along with a suite of physical, chemical, and biological factors that may influence the frequency of lateral gene transfer in newly formed first year Arctic sea ice of varying thicknesses relative to underlying seawater. The ice contained higher concentrations of dissolved DNA, including viruses (required for transduction) and extracellular free DNA (required for natural transformation), than have been reported before from the marine environment. Extremely high virus to bacteria ratios, up to 2820, were observed in the sea ice, with predicted virus to bacteria contact rates up to 844× those expected in the underlying seawater. Extracellular free DNA was up to 100× more concentrated in sea ice brine than in the underlying seawater, with a maximum of 135 μg L^{-1} brine in the upper sections of landfast ice in an enclosed bay. Higher concentrations of bacteria and the availability of numerous surfaces for attachment, known to stimulate LGT, suggest sea ice as a more favorable site for conjugation than in the underlying seawater. An analysis of multiple abiotic facilitators of lateral gene transfer and the abundant dissolved DNA in sea ice suggests that all these forms of LGT may be favored in sea ice, making it a potential hotspot for lateral gene transfer in the marine environment.

3.2 Introduction

Lateral gene transfer (LGT)—the movement of genetic material from one organism to another *sans* reproduction—has played a pivotal role in the evolution of extant microbial genomes, as revealed by massive sequencing efforts over the past decade (Brown, 2001; Goga-

rten et al., 2002; Lawrence and Ochman, 2002; Lawrence, 2002; Syvanen, 2002; Lawrence and Hendrickson, 2003; Beiko et al., 2005; Beiko and Hamilton, 2006). This genomic evidence has led to conclusions that LGT is both widespread among all three Domains of life and intensive, with ca. 15% (and perhaps much more) of the genes in any bacterial or archaeal genome likely obtained via LGT, depending on life history characteristics (Garcia-Vallve et al., 2000) and measurement technique. Several commentators on the importance of lateral gene transfer have focused on the past, questioning the reliability of the universal phylogenetic tree and how it has been shaped by LGT (Syvanen, 1994; Doolittle, 1999a,b; Gogarten et al., 2002; Brown, 2003; Lawrence and Hendrickson, 2003; Beiko and Hamilton, 2006). However, the hypothesis that LGT is an active mechanism in microbial evolution today—and not simply the relic of an earlier mode (Woese, 2002; Kurland et al., 2003)—has only recently been addressable by genomic techniques. The ability to sequence many closely related strains of a single 'species' has shown that for some microorganisms, like the abundant picophytoplankter *Prochlorococcus marinus*, there exists a 'pangenome' from which genetic diversity may be drawn via lateral gene transfer; the pangenome may be many times larger than the genome of any individual microorganism (Kettler et al., 2007; Tettelin et al., 2008). Current genomic approaches, like investigating the effect of microbial lifestyle (e.g. free-living versus obligately endosymbiotic) on the pangenome of a microorganism, are now being expanded into the field of microbial community ecology, which will soon enable the prediction of lifestyle characteristics and the impact of lateral gene transfer on as-yet uncultured microorganisms from the environment. Yet, there remains a need to amend gene sequence-based investigations with in situ experiments (using environmentally relevant microorganisms) and, ultimately, in situ experiments (with natural populations) to address the potential for and relative rates of LGT in the natural environment.

Unknown to date is the relative importance of the known mechanisms of LGT on the evolution of microbial genomes in any environment (Zaneveld et al., 2008), much less in perennially cold marine systems, which make up >90% of the habitat (by volume) of the world's oceans. Each of the mechanisms considered here—conjugation, transduction, and natural transformation—utilizes a different form of DNA: transducing phage, extracellular free DNA, and mobile genetic elements like plasmids, respectively. The relative availability of these particular forms of transferable DNA in a range of environments can provide one comparative measure of the potential for LGT in those habitats.

Conjugation, first described by Lederberg and Tatum (1946), is a biochemically complex system requiring direct contact between cells. Gene transfer occurs between a donor cell, containing a mobile genetic element (e.g. a conjugative plasmid), and a recipient cell (usually lacking the mobile genetic element). In the only existing study of plasmids in sea ice, Kobori et al. (1984) found plasmids ~50% more frequently in bacterial isolates from Antarctic sea ice than from seawater. Though conjugative capacity was not determined in that study, others report high frequencies of conjugation and conjugative plasmids in populations of marine bacteria (Frischer et al., 1994; Dahlberg et al., 1998a; Hausner and Wuertz, 1999). A number of previous reports on the frequency of conjugative gene transfer in environmental microcosms suggest that the frequency of recombination is proportional to the concentration of donor cells and the ratio of donor cells to recipient cells (Frischer et al., 1994; Dröge et al., 1998). Microcosm studies have reported the presence of surfaces for attachment to greatly increase the frequency of lateral gene transfer by conjugation (van Elsas et al., 1987; Yin and Stotzky, 1997). Surfaces may limit the degrees of spatial freedom and thus increase the frequency of finding a suitable partner.

The discovery of viruses as agents of LGT, called transduction, was first described by Zinder and Lederberg (1952). The process of transduction can be broken into the following steps: infection of host cell by a transducing phage, integration of phage into a donor genome (lysogeny) or stable mobile genetic element, induction of prophage and packaging of donor DNA (generalized transduction) or donor and phage DNA (specialized transduction) into phage capsid, lysis of donor cell and release of transducing phage, persistence of new transducing phage, adsorption of phage onto host cell, injection of donor DNA into host, and recombination of donor DNA into host genome. Transducing phage are known from the marine environment (Baross et al., 1978; Chiura, 1997; Jiang and Paul, 1998; Jiang et al., 1998; Wommack and Colwell, 2000), but have not yet been reported from sea ice. However, high abundances of viruses have been repeatedly observed in sea ice from a variety of sources (Maranger et al., 1994; Gowing et al., 2002, 2004; Wells and Deming, 2006c). Transducing phage are often specific in their host requirements (Wommack and Colwell, 2000), though some may be generalists capable of infecting a wider range of hosts (Olsen et al., 1974; Börsheim, 1993; Chiura, 1997; Comeau et al., 2006; Holmfeldt et al., 2007). The metabolic state of the host cell can affect the range of phage capable of infecting it, widening the range when under stress, presumably due to changes in membrane receptor composition (Wells,

2006).

An alternate form of gene transfer is mediated by a virus-like particle called a 'gene transfer agent' (GTA), present in some lineages of the Alphaproteobacteria (Lang and Beatty, 2007). In a recent metagenomic survey, the highest frequency of GTAs occurred in a hypersaline habitat (Biers et al., 2008), but the bacterial community in young sea ice is probably dominated by Alphaproteobacteria of the SAR11 clade (Collins, 2009), which do not appear to harbor GTA-like systems for LGT (Biers et al., 2008). This transduction-like mechanism of LGT may be more pertinent to seawater and spring or summer sea ice, which host alphaproteobacterial communities more likely to be dominated by GTA-containing *Roseobacters* (Zhao et al., 2009).

The first mechanism of LGT to be discovered, natural genetic transformation, involves the direct uptake and integration of extracellular DNA by a 'naturally competent' host cell (Griffith, 1928; Avery et al., 1944). The process of transformation can be broken into the following steps: the release and dispersal of DNA into the environment, the persistence of the DNA in the environment, the development of natural competence in host cells, uptake of DNA by competent cells, and expression of exogenous DNA by the host cell. Dissolved DNA can also be readily metabolized by bacteria (Redfield, 2001), some specializing in either low molecular weight or high molecular weigh eDNA (Lennon, 2007).

These mechanisms of gene exchange have been long utilized in the laboratory setting, but to date relatively few studies have investigated the frequency or even the potential for LGT in situ, which depends on, among other factors, the presence of a critical raw material: DNA.

Different pools of DNA can be measured as components of exogenous or total dissolved DNA (D-DNA) in seawater. The most recent method, by Brum et al. (2004), operationally defined D-DNA as the DNA that passed through a 0.22 μm filter but did not pass through a 10 kDa (about 15 bp)-cutoff filter and was quantifiable by a PicoGreen fluoresence assay. Thus, DNA from intact cells is excluded in this definition but viral DNA is included. Distinctions among the pools of total D-DNA have been defined as follows (Brum et al., 2004; Brum, 2005): 'viral DNA' is that fraction of the total D-DNA contained in viruses; 'enzymatically hydrolyzable dissolved DNA' (eh-DNA) is the fraction of the total D-DNA degradable by DNAse; and, 'uncharacterized bound dissolved DNA' is that which is neither viral DNA nor eh-DNA, and which may be bound to particles or otherwise inaccessible

to participate in LGT. Previous estimates of the concentration of eh-DNA in coastal and offshore seawater, using a variety of techniques, range from <1–31 μgL^{-1} (Deflaun et al., 1986; Karl and Bailiff, 1989; Dell'Anno et al., 1998; Brum et al., 2004), with median values near $10\mu gL^{-1}$. Older methods did not discriminate between viral DNA and uncharacterized bound dissolved DNA, reporting only the eh-DNA, which made up 75±19% of the total D-DNA in one early report (Deflaun et al., 1986). Here we report measurements of total D-DNA and viral DNA in sea ice, allowing an estimation of the total abundance of dissolved extracellular DNA (eh-DNA plus bound DNA), which we will refer to as extracellular DNA (eDNA).

Possible mechanisms of extracellular DNA release and accumulation in sea ice include concentration from source seawater, release of DNA-containing EPS by eukaryotic picoeukaryotes (Steinberger and Holden, 2005; Allesen-Holm et al., 2006; Bockelmann et al., 2006), sloppy feeding by grazers (Proctor and Fuhrman, 1990; Turk et al., 1992), or, as in Paul et al. (1988), bacterial production followed by excretion and cell lysis. Both dissolved DNA (Paul et al., 1987, 1991b) and DNA bound to sediment grains or particles (Lorenz et al., 1981; Lorenz and Wackernagel, 1987, 1990; Stewart et al., 1991) can act as suitable substrates for transformation, a fact which may be important in coastal sea ice or nepheloid layers, each of which may contain high amounts of resuspended sediments (Stierle and Eicken, 2002; Wells and Deming, 2003).

The quality of DNA and its suitability for LGT are related to its age. Older eDNA is more likely to be degraded by DNases and thus less likely to be taken up for transformation, since transformation occurs most efficiently with high molecular weight double-stranded DNA (Lorenz and Wackernagel, 1994). Previous measurements of eDNA in subtropical waters suggest that the molecular weight of eDNA ranges from 150 to 35,000 bp, with a half life of a few hours (usually <6 h) up to a few days (<4 d) in phosphorous-replete surface waters (DeFlaun et al., 1987). At the temperatures that characterize sea ice, the half-life of eDNA should be considerably longer. There are as yet no known reports on the existence, much less fate, of eDNA in sea ice, but viruses have previously been reported from this environment (Maranger et al., 1994; Wells and Deming, 2006c). Viruses can lose infectivity due to adsorption onto particles or surfaces (Suttle and Chen, 1992; Noble and Fuhrman, 1997) or exposure to UV radiation (Miller, 2001), but the production of viruses can offset these losses. Wells and Deming (2006c) measured the production of viruses at rates of the

order 10^4 viruses ml^{-1} h^{-1} in melted winter sea ice at −12°C.

As a first exploration into understanding the cycling of DNA in sea ice and its potential use in LGT, we sampled several different ice types in late autumn, including frazil, nilas, and pancake ice, as well as first year ice floes of varying thicknesses between 33 cm and 78 cm. At each station we measured the abundances of bacteria, viruses, and D-DNA in the ice and the underlying seawater, as well as various physical and chemical parameters for environmental context. Based on the results of this study we argue that sea ice is a potential hotspot for lateral gene transfer in the marine environment.

3.3 Methods

3.3.1 Sample collection

Sampling took place aboard the CCGS Amundsen between 10 November and 18 December 2007, as part of the Circumpolar Flaw Lead Systems Study (CFL) and the International Polar Year. Sea ice and seawater were collected in the vicinity of Amundsen Gulf, Beaufort Sea, Canada (Fig. 3.1). At stations with ice less than 20 cm thick, samples were collected by scooping or sawing chunks from the floe. For ice greater than 20 cm thick, a full 'physical' core was collected for measurements of temperature, taken in the field every 5 cm from the top (as before, see details in Collins et al., 2008), and bulk salinity, measured every 2.5 cm on melted samples. Sea ice microstructure analysis was conducted on many of the sections of the physical core by collaborators in CFL Team 2, led by Dr. David Barber (University of Manitoba). Briefly, vertical thin sections were prepared from cores by sawing and melting, followed by freezing onto glass plates. The thin sections were then photographed under polarized light to examine the ice crystal structure at each depth within the ice. Three 'biological' cores were cleanly drilled at the corners of a 1×1 m square with the physical core and sampled for the later enumeration of bacteria and viruses and quantification of D-DNA. The top 10 cm and bottom 10 cm of each core were removed and transferred sterilely to whirl-pak bags. At least 200 cm of full-length 'chemical' cores were also collected within 5 m of the physical core, for bulk measurements of suspended particulate matter (SPM), particulate organic carbon (POC), particulate organic nitrogen (PON), and chlorophyll *a*. At least 5 L of under-ice surface seawater was collected at each station using a hand-held Niskin bottle. At Station D7 additional seawater was sampled at depths of 10, 30, 100, 150, 250, and 441 m. Ice cores and seawater were stored at 2–4°C until processed within 24 h.

3.3.2 'Chemical' core analysis

The chemical cores were mechanically crushed and transferred to 20 L cubitainers to melt in a water bath at room temperature. Immediately upon completion of melting, continuously-stirred meltwater (and seawater) were pre-filtered through 140 μm mesh, then filtered with an in-line filtration system driven by a peristaltic pump. For C:N (POC PON^{-1}), two or three 2 L aliquots of meltwater or seawater were filtered through separate pre-combusted in-line 25 mm GF/F filters, then stored at −80℃ in pre-combusted foil. Samples were later dried at 60℃ fumed with HCl, and dried again before combustion in a Leeman Labs Model CEC440 Elemental Analyzer, using acetanilide and caffeine as standards. For chlorophyll *a*, one or two 2–8 L aliquots of meltwater or seawater were filtered in-line through separate 25 mm GF/F filters, which were extracted in 90% acetone at room temperature for 2 h aboard ship. The chlorophyll *a*, and, following acidification, phaeopigment, concentration was estimated fluorometrically with a Turner fluorometer. For suspended particulate material (SPM), a single aliquot of 2–16 L meltwater or seawater was filtered in-line through pre-combusted pre-weighed GF/C filters and rinsed with 10 ml 1% sodium formate to remove salts, then stored at −20℃. Filters for SPM were dried overnight at 60℃ and re-weighed to a precision of 0.0001 g. For each procedure, ASW or distilled water (pre-filtered by tangential flow filtration, TFF, with a 10 kDa cutoff) were used as negative controls. The chemical parameters were scaled to bulk melted ice volume because they were measured on whole cores rather than specific depth horizons.

3.3.3 'Biological' core analysis

The biological core sections were mechanically crushed and transferred aseptically to separate sterile melt jars, to which 2 volumes of TFF pre-filtered artificial seawater brine (made with ASW salts, Sigma Corp.) were added, so that after melting the final salinity was equivalent to the in situ brine salinity, calculated from the ice core temperature (Cox and Weeks, 1986, see also Collins et al., 2008). The volume of melted ice was measured immediately upon melting. For DAPI, 39 ml meltwater or seawater were fixed with 37% formaldehyde (2% final concentration) and stored at 2–4℃. For EPS, 150 ml meltwater or seawater were filtered through an in-line 24-mm diameter 0.4-μm filter, then stored at −20℃. Bacterial abundance and EPS concentrations were determined on these samples as in

Collins et al. (2008). For each procedure, ASW or distilled water (pre-filtered by TFF) were used as negative controls. The remainder of each meltwater or seawater sample was filtered through a 0.22 μm Sterivex in-line filter cartridge. For viral enumeration, a 14 ml aliquot of this filtrate was fixed with 0.2 μm-filtered 37% formaldehyde (1.5% final concentration) and stored at 2–4℃ until shipboard analysis within 1 week. Aboard ship, 1 mL of each fixed sample was filtered onto a 0.02 μm Anodisc filter and stained with SYBR Gold in accordance with standard protocols (e.g. see Wells and Deming, 2006c; Patel et al., 2007). At least 10 fields from each sample were photographed with a dedicated CCD camera attached to an epifluorescence microscope under blue excitation; viruses were manually counted from the photographs upon return to shore. Slides were stored at −20℃.

Calculation of virus-bacteria contact rates (J) proceeded as in Wells and Deming (2006c), following Murray and Jackson (1992), by the equation $J = 2\pi d D_v V B Sh$, where d is the spherical diameter of the average cell (here, $0.5{\times}10^4$cm), D_v is the viral diffusivity (cm^{-1}s^{-1}), V and B are the in situ concentrations of viruses and bacteria (ml^{-1}), respectively, and Sh is the Sherwood number, a nondimensional number describing the enhancement of transport due to fluid flow relative to simple diffusion. Accounting for cell shape and swimming speed, Murray and Jackson (1992) showed that even rapidly swimming bacteria (Arctic bacterium *C. psychrerythraea* 34H was reported to swim rapidly at subzero temperatures; Junge et al., 2003) had $Sh \sim 1$, indicating that the transport of viruses to bacteria is usually diffusion limited; here we use $Sh = 1$. The viral diffusivity is calculated as $D_v = \frac{kT}{3\pi\mu d_v}$, where k is Boltzmann's constant ($1.38{\times}10^{-9}$ g cm^2 K^{-1} s^2), T is the temperature (Kelvin), μ the viscosity (g cm^{-1} s^{-1}), and d_v the diameter of the average virus (here, $60{\times}10^{-7}$ cm). The following linear-squares best-fit equation for seawater and brine viscosity (μ) at low temperature (t, ℃) and high salinity (S, ‰) was computed with the curve-fitting program ZunZun (`http://zunzun.com`) using data available from Cox and Weeks (1975): $\mu = (0.62{+}0.020t{+}0.00014t^2{-}0.0012S{-}0.000030St)^{-1}$. Bacteria-virus contacts per bacterium expected in 1 d were calculated from the mean of the binomial distribution, which assumes sampling with replacement (e.g. no adsorption during contact), as $n \times p$, where n is the number of independent trials (here, the number of bacteria in the sample that may have had contact with a virus) and p is the probability of success on each trial (here, the total number of virus-bacteria contacts expected in 86400 s).

Concentrations of biological parameters in the ice were measured on individual horizons

and were therefore scaled to the volume of liquid brine in which they were presumed to be located in situ, as calculated from the equations of Cox and Weeks (1986). The assumption that bacteria reside in brine channels within ice (rather than being encased in the solid matrix) is well supported by microscopic observations (Junge et al., 2001); the other parameters have not been subjected to the same examination. Predictions of the brine concentrating effect were calculated for an 'average' ice column based on all paired temperature (T in ℃) and bulk salinity (S_{bulk}) measurements during CFL Leg 3, with the resulting relation: $S_{bulk} = -0.24T + 4.44$ (Fig. 3.2). Enrichment indices were calculated as follows:

$$E = \frac{\frac{C_{ice}}{C_{sw}} - \frac{S_{ice}}{S_{sw}}}{1 - \frac{S_{ice}}{S_{sw}}}$$

where C_{ice} and C_{sw} are the concentrations of the given parameter measured in a bulk sea ice sample and seawater, respectively, and S_{ice} and S_{sw} are the salinities measured in the bulk sea ice sample and seawater, respectively. Seawater values from the same site were used when available for new and bottom ice; otherwise the mean seawater values were used. The enrichment index is scaled such that $E = 0$ when $\frac{C_{ice}}{C_{sw}} = \frac{S_{ice}}{S_{sw}}$, indicating an entrainment into the ice equal to that of sea salts (i.e. passive entrainment). An enrichment of $E = 1$ occurs when $C_{ice} = C_{sw}$, indicating complete entrainment into the ice and no loss with brine expulsion (i.e. active entrainment). Enrichment indices of $E < 0$ indicate loss (e.g. decay or mortality; the minimal value $E = -1$ indicates complete loss); indices of $E > 1$ indicate gain (e.g. production) within the ice. The index is sensitive to differences between the assumed and actual initial (seawater) concentrations of a parameter, with discrepancies most likely to occur for the upper ice sections since they were furthest removed from the underlying source waters both spatially and temporally. Coefficients of variation of 35%, 59%, and 76% were observed for seawater concentrations of bacteria, viruses, and extracellular DNA, respectively, indicating that enrichment indices for viruses and eDNA in upper ice should be considered less robust than for bacteria.

Measurements of D-DNA followed broadly the method of Brum et al. (2004), using PicoGreen fluorescence to quantify D-DNA and a ^{35}S-labeled internal standard to determine yield, but several alterations were necessary to account for the high salinity of the sea ice brines. For each sample, a 13 ml aliquot of the biological core filtrate (0.22 μm) was collected,

1.6 M tetrasodium-EDTA was added to an excess of 100 mM to disrupt viral capsids, and the sample was stored at 2–4℃ until shipboard analysis within 1 week. ^{35}S-labeled λ-DNA was constructed aboard ship, using a nick-translation labeling kit (Amersham #N5000) according to the manufacturers instructions, and added to each sample to a final activity of 1200 dpm ml^{-1}. The sample was transferred to a Centricon Plus-20 centrifugal filter device (10 kDa nominal molecular weigh cutoff) and centrifuged for 60 min at 4000× g, after which the flowthrough was discarded and 5 ml filtered 10 mM tetrasodium EDTA (pH 10.5) was added. The sample was centrifuged again for 60 min at 4000× g and 500 μL 10 mM tetrasodium-EDTA (pH 10.5) was added. Concentrate was recovered by centrifugation at 600× g for 2 min. Total volume of concentrate was measured and transferred to a microfuge tube for storage at 4℃. Aliquots of each sample were mixed 1:1 (v/v) with 0.5% Pico Green and incubated for 15 min at room temperature in the dark. Fluorescence was measured on a Turner fluorometer at 75% sensitivity. Standard curves containing 0–100 ng ml^{-1} λ-DNA were created on a Turner fluorometer at 75% sensitivity. The remainder of the sample was combined with 20 ml scintillation fluid and ^{35}S radioactivity was measured on a liquid scintillation counter to correct for losses during processing. To estimate concentrations of extracellular DNA (eDNA), the D-DNA in viruses was subtracted from the total D-DNA using an estimated genome mass of 55 ag per virus (Steward et al., 2000). Slight negative concentrations of eDNA calculated in 2 of 23 sea ice samples (344 BB, 318 TC) suggested an overestimation of viral genome size. The true eDNA concentrations for these samples were assumed to be 0 μg L^{-1} for analytical purposes. The DNA content in bacteria was estimated using a genome mass of 2.5 fg per cell (Button and Robertson, 2001).

3.4 Results

3.4.1 'Physical' cores

Sea ice from a variety of ice types was successfully sampled from 8 stations in the western Amundsen Gulf (Fig. 3.1; Table 3.1). Three stations had new ice, including nilas (Stations 1800 and D3) and consolidated pancakes (Station 1200), with bulk salinities of 15–18.5, indicative of recent freezing. Stations R, 1117, and D4 had first year ice of medium thickness with bulk salinities of 6.0–10.3. Stations 437 and D7 had thicker first year ice with bulk salinities of 4.7–10.4. The calculated in situ brine salinities ranged from 41.3 (at −2.2 ℃) to 142.6 (at −11.3 ℃) in the sea ice, reaching maximal values in the colder surface layers of

the thick ice. Nine under-ice seawater samples were collected from these sites; all were at the freezing point (−1.7 ℃ at a salinity of 32).

3.4.2 'Chemical' cores

Measures of particulate organic matter (POM) in the cores indicated a concentration effect in ice over the values measured in seawater. The amount of total suspended particulate matter (SPM) decreased between seawater (1.6–8.9 mg L^{-1}) and sea ice (1.0–4.7 mg L^{-1}). Values of POC (~20–500 μg L^{-1}), the percentage of organic matter (POC SPM^{-1} × 100%; ~0.4–26%), and the C:N ratio (~7–27) each increased from low endpoints in seawater to high endpoints in thick ice (Fig. 3.3). The abundance of chlorophyll *a* was generally very low (<0.3 μg L^{-1}; Fig. 3.4), except in the case of a visible algal bloom in the bottom ice at Station 1117 (7.1 μg L^{-1}). The fraction of phaeopigment decreased slightly at increased concentrations of chlorophyll *a*, from 58 to 44%, but changes in chlorophyll *a* and phaeopigment were not related to ice type (Fig. 3.4). EPS was below detection limits (<10 μg xanthan gum equivalents L^{-1}) in all samples.

3.4.3 'Biological' cores and underlying seawater

Concentrations of bacteria increased upon entrainment and as temperature in the ice decreased further (Fig. 3.5), reaching a mean concentration ~8× greater in upper ice sections than the mean of 8.2×10^5 bacteria ml^{-1} observed in seawater. The observed enrichment (median 0.19, range −0.15–1.83) was in agreement with the enrichment expected due to passive or mildly active entrainment (Fig. 3.8A). Ice sections that had grown quickly (thin young ice) were more enriched in bacteria than slower-growing congelation ice sections (Table 3.1, Fig. 3.8A). Bacteria appeared to be passively incorporated into ice warmer than −5℃, but slightly enriched (relative to salt) in the ice at lower temperatures (Fig. 3.8).

Concentrations of viruses increased upon entrainment into the ice (Fig. 3.6), reaching a mean concentration ~ 37× greater in upper ice sections than the mean of 12.0×10^6 virues ml^{-1} observed in seawater. The observed median enrichment (1.23, range 0.25–12.6) was greater than that expected during complete entrainment of all seawater viruses into the ice. These large enrichments suggested production of viruses in samples derived from different ice types (Fig. 3.8). Viruses may have been passively incorporated into new ice but were highly enriched relative to salt in medium and thick ice, indicating production in situ (Figs. 3.6

and 3.8).

Virus-to-bacteria ratios (VBR) observed in seawater (160 ± 38) were significantly lower (one-way t-test, $p < 0.001$) than those in sea ice (846 ± 169; Fig. 3.9). The highest mean VBRs were found in the bottom 10 cm of thick ice (1654 ± 267), but the maximum VBR (2820) was observed in the bottom 10 cm of medium-thickness landfast ice collected within 500 m of the shore in Summers Harbour (Station R; Fig. 3.1). Calculated contact rates between bacteria and viruses were higher in sea ice than in underlying seawater in all cases (Fig. 3.10). The mean relative VBR (to seawater) was 102±36; the highest relative VBRs were found in upper sections of medium and thick sea ice and the lowest in new ice and the bottom 10 cm of medium and thick ice. Using the same data, virus-bacteria contact rates (Fig. 3.10) can additionally be interpreted as the mean number of viruses that one bacterium would be expected to contact per day (Fig. 3.11), or alternatively the average time between virus contacts for the average bacterium (Fig. 3.12). Thus, over the course of one day, the average seawater bacterium in this study would be expected to contact 13.7±1.2 viruses per day (~ 0.57 h^{-1}), while the average sea ice bacterium would be expected to contact 194±37 viruses per day (~ 8 h^{-1}).

Extracellular DNA (eDNA) concentrations increased upon entrainment into the ice in some ice types (Fig. 3.7), reaching a mean concentration ~ 13× greater in upper ice sections than the mean of 3.1 $\mu g\ L^{-1}$ observed in seawater. A wide range of enrichment indices was observed (–1.0–15.3; Fig. 3.8), with an overall positive enrichment into ice (median 0.72). The highest concentration of eDNA (135 $\mu g\ L^{-1}$) was observed in the upper 10 cm of landfast sea ice collected at the outflow of the Horton River (Station 1117; Fig. 3.1), exceeding the highest concentrations reported in seawater by 440% (Table 3.2). Concentrations of eDNA in sediment would exceed the reported values if scaled to volume of porewater, but eDNA is much more likely to be adsorbed to sediment grains, making such a calculation misleading. Except in thick ice, the amount of DNA in eDNA was greater than that in viruses (Figs. 3.13), though both were consistently greater than the amount of DNA estimated to be present within bacterial cells, which was less than 10% of the total DNA in every sample type (Fig. 3.14).

3.4.4 Seawater depth profile

The water column at Station D7 was stratified, as is typical for the Amundsen Gulf, with shallow Polar Mixed Layer water separated from the deeper, well-mixed Halocline Arctic Layer by an intrusion of warm Pacific water at 20–40 m (Fig. 3.15A). The transition to warmer, more saline Deep Atlantic Layer water occurred around 200 m. The concentrations of bacteria and extracellular D-DNA each exhibited a subsurface maximum within the Pacific-derived layer at 10–30 m (Fig. 3.15B,C), concomitant with a spike in C:N to 19, suggestive of ice algal EPS. Viral abundance decreased monotonically throughout the water column.

3.5 Discussion

Existing reports of lateral gene transfer (LGT) in the environmental literature derive primarily from the terrestrial environment, in the contexts of genetically modified agricultural products and bioremediation. Studies of LGT in the marine environment are relatively sparse and tend to pre-date genomic work (as reviewed by Gauthier and Briettmayer, 1990; Hermansson and Linberg, 1994; Lorenz and Wackernagel, 1994; Dröge et al., 1999). However, several studies (mostly in microcosms) have demonstrated that marine microbial communities undergo gene exchange under permissible conditions (Baross, 1972; Stewart and Sinigalliano, 1990; Paul et al., 1991a; Dahlberg et al., 1998a,b; Jiang and Paul, 1998), although the mechanism is not always known. Though we have not measured LGT in sea ice, the results of this study can be used to form hypotheses regarding the relative importance of different mechanisms of LGT in sea ice and its underlying seawater.

3.5.1 Seawater environment

The concentrations of bacteria we found in Amundsen Gulf seawater (0.4–1.0×10^5 cells ml^{-1}) were lower than expected in Arctic coastal seawater (e.g. 2.1–21×10^5 cells ml^{-1} in the Bering and Chukchi Seas, Steward et al., 1996; 2.0–9.5×10^5 cells ml^{-1} in the Chuckchi and Beaufort Seas, Yager et al., 2001; and 1.6–25×10^5 cells ml^{-1} in the Beaufort Sea and Amundsen Gulf, Payet and Suttle, 2008). However, the concentrations of viruses we found in seawater (4.8–27×10^6 cells ml^{-1}) were comparable to those expected (e.g. 2.5–36.0×10^6 viruses ml^{-1} in the Bering and Chukchi Seas, Steward et al., 1996; 0.8–7.9×10^6 cells ml^{-1} in the Chuckchi and Beaufort Seas, Yager et al., 2001; and 0.1–23×10^6 cells ml^{-1} in the

Beaufort Sea and Amundsen Gulf, Payet and Suttle, 2008). Due to the disparity in expected bacterial concentrations, the virus-to-bacteria ratios (VBRs) we detected in seawater were surprisingly high (45–340), considering that most reported VBRs in seawater fall close to 10 (Maranger and Bird, 1995), with VBRs up to 20 reported in Arctic seawater at the height of a spring algal bloom (Yager et al., 2001). Although the high VBRs reported here would suggest transduction as an important potential mechanism of LGT, the low concentrations of bacteria mean that each bacterium would only be expected to contact about one virus every two hours. Because only a small fraction of phage are transducing (probably < 10%) and many phage have restricted host ranges, the frequency of LGT by transduction in the Arctic seawater we sampled was likely low. We also expect conjugation to be similarly unlikely, since it requires direct cell-to-cell contact and there were few particulates in the water column on which to attract high concentrations of bacteria.

The primary form of D-DNA in the water column was as eDNA, with viral DNA representing up to 50% of the total only in deep waters collected below 100 m. DNA in bacterial cells made up less than 10% of the total DNA in the water column, much less than the ~70–90% observed in other studies (Paul and Carlson, 1984; Brum, 2005), indicating a higher ratio of dissolved DNA to cells in Arctic seawater than that measured in temperate or subtropical waters. The relative abundance of eDNA suggested that transformation may have been a more likely mechanism of LGT than transduction or conjugation in the Arctic seawater we sampled. However, the diffusivity of a given number of basepairs of eDNA is about an order of magnitude lower than that for viral DNA because viral DNA is highly efficiently packed within a protein capsid. Therefore, despite the greater fraction of eDNA in seawater samples, the amount of viral DNA available to a bacterium in seawater may be greater than the amount of eDNA, which may be expected to favor transduction.

At one station where we conducted a depth profile, peaks in bacterial abundance, C:N ratio, and eDNA concentration were observed in a subsurface layer between 10 and 50 m (Fig. 3.15), similar to subsurface peaks observed in temperate and subtropical water masses (Paul and Carlson, 1984; Brum, 2005). We would expect LGT to peak in this subsurface layer due to the relatively higher abundances of genetic material and particulate material upon which it could be concentrated.

3.5.2 Sea ice environment

Physical, chemical, and biological dynamics were observed within Arctic sea ice during the fall freeze-up period. Temperatures within the ice sections we sampled ranged from −1.7 ℃ to −11.3 ℃, while temperature gradients within newly formed ice were up to 2.2 ℃ cm^{-1}. The rapid time of formation of new ice from seawater suggests a temporal gradient up to several degrees per hour during the most intense period of ice growth, while seawater and winter ice may undergo changes of several degrees per day at the surface to only several degrees per month deeper in the water column or near the base of the ice sheet. When it has been tested, decreasing temperature has usually led to decreasing frequencies of natural transformation because DNA uptake mechanisms depend on cellular activity, though transformation has been observed at temperatures more than 20℃ below the host's optimal growth temperature (Frischer et al., 1993; Lorenz and Wackernagel, 1992). As temperature in the ice decreases during winter, the fraction of active bacteria decreases as well (Helmke and Weyland, 1995; Junge et al., 2004), suggesting that, based on temperature alone, the optimal location for transformation in autumn and winter sea ice is near the base of the ice sheet, where the temperature approaches that of the underlying seawater. In addition to heat and cold shock, other LGT-inducing stresses affecting sea ice microorganisms could be elevations in pH (Thomas and Dieckmann, 2002) and ultraviolet radiation (Murray and Jackson, 1993; Perovich, 1993) and high concentrations of divalent cations, reactive oxygen species (Gleitz et al., 1995), and mutagens or heavy metals like Hg (e.g. from snow deposition, Johnson et al., 2008).

The high salt concentrations we found in sea ice brine—between 1.3 and 4.5× the salinity of the underlying seawater—might influence the frequency of LGT in a number of ways. Divalent cations are generally required for DNA uptake during transformation, with frequencies of transformation increasing in a concentration-dependent manner in soil microcosms (Garcia et al., 1978), up to concentrations satisfied in Arctic seawater. The addition of monovalent cations, including Na^+, K^+, and NH_4^+, has also been shown to increase the frequency of transformation, up to the maximum amended concentrations of 100 mM (Garcia et al., 1978). Cations also bind to the negatively-charged phosphate backbone of DNA and bridge the gap between it and other negatively-charged surfaces, including mineral grains (Lorenz and Wackernagel, 1987), and potentially phage particles (Kokjohn, 1989) and EPS (Beveridge et al., 1997; Steinberger and Holden, 2005). Adsorption of eDNA to minerals

increases with cation concentration (Paget et al., 1992; Romanowski et al., 1991), though this adsorption does not necessarily render the eDNA inaccessible to uptake via transformation (Lorenz and Wackernagel, 1987, 1992). Higher salt concentrations can also induce autolysis in bacteria, particularly when a single monovalent cation increases disproportionally to the others, which may occur in very cold winter sea ice as the eutectic points for various sea salts are passed. Some bacteria have also been shown to autolyse, releasing eDNA into the environment, in response to low temperature, as well as during starvation (limitations in carbon and nitrogen Svarachorn et al., 1989, 1991; Yamanaka et al., 1997).

Attachment can also induce pathways for LGT in marine bacteria (Meibom et al., 2005), so we measured a number of parameters relating to the presence of surfaces available for attachment in first year sea ice, observing that older ice became increasingly enriched in particulate organic carbon, both in absolute terms and relative to particulate organic nitrogen (Fig. 3.3). Since the values in 'chemical' cores were scaled to bulk (melted) ice volume, a decrease in POC might have been expected due to the drainage of POC with brine during sea ice growth. The observed increase in POC implies de novo production in older ice (or higher values in the seawater from which it froze), consistent with the observed increase in the percentage of organic matter in older ice. Together with the observations of higher C:N ratios in older ice, these findings suggest the in situ production of carbon-rich material of high molecular weight, like extracellular polymeric substances (EPS). Although EPS was below detectable limits in the autumn ice examined here, previous work has demonstrated the production of EPS in sea ice from the same region during winter (Collins et al., 2008) and spring (Riedel et al., 2006). In addition to carbohydrates, DNA is frequently found as a component of EPS, up to 40% of the dry weight in some biofilm matrices (Catlin, 1956; Steinberger and Holden, 2005). This DNA can be released by cell lysis, secretion (Draghi and Turner, 2006), or via blebbing of membrane vesicles in Gram negative bacteria (Page, 1982; Schooling et al., 2009).

In addition to POC, sea ice is often replete with other biotic and abiotic surfaces, including the frozen ice matrix (to which bacteria have been observed to attach: Junge et al., 2001), large eukaryotic phototrophs or their debris, and entrained aggregates (common in coastal Arctic areas: Stierle and Eicken, 2002; Collins et al., 2008). The presence of surfaces and subsequent attachment has been shown to increase the frequency of gene transfer by conjugation, transduction, and natural transformation in microcosm and laboratory studies

by stabilizing extracellular DNA, attracting high cell densities (Hermansson and Linberg, 1994), and inducing changes in the metabolic state of resident bacteria (Ripp and Miller, 1995). DNA bound to minerals is more resistant to degradation than free DNA but can still be taken up by naturally competent microorganisms (Chamier et al., 1993; Lorenz and Wackernagel, 1990). In one case transformation was shown to be 50× more efficient at the solid-liquid interface than in the liquid alone (Lorenz et al., 1988).

Starvation provides another mechanism to induce pathways for LGT (Meibom et al., 2005). Though we did not measure concentrations of dissolved organic matter (DOM) in sea ice, the high concentrations of POM we found, and previous studies of DOM in sea ice (Thomas et al., 1995), indicate that they are likely to be higher than in seawater due to the brine concentrating effect (Riedel et al., 2006) and release of dissolved cellular material during cell lysis (Collins et al., 2008). The potential for an increased substrate requirement at low temperatures might induce starvation even at high concentrations of DOM (Wiebe et al., 1992, 1993), but this effect has not been conclusively demonstrated in environmental samples (Yager and Deming, 1999; Kirchman et al., 2005). Moreover, unusually high concentrations of organic or inorganic solutes could themselves induce a stress response in the oligotrophic microorganisms expected in autumn sea ice (Collins, 2009), which are adapted to lower, seawater concentrations of compounds like nitrate or organic acids and often fail to grow when exposed to higher concentrations (Könneke et al., 2005; Giovannoni et al., 2005).

If sea ice is in fact a hotspot for LGT, the dispersal of transformants from sea ice could be significant over large geographic and temporal scales. Arctic sea ice can entrain, transport and distribute particles and possibly pollutants (Nurnberg et al., 1994; Eicken et al., 1997; Pfirman et al., 1995; Stierle and Eicken, 2002; Eicken et al., 2005) throughout the Arctic Ocean faster than ocean currents alone and could be expected to do the same for recombinant microorganisms. This rapid and widespread distribution of a new lineage could potentially promote the fixation of the new recombinant genome into the metapopulation, for example by spreading genes during a selective sweep that might be detectable by metagenomic analyses (Cohan, 2001; Nesbø et al., 2005).

3.5.3 Conjugation in sea ice

Assuming that similar microbial communities (and their plasmids) are present in seawater and young sea ice (see Chapter 3), the higher concentrations of bacteria we observed (and others frequently observe) in autumn sea ice brine—nearly an order of magnitude greater than in the underlying seawater—should favor the occurrence of conjugation in sea ice relative to the underlying seawater. Conversely, the lower temperatures and higher salt concentration in sea ice brine should disfavor conjugation there relative to the underlying seawater. We predict the optimum location to investigate conjugation in sea ice would be from particle-rich coastal sea ice during the biologically productive spring and summer seasons.

3.5.4 Transduction in sea ice

High abundances of viruses have been repeatedly observed in sea ice from a variety of sources (Maranger et al., 1994; Gowing et al., 2002, 2004; Wells and Deming, 2006c), including the sea ice described here, which contained 4–150× more viruses than the underlying seawater. Extremely high VBRs were observed in the autumn sea ice we sampled (mean 105, maximum 2820), values up to two orders of magnitude greater than previously reported from the marine environment. Freshwater and saline lakes frequently have VBRs greater than in marine environemnts, including several Arctic and Antarctic lakes (Maranger and Bird, 1995; Laybourn-Parry et al., 2007; Säwström et al., 2008), one of which had VBRs up to 128 (Madan et al., 2005). Large VBRs were shown to increase the apparent transduction rate in a series of experiments with freshwater *Pseudomonas aeruginosa* microcosms (Saye et al., 1987; Replicon et al., 1995). Extremely high VBRs may increase the likelihood of abrupt cell lysis due to high multiplicities of infection, or the induction of lysogeny (Kokjohn, 1989), both of which would limit the production of new viruses in the system. Polylysogeny would increase the likelihood of high-frequency-of-transduction lysates (Kokjohn, 1989) in sea ice over that in the underlying seawater, increasing the potential for transduction there.

Predicted contact rates between cells and viruses in autumn sea ice were 7–844× higher than in the underlying seawater (Wells and Deming, 2006c). A comparison of the the absolute virus-bacteria contact rates between sea ice and seawater highlights the ecological importance of these differences. Over the course of one day, the average seawater bacterium

would be expected to contact about 14 viruses, or about 0.6 viruses h^{-1}. If a bacterium in seawater were susceptible to only 5% of the viruses that it contacted, it would take nearly two days to become infected. In contrast, the average sea ice bacterium would be expected to contact about 190 viruses per day, or about 8 h^{-1}; assuming the same 5% frequency of infection, it would take less than 3 h for a bacterium in sea ice to become infected.

However, the assumption of equal infectivities may not hold between sea ice and seawater: high salinity (161‰) was shown to inactivate cold-active bacteriophage 9A (isolated from an Arctic nepheloid layer; Wells and Deming, 2006a) up to 1000× faster than it was physically decayed (Wells and Deming, 2006b), dependent on temperature. In addition, the highest contact rates in the sea ice sample were observed in upper sections of ice, where the cells were likely to be least active (Junge et al., 2001). Metabolic activity by the host cell is required for viral production, but not for adsorption or injection of DNA into the host cell (Kokjohn, 1989). Furthermore, injected DNA can persist intracellularly until the phage follows the lytic or lysogenic pathway (pseudolysogeny, Miller, 2001), meaning that latent infections can persist until the return of favorable host growth conditions. Alternatively, lysogeny requires a minimum of energetic expense so it may be favored under stressful conditions (Payet and Suttle, 2008) like those present in sea ice, moreso than in the underlying seawater. In this case LGT would be favored by the vastly higher virus-bacteria contact rates in sea ice relative to its underlying seawater because of the increased probability of a cell contacting a virus capable of lysogenizing it.

The extremely high enrichment indices calculated for viruses in some sea ice samples strongly suggested viral production occurred within the ice. To explain the high concentrations of viruses in some upper sea ice samples without in situ production, at least 10× the mean seawater concentration of viruses would have had to be completely entrained during ice formation—an unlikely circumstance. The production of viruses in sea ice has been reported as an increase in viral abundance over time (Maranger et al., 1994) and of visibily infected cells (Gowing, 2003). Wells and Deming (2006c) observed production of sea ice viruses in natural brine incubations at temperatures as low as −12°C, comparable to the temperatures experienced by microorganisms in the upper sea ice sections we sampled. The continued presence of high viral loads in some sections of upper sea ice implied a low rate of viral decay there, likely attributable to the preservative effects of low temperatures (Noble and Fuhrman, 1997; Wells and Deming, 2006b). However, it must be emphasized that

many viruses may persist in sea ice but lose their infectivity (Wells and Deming, 2006b), potentially due to the denaturation of capsid proteins at high salinity or due to phenotyic changes in their host under extreme conditions. Additionally, while organic particles have been shown to adsorb viruses and render them inactive (Suttle and Chen, 1992; Noble and Fuhrman, 1997), cold-active bacteriophage 9A failed to adsorb to two types of clay at temperatures and salinities relevant to sea ice (Wells and Deming, 2006b). Instead, clays and sediment grains may enhance the lifetimes of viruses in seawater by reversibly adsorbing them (Murray and Jackson, 1992; Gantzer et al., 1994; Wells and Deming, 2006b).

3.5.5 Transformation in sea ice

Concentrations of eDNA in the Arctic seawater we sampled (0.3–12.7 μg L^{-1}) were comparable to typical values (0.1–28 μg L^{-1}), but much lower than in some sea ice samples, which had concentrations of eDNA up to 135 μg L^{-1}, higher than previously reported from any marine environment (Table 3.2). The frequency of transformation in experimental studies is often directly proportional to free DNA concentration (Frischer et al., 1993; Sikorski et al., 1998), so high concentrations of eDNA should favor transformation in sea ice relative to the underlying seawater. However, because of the long, flexible nature of free DNA, a 50 kbp fragment of eDNA is expected to have a diffusivity at least an order of magnitude lower than a virus with a comparable genetic complement. Smaller fragments of eDNA, circa 5 kbp, have diffusivities on the same order as a virus. Although in several ice types eDNA makes up a larger fraction of the total DNA than viral DNA does, the average bacterium will likely contact more viral DNA than eDNA over the course of one day, especially at lower temperatures, suggesting that transformation maybe of limited usefulness during colder parts of the winter.

Nearly half of the sea ice sections contained concentrations of eDNA consistent with depletion or passive accumulation from seawater (Fig. 3.8); most of the remaining samples exhibited concentrations suggestive of active accumulation, particularly within samples from the top 10 cm of medium-thickness ice (Fig. 3.8). Since the estimated pool of DNA within living bacterial cells was always less than 10% of the total eDNA (Fig. 3.14), it is unlikely that the 'excess' eDNA in enriched samples was produced exclusively by lysis of a static bacterial community. Rather, the enrichment indices (up to 15) suggested continued production over a period of time, for example by growth and lysis of an active bacterial

community over the course of a number of cell divisions, as part of a biofilm matrix, in response to stress or competence development. Although very low chlorophyll *a* values in most samples indicated that few photoautotrophic microorganisms were present, the effect of picoeukaryotic DNA on the concentration of eDNA could be disproportionally large due to their large genome sizes.

The presence of high concentrations of eDNA in upper sections of thick ice, which had likely been separated from communication with seawater for several weeks, suggests that eDNA can persist in sea ice at temperatures down to −11 ℃. Though we did not measure the quality or age of the eDNA, previous studies provide a wide range of half lives for D-DNA in warmer marine environments, from 3.4 h in estuarine conditions to 3.5 d in P-replete open ocean regions (DeFlaun et al., 1987). These half lives, measured in subtropical waters, are surely more rapid than in colder polar waters and sea ice, where the activity of extracellular nucleases would be temperature-limited. Because transformation rates tend to increase with higher molecular weight DNA, LGT by transformation should be favored in sea ice if eDNA has a longer half life there than in the underlying seawater.

Previous studies report naturally competent cells (genetically and phenotypically capable of taking up free DNA) comprised 10–16% of marine microbial communities (Frischer et al., 1990, 1994). Factors that induce host competence include quorum sensing (e.g. a diffusible 'competence factor' is required in Gram positives Petersen et al., 2005), stress, starvation, and attachment (Meibom et al., 2005; Blokesch and Schoolnik, 2008). As discussed above, the large surface areas, possible nutrient limitation and cold and salinity shock during initial entrainment may induce competence in cells within sea ice more frequently than in the seawater, favoring LGT by transformation in sea ice.

Once imported into the cell, exogenous DNA must be incorporated in the host genome to classify as a successful transformation event. Because some transformation systems require homologous DNA or the presence of specific binding sequences for uptake and recombination into the host genome (Danner et al., 1980, 1982), the presence of many closely-related but distinct phylotypes in winter sea ice (e.g. SAR11 clade Alphaproteobacteria and *Polaribacter* spp., Collins, 2009) suggests that transformation in sea ice may provide a suitable mechanism of genetic interaction for these populations. However, not all transformation systems require homologous DNA (Dubnau, 1991; Smith et al., 1981), so the presence of high concentrations of eDNA from a wide diversity of Bacteria and Archaea (Collins, 2009)

may provide unique possibilities for cross-Domain LGT in the marine environment.

3.6 Conclusions

Sea ice is a microbial habitat that embodies a number of physical, chemical, and biological characteristics that have previously been shown to increase the frequency of LGT by conjugation, transduction, or transformation. Here we report high concentrations of cells, viruses, extracellular DNA, and stress-inducing conditions like low temperature and high salinity in first year Arctic sea ice. Further inducers of LGT, including the production of phage, starvation, surfaces for attachment, antibiotics, and cell-to-cell communication (quorum sensing), likely occur in sea ice as well.

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Table 3.1: Summary of ice core characteristics.

Station	Day	Location	Thickness (cm)	Ice Type	Ice Class (top)	Ice Class (bottom)
D3	333	70.922 N 123.317 W	4	nilas	—	—
1800	330	72.088 N 127.592 W	9	nilas	frazil	—
1200	324	71.076 N 123.438 W	6	consolidated pancakes	—	—
D4	336	71.730 N 125.564 W	33	medium first year pack	transition	frazil, congelation
1117	320	69.872 N 126.542 W	35	medium first year landfast	frazil, transition	congelation
R	318	70.125 N 125.081 W	37	medium first year landfast	—	—
437	326	71.749 N 126.576 W	66	thick first year pack	frazil, transition	congelation
D7	344	71.258 N 125.299 W	78	thick first year pack	congelation	congelation

Table 3.2: Extracellular DNA concentrations (μg L^{-1}) measured in various marine environments. Techniques are abbreviated as follows: PG, PicoGreen fluorescence; Hoescht, Hoescht 33258 fluorescence; DABA, DABA fluorescence; ehDNA, extracellular hydrolyzable DNA.

Site	Location	Environment	Min	Max	Technique	Reference
Station 1117	Beaufort Sea	first year sea ice, medium	12	135	PG	this study
Station D7	Beaufort Sea	first year sea ice, thick	0	50	PG	this study
Station R	Beaufort Sea	first year sea ice, medium	0	48	PG	this study
Station D4	Beaufort Sea	first year sea ice, medium		40	PG	this study
	Mediterranean Sea	sediment	9	35	H-DNA	Dell'Anno et al. 2004
	Mediterranean Sea	offshore seawater	2	31	Hoescht	Dell'Anno et al. 1998
Kahana Bay	Hawaii	coastal seawater	12	28	DABA	Karl and Bailiff 1989
Station 437	Beaufort Sea	first year sea ice, thick	0	27	PG	this study
	Adriatic Sea	sediment		24	PG	Corinaldesi et al. 2005
Ala Moana Beach	Hawaii	coastal seawater	19	21	DABA	Karl and Bailiff 1989
	Mediterranean Sea W	sediment		21	PG	Corinaldesi et al. 2005
Tampa Bay	Florida	coastal seawater	10	19	Hoescht	DeFlaun et al. 1987
Magic Island	Hawaii	coastal seawater	14	19	DABA	Karl and Bailiff 1989
Kaneohe Bay	Hawaii	coastal seawater	10	16	DABA	Karl and Bailiff 1989
Station 1200	Beaufort Sea	consolidated pancake ice		16	PG	this study
Florida Bay	Florida	coastal seawater	10	15	Hoescht	DeFlaun et al. 1987
Tampa Bay	Florida	coastal seawater	2	15	Hoescht	Paul et al. 1988
Bransfield Strait	Antarctica	coastal seawater	6	15	DABA	Bailiff and Karl 1987
All stations	Beaufort Sea	Arctic coastal seawater, 0–30m	0.3	13	PG	this study
Tampa Bay	Florida	coastal seawater	9	12	Hoescht	Paul et al. 1986
Station D3	Beaufort Sea	nilas ice	5	8	PG	this study
Adriatic Sea	Adriatic Sea	coastal seawater	2	7	Hoescht	Turk et al. 1992
Pacific Ocean S	Pacific Ocean S	sediment		7	PG	Corinaldesi et al. 2005
Kahana Bay	Hawaii	coastal seawater		5	DABA	Karl and Bailiff 1989
Station D8	Beaufort Sea	Arctic coastal seawater, 100–441m	1	4	PG	this study
Gulf of Mexico SE	Gulf of Mexico SE	offshore seawater	1	4	Hoescht	Paul et al. 1986
Gulf of Mexico	Gulf of Mexico	offshore seawater	4	4	Hoescht	DeFlaun et al. 1987
Kaneohe Bay	Hawaii	coastal seawater	2	3	DABA	Karl and Bailiff 1989
Station 1800	Beaufort Sea	nilas ice		3	PG	this study
Chub Cay	Bahamas	coastal seawater		2	Hoescht	Paul et al. 1991
Mamala Bay	Hawaii	coastal seawater		1	DABA	Karl and Bailiff 1989
Loggerhead Key	Bahamas	coastal seawater		1	Hoescht	Paul et al. 1986
Station ALOHA	Station ALOHA	offshore seawater		1	PG	Brum et al. 2004
Pacific Ocean N	Pacific Ocean N	offshore seawater		1	DABA	Karl and Bailiff 1989
	Instant Ocean	artificial sea salts		0	PG	this study

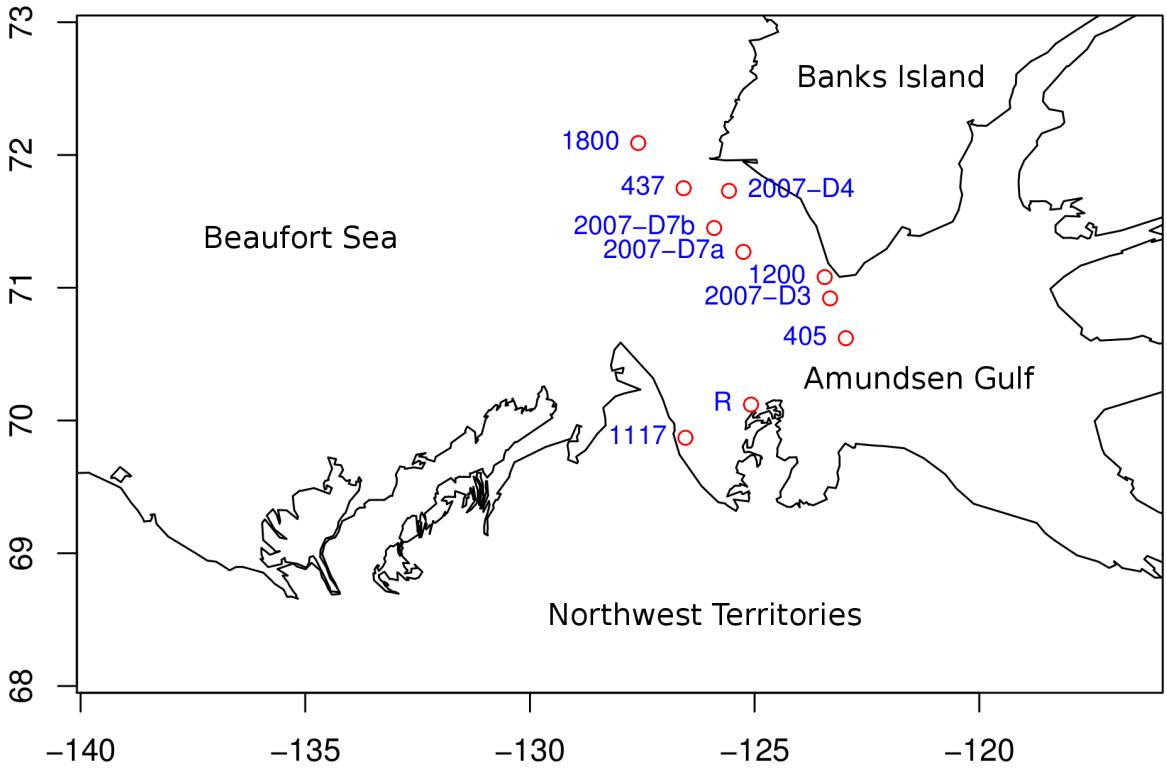

Figure 3.1: Map of sampling stations in the Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

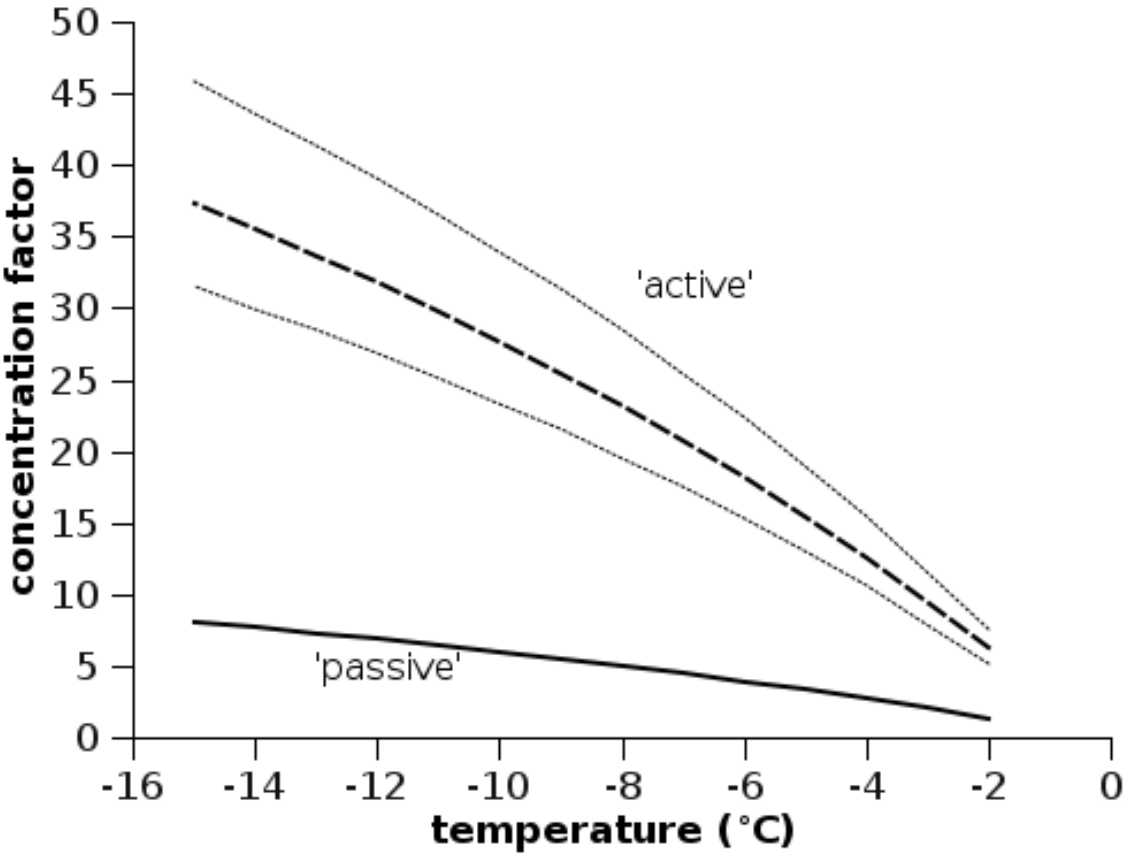

Figure 3.2: Concentration factors calculated for ice with a constant bulk salinity of 6.5±1.2, the mean for all sampled sea ice cores. The upper set of curves represents the brine concentrating factor (BCF) expected during complete entrainment of solutes from seawater into the ice during ice growth. Complete entrainment from seawater would only be expected by an 'active' ice-affine solute that would bind to or stick in the ice during brine expulsion and gravity drainage. 'Passive' solutes like salts instead follow the lower curve, indicative of partial entrainment and subsequent losses due to brine expulsion and gravity drainage.

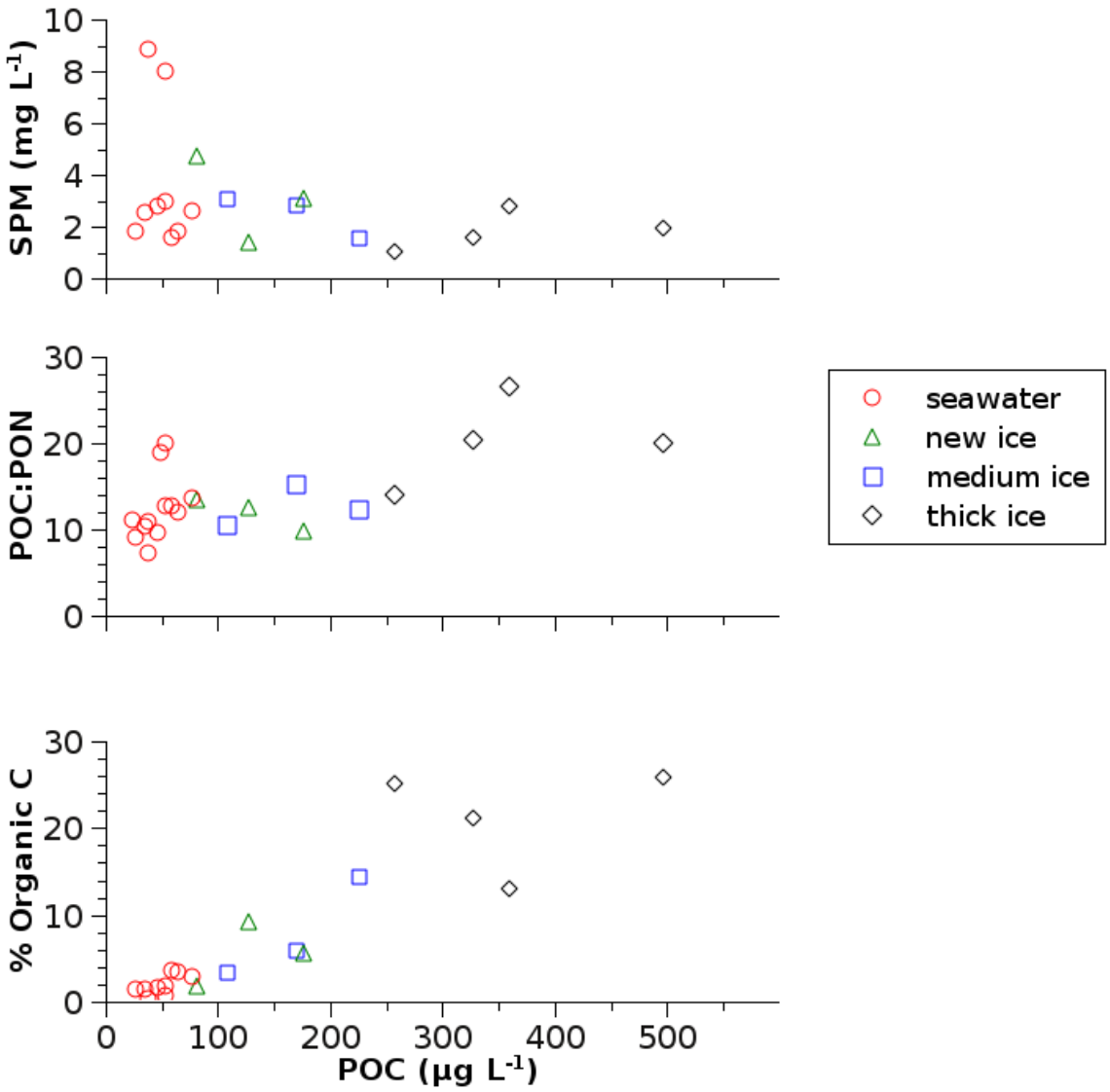

Figure 3.3: Total suspended particulate matter (SPM), C:N ratio (POC PON^{-1}) and percent organic carbon (POC × 1000 × SPM^{-1} × 100%) as a function of POC in seawater and sea ice from the Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

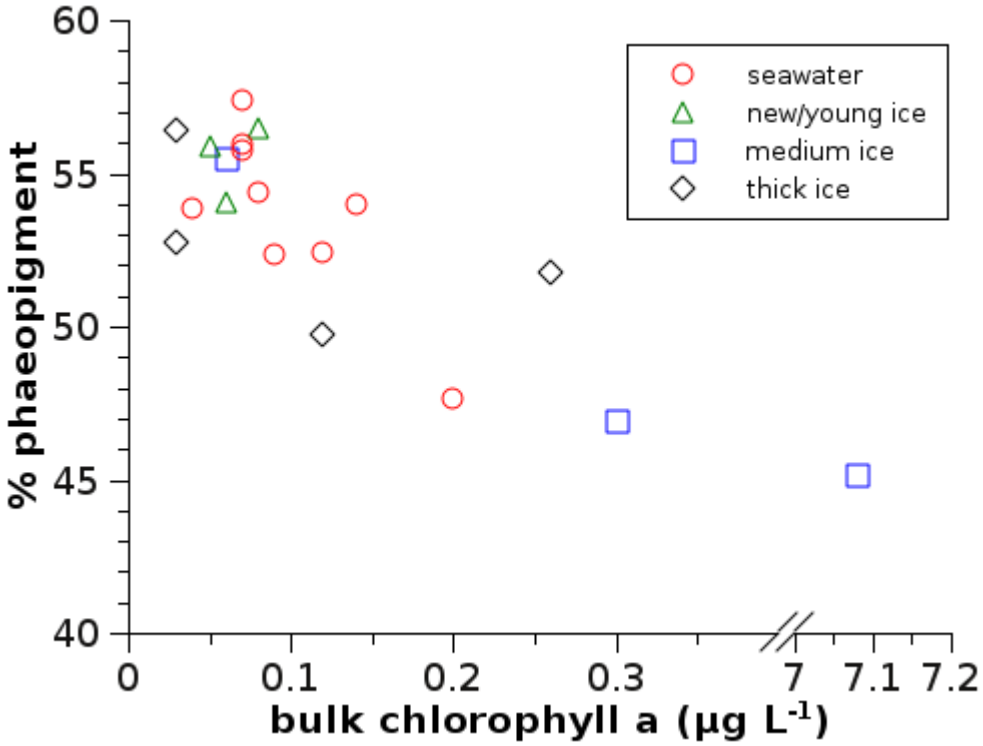

Figure 3.4: Chlorophyll *a* and percent phaeopigment in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

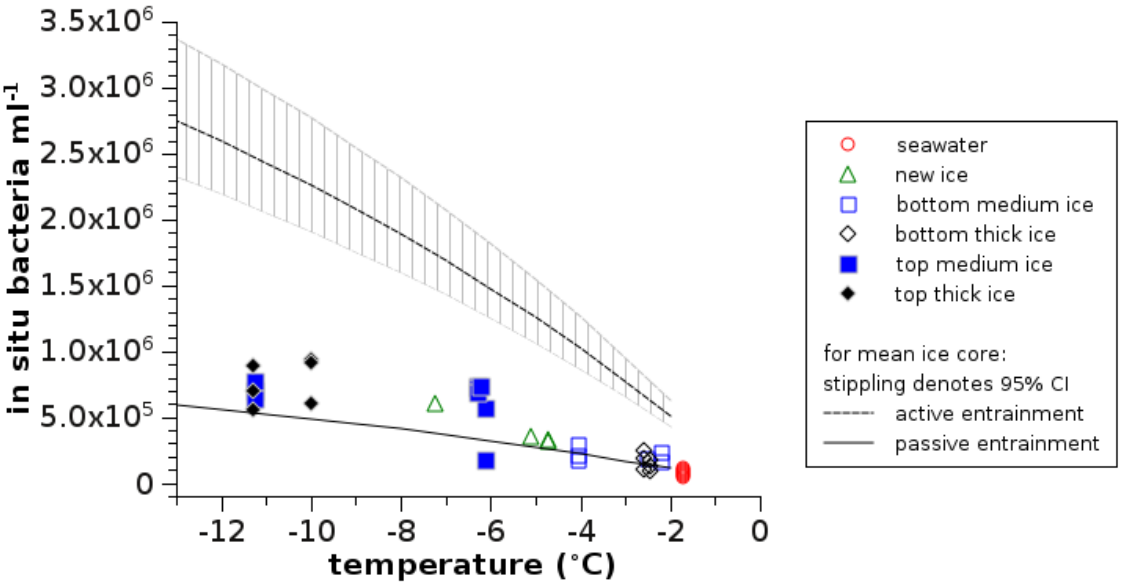

Figure 3.5: Concentrations of bacteria in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector, with predicted concentrations for passive and active entrainment (see Fig. 3.2)

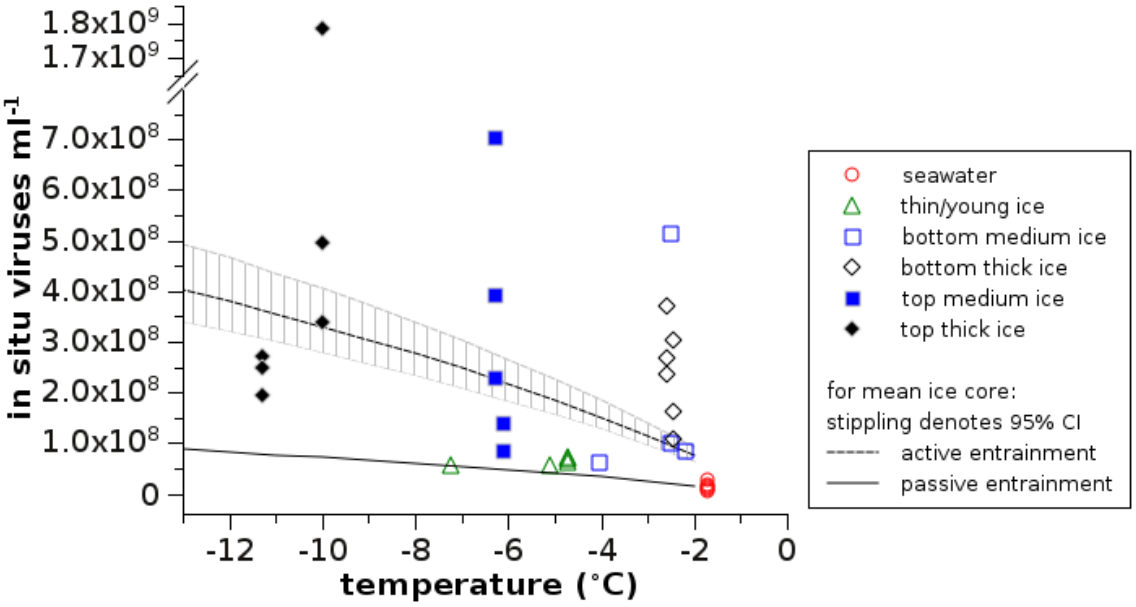

Figure 3.6: Concentrations of viruses in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector, with predicted concentrations for passive and active entrainment (see Fig. 3.2.

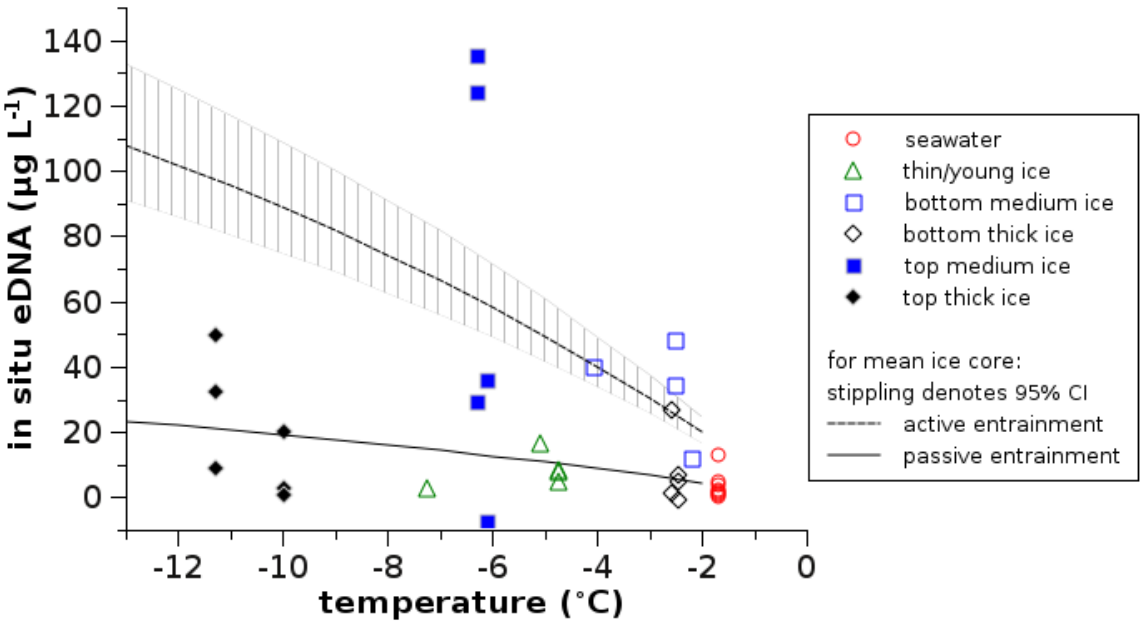

Figure 3.7: Abundance of extracellular dissolved DNA in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector, with predicted concentrations for passive and active entrainment (see Fig. 3.2.

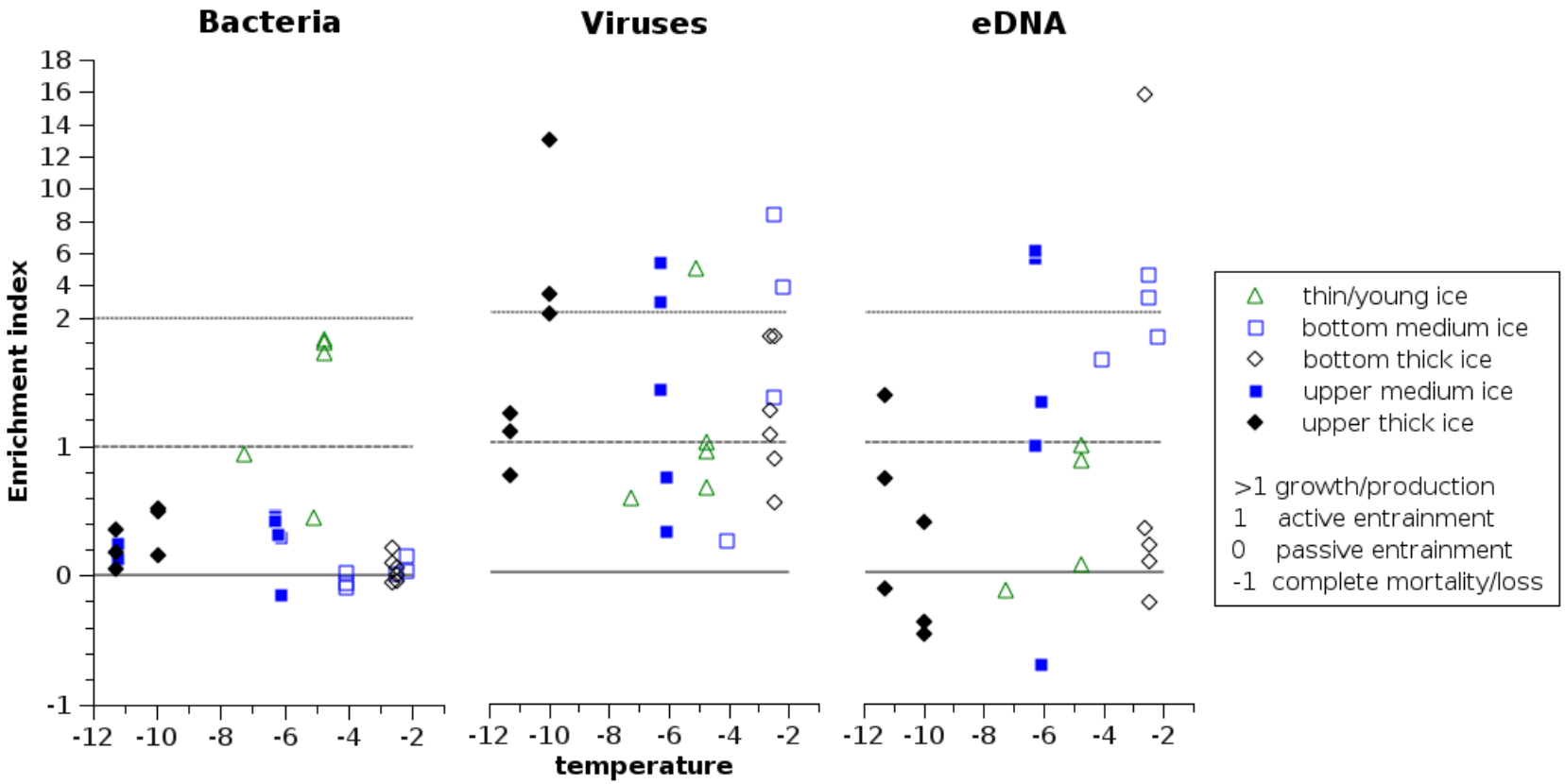

Figure 3.8: Enrichment indices of bacteria, viruses, and extracellular DNA in sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

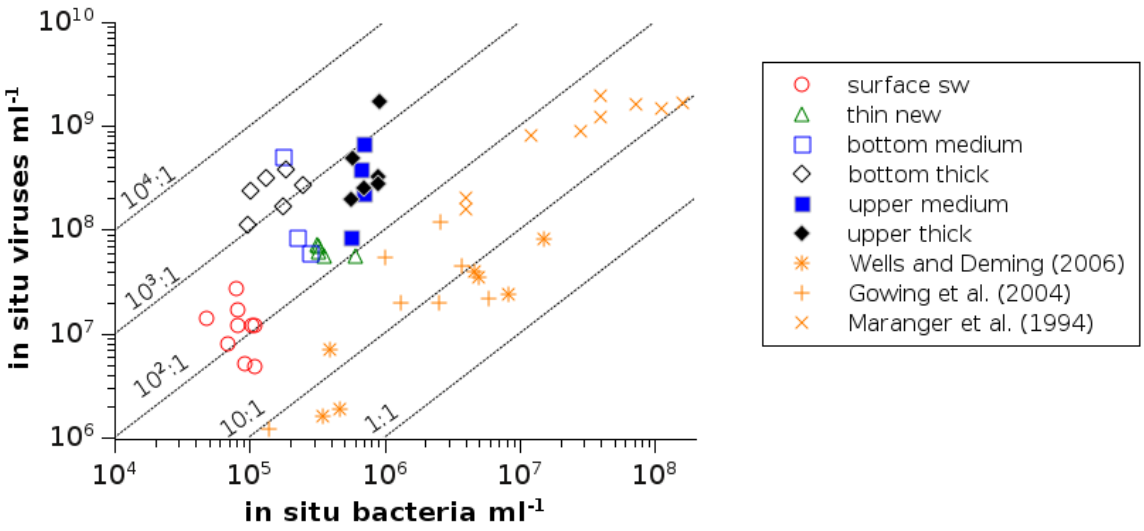

Figure 3.9: Concentrations of bacteria and viruses in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector. The parallel angled lines indicate lines of constant virus-to-bacteria ratio (VBR).

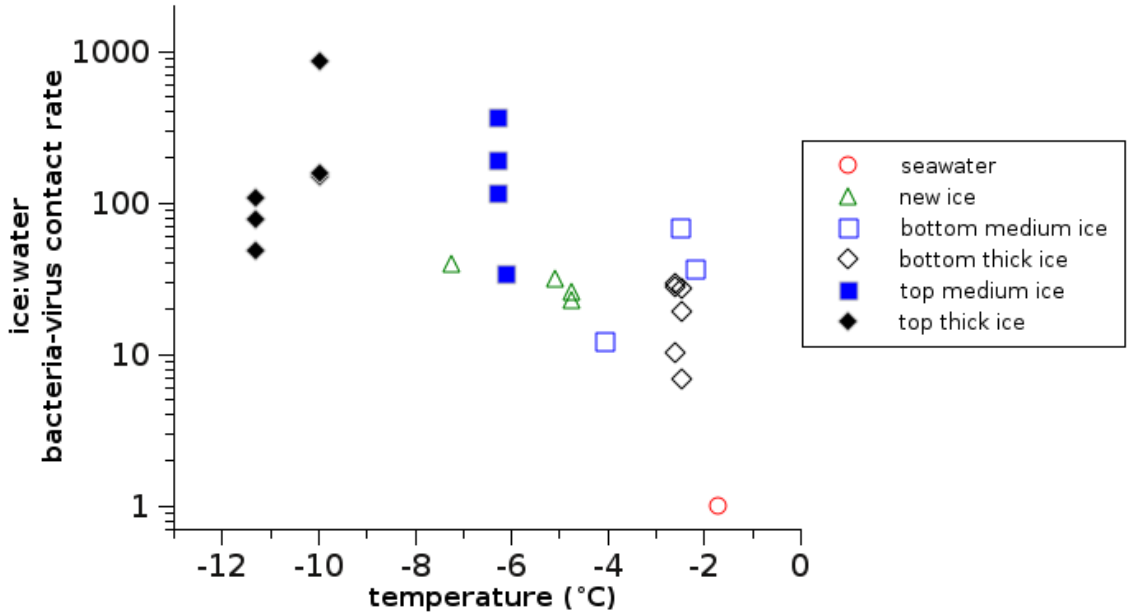

Figure 3.10: Virus-bacteria contact rates relative to seawater at −1.7℃ in sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

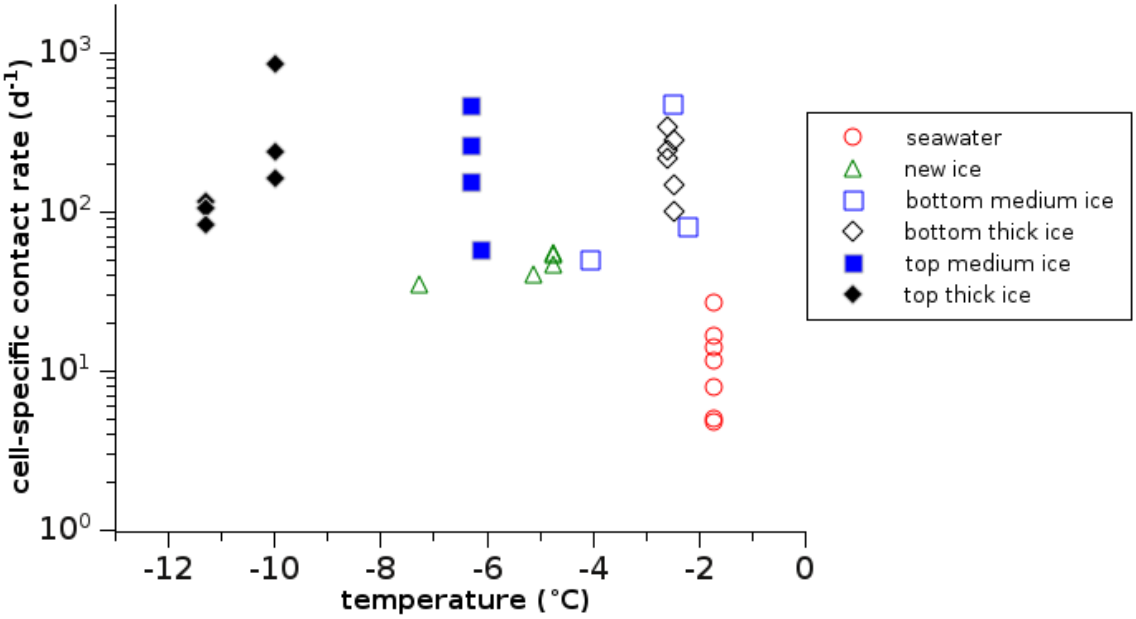

Figure 3.11: Average number of virus-bacteria contacts per bacterium per day expected in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

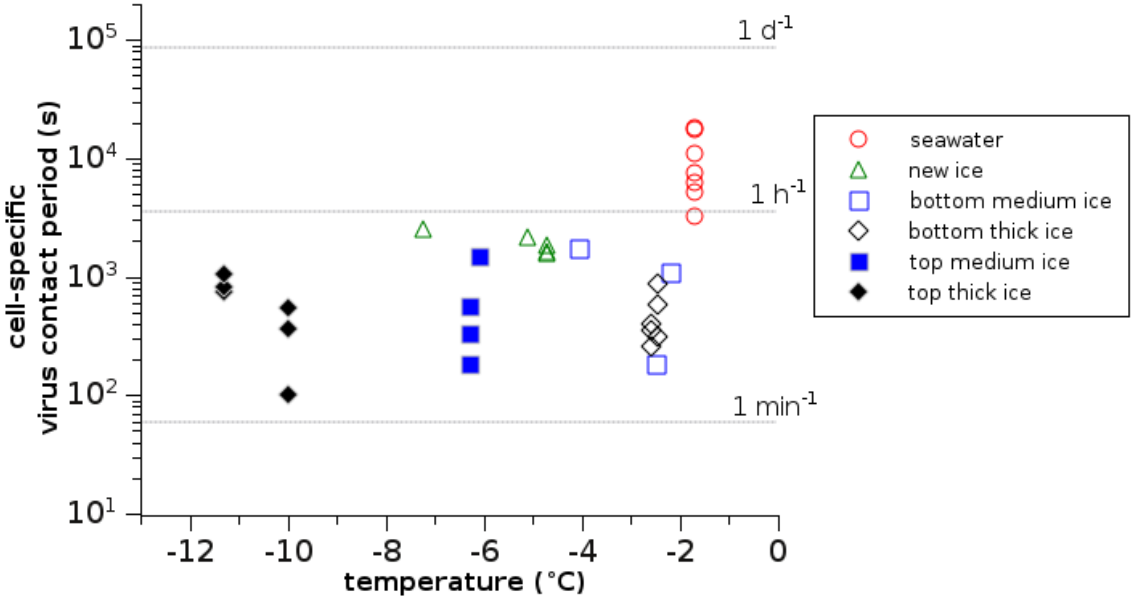

Figure 3.12: Average number of seconds between virus-bacteria contacts per bacterium expected in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

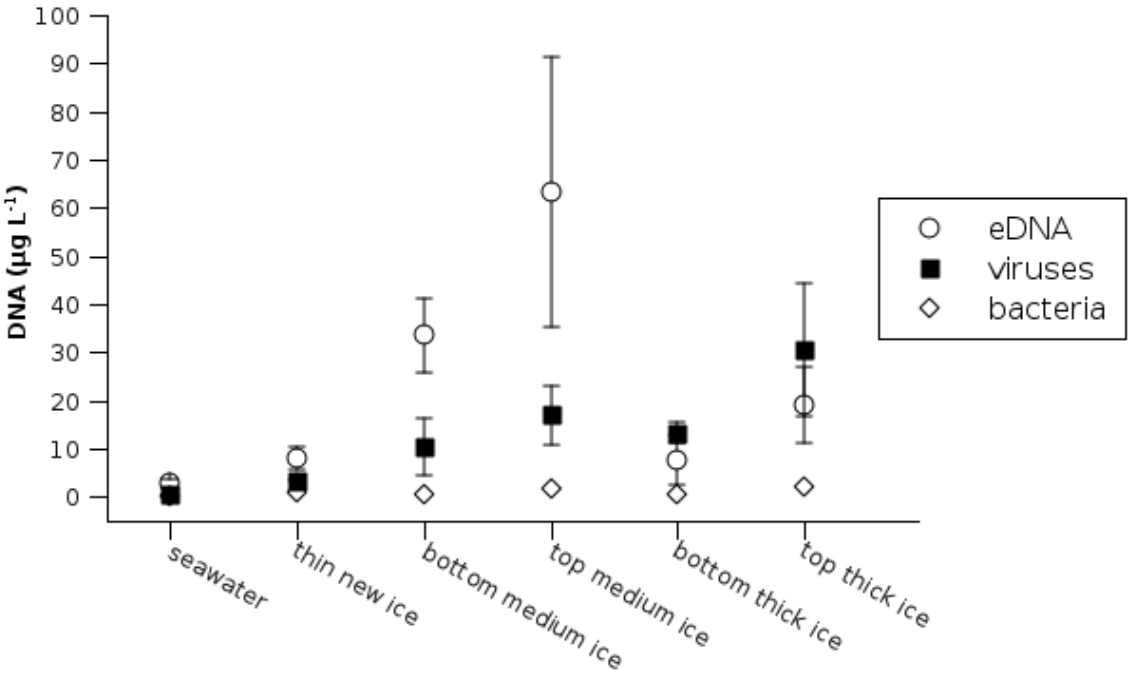

Figure 3.13: Concentrations of DNA in bacteria, viruses and extracellular DNA in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

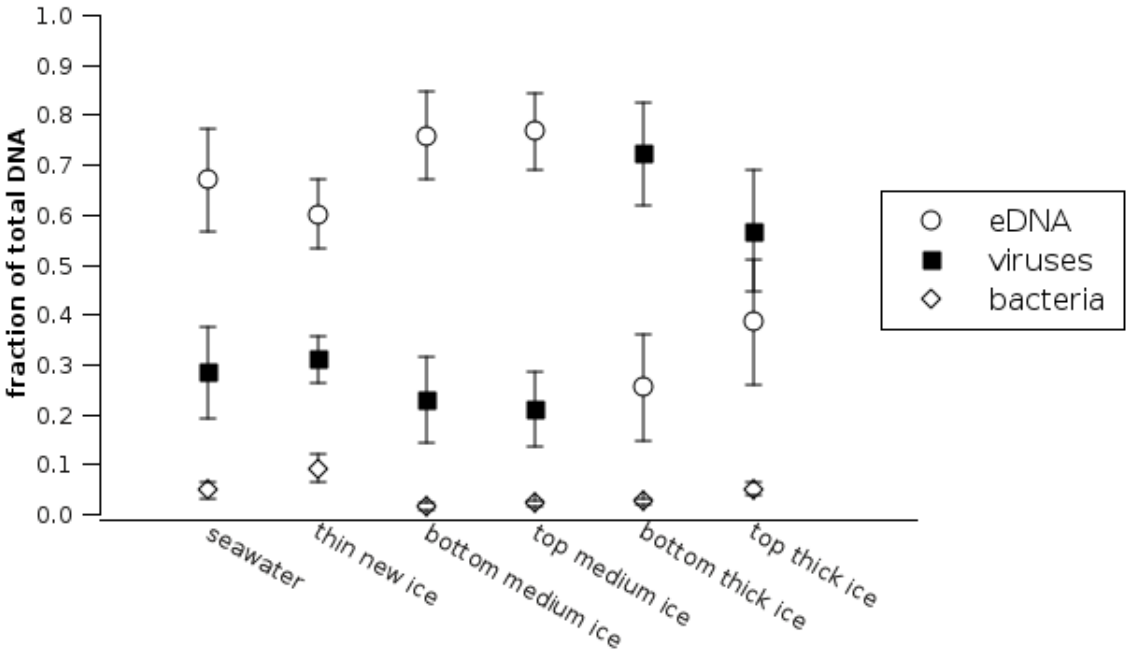

Figure 3.14: Relative abundances of DNA in bacteria, viruses and extracellular DNA in seawater and sea ice from Amundsen Gulf and Beaufort Sea, Canadian Arctic sector.

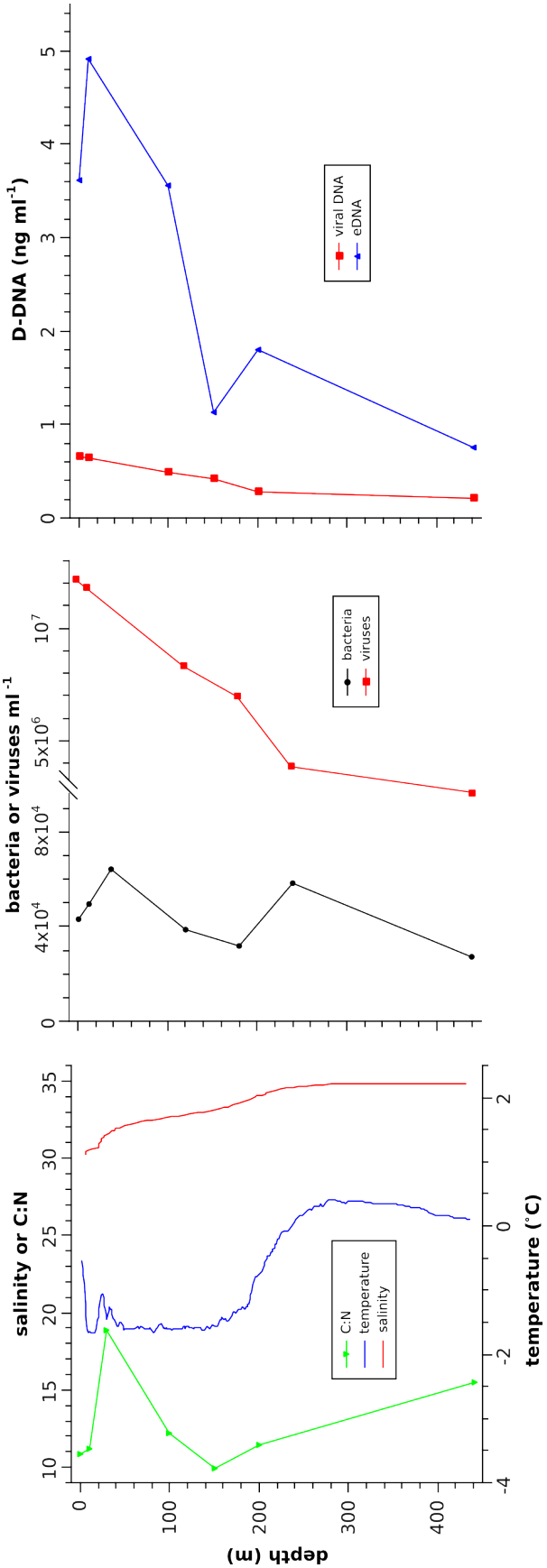

Figure 3.15: Seawater depth profile taken at Station D7a showing (A) temperature, salinity, and C:N ratio, (B) bacteria ml^{-1} and viruses ml^{-1}, and (C) D-DNA in viruses and dissolved free eDNA.

Chapter 4

IDENTIFICATION OF AN INTER-ORDER LATERAL GENE TRANSFER EVENT ENABLING THE CATABOLISM OF COMMON COMPATIBLE SOLUTES BY *COLWELLIA PSYCHRERYTHRAEA* 34H

4.1 Abstract

Colwellia is a genus of generally psychrophilic marine Gammaproteobacteria of the Order Alteromonadales that have been frequently isolated from polar marine sediments and sea ice. To date a single representative, *Colwellia psychrerythraea* 34H, has had its genome fully sequenced. Based on abnormally high GC content, we detected a duplicated genomic island containing an operon encoding for heterotetrameric sarcosine oxidase and additional genes used in the catabolism of sarcosine (*glyA-soxB-soxD-soxA-soxG-purU*), a derivative of the compatible solute glycine betaine. Phylogenetic analysis indicated that the original genomic island was likely acquired from *Marinomonas*, a genus of marine Gammaproteobacteria of the Order Oceanospirillales, which have also been isolated from sea ice. We detected expression of sarcosine oxidase in *C. psychrerythraea* 34H by positive growth on defined medium containing sarcosine (N-methylglycine) as the sole organic source of carbon and nitrogen, and by the detection of 40 peptides unique to sarcosine oxidase by tandem mass spectrometry. Growth of 90% of tested *Colwellia* species on a newly developed sarcosine-based defined medium for the rapid growth of *C. psychrerythraea* 34H suggested that the ability to catabolize sarcosine may be widespread in the genus *Colwellia*. This ability likely provides a selective advantage to *Colwellia*, many of which inhabit cold salty environments like sea ice.

4.2 Introduction

Lateral gene transfer is an important contributor to microbial evolution, allowing Bacteria and Archaea to adapt to new environments on relatively short time scales by importing useful genes from other, potentially unrelated, microorganisms. Large fragments of DNA containing multiple genes can be transferred from one microorganism to another by

conjugation, transduction, or transformation. Although little work has been done to localize and document the occurrence of lateral gene transfer in the environment, various chemical and biological parameters can be expected to influence the frequency of gene transfer and the functions of the genes transferred.

Sea ice is an extreme environment characterized by low temperature (−2 to −35℃) and high salinity (35 to 270‰) in its brine inclusions, where the microbes reside (Junge et al., 2001; Collins et al., 2008). Recent investigations of sea as a hotspot for lateral gene transfer have focused on its its high concentrations of bacteria, viruses, and extracellular free DNA, among other contributing factors (see Chapter 3). High salinity is known to induce several mechanisms for osmotolerance in Bacteria, Archaea, and Eukarya, one of the most common being the intracellular accumulation of molar quantities of compatible solutes like glycine betaine (Bremer and Krämer, 2000; Roberts, 2005), which could be released by microbial residents into sea ice brine channels from lysed cells during seasonal or diurnal changes in brine salinity or in the event of lysed cells. The rare ability to degrade these compounds might be expected to provide a selective advantage in such an environment. The most rapid mechanism by which such an ability could be acquired is lateral gene transfer of the necessary genes from a microorganism already equipped for the task. We have previously shown sea ice to contain high concentrations of the agents of lateral gene transfer, including bacteria, viruses, and extracellular dissolved DNA (Collins and Deming, 2009), in addition to conditions conducive to the induction of the various mechanisms involved.

Colwellia is a genus of heterotrophic marine Gammaproteobacteria (Deming et al., 1988) containing 7 described psychrophilic species (Bowman et al., 1998; Yumoto et al., 1998; Nogi et al., 2004) and one mesophile (Jung et al., 2006). Isolates belonging to this genus have been cultured from sea ice (Bowman et al., 1998; Zhang et al., 2008a), marine sediments, estuarine waters, and both epipelagic and hadalpelagic marine environments. The complete genome sequence for *Colwellia psychrerythraea* str. 34H, isolated from Arctic marine sediments (Huston et al., 2000), has been published (Methé et al., 2005). Prior work describing cold-active enzymes (Huston et al., 2000, 2004), motility at subzero temperature (Junge et al., 2003), activity in saline ice at very low temperature (Junge et al., 2006), increased production of EPS at low temperature (Marx et al., 2009), and a psychrophilic viral-host system (Wells and Deming, 2006a,b) complements plentiful genomic evidence of adaptation to cold salty environments by *C. psychrerythraea* 34H. Besides genes involved in maintaining

membrane fluidity at low temperature and synthesizing extracellular polysaccharides and enzymes, the genome also encodes for the production, transport, and degradation of compatible solutes (Methé et al., 2005).

Compatible solutes are small, chemically inert, neutral or zwitterionic molecules accumulated intracellularly by microorganisms to maintain turgor pressure and stabilize proteins in high osmolarity environments (Bremer and Krämer, 2000; Roberts, 2005). One of the most common compatible solutes is glycine betaine (trimethylglycine), which is used as an osmoprotectant by eukaryotes (including marine diatoms; Keller et al., 1999; Armbrust et al., 2004), and numerous bacteria (Imhoff and Rodriguez-Valera, 1984; Landfald and Strøm, 1986), which can retain intracellular concentrations on the order of 1 M. Glycine betaine can be produced from choline, a component of the common membrane lipid phosphotidylcholine (Sohlenkamp et al., 2000), by serial dehydrogenation via the intermediate betaine aldehyde (Fig. 4.1). The transport and catabolism of choline in bacteria is performed by a single operon, containing a betaine/carnitine/choline (BCCT) transporter (*betT*), choline dehydrogenase (*betA*), betaine aldehyde dehydrogenase (*betB*), and a transcriptional regulator (*betI*). Many bacteria that utilize glycine betaine as an osmolyte probably do not synthesize it themselves due to the energetic costs, since it is readily available in the environment from plant roots or the breakdown of cellular material (Bremer and Krämer, 2000); rather, they import it from their environment. Multiple glycine betaine import systems are known, even from a single organism (Kappes et al., 1996), including the BCCT secondary transporters, like BetL from *Listeria monocytogenes*, and a number of ATP binding casette (ABC) active transporters, including OpuAC from *Listeria monocytogenes*, ProU from *Vibrio parahaemolyticus*, and OpuABC from *Pseudomonas aeruginosa* (Bremer and Krämer, 2000; Wemekamp-Kamphuis et al., 2004; Ozcan et al., 2007). Investigation into *Vibrio cholerae* transporters has led to the hypothesis that they play an important role in the formation and persistence of biofilms in estuarine and marine environments (Kapfhammer et al., 2005).

Although most bacteria do not appear to utilize glycine betaine as an energy source, those that do may have a selective advantage in high-osmolarity environments. Glycine betaine degradation proceeds by serial demethylation of glycine betaine to dimethylglycine, sarcosine, and finally glycine, which can then be further catabolized to serine and pyruvate (Fig. 4.1). Although the pathway is conserved, there are multiple genes to perform

the reactions. Genes encoding demethylases that produce dimethylglycine (*gbcAB*) and sarcosine (*dgcAB*), and a transcriptional regulator (*gdbR*), were recently reported from *P. aeruginosa* PAO1 (Wargo et al., 2008), while different genes performing similar reactions have been reported from *Sinorhizobium meliloti* (Smith et al., 1988) and *Arthrobacter globiformis* (Meskys et al., 2001).

Sarcosine is oxidatively demethylated to glycine by a monomeric sarcosine oxidase (*soxA*), as in eukaryotes and *Bacillus* sp. B-0618 (Trickey et al., 1999), or by a heterotetrameric sarcosine oxidase (TSOX; encoded by *soxBDAG*), as in *Corynebacterium* sp. P-1 (Chlumsky et al., 1995). In the reaction of TSOX, O_2 is reduced to H_2O_2 and formaldehyde is released, except in the presence of tetrahydrofolate, when 5,10-methylenetetrahydrofolate is released instead (Fig. 4.1). These simple constituents can then be utilized by the cell for energetic or biosynthetic purposes: e.g., serine hydroxymethyltransferase (glyA) interconverts glycine and L-serine, which can then be interconverted with pyruvate by serine dehydratase; and energy in the form of reducing equivalents (i.e. NADH) can be obtained by the conversion of formaldehyde to formate and then to CO_2 by formaldehyde dehydrogenase and formate dehydrogenase (fdhGBAD), respectively.

Here we describe the lateral transfer and duplication in *C. psychrerythraea* 34H of an operon responsible for the catabolism of sarcosine, a derivative of the important compatible solute glycine betaine, to L-serine. Additionally, several genes enabling the importation and degradation of choline to glycine betaine and then sarcosine are described by analogy to experimentally verified genes. A defined medium for the growth of *C. psychrerythraea* 34H, based on sarcosine, lactate, and vitamins (SLV), was developed on the basis of this genomic evidence, suggesting the expression of the operon in vitro. Further evidence for expression was obtained by proteomic analysis of *C. psychrerythraea* 34H. Sarcosine oxidase may be widespread in the genus *Colwellia*, providing a selective advantage to these mostly psychrophilic, halophilic microorganisms.

4.3 Methods

4.3.1 Genomics

The genomic structures of bacteria encoding heterotetrameric sarcosine oxidase were investigated using tools available at the JGI Integrated Microbial Genomes (JGI-IMG) database version 2.8 (`http://img.jgi.doe.gov`; Markowitz and Kyrpides, 2007). Protein

coding sequences of 144 complete and partial genome sequences containing at least one soxBDAG operon were exported from JGI-IMG. The guanine-cytosine content (GC-content) of each ORF in each genome was calculated as $100 \times \frac{G+C}{A+T+C+G}$. The GC-content of genes comprising heterotetrameric sarcosine oxidase (TSOX, composed of the following genes: *soxB*, *soxD*, *soxA*, and *soxG*) were extracted from each genome and statistically compared to the GC-content of all remaining genes in the genome using a Student's t-test. To predict pathways and functions into which TSOX is incorporated, genes in the local neighborhood (± 10kbp) of *soxBDAG* from each genome were investigated by BLAST analysis, as were paralogs and homologs of other proteins known to participate in compatible solute metabolism. Operon predictions were performed using freely available online tools: MicrobesOnline Operon Predictions (`http://www.microbesonline.org/operons`) by the method of Price et al. (2005); and by the method of Dam et al. (2007). The online database Islandpath version 1.0 was used to identify putative genomic islands by deviations from mean GC-content and dinucleotide codon bias (Hsiao et al., 2003).

4.3.2 Phylogenetics

Small subunit 16S ribosomal RNA gene sequences from *Colwellia spp.*, each bacterial genome containing TSOX and additional bacterial reference sequences were imported into ARB, a software environment for sequence data (Ludwig et al., 2004), from version 98 of the SILVA database of quality-checked, pre-aligned rRNA gene sequences. Those 16S rRNA gene sequences not yet available in SILVA were exported from JGI-IMG and aligned using the SINA Webaligner before being imported into ARB, where they were added to the pre-computed SILVA guide tree by maximum parsimony. Tree topology and branch lengths were defined by neighbor-joining of Tamura-Nei distances after highly variable positions were removed.

Amino acid sequences of TSOX gene clusters (*soxBDAG*) from bacterial genomes were each concatenated and aligned using MAFFT version 6. Sequence alignments were imported into ARB and edited manually as necessary. Bootstrapped phylogenetic trees were constructed in PAUP* v4.10beta (Swofford, 2003).

4.3.3 Expression

Growth on sarcosine

Defined media containing glycine betaine (trimethylglycine; 2 g L^{-1}) or sarcosine (N-methylglycine; 2 g L^{-1}) were tested for their ability to support growth of *Colwellia* as the sole source of organic carbon and nitrogen. *C. psychrerythraea* 34H was grown at −1℃ from duplicate glycerol stocks in 50 ml of Marine Broth 2216. Growth was monitored by optical density at 600 nm; 15 mL aliquots were taken when an OD_{600} of 0.4 was reached. To rinse the inoculum of undefined medium these aliquots were centrifuged at 1000× g for 10 min at 8℃; the supernatant was discarded, 15 mL of 1× ASW (NaCl 23.4 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 4.9 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 1.1 g L^{-1}, KBr 0.2 g L^{-1}, KCl 0.75 g L^{-1}, $MgCl_2{\cdot}6H_2O$ 4.1 g L^{-1}) was added without re-suspending the pellet and the tubes were centrifuged again at 1000× g for 2 min. The supernatant was again discarded, a final 15 mL of 1× ASW was added and the pellet re-suspended. To three 50 mL aliquots of each experimental medium (and a positive control of Marine Broth 2216) was added 0.5 mL of rinsed inoculum, of which two were incubated at 4℃ and one at room temperature as a negative control.

An additional medium was developed to increase the growth rate of *C. psychrerythraea* 34H on sarcosine-based medium. The ability of this medium, containing sarcosine, lactate and vitamins (SLV) to support the growth of 10 additional *Colwellia* isolates at 8℃ was tested as above. The recipe for SLV medium is as follows: sarcosine (2 g L^{-1}) and calcium D-lactate (0.5 g L^{-1}) are added to 1 L of ASW and autoclaved. On cooling, 100× RPMI-1640 vitamin solution (D-biotin 0.02 g L^{-1}, choline chloride 0.3 g L^{-1}, folic acid 0.1 g L^{-1}, myo-inositol 3.5 g L^{-1}, niacinamide 0.1 g L^{-1}, p-amino benzoic acid 0.1 g L^{-1}, D-pantothenic acid 0.025 g L^{-1}, riboflavin 0.02 g L^{-1}, thiamine·HCl 0.1 g L^{-1}, vitamin B-12 0.0005 g L^{-1}, KCl 0.2 g L^{-1}, KH_2PO_4 0.2 g L^{-1}, NaCl 8 g L^{-1}, Na_2HPO_4 1.15 g L^{-1}) is added to a final concentration of 0.5 mL L^{-1}. To make solid SLV medium 4 g L^{-1} ultra-pure agarose may be added before autoclaving.

Mass spectrometry

C. psychrerythraea 34H was grown at −1℃ from glycerol stocks in 50 ml of Marine Broth 2216; growth was monitored by optical density at 600 nm. At an OD_{600} of 0.4, 20 mL of inoculum was added to 2 L Marine Broth 2216 at 8℃ with swirling. After growth to an

OD_{600} of 0.6, chloramphenicol was added to a final concentration of 100mg L^{-1} to prevent further protein translation while cells were harvested at 4℃ by centrifugation. Proteins were extracted from the cell pellet and digested concurrently with pepsin and trypsin, then purified with a strong cation exchange cleanup column. Mass spectrometry was performed at the Institute for Systems Biology (Seattle, WA) on a ThermoFinnigan LTQ Linear Ion Trap MS/MS.

4.4 Results and Discussion

In a previous analysis of the genome of *C. psychrerythraea* 34H, a psychrophilic, halophilic marine bacterium, Methé et al. (2005) identified a duplicated operon encoding for heterotetrameric sarcosine oxidase, an enzyme involved in the catabolism of glycine betaine, a common osmoprotectant molecule. In the present genomic analysis we have demonstrated the genetic potential for both choline and glycine betaine to be used by *C. psychrerythraea* 34H as substrates for the production of L-serine, which can be utilized in numerous biochemical pathways, and of formate, which can be used to generate energy by degradation to carbon dioxide. These predictions were tested by proteomic analysis, which confirmed the expression of numerous proteins involved in compatible solute catabolism. Furthermore, we successfully grew *C. psychrerythraea* 34H on medium containing sarcosine as the sole source of carbon and energy, which was then developed into SLV, a defined medium for the rapid growth of *Colwellia* species.

4.4.1 Phylogenetics and genomic structure

Compatible solute metabolism

Genes encoding heterotetrameric sarcosine oxidase were identified in 144 out of a total of 1284 partial and complete genome sequences available in the JGI-IMG database. While most genomes contained only a single copy of *soxBDAG*, a total of 240 *soxBDAG* gene clusters were identified, with some genomes encoding up to 5 copies of the genes (Fig. 4.2).

Two copies of the *soxBDAG* genes were located in the *C. psychrerythraea* 34H genome (Fig. 4.3). The first set, CPS_4032–CPS_4035, appeared as part of an larger 37 kb genomic island (base pairs 4236340–4273184), while the second set (CPS_2477–CPS_2472) appeared to be a copy of the first set, based on GC-content and codon bias in these regions both exceeding one standard deviation from the mean. Another unusual putative operon within

the genomic island (CPS_4043–CPS_4049) encoded for proteins required in the biosynthesis and utilization of coenzyme F_{420}, the key coenzyme in methanogenesis. The gene order within this second putative operon was conserved among *C. psychrerythraea* 34H and *Paracoccus denitrificans* PD1222, *Burkholderia phytofirmans* PsJN, and *Marinomonas* sp. MED121, which provided the best BLAST hits for each of the *C. psychrerythraea* 34H coding sequences, with amino acid identities of 48–78%.

Directly flanking the TSOX genes in *C. psychrerythraea* 34H were *glyA* and *purU*, both of which were predicted to belong to the *soxBDAG* operon in the arrangement *glyA-soxB-soxD-soxA-soxG-purU* (Fig. 4.3). Serine hydroxymethyltransferase (GlyA) catalyzes the reversible conversion of glycine to L-serine, while formyltetrahydrofolate deformylase (PurU) catalyzes the conversion of methylenetetrahydrofolate to tetrahydrofolate and formate (Fig. 4.1). A number of genes encoding proteins performing further reactions on the products of sarcosine catabolism were found near the second *soxBDAG* operon (Fig. 4.3), including formaldehyde dehydrogenase (CPS_4039) and formate dehydrogenase (CPS_4022–CPS_4026, another copy was found at CPS_2056–CPS_2060). In the same region, genes encoding the serial demethylation of choline to glycine betaine (CPS_4010–CPS_4012, another copy was found at CPS_1332–CPS_1334), dimethylglycine (CPS_4029–CPS_4030), and finally sarcosine (CPS_4016–CPS_4017) were identified (Figs. 4.1 and 4.3). Other genes in similar pathways were found elsewhere in the genome (CPS_3133, CPS_3620, CPS_3791, CPS_4357), including a truncated serine hydratase (CPS_2471) not expected to be functional, indicating that L-serine may not be converted directly to pyruvate in *C. psychrerythraea* 34H. Thus, the net reaction encoded by *C. psychrerythraea* 34H (Fig. 4.1) is the importation and complete degradation of choline (or any of its degradation product intermediaries) to L-serine (via glycine) and carbon dioxide (via formate).

Compatible solute transporters

Several transporters implicated in the importation of quarternary amine compounds were previously identified in the genome of *C. psychrerythraea* 34H (Methé et al., 2005), none of which were highly similar to previously characterized compatible solute transporters like ProU1 from *Vibrio parahaemolyticus*, OpuABC from *Pseudomonas aeruginosa* PAO1, or OpuAC from *Listeria monocytogenes*. Of the six predicted transporters, five were annotated as secondary transporters of the betaine-choline-carnitine transporter (BCCT) family, and

one as a primary transporter of the ATP-binding cassette (ABC) family. The putative quarternary amine ABC transporter, CPS_4933–CPS_4935, was highly similar to a pair of ABC transporters from *Pseudomonas aeruginosa* PAO1 (PA5096, PA5103). Other researchers have showed that neither of these *Pseudomonas* transporters were detected after induction by growth on glycine betaine (Diab et al., 2006) or by osmotic shock (Aspedon et al., 2006), nor were they required for growth on glycine betaine (Wargo et al., 2008).

The putative BCCT transporters fell into three distinct classes based on sequence similarity. The two members of the first class (CPS_1335, and, lying near *soxBDAG*, CPS_4009) were 80% identical at the amino acid level, and each were in apparent operons with choline dehydrogenase and betaine aldehyde dehydrogenase (*betABI*), which together convert choline to glycine betaine. It seems highly likely that the proteins in this first class of BCCT transporters are able to import choline, though some known choline importers have low-affinity and are able to import other quarternary amine compounds as well (Chen and Beattie, 2008), including glycine betaine. The best BLAST hit for CPS_4009 belonged to *Marinomonas* sp. MED121, with an identity of 80%.

The second class of BCCT transporters in *C. psychrerythraea* 34H included two protein coding sequences, CPS_2003 and, lying near *soxBDAG*, CPS_4027, which were only 43% identical at the amino acid level. Several of the top BLAST hits for CPS_4027, with 52–57% amino acid identity, belonged to BCCT transporters previously implicated in the metabolism of dimethylsulphoniopropionate (DMSP), deriving from *Marinomonas* sp. MED121, *Marinomonas* sp. MWYL1, *Marinobacter* sp. ELB17, and marine gamma proteobacterium HTCC2207. In these bacterial genomes the transporter is part of the *ddd* operon, which produces the gas dimethylsulfide (DMS) from DMSP (Todd et al., 2007; Johnston et al., 2008), but in *C. psychrerythraea* 34H these genes stand alone. There are no other indications that *C. psychrerythraea* 34H metabolizes DMSP or DMS, so the substrate for this class of BCCT transporters in *C. psychrerythraea* 34H remains speculative at this time.

The final class of BCCT transporter in *C. psychrerythraea* 34H was represented only by CPS_3860, which was located within a predicted operon of unknown function containing a LysR-family transcriptional regulator (CPS_3863, transcribed in the oppose direction from the other genes), an aldehyde dehydrogenase (CPS_3862), a dipeptidase (CPS_3861), and an endoribonuclease (CPS_3859). In a variety of configurations (which we've called

'types'), paralogs of these genes were found in other bacterial genomes. The *C. psychrerythraea* 34H gene order (BCCT3860 Type Ia) was observed to be conserved in the following: *Marinomonas* sp. MED121, marine gammaproteobacterium HTCC2207 (which encoded *soxBDAG* 15kb downstream), and *Roseobacter* Azwk-3b (in which the LysR-family regulator was not divergent from the other genes). A related type (BCCT3860 Type Ib) was found in additional genomes (*Silicibacter pomeroyii*, *Roseobacter* MED193, *Roseobacter* SKO9-2-6, *Phaeobacter gallaeaiensis* 2.10, and *Phaeobacter gallaeaiensis* BS107), which encoded *soxBDAG* and an AraC-type transcriptional regulator immediately upstream of a BCCT Type Ia operon. The most frequent gene order observed, BCCT3860 Type II, was similar to BCCT3860 Type Ia except that the endoribonuclease was found between the LysR-family regulator and aldehyde dehydrogenase, rather than at the end of the operon. Most of the gammaproteobacteria with the CPS_3860 paralog encoded this type: *Shewanella woodyi* 51908, *Psychromonas ingrahamii* 37 and *Moritella* PE36 in Order Alteromonadales, *Photobacterium* 3TCK, *Aliivibrio* LEI1238, *Vibrionales* SWAT-3, *Vibrio splendidus* LGP32, *Vibrio* Ex25, *Vibrio* MED222, *V. alginolyticus*, *V. harveyi* H401, *V. shilonii* AK1, and *V. campbelli* AND4 in Order Vibrionales, and *Psychrobacter* K5 and *Psychrobacter* PRwf-1 from Order Pseudomonadales. A final group of genomes had BCCT3860 Type III, which were paralogs of CPS_3860 that stood alone within the genome: *Halorhodospira* SL1, *V. parahaemolyticus* RIMD 2210633, *V. harveyi* BAA-1116, *Shewanella paeleana* 700345, and *Shewanella halifaxensis* HAW-EB4. The highly conserved nature of this operon argues for a conserved function in these diverse marine bacteria, but none has so far been elucidated. Possible roles may be as an importer for an undescribed precursor to sarcosine, or for a new or alternative compatible solute, e.g. the dipeptide N-acetylglutaminylglutamine amide, synthesized by *Rhizobium meliloti* and *Pseudomonas aeruginosa* (D'Souza-Ault et al., 1993). Heterotetrameric sarcosine oxidase has also been shown to catalyze the oxidative demethylation of a number of alternative substrates, including heterocyclic amines like L-proline and L-pipecolic acid (Zeller et al., 1989), so the specific association of this BCCT transporter with sarcosine remains to be determined.

Regulation of compatible solute metabolism

The expression of a niche-specific pathway like compatible solute metabolism in an environmentally gregarious generalist like *C. psychrerythraea* 34H can be expected to be well reg-

ulated. A number of putative regulators of choline and sarcosine metabolism were detected in the genome of *C. psychrerythraea* 34H, including a putative homolog (CPS_4021; 59% identity) of *gdbR*, an AraC-type transcriptional regulator from *Pseudomonas aeruginosa* PAO1. This protein has been shown to control the expression of GbcAB and DgcAB (Wargo et al., 2009), enzymes responsible for converting glycine betaine to dimethylglycine and dimethylglycine to sarcosine, respectively. Immediately following each *soxBDAG* operon, DNA-binding proteins (CPS_2482 and CPS_4036) were found which may be involved in the regulation of sarcosine oxidase activity as well.

Another class of regulators, acting via cyclic diguanylate (c-di-GMP), have been shown to control motility, attachment, EPS production and biofilm formation in a number of Gammaproteobacteria, including various species of *Pseudomonas* (Gjermansen et al., 2006), *Vibrio* (Beyhan et al., 2008; Ferreira et al., 2008), and *Shewanella* (Thormann et al., 2006). *C. psychrerythraea* 34H encodes for more regulatory elements associated with c-di-GMP than 90% of Gammaproteobacteria sequenced to date, 65 in all. Based on their close proximity, the first of the duplicate *soxBDAG* operons may be in part regulated by a GGDEF domain protein (CPS_2484). The mechanism of regulation of these newly-discovered regulatory elements is not well established, but it is clear that the two dominant domains, GGDEF and EAL, act together to modulate the intracellular c-di-GMP pool, with GGDEF acting as a diguanylate cyclase to produce c-di-GMP from GMP, and EAL acting as a phosphodiesterase to reverse the reaction (Beyhan et al., 2008; Ferreira et al., 2008). Most of the known proteins containing GGDEF/EAL domains, including CPS_2484, also contain transmembrane domains and signal reception and transduction domains with which they sense the external chemical environment. In *Gluconacetobacter xylinus*, c-di-GMP was shown to act as an allosteric activator of cellulose synthesis (Ross et al., 1987), while in *Vibrio parahaemolyticus*, a GGDEF-domain protein was shown to influence the transcription of genes involved in swarming motility and capsular polysaccharide production (Kim et al., 2007) by an unknown mechanism.

The presence of an extraordinary number of GGDEF/EAL domain proteins in the genome of *C. psychrerythraea* 34H suggests that the microorganism maintains close interactions with its environment and other microorganisms. Proteomic analysis indicated that *C. psychrerythraea* 34H expressed a complete pathway for the degradation of glycine betaine to L-serine and CO_2, even under conditions of replete nutrients and normal seawater salinity.

If it is true that this pathway is allosterically activated by c-di-GMP, the genes may be constitutively expressed but inactive until an external signal induces the diguanylate cyclase activity of a GGDEF-domain protein and allows the allosteric activation of the enzymes. In addition to compatible solute metabolism, a number of the GGDEF domain proteins in *C. psychrerythraea* 34H were in close proximity to genes controlling the production of EPS, antibiotics, and cyanocobalamin (vitamin B12), as well as motility, nitrate reduction, and chemotaxis. All of these functions could be usefully regulated during biofilm formation or dispersal. They could also aid in the formation of a symbiotic relationship with sea ice algae like diatoms, many of which have a strict requirement for vitamin B12 (Haines and Guillard, 1974).

Lateral gene transfer of soxBDAG

Several lines of evidence indicated that the genes encoding TSOX (*soxBDAG*) and other genes in the *C. psychrerythraea* 34H genome were laterally transferred from the Order Oceanospirillales, likely via a species of *Marinomonas*. Interestingly, when calculated for all 240 *soxBDAG* operons, both *soxA* and *soxG* had significantly higher GC-contents than the genomes from which they derived ($p << 0.001$, one-way t-test), while both *soxB* and *soxD* had similar GC-contents to their originating genomes (Fig. 4.4). Far greater than this deviation, however, the GC-contents of *soxBDAG* genes from *C. psychrerythraea* 34H (48.0, 48.7, 47.8, and 47.7% GC, respectively) were highly significantly greater (each $p < 0.001$, one-way t-test) than the mean GC-content of the remainder of the genome (38.0 ± 5.5% GC; Fig. 4.4). This large discrepancy, the largest of any genome-operon pair examined, strongly suggested a relatively recent lateral transfer event (Fig. 4.4). Described species of *Colwellia* have GC-contents of 35–46% (Deming and Junge, 2005), while described *Marinomonas* have GC-contents of 45–50% (Sanches-Amat and Solano, 2005).

Phylogenetic analysis of the concatenated *soxBDAG* genes demonstrated that the nearest relatives of the *C. psychrerythraea* 34H *soxBDAG* were from two Oceanospirillales (Fig. 4.5), *Marinomonas* MED121 and *Marinomonas* MWYL1. The *C. psychrerythraea* 34H and *Marinomonas* sequences clustered separately, with high bootstrap support, from another cluster comprising sequences from *Marinobacter* ELB17, *Chromohalobacter salexigens* DSM 3043, and 19 *Pseudomonas* genomes. These gammaproteobacterial *soxBDAG* genes were clearly divergent from those of the alphaproteobacteria and betaproteobacteria, where most

of the *soxBDAG* operons were observed to reside.

Phylogenetic analysis of the 16S rRNA genes of those bacteria containing *soxBDAG* showed that none of the 34 examined Alteromonadales contained *soxBDAG* (Fig. 4.6). The complete absence of *soxBDAG* in the genomes of *Pseudoaltermonas*, *Psychromonas*, *Shewanella*, *Alteromonas*, *Moritella*, *Idiomarina*, or *Aeromonas* suggests that the operon was not simply retained in *C. psychrerythraea* 34H while being lost in all of these related lineages. No sequenced members of the Enterobacteriales or Vibrionales contained *soxBDAG*, but a large number of Pseudomonadales were found to have them. The most taxonomically similar genome to contain *soxBDAG* was *Marinobacter* sp. ELB17, described as Order Alteromonadales but phylogenetically more similar to Order Oceanospirillales (Fig. 4.6), which was isolated from a permanantely ice-covered saline lake in Antarctica, Lake Bonney.

Of particular interest for further study is the origin of the duplicated *soxBDAG* operon involved in the metabolism of sarcosine in *C. psychrerythraea* 34H. Analysis of GC-content suggests that it arrived into the genome of *C. psychrerythraea* 34H via lateral gene transfer in the relatively recent past. Phylogenetic relationships of *soxBDAG* and BLAST analysis of other proteins encoded by the 37 kb genomic island suggest that the most likely source of the genomic island in *C. psychrerythraea* 34H was a species of *Marinomonas* (Fig. 4.5), several of which have been isolated from sea ice and polar seawater (Romanenko et al., 2003; Gupta et al., 2006; Zhang et al., 2008b). The closest sequenced relative containing *soxBDAG* was *Marinomonas* MED121, isolated from the Mediterranean Sea. Although it is impossible to unequivocally determine the specific source and direction of transfer between *Marinomonas* and *C. psychrerythraea* 34H, the presence of *soxBDAG* in a number of Oceanospirillales besides *Marinomonas* suggests that an Oceanospirillales genome was the proximal source, rather than recipient, of these genes. The ultimate source, however, remains to be determined, though the extent to which these genes permeate the Betaproteobacteria (e.g. *Burkholderia*) implicate that group. An alphaproteobacterial intermediate (e.g. *Roseobacter* or *Pelagibacter*) may be possible as well, considering the more direct overlap in habitat between this group and *Colwellia*. The mechanism of lateral gene transfer by which *C. psychrerythraea* 34H acquired its exogenous genes was not determined in this study, but the likelihood that *C. psychrerythraea* 34H is a lysogen has been explored by Wells (2006), suggesting transduction as a possible mechanism.

4.4.2 Expression of heterotetrameric sarcosine oxidase in C. psychrerythraea *34H*

Perhaps the most important qualification to define a successful lateral gene transfer event is endogenous expression of the transferred genes in the recipient. Sarcosine (N-methylglycine) was tested for its ability to support growth of *C. psychrerythraea* 34H (at the microorganism's optimal growth temperature, 8℃) as the sole source of organic carbon and nitrogen. Turbid growth was observed in duplicate aliquots of the sarcosine medium after 5 weeks. Successful growth after two consecutive transfers in the sarcosine medium demonstrated that growth was not dependent on co-factors remaining from prior growth in undefined medium. In an effort to increase the growth rate of *C. psychrerythraea* 34H on sarcosine we amended the medium with calcium D-lactate, vitamin solution, or a combination of both. We observed growth on the combined medium, denoted SLV (for sarcosine, lactate, and vitamins) after one week of incubation at 4℃. After 5 weeks no growth was detected in ASW containing only D-lactate or only vitamin solution without sarcosine. Time required for growth to turbidity of *C. psychrerythraea* 34H in SLV at −1℃ and 8℃ was comparable to growth on $\frac{1}{2}\times$ Marine Broth 2216 at the same temperatures.

To determine the taxonomic range of growth on sarcosine by members of the genus *Colwellia*, we tested the ability of 9 isolates to grow in liquid SLV at 8℃; all but one (*C. rossensis*) grew turbid within a week (Table 4.1). There appeared to be no phylogenetic signal in the distribution of phenotypes, except that growth was widespread throughout the genus (Fig. 4.7). Since *Colwellia* do not fix nitrogen or carbon dioxide, sarcosine must have been used for growth in *C. psychrerythraea* 34H and these additional *Colwellia* species.

A complete, defined medium for the rapid growth of *Colwellia* spp. was formulated and tested at temperatures between −1℃ and 8℃. This medium, denoted SLV (containing sarcosine, D-lactate, and a defined vitamin mixture in ASW) can be combined with ultra-pure agarose to provide a defined solid medium for the psychrophilic growth of *C. psychrerythraea* 34H. The ability of *Colwellia* spp. to grow on a compatible solute derivative as a sole source of organic carbon and nitrogen suggests the possibility of bringing into culture as-yet-uncultured microorganisms from environments expected to contain high levels of compatible solutes, including sea ice and other frozen saline environments (Deming, 2009). The medium could be further optimized by determining the specific vitamins necessary for growth of *C. psychrerythraea* 34H and other *Colwellia* spp. For example, the genome of *C. psychrerythraea* 34H contains genes encoding for the biosynthesis of

cyanocobalamin (vitamin B12), a component of SLV that may then be unnecessary. Four *Colwellia* strains that we tested for growth on sarcosine medium were originally isolated from Antarctic sea ice diatom assemblages (Bowman et al., 1998); three of these strains grew on the sarcosine medium (*Colwellia demingiae* ACAM 459T, *Colwellia psychrotropica* ACAM 179T, and *Colwellia hornerae* ACAM 607T). Previous research has shown that nutrients excreted from living diatoms or scavenged from expired cells can be used by bacteria to produce vitamin B12 (Haines and Guillard, 1974). Other sources of compatible solutes in sea ice are likely plentiful, including the lysis of high-osmolarity-adapted bacteria by phage, which are well-known from sea ice (Maranger et al., 1994; Wells and Deming, 2006c).

Tandem mass spectrometry was used to identify 40 peptide fragments unique to heterotetrameric sarcosine oxidase in *C. psychrerythraea* 34H, as well as those from a number of other proteins involved in glycine betaine catabolism (Table 4.2). Probabilities of 0.98–1.0 were calculated for the presence of these proteins, indicating with almost certain likelihood that they were expressed during growth on the complete medium Marine Broth 2216, near the optimal growth temperature of the microorganism. Based on the proteome results, *C. psychrerythraea* 34H expressed proteins enabling the complete metabolism of glycine betaine imported from the complex medium Marine Broth 2216, converting it to L-serine (via sarcosine and glycine) and carbon dioxide (via 5,10-methylenetetrahydrofolate and formate). Further useful experiments would be to determine the capability for *C. psychrerythraea* 34H to grow on choline, the metabolic precursor to glycine betaine (Fig. 4.1), and glycine betainse itself as a sole source of carbon and energy, since it is highly likely that the strain encodes choline transporting BCCTs in the duplicate *betTABI* operons.

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4.6 Tables and Figures

Table 4.1: Growth of *Colwellia* strains on a defined medium containing sarcosine, lactate, and vitamins (SLV) at 8℃. * indicates strains with 16S rRNA gene sequences available for phylogenetic analysis.

Strain	T_{opt} (℃)	$t_{turbidity}$ (days)	reference
C. psychrerythraea 34H *	9	2	Huston (2003)
C. demingiae ACAM 459T	11	2	Bowman et al. (1998)
C. maris ABE-1*	~15	2	Yumoto et al. (1998)
C. hornerae ACAM 607T*	12	3	Bowman et al. (1998)
C. demingiae strain ICP10	~11	3	J. Bowman, UT
Arctic sea ice isolate 21C	~5	3	Borriss et al. (2003)
C. psychrotropica ACAM 179T*	18	7	Bowman et al. (1998)
Arctic nepheloid layer isolate 75C3	unknown	7	This laboratory
C. rossensis ACAM 608T*	10	no growth	Bowman et al. (1998)

Table 4.2: Proteins involved in compatible solute metabolism that were detected by tandem mass spectrometry of *Colwellia psychrerythraea* strain 34H grown at 8℃ on a complete medium, Marine Broth 2216. 'Total' indicates the total number of detectable peptides predicted from the protein sequence. 'Unique' indicates the number of detectable and unique peptides predicted from the protein sequence. 'Identified' indicates the number of unique peptides actually identified during tandem mass spectrometry. '% coverage' indicates the percentage of the total amino acids in a protein that were actually detected in the identified peptides.

locus	gene	annotation	total	unique	identified	% coverage	p
CPS_4029	[gbcA]	iron-sulfur cluster-binding protein, Rieske family	22	13	10	33	1.00
CPS_4030	[gbcB]	oxidoreductase, FAD-NAD-binding-iron-sulfur cluster binding protein	4	4	4	14	1.00
CPS_4017	[dgcB]	oxidoreductase, FAD-FMN-binding	20	17	15	28	1.00
CPS_4032,CPS_2478	soxB2	sarcosine oxidase, beta subunit	16	13	10	35	1.00
CPS_4033,CPS_2479	soxD2	sarcosine oxidase, delta subunit	5	4	4	43	1.00
CPS_4034,CPS_2480	soxA2	sarcosine oxidase, alpha subunit	41	30	26	41	1.00
CPS_0728	glyA1	serine hydroxymethyltransferase	49	25	19	59	1.00
CPS_3133	folD1	FolD bifunctional protein	7	6	5	23	1.00
CPS_3791	folD2	FolD bifunctional protein	4	4	4	19	1.00
CPS_4039		putative glutathione-independent formaldehyde dehydrogenase	41	19	14	53	1.00
CPS_2057	fdhB1	formate dehydrogenase, beta subunit	1	1	1	6	0.98
CPS_4023	fdhB2	formate dehydrogenase, beta subunit	10	7	6	20	1.00
CPS_4024	fdhA2	formate dehydrogenase, alpha subunit	31	15	15	23	1.00
CPS_4026		hypothetical protein	3	3	3	52	1.00
CPS_4933		amino acid ABC transporter, periplasmic amino acid-binding protein	4	3	3	18	1.00

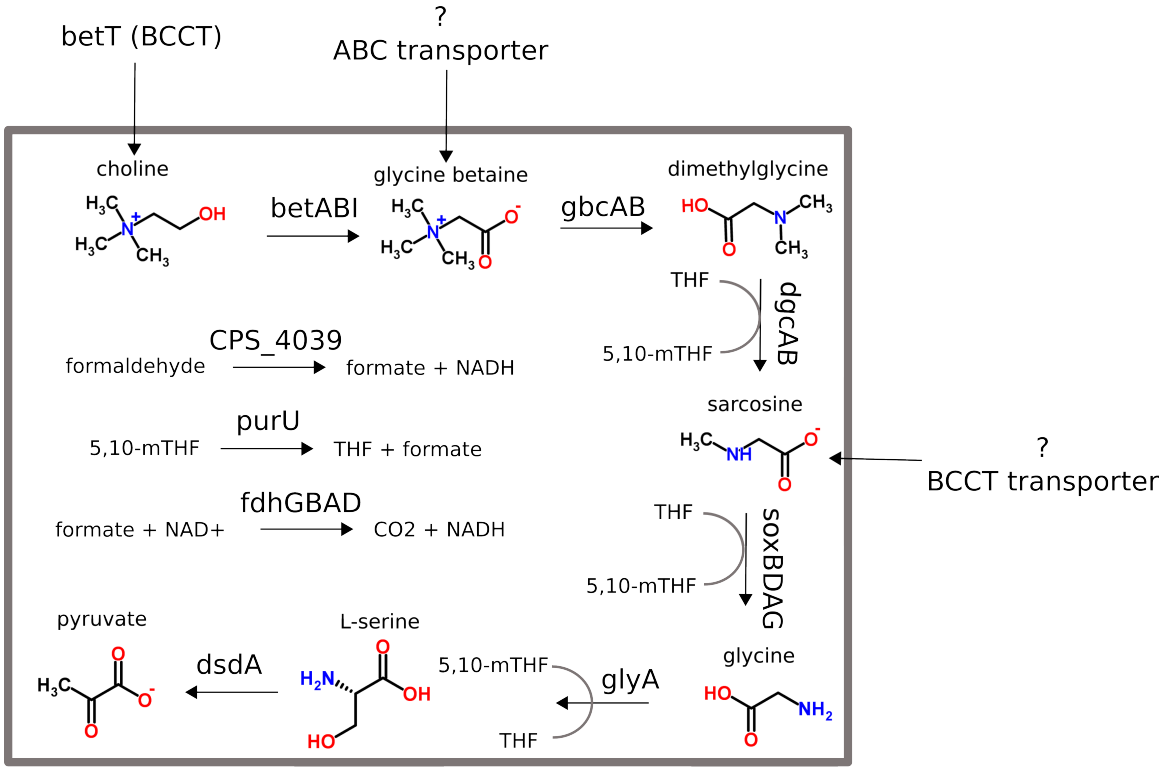

Figure 4.1: The pathway of choline degradation to L-serine encoded by *Colwellia psychrerythraea* strain 34H. Genes involved in the degradation process are: *betABI* (CPS_4010–CPS_4012, CPS_1332–CPS_1334), *gbcAB* (CPS_4029–CPS_4030), *dgcAB* (CPS_4016–CPS_4017), *soxBDAG* (CPS_2478–CPS_2481, CPS_4032–CPS_4035), *glyA* (CPS_2477, CPS_4031, CPS_0728, CPS_3844), and *dsdA*, a truncated serine dehydratase (CPS_2471). Genes involved in one-carbon cyling and folate metabolism are: *purU* (CPS_2482, CPS_4036, CPS_4357, CPS_3620), *folD* (CPS_3133, CPS_3791), formaldehyde dehydrogenase (CPS_4039), and *fdhGBAD* (CPS_4022-4026, CPS_2056-2060). Genes for putative transporters for quarternary amines are: choline BCCT *betT* (CPS_4009, CPS_1335), putative sarcosine BCCT (CPS_3860), and a putative glycine betaine ABC transporter (CPS_4932–CPS_4934). Abbreviations: tetrahydrofolate, THF; 5,10-mTHF, 5,10-methylenetetrahydrofolate.

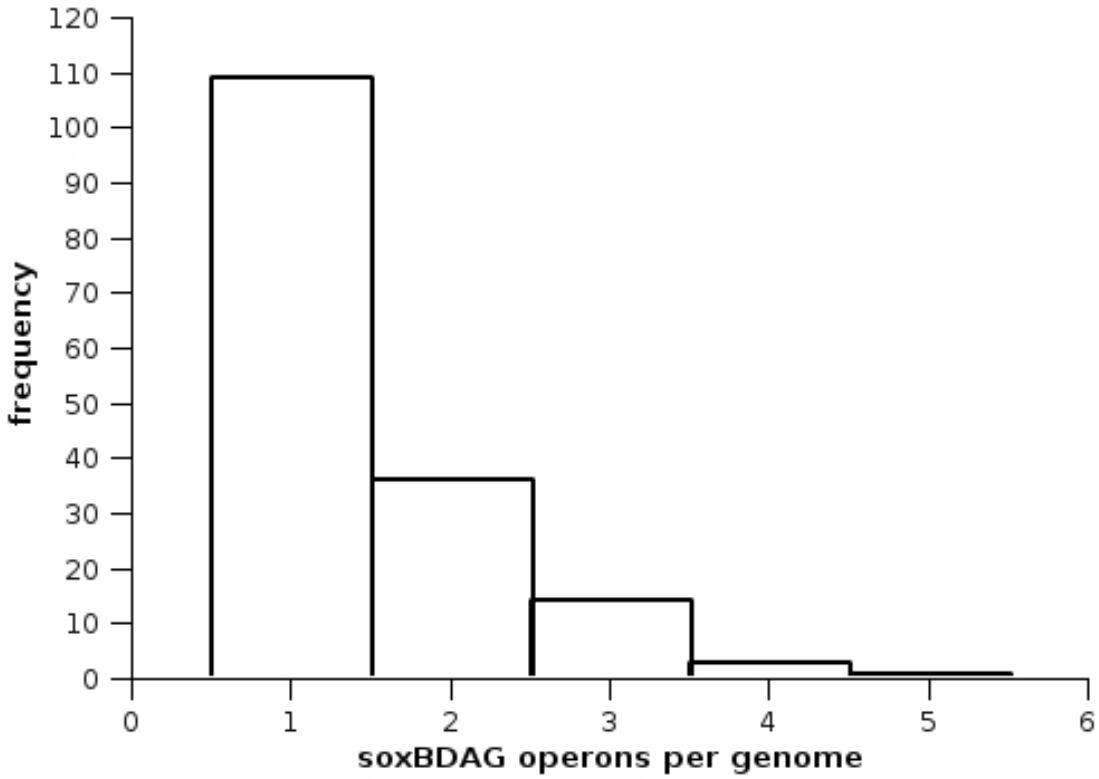

Figure 4.2: Number of copies of *soxBDAG* genes in 144 bacterial genomes obtained from the Joint Genome Institute Integrated Microbial Genomes database.

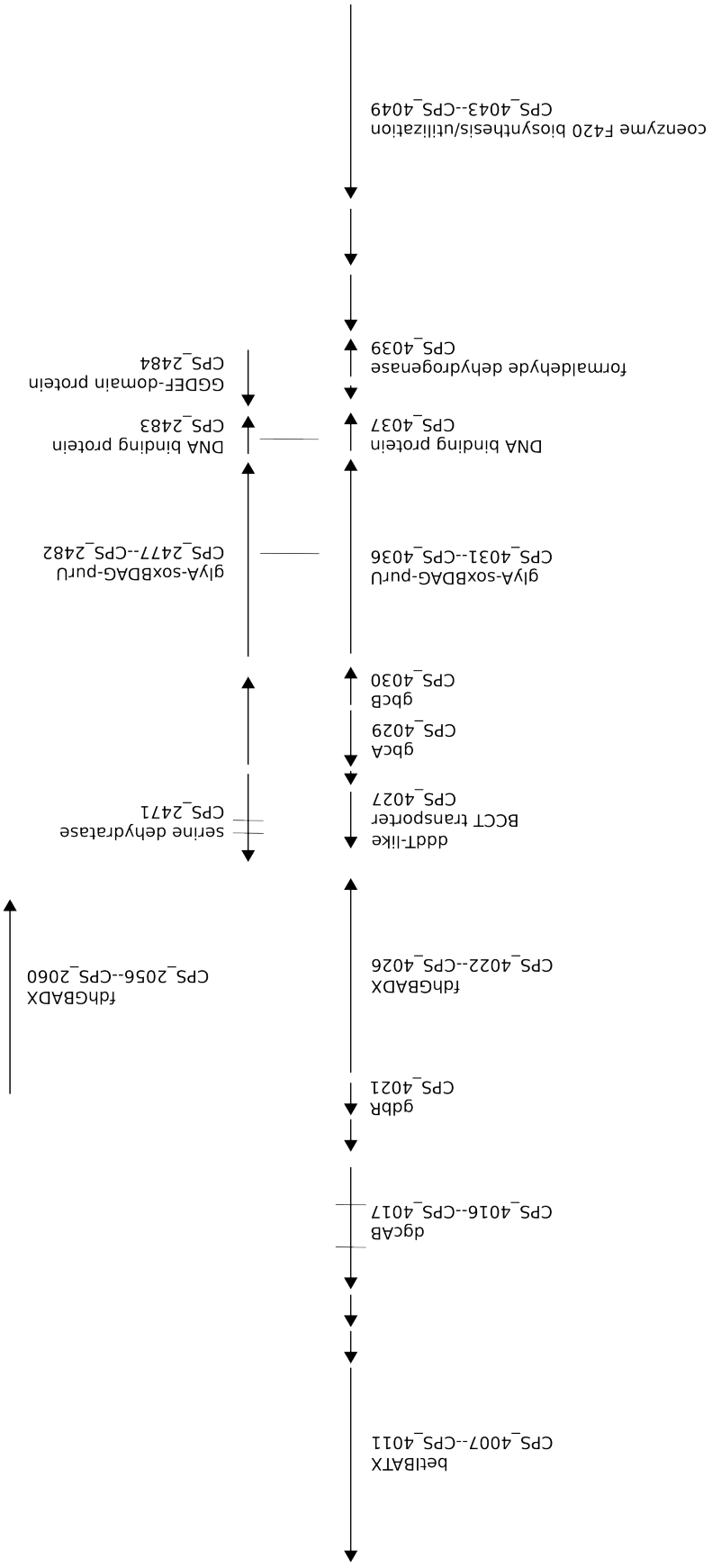

Figure 4.3: Genetic neighborhoods of duplicated *soxBDAG* operons in *Colwellia psychrerythraea* strain 34H, which encode multiple proteins involved in the pathway of choline degradation to L-serine. Each predicted transcriptional unit is illustrated by an arrow, orientation of which is determined by the strand of DNA upon which it is encoded: left-facing arrows are on the negative strand, right-facing arrows on the positive strand. Vertical bars through arrows indicate fractional transcriptional units identified by the corresponding label. Homologous transcriptional units in different regions of the chromosome are aligned with vertical bars.

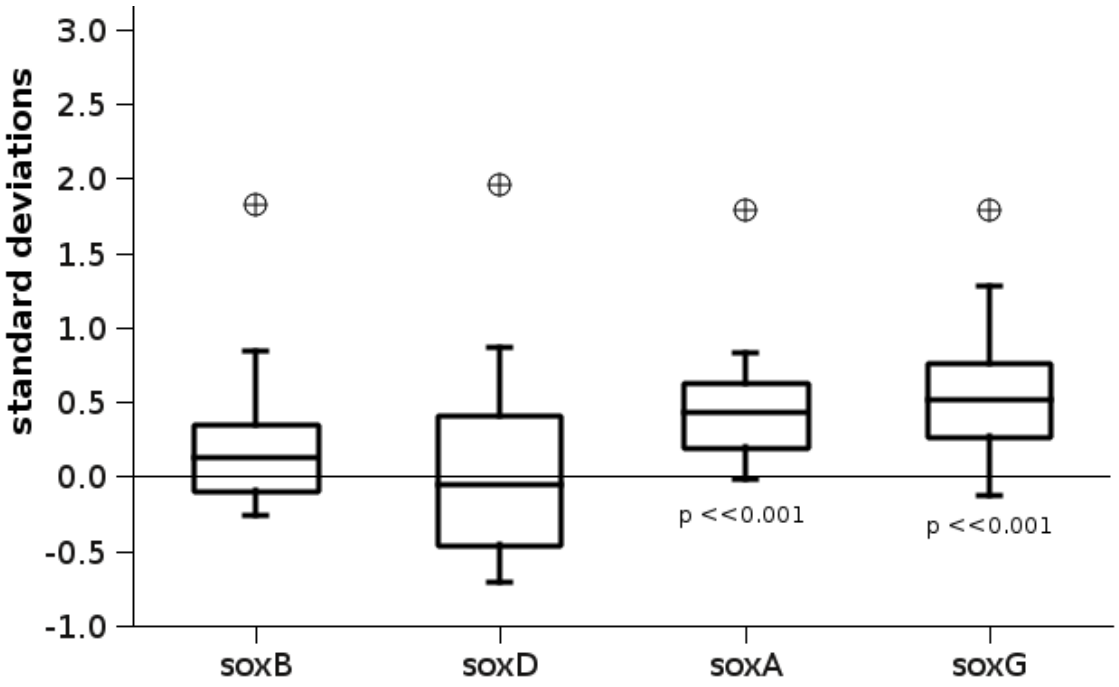

Figure 4.4: Boxplot of standard deviations from the mean genome GC-contents of each of the four genes encoding heterotetrameric sarcosine oxidase (*soxB*, *soxD*, *soxA*, *soxG*), calculated from all 240 *soxBDAG* operons in JGI-IMG. The horizontal line indicates a standard deviation of zero, equivalent to the mean genome GC-content. Overlapping open circles and plus symbols designate the duplicate copies of *soxBDAG* from *Colwellia psychrerythraea* strain 34H.

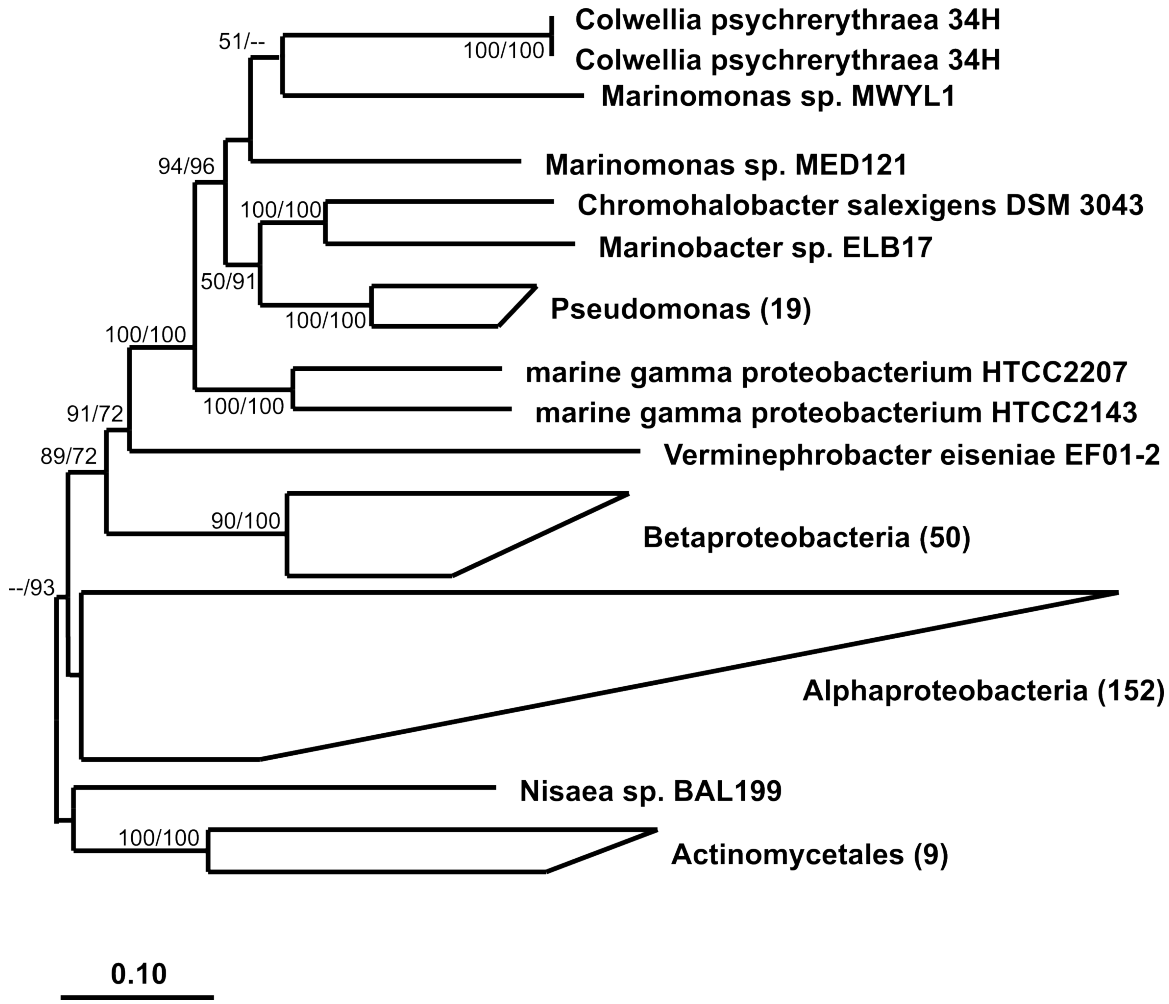

Figure 4.5: Phylogeny of a concatenation of amino acids sequences of the four genes encoding heterotetrameric sarcosine oxidase (*soxBDAG*) that were present in the Joint Genome Institute Integrated Microbial Genomes database. Tree topology was defined by the consensus of 100 maximum parsimony bootstrap replications utilizing 2366 nucleotides, of which 1798 were parsimony-informative. Branch lengths were defined by Tamura-Nei distances. Node values indicate percentage of 1000 distance and 100 maximum parsimony bootstrap replications, respectively; only bootstrap values greater than 50% are shown.

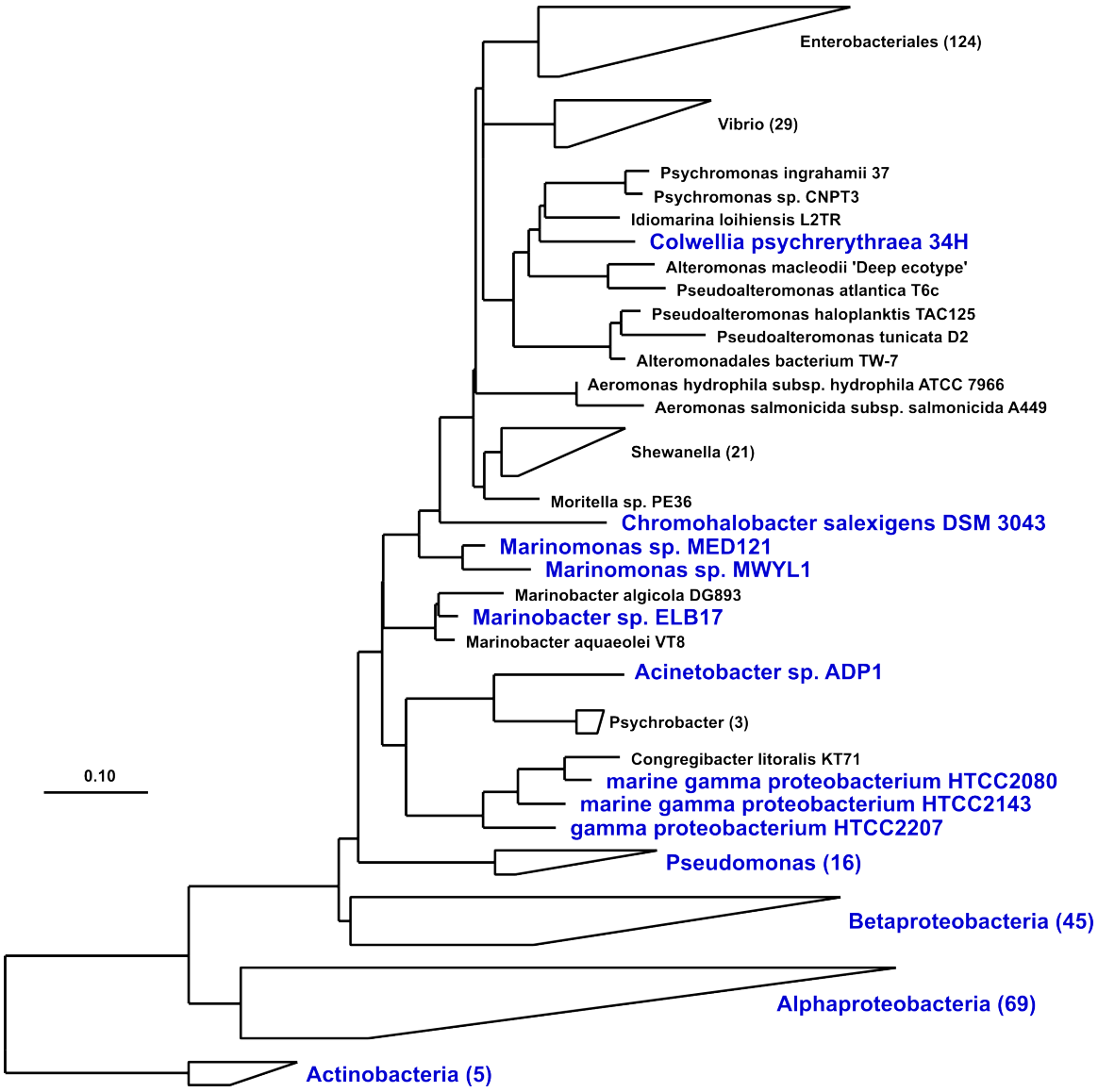

Figure 4.6: Phylogeny of 16S rRNA gene sequences from selected genomes in the Joint Genome Institute Integrated Microbial Genomes database. Alignment and neighbor-joining tree were acquired from SILVA version 98. Large blue leaf font indicates the genome included at least one copy of the genes encoding heterotetrameric sarcosine oxidase.

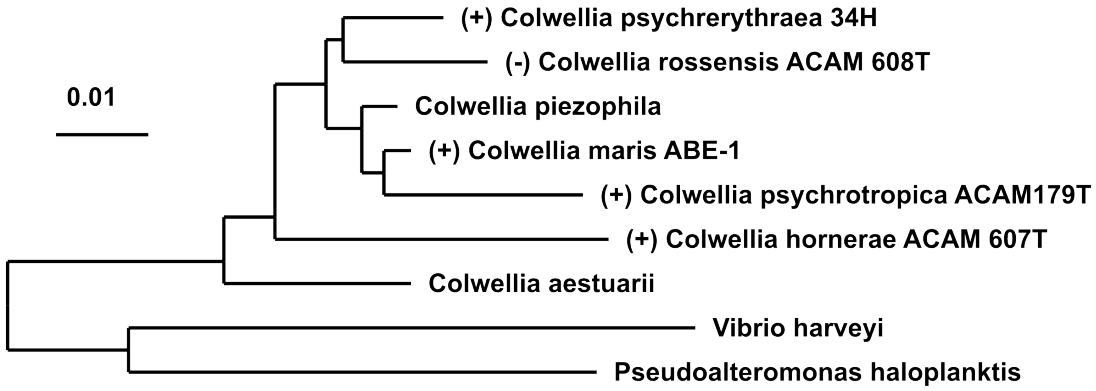

Figure 4.7: Phylogeny of 16S rRNA gene sequences from strains of *Colwellia*. Tree topology was defined by the consensus of 1000 maximum parsimony bootstrap replications performed on 145 full-length *Colwellia* sequences from the Ribosomal Database Project Release 10 Update 11, which were then pruned to the species shown here. The only two available sequences from typed strains of *Colwellia demingiae* were of poor quality and not usable for this analysis. Of 1394 nucleotide basepairs, 249 were parsimony-informative. Branch lengths were defined by Tamura-Nei distances. Positive (+) or negative (−) growth on SLV medium (sarcosine, lactate, and vitamins) is denoted for each of these strains that was tested.

Appendix A

REPK: AN ANALYTICAL WEB SERVER TO SELECT RESTRICTION ENDONUCLEASES FOR TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

A.1 Abstract

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a widespread technique for rapidly fingerprinting microbial communities. Users of T-RFLP frequently overlook the resolving power of well-chosen restriction endonucleases and often fail to report how they chose their enzymes. REPK (Restriction Endonuclease Picker) assists in the rational choice of restriction endonucleases for T-RFLP by finding sets of four restriction endonucleases that together uniquely differentiate user-designated sequence groups. With REPK, users can provide their own sequences (of any gene, not just 16S rRNA), specify the taxonomic rank of interest, and choose from a number of filtering options to further narrow down the enzyme selection. Bug tracking is provided, and the source code is open and accessible under the GNU Public License v.2, at http://code.google.com/p/repk. The web server is available without access restrictions at http://rocaplab.ocean.washington.edu/tools/repk.

A.2 Introduction

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a microbial fingerprinting technique capable of discriminating microbial communities quickly and relatively inexpensively(Liu et al., 1997; Osborn et al., 2000; Blackwood et al., 2003). T-RFLP is increasingly used in high-throughput studies of microbial communities in combination with or even in lieu of clone library analysis(Tom-Petersen et al., 2003; Moss et al., 2006). Briefly, the method involves PCR amplification of a gene of interest (often 16S rRNA genes) with fluorescent dye-labeled primers, followed by multiple single restriction digests done in parallel. The resulting fragments are then separated by capillary electrophoresis with an internal size standard to determine the lengths of the terminal (fluorescently-

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labeled) fragments. Each distinct terminal restriction fragment is considered an operational taxonomic unit (OTU), thus the choice of restriction enzymes can impact the number of OTUs observed in each sample and the calculation of diversity statistics.

When analyzing uncharacterized and very diverse bacterial communities, sufficient community discrimination can often be accomplished with multiple randomly-chosen tetrameric restriction enzymes (Engebretson and Moyer, 2003). However, a brief review of the literature indicates that there is still no standard in even this simplified case. We examined 26 papers (Liu et al., 1997; Chin et al., 1999; Dunbar et al., 2000; Osborn et al., 2000; Urakawa et al., 2000; Blackwood et al., 2003; Stepanauskas et al., 2003; Tom-Petersen et al., 2003; Gomez et al., 2004; Wolsing and Prieme, 2004; Hartmann et al., 2005; Pett-Ridge and Firestone, 2005; Yu et al., 2005; Chan et al., 2006; Danovaro et al., 2006; Gentile et al., in press; Hartmann and Widmer, 2006; Hjort et al., in press; Lazzaro et al., 2006; Moss et al., 2006; Nakanishi et al., 2006; Osborne et al., 2006; Pandey et al., 2007; Kvist et al., 2007; Siripong and Rittmann, in press) that were published between 1997 and 2007 and used T-RFLP. Of those papers, 38% used universal bacterial primers combined with a single restriction enzyme, but the choice of enzyme was not consistent. MspI was used most frequently (4 studies), followed by TaqI (2 studies), and one study each used AluI, CfoI, HhaI, and HaeIII. Overall, only 3 of the 26 papers included a rationalization of enzyme selection(Danovaro et al., 2006; Osborn et al., 2000; Liu et al., 1997).

An alternate approach to T-RFLP can be taken if the microbial community has been characterized (by clone library analysis or by prediction from previous studies) or if a particular taxonomic group is being targeted with specific primers. In this case a more reasoned choice of restriction enzymes can be conducted. In particular, specific species or microbial taxa of interest to the researcher—particularly closely-related taxa that may share some restriction sites—can often be differentiated if the proper restriction enzymes are selected.

There are however few resources available to narrow down the selection process. Over 600 Type II restriction enzymes are commercially available, accounting for 262 distinct specificities (Roberts et al., 2007). Existing computer programs for assisting in the choice of restriction enzymes include TAP-TRFLP (Marsh et al., 2000), MiCA Enzyme Resolving Power Analysis (http://mica.ibest.uidaho.edu), and TRF-CUT (Ricke et al., 2005). These programs perform *in silico* restriction digestions of a predefined sequence database or user

provided sequences, but these results must still be manually examined to determine which enzymes are best suited to discriminate that set of sequences. CLEAVER (Jarman et al., 2004), a stand alone program, provides the above features as well as the ability to assign sequences to taxonomic groups at multiple levels and to search for enzymes that cut one group but not another group. However, it is limited to comparing only two groups at once. Restriction Endonuclease Picker (REPK) addresses this gap by finding enzymes that are able to discriminate an unlimited number of user-designated sequence groups on the basis of their terminal restriction fragment lengths. If no single enzyme can discriminate all groups, REPK reports sets of four restriction enzymes that together are able to differentiate the groups of interest. An important component of REPK is this ability to specify the taxonomic rank of sequences to be differentiated, which is particularly useful in the case where a diverse microbial community has been characterized by clone library analysis or there is an existing database of several subgroups of sequences that amplify with the same specific primers.

A.3 Site Usage and Examples

A complete manual and example input files are provided on the REPK website (http://rocaplab.ocean.washington.edu/tools/repk). The example shown in Fig. 1 was prepared using REPK v.0.3.2, with the following operating parameters (also the defaults): example sequence file (`alignment5.txt`), all commercially available Type IIP enzymes (REBASE Version 704), taxonomic rank = 1, cutoff = 5, min fragment length = 75, max fragment length = 900, stringency = 'automatic', max missing groups = 0, max matches returned = 100.

A.3.1 User input

The user must provide a trimmed FASTA-formatted file with nucleotide sequences beginning at the 5'-end of the labeled primer used for PCR amplification and ending at the 5'-end of the unlabeled primer. Sequence groups can be designated in the description line of the FASTA file, by using a delimiter to separate taxonomic rank terms or optionally taxonomic identifications can be prepended to the description line using an output file from RDP-Classifier (Cole et al., 2005). Figure 1A shows *a subset of* the example sequence file provided on the website, `alignment5.txt`. Sequence groups are separated by a single underscore, and in this example 'taxonomic rank 1' was chosen, corresponding to the genus

of these Archaea.

A selectable list of commercially-available enzymes from the latest REBASE database (Roberts et al., 2007) is available and is automatically updated on the first day of each month. The enzymes available for selection include primarily Type IIP enzymes, which have symmetric recognition sequences and cleavage sites. Restriction enzymes of Type IIA (having asymmetric recognition sequences) and Type IIB (cleaving both sides of the recognition sequence on both strands) are at the present time not supported by REPK, although some are included in a separate enzyme file for advanced users willing to perform some manual processing. Users should be aware that some enzymes in the REBASE database may not be suitable for t-RFLP due to methylation specificities or requirements for multiple restriction sites to be present for effective digestion. Finally, users can define their own custom enzymes if they are not included in the standard list. The default (all standard enzymes) was used for the example in Fig. 1. For computational efficiency isoschizomers are grouped by cleavage site.

The final output is refined by setting several options. Some of these, the minimum and maximum allowable fragment lengths and the maximum difference in size between two fragments that will still be considered the "same" fragment, will be dependent on the specifications and resolving power of particular capillary electrophoresis systems. Users can also set the minimum threshold for the number of groups each enzyme must be able to discriminate on its own (the enzyme stringency), and the number of groups allowed to remain undifferentiated in the case that no "perfect" enzyme groups are discovered.

A.3.2 Program Operations

Sequences are first digested in both orientations by all selected enzymes to find the shortest labeled restriction fragment; these lengths are output as a table (and a downloadable tab-delimited text file, `fragfile.csv`), a subset of which is shown in Fig. 1B. In this example the sequences were cut by every enzyme except AasI, which resulted in full-length fragments.

Next, all terminal fragment lengths are binned within the chosen cutoff (here 5 bp) and a binary matrix of pairwise group differentiations is created. Bins containing a single sequence group yield a '1', while bins containing more than one sequence group yield a '0', indicating no differentiation between those groups. In the example in Fig 1, BanII failed to distinguish between sequence groups *Sulfurisphaera* and *Thermofilum* because the difference

between their fragment lengths (1 bp) was less than the chosen cutoff of 5 bp (Fig. 1B). However, AspLEI did distinguish between those groups because the difference in fragments lengths was 188 bp. It is not necessary for sequences from the same sequence group to have similar fragment lengths (e.g. *Sulfolobus*). Fragment lengths outside the boundaries set by the minimum and maximum fragment length options are binned together without regard for their actual lengths, decreasing the number of sequence groups discriminated by those enzymes (e.g. BmiI). The enzyme stringency filter is then applied to this matrix, allowing only enzymes that discriminate at least the specified fraction of sequence groups to proceed. The passing enzymes are output as a table (and a downloadable tab-delimited text file, `enzmatrix.csv`), a subset of which is shown in Fig 1C.

For computational efficiency, the enzymes are then sorted into 'enzyme bins' that produce identical differentiation patterns, although they may not produce the same terminal fragment lengths. In this example neoschizomers AspLEI and GlaI produce different fragment lengths but the same differentiation pattern so they were grouped together for the final analysis. It is important to note that the enzyme bins are dependent on the particular sequence file and taxonomic rank selected for the analysis. That is, two enzymes may have equal discriminatory power for a particular set of sequence groups but for a different set of sequences, one enzyme may be much better and the two enzymes would be placed in the same bin in the first but not the second case.

Finally, groups of 4 enzymes (a 'set') are logically summed (e.g. 101 + 011 = 111) to determine the coverage of the set, i.e. the number of sequence groups discriminated by the enzymes in the set. If this number is greater than the total number of sequence groups (less the max missing groups, here 0) then the set is saved. A score is calculated for each saved set and all saved sets are sorted before the highest-scoring sets are output to a text file, `finalout.txt`, *a subset of which* is shown in Fig. 1D. If more than 10,000 sets are found and the enzyme stringency is set to 'automatic', it is incremented by 10% (decreasing the number of passing enzymes and thus enzyme sets) and the analysis is repeated. The final output reports and summarizes those enzyme sets that best discriminated the sequence groups.

The final output consists of three parts: 'successful enzyme sets', 'enzyme picker key', and 'quick overview.' The successful enzyme sets (Fig. 1D.1) consist of a list of enzyme groups in each set, and a score indicating the frequency with which each set discriminated

the sequence groups. A perfect enzyme (one that discriminates 100% of the sequence groups) contributes a score of 1, so four perfect enzymes would produce the maximum score of 4. The enzyme picker key (Fig. 1D.2) lists the members of each enzyme group, with neoschizomers together in brackets. Each member of an enzyme group produces the same sequence group differentiation pattern but may differ in recognition site, terminal fragment lengths, etc. The quick overview (Fig. 1D.3) histogram summarizes the frequency with which each enzyme group appears in the printed results.

After submission the program generally takes less than one minute to complete, depending most heavily on the number of sequence groups, the number of enzymes selected, and the server load, respectively. The final choice of restriction enzymes is left to the researcher, and is likely to be based on practical factors such as cost, availability, reaction conditions, methylation sensitivity or requirements, star activity, and other specifics that are detailed at REBASE. An online manual detailing usage and options, bug tracking and the source code (open and accessible under the GNU Public License v.2) are available at http://code.google.com/p/repk.

A.4 Conclusions

We found that researchers often failed to report their rationale in choosing a particular set of restriction enzymes for T-RFLP analysis, yet this choice is crucial to resolving the microbial community and interpreting the results. We provide REPK in the hope that it will allow microbial ecologists to maximize their ability to discriminate terminal restriction fragments obtained during T-RFLP and thereby take greater advantage of this powerful community fingerprinting technique.

A.5 Acknowledgments

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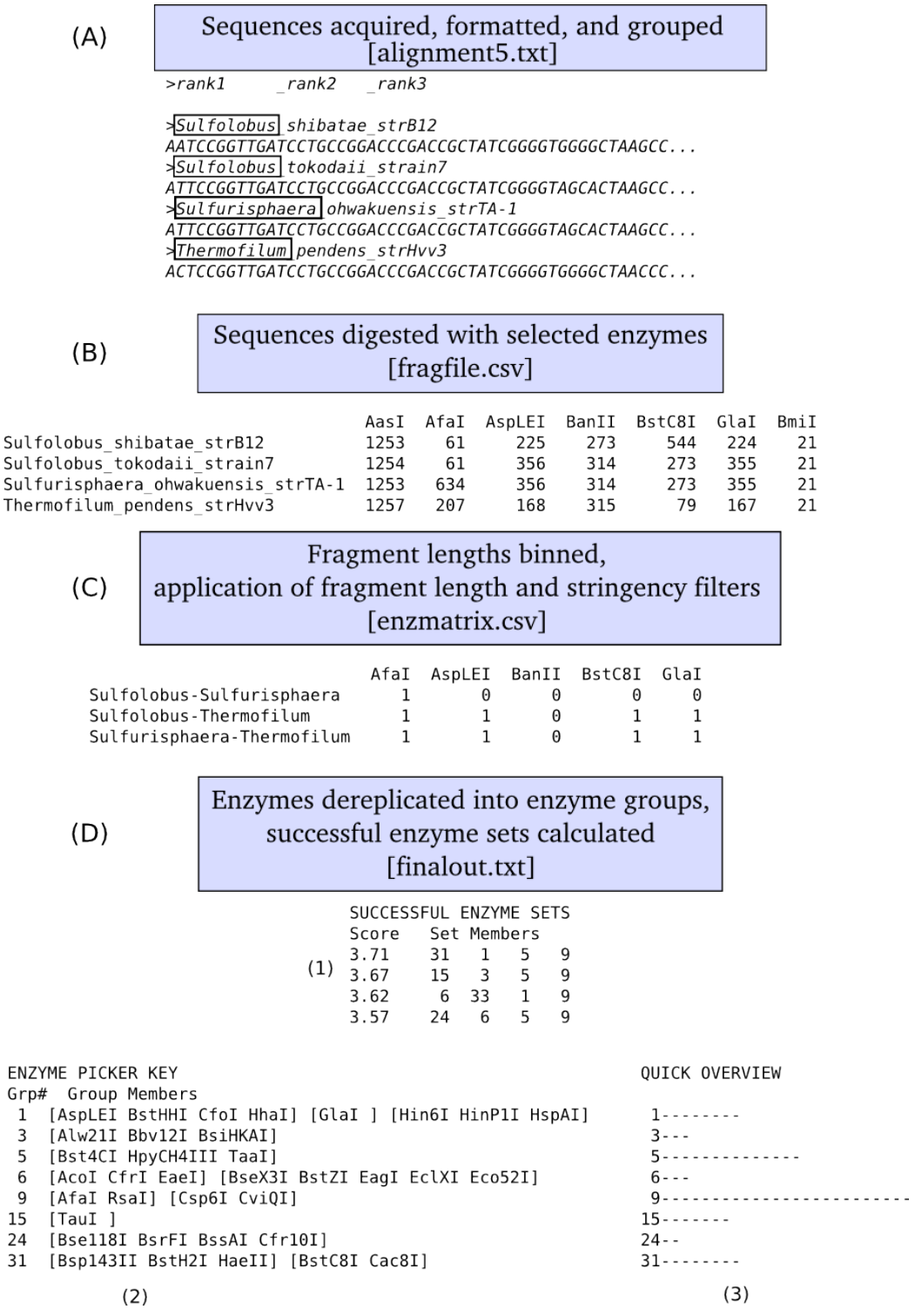

Figure A.1: Flow diagram illustrating REPK.

Appendix B

IN VITRO RATES OF ANAEROBIC METHANE OXIDATION IN MICROBIAL MATS FROM THE BLACK SEA

B.1 Introduction

Microsensors have become increasingly useful tools for investigating the biogeochemical cycling of various elements and nutrients in microbial mats, sediments, and biofilms. Examining microbial habitats on the microscale allows better comprehension of the diversity and distribution of life on Earth by revealing the niches in which microbial consortia evolved and in which they actually survive, details that were previously hidden. In the future, microsensors will be crucial in the effort to understand the (bio?)-geochemical cycling of elements on other planets, in situ, where the only laboratory in which to analyze the samples is the instrument itself.

In this study, microsensors were used to investigate microbial communities performing the anaerobic oxidation of methane (AOM). Because they are possibly living representatives of the biosphere of the early Earth, understanding the biogeochemistry of modern-day AOM-mediating mats will influence our models and understanding of the oceans and atmosphere of the early Earth and in turn influence our expectations for finding life on other planets in early stages of microbial habitation. Outstanding astrobiological questions include determining the relative rates of methanogenesis, methane oxidation, and sulfate reduction, whether these processes might occur simultaneously, and how different Archean atmospheric compositions might have influenced these processes. Large samples of microbial mats from the anaerobic bottom layer of the Black Sea were collected by researchers at the Max Planck Institute for Marine Microbiology (Bremen, Germany) during a cruise to the Crimean shelf of the Black Sea in February 2007. Located atop methane cold seeps at a depth of 250 meters, these mats grew in carbonate chimney reefs and were likely thousands of years old. In vitro, these mats display high methanotrophic activity and are thus active in AOM, using sulfate as a terminal electron acceptor.

[0]Research conducted in association with Anna Lichtschlag, Thomas Holler, Antje Boetius, and Dirk deBeer at the Max Planck Institute for Marine Microbiology in Bremen, Germany in partial fulfillment of requirements for a Certificate in Astrobiology.

The anaerobic oxidation of methane by microbial communities was first discovered in the 1970s when it was observed that methane from deep sources often failed to reach the oxic zone, indicating that it was being oxidized anaerobically, in a zone concomitant with sulfate reduction.

(1) $CH_4 + SO_4^{2-} \longrightarrow HCO_3^- + HS^- + H_2O$

In reaction (1) the local alkalinity increases, and, if present, calcium ions can enter into it, precipitating carbonates, as in reaction (2).

(2) $CH_4 + SO_4^{2-} + Ca^{2+} \longrightarrow CaCO_3 + H_2S + H_2O$

However, reaction (2) occurs only in anoxic environments because the re-oxidation of sulfide to sulfuric acid in the presence of oxygen impedes the precipitation. As a result of this precipitation, large carbonate structures can be built up over time, and chimney structures resulting from this process have been observed in the anoxic part of the Black Sea, covered with thick microbial mats (Michaelis et al., 2002).

Microbial mats mediating AOM in the Black Sea, and others like them (Knittel et al., 2005; Nauhaus et al., 2005; Lloyd et al., 2006; Brazelton et al., 2006), contain uncultured methanotrophic members of Euryarchaeal clades designated ANME-I and ANME-II, which are related to the Methanosarcinales, a diverse group of methanogens. These Archaea perform the anaerobic oxidation of methane essentially by reversing the pathway of methanogenesis, using most of the same enzymes in the process. In the marine environment these Archaea are often found in syntrophic relationships with sulfate reducing Bacteria, and as yet only sulfate is known to act as a terminal electron acceptor for AOM there. The redox potential for nitrate is much greater than for sulfate, and nitrate is thought to be utilized for the oxidation of methane (Raghoebarsing et al., 2006) in one freshwater system, but this process has yet to be confirmed. Although various members of the δ-proteobacteria are known to use nitrate or ferric iron (in addition to sulfate) to oxidize other hydrocarbons anaerobically, these Bacteria are not known to be capable of oxidizing methane anaerobically.

A recent study (Nauhaus et al., 2005) reported that ANME-II communities from marine sediments at Hydrate Ridge (a methane seep off the coast of Oregon in the NE Pacific) were incapable of using several alternative electron acceptors for methane oxidation, including nitrate, Fe-citrate, and ferrihydrite. However, ANME-I communities, like those in microbial

mats from the Black Sea (Michaelis et al., 2002), have yet to be tested for their ability to use alternative electron acceptors for methane oxidation.

The aim of this study was to determine the depth of maximum sulfate reduction in highly active microbial mats from the Black Sea, predicted to coincide with the depth of maximum methanotrophy, and to measure the in vitro rate of sulfate reduction (and thus methane oxidation) in these mats.

B.2 Methods

B.2.1 Mat Collection

Microbial mats were collected by ROV from the anoxic bottom layer of the Crimean Shelf in the Black Sea in the summer of 2007 during the GHOSTDABS cruise. The mats were maintained anoxically in the dark in BS-M at 10℃ until use in these experiments.

B.2.2 LIX sensor preparation

The preparation of liquid ion exchange (LIX) sensors was based on de Beer et al. (1997). Green soda lime glass tubes were drawn to microcapillaries of ~10 μm in 3 steps. First the glass was pulled after melting on an open flame. Second the tip size was decreased using a heating coil, and the final tip was produced using a heated platinum wire. The glass was then baked in an open dessicator for 4 hours at 200℃ to remove any traces of water. The capillaries were then silanized (for better adherence of the LIX membrane) with the addition of 200 μl N-N-dimethyltrimethylsilylamine and immediate closure, followed by a return to 200℃ overnight. Silane fumes were then vented for 15 minutes and the open container was baked for an additional 4 hours at 200℃. After cooling, the microcapillary tip was broken to the proper width and filled with electrolyte solution (300 mM KCl, 50 mM $NaH_2PO_4 \cdot 2H_2O$ pH 7). The LIX membrane was added in two steps. First, the LIX membrane, a solution of 1% (wt/wt) Ionophore III, 0.3% (wt/wt) [tetraphenylborate] in [phenyloctylether] was drawn into the microcapillary to a thickness of ca. 300 μm. Second, PVC (10 mg in 300 μl tetrahydrofuran) was added to the LIX membrane solution and quickly drawn into the microcapillary to an additional 300–400 μm. After drying for several minutes, the body of each sensor was enclosed in a casing made from a Pasteur pipette with about 2 cm of capillary protruding. The casing was cemented into place with epoxy. KCl solution and an Ag/AgCl reference wire were added to the casing to dampen electrical noise, before sealing

with epoxy. The microsensors can be stored in glass test tubes for a maximum of one week before use.

B.2.3 Culture Media

Black Sea Medium (BS-M) was based on $\frac{2}{3}\times$ artificial seawater, simulating the salinity of the Black Sea (~22‰). BS-MOS is sulfate-free BS-M, designed for future experiments testing the utilization of alternative electron acceptors by the Black Sea mats. Media was made to 950 ml deionized distilled water with the salts in Table B.1, autoclaved in side-valve flasks with a butyl stopper and removed while still hot (90°C). The side valve was opened slightly and the headspace flushed with N_2 through the stopper for at least 20 minutes. The headspace was then replaced with N_2:CO_2 (90:10 v/v) and the side valve closed. The media was cooled overnight and the following solutions added the next day:

30 ml $NaHCO_3$ (1 M, anoxic, with N_2:CO_2 (90:10 v/v)); final 30 mM

25 ml NH_4Cl (187 mM; final 4.67 mM) + KH_2PO_4 (58.8 mM; final 1.47 mM), anoxic with N_2 headspace

1 ml trace element mixture (modified from Widdel et al., 1983) [H_2O 50 mL, $FeSO_4{\cdot}7H_2O$ 2100 mg (7.5 mM), HCl (25% = 7.68 M) 13mL, H_3BO_3 60 mg (1.0 mM), $MnCl_2{\cdot}4H_2O$ 1000 mg (5.0 mM), $CoCl_2{\cdot}6H_2O$ 380 mg (1.6 mM), $NiCl_2{\cdot}6H_2O$ 240 mg (1.0 mM), $CuCl_2{\cdot}2H_2O$ 2 mg (0.01 mM), $ZnSO_4{\cdot}7H_2O$ 288 mg (1.0 mM), $Na_2MoO_4{\cdot}2H_2O$ 72 mg (0.3 mM), H_2O to 1000 ml]

1 ml vitamin mixture [NaH_2PO_4 (10 mM, pH 7.1) 100 mL, 4-aminobenzoic acid 4 mg, D(+)-biotin 1 mg, nicotinic acid 10 mg, Ca-D(+)-pantothenic acid 5 mg, pyridoxin dihydrochloride 15 mg, folate 4 mg, lipoic acid 1.5 mg,]

1 ml riboflavin solution [NaH_2PO_4 (25 mM, pH 3.2) 100 ml, riboflavin 2.5 mg]

1 ml thiamin solution [NaH_2PO_4 (25 mM, pH 3.4) 100 ml, thiamin·2HCl 10 mg]

1 ml cyanocobalamin solution (vitamin B12) [cyanocobalamin 5 mg, H_2O 100 mL]

1 ml selenium/tungsten solution [NaOH 400 mg (10 mM), $Na_2SeO_3 \cdot 5H_2O$ 6 mg (0.02 mM), $Na_2WO_4 \cdot H_2O$ 8 mg (0.02 mM), H_2O to 1000 ml]

trace of sodium dithionite (~ 0.1 mM) as reductant

pH 7.5 (with 1 M HCl or 1 M Na_2CO_3)

redox indicator to sulfate-containing media only

B.2.4 Cline sulfide assay

Using the method of Cord-Ruwisch (1985), 2 ml of sample were added to 4 ml of copper reagent [$CuSO_4$ (5 mM) dissolved in HCl (50 mM)] and measured spectroscopically.

B.2.5 Experimental setup

The depth of maximum sulfide production (correlating to sulfate reduction) in Black Sea microbial mats was measured using microsensors prepared in-house. Depth profiles were taken at intervals of 100 μm using pH and H_2S microsensors. The pH is measured concurrently with sulfide to obtain a total sulfide concentration because the sulfide microsensor measures only a single species, H_2S, though HS^- and S^{2-} are also present in the solution in concentrations that are pH-dependent. In the first, 'Positive' experiment, methane was added to the headspace (Table B.2). In the second, 'Negative' experiment, methane was excluded from the headspace and purged from the mat over the course of two weeks by frequent exchanges of the headspace with N_2:CO_2 (90:10 v/v).

B.2.6 Computational analysis

The computer program PROFILE (v1.0 Berg et al., 1998) was used to calculate sulfide fluxes within the mats using the input parameters listed in Table B.3.

B.3 Results and Discussion

Microsensors were used to measure sulfide production, as a proxy for methane oxidation, on two samples of Black Sea mats. Since AOM involves a 1:1 stoichiometry between sulfate reduction and methane oxidation, the methane oxidation rate is equivalent to the sulfate reduction rate, which in turn is in a 1:1 stoichiometry with sulfide production. Excepting

a very small amount of sulfide produced using residual methane, no additional production was evident in the methane-free mat (Fig. B.2). In contrast, the methane-containing mat was highly sulfidic, producing sulfide at a rate of 4.5×10^4 nmol cm^{-3} d^{-1} in the upper 1 mm, with a smaller but definite production of 3.4×10^3 nmol cm^{-3} d^{-1} in the mat below 1 mm (Fig. B.3). Higher AOM rates were expected in the surface of the mat based on FISH probing that localized the ANME-I Euryarchaeota thought to mediate AOM to the upper two millimeters of the mat. While it is clear that methane oxidation occurred to a much greater extent in the surface of the mat, some smaller amount of methane oxidation must still have occurred deeper in the mat to prevent the sulfide concentration from reaching an equilibrium concentration at depth. At either depth, the methane oxidation rates are extremely high compared to previous measurements of methane oxidation rates in the environment: 2–30× the highest rates measured before (Boetius and Suess, 2004; Treude et al., 2005, 2007).

B.4 Acknowledgments

I thank Anna Lichtschlag, Thomas Holler, Antje Boetius, and Dirk deBeer for assistance in Bremen, Bonnie Change for help with flux calculations, and the UW Astrobiology Program for funding to go to Bremen to undertake this rotation experience.

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Table B.1: Recipes for Black Sea Media with (BS-M) and without (BS-MOS) sulfate.

		BS-M		BS-MOS	
	MW	mM	g/L	mM	g/L
$MgSO_4$	246.48	18.20	4.49	0	0
KBr	119.01	0.50	0.06	0.50	0.06
KCl	74.56	5.37	0.40	5.37	0.40
$CaCl_2{\cdot}2H_2O$	147.02	6.60	0.97	6.60	0.97
$MgCl_2{\cdot}6H_2O$	203.30	18.40	3.74	36.60	7.44
NaCl	58.44	297.70	17.40	297.70	17.40

Table B.2: In vitro conditions for sulfate reduction experiments.

	Positive	Negative
basal medium	BS-M	BS-M
N_2	0%	90%
CH_4	90%	0%
CO_2	10%	10%
SO_4^{2-}	16 mM	16 mM
H_2S	0.5 mM	0.5 mM

Table B.3: Parameters used in PROFILE analysis used to calculate AOM mat sulfide production (mmol m^{-2}). Details of parameter options can be found in the PROFILE manual.

Value	Parameter	Units
-0.06	Depth at top of calculation domain	cm
0.3	Depth at bottom of calculation domain	cm
7	Max number of equally spaced zones in interpretation	—
1	Type of boundary conditions (1:t=C b=C)	—
515	First boundary condition	nmol cm^{-3}
1400	Second boundary condition	nmol cm^{-3}
15.3E-06	Diffusivity in water (D)	cm^2 s^{-1}
3	Expression for sediment diffusivity (Ds) (3: Ds=D/(1+3*(1-FI))	—
0	Concentration in water column (C0) [not used]	—
-1.0E+20	Minimum for production rate	nmol cm^{-3} s^{-1}
1E+10	Maximum for production rate	nmol cm^{-3} s^{-1}
0.001	Maximum deviation (in %) when accepting a calculated minimum	—
0.01	Level of significance in the F statistics	—

Figure B.1: Experimental setup, showing the mat-containing anaerobic incubation chamber, sulfide microsensor, and gas-exchange lines.

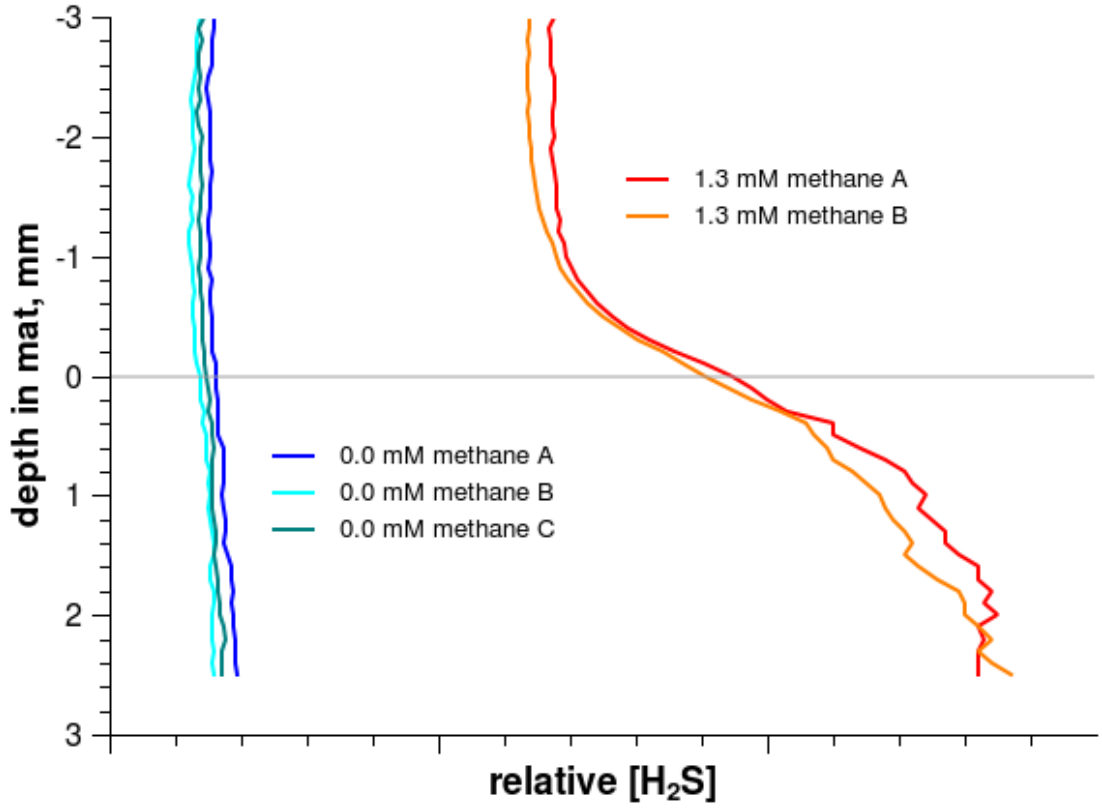

Figure B.2: Comparison of sulfide concentrations in methane-containing and methane-starved Black Sea mats. Total sulfide species were not measured for the profiles shown here, so only relative sulfide concentration is shown.

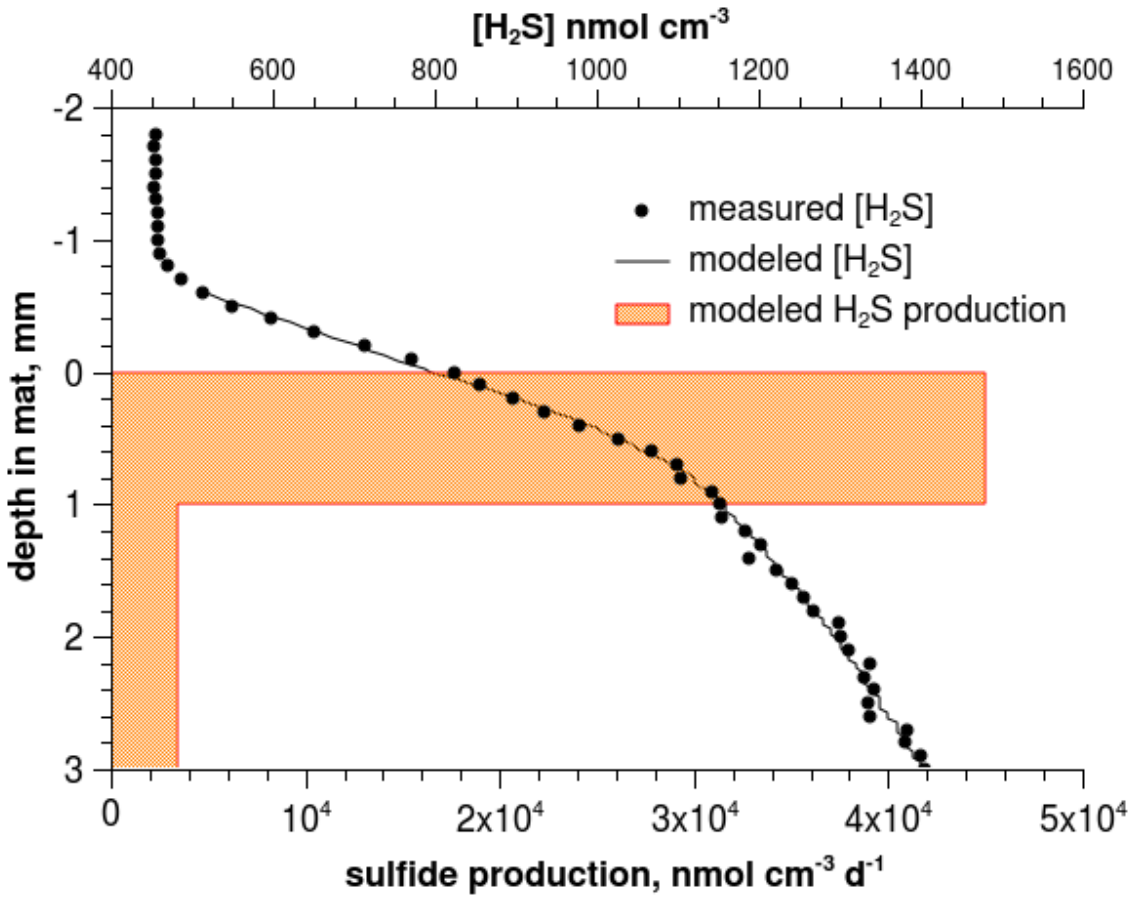

Figure B.3: Sulfide concentration and production rates in Black Sea mats.

Appendix C

GROWTH RATES OF *COLWELLIA PSYCHRERYTHRAEA* STRAIN 34H AT PRESSURES SIMULATING THE DEEP SEA

Colwellia is a genus of marine Gammaproteobacteria commonly found in marine sediments and sea ice. One species, *Colwellia piezophila*, is an obligate piezophile isolated from sediments of the Japan Trench at a depth of 6278 m (Nogi et al., 2004), and has an optimal growth pressure of 592 atm at 10℃. Another isolate, *C. psychrerythraea* strain 34H, was isolated from Arctic marine sediments at a depth of 305 m (Huston et al., 2000). Here we measured the growth of *C. psychrerythraea* strain 34H under atmospheric pressures ranging from surface seawater (0 m = 1 atm) to that expected in the deep sea (3950 m = 395 atm).

C. psychrerythraea strain 34H was grown to early-exponential phase at 8℃ (T_{opt} = 9℃ at 1 atm) in Marine Broth 2216 at standard atmospheric pressure, diluted 1:10 into fresh medium, then aliquoted into 12 ml round bottom polycarbonate centrifuge tubes, which were filled completely and covered tightly with parafilm foil. For each pressure, two stainless steel pressure vessels were filled with about 20 aliquots of *C. psychrerythraea* strain 34H and distilled deionized water. The pressure vessels were pressurized using a hand-driven hydraulic pump and incubated in an upright cooler. At each time point, 0–2 aliquots were sacrificed from each vessel, which were each depressurized, sampled, and repressurized within 3 minutes. From each sampled aliquot 1 mL was immediately transferred to a plastic cuvette and the optical density measured at 600 nm.

While *C. psychrerythraea* strain 34H was found to grow to highest optical densities at 1 atm of pressure, it grew even at the highest pressures tested (Fig. C.1), with a trend towards decreased densities at increased pressures (Fig. C.2). However, density did not decrease monotonically with pressure, rather, an increase in maximal optical density was detected at a mid-range pressure of 245 atm. These results suggest that *C. psychrerythraea* strain 34H encodes variable phenotypic responses to pressure, perhaps inducing pressure-tolerance genes after passing an undefined threshold pressure between 122 and 245 atm.

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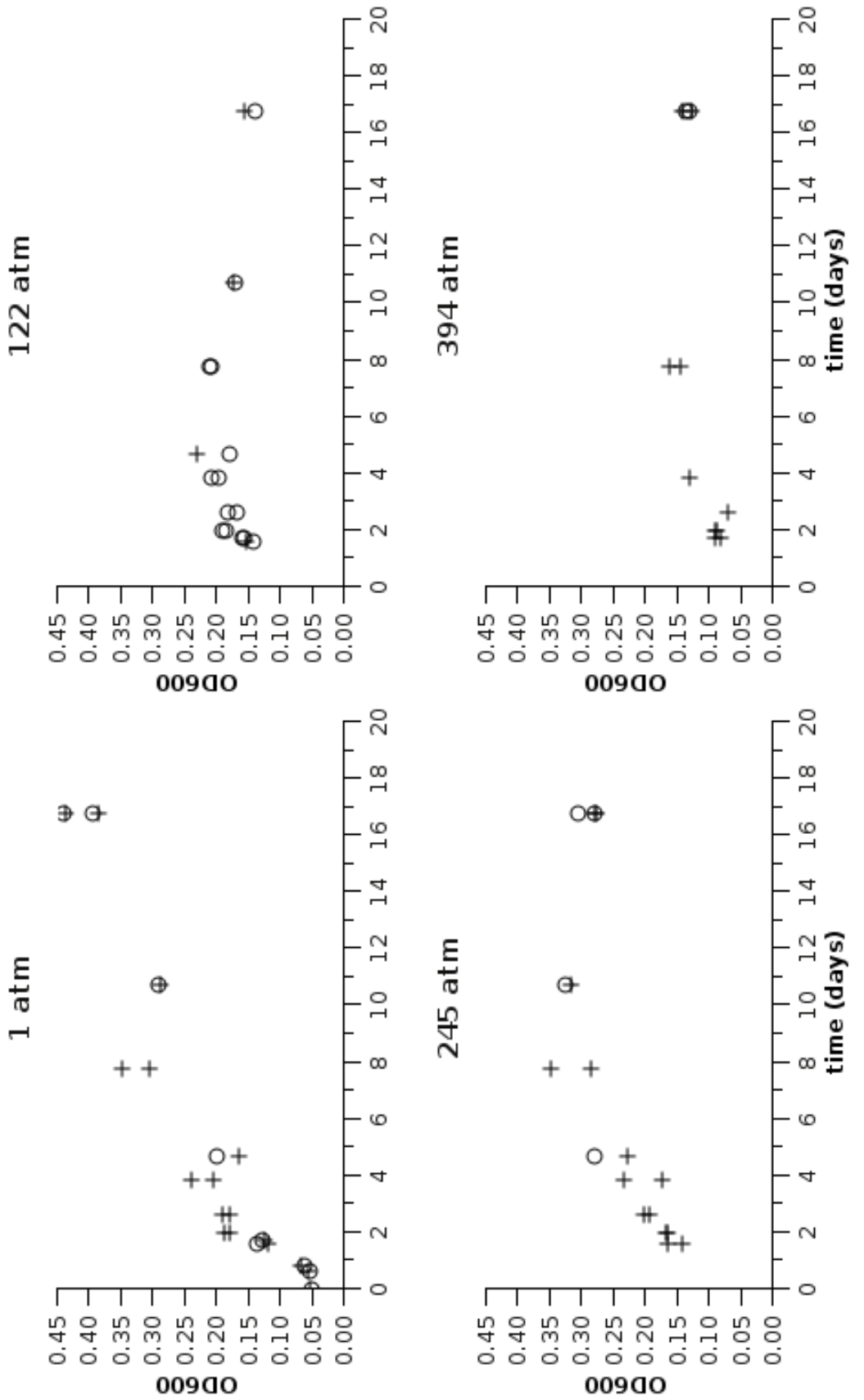

Figure C.1: Optical density at 600 nm of *C. psychrerythraea* strain 34H grown at 8°C under varying hydrostatic pressures.

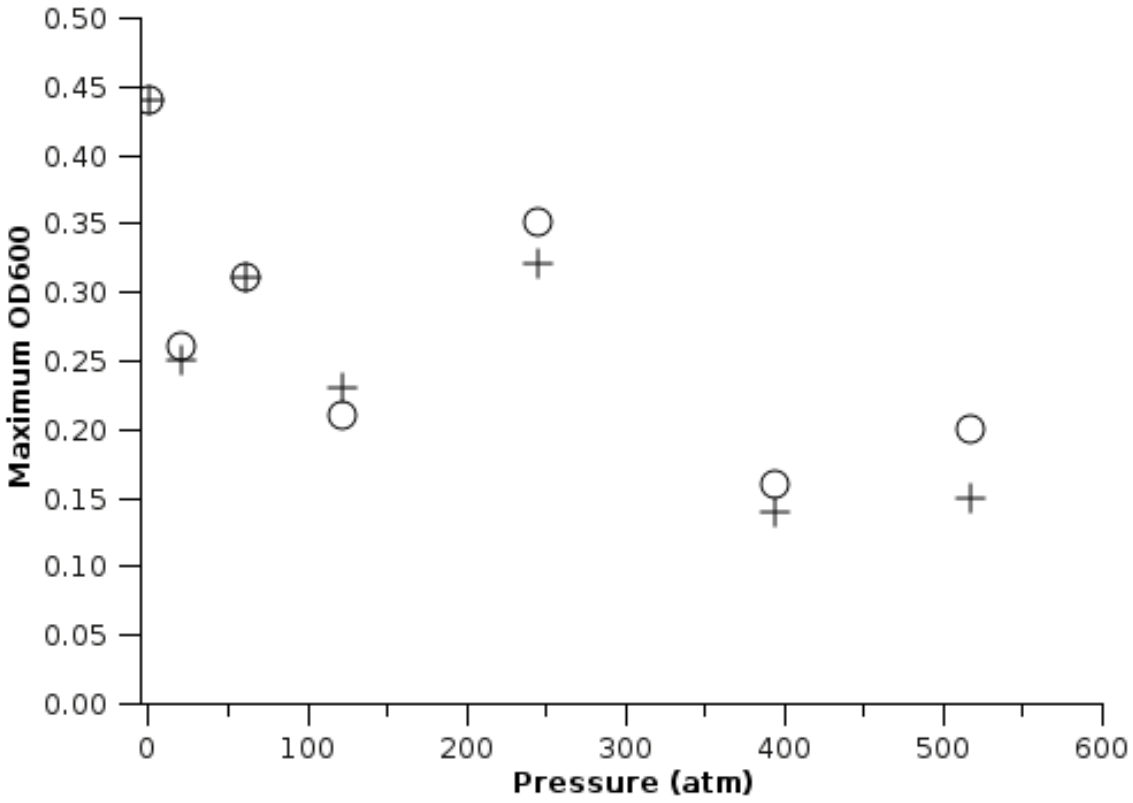

Figure C.2: Maximum optical density at 600 nm achieved by *C. psychrerythraea* strain 34H grown at 8℃ under varying hydrostatic pressures.

Appendix D

PHYLOGENETIC ANALYSIS OF PSYCHROPHILIC BACTERIAL ISOLATES FROM THE ARCTIC

Partial-length 16S rRNA gene sequences were obtained for 15 isolates of Bacteria previously isolated from Arctic sea ice, gut flora, and sediment. Sequences obtained from these isolates were most similar to those of other microbes often found in Arctic environments, including *Psychrobacter* and several Alteromonadales: *Colwellia*, *Pseudoalteromonas*, *Moritella* and *Shewanella* (Fig. D.1).

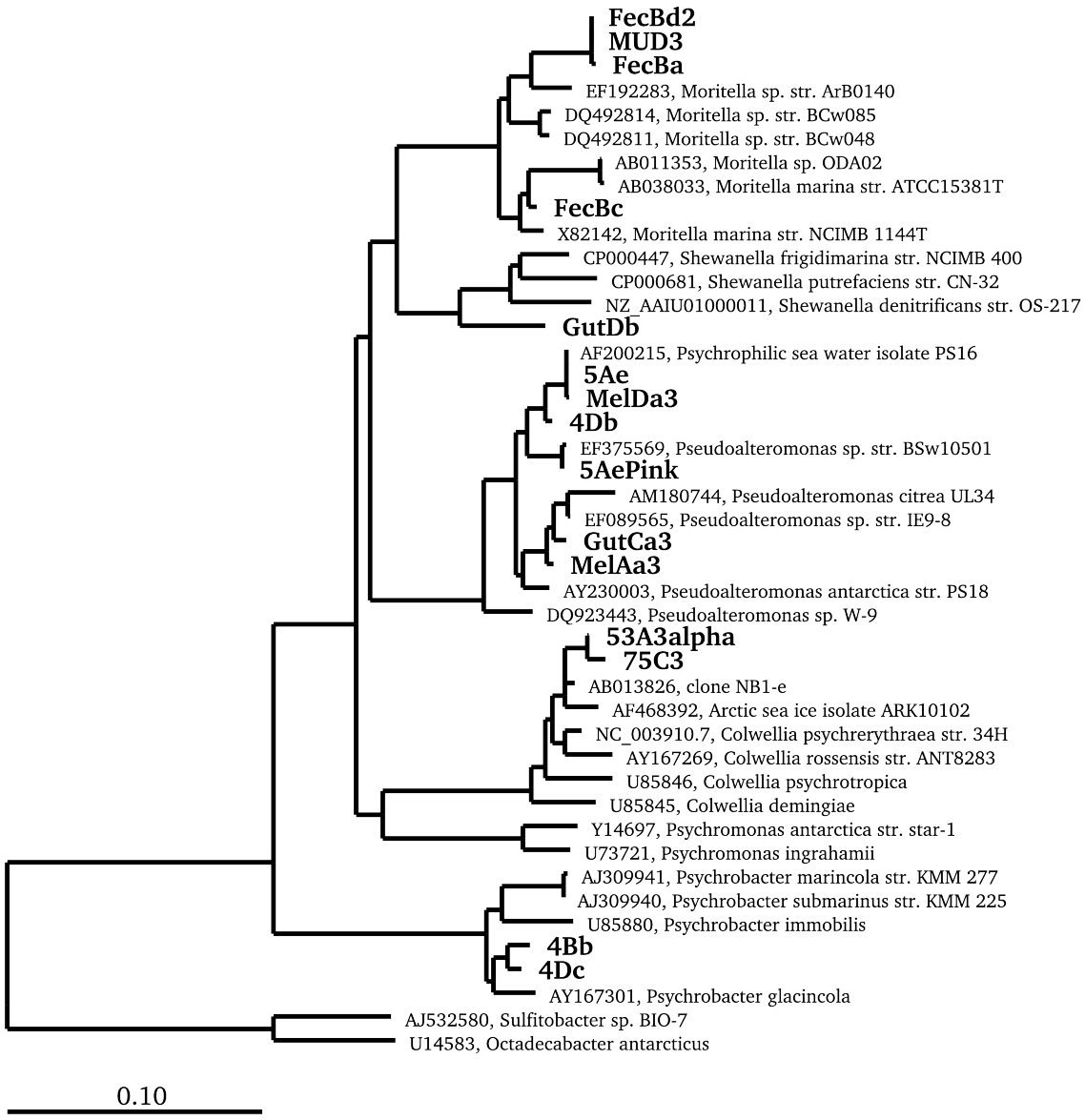

Figure D.1: Phylogenetic tree of 16S rRNA gene sequences; sequences from Arctic isolates in this study shown in large bold font. Tree was constructed in ARB using 'dnadist' and 'fitch' from PHYLIP 3.6. Sites 432–6525 were used in the alignment and hypervariable regions were removed before phylogenetic analysis.

Appendix E

MUTAGENESIS OF *COLWELLIA PSYCHRERYTHRAEA* STRAIN 34H

Mutagenesis of *C. psychrerythraea* strain 34H was attempted with the goal of obtaining auxotrophic mutants to be used in experiments testing the ability of this strain to undergo natural competence for transformation.

C. psychrerythraea strain 34H was grown in SLV minimal medium to an OD600 of 0.6 at 8℃ (Fig. E.1). To each 10 mL culture, 1 ml of 10 μg mL mytomycin C was added (Fig. E.2). Cells were incubated at 8℃ for about one cell doubling period, then centrifuged at 5000× g for 5 minutes to pellet cells. Cells were resuspended in 10 mL SLV minimal medium and grown to early exponential phase, OD600 = 0.1. Penicillin was added to 10,000 U per mL and cells incubated until the turbidity increase leveled off. Cells were then centrifuged at 5000× g for 5 minutes to pellet cells, which were washed once with fresh SLV and resuspended in 10 mL of SLV. Each culture was serially diluted and 200 μL of each dilution was plated onto marine broth 2216 plates. These plates were incubated until colony formation, then replicated onto SLV minimal medium plates with autoclave-sterilized velveteen. The replica plates were incubated until colony formation, then screened by comparison with the originals, looking for colonies that grew on rich medium but not SLV. Potential auxotrophs were plated onto rich medium plates and the replica plating and screening were repeated. Several dozen potential auxotrophs were found from hundreds of plates using this technique, but after a secondary screen no true auxotrophs were detected.

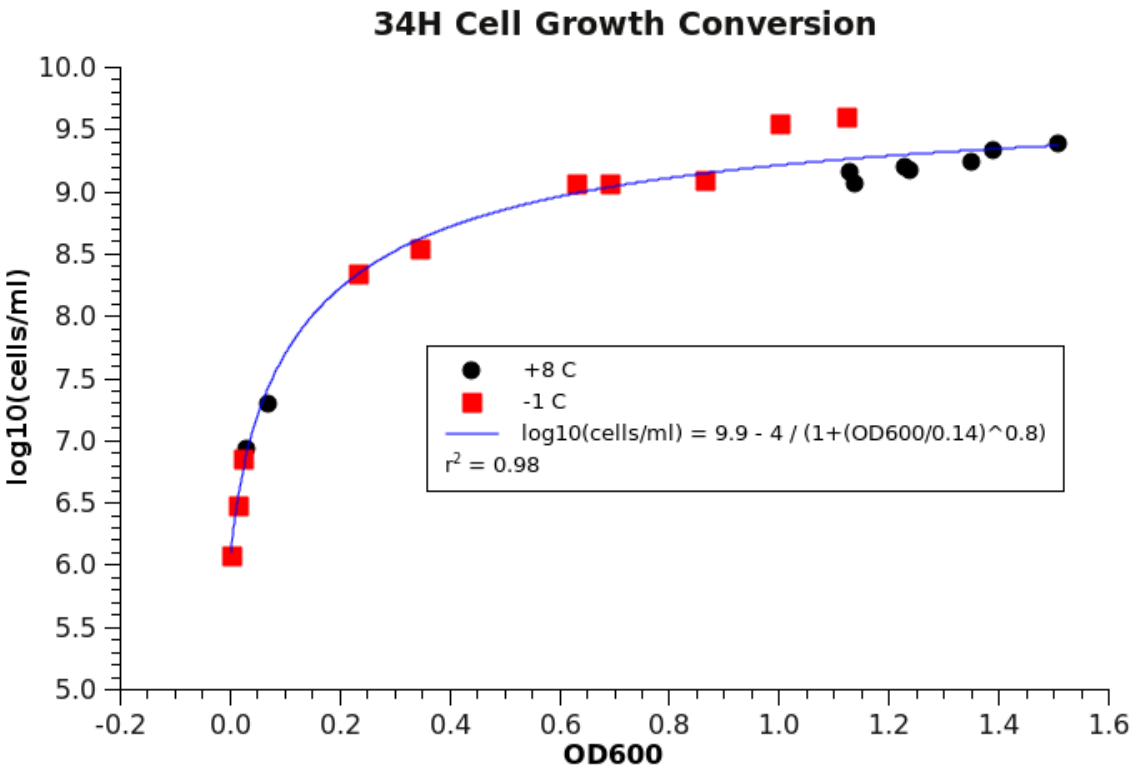

Figure E.1: Measurements of OD600 and microscopic counts of *C. psychrerythraea* strain 34H in Marine Broth at two temperatures: −1℃ and 8℃, near its optimal growth temperature (made by A. Huston, unpublished data; plotted here for comparative purposes). Line of best fit, calculated in this work using Qtiplot: $y = 9.9 - \frac{4}{1 + \left(\frac{x}{0.14}\right)^{0.8}}$, had an $r^2 = 0.98$ for the combined datasets.

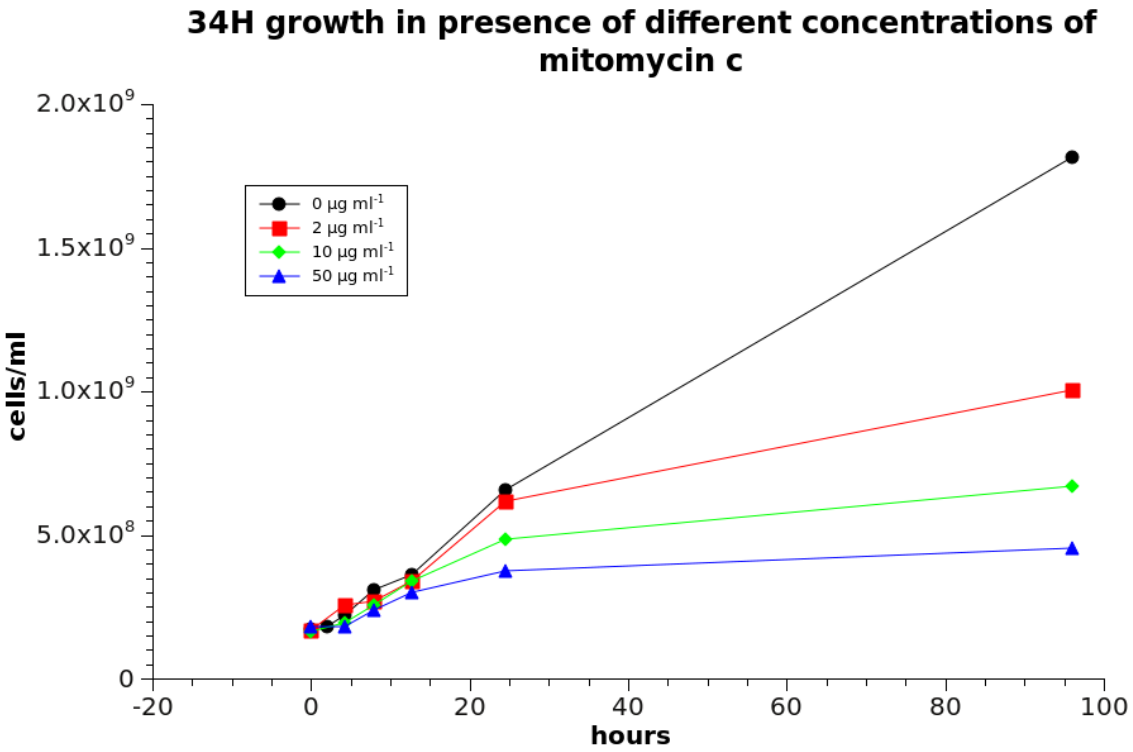

Figure E.2: Growth of *C. psychrerythraea* strain 34H in minimal medium with different concentrations of the mutagen mitomycin c.

Appendix F

AN IMAGEJ SCRIPT FOR THE AUTOMATION OF VIRAL COUNTING FROM DIGITAL IMAGES

Counting viruses from marine samples is a time-intensive process that could be usefully simplified by digital automation. Open source scripts for ImageJ and Matlab software were written to automatically and repeatably count viruses (or bacterial cells, but not both simultaneously) automatically from digital image files (e.g. Fig. F.1). Automated counting may enable more complete statistical analyses of viral or bacterial concentrations by allowing more fields to be counted with the same input of operator time. The program presented here takes as input JPEG image files, but most microscope-mounted cameras return TIFF files, which can be converted in batch to JPEG using open source ImageMagick software with the following command:

```
mogrify -format jpg *.tif
```

The counting program consists of two scripts. The first, `JVirusCount`, is to be used with the image-processing software ImageJ. After opening any image in the desired directory and running the script, it iteratively adjusts the noise threshold and uses the "Find Maxima" command to count the number of cohesive particles (e.g. cells, viruses) in every image file in the desired directory. At very low noise thresholds, non-particles are detected as particles (type I error); at very high noise thresholds, true particles are not detected (type II error). The best estimation for the particle count (defined presently as that which best matches the human estimation) lies between these two extreme thresholds. A rapid change in the slope of this curve (particles counted versus noise threshold, Fig. F.2) was observed during the transition from 'noise' to 'particles', but the location and slope of the curve depended on the cleanliness and quality (brightness, contrast) of the image.

The best match to human counts was observed at the maximum of the first derivative of the curve (Fig. F.2), estimated by the intersection of a two-line regression. The output of `JVirusCount` is a number of tab-delimited text files, each named after an image, summarizing the number of particles detected at each threshold. This file is used as the

input to the second script, `MVirusCount`, which uses an external function, `regress2lines`, to estimate the number of human-countable particles by determination of the intersection of the two regression lines and the abundance of particles at that point.

In testing with field samples (Fig. F.1), the program estimated counts about 8% greater than human counts, which is within the ~15% error estimated among human counters in our laboratory (Fig. F.3). The samples used in this pilot study were field samples taken from an extreme environment, so usage in a laboratory setting is expected to be more precise. The lowest relative error rates were observed at higher concentrations of viruses, indicating that the program requires more extensive optimization before being used at concentrations of less than 100 viruses per field (Fig. F.3).

F.1 JVirusCount: a script for ImageJ

```
list = getFileList(File.directory);

for (i=0; i<list.length; i++) {
run("Clear Results");
run("Set Measurements...", "  decimal=9");

for (noise=1; noise<42; noise++) {
 run("Find Maxima...", "noise=" + noise + " output=Count");
 rows = nResults-1;
 setResult("Noise", rows, noise);
 counts = getResult("Count", rows);
 setResult("logcount", rows, log(counts));
}

title = getTitle();
saveAs("Measurements", File.directory + title +".csv");
run("Open Next");
}
```

F.2 MVirusCount: a script for Matlab

```
clear all;
d=dir("*jpg.csv");

for k=1:length(d);

  fname=d(k).name;
counts = dlmread(fname,"\t",1,0);
counts(end,:)=[];
counts(:,end)=[];

% from 10:end because the curve has a sigmoidal shape
[m, R, idiv, G] = regress2lines(counts(10:end,3),counts(10:end,4));

xy = [1, m(5);
(m(1)*1+m(2)), (m(1)*m(5)+m(2));
m(5), 40;
(m(3)*m(5)+m(4)), (m(3)*40+m(4));];

store_fname{k,1} = fname;
store_intercept(k,1) = m(5);
store_count(k,1) = exp((m(1)*m(5)+m(2)));

hold off
plot(counts(:,3),counts(:,4),"bs")
hold on
plot(xy(1,:),xy(2,:),"b-")
plot(xy(3,:),xy(4,:),"r-")
title({fname});

drawnow
```

```
pause
end

fid = fopen("output.csv","a");
for k=1:length(store_fname);
fprintf(fid,"%s\t%0.5g\t%0.5g\n", store_fname{k},
store_intercept(k), store_count(k));

end
```

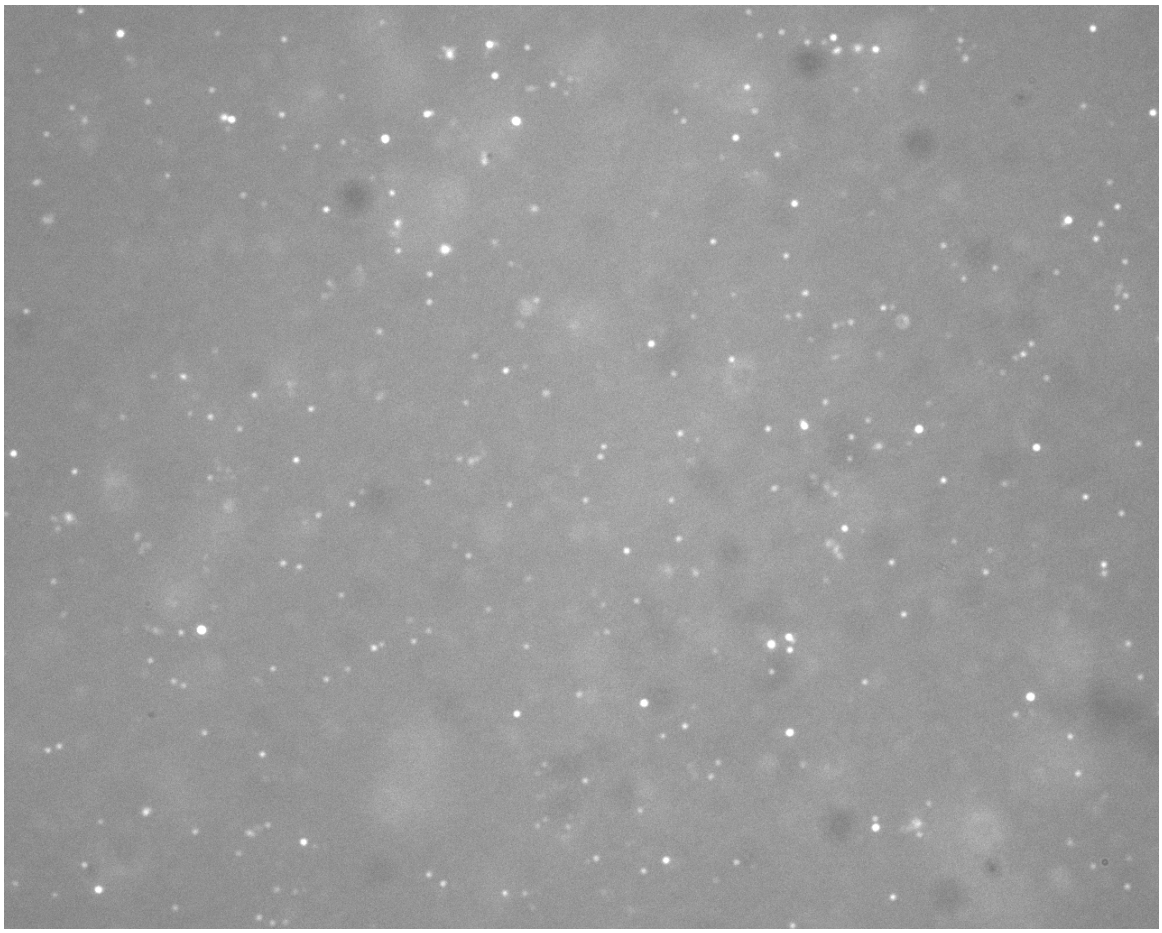

Figure F.1: Viruses stained with SYBR Gold and observed under epifluorescence microscopy, from Arctic seawater below nilas ice, sampled at Station D3 (Table 3.1) during the Circumpolar Flaw Lead System Study.

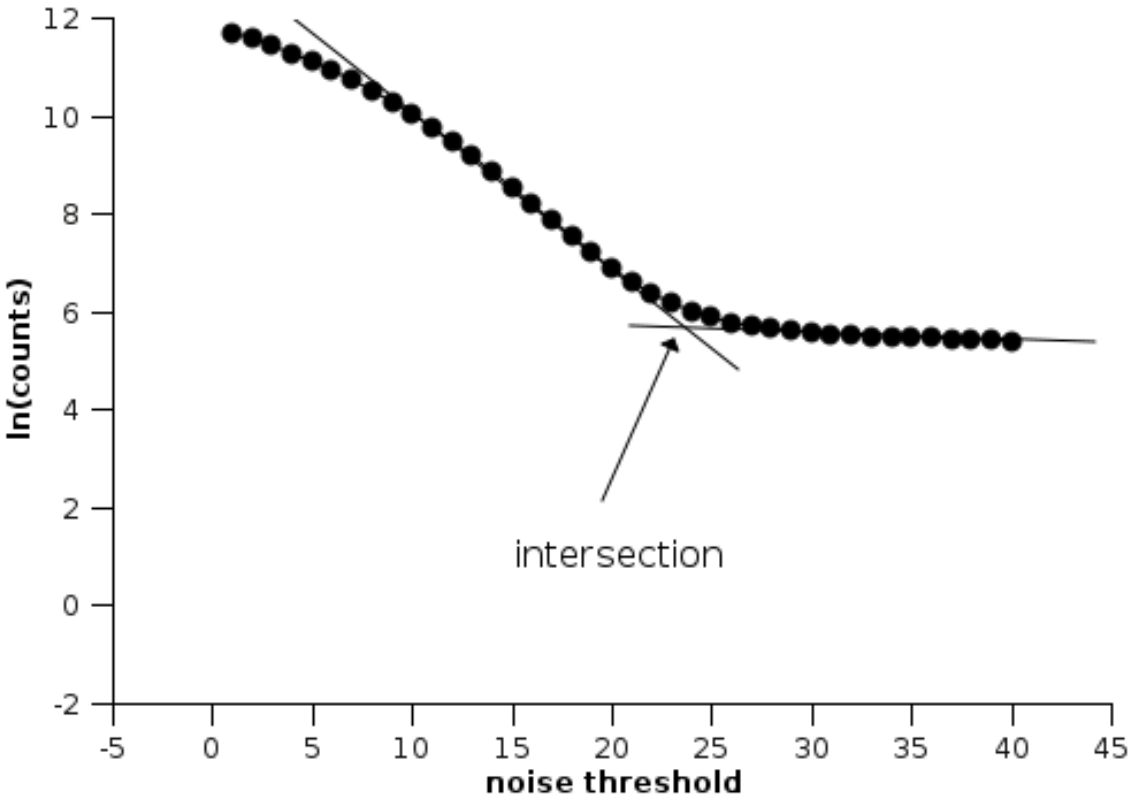

Figure F.2: Particle abundance as a function of noise threshold for a stained virus sample from Arctic seawater.

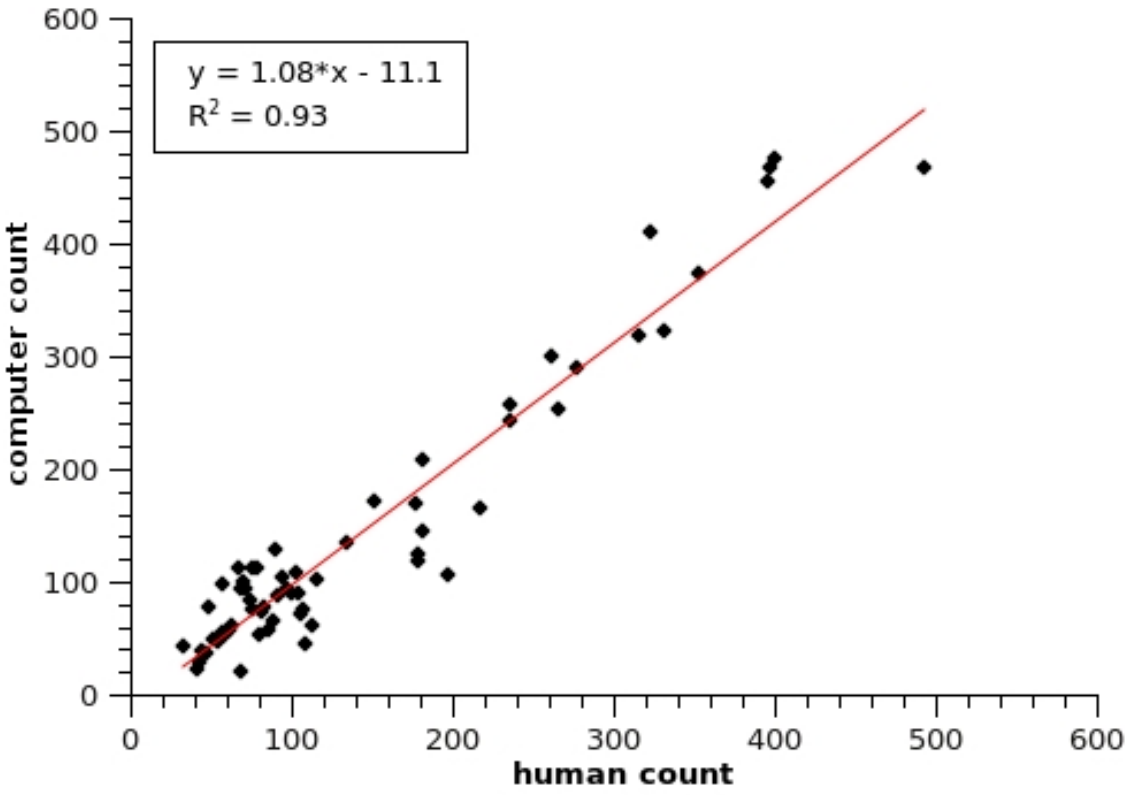

Figure F.3: A comparison of virus samples counted both manually and by the automated scripts presented here.

Appendix G

VERTICAL DESCENT OR LATERAL TRANSFER? UNRAVELLING THE LARGE NUMBER OF WHOLE-GENOME RECIPROCAL BLAST HITS BETWEEN ANAEROBIC, THERMOPHILIC BACTERIA AND ARCHAEA

Abstract

Whole genome comparisons using reciprocal BLAST hits (RBHs) can provide a measure of the number of shared genes between two genomes, even across domains. Reciprocal BLAST, however, does not distinguish between genes that are shared because of evolutionary vertical descent and those shared due to lateral gene transfer. In a reciprocal BLAST dataset of pairwise comparisons among 132 Bacterial genomes and 18 Archaeal genomes, thermophilic and anaerobic Bacteria had significantly more reciprocal BLAST hits to Archaeal genomes than did other Bacteria when adjusted for genome size. Several lines of evidence indicate that the Last Common Ancestor may have evolved in a thermophilic, anaerobic environment – environmental conditions in which many of the Archaea used in our dataset are found today. We initially tested the hypothesis that the genomic relationship was explainable by vertical descent (and not lateral gene transfer) by statistically comparing a distance matrix based on numbers of RBHs among Bacteria and Archaea to a 16S rRNA distance matrix, and found a significant correlation, though we also found a significant correlation between the RBH distance and a 'phenotypic' distance matrix generated using environmental parameters of temperature, oxygen requirements, and habitat. To further investigate these findings we sorted RBHs into clusters of orthologous groups (COGs) to determine which COGs were driving the genomic relationships. In general, COGs involved in 'core processes' (e.g. replication and transcription) better explained the relationships than did COGs expected to be more susceptible to lateral gene transfer (e.g coenzyme metabolism and transport), though important exceptions were evident. Phylogenetic analyses of individual genes from influential COGs that are more frequently shared between thermophilic or anaerobic

[0]Abstract previously published as Fuchsman CA, Brazelton WJ, Collins RE, Horner-Devine MC, and G Rocap (2007) Vertical descent or lateral transfer?: Unravelling the large number of whole-genome reciprocal BLAST hits between anaerobic, thermophilic Bacteria and Archaea. American Society for Microbiology General Meeting, Toronto, Canada.

Bacteria and Archaea will provide a higher resolution view of the combined influences of vertical descent and lateral gene transfer on microbial evolution.

Methods

To account for variation in reciprocal best hits (RBHs) due to genome size, a four-parameter log-logistic function of the form

$$f(x) = c + \frac{d-c}{1+e^{b(log(x)-log(e))}} \tag{G.1}$$

was fitted for each archaeon, with bacterial genome size as the predictor and RBHs as the response variable. The parameters b,c,d, and e were estimated in R (`http://www.r-project.org`) using the non-linear fitting function 'drm' in the 'drc' package. Using these estimates, 95% confidence intervals on observation predictions were calculated by the delta method in Matlab using the functions 'nlinfit' and 'nlpredci'. Residuals from the best fit line were calculated and genome pairs falling outside the 95% confidence intervals were considered significantly different (Fig. G.1).

G.1 getcoefs: a script for R

```
library(drc)

rbhmatrix <- read.csv("bactarch.csv", header=T,row.names=1,
check.names=T,blank.lines.skip=T)

attach(rbhmatrix)

#initialize
cf<-cbind(1:4)
rbhrows <- nrow(rbhmatrix)
rbhcols <- ncol(rbhmatrix)

for (i in 2:rbhcols) {
rbhmodel <- drm(rbhmatrix[,i] ~ rbhmatrix[,1],fct=LL.4())
plot(rbhmodel,main=colnames(rbhmatrix[i]),xlab="genes in bacterial genome",
ylab="reciprocal best BLAST hits")
```

```
cf <- cbind(cf,coef(rbhmodel))
}

cf <- cbind(cf,c(mean(cf[1,]),mean(cf[2,]),mean(cf[3,]),mean(cf[4,])))
colnames(cf) <- c(colnames(rbhmatrix),"mean")
write.csv(cf, file="cf.csv")

quit()
```

G.2 getresiduals: a script for Matlab

```
clear all
rbh = csvread("input.csv");

%initial guesses calculated in R, input from cf.csv
beta0 = [-2.3 62.1 351.6 1574.8];

[rbhrows,rbhcols] = size(rbh);
userows = 1:rbhrows;
xes = rbh(:,1);

for i=2:rbhcols
[Beta(:,i),resid(:,i),J] = nlinfit(rbh(userows,1),rbh(userows,i),
@l4,beta0);
[ypred(:,i), Delta(:,i)] = nlpredci(@l4,xes,Beta(:,i),resid(:,i),
J,0.05,"off","observation");

plot(rbh(userows,1),rbh(userows,i),".");
hold on;
plot(xes,l4(Beta(:,i),xes));
plot(xes,(ypred(:,i)+Delta(:,i)));
plot(xes,(ypred(:,i)-Delta(:,i)));
hold off;
xlabel("Genes in Genome");
ylabel("Reciprocal Best BLAST Hits");
pause(.25)
```

```
end

over = rbh - ypred - Delta;
over = over(:,2:end);
csvwrite("archbact_overs.csv",over);

under = ypred - Delta - rbh;
under = under(:,2:end);
csvwrite("archbact_unders.csv",under);
```

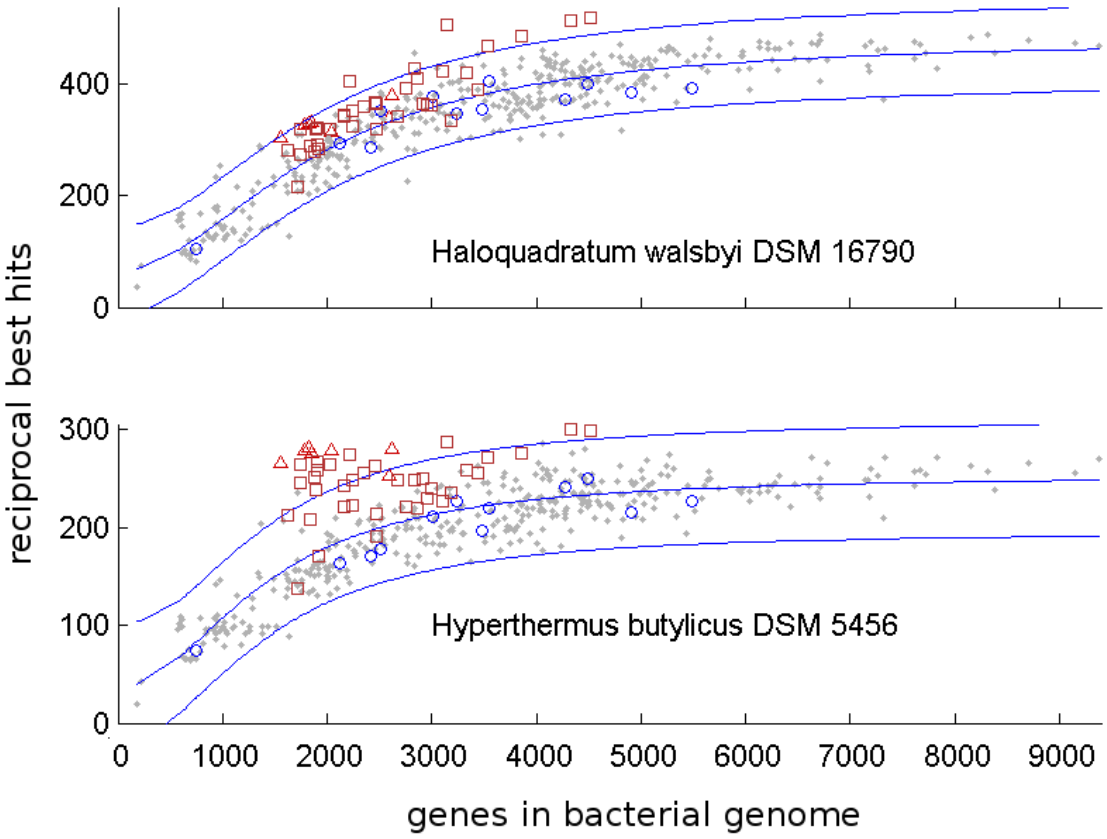

Figure G.1: Reciprocal best BLAST hits for each bacterium against two archaeons, plotted by temperature classification: red triangle, hyperthermophile; red square, thermophile; gray filled circle, mesophile; blue circle, psychrophile.

Appendix H

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VITA

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Education and Research Experience

Ph.D. Biological Oceanography, University of Washington (expected Summer 2009)

Sea ice as a hotspot for horizontal gene transfer: I investigated the diversity of bacterial and archaeal communities in winter sea ice with fragment analysis, utilizing software I wrote for this project to aid in fingerprinting analyses of environmental communities. I found Archaea and Bacteria from seawater and possibly other environments were found to persist through the winter season with no change in richness and limited changes in the structure of either community. During the Circumpolar Flaw Lead System Study of the International Polar Year I measured the abundances of parameters relevant to the frequency of horizontal gene transfer in newly formed sea ice. Subsequently, I performed in silico analyses of genome sequences for evidence of horizontal gene transfer, including that of a model psychrophile, *Colwellia psychrerythraea* strain 34H. Advisor: Dr. Jody W. Deming.

Certificate in Astrobiology, University of Washington (expected Summer 2009)

In vitro microsensor measurements of anaerobic oxidation of methane: While conducting my research rotation at the Max Planck Institute for Marine Microbiology in Bremen, Germany, I used pH and sulfide microsensors to measure the rate of anaerobic oxidation of methane (AOM) in samples of a microbial mat from

the Crimean Shelf of the Black Sea. This study confirmed expectations from stable and radioactive isotope analyses that AOM occurs primarily in the surface layer of the mats, where ANME-1 Archaea dominate the microbial community. Advisors: Dr. Dirk de Beer, Dr. Antje Boetius.

M.S. Biological Oceanography, University of Washington (2006)

Microbial persistence over an Arctic winter season: I investigated the abundances of microorganisms, particles, and particulate extracellular polymeric substances (pEPS) in Arctic winter sea ice during the Canadian Arctic Shelf Exchange study. Although a significant decline in microorganismal abundance was observed in the coldest ice, there was also significant production of pEPS in all of the ice measured, interpreted as evidence for microbial adaptation to this extremely cold and salty environment. Advisor: Dr. Jody W. Deming.

B.S. Biochemistry with Honors, Washington State University (2002)

I performed research on the biochemistry and molecular biology of the phenylpropanoid pathway in vascular plants, gaining skills with enzyme assays, PCR, molecular cloning, and bioinformatics. Advisor: Dr. Norman G. Lewis.

Field Experience

I have spent a total of 20 weeks in the field for various projects, primarily in the Arctic or other cold regions, including 3 months in the Northwest Territories during fall and winter, and several weeks in Barrow, Alaska, and Hokkaido, Japan, during winter:

Autumn 2007: Six-week cruise in the Beaufort Sea aboard the CCGS *Amundsen* as part of the International Polar Year (IPY) Circumpolar Flaw Lead System Study (CFL).

Winter 2004: Six-week overwintering cruise in Franklin Bay, Northwest Territories aboard the CCGS *Amundsen* as part of the Canadian Arctic Shelf Exchange Study (CASES).

Winter 2006: Two-week sea ice field course in Hokkaido, Japan, through the University of Alaska–Fairbanks. Instructors: Hajo Eicken (UA-Fairbanks), Rolf Gradinger (UA-Fairbanks), and Kunio Shirasawa (Hokkaido University).

Summer 2003: Two-week cruise in the Chukchi Sea on the R/V *Xuelong* with the Chinese National Arctic/Antarctic Research Expedition (CHINARE) program.

Winter 2003: One-week workshop with the NASA Astrobiology Institute Europa Focus Group in Barrow, Alaska.

Spring 2008: Two-week cruise to Glacier Bay National Park, Alaska, aboard the R/V Thomas G. Thompson as a teaching assistant for the UW Oceanography Senior Undergraduate Thesis course.

Spring 2004: One week of daily student cruises in Puget Sound aboard the R/V Barnes as a teaching assistant for Oceanography 101.

Summer 2007,2009: Instructor on one-day education and outreach cruises in Puget Sound organized by the Ocean Inquiry Project (Seattle, USA).

Autumn 2002-2008: UW Astrobiology Workshops at Easton Glacier on Mt. Baker, Mount St. Helens, Yellowstone National Park, University of Arizona Kitt Peak Observatory, and the Channeled Scablands.

Computer Proficiences

Applied experience with PAUP*, ARB, Matlab, R, PRIMER-E, Perl, Javascript, PHP, HTML, XML, LaTeX, MySQL database administration, and Linux system administration.

Two web-accessible programs were written to aid in fingerprinting analyses of complex microbial communities, gaining frequent use both within the UW Center for Environmental Genomics and by external users. REPK calculates the best enzymes for use with T-RFLP given a user-inputted sequence file; Dakster performs binning of electropherogram peaks given a user-inputted fragment list.

REPK: http://rocaplab.ocean.washington.edu/tools/repk

Dakster: http://rocaplab.ocean.washington.edu/cgi/dakster/index.html

Two interactive web-accessible databases were engineered to facilitate data-sharing both among scientists and between scientists and the public. The Deming Lab Sampling Database will make information about our group's sampling efforts easily accessible to the public and to other scientists with whom collaborations might be initiated. The Seattle Crow Project collects cultural and scientific evidence for the extensive interactions between humans and our corvid neighbors.

Deming Lab Sampling Database: http://staff.washington.edu/rec3141/deminglab/

Seattle Crow Project: http://staff.washington.edu/rec3141/crows/

Professional Service

Student representative to the UW Astrobiology Steering Group (2006) and UW Oceanography Faculty Search Committee (2007).

Public Outreach

I consider science outreach an important part of my scientific development and have volunteered my time to a variety of outreach activities, including dozens of hours giving demonstrations at Polar Science Weekend at the Seattle Science Center, the annual School of Oceanography Open House, inside K-6 classrooms, and at sea with the Ocean Inquiry Project. In partnership with the Centers for Ocean Sciences Educational Excellence–Ocean Learning Communities (COSEE-OLC), a fellow student and I designed and implemented an ocean sciences curriculum with a local grade school teacher. I participated in an event to improve my ability to communicate with citizen scientists, a goal shared by another project I developed to allow engaged amateurs to report and track crows banded by Dr. John Marzluff in the College of Forest Resources. I have also written articles and shared photographs for publication in non-technical forums, one of which was subsequently translated and published in Catalan.

Teaching Experience

Autumn 2008: Teaching Assistantship, Oceanography 430: Senior undergraduate core course in Biological Oceanography. Led weekly review sessions and gave lectures. Assisted in adaptation of curriculum towards discovery-based learning approaches.

Winter/Spring 2008: Teaching Assistantship, Oceanography 443/444: Senior undergraduate thesis course. Mentored students in the development and execution of individual research projects which were then carried out aboard the R/V Thomas G. Thompson in March 2008.

Spring 2007: Communicating Ocean Sciences outreach course. Constructed and presented pre-planned kits for science outreach to 2nd grade classroom, then designed, constructed, presented, and evaluated implementation of a new kit with a team member.

Autumn 2005: Astrobiology Exchange Workshop at Friday Harbor Labs. Assisted in planning and execution of 4-day hands-on scientific workshop involving ~20 students and faculty from the Astrobiology programs at the University of Washington and the University of Arizona.

Spring 2004: Teaching Assistantship, Oceanography 101: Introduction to Oceanography for non-majors. Lectured and taught 3 lab sections of 15 students each, culminating in a research cruise in Puget Sound.

Publications

Miller LA, Papakyrakou TN, Owens O, Sutherland N, Macdonald R, Mucci A, **Collins RE**, and JW Deming (in preparation) Carbon dynamics in sea ice: a winter flux time series.

Collins RE, Deming JW (in preparation) Abundant dissolved genetic material in sea ice: a hot spot for lateral gene transfer?

Collins RE, Deming JW (in preparation) Identification of an inter-Order lateral gene transfer event enabling the catabolism of common compatible solutes by *Colwellia*

psychrerythraea 34H

Fuchsman CA, Brazelton WJ, **Collins RE**, Horner-Devine MC, Rocap G (in preparation) Vertical descent or lateral transfer? Unravelling the large number of whole-genome reciprocal BLAST hits between anaerobic, thermophilic Bacteria and Archaea.

Collins RE, Rocap G, Deming JW (submitted) Persistence of bacterial and archaeal communities in sea ice through an Arctic winter.

Collins RE, Carpenter S, Deming JW (2008) Spatial and temporal dynamics of particles, bacteria, and extracellular polymeric substances in Arctic winter sea ice. Journal of Marine Systems 74:902–917. doi:10.1016/j.jmarsys.2007.09.005.

Collins RE, Rocap G (2007) REPK: an analytical web server to select restriction endonucleases for terminal restriction fragment length polymorphism analysis. Nucleic Acids Research 35 (Database issue): W58-W62. doi:10.1093/nar/gkm384

Ehlmann BL, Chowdhury J, Marzullo TC, **Collins RE**, Litzenberger J, Ibsen S, Krauser WR, DeKock B, Hannon M, Kinnevan J, Shepard R, Grant FD (2005) Humans to Mars: a feasibility and cost-benefit analysis. Acta Astronautica 56:851.

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Presentations

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