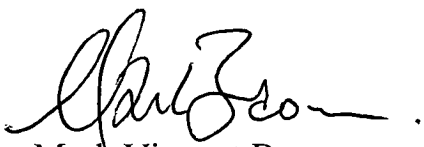
A Molecular Phylogenetic Survey of Polar Sea Ice Microbial Communities

Mark Vincent Brown, B.Sc.(Hons)

Submitted in fulfillment of the requirements
for the degree of Doctor of Philosophy
The University of Tasmania
Hobart, October, 2000

Declaration

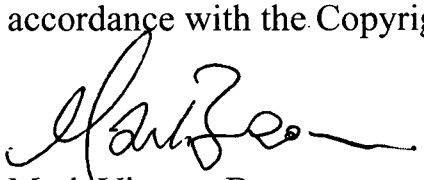
I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of this thesis.



Mark Vincent Brown

Authority of Access

This thesis may be made available for loan and limited copying in accordance with the Copyright Act, 1968



Mark Vincent Brown

Abstract

The structure of the Antarctic sea ice cover is highly heterogenous, with composition in a given region depending on extremely localised physical processes. Past studies have revealed a wide range of sea ice microbial communities (SIMCO's), associated with a variety of ice types. Many of these assemblages are highly productive. Given the enormous extent of the ice cover these localised but highly productive communities constitute an important component of the regions biological processes. To date, our knowledge of the taxonomical composition of SIMCO's extends from culture based studies. This study employed culture independent molecular techniques to identify biodiversity in a variety of SIMCO's. Clone libraries of 16S rRNA genes were constructed from the total environmental DNA extracted from one Arctic and seven Antarctic sea ice samples using universally-conserved, *Archaea*-specific and *Bacteria*-specific 16S rDNA primers. A total of 539 recombinant clones were obtained. Restriction fragment length polymorphism (RFLP) and sequence analysis grouped the clones into 100 distinct phylotypes (a unique clone or group of clones with sequence similarity >0.98) representing sympagic organisms of *Bacterial* and Eukaryotic origin. *Bacterial* clones were affiliated with the alpha and gamma *Proteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* Group, the *Chlamydial/Verricomicrobia* and the Gram positive bacteria. One clone was not closely affiliated with any *Bacterial* Division. Eukaryotic clones were affiliated with a variety of autotrophic and heterotrophic nanoplankton and included a large number of plastid genes. A number of sequences from both groups represented putatively novel organisms. The findings of this examination corroborate data previously collected during culture based studies indicating bacterial biodiversity increases in SIMCO's displaying high levels of primary productivity. Shifts in community composition appear to be associated with alterations in the carbon budget. A comparison of Arctic and Antarctic sea ice communities revealed several common genera occuring at both poles.

The information gained from this study provides a focus for a number of important future studies including *in situ* based analyses of SIMCO composition, cultivation of novel organims identified by sequence anlaysis, and the examination of biogeography of polar sea ice microbial bacteria.

Acknowledgements

Id like to sincerely thank the following people

John Bowman for providing funding and excellent guidance, and, along with Tom McMeekin, for understanding and supprt during difficult times.

Sharee McCammon for her supreme efforts in the lab and along with Sue Dobson, Suzy Rae, Mathew Smith and Jodie van de Kamp for assistance in all things molecular biological.

Peter Grewe and Sharon Appleyard for their automated DNA sequence electrophoresis.

Andrew McMinn and Connie Lovejoy for providing the McMurdo and Arctic samples.

The Tasmanian Branch of the ASM and particularly David Nichols for conference funding.

Everyone associated with ANARE expeditions, especially Andrew Irvine, Ingmar Janse, Andrew Davidson and the crews of the Aurora Australis and the Polar Bird.

Everyone asociated with the School of Agricultural science for making my years here so enjoyable. Including but not solely and in no particular order James Benson, David Miles, Mark Salter, Suzy Rae, Nadine Donovan, Mathew Smith , Craig Shadbolt, Andrew Greenhill, Kylie Lance, Fiona Giblin, Sven Rassmussen, Jodie van de Kamp, Rachel Walker and Janelle Brown.
All friends outside the department but especially Becky Brown for her faithful and entertaining email correspondance and Simona Timmins for her friendship and support during the hardest stretch and especially for not letting me come to uni on my birthday.

Finally thanks to my parents for their neverending love and support.

Table of Contents

List of Tables

List of Figures

1. Literature Review

1.1 Sea Ice Formation

The Southern Ocean comprises 10% of the earth's ocean area and has immense impacts on the climate of the southern hemisphere. Its high latitude (50-80°S) means it receives markedly asymmetric radiation regimes over the course of the year. During the winter months, beginning around March, day length is short and the angle of incidence of sunlight is low. The heat input to the ocean is so low that there is a net loss of heat from the surface water, which drops below its freezing point (Priddle, *et al.* 1996). High wind speeds experienced at this time of year cause turbulent mixing leading supercooling in near surface waters. Supercooling in the order of only a few hundredths of 1°C is required for ice formation (Maykut, 1985; Freidmann, 1993; Wadhams, 1994). Frazil ice, small cylindrical ice disks approximately 1 mm in diameter and generally not exceeding 3-4 mm, are formed rapidly in open water. These coalesce to form a thin layer of "grease ice" covering the water surface. The ice suspension then congeals by wave-induced compression, to produce pancake ice (Wadhams, 1994), rounded masses of semi consolidated slush. These are initially only small but grow gradually through accretion of frazil ice crystals until they reach 3- 5 m in diameter and around 50 cm thickness (Maykut, 1985; Wadhams, 1994). Grease ice then forms between these pancakes causing them to coalesce into a consolidated ice sheet (Maykut, 1985). Further growth of the ice sheet may occur in a variety of ways and is primarily regulated by local physical regimes, resulting in large scale heterogeneity of the ice habitat (Garrison, 1991).

In areas where the sea surface is completely ice covered, additional heat loss takes place through the ice sheet. Due to the lack of available free water, air temperatures at the ice surface drop from around -10° to the -20°C range, causing the slow removal of heat from the water beneath and freezing at the ice/water interface. This results in the formation of congelation ice (Vincent, 1988; Freidmann, 1993). Congelation ice consists of an organised crystal lattice in which ice crystals assume a uniform orientation, with their principal axis of symmetry (the C-axis) horizontal. Growth occurs perpendicular to this axis, producing interlocking columns about 1-2 cm in diameter (Lewis and Weeks, 1971 cited in Vincent, 1988). As the seawater freezes, particulate matter is rejected from the ice matrix and as a result, less biological

material is incorporated in congelation ice than in frazil ice. This rejected material increases the density of surrounding waters, causing it to sink and carry heat away from the region of ice growth. Less dense seawater then rises up to the freezing ice face (Vincent, 1988).

In regions influenced by the oceanic environment, turbulent wind and wave action maintains considerable amounts of open water, referred to as polynyas. The presence of these regions allows continued cycles of frazil and pancake ice production (Weeks and Ackley, 1982, Clarke and Ackley, 1984). This is followed in calmer periods by consolidation and accretion into a closed pack ice cover. Here, congelation ice accounts for only ~20% of multi-year ice and 40% of first-year ice, with frazil ice making up the remainder (Clarke and Ackley, 1982; Vincent, 1988; Garrison and Close, 1993; Wadhams, 1994).

Platelets of ice typically 10-15 cm in diameter and 0.2-0.3 cm in thickness may form below the ice sheets at considerable depths in the water column. This frequently occurs in coastal regions as water flowing out from under ice shelves rises and undergoes supercooling due to decreasing pressure (Maykut, 1985). These platelets rise and accumulate under established ice floes where freezing occurs at the platelet-congelation interface (Horner, 1985). This platelet layer is continuous with the brine of the congelation ice above and the water column below. Platelet ice may form porous layers up to 5 m thick (Lange, 1988).

Infiltration ice forms when heavy snow cover or ice piled up during pressure-ridge formation causes the ice sheet to become depressed or bent resulting in flooding by seawater. A layer of ice then forms at the snow-ice interface.

The sea ice environment in the Antarctic is a heterogeneous one composed to greater or lesser degrees by some, or all, of these ice types. Variations in local physical regimes during ice formation result in variations in ice structure, in turn producing very different types of microenvironment for microbial growth and survival. Also, due to dynamic processes such as drifting, divergence and rafting, even adjacent floes may have evolved completely differently (Helmke and Weyland, 1995).

1.2 Southern Ocean Phytoplankton Blooms and Bacterial Associations.

Sea ice is a transient phenomenon. At its maximum extent in September the ice extends to cover about 20 million km^2, 60% of the southern ocean, encircling the Antarctic continent in a 400-1900 km wide ring of ice (Zwally, *et al.*, 1983). The majority of this ice cover melts over the course of spring and summer when daylight hours are long and the angle of incidence of irradiance high. During this time, low density meltwater from the ablating ice sheet and a decrease in wind driven turbulent mixing result in increased water column stability along the ice edge or marginal ice zone (MIZ). The Southern Ocean is characterised by relatively high nutrient concentrations which are well above limiting values even during periods of intense phytoplankton growth (El-Sayed, 1971). However, it has been suggested that efficient utilisation of macronutrients by phytoplankton is prevented by deep vertical mixing (Mitchell and Holm-Hansen, 1991). This mixing also influences the radiation climate experienced by individual phytoplankton cells. Primary production is enhanced when the maximum depth to which phytoplankton cells are taken during mixing (Zmix) is shallower than the critical depth (Zcr) at which irradiance is limiting, as cells will be mixed in a constantly favourable light environment (Dower, *et al.* 1996). During most of the year phytoplankton cells are mixed to depths where the photon flux is low resulting in populations which are highly adapted to low light levels. The stabilisation of the water column results in mixing depths around 20-40m, which, if maintained for long enough periods (i.e. weeks), facilitate phytoplankton blooms, where primary production exceeds the mean total loss rate through algal respiration, excretion of dissolved organic compounds, grazing losses and sedimentation (Gleitz, *et al.* 1994). These blooms are highly productive and may quickly result in large standing stocks of organic matter in localised areas.(e.g. Holm-Hansen and Vernet, (1990) observed an increase in maximum chlorophyll *a* (Chl*a*) concentrations from <0.5 $\mu g\ L^{-1}$ to >16 $\mu g\ L^{-1}$ over approximately one month in the northern Gerlache Strait and southwestern Bransfield Strait). They dominate annual productivity in many Antarctic marine habitats. A number of studies have reported a distinct absence of phytoplankton blooms in the MIZ (Bathmann, *et al.*, 1997, Savidge, *et al.*, 1995), highlighting the extreme spatial and temporal variability inherent in such incidents and the complexity of factors involved. Extensive phytoplankton blooms occur in other areas, most notably the Polar Frontal Zone (PFr) (Bathmann, *et al.*, 1997), in the absence of a shallow mixed layer. Giesenhagen, *et al.* (1999) performed microcosm experiments to study the effect of melting sea ice on planktonic microbial food webs. They used first-year ice and water from the

Antarctic Circumpolar Current (ACC) and the ACC-Weddell Grye Boundary (AWB), a region where ice edge blooms do not usually occur. Their results indicated that stratification alone was insufficient to induce phytoplankton bloom formation, although their experiment ran for only two weeks, which may not be sufficient time to test this. They found that phytoplankton blooms were stimulated by the seeding of the water column by sympagic organisms in the absence of metazoan grazers. When grazers were present, high levels of DOM (which would be amplified by 'sloppy feeding' of grazers) resulted in the stimulation of pelagic bacterioplankton but no accumulation of algae, indicating that in regions with the potential for phytoplankton blooms, this process is controlled by grazing activity.

Nitrate uptake is an enzyme mediated process. The enzyme involved, Nitrate-reductase, contains iron which is in low concentrations in the southern ocean but which is concentrated in fronts (de Baar, *et al.* 1995). Bathmann, *et al.* (1997) suggest higher iron concentration is a factor leading to the accumulation of high phytoplankton stocks in the PFr but not in the melt-water zones of the MIZ. It may also explain the preferential utilisation of regenerated sources of nitrogen, such as ammonium, by phytoplankton in the presence of high nitrate concentrations, as was observed by Gleitz, *et al.* (1994). Indeed in some cases more than 50% of primary production may be attributed to microbial regeneration of nutrients (Delille and Drosiers 1996).

Regeneration of nutrients occurs via a microbial loop, involving energy flow through microheterothroph and protozooplankton assemblages. Heterotrophic Bacteria are ubiquitous in Antarctic waters but at relatively low concentrations compared to temperate regions. The bacteria utilise high and low molecular weight dissolved organic material produced by phytoplankton, regenerating nitrogen, and through grazing, death and autolysis, returning carbon and energy to the nutrient pool. Bacterial growth and production appears to co-vary with phytoplankton over the course of the year (Delille, *et al.* 1995). However a lag in bacterioplankton response during and immediately after the spring phytoplankton blooms, resulting in an uncoupling of bacterioplankton and phytoplankton production, has been reported (Lancelot, *et al.* 1989, Cota, *et al,* 1990, Karl, *et al.* 1991). The cause of this phenomenon, which has been observed on both eastern and western sides of the continent, is not understood, however a number of hypotheses have been offered. The Pomeroy /Deibel hypothesis suggests a differential temperature dependency for phytoplankton and bacterial production. The thermal budget of the top 100m of the water column is dominated by ice formation and ice melt (Priddle, *et al.* 1996) and as a consequence, the surface waters are constantly cold, with temperatures varying by as little as one or two degrees over the year at high latitudes. Delille (1996) found

that seawater temperature ranged from +0.5 to –1.7°C during a 2 year study period. Low temperature is probably the ultimate rate limiting factor in potential phytoplankton primary production. The phytoplankton inhabiting the Southern Ocean appear to be obligate psychrophiles, physiologically adapted to exhibit near maximal growth rates at low temperatures (El-sayed and Fryxell, 1993). However even at the greatest recorded values, when other potentially limiting factors are optimal, growth rates fail to reach optimal levels as derived from the Arrhhenius' law (Jacques, 1983). Cold adapted bacteria may be grouped into two categories. Psychrophiles comprise those organisms whose minimum, optimum and maximum (cardinal) growth temperatures are <0, <15 and <20°C respectively, whilst psychrotrophs comprise those organisms whose cardinal temperatures are 0-5, >15 and >20°C (Morita, 1975). Some bacteria fall between these two definitions (Russell, 1992). The bacterial assemblages of the Southern Ocean are predominantly psychrotrophic, not psychrophilic (eg Delille, *et al.* 1988, Vincent, 1988, Delille and Perret, 1989, Delille, *et al.* 1996). which may suggest greater low temperature inhibition than the psychrophilic phytoplankton assemblages. However this is not the case, and despite the extreme cold, the temperature appears to have very limited influence on Antarctic bacterioplankton populations (Delille and Rosiers, 1996).

During the initial phases of a bloom phytoplankton standing stocks develop quickly whilst zooplankton grazing rates and bacterial consumption are still low. Bacterial consumption is based on dissolved organic matter and degradation of dead or moribund cells. Gleitz, *et al.* (1994) found that bacterial metabolism was limited during a phytoplankton bloom but stimulated during bloom demise when natural mortality, in conjunction with zooplankton grazing and storm induced sedimentation, raised concentrations of phytoplankton derived dissolved organic carbon and moribund cells in the water considerably. This perhaps suggests a delay in production of extracellular carbon by healthy phytoplankton during blooms as a cause for the uncoupling. However it cannot rule out the prospect that the composition of DOM released during the early stages of a bloom is of high molecular weight thus requiring hydrolysis by bacterial produced exoenzymes before utilisation, a process which has been suggested could take about 1 month (Billen and Becquevort, 1991).

These stocks rapidly decline during late summer and early autumn. Grazing pressure from proto- and meta-zooplankton is likely to remove substantial proportions of phytoplankton biomass (Gleitz, *et al.* 1994). Increasing wind stress will result in dispersal of blooms, sedimentation and, along with decreased irradiance levels, removal of phytoplankton from favorable light conditions due to and deep mixing in the water (Dower, *et al.* 1996). So at the time of new ice formation standing stocks of

organic matter are generally low. The scavenging of this remaining matter during sea ice formation effectively end the seasonal production of the water column.

1.3 Development and Growth of Sea Ice Microbial Communities.

The physical mechanisms involved in the formation of sea ice provide not only varied environments for microbial habitation but are also avenue for the incorporation and enrichment of microorganisms, and a variety of other materials, in the sea ice structure. The surface properties of frazil ice crystals facilitate the adhesion of particles from the water column. As the crystals rise through the water column or move horizontally across the ocean surface to form new ice, materials accumulate on and between them, so that the ice matrix contains not only the ice crystals which form its structure but also the associated organic and inorganic materials (Garrison, *et al.* 1983). Incorporation of materials including silt, clay, sand-sized particles, diatom frustules, foraminiferal tests, live plankton and large bacteria into sea ice via harvesting or scavenging by frazil ice crystals has been identified (Reimnitz, *et al.* 1993, Garrison, *et al.* 1983). Concentrations of organic matter in newly formed ice will thus initially reflect the concentrations remaining in the water column at the time of ice formation Gleitz and Thomas (1993). Enrichment factors for algal cells in newly formed sea ice up to 2.5 times sea water concentrations have been found in both laboratory and field investigations (Gleitz and Thomas, 1993, Grossmann and Gleitz, 1993, Garrison and Buck, 1989). Scavenging is a size dependent process (Grossmann and Dieckmann, 1994, Weissenberger and Grossmann, 1998) and not effective for the majority of bacterioplankton (cells $< \sim 1 \mu m^3$). However, it has been found (e.g. Grossmann and Dieckmann, 1994; Grossmann, 1994; Grossmann and Gleitz, 1993) that bacterial concentrations in newly formed ice are much greater than can be accounted for by biological growth over the short time span involved. Co-incorporation of bacteria with algae is considered the predominant mechanism of bacterial enrichment in sea ice. Planktonic bacteria concentrate on marine aggregates (Caron, *et al.* 1982, Sullivan and Palmisano, 1984; Grossi, *et al.* 1984; Putt, *et al.* 1994; Grossmann, 1994), and are incorporated in the ice matrix along with these (Grossmann, 1994, Grossmann and Gleitz, 1993, Weissenberger and Grossmann, 1998). In support of this, strong correlations have been reported between the numbers of bacterial cells incorporated into sea ice and the concentration of phytoplankton in

the sea ice and water column. In experimental sea ice formation, Weissenberger and Grossmann (1998) found that physical enrichment of bacteria within new ice was negligible and bacterial biomass was only enhanced when the concentration of algae was high. Grossmann (1994) found that when sea ice was forming in regions of high phytoplankton concentrations, bacterial cells experienced strong enrichment in the new ice. Conversely, when phytoplankton populations in the vicinity of sea ice formation were low, there was little enrichment in bacterial concentration. Further to this, Grossmann and Dieckmann (1994) reported an enrichment of bacterial cells in newly formed sea ice only in conjunction with algae. This co-incorporation may result in selective enrichment of viable bacteria (Helmke and Weyland, 1995) through their attachment to larger nutritious particles such as algae or detritus. Another possible mechanism of cell enrichment in sea ice is through percolation pumping. This occurs in the outer realms of pack ice where ocean swells still influence the environmental conditions. Heavy swells cause bending of the ice sheet and percolation by seawater leading to the enrichment of adhesive particles which are retained preferentially in the pore system (Ackley, *et al.* 1987, Shen and Ackermann, 1990)

The physico-chemical stresses experienced by pelagic biota during the transition from an open water to a sea ice habitat result in the development of a community which is very much characteristic of the sea ice environment. Phytoplankton and associated bacteria growing unconstrained in seawater at around +0.5 to −1.7°C are exposed to varying light regimes, restricted to small brine channels and exposed to salinities up to 150‰ and temperatures down to −10°C. The ability to acclimate to these conditions will determine which components of the initial sea ice community become dominant. Gleitz and Thomas (1993) suggest that size may be the critical determining factor. Polar diatom species appear have the ability to acclimate to a wide range of light, salinity and temperature conditions, however spatial confinement within the sea ice matrix appears to inhibit growth of species exceeding 40um in any dimension, thus preferentially selecting smaller species (Gleitz and Thomas, 1993).

Phytoplankton exhibit impaired photosynthetic capacity within newly formed ice. This initially may be a response to increases in salinity and or decreases in temperature (Palmisano, *et al.* 1987, Kottmeier and Sullivan, 1987) and is further compounded in established ice flows by low light availability. Increasing ice

thickness, accumulation of snow on the ice surface and ever declining irradiance levels result in decreases in photosynthetic saturation levels (Palmisano, *et al.* 1987, Watanabe and Satoh, 1987), as algae increase cellular pigment concentration, thereby increasing the number or size of photosynthetic units, in order to maximize utilisation of available light. This is evidenced by increases in Chl*a* concentrations (Gleitz and Thomas, 1993). The higher cell concentration found in sea ice as a result of physical enrichment and concomitant growth in confined regions may result in an increase in mean primary production.

Bacterial metabolism experiences a concomitant downturn during new ice formation. Using incorporation rates of the amino acid [^{3}H] leucine as an indicator of general heterotrophic activity in an experiment simulating ice formation, Grossmann and Gleitz (1993) found the physiological state of bacteria declined substantially in the early stages of ice formation. After three days of freezing, uptake rates of [^{3}H] leucine had decreased significantly compared to those found in water at the start of the experiments, indicating a decrease in specific bacterial activity. In similar experiments in the sea ice and open water of the Weddell Sea, Grossmann (1994) found that in ice forming in a region of high phytoplankton concentration, bacterial production exceeded that of open water. However, due to the high bacterial biomass as a result of physical enrichment, the specific bacterial activity was actually lower in ice than in water, and estimated growth rates of active bacteria decreased from 0.15 day^{-1} to about 0.017 day^{-1}. Grossmann and Dieckmann (1994) found a substantial decline in both total and specific bacterial activity in young sea ice forming in a region of low phytoplankton concentration where bacterial enrichment did not occur. Here, the average molar ratio of leucine to thymidine was five times lower than that of the open water, suggesting frequent division of cells but low rates of protein synthesis. Clearly acclimation to the prevailing conditions results in the formation of productive microbial communities within the ice matrix.

Whilst levels of major nutrients in the brine (e.g. nitrate, silicate and phosphate) are high due to initial high levels in the water column and further concentration effects during freezing. they are not sufficient to sustain phytoplankton production for long in isolation. However, phytoplankton standing stocks have been observed at concentrations far in excess of maximums predicted from complete utilization of the major nutrients initially present (Gunther, *et al.* 1999). Gosselin, *et al.* (1985 cited in

Knox, 1994) reported that nutrient enrichment of ice occurs via an upward flux of nutrients from the underlying water column when the boundary layer beneath the ice is convectively unstable. This boundary layer is destabilized by factors such as brine rejection, tidal movement and current variability so it is these factors which determine the rate of nutrient exchange (Knox, 1994). As algal cells are generally attached to the ice crystal matrix they are retained within the ice habitat, preventing a concomitant decrease in community standing stocks during flushing. Nutrients may also be regenerated *in situ* by heterotrophic remineralization by bacteria and phagocytic flagellates (Griffiths, 1982).

In pack ice regions which make up >90% of the ice cover, ice development is greatly influenced by the oceanic environment. Turbulent wind and wave action leads to continued cycles of frazil and pancake ice production. This is followed in calmer periods by consolidation and accretion into a closed pack ice cover. Hydraulic pumping by wave action leads to perfusion of the ice by seawater resulting in an influx of both nutrients and microbial biomass. This, along with the continued incorporation of matter via harvesting during ice formation, leads to the presence of substantial microbial communities throughout the ice column (e.g. Kottmeier and Sullivan, 1987), and in some regions, at the ice surface (Garrison and Buck, 1989). Grossmann (1994) found pack ice in the Weddell Sea to constitute regions of substantial heterotrophic potential, with both bacterial biomass and production exceeding significantly that of the water column. Similar regions were observed by Helmke and Weyland (1995). They found bacterial counts during their investigation ranged over four orders of magnitude, correlating with the ice type present at each station studied. 'Mainly frazil' and 'mixed frazil/ congelation' ice had the highest bacterial counts (~2 x 10^7 cells.ml^{-1}), whilst predominantly congelation ice had the lowest counts (4 x 10^3 cells.ml^{-1}).

The regions of highest production in pack ice develop in the surface layer near the snow-ice interface during summer. Here, photosynthetically available radiation (PAR) is at its maximum and the ice surface temperatures are much milder than the extremes occurring during the winter months. Garrison and Buck (1989) observed Chl*a* concentrations in such assemblages more than five times greater than the average for pack ice. These communities form when heavy snow cover or ice, piled up during pressure-ridge formation, causes the ice sheet to become depressed or bent

resulting in flooding by seawater, introducing nutrients and biological matter to the habitat. A layer of ice then forms at the snow-ice interface.

In fast ice regions (i.e. floes attached to the coast of Antarctica), congelation ice is the dominant form. In the upper ice layers brine channels are very small and are effectively isolated systems, closed off from the surface and from the water column. There is therefore no influx of fresh nutrients by processes such as convective flushing (Clarke and Ackley, 1984; Sullivan and Palmisano, 1984; Grossmann and Dieckmann, 1994). Watanabe and Satoh (1987) observed algal communities in fast ice near Syowa Station during winter. Initial ice formation lead to the development of a microbial community in a number of regions of the ice/ water interface. Upon incorporation into a consolidated ice layer, standing crops of Chl*a* greatly decreased in all station studied. In one station Chl*a* levels in a five centimetre layer at the bottom of a floe were 40 $mg.m^{-3}$. When this layer was internalised by consolidated ice growth, levels dropped to 2.55 $mg.m^{-3}$ in five days and to 0.66 $mg.m^{-3}$ after 15 days. During periods such as this, when algal biomass is breaking down and photosynthetic activity is limited, there is likely to be an increase in DOC availability and a corresponding increase in secondary production by the bacterial component of the sea ice community. This production might possibly achieve levels greater than the initial primary production. Such a situation was observed by Grossmann and Dieckmann (1994) in thick pack ice of the Weddell Sea where congelation ice was predominant, resulting in a negative carbon balance for a short period. Without a source of nutrient input these communities recede quickly into unproductive layers of the ice matrix. The continued downward growth of ice is mirrored by a downward shift in chlorophyll maxima, as new productive bottom layers develop, whilst older layers are internalised, resulting ultimately in a dispersed interior assemblage of low productivity and a thin bottom assemblage characterised by high productivity. At the ice/ water interface the temperature is fairly stable at around -1.9°C and does not drop to the severe levels experienced nearer the ice/ air interface (Vincent, 1988) and, given the thin snow cover occurring over fast ice, irradiance levels are sufficient for photosynthesis (Ackley and Sullivan, 1994). There is plenty of environmentally available space in the numerous brine channels and interstitial spaces of the platelet layer, and fresh nutrients are regularly supplied by the convective flushing of seawater. The development of the sea ice and associated microbial communities of

McMurdo Sound appear consistent with this scenario. Sullivan and Palmisano (1981, 1984) found a sharp gradient in the distribution and production of biological matter within the ice matrix. The lower 20 cm of the sea ice contained 99% of the chlorophyll content of the ice, 47% of bacterial numbers and 93% of bacterial biomass. These bacterial cells were morphologically diverse, appeared as dividing cells and in long chains, indicating a physiologically active population, and were at least 10 times larger than those found in the upper ice layers, where bacterial concentrations were an order of magnitude lower, cells were small, usually singular, and were similar to bacterioplankton found in the water column which is similarly distrophic.

1.4 Pathways for Nutrient Replenishment in SIMCO's

It is apparent that the mostly highly productive environments in the sea ice cover are those where replenishment of nutrients from surrounding seawater is sufficient to maintain nutrients at non-limiting concentrations. Where this occurs production can be sustained until self shading due to the dense concentration of algal pigments terminates the bloom (Gunther, *et al.* 1999). However, ice/water boundary stabilization caused by density stratification due to meltwater input, or ice dynamics may result in a physical barrier disconnecting the sea ice community from the surrounding sea water. Subsequently, ice algal demand may exceed rates of replenishment. When nutrient exchange has completely subsided algal growth relies on rates of *in situ* nutrient regeneration.

Azam, *et al.* (1983) described an ecological role for water column bacteria in the return of energy, released as EOC by phytoplankton, to the main food chain. Bacteria utilise EOC for growth and production, resulting in high bacterial biomass and the regeneration of nutrients required for phytoplankton growth. This high biomass also supports bacteriovorous protozoa, including microflagellates and ciliates, which in turn support higher trophic levels of the classic food chain. Such microbial loops have been reported to operate within both Antarctic fast ice (Sullivan and Palmisano, 1984) and pack ice (Miller, *et al.*, 1984; Kivi and Kuosa, 1994). Components of the microbial loop release CO_2, PO_4^{3-}, NH_4^+, SO_4^{2-}, Si, and other inorganic nutrients required by diatoms and algae (Bidle and Azam, 1999, Chróst, 1992). Consequently

the microbial loop may be an important contributor to the steady supply of phytoplankton nutrients. Although the exclusion of bacteriovores from brine channels due to size restriction has been cited as a possible reason for enlarged bacterial cell size in sea ice (e.g. Grossi, *et al.* 1984), a number of reports have described significant numbers of protozoans from sea ice microbial communities (Garrison, *et al.* 1984, Garrison and Buck, 1986, Dieckmann, *et al.* 1986, Kottmeier, *et al.* 1987, Kivi and Kuosa, 1994). Krembs, *et al.* (1999) reported that large predators may be excluded from brine channels depending on the architecture of the channel network, itself dependent on ice temperature. However, by conforming to the osmotic pressure of the brine, more elastic predators, such as turbellaria, are able to traverse structural impasses in the brine channel network.

Gunther, *et al.* (1990) have proposed that the bacterial regeneration of nutrients within Antarctic sea ice had only a limited impact. Evidence for this comes from the presence of extremely high phosphate levels in tested samples. These levels could not have been generated by heterotrophic (bacterial) production as this results in the concomitant release of carbon dioxide, and levels of dissolved inorganic carbon were significantly depleted. It was proposed that, in certain environments, 'liberation', rather than heterotrophic remineralization, is the major source of nutrient replenishment. This term refers to the process whereby algal lysis, due to cell mortality and inefficient feeding by metazoan grazers, results in the release of dissolved matter stored in the large biomass pool accumulated during periods when nutrient replenishment exceeded algal uptake. When the processes of convective flushing and 'liberation' are inactive then bacterial regeneration would assume a primary role, however this may not be sufficient to sustain a microbial community for extended periods. When it does not operate efficiently, phytoplankton are a self-limiting, nutrient sink and their populations crash when nutrients are exhausted (Pomeroy and Weibe, 1988). This would be likely to occur in fast ice regions where there is little wave action to fuel convective flushing and consolidated ice structures does impose physical limitations on the presence of grazers. McMinn, *et al.* (1999) reported severe and progressive nutrient limitation with distance from the ice/ water interface in fast ice from McMurdo Sound. Given the presence of well developed microbial communities in multi-year pack ice (e.g. Kottmeier and Sullivan, 1990) it appears that processes of nutrient regeneration are more reliable in this environment which is more influenced by oceanic dynamics.

1.5 Bacterial Interactions with the Sea Ice environment

Bacteria in sea ice may occur as free-living organisms, or as epiphytic bacteria, attached to the surfaces of living algal cells or detritus. Free living bacteria comprise approximately 70% of the total bacterial community whilst epibacteria make up the remainder (Sullivan and Palmisano, 1981; Sullivan and Palmisano, 1984, Grossi, *et al.* 1984). The different microenvironments occupied by these groups result in a diverse range of sea ice bacteria, with a variety of morphological and physiological states.

Significant increases in growth and metabolism of epibacteria over free-living bacteria, correlated to microalgal production, indicate a mutualistic association between epibacteria and algae in the sea ice microbial community. Evidence for this hypothesis has come from a number of researchers. Sullivan and Palmisano (1981) were among the first to recognise the complexity of the sea ice microbial community. They observed bacteria distributed throughout the ice zone but found by far the highest concentrations (6×10^9 cells/ L) in the bottom 20 cm. This region contained 10-1000 times more Chl*a* than the remainder of the sea ice. Here the bacteria were large, often paired or seen as dividing cells and often in chains of up to 30 cells. This indicated an actively growing and metabolising bacterial community. In contrast, in the upper region of the ice sheet where algal growth was negligible, the bacterial concentration was an order of magnitude less and the cells were small and single. The bacteria of the bottom community were observed to be in close association with living diatoms. Grossi, *et al.* (1984) reported a positive correlation between the growth of algae and that of bacteria in sea ice during a spring bloom in the McMurdo Sound. Using light perturbation experiments, during which snow cover over quadrats was varied in order to control light attenuation and hence PAR, they found that decreased light levels not only reduced the rates of algal growth but also significantly reduced the bacterial growth rate, indicating a potential coupling between the two. Furthermore they found that in regions where light was not limited, epiphytes increased at a rate twice that of non-epiphytes (0.08 day^{-1} as compared to 0.04 day^{-1}), doubling in numbers per algal cell even during the most rapid phase of algal growth. The epiphytes were also observed to be significantly larger than the free-

living bacteria. Further evidence stems from the finding of significant positive correlation's between Chl*a* and bacterial numbers in McMurdo Sound (Sullivan and Palmisano, 1984; Staley, *et al.* 1989) and diatom and bacterial numbers in the western Weddell Sea (Kivi and Kuosa, 1994). Kottmeier, *et al.* (1987) studied the dominant bottom community of congelation and platelet ice in McMurdo Sound over the course of a two and a half month period including a spring microalgal bloom. Bacterial production lagged behind the onset of primary algal production by 10 days, after which it increased logarithmically, stimulated by the onset of the bloom. Secondary production paralleled the rate of primary production. Significant correlations were found between a number of parameters measured including bacterial production and growth and microalgal biomass, production and growth. These lines of evidence suggest that there may be a coupling of microalgal photosynthetic activity and the rate of bacterial production in Antarctic sea ice. It has been suggested (Grossi, *et al.* 1984) that this relationship falls within the parameters of the hypothesised phycosphere in aquatic ecosystems (Bell and Mitchell, 1972), whereby there exists a zone extending outward from algal cells in which bacterial growth is stimulated by the extracellular products of the alga.

1.5.1 Bacterial Assimilation of Extracellular Organic Carbon

Phytoplankton production is the ultimate source of organic matter for bacterial metabolism and growth in the marine environment (Williams and Yentsch, 1976; Kirchman, 1990). Extracellular material released from phytoplankton (Hellebust, 1965) exists in two states; dissolved (DOC) and particulate (POC) organic carbon. These are often referred to collectively as extracellular organic carbon (EOC). DOC consists of simple compounds of low molecular weight, such as amino acids and sugars, which are easily assimilated by bacteria. POC consists of high molecular weight macromolecules such as proteins and polysaccharides. The secretion of extracellular enzymes mediates microbial transformations of POC. Bacterial growth rates may be limited by both the quantity and the quality of these materials (Kirchman, 1990). Sea ice microbial assemblages have temperature optima for the assimilation of dissolved organic substrates near ambient temperatures (Hodson, *et al.* 1981). In environments with high nutrient concentrations, such as in sea ice with

associated phytoplankton biomass, bacterial growth and production is stimulated and rapid turnover of DOC occurs, often leading to relatively high concentrations of POC within sea ice (Zdanowski and Donachie, 1993; Helmke and Weyland, 1995). Annual phytoplankton blooms in the Antarctic provide substrate for substantial bacterial production in sea ice throughout the year. At the height of such blooms, when phytoplankton production is maximal, bacterial production may be only a small percentage of overall production. However, in the advancing stages of algal blooms, when nutrients are limiting, there are high rates of DOM production (Gunther, *et al.* 1999) which, along with algal detritus, provides ample substrate to support bacterial production into the winter months, when primary production is at a minimum. During these periods, primary and secondary production may become uncoupled (Kottmeier and Sullivan, 1987), with bacterial production becoming a more substantial percentage of overall production (Kottmeier, *et al.* 1987). High nutrient levels in sea ice comprise a wide range of organic materials, which require specific enzymes to effect full utilisation (Zdanowski and Donachie, 1993). Coupling of primary and secondary production may involve selection for bacteria capable of utilising certain biosynthetic pathways, or possessing certain enzymes. Grossi, *et al.* (1984) observed a change in the size and morphology of bacterial epiphytes during the course of a spring bloom in the Antarctic sea ice. These changes may be a result of microbial succession, or they may reflect adaptations to differing spectra of available carbon sources (Zdanowski and Donachie, 1993). Changes in the quantity and quality of EOC available to bacteria are likely over the course of an algal bloom. As algae age, the rate at which they release EOC increases, resulting in higher nutrient levels. The composition of algal exudate also alters with age. Qualitative and quantitative changes to the natural bacterial population occur in response to such alterations in substrate. As new compounds become available, either the appropriate transport system is induced, or there is an increase in the number of microheterotrophs that are capable of utilising that substrate. Thus the composition of microbial populations responds very rapidly to qualitative changes in nutrient availability (Griffiths, *et al.* 1982).

1.5.2 Nitrogen metabolism

Continued maintenance of photosynthetic machinery after nutrient exhaustion is accompanied by excretion of carbohydrates that have a high C:N ratio (Hellebust,

1965). Phytoplankton in the later stages of a bloom, senescent, and/ or nitrogen limited, incorporate a high percentage of carbon fixed from photosynthesis into a polysaccharide fraction (Palmisano and Sullivan, 1985). When phytoplankton excrete compounds of low mineral content, bacterial growth will require the concomitant uptake of dissolved phosphate and/ or nitrogen compounds for the synthesis of bacterial biomass (Bratbak and Thingstad, 1985). Bacterial nitrogen is supplied as either inorganic nitrogen, such as ammonia, or as dissolved organic nitrogen, generally in the form of amino acids. Tupas (1994) found that ammonia supplied 35-60% of bacterial nitrogen requirements in the northern Gerlach Strait region of the Antarctic Peninsula. The large surface area to volume ratio of bacteria makes them excellent competitors with phytoplankton for nutrient elements such as nitrogen and phosphate (Elser, *et al.* 1995). As ammonia is the principle nitrogen source utilised by many Antarctic phytoplankton species (Olson, 1980), it may appear that competition should lead to a deficiency of ammonia. However, bacteria not only use ammonia but also simultaneously produce it by remineralisation of amino acids, resulting in net ammonia production. Bacterial utilisation of amino acids is therefore vital to the cycling of nitrogen and consequently other dissolved substrates in the ice ecosystem (Tupas, *et al.* 1994).

1.5.3 Amino acid utilisation

The addition of amino acids has been found to stimulate the growth of Antarctic bacteria to a greater extent than addition of ammonium and glucose. This is contrary to findings in more temperate latitudes (Kirchmann, 1990). Christian and Karl (1995) showed that protease activity increased in importance with increasing latitude. In Antarctica, relative protease activity was greatest in summer, and dropped off in winter, whilst relative glucosidase activity showed an opposite trend. This indicates that glucose is more heavily utilised only when the supply of dissolved proteins and peptides produced by the spring summer bloom process is exhausted; a period corresponding to phytoplankton senescence and the release of polymeric substances with low C:N ratios. Such preferential utilisation of amino acid containing compounds suggests that Antarctic bacteria use extracellular amino acid precursors to synthesise cellular proteins, rather than amino acids derived from *de novo* synthesis.

1.6 Psychrophilic enzymes

Extracellular enzymes are inducible, catabolic enzymes which enable microorganisms to utilise POC (Chróst, 1992). Although the environmental conditions in Antarctic sea ice would, at first glance, appear to be unfavorable to the operation of these enzymes, this is not necessarily the case (Zdanowski and Donachie, 1993). The sea ice structure provides a more physically stable environment than the water column, reducing the chances that an enzyme and bacterium will be separated, and temperatures in the lower (more active) part of the sea ice are fairly constant (around -1.9°C) (Zdanowski and Donachie, 1993). The major class of ectoenzymes concerned in bacterial metabolism of EOC are hydrolases. These convert POC to DOC, permitting its transport across microbial cell membranes (Freidmann, 1993). A number of hydrolases have been reported from Antarctic isolates. Those of most importance include chitinases, uricases, keritinases and other proteases, lipases and cellulases (Freidmann, 1993). A number of more specific enzymes, such as β-galactosidase, are also prevalent in sea ice. This indicates the presence of a complex spectrum of available nutrients, which requires specific enzymes to effect full utilisation (Zdanowski and Donachie, 1993).

The extracellular enzymes of Antarctic sea ice bacteria show a reduced response to temperature as an adaptation to the cold environment (Christian and Karl, 1995, Helmke and Weyland, 1995). Although they do not display temperature optima at *in situ* temperatures, they have optima and maxima 10°- 20°C, or even 30°C lower than mesophilic bacteria. These enzymes are also generally thermolabile, some becoming completely inactive at temperatures of 45°C, whilst displaying some inactivation at temperatures as low as 25°C (Russell, 1998). The loss of activity of one or more key enzymes will determine the upper growth temperatures of psychrophilic organisms. In order to function at low temperatures, psychrophilic enzymes must balance the potentially conflicting requirements for structural stability and conformational flexibility. Until recently there has been a paucity of studies concerning the enzyme structure of cold adapted microorganisms. Indeed it was not until 1996 that the first crystallisation of an enzyme from a psychrophilic bacterium was available (Aghajari, *et al.* 1996). Until then theories of psychrophilic enzyme structure had mostly relied upon extrapolation of the trends seen in thermophilic and mesophilic bacteria. It was

believed that, as the requirements for flexibility at low temperatures were opposite the requirements for conformational stability at high temperature, the structural differences between the enzymes of psychrophiles and thermophiles would present mirror images of each other (Russell, 1998). This has proven not to be the case as comparative examination of a range of psychrophilic enzymes has highlighted a wide spectrum of molecular changes which are not necessarily the opposite of those in thermophilic proteins (Russell, 1998). The range of molecular adaptations within psychrophilic enzymes includes the presence of additional glycine residues, a low arginine/lysine ratio, more hydrophilic surfaces, the lack of several salt bridges and fewer aromatic interactions (Russell, 1998). Further analysis of psychrophilic enzyme structure will enable not only insights into the nature of psychrophily but also add to the knowledge of processes of evolution and biogeography. Comparative enzyme analysis of psychrophilic organisms from a variety of cold environments will determine whether or not these adaptations have occurred in isolation.

POM utilisation by bacteria in cold environments is not only controlled by temperature adaptations of the enzymes *per se*, but also by the adaptations of bacteria which allow the formation of enzymes at low temperature. The ribosomes of cold adapted bacteria are, in themselves, adapted to functioning at low temperatures. Studies of cell-free protein-synthesising systems by Krajewska and Szer (1967) have shown that ribosomes from psychrophilic bacteria have very low miscoding rates at low temperatures compared to those of mesophiles and thermopiles. Furthermore, the initiation of protein synthesis has been demonstrated to be more resilient to decreases in temperature in cold adapted bacteria than in mesophiles (Broeze, *et al.* 1978), suggesting a role for translation in cold adaptation.

1.7 Psychrophiles in Antarctic sea ice

There is a considerable difference in the distribution of psychrophiles and psychrotrophs between the water column and sea ice. The majority of bacteria isolated from the water column are psychrotrophic, whilst those from consolidated sea ice are predominantly psychrophilic (Delille, 1992, Helmke and Weyland, 1995). Bowman, *et al.* (1997a, 1997b) performed a survey on cultures isolated from Antarctic fast ice and underlying seawater. The seawater strains were generally

psychrotrophic whilst 45% of sea ice strains were psychrophilic, and psychrophilic bacterial diversity was significantly enriched in sea ice communities from algal rich platelet or bottom ice communities as opposed to samples containing low algal standing stocks. Further to this, Helmke and Weyland (1995) found that the psychrophilic bacteria cultured from consolidated sea ice comprised a very high percentage of total bacterial numbers, indicating they were a true representation of the *in situ* microbial assemblage. There appears therefore, to be selection occurring for psychrophiles in the consolidated sea ice habitat however, the exact mechanisms of this selection process are unknown. The presence of both psychrophiles and psychrotrophs in young sea ice indicate that temperature alone cannot be the decisive factor (Helmke and Weyland, 1995). Salinity also cannot be responsible as psychrophiles have a smaller range of salinity tolerance than psychrotrophs (Helmke and Weyland, 1995; J. Bowman, pers. comm.). Harder and Veldcamp (1971) observed that psychrophiles consistently outgrew psychrotrophs at low temperatures in the presence of high nutrients. Given that psychrophiles may predominate in plankton-rich water (Helmke and Weyland, 1995) and that the consolidated sea ice assemblages from which psychrophiles have been isolated are similarly nutrient rich, nutrient availability would appear to have some influence over the distribution of psychrophiles in sea ice. Other factors such as a physically stable environment are also likely to be important.

1.8 The Fate of SIMCO's

The majority of Antarctic sea ice is seasonal and consequently provides only transient habitation for developing microbial communities. Even during winter, oceanic heat flux causes the slow ablation of pack ice, resulting in constant exposure of interior communities to the peripheral surfaces of ice floes (Ackley and Sullivan, 1994). Here they may serve as an especially important nutrient source for grazers such as juvenile and adult krill (Kottmeier and Sullivan, 1987). Larval krill feeding on sea ice microbiota over winter in the Weddell Sea have been found (Daly, 1990) to maintain high growth rates compared to those inhabiting the relatively distrophic water column. Given the relatively low rates of primary production, the timing and extent of winter bacterial production may thus be a most important contribution to food web relationships.

Increasing irradiance levels during spring and summer result not only in high productivity within sea ice but also in ice melting. The sea ice microbial communities have a major effect on the integrity of the platelet ice layer. Melting of this region is mediated by darkly pigmented algae which absorb light energy (Sullivan, *et al.* 1983) and as a result this highly productive layer may rapidly disintegrate. Retention time for this layer is thus an important determinant in the overall level of sea ice production (Kottmeier and Sullivan, 1987).

Melting of sea ice results in the release of high levels of organic matter, as sea ice microbial communities are deposited at their highest concentration into the water column. Communities inhabiting sea ice are well adapted to the *in situ* conditions. Exposure to the water column environment will therefore result in significant changes in species abundance, as those better adapted to the new environmental conditions, such as higher irradiance and lower salinity, are favoured. Matsuda, *et al.* (1990) observed salinity stratification in the water column directly beneath summer sea ice caused by ice melting. A band of extreme low salinity extended 1 metre below the sea ice /water interface. Exposure to this band may severely compromise psychrophilic organisms which have a smaller range of salinity tolerance than psychrotrophs (Helmke and Weyland, 1995; J. Bowman, pers. comm) and may be one explanation for shifts in microbial populations between sea ice and sea water during ice melt (Garrison and Buck, 1986). However, Giesenhagen, *et al.* (1999) reported that, upon seeding of the water column with sympagic microbial communities, initial high activities were restricted to ice derived auto and heterotrophs, whilst addition of DOC stimulated the pelagic bacterial population. High metabolic rates were maintained by these sympagic organisms for several days, which may account for the observations of Garrison, *et al.* (1987) who found a high degree of similarity between sea ice and ice edge assemblages. It appears that in water columns stratified by ice melt, phytoplankton blooms may be initiated by the input of active sympagic microbial communities in the absence of grazers (Giesenhagen, *et al.* 1999). In the presence of grazers, bacterioplankon blooms occur without any associated phytoplankton accumulation.

1.9 Bacterial Diversity

Until the last five years, studies on sea ice bacteria had generally focused on community function and physiology. However elucidation of community composition is vital to understanding specific bacterial interactions in the region, especially in association with ice algae. It will also allow insight into the adaptations required for survival in such extreme habitats and highlight potential biotechnological applications for novel bacteria. The morphologically diverse range of bacteria colonising diatoms has been observed through scanning electron micrographs (Sullivan and Palmisano, 1984). Prosthecate, straight and branched filamentous forms dominate the epiphytes associated with algae, whilst cocci, short and long rods and fusiform bacteria are also abundant. Many epiphytic bacteria contain structural modifications of the cell wall or exopolysaccharides (EPS) which may facilitate attachment to diatoms. One chain forming bacterium was shown to have cell surface modifications at one pole to aid attachment, whilst an EPS layer of another has been shown embedded in the puncta of an *Entomoneis* cell wall, serving to anchor the bacterium to the host. This observed diversity has been examined in recent culture based studies (Bowman, *et al.* 1997a and 1997b, Gosink, *et al.* 1997). Cultured bacteria were associated with four major phylogenetic groups, the Proteobacteria, the *Cytophaga-Flexibacter-Bacteroides* group and the high and low G+C gram positive branch. Psychrophilic strains belonged to the genera *Colwellia*, *Shewanella*, *Marinobacter*, and *Planacoccus* along with novel phylogenetic lineages adjacent to *Colwellia* and *Alteromonas* and within the alpha Proteobacteria and the *Cytophaga-Flexibacter-Bacteroides* group. Psychrotrophic strains were found to be members of the genera *Pseudoalteromonas*, *Psychrobacter*, *Halomonas*, *Pseudomonas*, *Sphingomonas*, *Arthrobacter*, *Planococcus* and *Halobacillus*.

Highlighting the novel nature of these organisms, seven new genera have been described from bacteria inhabiting Arctic and Antarctic sea ice. These include *Gelidibacter* (Bowman, *et al.* 1997c), *Glaciecola* (Bowman, *et al.* 1998b), *Octadecabacter* (Gosink, *et al.* 1997), *Polaribacter* (Gosink, *et al.* 1998), *Polaromonas* (Irgens, *et al.* 1996), *Psychroflexus* (Bowman, *et al.* 1998c) and *Psychroserpens* (Bowman, *et al.* 1997c). Novel species isolated form the sea ice environment include *Colwellia demingiae*, *Colwellia hornerae*, *Colwellia rossensis*, *Colwellia psychrotropica* (Bowman, *et al.* 1998a), *Glaciecola punicea*, *Glaciecola*

pallidula (Bowman, *et al.* 1998b), *Pseudoalteromonas prydzensis* (Bowman 1998), *Planococcus Mcmeekinii* (Junge,. *et al.* 1998), *Psychroflexus torquis* (Bowman, *et al.* 1998c), *Polaribacter irgensii*, *Polaribacter franzmannii*, *Polaribacter filamentus* (Gosink, *et al.* 1998), *Polaromonas vacuolata* (Irgens, *et al.* 1996), *Shewanella gelidimarina*, *Shewanella frigidimarina* (Bowman, et al. 1997d), *Octadecabacter arcticus, Octadecabacter antarcticus* (Gosink, *et al.* 1997), *Psychroserpens burtonensis*, *Gelidibacter algens* (Bowman, *et al.* 1997c), *Psychrobacter glacincola* (Bowman, *et al.* 1997e) and *Celluliphaga algicola* (Bowman, 2000 in press).

In line with the view that only approximately 1% of environmental organisms are culturable (e.g. Pace, 1997), this study seeks to use molecular teqhniques to by-pass the bias of culture methods and to further analyse the commnity composition of polar sea ice ecosystems.

1.10 Biogeography of Sea Ice Bacteria.

Biogeography is defined as the study of the global distribution of species, living or extinct (Staley and Gosink, 1999). Such study seeks to elucidate the existence or otherwise of (potentially) endemic species within an ecosystem and is required if we are to fully understand aspects of biodiversity, ecophysiology and extinction. From a microbial perspective, the study of biogeography is particularly challenging. The concept of bacterial endemism is anathema to the prevailing hypothesis that free living bacteria are cosmopolitan in their distribution (Staley and Gosink, 1999). Detailed comparison of the community composition of analogous but geographically separate environments is required to reconcile these ideas. In the past, community comparisons were based on culture based studies, however, molecular techniques have now opened the way for more sensitive analyses. Recent reviews of the subject as it pertains to the microbiota of Antarctica have been written by Staley and Gosink (1999) and Vincent (2000). They suggest polar sea ice as an ideal habitat for examination of bacterial biogeography. This extreme environment provides selective pressure for certain types of well adapted bacteria such as psychrophiles. The cosmopolitan theory would require that the same species of psychrophiles be found within the sea ice of both poles. This would depend on whether there exist sufficient pathways for the continual exchange of psychrophilic microorganisms between the poles and if so whether such

organisms are capable of surviving transit within these pathways. A number of pathways for global traverse have been proposed, including animal vectors (such as the arctic terns and skuas), cold deep sea currents, aerial transport, and of course anthropomorphic activity. This last, although only occurring recently, may prove to be a defining factor e.g. the ballast water of ships has acted as a vector for the transport of many organisms around the globe.

Staley and Gosink (1999) examined three bacterial genera which were described from sea ice from both the Arctic and the Antarctic. These included the genera *Octadecabacter*, *Polaribacter*, and the as yet officially unnamed genus "*Iceobacter*", which appears synonomous with genus *Psychromonas*. Their preliminary examinations led them to declare that although members of these genera occur at both poles there was no bi-polar distribution of species. Evidence for this was based primarily on DNA-DNA hybridization experiments, along with some phenotypic data. Sequence analysis of 16S rDNA did not however produce such clear results. No evidence was found that the species from each pole were more closely related than to the species from the other pole, as would be expected if speciation were occurring. They interpreted this result as an indication that the 16S rDNA sequences are too highly conserved to allow for the assessment of endemic polar strains and species.

1.11 Conclusion

Until recently, Antarctic sea ice has been considered an inhospitable environment whose inhabitants contributed little to the ecology of the polar region. However, it is now accepted that microenvironments exist in sea ice suitable for microbial growth and production and which are utilised by a variety of organisms. Bacteria, via their close association with sea ice algae and through the dynamics of the microbial loop, are vital links within the biological cycles of Southern Ocean. A complete understanding of these cycles will therefore require the elucidation of the ecology of these bacterial communities. To this end, research thus far has been somewhat inadequate in certain areas. There is a paucity of information concerning the species composition of sea ice bacterial assemblages. This is a situation that must be remedied for it may provide insights not evident through other scientific approaches.

2. Molecular Phylogenetic Survey of Polar Sea Ice Microbial Communities

2.1 Introduction to Methods

It is only relatively recently that microbiologist have begun focusing their efforts on elucidating microbial processes in extreme environments. Developments in both the understanding of the tolerance levels of microorganisms and the technologies crucial to science support have resulted in the discovery of many novel bacteria inhabiting environments previously assumed uninhabitable. These include deep sea hydrothermal vents, where bacteria live in temperatures above 100°C, deep sea sediment regions where barometric pressures are extreme, and of course polar regions. This study examines the communities inhabiting sea ice, which is an annual feature forming at both poles

The study of microbial processes in an ecosystem is a multifaceted affair requiring attack from many angles and utilising a wide variety of techniques. Only with such an approach can we hope to unravel the complexities of even the simplest communities. With this in mind it is important to ensure each angle studied is not to the detriment of another, as only a thorough examination of any given area can be of use. One important aspect is the determination of community composition, the identification of organisms living in an environment. In the last few years some work has examined the community composition of Antarctic and arctic sea ice microbial communities via culture dependent methods (e.g. Bowman, *et al.* 1997a and 1997b, Helmke and Weyland, 1995, Gosink, *et al.* 1993). These studies identified a wide range of organisms inhabiting the sea ice environment, many of which have proved to be novel species. Characterisation of these organisms has shown a diverse array of metabolic strategies and highlighted the potential for many interesting biotechnological applications. There is a general view held that less than 1% of organisms in the environment can be cultivated by standard techniques (e.g. Pace, *et al.* 1997). This indicates that the organisms obtained in previous culture based studies of the sea ice environment may not be very representative of the actual community composition.

The advent of powerful tools in molecular biology has provided a means to circumvent the need for cultivation when studying community composition. The ability to extract DNA from an entire environmental sample then amplify and sequence a specific gene means that all organisms, cultivable or not, can be detected. This study employs such techniques in a bid to provide further insight into the inhabitants of the sea ice microbial assemblages. The gene involved in this study is that encoding for 16S rRNA. This gene has been widely used in other studies and forms the basis of the phylogenetic view of life described by Woese (1987) wherein all organisms reside in one of three kingdoms, the *Bacteria*, the *Archaea* and the *Eukarya*, as defined by comparison of their 16S rDNA to that of other organisms. The library of 16S rDNA sequences is extensive and growing rapidly as new techniques become standard practice in most laboratories. This is important as identification or at least classification of sequences requires comparison to previously characterised organisms. The comparative similarity between the nucleotide bases at corresponding positions can be expressed as evolutionary distance indicating the number of nucleotide substitutions or sequence divergence. Sequence similarity can then be converted to an estimate of evolutionary distance (Devereux, *et al.* 1993). As ribosomes are requisite to life there exist functional and structural homologues to the 16S rDNA in all cellular organisms, and mutations due to selection are rare. Critical to the establishment of phylogenetic relationships, it appears that there is no lateral transfer of this gene between organisms. The size of the gene and the presence of both highly conserved and highly divergent regions allow scope for broad phylogenetic analyses which are statistically significant. Also, highly conserved regions provide convenient sites for primer directed amplification and sequencing whilst lesser-conserved regions allow the targeting of specific phylogenetic lineages.

Hugenholtz *et al.* (1998a) defined a bacterial division (in phylogenetic terms) as a lineage consisting of two or more 16S rRNA sequences that are reproducibly monophyletic and unaffiliated with all other division-level relatedness groups that constitute the bacterial domain. Use of molecular techniques over the past decade has seen the Bacterial domain expanded from the 10 divisions suggested by Woese (1987) to 36 divisions (Dojka, *et al.* 1998, Hugenholtz, *et al.* 1998b). Several more potential divisions are represented by single environmental sequences. Thirteen of the recognised divisions do not contain cultivated representatives and are hence classified as "candidate divisions". Although phylogenetic classifications are a basis for a stable

framework for the construction of bacterial taxonomy, phenotypic, chemotaxonomic and genotypic characteristics are still essential in describing new taxa.

2.2 Materials and Methods

2.2.1 Sea Ice Sampling

Table 2.1 displays the sample collection dates, site coordinates and PCR primers used for creation of clone libraries.

Table 2.1: Sample Collection Details and Primer Designation

Sample	Date	Location		PCR Primers Used		
				Universal	Bacterial	Archaeal
1	5/10/'96	64° 51'S	109° 37'E	✔	✘	✔
4	14/10/'96	62° 55'S	92° 37'E	✔	✘	✔
7	17/10/'96	64° 18'S	74° 07'E	✔	✘	✔
8	23/10/'96	66° 21'S	76° 54'E	✔	✔	✔
9	25/10/'96	66° 21'S	76° 58'E	✔	✔	✔
10	28/10/'96	68° 35'S	77° 58'E	✔	✘	✔
McMurdo Sound		77 5°S	165°W	✔	✘	✔
Sample Arctic	14/6/'98	73°N	66°W	✔	✘	✔

Sea ice cores 1, 4, 7, 8, 9 and10 were collected at various locations in the Antarctic pack ice between Casey and Davis stations during the month of October on ANARE Voyage 2 of the Aurora Australis, 1996. Cores were obtained using a SIPRE corer with care taken not to disturb the lower platelet and algal assemblage if present. Cores were immediately transferred to sterile plastic bags in which they were melted the same day at 4°C in equivalent volumes of sterile seawater to avoid hypotonic shock of halophilic bacteria. Samples were then filtered onto 0.2μm pore filters (millipore) which were subsequently stored at -80°C until processed.

Samples 1-9 were taken from pack ice regions. Sample 1 was a clear core with very consolidated ice structure and no visible algae, indicating the presence of no or only a

poorly formed sea ice microbial community. Samples 4, 7, 8 and 9 all contained visible colouration in the bottom 10-20cm or the cores, with samples 8 and 9 containing extreme colouration. Sample 10 was taken from the fast ice of Prydz Bay, near Davis Station. This ice was very thick and highly consolidated, with remnants of productive microbial assemblages obvious throughout

The McMurdo Sound sample was collected by Andrew McMinn (University of Tasmania, Australia) from fast ice near McMurdo Station, Antarctica. The filter of this sample contained large quantities of algae.

The Arctic sample was collected from the Northern Water Polynya region of Baffin Bay, by Connie Lovejoy (University of Laval, Quebec, Canada) and appeared to contain a flourishing microbial community (Connie Lovejoy, pers. comm.).

2.2.2 DNA Extraction and Purification

Extraction of DNA from the filtered samples followed a modification of the method of Furhman *et al.* (1988). Frozen filters were thawed, cut into small strips with a clean, sterile razorblade, suspended in 2ml STE buffer (10mM Tris HCl (pH 8); 1mM EDTA; 100mM NaCl) in a conical bottom 50ml polypropylene centrifuge tube and vortexed to remove biological material. To lyse cells, 0.1 volume of 10% sodium dodecyl sulphate (SDS) was added and the tubes placed in a boiling water bath for 2 min. The liquid was poured into a clean 15ml centrifuge tube and the filters washed with an additional 1ml of STE buffer which was added to the 15ml tube. Cellular debris was pelleted by centrifugation (10 min at 10,000 x g) (5417c, Eppendorf, Hamburg, Germany) at room temperature. The supernatant was removed to a new 15ml tube. To precipitate DNA, 0.7ml 10.5M ammonium acetate plus 7ml 95-100% ethanol was added and the tubes placed at -20°C for at least 2 hrs. DNA was pelleted by centrifugation (30min at 10,000 x g) in a swinging bucket rotor (Sorvall Super T 21, DuPont). The supernatant fluid was poured off, the DNA pellet air dried, resuspended in 0.5ml TE buffer (10mM Tris HCl (pH 8); 1mM EDTA) and transferred to a 1.5ml centrifuge tube. Phenol (equilibrated to TE (pH 8)) was added to a volume of 1ml, the sample mixed by gentle inversion and then centrifuged in a microcentrifuge (2min at 15,000 x g) at room temperature. The lower organic phase was removed 0.1µl at a time and the remaining interface and aqueous phase re-extracted as described above, but with 0.6ml phenol: chloroform (3:1) and then finally

with 0.5ml chloroform and the interface removed. The DNA was precipitated with 0.12ml 10.5M ammonium acetate plus 1ml of ice-cold 95-100% ethanol for at least 1hr at -20°C and pelleted (10 min., 15,000 x g) at 4°C. The supernatant was poured off and the pellet air dried then resuspended in 0.3ml H_20 at 37°C for 2 hrs or overnight at room temperature.

2.2.3 Agarose Gel Electrophoresis

For examination, nucleic acid materials were fractionated by electrophoresis through 0.8% - 2% (w/v) agarose gel with ethidium bromide (0.5 µg/ml) in Tris-acetate EDTA buffer (40 mM Tris-acetate; 1mM disodium EDTA; pH 8) (TAE), in a minigel apparatus (Horizon 58, Horizontal Gel Electrophoresis, BRL, Gaithersbug, MD, USA). Samples were mixed with 6x gel loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol FF; 40% sucrose) in a ratio of 5:1 and loaded into the gel. Electrophoresis was carried out using Power Pack 300 power supply (Bio-Rad, Richmond, CA, U.S.A.). The DNA/EtBr complex was visualised under short wavelength ultra-violet radiation on an electronic ultraviolet light transilluminator (Ultra. Lum. Inc, Carson, CA U.S.A.).

2.2.4 Determination of DNA Concentration

The absorbance of DNA solutions at 260 nm (A_{260}) was measured using a spectrophotometer (GeneQuant RNA/DNA Calculator, Pharmacia Biotech, Cambridge, U.K.). DNA concentrations were determined by use of the equation:
[DNA] mg/ml = (A_{260} x 50 mg/ml x D) / 1000 µg/mg; where D = dilution factor (Dobson 1993).

2.2.5 Polymerase Chain Reaction

The 16S rRNA genes from environmental genomic DNA samples were amplified using the polymerase chain reaction (PCR) (Saiki, *et al.* 1988). Details of the oligonucleotide PCR primers used are given in Figure 1. Universally-conserved primers REB519(f)/REN1513(r) (Lane, 1991; modified) and *Archaea*-specific primers Arch-Bst 21(f)/Arch-Not1 958(r) (DeLong, 1992; modified) were used in PCR

reactions for all samples. In addition, *Bacteria*-specific primers A-Bst 8(f) and 1492-Not1(r) (Lane, 1991; modified) were used in PCR reactions for samples 8 and 9. The PCR reactions were performed in a thermosequencer model FTS-960 (Corbett Research, Mortlake, N.S.W., Australia). The reaction mixure contained 1.25 mM dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, reaction buffer (20 Mm $(NH_4)_2SO_4$; 75 mM Tris-HCl pH 9.0; 0.01 % (w/v) Tween), 0.75ng Primer (F), 0.75ng Primer (R), 5% (w/v) dimethylsulfoxide, 200-500ng genomic DNA and 2.5U of thermostable DNA polymerase (Advanced Biotechnologies, Epsom, Surrey, U.K.). Reaction parameters included an initial 3 min incubation at 94°C followed by 24 cycles of 94°C for 1 min, 45°C for 2 min and 72°C for 6 min. The final extension step (72°C) was extended to 12 min to allow full extension of any partly amplified DNA fractions.

2.2.6 Construction of Clone Library

PCR products were purified using QIAquick-spin PCR purification columns (Qiagen, Chatsworth, CA, U.S.A.) following the manufacturers instructions. Purified PCR product and pGEM-5Zf(+) vector (Promega, Madison, WI., U.S.A.) were prepared separately for cloning in the following way : 20µl reactions were set up containing 2µg of PCR product or vector, 10U BSA (New England Biolabs, Beverly, MA, U.S.A.) and 10U of Not1 (New England Biolabs) using the recommended buffer (NEB3). Reactions were incubated at 37°C for 2 hrs, following which 10U BstX1 (New England Biolabs) was added and the solution incubated at 55°C for 2 hrs. DNA was then purified using the Prep-a-Gene kit (Bio-Rad, Richmond, CA, U.S.A.) following the manufacturers instructions. Utilising an insert : vector molar ratio of 3:1, the ligation mixture contained 50 ng cut, purified PCR product, 50 ng cut, purified vector and 1U T4 DNA ligase (Boehringer Mannheim, Mannheim, Germany) in the recommended buffer conditions. Reactants were incubated at 16°C for 2hours. Epicurian Blue Ultracompetent *Escherichia coli* cells (Stratagene, La Jolla, CA, U.S.A.) were transformed following the manufacturers instructions. An 100µl aliquot of cells was thawed on ice in a pre-chilled 1.5ml microcentrifuge tube and 1.7µl of β-mercaptoethanol (Stratagene) was added giving a concentration of 25mM. The tube was incubated on ice for 10 min and the contents mixed gently every 2 min after which 50ng of DNA from the ligation reaction was added. After a further 30 min incubation on ice the solution was heat shocked for 30 sec in a 42°C water bath then

returned to ice for a further 2 min. Luria Broth (1% NaCl, 1% tryptone, 0.5% yeast extract) was preheated to 42°C and 0.9mls added to the solution and the tubes incubated at 37°C for 1 hr with shaking at 225-250 rpm. Aliquots (20µl) of the transformation mixture were plated onto Luria Broth agar plates (1% NaCl, 1% tryptone, 0.5% yeast extract, 2% bacto-agar) containing the antibiotic ampicillin (50µg l^{-1}) and coated with 100µl IPTG (iso-propyl-beta-D-thio-galactopyranoside) (120mg ml^{-1}) and 20µl X-gal (5-bromo-4-chloro-3-indoyl-beta-D-thio-galactopyranoside) (50 mg ml^{-1}), to facilitate blue/white colour screening of colonies, and incubated overnight at 37°C.

Cloning of 16S rRNA genes utilised blue/ white colour screening for initial detection of recombinant plasmids. The Epicurian Ultracompetent *E. coli* cells carry a deletion of the chromosomal *lac* Z genes. This gene, encoding for β galactosidase production, is carried on the pGEM –5Zf+ plasmid along with suitable RNA polymerase promoter sites. Expression is inducible in the presence of IPTG. A multiple cloning region is contained within the α peptide coding region of the *lac* Z gene. Expression of the gene results in cleavage of X-Gal and the resulting halogenated indole stains the cells blue. Colonies containing recombinant plasmids, however, appear white due to the insertional inactivation of the *lac* Z gene. A fairly large proportion of white colonies turned out to be "false positives". This may have resulted from inactivation of the X-Gal or IPTG when many blue colonies were present, or from incomplete ligation. The Lac Z gene may have been disrupted, but the 16S rRNA gene fragment may not have been successfully inserted or excess primers were inserted instead. This initially resulted in a large amount of time and money being spent extracting non recombinant plasmid DNA from white colonies. Consequently a limit of about 100 recombinant plasmids per sample were examined. White colonies were subcultured onto fresh LB/ ampicillin plates and incubated overnight at 37°C. The percentage of white colonies containing recombinant plasmids was often very low so an abbreviated plasmid extraction protocol was employed for confirmation of recombination. Cell cultures were harvested from agarose plates and suspended in 100µl resuspension solution (50mM glucose; 25mM Tris HCl (pH 8); 10mM EDTA) in a 1.5ml microcentrifuge tube, 100µl phenol:chloroform added and the sample vigorously vortexed then centrifuged for 1 min (10,000 x g at room temperature). The upper aqueous layer containing plasmid DNA was examined by agarose gel electrophoresis. Samples

containing recombinant plasmid DNA were identified by correlation of their position on the gel with a plasmid known to contain the correct size insert.

Following confirmation of recombination, plasmid DNA was extracted from samples in preparation for sequencing. Cell cultures were harvested from agarose plates and suspended in 100µl resuspension solution in a 1.5ml microcentrifuge tube. Cell lysis solution (0.2M NaOH; 1% SDS) was added to a volume of 300µl and the sample inverted until clear, at which point 150µl neutralisation solution (3M Potassium Acetate; 2M Acetic Acid pH 4.8) was added. The tube was centrifuged in a microcentrifuge for 5 min (10,000 x g at room temperature) and the supernatant transferred to a clean 1.5ml microcentrifuge tube. An equivalent volume of phenol was added, the tube vortexed and then centrifuged for 5min (7,500 x g at room temperature). The upper aqueous layer was transferred to a clean 1.5ml microcentrifuge tube and 1ml ice cold 95-100% ethanol and 40µl ammonium acetate added. The tube was incubated at -20°C for at least 2 hrs to precipitate the DNA. DNA was pelleted by centrifugation (10,000 x g, 4°C) for 30 min. and the pellet dried in a vacuum centrifuge (DNA mini, Heto) (5 min.) before resuspension in 30µl H_20. Samples were quantified and stored at -20°C.

2.2.7 16S rRNA Gene Sequencing

A number of sequencing protocols were used over the duration of the study, the most effective being the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA., U.S.A.). Between 0.3 – 0.5 µg of plasmid DNA was added to 4µl Terminator Ready Reaction Mix and 3.2 pmol primer M13R and the reaction volume made up to 20ml with deionised water. Reactions were performed in an FTS-960 model thermosequencer (Corbett Research). Denaturation of DNA at 96°C for 30 s. was followed by primer –template annealing at 50°C for 15 s. then extension at 60°C for 4 min. This cycle was repeated 24 times after which the products were held at 4°C until purification. For purification, reaction products were added to a 1.5ml microcentrifuge tube containing 2µl 3M sodium acetate (pH 4.6) and 50µl 95% ethanol. The tubes were vortexed to mix the contents and incubated at room temperature for 15 min (incubation at room temperature was sufficient to precipitate extension products but stopped excessive precipitation of dyes

which may have interfered with the sequencing gel). Tubes were centrifuged in a microcentrifuge for 30 mins. (14,000 x g at room temperature) and the supernatant aspirated with a pipette and discarded. The remaining pellet was rinsed with 250 µl 70% ethanol and the tubes centrifuged in a microcentrifuge for 10 mins (14,000 x g at room temperature). The supernatant was aspirated with a pipette, the sample spun again briefly and any remaining ethanol removed. The pellet was dried in a vacuum centrifuge for 10 mins.

Samples were analysed on an AB377 automated sequencer (CSIRO-Marine Research Division, Castray Esplanade, Hobart, Tasmania).

2.2.8 Phylogenetic Analysis

Sequence electrophoretograms were examined using the program Sequence Navigator (PE Applied Biosystems) in order to resolve ambiguous base positions. The CHECK-CHIMERA program (Maidak, *et al.* 1999) was used to check for the existence of PCR amplified hybrid sequences. Sequences were submitted to the BLAST (baArctic lignment search tool) and GAPPED BLAST on-line services (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) (Altschul, *et al.* 1997) for comparison to the National Center for Biotechnology Information database (Genbank) and to the Ribosomal RNA Database Project II (RDP) database (http://www.cme.msu.edu/RDP/html/index.html) (Maidak, *et al.* 1999). Sequences were then manually aligned against all relevant downloaded sequences. Genbank accession numbers for reference strains are cited in Appendix 1. Phylogenetic analysis was done using the PHYLIP (version 3.572c) (Felsenstein, 1993) suite of programs. Evolutionary distances were determined using the Maximum likelihood algorithm in the program DNADIST and phylogenetic trees constructed using the neighbor-joining method in the program NEIGHBOR. The robustness of tree topology was estimated through bootstrapping analysis using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE, with 500 replicate analyses performed for each dataset.

Figure 2.1: PCR Primer Sequences

Universally-conserved primers (Lane, 1991; modified)

(a) Primer REB519(f): M=A/C (equimolar amounts)

GC Clamp BSTX1 Restriction site

5' ACg | CCC | AAC | gCg | TTg | gCA | gCM | gCC | gCg | gTA | ATA | C 3'

(b) Primer REN1513(r): R=A/g (equimolar amounts)

GC Clamp Not 1 Restriction site

5' ACg | CgC | gCg | gCC | gCT | ACg | gRT | ACC | TTg | TTA | CgA | CTT 3'

Bacteria-specific primers (Lane, 1991; modified)

(c) Primer A-Bst 8(f):

GC Clamp BSTX1 Restriction site

5' ACg | CCC | AAC | gCg | TTg | gAg | Agt | TTg | ATC | CTg | gCT | gAC 3'

(d) Primer 1492-Not-1(r): Y=C:T (equimolar amounts)

GC Clamp Not 1 Restriction site

5' ACg | CgC | gCg | gCC | gCT | ACg | gYT | ACC | TTg | TTA | CgA | CTT 3'

Archea-specific primers (DeLong, 1992; modified)

(e) Primer Arch-Bst 21(f): Y=C:T (equimolar amounts)

GC Clamp BSTX1 Restriction site

5' ACg | CCC | AAC | gCg | TTg | gTT | CCg | gTT | gAT | CCY | gCC | ggA 3'

(f) Primer Arch-Not1 958(r): Y=C:T, M=A/C (equimolar amounts)

GC Clamp Not 1 Restriction site

5' ACg | CgC | gCg | gCC | gCY | CCg | gCg | TTg | AMT | CCA | ATT 3'

2.2.9 Calculation of Diversity indices

For calculations of diversity indices, the libraries were normalised to 50 clones using the rarefaction method (Simberloff, 1972) by utilising the program RAREFACT.FOR written by C. J. Krebs (University of British Columbia) and which is available through the internet at http://gause.biology.ualberta.ca/jbrzusto/rarefact.html.

Estimates of Diversity (*H'*) were determined using the Shannon-Weaver (or Shannon Wiener) Index (Krebs 1989). This index measures the average degree of uncertainty (synonymous with diversity) of predicting the species (or phylotype) of a given individual picked at random from a community The model takes the form:

$$H' = \frac{n \log n - \sum_{i=1}^{k} N \log N}{n}$$

where k is the total number of unique phylotypes, n is total number of clones and N is the number of observations of each phylotype (i).

Measures of dominance concentration were determined using the Simpson Index (SI'): (Krebs 1989). This index is based on the probability of drawing a pair of individuals of the same species from a sample. The model takes the form:

$$SI' = \sum_{i=1}^{k} \frac{Ni\ (Ni - 1)}{n\ (n - 1)}$$

Equitability indices (J') were based on the Shannon-Weaver index data. This index is concerned with the evenness with which individuals are divided among the species present in a sample. The model takes the form:

$$J' = \frac{H'}{H\max}$$

where Hmax is equal to log$k$.

Species richness (the estimate of the total number of species in an ecosystem based on the number of species present and the abundance of these species in a sample of given size) was extrapolated from the data by using the non-parametric model of Chao (1984), available at http://gause.biology.ualberta.ca/jbrzusto/rarefact.html.

Biodiversity coverage (C) (Mullins, *et al.* 1995) (the portion of a clone library of infinite size that would be sample by the smaller clone library obtained) was calculated using the equation:

$$C = 1 - (\frac{n_1}{N})$$

Where n_1 is the number of phylotypes containing only one clone, and N is the total number of clones.

Pairwise comparisons of clone libraries were carried out using the Similarity Coefficient (S) (Odum, 1971). The model takes the form:

$$S = \frac{2C}{A + B}$$

where A and B are the numbers of phylotypes in libraries A and B respectively, and C is the number of shared phylotypes.

2.3 Limitations and Evaluation of Methods.

2.3.1 Sea Ice Sampling

Assessing microbial diversity in an heterogenous environment such as sea ice is a difficult task especially when working within the rigorous confines of a sampling regime dictated by the requirements of a non-microbiological scientific survey (ship stops were ordered by a Crabeater seal survey team when they wished to capture a seal). Microbial diversity will change on crossing ecological boundaries on all physical and temporal scales. The approach to collection taken in this study was simply to take samples whenever physically possible and then examine what appeared to be the most diverse array of samples both in ice type and biological content. Obviously this is not an ideal sampling procedure. It encompasses only two months of the climatologically dynamic Antarctic year and has no set spatial structure, samples being taken somewhat randomly. However the different community levels as broadly determined by presence and development of biological communities can provide an insight into overall diversity when viewed in context of the cycles of sea ice formation and community development which have been studied over the past decade.

In order for the clone libraries to be representative of *in situ* communities it was vital that the composition of the community not change between the time of sampling and the time of processing, and that any losses during the DNA extraction process be non-selective. In this study samples were processed immediately upon collection. The addition of sterile seawater to samples reduced the osmotic shock on halophilic bacteria expected to be present based on results of culture based surveys. Melting took between 10 and 20 hours at 4°C. Samples were filtered on 0.2μm pore filters without pre-filtration, thus retaining all organisms above this size.

2.3.2 DNA Extraction and Purification

The DNA extraction method followed that of Fuhrman *et al.* (1988). To provide a true representation of community composition it is imperative that DNA be obtained from all cells in a sample and that any downstream losses of DNA be non-selective. Fuhrman *et al.* (1988) found the lysing procedure employed here had a >99% success rate for suspended marine bacteria in their study. Owing to the spatial and temporal

dislocation of sample filtering and DNA extraction, no direct observation of efficiency of cell lysis was performed in this study. Fuhrman *et al.* (1988) suggested that high DNA yield inferred efficient cell lysis. DNA yields were sufficiently high in this study to be confident that samples were representative of total microbial populations.

2.3.3 Polymerase Chain Reaction

The 16S rRNA genes from DNA extracts of the samples were amplified using the PCR. This is an extremely sensitive technique capable of exponentially replicating very small quantities of DNA. However the beauty of this exquisite tool is also its tyranny. The results of such a culture independent study are fraught with potential artifacts.

2.3.3.1 Contamination Effects

The introduction of foreign DNA to a sample at any stage during the procedure prior to PCR may mean that contaminants appear in the clone library and are mistaken for inhabitants of the study community. Tanner (1998) identified numerous sequences which commonly appeared in molecular examinations of a diverse array of environmental habitats and also in negative controls of PCR. He concluded these organisms were probably common contaminants in reagents used to prepare genomic DNA. Among these were many sequences with 97%-99% similarity to *Echerichia coli*. A small number of such sequences appeared in this study. These sequences were omitted from analysis

2.3.3.2 Chimeric Sequence Formation

Chimeric sequences occur when a DNA fragment of one gene anneals with another strand of template DNA rather than with an oligonucleotide primer. The next cycle of DNA synthesis then forms a hybrid gene containing segments of both templates. Wang and Wang (1996) found that up to 32% of the products of PCR amplification of simple DNA samples were chimeric molecules. Thus it is imperative that steps be taken to identify such chimeric sequences lest they be mistaken as indicators of novel organisms in the study environment. The software program CHECK_CHIMERA was utilised in this study, however such programs may not detect chimeras when the component sequences are derived from closely related organisms. This problem is

exacerbated by the potential heterogeneity of rRNA genes within a single genome. Cells generally contain multiple copies of the 16S rRNA gene. A number of studies (e.g. Mylvaganam *et al.* 1992, Leifting *et al.* 1996, Wang *et al.* 1997, Ueda *et al.* 1999) have identified sequence differences between rRNA operons, some of which may be as great as 6.4% (Wang, *et al.* 1997). Intercopy chimeric formation would result in extremely closely related sequences not detectable within a mixed DNA experiment but which may compromise the integrity of phylogenies and ultimately lead to misinterpretation of the extent of microbial diversity. Thus the definitive proof for the occurrence of an organism indicated by a cloned rDNA sequence requires explicit identification of that organism *in situ*.

2.3.3.3 PCR Bias

Reysenbach *et al.* (1992) illustrated the potential for differential amplification of rRNA genes from mixed populations. This results from an increased efficiency of replication by DNA with specific properties such as low G+C content (templates with a low G+C content dissociate into single stranded molecules with a greater efficiency than high G+C templates) or an enhanced primer binding efficiency. Other biases may occur when a particular sequence reaches concentrations where template reannealing inhibits primer binding (Suzuki and Giovannoni, 1996), allowing less abundant sequences to be preferentially amplified. Biased amplification can also lead to an increase in the frequency of chimeric sequences (Wang, *et al.* 1997). The implications are that the abundance of a particular product in an amplified sample may be a distorted representation of its actual abundance in the original sample. Similarly, given that there exist multiple copy numbers of 16S rRNA genes in a single cell, it is important to realise that the molecular phylogenetic analysis of environmental clone libraries is reflective of the number of 16S rRNA gene copies present in the sample and not directly of the numbers of different kinds of organisms (Gray and Herwig, 1996). This said however, several studies have found encouragingly good correlations between PCR-based clone libraries and other quantitative methods (Dojka, *et al.* 1998).

The effects of these PCR artifacts are heavily dependent on the number of cycles in a reaction. To alleviate these as much as possible reaction conditions were optimised, the number of cycles in each reaction was limited to 28 and multiple reactions were carried out for each sample.

2.3.4 Phylogenetic analysis

Notions of diversity come from the ability to tell things apart. In phylogenetic analysis it is imperative that sequence alignments be completely accurate, as mismatched base pairs will be construed as "different" and measures of diversity will be erroneously inflated. To this end all alignments in this study were performed manually.

3. Results and Discussion

3.1 Overerview of Phylogenetic Analysis

16S rDNA clone libraries were constructed from DNA extracted from seven Antarctic sea ice cores and one Arctic sea ice core. Samples were chosen to cover a range of sea ice types and apparent community complexities (as gauged from visible organic growth) within the boundaries of the sampling regime. Libraries from all samples were constructed with universal primers and comparative libraries using bacterial primers were constructed for samples 8 and 9. Approximately 50-80 clones per library were screened by analysis of RFLP patterns and selected representatives were then sequenced. Sequences greater than 400 base pairs were included in phylogenetic analysis. Groups of two or more highly related sequences ($\geq$ 98% identical) were considered to belong to the same sequence type. A total of 539 putatively nonchimeric clones were obtained. Of these, 386 were affiliated with the domain *Bacteria* and 152 with the domain *Eukaryota*. One clone, sic 10310 had a sequence which was not comparable to any sequences in the GENBANK or RDP II databases and hence was not used in further analysis. Table 3.1 provides a summary of the representative sequences and their phylogenetic affiliations. The majority of bacterial clones fell into five major phylogenetic groups: the Proteobacteria, the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, the high and low G+C gram positive bacteria and the *Chlamydia/ Verrucomicrobia* group. The eukaryote clones belonged to a range of sympagic organisms including autotrophic and heterotrophic nanoplankton, amoebae and Acoel Turbellarian flatworms.

Table 3.1: Summary of Phylotype Abundance and Phylogenetic Affiliations

Phylotype[a]	Number of Clones From Sample[b]:								Closest Cultured Relative[c]	Dissimilarity
	1	4	7	8	9	10	McMurdo	Arctic		
									Cytophaga-Flexibacter-Bacteroides	
Sic 917					1				*Polaribacter* sp. IC066	0.0206
Sic 127	3								*Polaribacter irgensii*	0.0239
Sic B9029					8				*Polaribacter irgensii*	0.0047
McMurdo 20							8		*Polaribacter franzmannii*	0.0894
Sic 10332						1			*Polaribacter franzmannii*	0.0465
Sic 42406		2							*Polaribacter franzmannii*	0.0132
Sic 8119				4					*Gelidibacter algens*	0.0488
Sic 815				1					*Gelidibacter algens*	0.0734
Arctic 123								3	*Gelidibacter algens*	0.0453
Sic B8217				23					*Gelidibacter algens*	0.0642
Sic B9002					49				*Gelidibacter algens*	0.0814
McMurdo 124							1		*Gelidibacter algens*	0.0806
Sic 42372		1							*Psychroserpens burtonensis*	0.0309
Sic 8118				6					*Psychroserpens burtonensis*	0.0411
Arctic 121								6	*Psychroserpens burtonensis*	0.0373
Sic 834				1					*Celluliphaga uliginosa*	0.0389
Sic B8008				1					*Celluliphaga uliginosa*	0.0630
Sic B8233				1					*Celluliphaga uliginosa*	0.0416
Sic B8113				2					*Celluliphaga uliginosa*	0.0501
McMurdo 201							1		*Celluliphaga uliginosa*	0.0441
Sic 946					10				*Celluliphaga fucicola*	0.0716
Sic B8018				8					*Celluliphaga fucicola*	0.0730
Sic B8232				5					*Celluliphaga fucicola*	0.0662
Arctic 117								1	Sea ice strain IC076	0.0041
Sic 165	1								*Lewinella cohaerens*	0.0509
Arctic 161								1	*Lewinella persicus*	0.0873
Sic 10379							1		*Lewinella persicus*	0.0380
Arctic 156								1	*Flavobacterium xylanivorum*	0.0077
Arctic 16								2	*Cytophaga latercula*	0.0702
Sic B8012				1					Antarctic seawater strain SW17	0.1072
Sic B8236				9					Antarctic seawater strain SW17	0.0576
Sic 42370		3							Deep sea sediment strain BD2-2	0.1647

Continued following page

Phylotype[a]	Number of Clones From Sample[b]								Closest Cultured Relative[c]	Dissimilarity
	1	4	7	8	9	10	McMurdo	Arctic		
									gamma Proteobacteria	
Sic 114	32								*Shewanella frigidimarina*	0.0102
Sic 10368						15			*Shewanella frigidimarina*	0.0161
McMurdo 10							5		*Shewanella gelidimarina*	0.0041
Sic 10363						9			*Shewanella baltica*	0.0592
Sic 153	10								*Pseudoalteromonas nigrefaciens*	0.0132
Sic 71302			1						*Pseudoalteromonas atlantica*	0.0130
Sic 999					1				*Pseudoalteromonas tunicata*	0.0128
Sic 8123				1					*Pseudalteromonas gracilis*	0.0227
Sic 10345						4			*Pseudoalteromonas elyakovii*	0.0395
Sic 1059						1			*Pseudoalteromonas elyakovii*	0.0245
Sic 10409						4			*Pseudoalteromonas elyakovii*	0.0271
Sic 42105		1							*Pseudoalteromonas elyakovii*	0.0284
Sic129	3								*Marinomonas protea*	0.0324
Sic163	1								*Marinomonas protea*	0.0432
Sic 8125				5					Sea ice strain IC169	0.0188
Sic 10369						1			Sea ice strain IC169	0.0686
Sic 42384		2							Sea ice strain IC169	0.0046
Sic 42402		1							Candidatus *Endobugula sertula*	0.0834
Sic 9092					5				*Neptunomonas napthavorans*	0.0393
Sic 8124				5					*Colwellia hornerae*	0.0323
Sic 42396		2							*Clowellia hornerae*	0.0071
McMurdo 541							1		*Glaciecola punicea*	0.0000
Sic 7265			1						*Citrobacter brakii*	0.0453
Sic 9311					1				Sea ice strain IC038	0.0101
Sic 7153			1						Sea ice strain IC038	0.0112
Sic 10359						1			*Psychrobacter* sp. QSSC5-4	0.0053
Sic 42393		1							*Psychrobacter* sp. QSSC5-4	0.0108
Sic 10360						11			*Psychrobacter glacincola*	0.0056
Sic 42334		1							*Halomonas aquamarina*	0.0039
Sic B9065					19				*Pseudomonas gessardii*	0.0035
Sic 711			4						*Pseudomonas pseudoalcaligenes*	0.0081
Arctic 234								1	*Microbulbifer hydrolyticus*	0.0515
McMurdo 211							1		*Psychromonas antarcticus*	0.0235

Continued following page

Phylotype[a]	Number of Clones From Sample[b]								Closest Cultured Relative[c]	Dissimilarity
	1	4	7	8	9	10	McMurdo	Arctic		
									alpha Proteobacteria	
Sic 42338		1							*Sulfitobacter* sp. EE-36	0.0153
Sic 10342						1			*Sulfitobacter* sp. EE-36	0.0400
Sic 8141				4					*Sulfitobacter pontiacus*	0.0103
Sic 174	1								*Ruegeria atlantica*	0.0182
Arctic 235								8	*Octadecabacter antarcticus*	0.0170
Arctic 128								6	*Octadecabacter antarcticus*	0.0165
Arctic 153								14	*Octadecabacter arcticus*	0.0082
Sic 8142				1					*Octadecabacter arcticus*	0.0886
Sic 8163				2					*Roseobacter* sp. *shippagan*	0.0207
Sic 42383		1							*Orientia tsutsugamushi*	0.4345
Sic 926					1				*Orientia tsutsugamushi*	0.4353
Arctic 437								1	*Orientia tsutsugamushi*	0.4695
Sic 42340		2							*Orientia tsutsugamushi*	0.4723
Sic 8120				1					*Orientia tsutsugamushi*	0.4638
									Firmicutes	
Sic 7252			4						*Rubrobacter radiotolerans*	0.0721
Sic 950					9				*Rubrobacter radiotolerans*	0.0688
Sic 42381		6							*Bacillus marismortui*	0.0194
									Chlamydia/ Verrucomicrobia	
Sic 812				1					*Prosthecobacter* sp. FC3	0.1365
Sic 816				1					*Prosthecobacter fusiformis*	0.1358
Sic 10401						1			*Verrucomicrobium spinosum*	0.1565
Arctic 240								1	*Parachlamydia acanthoamoebae*	0.1312
									Unaffiliated clones	
Sic 7255			1						*Rubrobacter xylanophilus*	0.2274
									Autotrophic plankton	
Sic 42345		1							*Phaeocystis antarctica*	0.0380
Sic 928					18				*Odontella sinensis* plastid	0.0373
Sic 103602						7			*Odontella sinensis* plastid	0.0108
Sic 8113				11					*Odontella sinensis* plastid	0.0229
Sic 813				1					*Odontella sinensis* plastid	0.0219
Sic 42333		17							*Odontella sinensis* plastid	0.0223
Sic 42330		1							*Emiliania huxleyi* plastid	0.0738
Arctic 149								7	*Odontella sinensis* Plastid	0.0200
McMurdo 54							33		*Skeletonema pseudocostatum* plastid	0.1430

Continued following page

Phylotype[a]	Number of Clones From Sample[b]								Closest Cultured Relative[c]	Dissimilarity
	1	4	7	8	9	10	McMurdo	Arctic		
									Heterotrophic plankton	
Sic 4276		7							*Pentapharsodinium tyrrhenicum*	0.0199
Sic 7235			38						*Cercomonas* ATTC50318	0.1304
									Acoel Turbellaria	
Sic 1028						2			*Atriofonta polyvacuola*	0.1173
Sic 8114				4					*Atriofonta polyvacuola*	0.1555
Sic 818				1					*Atriofonta polyvacuola*	0.1030
Sic 9019					4				*Atriofonta polyvacuola*	0.2035
Sic 42342		2							*Atriofonta polyvacuola*	0.1038

[a] A unique sequence or group of highly related sequences (>98% identical) from the same library considered to belong to the same sequence type

[b] Based on direct sequence comparisons or inferred from RFLP patterns.

[c] inferred from direct sequence comparison

3.2 The Archaea

No product was obtained in any PCR reactions using archaeal-specific primers. Delong *et al.* (1994) reported a high abundance of archaeal RNA in Antarctic surface seawater and under ice communities in late winter. However, with the onset of spring and increased daylengths, archaeal RNA levels dropped precipitously. They then rose again the next winter. Glockner *et al.* (1999) and Simon *et al.* (1999) failed to detect archaea in Antarctic Ocean surface water sampled during a *Phaeocystis* bloom. This bloom was associated with high nutrient levels and a corresponding increase of members of the *Cytophaga-Flexibacter-Bacteroides* group. It appears that with the onset of increased daylight hours and the associated increase in primary productivity, archaea fail to compete successfully with other microorganisms. Hence at the time of ice formation there would be little archaeal activity within the water column and they would not be incorporated into the sea ice matrix.

3.3 The Bacteria

3.3.1 *Cytophaga-Flexibacter-Bacteroides* (CFB) group

The highest proportion of phylotypes detected in this study grouped within the *Cytophaga-Flexibacter-Bacteroides* (CFB) group (30.2% of all clones). The majority of clones fell within the [*Flexibacter*] *maritimus* branch of the family *Flavobacteriaceae*. The affiliations of sea ice clones within the CFB are displayed in Figure 3.1

Two phylotypes were affiliated with genus *Lewinella* in the *Saprospira* group of the CFB. This genus contains three species of marine, gliding, chemoorganotrophs that were isolated from the coasts of France, Ireland and Nigeria (Sly, *et al.* 1998). The phylotypes obtained in this study are not closely related to the existing *Lewinella* species however they group with strong bootstrap support. One phylotype, represented by sic 165 grouped (86% bootstrap support) with *Lewinella cohaerens* at an evolutionary distance of 0.0509. The other two clones, represented by sic 10379 and Arctic 161, grouped (100% bootstrap support) with *Lewinella persicus* at a distances of 0.0873 and 0.0380 respectively. Although these distances are quite large, the distances between the 16S rDNA sequences of the existing *Lewinella* species are even greater at a range of 86-88% (Sly, *et al.* 1998). It is likely the organisms represented by these sea ice clones reflect an expansion of the *Lewinella* genus.

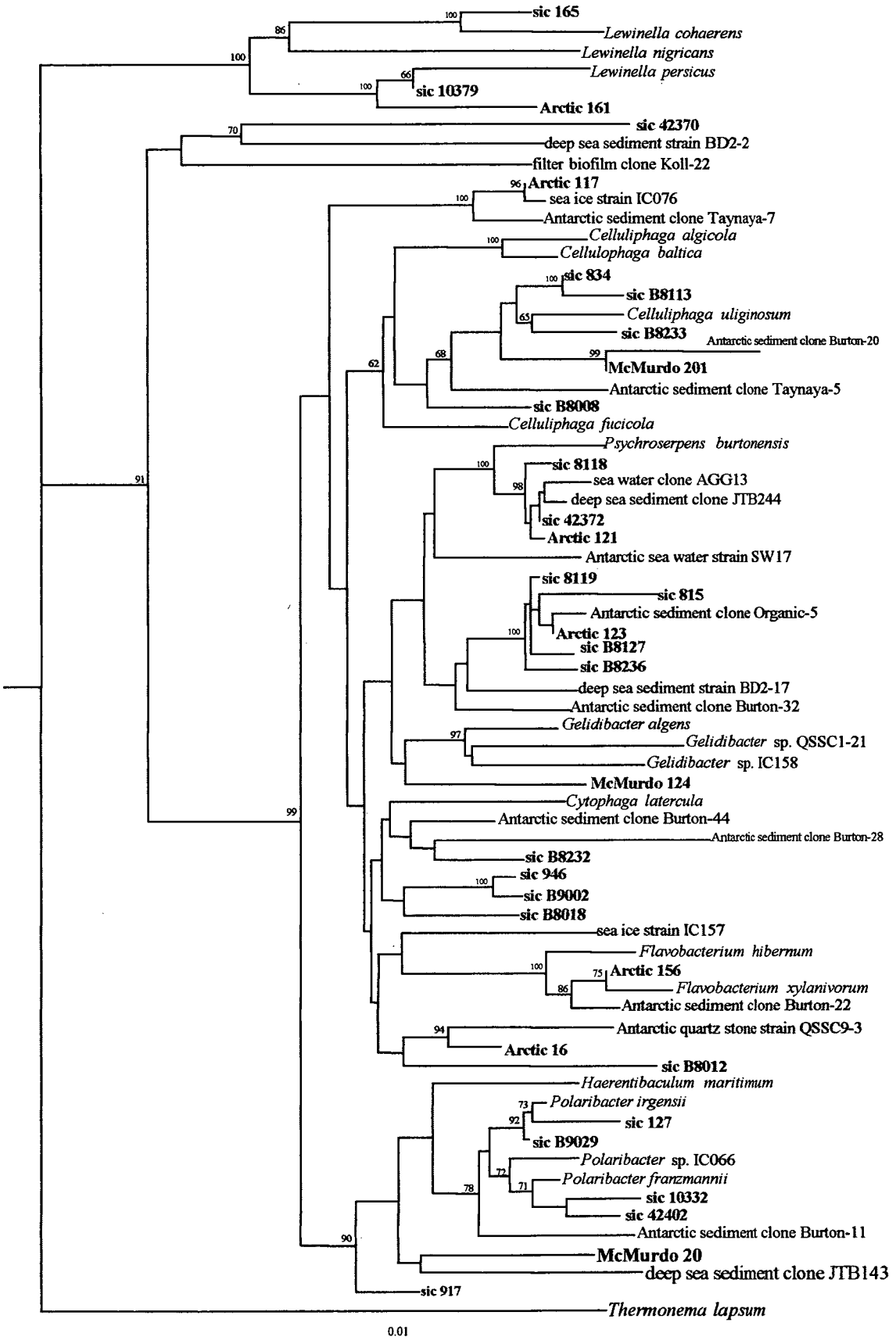


Figure 3.1: Evolutionary distance dendrogram created from 16S rDNA sequences of some members of the *Cytophaga-Flexibacter-Bacteroidies* Group and associated sea ice clones. *Thermonema lapsum* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

One phylotype, represented by Arctic 117, grouped (100% bootstrap support) with the Antarctic sea ice strain IC076, at an evolutionary distance of 0.0077. This strain is obligately psychrophilic, not growing at temperatures above 15°C and with an estimated optimum temperature of between 4-8°C (Bowman, *et al.* 1997b).

Five phylotypes, represented by sic clones 834, B8113, B8233 and B8008 and McMurdo 201 were affiliated with the newly described genus *Celluliphaga.* Interestingly, these phylotypes were more closely associated with *Celluliphaga uliginosa* at evolutionary distances of between 0.0389 – 0.0630 rather than *Celluliphaga algicola* which was isolated directly from Antarctic sea ice diatoms and macrophytes (Bowman, *et al.* 1997b). Two phylotypes were most closely related to clones detected from Antarctic sediment. The phylotype represented by sic B8008 was most closely related to Taynaya-5 whilst McMurdo 201 was most closely related to Burton-20. These Antarctic sediment clones originate from Taynaya Bay and Burton Lake respectively. Both regions are seasonal marine basins on the coast of the Vestfold Hills, East Antarctica (68°S 78°E). The characterised strains of the genus *Celluliphaga* produce a number of extracellular enzymes and are known to attack cells. *Celluliphaga uliginosa* is capable of hydrolysing agar, gelatin, chitin, DNA, casein and starch (Bowman, 2000 in press).

Nine phylotypes fell within the branch of the *Flavobacteriaceae* containing the psychrophilic genera *Psychroserpens* and *Gelidibacter*, both isolated from Antarctic marine and marine derived ecosystems (Bowman, *et al.* 1997c). Three phylotypes, represented by sic clones 8118, 42372 and Arctic 121, grouped in a coherent cluster (100% bootstrap support) with clones from deep sea sediment (JTB244) and marine phytodetrital macroaggregates (AGG13). These phylotypes all had *Psychroserpens burtonensis* as their closest characterised relative at evolutionary distances of 0.0311, 0.0309 and 0.0373 respectively. *Psychroserpens burtonensis* is non-motile, strictly aerobic chemoheterotroph isolated from the pycnocline of Burton Lake. It is psychrophilic with an optimum growth temperature of between 15-18°C and requires seawater for growth (Bowman, *et al.* 1997c). The close relationship between the clones in this cluster (including JTB244 and AGG13) (evolutionary distances between

0.0029 and 0.0213) and the strong bootstrap support suggest this group represents a novel species of the genus *Psychroserpens.*

Five phylotypes, represented by sic clones 8119, 815, B8217, B8236 and Arctic 123, formed a coherent cluster together with the Antarctic sediment clone Organic-5 (Bowman, *et al.* 2000b) which originates from Organic Lake, a meromictic hypersaline lake in the Vestfold Hills, East Antarctica. All these clones (including Organic-5) are closely related with evolutionary distances between 0.000 and 0.0214, except for sic 815 which is slightly more removed with distances from the other five clones of between 0.0199 and 0.0414. The most closely related cultivated strains (*Gelidibacter algens* for sic clones 8119, 815, B8217 and Arctic 123, and the Antarctic seawater strain SW17 for sic B8236 and Organic-5) are further removed at distances between 0.0453 and 0.0734. These distances suggest the clones in this cluster represent a possible new genus, related to the genera *Psychroserpens* and *Gelidibacter.* Given that all characterised isolates in this branch are psychrophilic, seawater requiring, chemoheterotrophs it seems feasible that this cluster also posses such characteristics. One other phylotype represented by McMurdo 124 was also affiliated with this branch. This clone branched on the periphery of the *Gelidibacter* genus with no other reference strains and was mostly closely related to *Gelidibacter algens* at an evolutionary distance of 0.0806. Such a distance suggests this phylotype may also represent a novel genus with similar characteristics to those described above.

One phylotype, represented by Arctic 156 grouped closely (88% bootstrap support) with *Flavobacterium xylanivorum,* at a distance of only 0.0077. *Flavobacterium xylanivorum* is a psychrophilic, xylanolytic bacterium isolated from British Antarctica (Humphry, *et al.* Unpublished).

Four phylotypes grouped within the genus *Polaribacter.* The phylotypes represented by sic clones 127 and B9029 grouped closely with *Polaribacter irgensii* at distances of 0.0239 and 0.0047 respectively, whilst the phylotypes represented by sic clones 10332 and 42406 grouped (with 72% bootstrap support) with *Polaribacter franzmannii* at distances of 0.0465 and 0.0132. Both *Polaribacter irgensii* and *Polaribacter franzmannii* were isolated from the seawater : sea ice interface in McMurdo Sound, West Antarctica (Irgens, *et al.* 1989, Gosink *et al.* 1993). They are non motile, produce

gas vesicles and are very psychrophilic, not growing at temperatures above 10°C on agar (Gosink, *et al.* 1998). Two other phylotypes grouped in this branch of the *Flavobacteriaceae*, both having members of the *Polaribacter* genus as their closest relatives. Clone sic 917 was quite closely related to *Polaribacter* sp. IC066 at a distance of 0.0206. *Polaribacter* sp. IC066 was isolated from the sea ice of Eastern Antarctica and like the other *Polaribacter* species this strain is very psychrophilic, having an estimated optimum temperature of about 10°C (Bowman, *et al.* 1997b). The phylotype represented by McMurdo 20 was fairly distantly related to its closest relative, *Polaribacter franzmannii*, at a distance of 0.0894. It grouped loosely outside the *Polaribacter* genus with the deep sea sediment clone JTB143.

A number of phylotypes including those represented by sic clones 8232, 946, 9002, B8018 and 42355, grouped in a branch of the *Flavobacteriaceae* which also contained the generically misclassified species [*Cytophaga*] *latercula*. These phylotypes did not form a coherent group and were fairly removed from any reference strains, their closest characterised relative being *Celluliphaga fucicola* at evolutionary distances of between 0.0662 and 0.0964, except for sic B9002 which was closest to *Gelidibacter algens* at a distance of 0.0814. All clones however were most closely related to the Antarctic sediment clone Burton-44. Such distant relationships to any classified organism prohibits inferences as to their potential characteristics. Two phylotypes, represented by sic B8012 and Arctic 16, grouped loosely with the *Cytophaga*-like bacterium QSSC9-3, branching from a lineage containing several *Flavobacteria* species. These phylotypes had only very distant relationships with characterised bacteria. Clone Arctic 16 was most closely related to [*Cytophaga*] *latercula* at a distance of 0.0702 but its closest sequence was Burton-44 at a distance of 0.0500. The clone Burton-44 was retrieved from an Antarctic lake sediment sample (Bowman, *et al.* 2000). The sic B8012 was most closely related (however only distantly) to the Antarctic seawater strain SW17 at a distance of 0.1072. SW17 is a yellow pigmented psychrotolerant heterotroph found in abundance in Antarctic coastal water, according to most probable number counts (Bowman, unpublished data).

One phylotype, represented by sic 42370, branched very deeply and was most closely related to the deep sea sediment strain BD2-2 (Li, *et al.* 1999) at an evolutionary

distance of 0.1647. This distance prohibits any speculation as to the characteristics of the organism this clone represents.

3.2.2 The *Proteobacteriacea*

After the CFB group, the next most common group within the clone libraries was the Proteobacteria. Members from this group are readily cultivated and many strains were isolated by Bowman *et al.* (1997b) in their survey of Antarctic sea ice. In this study representatives of the alpha and gamma subclasses of the Proteobacteria were detected. No clones belonged to the beta, delta or epsilon subclasses.

3.2.2.1 The Gamma Subclass

Figures 3.2 displays an evolutionary distance dendrogram of some members of the gamma subdivision of the *Proteobacteriacea* and associated sea ice clones. A total of 162 clones, including representatives from all 10 libraries, fell into this group. Many sequences from this study group closely with strains previously isolated from Antarctic sea ice.

Five phylotypes were closely associated with the genus *Shewanella*. Two phylotypes, represented by sic clones 114 and 10368, clustered with strong bootstrap support with strains of the species *Shewanella frigidimarina*. These phylotypes accounted for 47% and 25% of the clones in their respective libraries. A third phylotype, represented by McMurdo 10, clustered with the species *Shewanella gelidimarina* (bootstrap support 61%). The genus *Shewanella* contains facultative anaerobic, gram negative, motile, rod-like cells with versatile metabolism. Both species referred to above are iron reducers originally isolated from congelation fast ice in the coastal Vestfold Hills region of Antarctica (Bowman, *et al.* 1997d). *Shewanella frigidimarina* is psychrotolerant (growth from <0 to 28°C) and halotolerant and unable to degrade polysaccharides. It is apparently cosmopolitan in Antarctic marine and marine derived environments, having been isolated from sea ice, seawater, lake ice and oxic and anoxic lake water and sediment (Bowman, *et al.* 1997d, Rea, 1999). *Shewanella gelidimarina* is psychrophilic (growth from <0 to 23°C), halophilic and chitinolytic. This species appears to be restricted to the sea ice ecosystem, not having been detected in extensive investigations of Antarctic lake and seawater environments (Bowman, *et*

al. 1997d). The phylotype represented by sic 10363 branched alone within the genus *Shewanella.* Its closest characterised relative was *Shewanella putrefaciens* at a distance of 0.0501. This organisms represents a novel lineage within the genus *Shewanella.*

Four phylotypes were associated with the genus *Colwellia* and *Colwellia*-like organisms. Three phylotypes, represented by sic clones 8124, 8125 and 42384, grouped (99% bootstrap support) with the Antarctic sea ice strain IC169 forming a novel phylogenetic lineage adjacent to the genus *Colwellia.* Given the phylogenetic positioning of this group and the fact that IC169 has a different fatty acid profile to members of the *Colwellia,* this group may represent a novel genus (Bowman pers comm.). IC169 is a facultatively anaerobic, motile, psychrophilic organism requiring seawater for growth (Bowman, *et al.* 1997b). Rea (1999) detected two clones (not shown in phylogenetic analysis) in sediment from Clear lake, a stratified brackish lake in the Vestfold Hills, Antarctica which also grouped with this strain. She surmised these organism occupied the oxic and oxycline zones of the lake.

One phylotype, represented by sic 42396, grouped within the genus *Colwellia,* and was closely affiliated (phylogenetic distance 0.0071) with the species *Colwellia hornerae* which was isolated from the fast ice of the coastal Vestfold hills region, Antarctica. This species is psychrophilic, halophilic and synthesises docosahexaenoic acid (Bowman *et al.* 1998a), an attribute common to the *Colwellia* species and one proposed to enhance their ability to colonise environments characterised by constant extreme low temperatures and high hydrostatic pressure (DeLong and Yayanos, 1986).

One phylotype, represented by sic 10369, grouped (bootstrap support 77%) with clones Clear-11 and Pendant-8, derived from Antarctic marine salinity, meromictic lakes sediments (Bowman, *et al.* 2000). The evolutionary distance between any of these organisms and its closest characterised relative prohibits speculation as to their possible characteristics.

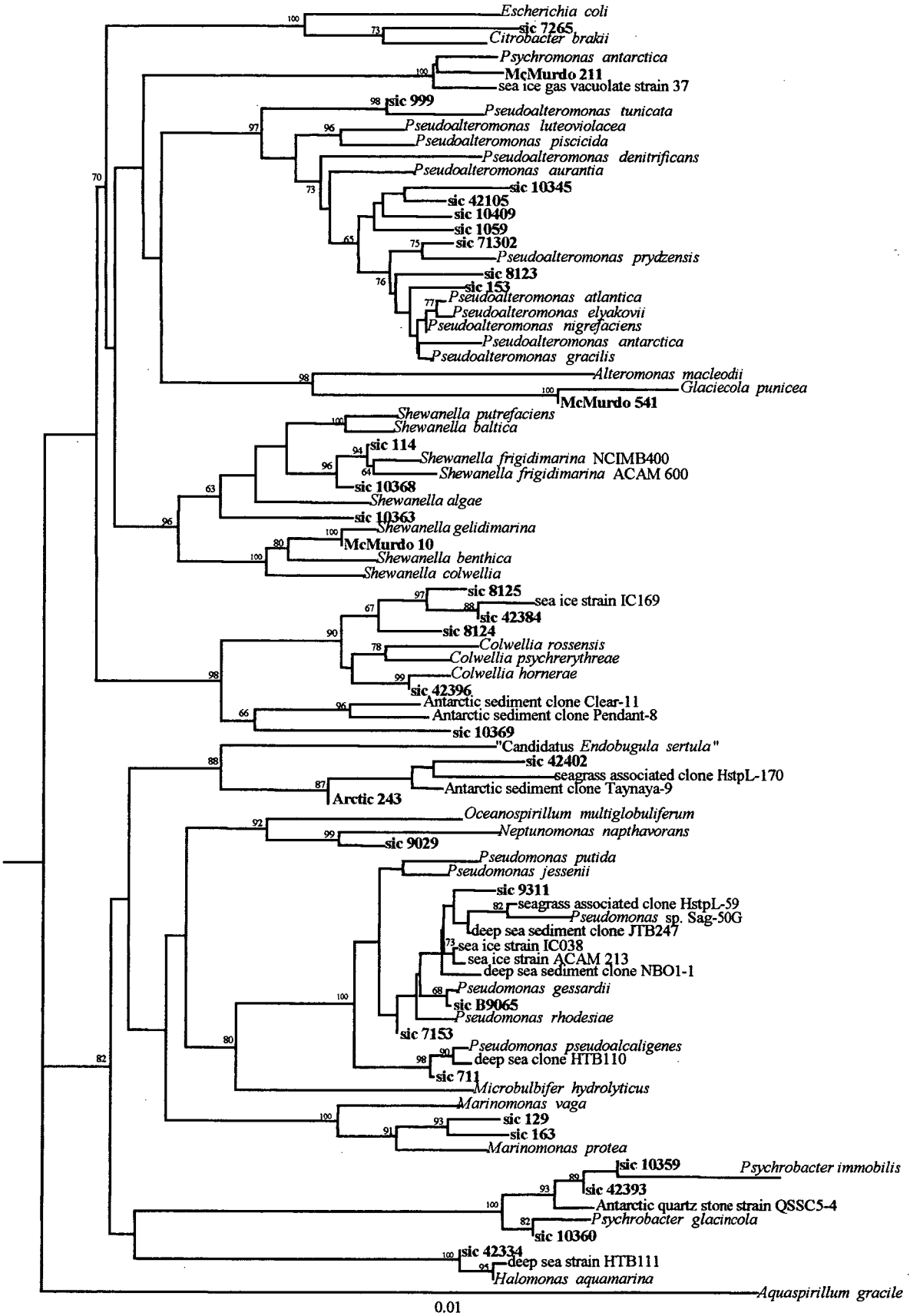


Figure 3.2Evolutionary distance dendrogram created from 16S rDNA sequences of some members of the gamma subclass of the *Proteobacteria* and associated sea ice clones.*Aquaspirillum gracile* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

One phylotype, represented by sic 7265, fell within the *Enterobacteriaceae*, its closest relative being *Citrobacter brakii* at a distance of 0.0453. It is possible this phylotype represents a contaminant. It seems incongruous that an enteric bacterium should appear in an environment such as the Antarctic sea ice. However, it is possible that it represents a member of the intestinal biota of one or more of the sympagic biota which commonly occupy this ecosystem and hence it was included in this analysis.

One phylotype, represented by McMurdo 211, grouped in a deep monophyletic lineage containing *Psychromonas antarctica* and the Arctic gas vacuolate sea ice strain 37 (this strain was identified by Staley and Gosink (1999) as belong to as yet officially unnamed genus "*Iceobacter*". The genus appears to be synonymous with the genus *Psychromonas*). Clone McMurdo 211 was related to these isolates at distances of 0.0235 and 0.236 respectively. *Psychromonas antarctica* is a motile, aerotolerant, anaerobic bacterium which was isolated from an anaerobic enrichment inoculated with sediment taken from below the cyanobacterial mat of a high-salinity pond near Bratina Island on the McMurdo Ice Shelf, Antarctica (Mountfort *et al.* 1998). The gas vacuolate sea ice strain 37 was isolated from Arctic sea ice (Gosink et al. 1993). It is highly psychrophilic, not growing at temperatures above 15°C.

No single genus was represented in all sea ice samples analysed however clones clustering within the genus *Pseudoalteromonas* were described from all samples obtained from the Eastern Antarctic coast. *Pseudoalteromonas* species are extremely common in seawater worldwide and Bowman *et al.* (1997b) reported them to be the most commonly isolated psychrotolerant bacteria from Antarctic sea ice. The genus has a number of distinct branches, one of which contained the phylotype represented by sic 999, grouped with the species *Pseudoalteromonas tunicata*, a position strongly supported by bootstrap analysis (87%). *Pseudoalteromonas tunicata* is a dark-green pigmented marine bacterium which produces a number of antilarval and antibacterial extracellular metabolites (Holstrom, *et al.* 1998). Given the strength of this grouping and the small evolutionary distance between the two sequences (0.0128) it is possible that sic 999 shares these characteristics, more-so perhaps given that its next two closest relatives, *Pseudoalteromonas luteoviolacea* and *Pseudoalteromonas piscicida* are also pigmented and produce bioactive extracellular compounds (Baumann, *et al.*

1984). Three phylotypes, represented by sic clones 71302, 8123 and 153 are affiliated with a very shallow clade containing non-pigmented *Pseudoalteromonas* species. Although two of these phylotypes (8123 and 71302) grouped loosely with *Pseudoalteromonas prydzensis*, a species isolated from Antarctic fast ice (Bowman, 1998) which clusters at the periphery of this clade, all three were most closely related to species falling well within this clade. The species in this clade are so closely related that other procedures such as phenotypic analysis and DNA-DNA hybridization are required to determine their interspecies relationships (Bowman, 1998). Such procedures are obviously outside the scope of this study. It was within this clade that the majority of Antarctic sea ice strains isolated by Bowman, *et al.* (1997b) fell. Four phylotypes, represented by sic clones 42105, 10345, 10409 and 1059, clustered together in a branch containing no reference strains. All four phylotypes had *Pseudoalteromonas elyakovii* as their closest characterised relative at distances of between 0.0245 and 0.0395. This species was isolated from lesions in the fronds of a brown algae (*Laminaria* sp.) and so obviously possesses agarolytic ability. Given that many other *Pseudoalteromonas* species possess similar agarolytic characteristics it is probable that the organisms represented by this phylotype cluster are also agarolytic. It is likely they also possess other common characteristics of genus *Pseudoalteromons*, including motility, psychrotolerance, halotolerance, and a requirement of salt for growth.

One phylotype, represented by McMurdo 541, grouped with the species *Glaciecola punicea*. There were no sequence differences between the two in the region examined. *Glaciecola punicea* is a pink-red pigmented, strictly aerobic, psychrophile originally isolated from coastal fast ice in eastern Antarctica. It has an absolute requirement for seawater and is slightly halophilic (Bowman, *et al.* 1998b).

Four phylotypes, represented by sic clones 711, 7153, 9311 and B9065, grouped within the *Pseudomonas* assemblage. This group contains a large number strains and clones with very similar 16S rDNA sequences which have been isolated or detected from a wide variety of environments. The phylotype represented by sic 711 grouped (62% bootstrap support) with *Pseudomonas pseudoalcaligenes* which has been isolated from lake sediments (Venkateswaran. and Nealson, unpublished) and the clone HTB110, detected in a deep sea site near the south of Japan (Takami and

Nagahama, unpublished). Clone sic 711 was very closely related to both at an evolutionary distance of 0.0081 from each. The remaining three phylotypes grouped within a shallow clade containing a number of clones from permanently cold or marine environments as well as two strains isolated from Antarctic sea ice (Bowman, *et al.* 1997). It was to one of these sea ice strains, IC038, that clones sic 9311 and 7153 were most closely related. Clone sic 9311 was related at an evolutionary distance of 0.0101 whilst clone sic 7153 was identical to IC038 over the sequence length analysed. At the periphery of this *Pseudomonas* clade lie a number of species isolated from natural mineral waters (Verhille, *et al.* 1999). Clone sic 9065 was very closely related to one of these species, *Pseudomonas gessardii,* at an evolutionary distances of just 0.0035.

Two phylotypes represented by sic 42402 and Arctic 243, grouped with environmental clones HstpL-70 and Taynaya-9 in a deeply branching cluster strongly supported by bootstrap analysis. Clone HstpL-70 was detected in association with leaves of the seagrass *Halophila stipulacea.* Taynaya-9 was detected from Taynaya Bay sediment. Their only close relative, Candidatus *Endobugula sertula*, is a symbiont of bryzoan larvae (Haygood and Davidson, 1997). This organism has been identified but not cultured suggesting this radiation of the gamma Proteobacteria may require special conditions for isolation, possible due to their symbiotic nature. Although the evolutionary distance between these organisms is large, given that they share a deeply branching origin with this symbiotic bacterium it is possible the environmental clones represent similar endosymbionts to a common marine organism.

One phylotype, represented by sic 9092 grouped closely with the obligately marine organism *Neptunomonas naphthavorans* at an evolutionary distance of 0.0393, forming a distinct lineage in the *Halomonadaceae*, most closely related to members of the genus *Oceanospirillum. Neptunomonas napthavorans* was isolated from coal tar creosote-contaminated marine sediment from Eagle Harbor, Washington (Hedlund, *et al.* 1999). It is a halophilic chemoheterotroph which is capable of degrading naphthalene and 2-methylnaphthalene with concomitant growth. In addition, it significantly transforms 1-methylnaphthalene, although it is not capable of using this compound as a sole carbon and energy source. The minimum temperature for growth of this organism is unknown, however it is capable of growing at 4°C.

Two phylotypes, represented by sic clones 129 and 165, formed a strongly supported group (bootstrap support 100%) within the genus *Marinomonas*, clustering with the species *Marinomonas protea.* This organism was originally isolated from Ace Lake in the Vestfold Hills region of eastern Antarctica (Mills, *et al.* Unpublished) and Bowman *et al.* (1997b) isolated it from under-ice seawater. *Marinomonas* species are motile, free living marine bacteria, which are highly nutritionally versatile. The other two species in this genera, *Marinomonas vaga* and *Marinomonas communis*, do not grow at 4°C (Kita-Tsukamoto, *et al.* 1993). *Marinomonas protea* is a cold adapted relative, apparently common in Antarctic marine and marine derived ecosystems.

One phylotype, represented by sic 42334, grouped within the genus *Halomonas.* It was extremely closely related to *Halomonas aquamarina* (evolutionary distance 0.0039), a common marine species, and to the clone HTB111 (evolutionary distance 0.0038) detected in a deep sea site near the south of Japan. *Halomonas aquamarina* is a motile, seawater requiring, strictly aerobic chemoorganotroph (Dobson, *et al.* 1993).

Three phylotypes, represented by sic clones 10359, 10379 and 42393, grouped with 96% bootstrap support with the genus *Psychrobacter.* The *Psychrobacter* species are non-motile, psychrotolerant and halotolerant, adaptions which have enabled their colonisation of diverse Antarctic habitats including sea ice, ornithogenic soils and quartz stone rock habitats (Bowman, *et al.* 1997e, Smith, *et al.* 2000). One phylotype, represented by sic 10379, branched (100% bootstrap support) deeply at the periphery of the genus and given the evolutionary distance to its closest relative, *Psychrobacter* sp. QSSC5-4, was 0.0791 it appears likely that this phylotype represents at least a new species of the *Psychrobacter* genus. The other two phylotypes also had *Psychrobacter* QSSC5-4 as their closest relative however the evolutionary distances between their sequences were much closer at 0.0053 for sic 10359 and 0.0108 for sic 42393. *Psychrobacter* QSSC5-4 was isolated from an Antarctic quartz stone habitat. It is psychrophilic and moderately halophilic (Smith, *et al.* 2000).

3.2.2.2 The Alpha Subclass

Figure 3.3 displays an evolutionary distance dendrogram of some members of the alpha subclass of the *Proteobacteria* and associated sea ice clones. All clones affiliated with this subclass fell within the "Marine alpha group" of the *Rhodobacter* branch. This group has been found to comprise a large fraction of the marine bacterial communities from the south-east coast of the U.S.A. and is readily culturable (Gonzalez and Moran, 1997). The affiliations of sea ice clones within the alpha *Proteobacteria* are displayed in Figure 3.

Three phylotypes from the Arctic sea ice, represented by Arctic clones 153, 128 and 235, grouped within the genus *Octadecabacter*. This genus was described on the basis of two isolates, one from arctic sea ice and one from the Antarctic sea ice, which had closely related 16S rDNA sequences but a low DNA:DNA hybridisation value (Gosink, *et al.* 1997). Indeed the two species were related by an evolutionary distance of only 0.0080 in this analysis. Such a lack of clear distinction between 16S rDNA sequences makes it difficult to resolve the affiliations of the clones obtained in this study. The cut off limit of replicate clones in this study was set at an evolutionary distance of 0.0200, which is clearly much greater than the distance between *Octadecabacter arcticus* and *Octadecabacter antarcticus*. This anomaly leads to an interesting situation whereby Arctic 153 is most closely related to *Octadecabacter arcticus* (evolutionary distance 0.0082), Arctic 235 is most closely related to *Octadecabacter antarcticus* (evolutionary distance 0.0169) and Arctic 128 is related to both species equally. The genus *Octadecabacter* contains psychrophilic, halophilic gas vacuolate bacteria that have octadecanoic acid as a very high proportion of their total cellular fatty acids (Gosink, *et al.* 1997).

One phylotype, represented by sic 8163, grouped (74% bootstrap support) with and was most closely related to the *Roseobacter* sp. shippagan (evolutionary distance 0.0207), an unculturable bacterial species associated with excessive mortality in larval haddock (Griffiths and Melville, unpublished). Another phylotype, represented by sic 8141, grouped within the genus *Sulfitobacter*, and was most closely related to *Sulfitobacter pontiacus* at an evolutionary distance of 0.0103. *Sulfitobacter pontiacus* is an halotolerant, aerobic sulfite oxidiser isolated from the Black Sea (Sorokin, 1995). Two phylotypes, represented by sic clones 10342 and 8142, branched (76% bootstrap

support) with the Antarctic sediment clones Taynaya-15 and Burton-9. These sea ice clones were not closely affiliated with any of the characterised species in the marine alpha group. The closest characterised relatives of sic 10342 were two *Sulfitobacter* strains, EE-36 and DSS-2, at a distance of 0.0400, whilst sic 8142 was closest to *Octadecabacter arcticus* at a distance of 0.0903. Two other phylotypes, represented by sic clones 174 and 42338, also clustered near the genus *Sulfitobacter*. The sic 174 was most closely related to *Ruegeria atlantica* at a distance of 0.0186. *Ruegeria atlantica* is a non-motile, halophilic, nitrate reducing bacterium isolated from marine sediments of the northwestern Atlantic Ocean (Uchino, *et al.* 1998). The closest characterised relative to sic 42338 was the *Sulfitobacter* strain EE-36 at a distance of 0.0153. *Sulfitobacter* strain EE-36 was a numerically important culturable bacteria from a lignin enriched marine community (Gonzalez, *et al.* 1996)

Five phylotypes, represented by sic clones 42340, 42383, 8120, 926 and Arctic 437, branched deeply within the *Rickettsiales* forming a coherent cluster with the Antarctic lake sediment clone Pendant-24. Their closest characterised relative was *Orientia tsutsugamushi* at distances of between 0.4345 and 0.4723. The only other organsims associated with this deeply branching group were members of the SAR11 cluster. The SAR11 cluster contains to date uncultivated organisms that constitute a significant component of the bacterioplankton community in seawater of the Atlantic and Pacific Oceans (Field, *et al.* 1997). Given their abundance, it is probable the organisms represented by the SAR11 cluster play an important role in carbon and nutrient recycling of these regions. The members of the cluster most closely associated with the Antarctica and Arctic clones obtained in this study are SAR220 and SAR203, both of which were detected in the Atlantic Ocean at a depth of 250m (Field, *et al.* 1997). The relationship between these clusters suggests the polar clones may represent a cold adapted radiation of this ecologically important novel bacterial lineage.

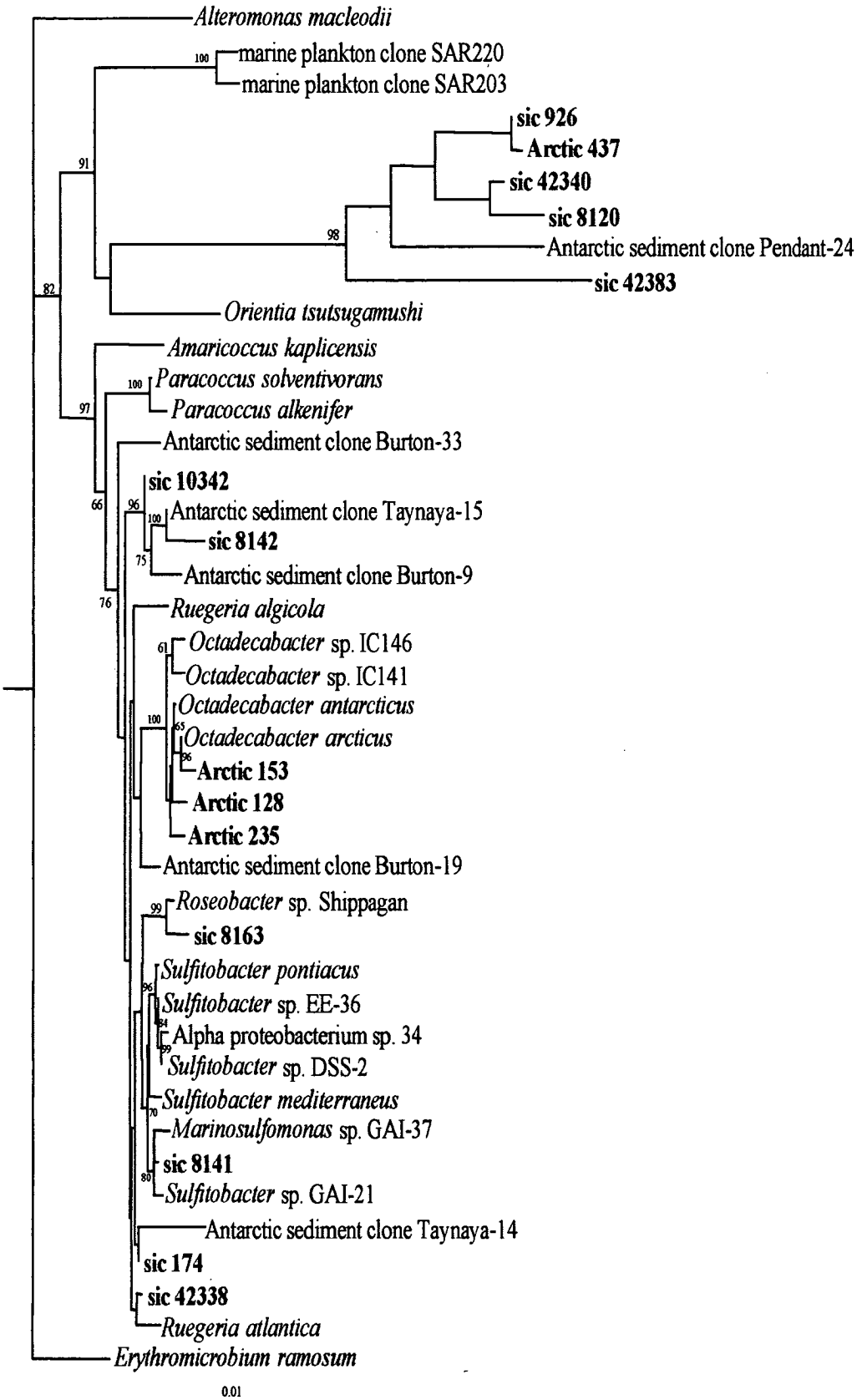


Figure 3.3 Evolutionary distance dendrogram created from 16S rDNA sequences of some members of the Alpha subclass of the *Proteobacteriaceae* and associated sea ice clones. *Erythromicrobium ramosum* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

3.2.3 The *Chlamydia/Verrucomicrobia*

Figure 5 displays an evolutionary distance dendrogram of some members of the orders *Chlamydiales* and the *Verrucomicrobiales* and associated sea ice clones.

Three phylotypes, represented by sic clones 812, 816 and 10401, grouped within the division *Verrucomicrobia*, clustering with clones derived from Antarctic lake sediments and from bacteria colonising the leaves of the sea grass *Halophila stipulacea* (Weidner, *et al.* unpublished). These clones appear to belong to a novel monophyletic subdivision of the *Verrucomicrobia*, with the deep branching supported by 100% bootstrap analysis. The sea ice clones had evolutionary distances from their nearest characterised relatives of between 0.1312 to 0.1565.

Although the division *Verrucomicrobia* contains very few cultured representatives, it does contain a large number of clones from extremely diverse environments including soybean fields (Ueda, *et al.* 1995), rice paddy field soil (Ueda, *et al.* 1995), forest soil (Liesack and Stackbrandt, 1992), and lake (Hiorns, *et al.* 1996) and maritime (Wise, *et al.* 1997, Fuhrman, *et al.* 1993) environments. This diversity prompted Hugenholtz *et al.* (1998b) to declare they represent a ubiquitous branch of the domain *Bacteria.* The two named species of the division, *Verrucomicrobium spinosum* and *Prosthecobacter fusiformis*, are heterotrophic prosthecate bacteria, which are heavily fimbriate. Prosthecae are narrow extensions of the bacterial cell wall containing cytoplasm (Staley, 1968). Its has been proposed these structures confer a variety of benefits to aerobic heterotrophic bacteria including a mechanisms for attachment to solid substrates and enhanced respiration and nutrient uptake (Hedlund, *et al.* 1996). Such characteristics would make these bacteria ideal candidates for colonisation of sea ice algal communities, especially given that the closely related HstpL clones were found associated with seagrass leaves. Prosthecate form bacteria have been observed in microscopic examinations of Antarctic sea ice microbial communities (e.g. Bowman, *et al.* 1997b, Helmke and Weyland, 1995, Zdanowski, 1988), however none have been isolated. It is clear that members of this division are difficult to isolate. Hedlund *et al.* (1996) reported a failed attempt to isolate such organisms from the Antarctic sea ice, an environment in which they obviously exist. Janssen *et al.* (1997) isolated

ultramicrobacteria (with mean volumes of between 0.03 and 0.04μm^3), phylogenetically affiliated with the division *Verrucomicrobia*, from rice paddy soil using serial dilution methods. These organisms, like the other isolates of this division (Hugenholtz, *et al.* 1998b), preferentially utilised sugars and some sugar polymers as substrates for growth (Janssen, *et al.* 1997). It appears those characterised representatives of the division *Verrucomicrobia* have complex nutritional requirements which may be difficult to replicate in a laboratory environment.

One phylotype was, interestingly, associated with the order *Chlamydiales*. The bulk of the representatives in the genera *Chlamydia* and *Parachlamydia* are cultured or observed isolates. This phylotype, represented by Arctic 240, was most closely related to the observed organism *Parachlamydia acanthoamoebae*, an obligate intracellular parasite of the protozoan *Acanthamoeba castellanii* (Amann, *et al.* 2000). Similar clones have been reported from molecular surveys of Antarctic lake sediments (Bowman, *et al.* 2000). One of these clones had the most closely related sequence to that of Arctic 240. It is possible the organism represented by this phylotype is also an intracellular metazoan parasite inhabiting the Arctic sea ice ecosystem and that such metazoans may serve as hosts to other, presently uncultured, bacterial endosymbionts.

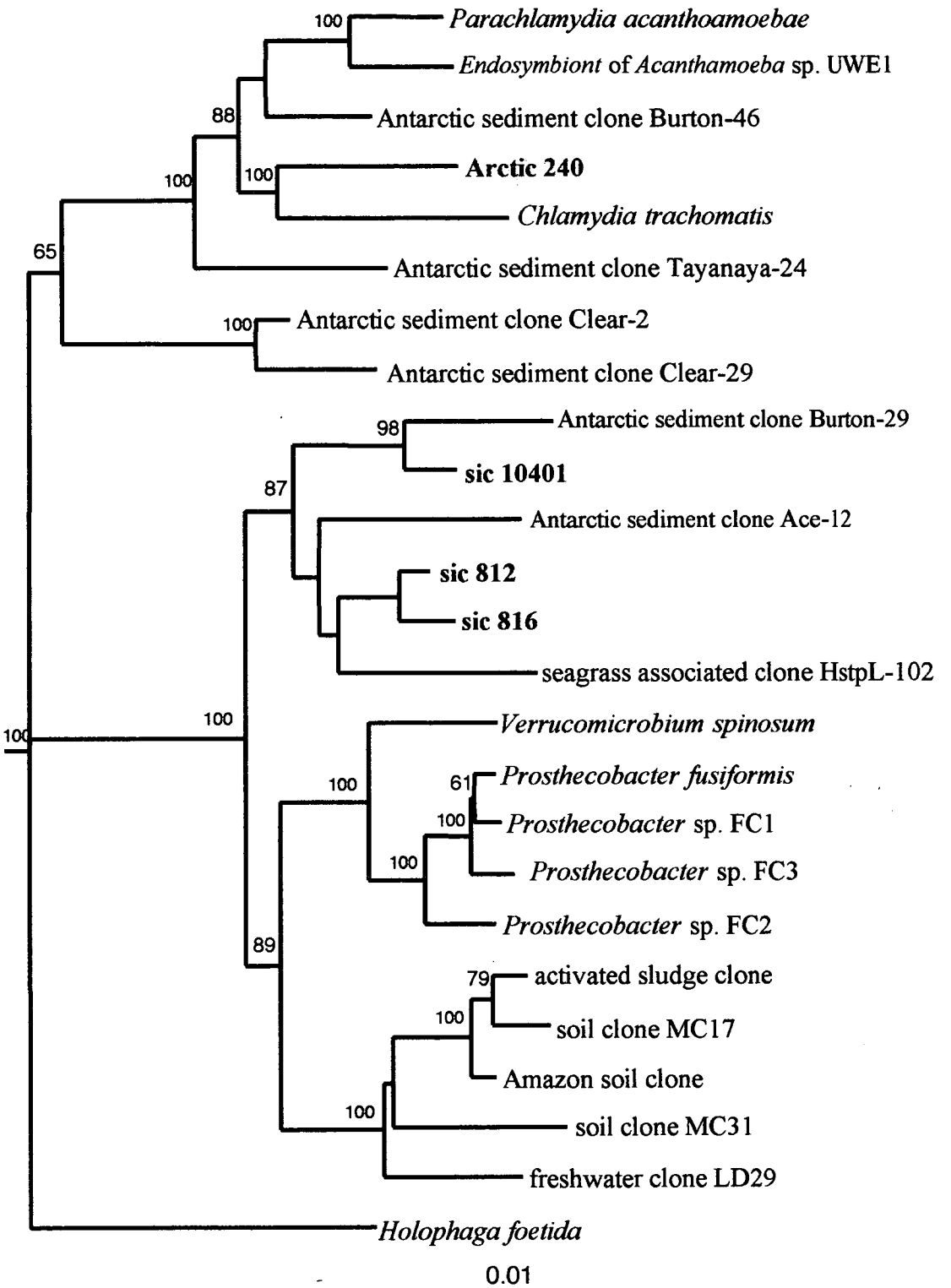


Figure 3.4: Evolutionary distance dendrogram created from 16S rDNA sequences of some members of the Orders *Chlamydiales* and *Verrucomicrobiales* and associated sea ice clones. *Holophaga foetida* was used as the outgroup for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar represents 0.01 changes per nucleotide.

3.2.4 The Firmicutes (Gram-positive Bacteria)

The phylogenetic affiliations of sea ice clones within the Gram positive bacteria is displayed in Figure 6.

3.2.4.1 The *Actinobacteria*

Two phylotypes, represented by sic clones 7252 and 950, grouped within the family *Rubrobacteridae*, in the genus *Rubrobacter*, branching from the lineage containing the species *Rubrobacter radiotolerans* (99% bootstrap support) and forming a coherent cluster with the Antarctic quartz stone clone QSSC8L-10 (Smith, *et al.* 2000). These three clones were closely inter-related at evolutionary distances of between 0.0036 and 0.0349, whilst being fairly removed from their closest characterised relative, *Rubrobacter radiotolerans* at distances of between 0.0687 and 0.0972. This information suggests the Antarctic clones represent a novel species of the genus *Rubrobacter*. The only characterised *Rubrobacter* species are *Rubrobacter radiotolerans* and *Rubrobacter xylanophilus*, both thermophilic bacteria, with optimum growth temperatures of 48°C and 60°C respectively (Kausar, *et al.* 1997). It would be reasonable to assume these Antarctic clones represent a novel, cold adapted member of the genus. *Rubrobacter radiotolerans* is known to be extremely resistant to ionizing radiation (Kausar, *et al.* 1997). Such a characteristic may be of value to Antarctic bacteria if exposed to high levels of ultraviolet radiation.

Whether the Antarctic *Rubrobacter* are of marine or terrestrial origin is unsure. The only other description of *Rubrobacter* from the Antarctic environment is by Smith *et al.* (2000) who detected the organisms in a clone library of quartz stone rock sublithic communities in the Vestfold Hills. It may be expected that inoculation of quartz stone rock habitats by windblown marine particles either directly or from snow melt would be a reasonably common event in this largely exposed coastal region. Indeed a number of the quartz stone rock habitats studied by Smith *et al.* (2000) contained marine bacterial taxa, many of which were psychrophilic. Little can be surmised by examining the environments from which the cultured *Rubrobacter* species were isolated, which include a radon-containing hot spring and a thermally polluted industrial runoff.

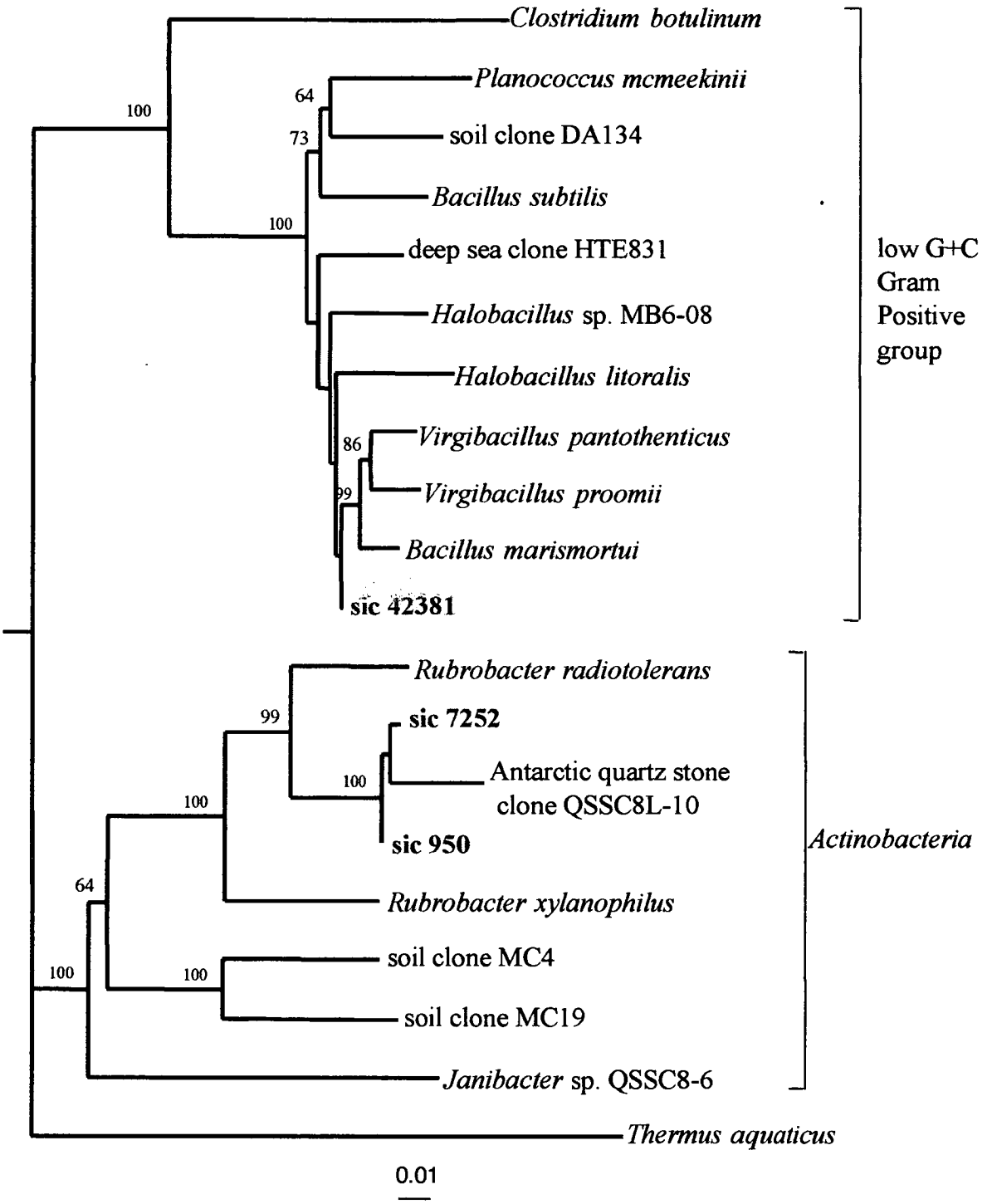


Figure 3.5: Evolutionary distance dendrogram created from 16S rDNA sequences of some members of the *Actinobacteria* and the *Bacillus/ Clostridium* Group and associated sea ice clones. *Thermus aquaticus* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

The *Rubrobacteridae* appear to be part of a broad monophyletic group within the *Actinobacteria* consisting of to date largely uncultivated organisms (Rheims, *et al.* 1996). Studies using molecular techniques have detected members of this group inhabiting a diverse array of environments including peat bog (Rheims, et al. 1996), forest soil (e.g. Leisack and Stackebrandt, 1992, Saano *et al.* 1995), geothermal soil (Rainey, *et al.* 1993), paddy and soybean fields (Ueda, *et al.* 1995), and marine habitats (Fuhrman, *et al.* 1993). The fact that these studies were carried out in geographic regions far removed from one another indicates this group seems to represent a ubiquitous, ecologically significant radiation of the *Actinobacteria.*

3.2.4.2 *Bacillus/Clostridium*

One phylotype, represented by sic 42381 was closely related to *Bacillus marismortui* at an evolutionary distance of 0.0194. Although this distance is relatively close, the sea ice clone does not group with this *Bacillus* species, branching by itself from a lineage containing *Bacillus marismortui* and two species of the genus *Virgibacillus*, *Virgibacillus proomii* and *Virgibacillus pantothenticus*. This branch however is not strongly supported by bootstrap analysis. *Bacillus marismortui* and the *Virgibacillus* species are strictly aerobic, moderately halophilic and form terminal endospores. They are able to able to utilise a wide variety of substrates and all are capable of hydrolysing casein and gelatin (Heyndrickx, *et al.* 1999, Arahal, *et al.* 1999). It is likely that the organism represented by sic 42381 shares these characteristics. However the aforementioned species are mesophilic, growing only at temperatures above 15°C. Thus it is possible sic 42381 could represent a novel cold adapted species within the *Bacillus* rRNA group 1 (*Bacillus sensu stricto*). Again the question of habitat origin is raised. The *Bacillus* group are primarily soil taxa and it is possible that this organsim could derive from soil or dust blown or washed into the sea from the Antarctic continent.

3.2.5 Unaffiliated clones

One clone, sic 7235 was not clearly affiliated with any known bacterial lineages. A GAPPED BLAST search of this sequence returned clones from Candidate divisions OP8 and OP9 as the closest relatives. However in a large scale analysis containing representatives from every bacterial group (Bowman pers. comm. Data not shown), and in the smaller scale analysis shown in Figure 7, this clone groups with clones from Candidate divisions OP3 and OP6, with an evolutionary distance to its closest relative, the candidate division OP6 clone OPS152, of 0.2136. The OP cones were obtained from Obsidian Pool, a hot spring in Yellowstone National Park rich in reduced iron, sulfide, CO2 and hydrogen (Hugenholtz, *et al.* 1997). The distance of this clone from any other sequences indicates it may represent a novel (candidate) division level representative of the bacterial domain. The typical interdivisional rRNA sequence difference is 20 to 25%, a range into which this clone falls. However, to qualify for "candidate division" status a lineage must contain two or more 16S rDNA sequences.

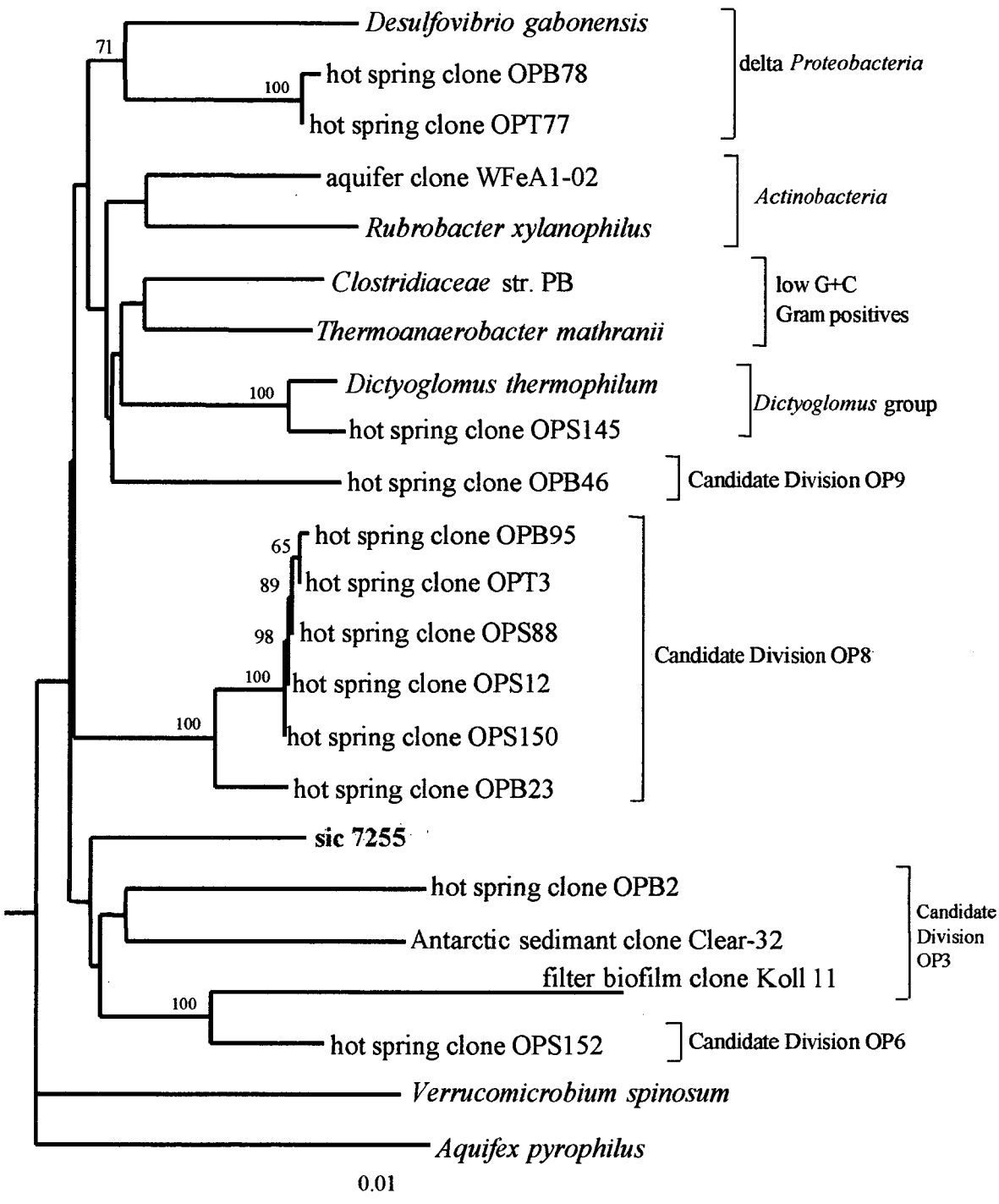


Figure 3.6: Evolutionary distance dendrogram created from 16S rDNA sequences of some members of the *Verrucomicrobiae*, *Actinobacteria*, *Bacillus/Clostri*dium group, delta *Proteobacteria*, Candidate Divisions OP3, OP6, OP8 and OP9 and sic 7255. *Aquifex pyrophilus* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

3.2.6 Summary of Bacterial Clones

Molecular surveys of previously well-examined environments often uncover a diversity of bacteria not identified through culture dependent methods. This study has identified novel lineages within the *Actinobacteria*, the *Verrucomicrobiae/Chlamydia* and amongst the Candidate division bacteria. Organisms from these groups had not previously been identified as inhabiting the sea ice ecosystem. Similarly, potentially novel organisms were detected amongst those groups from which sea ice strains have previously been isolated, including the alpha and gamma *Proteobacteria* and the *Cytophaga-Flexibacter-Bacteroides* group. Amongst these groups many sequences were closely related to characterised species. This finding is testament to the thoroughness of the culture investigation and may also indicate that many bacteria inhabiting the sea ice ecosystem are able to utilise a broad range of substrates, and do not have the complex nutritional requirements which characterise many difficult or unculturable bacterial groups. This similarity to the culture based study was particularly evident within the gamma *Proteobacteria*, where most clones were closely related to *Pseudoalteromonas*, *Shewanella*, *Colwellia*, *Marinomonas*, *Pseudomonas*, *Psychrobacter* or *Halomonas* species. This was not surprising considering that members of the gamma *Proteobacteria* often grow inordinately well under laboratory conditions. Many sea ice clones were closely related to clones obtained from sediment samples of Antarctic lake and marine derived ecosystems. Given that the majority of these clones are most closely associated with predominantly aerobic bacteria, it is likely the sediment clones represent organisms that are active in the water column or surface ice of the habitat examined. The similarities to sea ice clones indicate that many similar organisms are active in most marine and marine derived ecosystems in Antarctica.

3.3 The Eukaryotes

The protocol followed in this study was designed to survey the bacterial composition of the sea ice community. However it is not unusual for eukaryotic genes, particularly plastid genes, to appear in such analyses (eg Rappé *et al.* 1995, DeLong, et al. 1993). The eukaryotes appearing in this study are restricted to those susceptible to detection by the methods utilised and as such should not be considered strictly representative of the sea ice eukaryotic assemblages. For example, phytoplankton species which are susceptible to lysis on contact with filter membranes, and species from which algal cell walls and extracellular materials interfere with recovery of genomic DNA, may be selectively overlooked (Rappé, et al. 1995). The results described here do however provide insight into the phylogenetics of some common sympagic eukaryotes and may facilitate future biodiversity studies.

3.3.1 Phytoplankton Plastid genes

Large numbers of plastid genes from eukaryotic autotrophic nanoplankton have previously been reported in molecular diversity studies of marine environments (DeLong, *et al.* 1993, Rappé, *et al.* 1995, Rappé, *et al.* 1997, Rappé, *et al.* 1998). Such organisms are important components of the phytoplankton assemblages occupying most pelagic marine habitats (Courties, *et al.* 1994). Clones representing chromophyte plastid genes comprised 16% of the sequences obtained in this study. The study of plastid genes provides a unique insight into the phylogenetics of phytoplankton. Plastid genes are sufficiently conserved to allow amplification in PCR reactions with bacteria-specific primers, and to identify their apparent monophyletic origins from within the cyanobacterial phylum. However, their diversity allows for identification between phenotypically distinct chlorophyte groups (Rappé et al. 1995) and, given they are not subject to sexual recombination, they may offer advantages over nuclear genes for some studies of phytoplankton biogeography and speciation, particularly when the divergence of subpopulations is in question (Rappé, *et al.* 1995). The phylogenetic affiliations of sea ice clones within the phytoplankton plastid group are displayed in Figure 8. The majority of plastid clones in this study, including seven phylotypes represented by sic clones 42333, 813, 8113, 928, 103602, Arctic 149 and McMurdo 54, were affiliated with the *Bacillariophyceae*. All but McMurdo 54 were

closely related to the *Odontella sinensis* plastid at evolutionary distances of between 0.0108 and 0.0373. *Odontella sinensis* is a temperate marine diatom. It is possible these sea ice clones represent plastids from the Antarctic species, *Odontella weissflogii*, however this is purely speculative as a sequence for the 16S rDNA of this plastid is not currently available. The phylotype represented by McMurdo 54 was most closely related to the seagrass associated clone HstpL-35 (Weidner, *et al.* Unpublished) at a distance of 0.0184. One phylotype, represented by sic 42330, was affiliated with the plastid genes of the *Prymnesiophyceae*, branching alone at the periphery of this group (100% bootstrap support). This sequence was most closely related to the environmental clone OCS 50, a plastid gene detected from the Pacific Ocean off the mouth of Yaquina Bay, Oregon (Rappé, *et al.* 1998). The closest related plastid from a cultured organism was that from *Emiliana huxleyi*, an alga common in the Southern Ocean north of the Polar Front, at a distance of 0.0738.

3.3.2 Phytoplankton nuclear genes

Phylotype sic 42345 was closely affiliated with the 18S rDNA of the genus *Phaeocystis* in the *Prymnesiales* (Figure 9). This clone grouped (75% bootstrap support) with two strains of the species *Phaeocystis antarctica* at an evolutionary distance of 0.0380 from each. *Phaeocystis antarctica* is common in Antarctic waters and was the dominant haptophyte in spring phytoplankton blooms during a study of the Ross Sea by Smith, Jr *et al.* (1999)

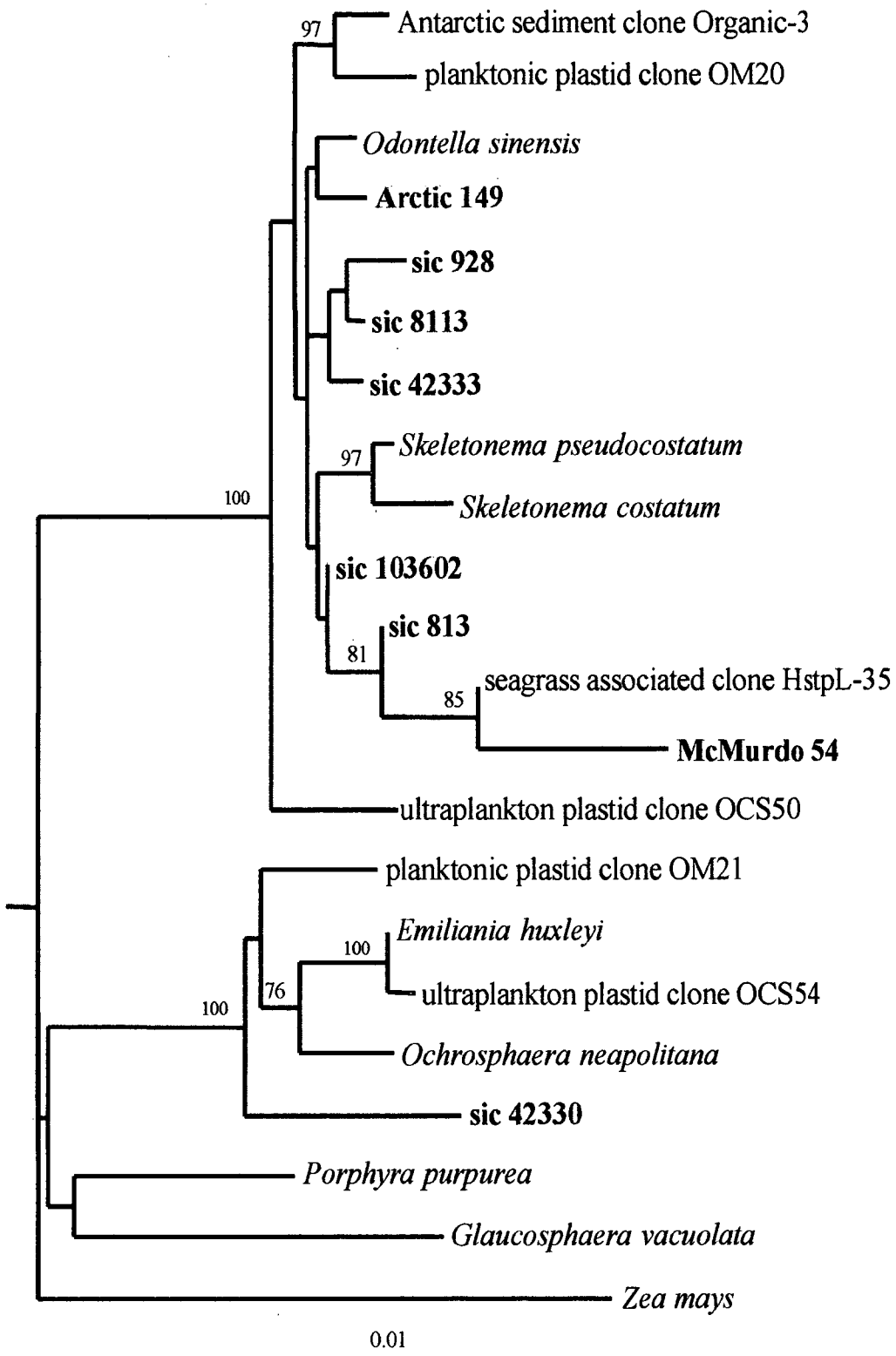


Figure 3.7: Evolutionary distances dendrogram created from 16S rDNA sequences from the chloroplasts of some members of the *Bacillariophyceae*, *Prymnesiophyceae*, *Bangiophyceae* and *Glaucocystophyceae* and associated sea ice clones. *Zea mays* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

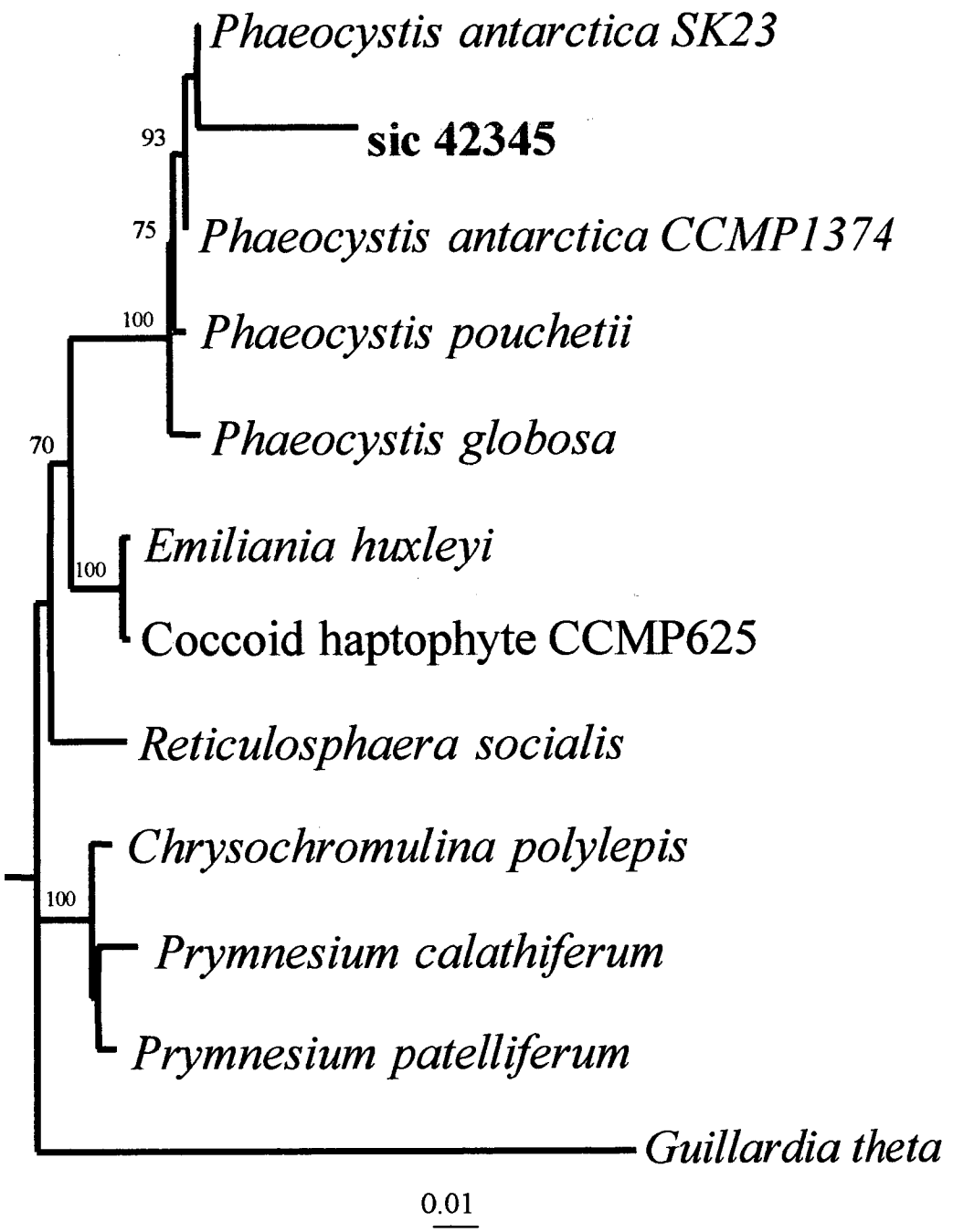


Figure 3.8:Evolutionary distance dendrogram created from 18S rDNA sequences of some members of the *Haptophyceae* and sic 42345. *Guillardia theta* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shjown at nodes. The scale bar indicates 0.01 changes per nucleotide.

3.3.3 The Acoel Turbellaria

The phylogenetic affiliations of sea ice clones with the Acoel Turbellaria are shown in Figure 10.

Five phylotypes, represented by sic clones 42343, 8114, 818, 9019 and 1028 formed a discrete branch within the Acoel group of the Turbellaria. Due to the high degree of variability in the hyper-variable regions of this data set only unambiguously alignable regions were used in the analysis. The sea ice clones, whilst closely inter-related, were only distantly related to *Atriofonta polyvacuola*, their common closest characterised relative, at evolutionary distances of between 0.1030 to 0.2035. The Acoel flatworms represent the extant members of the earliest divergent Bilateria (Ruiz-Trillo *et al.* 1999). It is possible that these sequences represent one of the two putatively novel species of Acoel Turbellaria described by Janssen and Gradinger (1999) as inhabiting the Antarctic sea ice ecosystem (although without comparative sequence data this is purely speculative). Krembs et al. (1999) found that by conforming to the osmotic pressure of brine, turbellaria match their body dimensions to the fluctuating dimensions of the brine channel system. This allows them to traverse structural impasses within the brine channels of established sea ice floes enabling them to seek out diatoms and detritus upon which to graze. Indeed, Janssen and Gradinger (1999) found their distribution to be positively correlated with the accumulation of algal biomass. This corresponds to their appearance in this study within clone libraries from samples containing significant algal biomass, signifying that an active microbial loop had formed in these ice floes at the time of sampling. Similar Acoel-type organism have been described as significant contributors to the grazing of primary production within arctic multi year ice floes (Gradinger, 1999) and given their prevalence within Antarctic sea ice (Janssen and Gradinger, 1999), Aceol Turbellaria may play a similar role in this ecosystem.

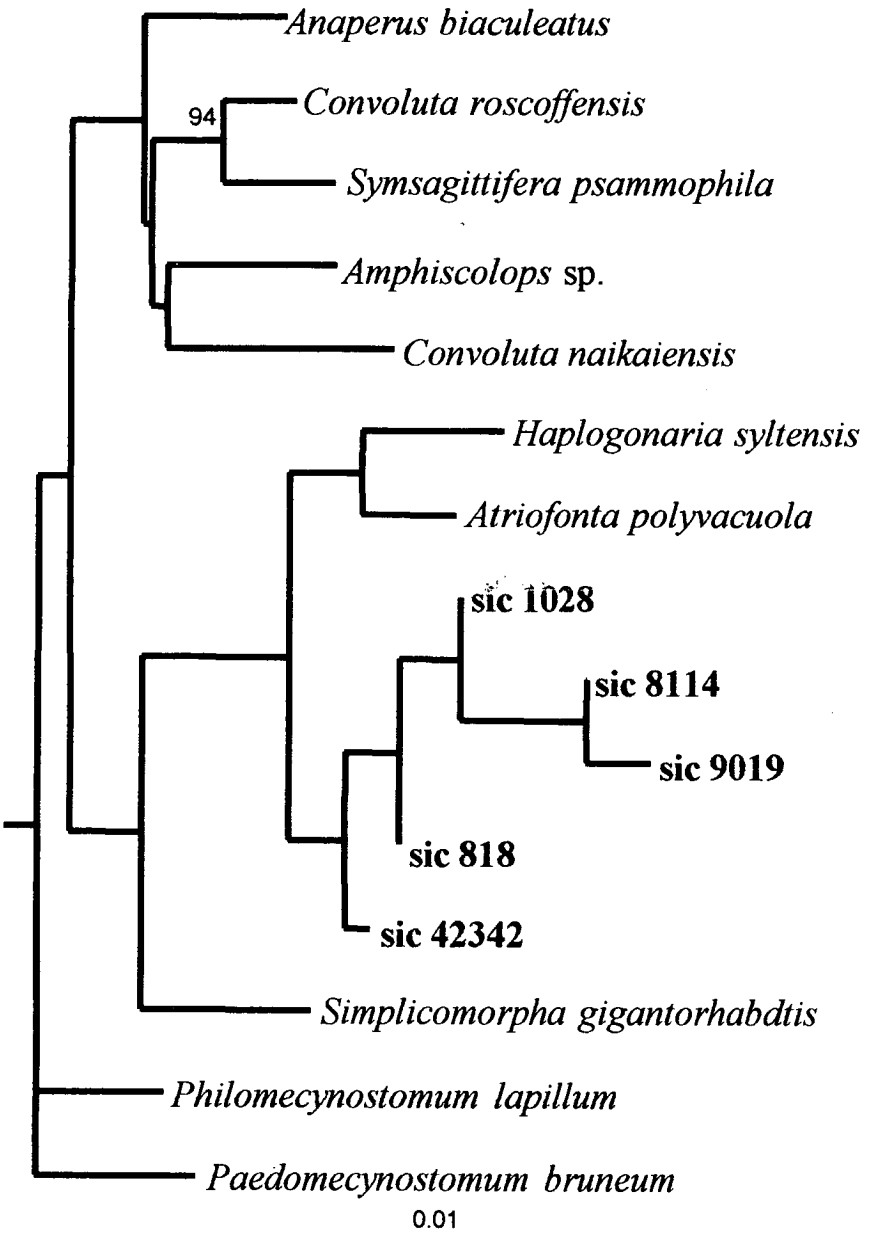


Figure 3.9: Evolutionary distance dendrogram created from 18S rDNA sequences of some members of the Turbellaria and associated sea ice clones.*Paedomecynostomum byuneum* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucloetide.

3.3.4 The Dinoflagellates

The phylogenetic affiliations of sea ice clones with the dinoflagellates are shown in Figure 11. One phylotype, represented by sic 4276, was affiliated with the heterotrophic dinoflagellates of *Dinophyceae*. This clone was closely related to a number of dinoflagellates species including *Gyrodinium* and *Gymnodinium* species, at distances of between 0.0199 and 0.0222, however its exact phylogenetic position is not accurately determined by this analysis. *Gyrodinium* and *Gymnodinium* are common heterotrophic nanoplankton in the Antarctic marine ecosystem. Indeed they were the dominant genera in the heterotrophic nanoplankton assemblages in the Western Ross Sea during the study by Vanucci and Bruni (1999).

3.3.5 The Cercomonadidae

One phylotype, represented by sic 7235, was affiliated with the amoeboid heterotrophic flagellates of the *Cercomonadidae* (Figure 12). These organisms switch reversibly from flagellate to non-flagellated amoebae and are capable of forming cysts. They grow aerobically and persist but do not replicate under anaerobic conditions (Zaman, *et al.* 1999). Although grouping within the genus *Cercomonas*, this clone was not closely related to the characterised representatives of this group and appears to represent a novel species. This phylotype dominated the clones obtained from sea ice sample 7, representing 65% of the library.

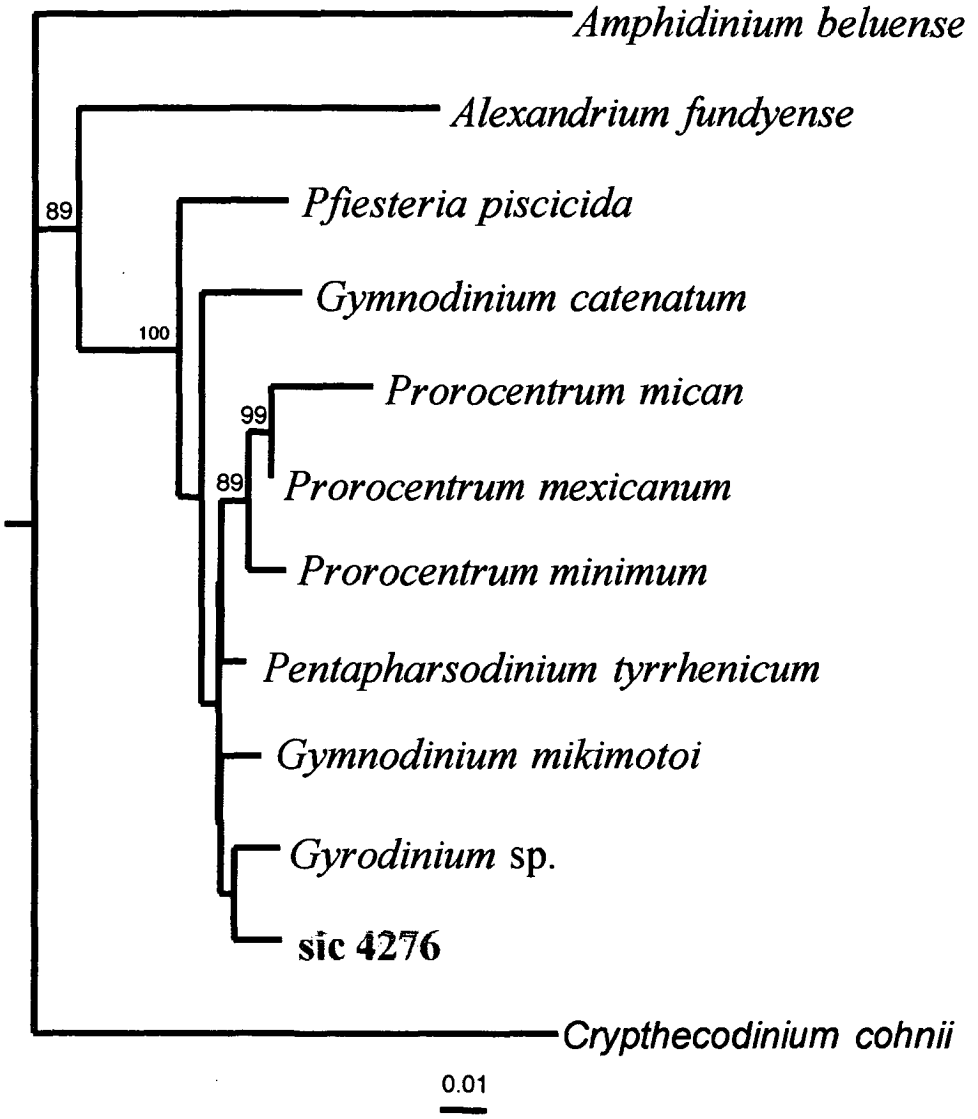


Figure 3.10: Evolutionary distance dendrogram created from 18S rDNA sequences of some members of the *Dinophyceae* and sic 4276. *Crypthecodinium cohnii* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

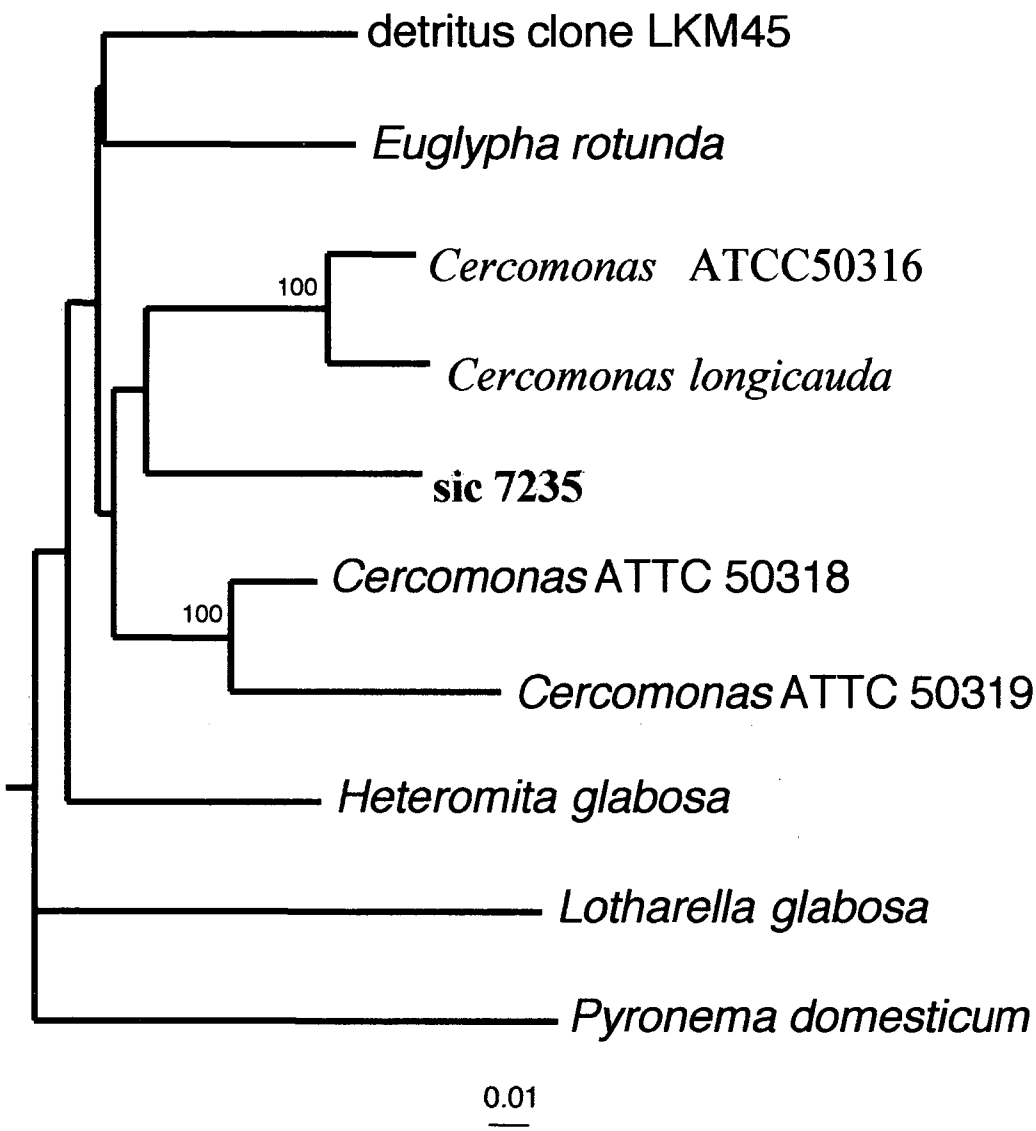


Figure 3.11:Evolutionary distance dendrogram of some members of the *Cercomonadidae*, *Euglyphidae*, *Heteromitidae* and *Chlorarachniophyceae* and sic 7235. *Pyronema domesticum* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (500 replicates) have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

3.4 Biodiversity and Ecophysiology

Measures of genetic diversity were determined following normalisation of the clone libraries by the rarefaction method. Indices indicating biodiverity coverage (C) (Mullins, *et al.* 1995), diversity (Shannon-Weaver index H'), dominance (Simpson index SI'), evenness (J') and species richness (Chao) are displayed in Table 3.

Table 3.2: Biodiversity indices for Antarctic and Arctic sea ice samples. NA indicates the indice could not be calculated due to the absence of doublets in the sample.

Clone Library	Coverage (%)	Diversity (H')	Dominance (Si)	Evenness (J')	Species Richness (Chao)
1	57.1	0.495	0.45	0.586	11.5 ± 5.4
4	50.0	1.037	0.13	0.826	26.1 ± 5.8
7	42.9	0.402	0.58	0.476	NA
8	47.1	1.128	0.09	0.917	57.5 ± 27.8
B8	62.5	0.674	0.27	0.746	12.5 ± 5.4
9	55.5	0.757	0.18	0.794	NA
B9	100.0	0.378	0.48	0.793	NA
10	53.8	1.050	0.13	0.943	38.5 ± 19.1
McMurdo	42.9	0.482	0.46	0.570	NA
Arctic	53.9	0.945	0.13	0.849	31.0 ± 15.17

The coverage of biodiversity was quite high. Values ranged between 42.9-62.5% except for library B9 for which coverage was calculated at 100%. Library B9 was created from *Bacteria*-specific primers in PCR reactions using the same environmental DNA as Library 9. The 100% coverage value obtained here is obviously an aberration, possibly reflecting the potential bias of the PCR method. Only three phylotypes were detected in the library and all occurred with a frequency greater than one. Comparison with the universally-primed library from the same sample (Library 9) shows the true biodiversity of the sample is much greater. Such results prove as timely reminders of the inherent limitations of these molecular techniques and the importance of remembering that the phylotype abundance, diversity and richness values of all samples are not to be taken as strictly accurate reflections of the actual community structure.

Five of the libraries (1, 7, B8, B9 and McMurdo) had very high dominance values indicating that one or two phylotypes accounted for most of the clones in these libraries. Consequently these libraries also had the lowest diversity values and also low evenness values. Again these results may be a reflection of bias in the PCR method, however it is also possible these results reflect high *in situ* abundance of the dominant genes. A number of multifaceted studies have reported good correlations between clone library findings and actual community species abundance (eg Sahm, *et al.* 1999, Knoblauch, *et al.* 1999, Snaidr, *et al.* 1997, Giovannoni, *et al.* 1996, Gordon, *et al.* 1996). Although the methods used in this study were optimised towards the recovery of prokaryotic DNA, genes from a number of Eukaryotic organisms were also recovered. Indeed clones representing plastid 16S rDNA were prevalent in all clone libraries except that of sample 1, which was used as an example of sea ice without a developed microbial community, and sample 7. Given that phytoplankton cells are likely to have high copy numbers of chloroplasts (eg *Heterosigma akashiwo* contains approximately 650 chloroplast genomes per cell (Ersland, *et al.* 1981) it is expected there would indeed be large quantities of plastid DNA in any community sample where phytoplankton susceptible to the recovery procedure were present. Similarly, in sample 1, genes from *Shewanella frigidimarina* and *Pseudoalteromonas* species dominated the library. These organisms are abundant in Antarctic seawater (Bowman, *et al.* 1997b) and would likely be present in similar abundance in a sea ice community where, after initial formation and colonisation, there has been little or no community development.

The greatest diversity and species richness values were observed in sample 8. This diversity appears to stem from the presence of a well developed microbial community, evidence of which was visible upon collection in the form of colouration throughout the core structure. Over 20 bacterial phylotypes were detected (from both *Bacterial* and universally primed libraries) along with autotrophic and heterotrophic eukaryotic organism, indicating the presence of a functional microbial loop.

Evidence of broad species diversity within the sea ice ecosystem comes from pairwise comparisons of organismal composition (Table 4). Very few phylotypes are shared by any two libraries and none by more than two libraries.

Table 3.3: Pairwise comparisons of sea ice clone library phylotype composition.

Sample 1									
Sample 4	0.000								
Sample 77	0.000	0.000							
Sample 8	0.000	0.154	0.000						
Sample B8	0.000	0.000	0.000	0.286					
Sample 9	0.000	0.000	0.111	0.000	0.000				
Sample B9	0.000	0.000	0.000	0.000	0.000	0.154			
Sample 10	0.111	0.111	0.000	0.000	0.000	0.000	0.000		
McMurdo	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
Arctic	0.000	0.111	0.000	0.286	0.154	0.000	0.000	0.000	0.000
	S1	S4	S7	S8	SB8	S9	SB9	S10	MCMurdo

In essence this expands the observed sea ice microbial diversity to encompass nearly all the phylotypes recovered. This diversity speaks to the potential for microbial community adaption to changing environmental condition, which in turn increases the efficiency of processes such as nutrient cycling. Such adaption is particularly important in an environment such as sea ice. From the onset of formation to the final stages of melting the environment is in a continual state of flux. Physical processes (such as accretion and consolidation of ice structure, melting and thawing cycles and convective flushing of water through the system) and biological processes (such as alterations in composition of primary production, competition and predation) combine to create large and small scale heterogeneities within the ecosystem, providing a diverse array of microbial niches for occupancy by the well adapted organisms present.

A graph of the distribution of major phylogenetic groups (Figure 13) further highlights the differences in community composition over the samples examined.

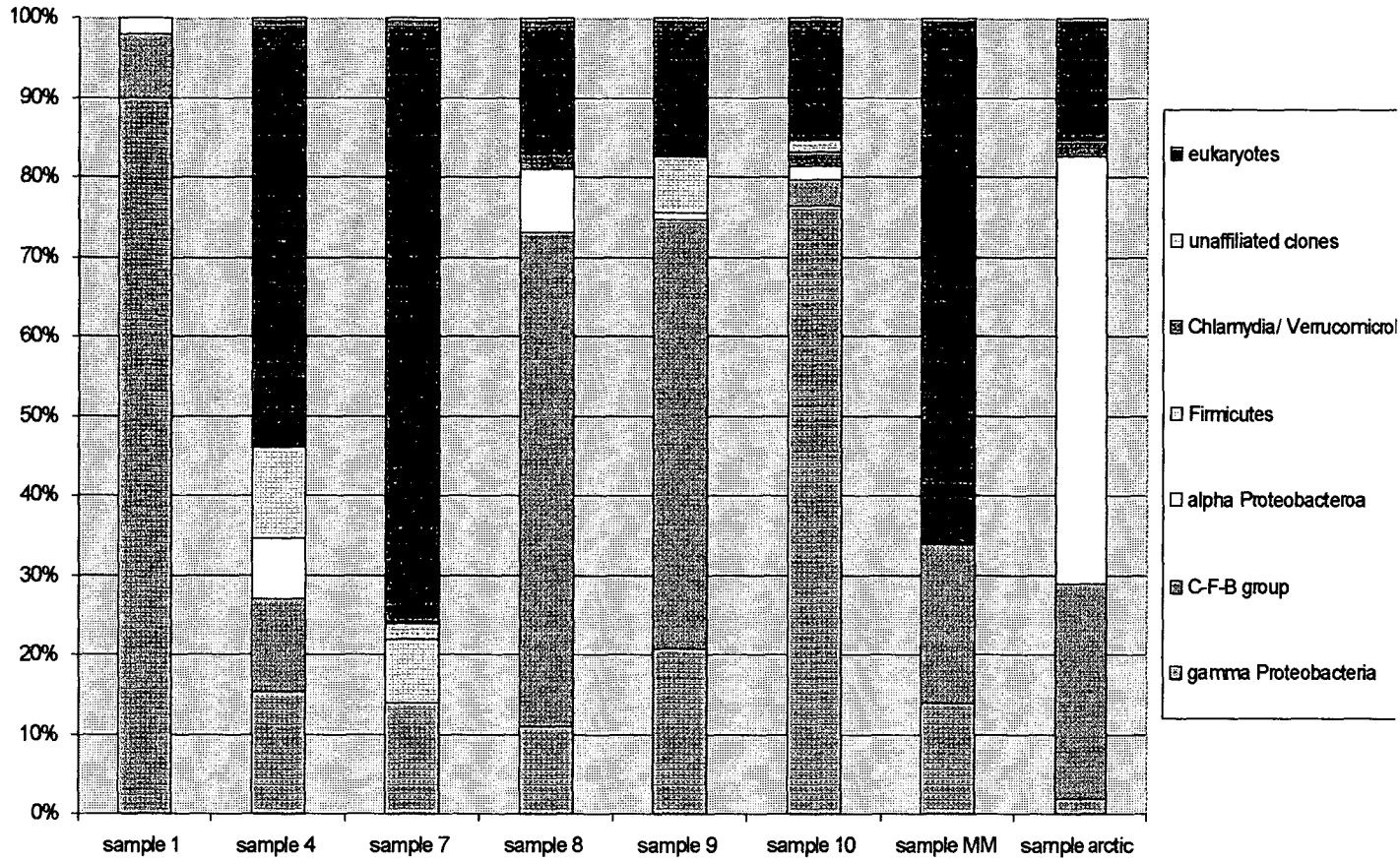


Figure 3.12: Graph displaying the percentage of clones in each sample associated with distinct phylogenetic lineages.

Both diversity and community structure change dramatically when phytoplankton is associated with the sample. An increase in the occurrence and diversity of organisms from the *Cytophaga-Flexibacter-Bacteroides* group, especially in the marine *Flavobacteriaceae*, is apparent in the pack ice samples associated with developed microbial communities. This was expected given the results of previous studies. Glockner *et al.* (1999) and Simon *et al.* (1999), in a fluorescent *in situ* hybridization study, found that a uniform and clearly detectable bloom of members of this phylogenetic lineage coincided with a *Phaeocystis* algal bloom. Indeed during this bloom the *Cytophaga-Flexibacter-Bacteroides* group accounted for 72% of all DAPI stained bacteria. They concluded that the mucus- and substrate-rich environment presented by the algae favoured the establishment of blooms of psychrophilic *Cytophaga-Flavobacterium* populations (Simon *et al.* 1999). Many members of this group are non-motile or capable of gliding motility and therefore usually inhabit the

surface of diatoms, phytoplankton, heterotrophic zooplankton, organic detritus and phyto-aggregates (Rath, *et al.* 1998). They are also characterised by the production of extracellular enzymes (e.g. proteases, glucosidases, phosphatases, lipases and chitinases) which enable them to degrade a wide variety of substrates ranging from proteins and carbohydrates to complex macromolecules (Bernardet, *et al.* 1996). It appears likely therefore that these organisms are responsible for the initial breakdown of primary production from sea ice microbial assemblages and are hence critical to the nutrient cycling within these ecosystems. Bacteria such as the *Celluliphaga* species, that are capable of attacking cells using hydrolytic enzymes, are also likely to be important in regeneration of silicon, a process vital to the sustained production of diatom assemblages. Biddle and Azam (1999) reported that bacteria-mediated silicon regeneration rates could explain most of the reported upper-ocean silicon regeneration. Many species from the family *Flavobacteriaceae* are psychrophilic, including species from the genera *Polaribacter*, *Psychroserpens*, and *Gelidibacter*, along with other unclassified strains. Such selection for psychrophiles within sea ice microbial communities has been observed in several culture based studies (Bowman, *et al.* 1997a, 1997b, Delille, 1992, Helmke and Weyland, 1995). The colonisation of phytoplankton by these bacteria provides a relatively stable environment with high substrate levels. Theoretically, under such conditions, in a permanently cold environment, psychrophiles will grow more rapidly and compete successfully with psychrotolerant organisms (Harder and Veldcamp, 1971, Nedwell, 1999). Similarly, there is a transition from psychrotolerant to predominantly psychrophilic organisms within the gamma *Proteobacteria.* The psychrotolerant gamma Proteobacterial species which dominated the clear ice sample 1, *Shewanella frigidimarina* and *Marinomonas* species, are replaced in the more developed communities by psychrophilic organisms, such as *Colwellia hornerae*, *Psychrobacter glacincola*, the psychrophilic species *Shewanella gelidimarina* and sea ice strains IC169 and IC038. Again it is likely that the psychrophiles compete successfully with the psychrotolerant organisms in the high nutrient environment.

Species of the psychrotolerant genus *Pseudoalteromonas* were common in all samples. This finding is in agreement with that of Bowman *et al.* (1997a) who found these bacteria to be the most commonly isolated psychrotrophs from the Antarctic sea ice environment. It appears these bacteria are capable of competing with psychrophilic

organisms in both high and low nutrient conditions, perhaps because of their ability to attack cells, produce a number of bioactive extracellular compounds, including antimicrobial compounds, and utilize a wide variety or organic substrates (Holmstrom and Kjelleberg 1999).

The process of psychrophilic enrichment conceivably starts during the process of sea ice formation. In regions where ice is forming in the absence of marine phytoplankton assemblages there is negligible bacterial enrichment. Free living pelagic bacteria, which are predominantly psychrotolerant (Bowman *et al.* 1997b, Delille, 1993, Helmke and Weyland, 1995, Zdanowski and Donachie, 1993) are incorporated into the ice matrix by the scavenging action of frazil ice crystals or particulate organic matter. A combination of factors including high salinities and processes such as brine drainage cause a suppression of bacterial activity during early ice formation (e.g. Grossmann and Gleitz, 1993). This environment would favour certain bacteria, e.g. those capable of withstanding the increased salinities, and lead to a decrease in diversity as seen in sample 1. Where sea ice forms in the presence of high algal concentrations, incorporation of algae into the ice matrix results in a concomitant enrichment of bacteria (possibly psychrophilic) which are attached to the cell surfaces (e.g. Weissenberger and Grossmann, 1998). Again these organisms will experience extreme physical conditions, including (importantly for phytoplankton and diatom assemblages) physical confinement within the ice matrix, and experience a decline in production. However those that survive will form the basis of a productive microbial community leading to those conditions described above which are selective for psychrophilic bacteria.

The impact of bacterial regeneration of nutrients within Antarctic sea ice was questioned by Gunther *et al.* (1990). They suggested that microbial metabolism of primary production may not represent a major pathway of nutrient regeneration. It was speculated that high *in situ* nutrient levels result from the 'liberation' of dissolved matter during algal cell lysis, rates of which would be amplified by inefficient feeding by metazoan grazers. It is likely that this process, and physical enrichment by convective flushing of sea ice by nutrient rich seawater, are indeed major pathways of nutrient replenishment in sea ice microbial communities. When these processes are inactive then bacterial regeneration would assume a primary role, however this may

not be sufficient to sustain a microbial community for extended periods. This would be likely to occur in fast ice regions where there is little wave action to fuel convective flushing and consolidated ice structures impose physical limitations on the presence of grazers. McMinn *et al.* (1999) reported severe and progressive nutrient limitation with distance from the ice/ water interface in fast ice from McMurdo Sound. The effect of this on community structure is evident in the fast ice sample 10 taken from Prydz Bay. This ice was very thick and highly consolidated, with remnants of productive microbial assemblages obvious throughout. Decreased nutrient levels resulting from the breakdown of nutrient replenishment mechanisms see a return to prominence of heterotrophic, psychrotolerant bacteria, such as *Shewanella frigidimarina*, *Psychrobacter* and *Pseudoalteromonas* species, along with a dramatic decrease in members from *Cytophaga-Flexibacter-Bacteroides* group. Here it appears bacterial nutrient replenishment was not sufficient to sustain a microbial community such as those evident in the pack ice samples.

The community structure of the sample from the arctic sea ice made an interesting comparison to the Antarctic samples. The appearance of this sample indicated the presence of a developed microbial community (Connie Lovejoy, pers. comm.). Similar to the analogous Antarctic samples, this sample showed quite high numbers and diversity of potentially psychrophilic organisms from within the *Cytophaga-Flexibacter-Bacteroides* group. However the vast majority of clones were associated with the psychrophilic, gas vacuolate organisms of the genus *Octadecabacter* from the alpha *Proteobacteria.* The dominance of these organisms in the clone library is inconsistent with the report of Gosink *et al.* (1993) who found only low levels of gas vacuolate bacteria within the arctic sea ice using traditional cultivation techniques. The high occurrence of *Octadecabacter* clones in this study may be due to natural population variations within the sea ice ecosystem or may be a result of PCR bias.

3.5 Biogeography of Sea Ice bacteria.

The environmental pressures of the sea ice habitat select for highly adapted bacteria. This selection implies potential for the evolutionary divergence of these organisms from temperate latitude microbiota (Vincent, 2000). Whether this evolution has occurred in isolation from other sea ice environments will determine whether or not

these organisms are endemic. Such is the focus of studies in biogeography, which seek to fully elucidate aspects of biodiversity, ecophysiology and extinction.

Staley and Gosink (1999) described three genera of sea ice microorganisms common to both poles, including *Octadecabacter*, *Polaribacter*, and the, as yet, officially unnamed genus "*Iceobacter*", which appears synonymous with genus *Psychromonas*. However they observed no bi-polar species distribution within these genera. They also asserted that 16S rDNA is too highly conserved to allow for the assessment of polar strains and species.

A 16S rDNA clone library of one sample from the Arctic sea ice environment was created to provide a comparison of community structures between the polar sea ice environments and to highlight common lineages representing organisms which may warrant further study regards their biogeography. Interestingly, over the sequence lengths analysed, the majority of phylotypes within the Arctic clone library were very and most closely related to strains or clones of Antarctic origin. This finding may reflect the lack of intense, taxonomically directed culture based studies from the Arctic sea ice environment. However, further to this finding, in the pairwise comparisons of libraries (Table 3), the Arctic library displayed greater phylotype similarity to the libraries from Antarctic sea ice than did any of the Antarctic sea ice libraries to the others. Six Arctic phylotypes fell within the *Cytophaga-Flexibacter-Bacteroides* group. Of these, five were most closely related to Antarctic organisms, although one, represented by Arctic 16 was related only at a relatively large evolutionary distance. The phylotype represented by Arctic 117 grouped (100% bootstrap support) with the Antarctic sea ice strain IC076 at an evolutionary distance of 0.0077. The phylotype represented by Arctic 121 grouped (91% bootstrap support) with the Antarctic sea ice clone 42372 from this study along with seawater and deep sea sediment clones. It was most closely related to the sic 42372 at an evolutionary distance of 0.0046. The phylotype represented by Arctic 123 grouped (100% bootstrap support) with three Antarctic sea ice clones from this study (sic clones 815, 8119 and B8127) along with a clone from Antarctic lake sediments (Organic-5). It was most closely related to sic 8119 at an evolutionary distance of 0.0045. The phylotype represented by Arctic 156 grouped (86% bootstrap support) with *Flavobacterium xylanivorum* and was most closely related to this species at an evolutionary distance of 0.0077. *Flavobacterium*

xylanivorum was isolated from British Antarctic territory. Within the gamma *Proteobacteria*, the phylotype represented by McMurdo 211 (derived from Antarctic sea ice from McMurdo Sound) was equally closely related to *Psychromonas antarctica* and the Arctic gas vacuolate sea ice strain 37. Within the alpha Proteobacteria, three phylotypes, represented by Arctic clones 128, 153, 235, were associated with the genus *Octadecabacter*. Two of these phylotypes were more or equally closely related to *Octadecabacter antarcticus* than to the Arctic species of the genus. Within the eukaryotes, the plastid clone Arctic 149 was closely related to the plastid clones or Antarctic origin. Rappé *et al.* (1995) suggest that plastid genes may be more suitable than nuclear genes in the study of phytoplankton biogeography. If it were shown that species of phytoplankton within the polar sea ice zones were endemic or otherwise it would have implications for the status of the associated bacterial assemblages.

As described previously, the species of the genus *Octadecabacter* have highly similar 16S rDNA sequences, however, sequences alone cannot elucidate clearly the interspecific relationships. This highlights the lack of sensitivity associated with using single genes for phylogenetic analyses and the absolute requirement for isolation of bacteria for the further study of biogeography. However, by identifying additional diversity within those lineages examined by Staley and Gosink (1999) (i.e. the genera *Octadecabacter*, *Polaribacter* and *Psychromonas*), this study has underlined the need for further examination of the biogeography of these organisms. The results herein have also highlighted at least four further bacterial genera common to both poles and containing organisms with very closely related 16S rDNA sequences. Examination of the biogeography of these organisms will further the understanding of potential bacterial endemism within polar sea ice.

3.6 Further work.

The real value of a study such as this lies in the foundations it builds for future research. Information highlighting the affiliations of uncultured microorganisms within an environment may, together with information on the physico-chemical conditions of the habitat, facilitate more directed cultivation attempts. Such work will be particularly important when considering the biogeography of polar sea ice

organisms and the ecological impact of lineages such as the *Verrucomicrobia* and the *Rubrobacteridae* which are hitherto poorly represented by cultivated organisms. Clearly, given the potential biases inherit in the molecular methods utilised in this study, further work is required to determine more accurately the community composition of the sea ice environment. Species diversity will be affected by temporal and spatial heterogeneities, competition, predation, environmental stability and levels of community production. Understanding the impacts of these forces on microbial communities requires quantitative information. This will come from using tools such as substrate-tracking autoradiographic fluorescent in situ hybridization (STARFISH) (Ouverney and Fuhrman, 1999), whereby total direct counting, fluorescent in situ hybridization using group specific 16S rDNA probes, and microautoradiographic tracking of H^3-substrate uptake are combined on a single slide. This technique allows determination of which specific groups of bacteria are dominant and active within a population at any given time, the study of the effects on these bacteria of environmental pressures such as alterations in nutrient availability and composition, and mortality due to grazing or virus infection. The focus of such efforts is largely established by studies such as this, which serve to identify abundant lineages within a community and to provide additional nucleic acid sequences for 16S rDNA probe design.

In addition to examination of genetic diversity through conserved genes, it will also be beneficial in the future to study the functional diversity of a community, i.e. the presence of other genes used in direct interactions with the environment. Although genetic diversity may imply functional diversity it is only through determination of the genes utilised by well adapted bacteria to survive in an environment that full appreciation of a systems ecology will be realised. Such studies will also serve to identify potentially novel genes for use in biotechnological applications, and so serve to justify scientific expenditure to the general community.

4. References

Ackley, S. F., Diekmann, G. and Shen, H. 1987 Algal and foram incorporation into new sea ice. *EOS* **68**: 1736

Ackley, S. F. and Sullivan, C. W. (1994) Physical controls on the development and characteristics of Antarctic sea ice biological communities - a review and synthesis. *Deep Sea Res. I* **41** : 1583 - 1604

Aghajari, N., Feller, G., Gerday, C. and Haser, R. (1996) Crystallization and preliminary x-ray diffraction studies of alpha-amylase from the Antarctic psychrophile *Alteromonas haloplanctis* A23. *Prot. Sci.* **5**: 2128-2129

Altschul, S. F., Madden, T. L., Sch ffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* **25**:3389-3402

Amann, R., Springer, N., Schoenhuber, W., Ludwig, W., Schmid, E. N., Mueller, K. D. and Michl, R. (2000) Obligate intracellular bacterial parasites of *Acanhamoebae* related to *Chlamydia Appl. Environ. Microbiol.* In press

Arahal, D. R., Marquez, M. C., Volcani, B. E., Schleifer, K. H. and Ventosa, A. (1999) *Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea. *Int. J. Syst. Bacteriol.* **49**: 521-530

Azam, F., Fenchel, T., Field, J. G., Gray, J. S., Meyer-Reil, L. A. and Thingstad, F. (1983) The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* **10** : 257 - 263

Bathmann, U. V., Scharek, R., Klaas, C., Dubischar, C. D. and Smatacek, V. (1997) Spring development of phytoplankton biomassand composition in major water masses of the Atlantic sector of the Southern Ocean. *Deep Sea Res. II* **44**: 51-67

Bell, W. and Mitchell, R. (1972) Chemotactic and growth responses of marine bacteria to algal extracellular products. *Biol. Bull.* **143**: 265 - 277

Bernardet, J. F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme. (1996) Cutting a gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Stohl and Tait 1978). *Int. J. Syst. Bacteriol.* **46**:128-148

Bidle, K. D. and Azam, F. (1999) Accelerated dissolution of diatom silica by marine bacterial assemblages. *Nature* **397**: 508-512

Billen, G. and Becquevort, S. (1991) Phytoplankton-bacteria relationship in the Antarctic marine ecosystem. Pro Mare Symposium on Marine Arctic Ecology, Trondheim, Norway.

Bowman, J. P. (1998) *Pseudoalteromonas prydzensis* sp. nov., a psychrotrophic, halotolerant bacterium from Antarctic sea ice. *Int J. Syst. Bacteriol.* **48**:1037-1041

Bowman, J. P., Brown, M. V. and Nichols, D. S. (1997a) Biodiversity and ecophysiology of bacteria associated with Antarctic sea ice. *Antarctic Science* **9**: 134-142

Bowman, J. P., Gosink, J. J., Mccammon, S.A., Lewis, T.E., Nichols, D. S., Nichols, P. D., Skerratt, J. H., Staley, J. T. and McMeekin, T. A. (1998a) *Colwellia demingiae* sp. nov., *Colwellia hornerae* sp. nov., *Colwellia rossensis* sp. nov. and *Colwellia psychrotropica* sp. nov - psychrophilic Antarctic species with the ability to synthesize docosahexaenoic acid (22/6-omega-3). *Int. J. Syst. Bacteriol.* **48**: 1171-1180

Bowman, J. P., McCammon, S. A., Brown, M. V., Nichols, D. S. and McMeekin, T. A. (1997b) Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl. Environ. Mircobiol.* **63**: 3068-3078

Bowman, J. P., Mccammon, S. A., Brown, J. L., Nichols, P. D. and McMeekin, T. A. (1997c) *Psychroserpens burtonensis* gen. nov, sp. nov, and *Gelidibacter algens* gen. nov, sp. nov, psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. *Int. J. Syst. Bacteriol.* **47**: 670-677

Bowman, J. P., Mccammon, S. A., Brown, J. L. and McMeekin, T. A. (1998b) *Glaciecola punicea* gen. nov., sp. nov. and *Glaciecola pallidula* gen. nov., sp. nov - psychrophilic bacteria from Antarctic sea-ice habitats. *Int. J. Syst. Bacteriol.* **48**: 1213-1222

Bowman, J. P., Mccammon, S. A., Lewis, T., Skerratt, J. H., Brown, J. L., Nichols, D. S. and McMeekin, T. A. (1998c) *Psychroflexus torquis* gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of *Flavobacterium gondwanense* (Dobson et al. 1993) as *Psychroflexus gondwanense* gen. nov., comb. nov. *Microbiology.* **144**: 1601-1609

Bowman, J.P., McCammon, S.A. and McMeekin, T.A. (2000) Diversity and community structure of Antarctic maritime lake and fjord benthic zones. *Environmental Microbiology* In Press.

Bowman, J. P., Mccammon, S. A., Nichols, D. S., Skerratt, J. H., Rea, S. M., Nichols, P. D. and McMeekin, T. A. (1997d) *Shewanella gelidimarina* sp. nov. and *Shewanella frigidimarina* sp. nov., novel antarctic species with the ability to produce eicosapentaenoic acid (20/5-omega-3) and grow anaerobically by dissimilatory Fe(iii) reduction. *Int. J. Syst. Bacteriol.* **47**: 1040-1047

Bowman, J. P., McCammon, S. A., Rea, S. M. and McMeekin, T. A. (2000b) The microbial composition of three limnologically disparate hypersaline Antarctic lakes ?? in press

Bowman, J. P., Nichols, D. S. and McMeekin, T. A.(1997e) *Psychrobacter glacincola* sp. nov, a halotolerant, psychrophilic bacterium isolated from Antarctic sea ice. *Syst. Appl. Microbiol.* **20**: 209-215

Bratbak, G. and Thingstad, T. F. (1985) Phytoplankton-bacteria interactions: an apparent paradox? Analysis of a model system with both competition and commensalism. *Mar. Ecol. Prog. Ser.* **25**: 23 - 30

Broeze, R. J., Solomon, C. J. and Pope, D. H. (1978) Effects of low temperature on *in vivo* and *in vitro* protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. *J. Bacteriol.* **134**: 861 - 874

Caron, D. A., Davis, P. G., Madin, L. P. and Sieburth, J. McN. (1982) Heterotrophic bacteria and bacterivorous protozoa in oceanic macroaggregates. *Science.* **218** : 795 - 797

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scand. J. Stat.* **11**: 265-270

Christian, J. R. and Karl, D. M. (1995) Bacterial ectoenzymes in marine waters: Activity ratios and temperature responses in three oceanographic provinces. *Limnol. Oceanogr.* **40**: 1042 - 1049

Chróst, R. J. (1992) Significance of bacterial ectoenzymes in aquatic environments. *Hydrobiologia* **243**: 61-70

Clarke, D. B. and Ackley, S. F. (1984) Sea ice structure and biological activity in the Antarctic marginal ice zone. *Journal of Geophysical Research* **89**: 2087-2095

Cota, G. F., Kottmeir, S. T., Robinson, D. H., Smith, Jr, W. O. and Sullivan, C. W. (1990) Bacterioplankton in the marginal ice zone of the Weddell Sea: biomass, production and metabolic activities during austral autumn. *Deep Sea Research* **37**: 1145-1167

Courties, C., A. Vaquer, M. Troussellier, J. Lautier, M. J. Chretiennot-Dinet, J. Neveux, C. Machando, and H. Claustre. (1994). Smallest eukaryotic organism. *Nature* **370**: 255

Daly, K. L. (1990) Overwinter development, growth and feeding of larval *Euphausia superba* in the Antarctic marginal ice zone. *Limnol. Oceanogr.* **35**: 1564-1576

De Baar H. J. W., De Jong, J. T. M., Bakker, D. C. E., B. M. Loscher, Veth, C, Bathmann, U. and Smatacek, V. (1995) Importance of iron for plankton blooms and carbon dioxide drawdown in the Southern Ocean. *Nature* **373**: 412-415

Deickmann, G. S., Lange, M. A. and Ackley, S. F. (1986) Sea ice biota and ice formation processes in the Weddell Sea during winter. *EOS* **67**: 1005

Delille, D. (1992) Marine bacterioplankton at the Weddell Sea ice edge: distribution of psychrophilic and psychrotrophic populations. *Polar Biol.* **12**: 205-210

Delille D. (1996) Biodiversity and function of bacteria in the Southern Ocean *Biodivers. Conserv.* **5**: 1505-1523

Delille, D., Bouvy, M. and Cahet, G. (1988) Short-term variations of bacterioplankton in Antarctic zone: Terre Adelie area. *Microb. Ecol.* **15**: 293-309

Delille, D. and Perret, E. (1989) Influence of temperature on the growth potential of southern polar marine bacteria. *Microb. Ecol.* **18**: 117-123

Delille, D., Fiala, M. and Rosiers C. (1995) Seasonal changes in phytoplankton and bacterioplankton distribution at the ice-water interface in the Antarctic neritic area. *Mar. Ecol.-Prog. Ser.* **123**: 225-233

Delille, D., Mallard, L. and Rosiers, C. (1996) Inter-annual variability in marine coastal Antarctic bacterioplankton. *Polar Biol.* **16**: 19-25

Delille, D. and Rosiers, C. (1996) Seasonal changes of Antarctic marine bacterioplankton and sea ice bacterial assemblages. *Polar Biol.* **16**: 27-34

Delong, E. F. (1992) Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA.* **89**: 5685-5689

DeLong, E. F., Franks, D. G. and Alldredge, A. L. (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol. Oceanogr.* **38**: 924-934

DeLong, E. F. and Yayanos, A. A. (1986) Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. *Appl. Environ. Microbiol.* **51**: 730-737

Delong, E. F., Ke Ying Wu, Prezelin, B. B. and Jovine, R. V. M. (1994) High abundance of Archaea in Antarctic marine picoplankton. *Nature* **371**: 695-697

Devereux, R. and Stahl, D. A. (1993) Phylogeny of Sulfate-Reducing Bacteria and a Perspective for Analyzing Their Natural Communities. *The Sulfate-Reducing Bacteria: Contemporary Perspectives*. J. M. Odom and R. Singleton Jr. New York, Springer-Verlag: 131-160

Dobson, S. J. (1993) The phylogeny of members of the *Halomonadaceae* and some Antarctic *Flavobacteria*. PhD. thesis. Dept. of Agricultural Science, University of Tasmania

Dobson, S. J., McMeekin, T. A. and Franzmann, P. D. (1993) Phylogenetic relationships between some members of the genera *Deleya*, *Halomonas*, and *Halovibrio*. *Int. J. Syst. Bacteriol.* **43**: 665-673

Dojka, M. A., Hugenholtz, P., Haack, S. K. and Pace, N. R. (1998) Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl. Environ. Microbiol.* **64**: 3869-3877

Dower, K. M., Lucas, M. I., Phillips, R., Diekmann, G. and Robinson, D. H. (1996) Phytoplankton biomass, P-I relationships and primary production I the Weddell Sea, Antarctica, during the austral autumn. *Polar Biol.* **16**: 41-52

El-Sayed, S. Z. (1971) Biological aspects of the pack ice ecosystem. In Deacon, G. (ed.), *Symposium on Antarctic Ice and Water Masses.* Pp.34-554 Tokyo: Scientific Committee on Antarctic Research

El-Sayed, S. Z. and Fryxell, G. A. (1993) Phytoplankton. in Friedmann, E. I. (ed.) *Antarctic Microbiology* Wiley-Liss, inc. New York

Elser, J. J., Stabler, L. B. and Hassett, R. P. (1995) Nutrient limitation of bacterial growth and rates of bactivory in lakes and oceans: a comparative study. *Aquat. microb. Ecol.* **9**: 105-110

Ersland, D. R., Aldrich, J. and Cattolico, R. A. (1981). Kinetic complexity, homogeneity and copy number of chloroplast DNA from the marine alga *Olisthodiscus luteus. Plant. Physiol.* **68**: 1468-1473

Felsenstein, J. (1993) PHYLIP v. 3.57c (phylogenetic inferece program package). University of Washington, Seattle, USA

Field, K. G., Gordon, D., Wright, T., Rappé, M., Urbach, E., Vergin, K. and Giovannoni, S. J. (1997) Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. *Appl. Environ. Microbiol.* **63**: 63-70

Freidmann, E. I. (1993) (ed.), *Antarctic microbiology.* Williams and Wilkins, Baltimore.

Fuhrman, J. A., Comeau, D. E., Hagstrom, A. and Chan, A. M. (1988) Extraction of natural planktonic microorganisms of DNA suitable for molecular biological studies. *Appl. Environ. Microbiol.* **54**: 1426-1429

Fuhrman, J. A., McCallum, K. and Davis, A. A. (1993) Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific oceans. *Appl. Environ. Microbiol.* **59**: 1294-1302

Garrison, D. L. (1991) Antarctic sea ice biota. *Amer. Zool.* **31**: 17-33

Garrison, D. L., Ackley S. F., Buck, K. R. (1983) A physical mechanism for establishing algal populations in frazil ice. *Nature* **306**: 363–365

Garrison, D. L. and Buck, K. R. (1989) The biota of Antarctic pack ice in the Weddell Sea and Antarctic Peninsula regions. *Polar Biol.* **10**: 211-219

Garrison, D. L. and Buck, K. R. (1986) Organism losses during ice melting: a serious bias in sea ice community studies. *Polar Biol.* **10**: 211-219

Garrison, D. L., Buck, K. R. and Fryxell, G. (1987) Algal assemblages in Antarctic pack ice and in ice edge plankton. *J. Phycol.* **23**: 564-572

Garrison, D. L., Buck, K. R. and Silver, M. W. (1984) Microheterotrophs in the ice-edge zone. *Ant J. U.S.* **19**: 109-111

Garrison, D. L. and Close, A. R. (1993) Winter ecology of the sea ice biota in the Weddell Sea pack ice. *Mar. Ecol. Prog. Ser.* **96**: 17-31

Giesenhagen, H. C., Detme, A. E., de Wall, J., Weber, A., Gradinger, R. R. and Jochem, F. J. (1999) How are Antarctic planktonic microbial food webs and algal blooms affected by melting of sea ice? Microcosm simulations. *Aquatic Microbial Ecology.* **20**: 183-201

Giovannoni, S. J., M. S. Rappe, K. L. Vergin, and N. L. Adair. (1996) 16S rRNA genes reveal stratified open ocean bacterioplankton populations related to the green non-sulfur bacteria. *Proc. Natl. Acad. Sci. USA* **93**: 7979-7984

Gleitz, M., Bathmann, U. V. and Lochte, K. (1994) Build-up and decline of summer phytoplankton biomass in the eastern Weddell Sea, Antarctica. *Polar Biol.* **14**: 413-422

Gleitz, M. and Thomas, D. N. (1993) Variation in phytoplankton standing stock, chemical composition and physiology during sea ice formation in the southeastern Weddell Sea, Antarctica. *J. Exp. Mar. Biol. Ecol* **173**: 211-230

Gleitz M., Vonderloeff, M. R., Thomas, D. N., Dieckmann, G. S. and Millero, F. J. (1995) Comparison of summer and winter inorganic carbon, oxygen and nutrient concentrations in Antarctic sea ice brine. *Mar. Chem.*. **51**: 81-91

Glockner, F. O., Fuchs, B., M. and Amann, R. (1999) Bacterioplankton composition of lakes and oceans: a first comparison based on fluorescence *in situ* hybridization. *Appl. Environ. Microbiol.* **65**: 3721-3726

Gonzalez, J. M. and Moran, M. A. (1997) Numerical dominance of a group of marine bacteria in the alpha-subclass of the class proteobacteria in coastal seawater *Appl. Environ. Microbiol.* **63**: 4237-4242

Gonzalez, J. M., Whitman, W. B., Hodson, R. E. and Moran, M. A. (1996) Identifying numerically abundant culturable bacteria from complex communities: an example from a lignin enrichment culture. *Appl. Environ. Microbiol*, **62**: 4433-4440

Gordon, D A., and S. J. Giavannoni. (1996) Detection of stratified microbial populations related to *Chlorobium* and *Fibrobacter* in the Atlantic and Pacific oceans. *Appl Environ. Microbiol.* **62**: 1171-1177

Gosink, J. J., Irgens, R. L. and Staley, J. T. (1993) Vertical distribution of bacteria in arctic sea ice *FEMS microbial Ecology* **102**: 85-90

Gosink, J. J., Herwig, R. P. and Staley, J. T. (1997) *Octadecabacter arcticus* gen. Nov., sp. Nov., and *O. antarcticus*, sp.nov., Nonpigmented, psychrophilic gas vacuolate bacteria from polar sea ice and water. *System. Appl. Microbiol.* **20**: 356-365

Gosink, J. J., Woese, C. R., and Staley, J. T. (1998) *Polaribacter* gen. nov., with three new species, *P-irgensii* sp. nov., *P-franzmannii* sp. nov., and *P-filamentus* sp. nov., gas vacuolate polar marine bacteria of the *Cytophaga-Flavobacterium-Bacteroides* group and reclassification of *Flectobacillus glomeratus* as *Polaribacter glomeratus* comb. nov. *Int. J. Syst. Bacteriol.* **48**: 223-235

Gray, J. P. and Herwig, R. P. (1996) Phylogenetic analysis of the bacterial communities in marine sediments. *Appl. Environ. Microbiol.* **62**:4049-4059

Griffiths, R. P., Caldwell, B. A. and Morita, R. Y. (1982) Seasonal changes in microbial heterotrophic activity in subarctic marine waters as related to phytoplankton primary productivity. *Mar. Biol.* **71**: 121-127

Grossi, S. M., Kottmeier, S. T., Sullivan, C. W. (1984) Sea ice microbial communities. III. Seasonal abundance of microalgae and associated bacteria, McMurdo Sound, Antarctica. *Microb. Ecol.* **10**: 231-242

Grossmann, S. (1994) Bacterial activity in sea ice and open water of the Weddell Sea, Antarctica : A microautoradiographic study. *Microb. Ecol.* **28**: 1-18

Grossmann, S. and Gleitz, M. (1993) Microbial responses to experimental sea ice formation : implications for the establishment of Antarctic sea ice communities. *J. Exp. Mar. Biol. Ecol* **173**: 273-289

Grossmann, S. and Diekmann, G. S. (1994) Bacterial standing stock, activity, and carbon production of sea ice in the Weddell Sea, Antarctica. *Appl. Environ. Microbiol.* **60**: 2746-2753

Gunther, S., Gleitz, M. and Dieckmann, G. S. (1999) Biogeochemistry of Antarctic sea ice: a case study on platelet ice layers at Drescher Inlet, Weddell Sea. *Mar. Ecol. Prog. Ser.* **177**: 1-13

Harder, W. and Veldkamp, H. (1971) Competition of marine psychrophilic bacteria at low temperatures. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **37**: 51-63

Haygood, M. G. and Davidson, S. K. (1997) Small-subunit rRNA genes and in situ hybridization with oligonucleotides specific for the bacterial symbionts in the larvae of the bryozoan *Bugula neritina* and proposal of 'Candidatus endobugula sertula'. *Appl. Environ. Microbiol.* **63**: 4612-4616

Hedlund, B. P., Geiselbrecht, A. D., Bair, T. J. and Staley, J. T. (1999) Polycyclic aromatic hydrocarbon degradation by a new marinebacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov. *Appl. Environ. Microbiol.* **65**: 251-259

Hellebust, J.A. (1965) Excretion of some organic compounds by marine phytoplankton. *Limnol. Oceanogr.* **10**: 192-206

Helmke, E. and Weyland H. (1995) Bacteria in sea ice and underlying water of the eastern Weddell Sea in midwinter. *Mar. Ecol. Prog. Ser.* **11**: 269-287

Heyndrickx, M., Lebbe, L., Kersters, K., Hoste, B., De Wachter, R., DeVos, P., Forsyth, G. and Logan, N. A. (1999) Proposal of *Virgibacillus proomii* sp. nov. and emended description of *Virgibacillus pantothenticus* (Proom and Knight 1950) Heyndrickx *et al. 1998 Int. J. Syst. Bacteriol.* **49**: 1083-1090

Hirons, W. D., Methe, B. A., Nierzwicki-Bauer, S. A. and Zehr, J. P. (1997) Bacterial diversity in Adirondack mountain lakes as revealed by 16S rRNA gene sequences. *Appl. Environ. Microbiol.* **63**: 2957-2960

Hodson, R. E., Azam, F., Carlucci, A. F., Fuhrman, J. A., Karl, D. M. and Holm-Hansen, O. (1981) Microbial uptake of dissolved organic matter in McMurdo Sound, Antarctica. *Mar. Biol.* **61**: 89-94

Holm-Hansen, O. and Vernet, M. (1990) RACER: phytoplankton distribution and rates of primary production during the austral spring bloom. *Antarct. J. U.S* **25**: 141-144

Holmstrom, C., James, S., Neilan, B. A., White, D. C. and Kjelleberg, S. (1998) *Pseudoalteromonas tunicata* sp. nov., a bacterium that produces antifouling agents. *Int. J. Syst. Bacteriol.* **48**:1205-1212

Holmstrom, C. and Kjelleberg, S. (1999) Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol. Ecol.* **30**: 285-293

Horner, R. A. (1985) (ed.) *Sea ice biota* CRC Press, Baco Raton, Florida :

Hugenholtz, P., Pitulle, C., Hershberger, K. L. and Pace, N. R. (1998a) Novel division level bacterial diversity in a Yellowstone hot spring. *J. Bacteriol.* **180**: 366-376

Hugenholtz, P., Goebel, B. M. and Pace, N. R. (1988b) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180**: 4765-4774

Irgens, R. L., Gosink, J. J. and Staley, J. T. (1996) *Polaromonas vacuolata* gen nov, sp nov, a psychrophilic, marine, gas vacuolate bacterium from Antarctica. *Int. J. Syst. Bacteriol.* **46**: 822-826

Irgens, R. L., Suzuki, I. and Staley, J. T. (1989) Gas vacuolate bacteria obtained from marine waters of Antarctica. *Curr. Microbiol.* **18**: 261-265

Jacques, G. (1983) Some eco-physiological aspects of the Antarctic phytoplankton. *Polar Biol.* **2**: 27-34

Janssen, P. H., Schuhmann, A., Morschel, E. and Rainey, F. A. (1997) Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. *Appl. Environ. Microbiol.* **63**: 1382-1388

Junge, K., Gosink, J. J., Hoppe, H. G. and Staley, J. T. (1998) *Arthrobacter, Brachybacterium* and *Planococcus* isolates identified from Antarctic sea ice brine - description of *Planococcus McMeekinii*, sp. nov. *Syst. Appl. Microbiol.* **21**: 306-314

Karl, D. M., Holm-Hansen, 0., Taylor, G. T., Tien, G.and Bird, D. F. (1991) Microbial biomass and productivity in the western Bransfield Strait, Antarctica during the 1986-87 austral summer. *Deep Sea Res.* **38**: 1029-1055

Kausar J. Ohyama Y. Terato H. Ide H. Yamamoto O. (1997) 16S rRNA gene sequence *of Rubrobacter radiotolerans* and its phylogenetic alignment with members of the genus *Arthrobacter*, gram-positive bacteria, and members of the family Deinococcaceae. *Int. J. Syst. Bacteriol.* **47**: 684-686

Kirchman, D. L. (1990) Limitation of bacterial growth by dissolved organic matter in the subarctic Pacific. *Mar. Ecol. Prog. Ser.* **62**: 47-54

Kita-Tsukamoto, K., Oyaizu, H., Nanba, K. and Simidu, U. (1993) Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae*, determined on the basis of 16S rRNA sequences *Int. J. Syst. Bacteriol.* **43**: 8-19

Kivi, K. and Kuosa, H. (1994) Late winter microbial communities in the western Weddell Sea (Antarctica). *Polar Biol.* **14**: 389-399

Knox, G. A. (1994) The biology of the Southern Ocean. *Stud. Polar. Res.* New York, USA, Cambridge University Press.

Kottmeier, S. T. and Sullivan, C. W. (1987) Late winter primary production and bacterial production in sea ice and seawater west of the Antarctic Peninsula. *Mar. Ecol. Prog. Ser* **36**: 287–298

Kottmeier, S. T. and Sullivan, C. W. (1990) Bacterial biomass and production in pack ice of Antarctic marginal ice edge zones. *Deep-Sea Res.* **37**: 1311-1330

Kottmeier, S. T., Grossi, S. M. and Sullivan, C. W. (1987) Sea ice microbial communities. VIII. Bacterial production in annual sea ice of McMurdo Sound, Antarctica. *Mar. Ecol.-Prog. Ser.* **35**: 175-186

Krajewska, E. and Szer, W. (1967) Comparative studies of amino acid incorporation in a cell-free system from psychrophilic *Pseudomonas* sp. **412**. *Eur. J. Biochem.* **2**: 250-256

Krebs, C. J. (1989) *Ecological Methodology*. Harper and Row, New York.

Krembs, C., Gradinger, R. and Spindler, M. (1999) Implications of brine channel geometry and surface area for the interaction of sympagic organisms in Arctic sea ice. *J. Exp. Mar. Biol. Ecol.* **243**: 55-80

Knoblauch, C., B. B. Jorgensen, and J. Harder. (1999) Community size and metabolic rates of psychrophilic sulfate-reducing bacteria in Arctic marine sediments. *Appl. Environ. Microbiol.* **65**: 4230-4233

Lane, D. J. (1991) 16S/23S rRNA Sequencing. In Stackebrandt, E. (ed.) *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, Ltd.

Lancelot, C., Billen, G. and Mathot, S. (1989) Ecophysiology of phyto- and bacterioplankton growth in the Southern Ocean. Belgian Scientific Research Programme on Antarctic Scientific Results of phase One (October 1985 – January 1989), vol. 1. Plankton Ecolgy. pp 97

Lange, M. A. (1988) Basci properties of Antarctic sea ice as revealed by textural analysis of ice cores. *Ann. Glaciol.* **10**: 95-101

Liefting, L. W., Andersen, M. T, Beever, R. E., Gardner, R. C. and Forster, R. L. S. (1996) Sequence heterogeneity in the two 16S rRNA genes of Phormium yellow leaf phytoplasma. *Appl. Environ. Microbiol.* **62** : 3133-3139

Li, L., Kato, C. and Horikoshi, K. (1999) Bacterial diversity in deep-sea sediments from different depths *Biodivers. Conserv.* **8**: 659-677

Liesack, W. and Stackebrandt, E. (1992) Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J. Bacteriol.* **174**: 5072-5078

Lovejoy, C., Bowman, J. P. and Hallegraeff, G. M. (1998) Algicidal effects of a novel marine *Pseudoalteromonas* isolate (Class *Proteobacteria*, gamma subdivision) on harmful algal bloom species of the genera *Chattonella*, *Gymnodinium*, and *Heterosigma. Appl. Environ. Microbiol.* **64**: 2806-2813

McMinn, A., Skerrat, J., Trull, T., Ashworth, C. and Lizotte, M. (1999). Nutrient stress gradient in the bottom 5 cm of fast ice, McMurdo sound, Antarctica. *Polar Biol.* **21**: 220-227

Maidak, B. L., Cole, J. R., Parker, C. T., Garrity, G. M., Larsen, N., Li, B., Lilburn, T. G, McCaughey, M. J., Olsen, G. J., Overbeek, R., Pramanik, S., Schmidt, T. M., Tiedje, J. M. and Woses, C. R. (1999) A new version of the RDP (Ribosomal Database Project). *Nucl. Acids Res.* **27**: 171-173

Matsuda, O., Ishikawa, S. and Kawaguchi, K. (1990) Fine-scale onservation on salinity stratification in an ince hole during melting season of Antarctic sea ice. *Antarctic Record* **34**: 357-362

Maykut, G. A. (1985) The ice environment. in Horner R. A. (ed.) *Sea ice Biota.* CRC Press, Boca Raton, Florida

Miller, M. A., Krempin, D. W., Manahan, D. T. and Sullivan, C. W. (1994) Growth rates, distribution, and abundance of bacteria in the ice-edge zone of the Weddell and Scotia Seas, Antarctica. *Antarct. J. U.S.* **19**: 103-105

Mitchell, B. G. and Holm-Hansen, O. (1991) Observations and modelling of the Antarctic phytoplankton crop in relation to mixing depth. *Deep Sea Res.* **38**: 981-1007

Mountfort, D. O., Rainey, F. A., Burghardt, J., Kaspar, H. F. and Stackebrandt E. (1998) *Psychromonas antarcticus* gen. nov., sp. nov., a new aerotolerant anaerobic, halophilic psychrophile isolated from pond sediment of the McMurdo ice shelf, Antarctica. *Archiv. Microbiol.* **169**: 231-238

Mullins, D. T., Britschgi, T. B., Krest, R. L. and Giovannoni, S. J. (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. *Limnol. Oceanogr.* **40**: 148-158

Mylvaganam, S. and Dennis, P. P. (1992) Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. *Genetics* **130** : 399-410

Nedwell, D. B. (1999) Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature. *FEMS Microbiol. Ecol.* **30**: 101-111

Odum, E. P. (1971). Principles and concepts pertaining to organisation at the community level, p. 140-161. In *Fundamentals of ecology*. Saunders College Publishing, Philadelphia, Pa.

Olson, R. J. (1980) Nitrate and ammonium uptake in antarctic waters. *Limnol. Oceanogr.* **25**: 1064-1074

Ouverney C. C. Fuhrman J. A. (1999) Combined Microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types *in situ*. *Appl. Environ. Microbiol.* **65**: 1746-1752

Pace, N. R. (1997) A molecular view of microbial diversity and the biosphere. *Science* **276**: 734-740

Palmisano, A. C., SooHoo, J. B., Moe, R. L. and Sullivan, C. W. (1987) Sea ice microbial communities. VII. Changes in under-ice spectral irradiance during the

development of Antarctic sea icemicroalgal communities. *Mar. Ecol.-Prog. Ser.* **35**: 165-173

Palmisano, A. C. and Sullivan, C. W. (1985) Pathways of photosynthetic carbon assimilation in sea ice microalgae from McMurdo Sound, Antarctica. *Limnol. Oceanogr.* **30**: 674-678

Pomeroy, L. R. and Weibe, W. J. (1988) Energetics of microbial food webs. *Hydrobiologia* **159**: 7-18

Priddle, J., Leakey, R. K. G., Archer, S. D. and Murphey, E. J. (1996) Eukaryotic microbiota in the surface waters and sea ice of the Southern Ocean: aspects of physiology, ecology and biodiversity in a 'two-phase' ecosystem. *Biodivers. Conserv* **5**: 1473-1504

Putt, M., Miceli, G. and Stoeker, D. K. (1994) Association of bacteria with *Phaeocystis* sp. in MCMurdo Sound, Antarctica. *Mar. Ecol.-Prog. Ser.* **105**: 179-189

Rainey, F. A., Ward, N. L. and Stackebrandt, E. (1993) Molecular ecology study of a New Zealand acidothermal soin. In Thermophiles '93, Hamilton, New Zealand, Abstract A7. Hamilton: University of Waikato

Rappe, M. S., Kemp, P. F. and Giovannoni, S. J. (1995) Chromophyte plastid 16S ribosomal RNA genes found in a clone library from atlantic ocean seawater. *J. Phycol.* **31**: 979-988

Rappe M. S. Kemp P. F. Giovannoni S. J. (1997) Phylogenetic diversity of marine coastal picoplankton 16s rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. *Limnol. Oceanog.* **42**: 811-826

Rappé M. S. Suzuki M. T. Vergin K. L. Giovannoni S. J. (1998) Phylogenetic diversity of ultraplankton plastid small-subunit rRNA genes recovered in environmental nucleic acid samples from the Pacific and Atlantic coasts of the United States. *Appl. Environ. Microbiol.* **64**: 294-303

Rath, J., Ying Wu, K., Herndl, G. J. and DeLong, E. F. (1998) High phylogenetic diversity in a marine-snow-associated bacterial assemblage. *Aquat. Microbial. Ecol.* **14**: 261-269

Rea, S. (1999) A Polyphasic Study of Sulfate and Iron Reducing Bacteria from the Vestfold Hills, Antarctica and Phylogenetic Survey of Lake Sediments. PhD Thesis School of Agricultural Science, University of Tasmania.

Reimnitz, E., Clayton, J. R., Kempema, E. W., Payne, J. R. and Weber, W. S. (1993) interaction of rising frazil with suspended particles: tank experiments with application to nature. *Cold Reg. Sci. Technol.* **21**: 117-135

Reysenbach, A.L., Giver, L. J., Wickham, G. S. and Pace, N. R. (1992) Differential amplification of rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* **58**: 3417-3418

Rheims, H., Rainey, F. A. and Stackebrandt ,E. A. (1996) Molecular approach to search for diversity among bacteria in the environment. *J. Ind. Microbiol.* **17**:159-169

Ruiz-Trillo, I., Riutort, M., Littlewood, D. T. J., Herniou, E. A. and Baguñà, J. (1999) Acoel flatworms: Earliest extant bilaterian metazoans, not members of Platyhelminthes. *Science* **283**: 1919-1923

Russell, N. J. (1998) Molecular adaptations in psychrophilic bacteria: Potential for biotechnological applications. *Advances in Biochemical Engineering/ Biotechnology* **61**: 1-21

Saano, A., Lindstrom, K. and Van Elsas, J. D. (1995) Eubacterial diversity in Finnish forest soil. In 7th International Symposium on Microbial Ecology, Santos, Brazil, Abstract P1-3.9. Santos: Brazilian Society for Microbiology.

Sahm, K., C. Knoblauch, and R. Amann. (1999) Phylogenetic affiliation and quantification of psychrophilic sulfate-reducing isolates in marine artic sediments. *Appl. Environ. Microbiol.* **65**: 3976-3981.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) Primer-directed enzymatic amplificatino of DNA with thermostable DNA polymerase. *Science* **239**: 487-491

Savidge, G., Harbour, D., Gilpin, L. C. and Boyd, P. W. (1995) Phytoplankton distribution and production in the Bellingshausen Sea, Austral spring 1992. *Deep Sea Res. II* **42**: 1202-1224

Sawabe, T., Tanaka, R., Iqbal, M. M., Tajima, K., Ezura, Y., Ivanova, E. P. and Christen, R. (2000) Assignment of *Alteromonas elyakovii* KMM 162T and five strains isolated from spot-wounded fronds of *Laminaria japonica* to *Pseudoalteromonas elyakovii* comb. nov. and the extended description of the species. *Int. J. Syst. Evol. Microbiol.* **50**: 265-271

Shen, H. T. and Ackermann, N. L. (1990) Wave-induced sediment enrichment in coastal ice covers. in Ackley, S. F. and Weeks, W. F. (eds.) Sea ice properties and processes. Proceedings of the W. F. Weeks Sea Ice Symposium, CRREL Monograph 90-1, Cold Reg Res Eng Lab, Hanover, NH, pp 100-102

Simberloff, D. S. (1972). Properties of the rarefaction diversity measurement. *Am. Natur.* **106**: 414-418

Simon, M., Glockner, F. O. and Amann, R. (1999) Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. *Aqut. Microbial. Ecol.* **18**: 275-284

Sly, L. I., Taghavi, M. and Fegan, M. (1998) Phylogenetic heterogeneity within the genus *Herpetosiphon*: transfer of the marine species *Herpetosiphon cohaerens*, *Herpetosiphon nigricans* and *Herpetosiphon persicus* to the genus *Lewinella* gen.nov. in the *Flexibacter-Bacteroides-Cytophaga* phylum. *Int. J. Syst. Bacteriol.* **48**: 731-737

Sorokin, D. Yu. (1995) Sulfitobacter pontiacus gen nov., sp. nov. – A new heterotrophic bacterium from the Black Sea, specialized on sufite oxidation. *Microbiology* **64**: 295-305

Smith, M C., Bowman, J. P. and Line, M. A. **(2000) Cyanobacterial and heterotrophic bacyerial composition of Antarctic quartz stone sublithic communities. *Polar Biol.* in press**

Smith, Jr, W. O., Nelson, D. M. and Mathot, S. (1999) Phytoplankton growth rates in the Ross Sea, Antarctica, determined by independent methods: temporal variations. *J. Plankton. Res.* **21**: 1519-1536

Snaidr, J., R. Amann, I. Huber, W. Ludwig, and K.-H. Schleifer. (1997). Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Appl. Environ. Microbiol.* **63**: 2884-2896

Staley, J.T. (1968) *Prosthecomicrobium* and *Ancalomicrobium*: new freshwater prosthcate bacteria. *J. Bacteriol.* **95**: 921-1942

Staley, J. T. and Gosink, J. J. (1999) Poles apart: Biodiversity and biogeography of sea ice bacteria. *Ann. Rev. Microbiol.* **53**: 189-215

Staley, J. T., Irgens, R. L. and Herwig, R. P. (1989) Gas vacuolate bacteria from the sea ice of Antarctica. *Appl. Environ. Microbiol.* **55**: 1033-1036

Sullivan, C. W. and Palmisano, A. C. (1981) Sea ice microbial communities in McMurdo Sound. *Ant. J. U.S.* **16**: 126-127

Sullivan, C. W. and Palmisano, A. C. (1984) Sea ice microbial communities : Distribution, abundance, and diversity of sea ice bacteria in McMurdo Sound, Antarctica, in 1980. *Appl. Environ. Microbiol.* **47**: 788-795

Sullivan, C. W., Palmisano, A. C., Kottmeier, S., Grossi, S. M., Moe, R. and Taylor, G. T. (1983) The influence of light on development and growth of sea ice microbial communities in McMurdo Sound. *Ant. J. Rev. U.S.* 177-179

Suzuki, M. T. and Giovannoni, S. T. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Micorbiol.* **62**: 625-630

Suzuki M. Rappe M. S. Giovannoni S. J. (1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR
amplicon length heterogeneity. *Appl. Environ. Microbiol.* **64**: 4522-4529

Tanner, M. A., Goebel, B. M., Dojka, M. A. and Pace, N. R. (1998) Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. *Appl. Environ. Microbiol.* **64**: 3110-3113

Tupas, L. M., Koike, I., Karl, D. M. and Holm-Hansen, O. (1994) Nitrogen matabolism by heterotrophic bacterial assemblages in Antarctic coastal waters. *Polar Biol.* **14**: 195-204

Ueda, T., Suga, Y. and Matsuguchi, T. (1995) Molecular phylogenetic analysis of a soil microbial community in a soybean field. *Eur. J. Soil Sci.* **46**: 415-421

Ueda, K., Seki, T., Kudo, T., Yoshida, T. and Kataoka, M. (1999) Two distinct mechanisms cause heterogenetity of 16S rRNA. *J. Bacteriol* **181** : 78-82

Uchino, Y., Hirata, A., Yokota, A. and Sugiyama, J. (1998) Reclassification of marine *Agrobacterium* species: Proposals of *Stappia stellulata* gen. nov., comb. nov., *Stappia aggregata* sp. nov., nom. rev., *Ruegeria atlantica* gen. nov., comb. nov., *Ruegeria gelatinovora* comb. nov., *Ruegeria algicola* comb. nov. and *Ahrensia kieliense* gen. nov., sp. nov., nom. rev. *J. Gen. Appl. Microbiol.* **44**: 201-210

Vanucci, S. and Bruni, V. (1999) Small nanoplankton and bacteria in the western Ross Sea during sea-ice retreat (spring 1994). *Polar Biol.* **22**: 311-321

Verhille, S., Baida, N., Dabboussi, F., Hamze, M., Izard, D. and Leclerc, H. (1999) *Pseudomonas gessardii* sp. nov. and *Pseudomonas migulae* sp. nov.,two new species isolated from natural mineral waters *Int. J. Syst. Bacteriol.* **49**: 1559-1572

Vincent, W. F. (1988) Microbial ecosystems of Antarctica. Cambridge University Press. London.

Vincent, W. F. (2000) Evolutionary origins of Antarctic microbiota: invasion, selection and endemism. *Antarctic Science* in press

Wang, G. C.-Y. and Wang, Y. (1996) The frequencey of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. *Microbiology* **142**: 1107-1114

Wang, Y., Zhang, Z. and Ramanan, N. (1997). The actinomycete *Thermobispora bispora* contains two distinct types of transcriptionally active 16S rRNA genes. *J. Bacteriol.* **179** : 3270-3276

Watanabe, K. and Satoh, H. (1987) Seasonal variations of ice algal standing crop near Syowa Station, East Antarctica. *Bulletin of Plankton Society of Japan* **34**: 143-164

Weeks, W. F. and Ackley, S. F. (1982) The growth, structure and photosynthesis of sea ice. Monograph 82, Cold Reg Res Eng Lab, Hanover, NH pp1-13

Weissenberger, J. and Grossmann, S. (1998) Experimental formation of sea ice: importance of water circulation and wave action for incorporation of phytoplankton and bacteria. *Polar. Biol.* **20**: 178-188

Wadhams, P. (1994) The Antarctic sea ice cover. In *Antarctic Science.* Global Concerns. Hempel, G. (ed) Springer-Verlag, Berlin. pp 45-59

Williams, P. J. leB. and Yentsch, C. S. (1976) An examination of photosynthetic production, excretion of photosynthetic products and heterotrophic utilisation of dissolved organic compounds with reference to results from a coastal sub- tropical sea. *Mar. Biol.* **35**: 31-40

Wise, M. G., M. G., McArthur, J. V. and Shimkets, L. J. (1997) Bacterial diversity of a Carolina bay as determined by 16S rRNA gene analysis: confirmation of novel taxa. *Appl. Environ. Microbiol.* **63**: 1505-1514

Woese, C. R. (1987) Bacterial evolution. *Microbiol. Rev.* **51**: 221-271

Zaman V., Zaki M., Howe J., Ng M., Leipe D. D., Sogin M. L. and Silberman J. D. (1999) Hyperamoeba isolated from human feces: Description and phylogenetic affinity. *Eur. J. Protistol.* **35**: 197-207

Zdanowski, M. K. (1988) Bacteria in pack-ice north of Elephant Island (BIOMASS III, October 1986). *Polish Polar Res.* **9**: 203-216

Zdanowski, M. K. and Donachie, S. P. (1993) Bacteria in the sea ice zone between Elephant Island and the South Orkneys during the Polish sea-ice zone expedition, (December (1988) to January 1989). *Polar Biol.* **13**: 245-254

Zwally, H. J., Parkinson, C. L. and Comiso, J. C. (1983). Variability of Antarctic sea ice and changes in carbon dioxide. *Science* **220**: 1005-1012

Appendix 1

Genbank Accesion Numbers of Reference Strains

Figure 3.1: The *Cytophaga-Flexibacter Bacteroides*	
Cellulophaga baltica	AJ005972
Lewinella cohaerens	AF039292
Lewinella nigricans	AF039294
Lewinella persicus	AF039295
deep sea sediment clone BD2-2	AB015532
filter biofilm clone Koll-22	AJ224942
Antarctic sediment clone Burton-20	AF142840
sea ice strain IC166	AF001366
Flavobacterium uliginosum	M62799
Antarctic sediment clone Taynaya-5	AF142951
Cellulophaga fucicola	AJ005973
Cytophaga latercula	M58769
Antarctic sedimane clone Burton-44	AF142862
Antarctic sediment clone Burton-28	AF142846
sea ice strain IC157	AF001370
Flavobacterium hibernum	L39067
Flavobacterium xylanivorum	AF162266
Antarctic sediment clone Burton-22	AF142841
Antarctic quartz stone clone QSSC9-3	AF170749
Psychroserpens burtonensis	U62913
sea water clone AGG13	L10948
deep sea sediment clone JTB-244	AB015262
Antarctic sea water clone SW17	AF001368
Antarctic sediment clone Organic-5	AF142907
deep sea sediment clone BD2-17	AB015545
Antarctic sediment clone Burton-32	AF142850
Gelidibacter algens	U62914
sea ice strain IC076	U85882
Antarctic sediment clone Taynaya-7	AF142953
Haerentibaculum maritimum	D14023
Polaribacter irgensii	M61002
Polaribacter sp. IC066	U85884
Polaribacter franzmannii	U14586
Antarctic sediment clone Burton-11	AF142831
deep sea sediment clone JTB143	AB015261
Thermonema lapsum	L11703
Figure 3.2: The Gamma Proteobacteria	
Shewanella putrefaciens	X81233
Shewanella baltica	AJ000216
Shewanella frigidimarina ACAM 600	U85906
Shewanella frigidimarina NCIMB400	Y13699
Shewanella algae	X81621
Shewanella gelidimarina	U85907
Shewanella benthica	AB008796
Shewanella colwellia	AF170794
sea ice strain IC169	AF001376

Colwellia rossensis	UI4581
Colwellia psychrerytreae	AF001375
Colwellia hornerae	U85843
Antarctic sediment clone Clear-11	AF146235
Antarctic sediment clone Pendant-8	AF142921
Escherichia coli	
Citobacter brakii	AF025368
Psychromonas antarctica	Y14697
Pseudoalteromonas denitrificans	X82138
Pseudoalteromonas tunicata	Z25522
Pseudoalteromonas piscicida	AF144036
Pseudoalteromonas luteoviolacea	X82144
Pseudoalteromonas aurantia	AF025570
Pseudoalteromonas prydzensis	U85855
Pseudoalteromonas atlantica	X82134
Pseudoalteromonas elyakovii	AF116188
Pseudoalteromonas nigrefaciens	X82146
Pseudoalteromonas antarctica	AF045560
Pseudoalteromonas gracilis	AF038846
Alteromonas mccleodii	X82145
Glaciecola punicea	U85853
Pseudomonas putida	U70977
deep sea sediment clone JTB 247	AB015251
sea ice strain IC038	U85869
sea ice strain ACAM 213	U85868
Pseudomonas putida	D84020
Pseudomonas pseudoalcaligenes	Z76666
Pseudomonas sp.Sag-50G	AF098466
seagrass associated clone HstpL-59	AF159678
Pseudomonas rhodesiae	AF064459
deep sea sediment clone NBO.1-1	AB013844
Pseudomonas gessardii	AF074384
Pseudomonas jessenii	AF068259
Microbulbifer hydrolyticus	U58338
Endobugula sertula	AF006608
seagrass associated clone HstpL170	AF159681
Antarctic sediment clone Taynaya-9	AF142955
Oceanospirillum multiglobuliferum	AB006764
Neptunomonas napthovorans	AF053734
Marinomonas vaga	X67025
Marinomonas protea	AJ238597
deep sea strain HTB111	AB010870
Halomonas aquamarina	AF199439
Antarctic quartz stone strain QSSC5-4	AF170743
Psychrobacter immobilis	U39399
Psychrobacter glacincola	U46145
Aquaspirillum gracile	AF078753
deep sea strain HTB110	AB010851
gas vacuolate str. 37.	U73721
Psychromonas antarctica	Y14697
Figure 3.3: The Alpha Proteobacteria	
alpha proteobacterium S34	U87407
Ruegeria atlantica	AF124521
Alpha proteobacterium sp. MBIC1876	AB026194

Sulfitobacter mediterraneus	Y17387
Octadecabacter arcticus	U73725
Octadecabacter antarcticus	U14583
Marinosulfomonas sp. GAI-37	AF007260
Octadecabacter sp. IC141	U85840
Sulfitobacter sp. EE-36	AF007254
Alpha proteobacterium sp. S34	U87407
Alteromonas mccleodii	X82145
Antarctic sediment clone Burton-33	AF142851
Antarctic sediment clone Burton-19	AF142839
Sulfitobacter pontiacus	Y13155
Sulfitobacter sp. Dss-2	AF098490
Sulfitobacter sp. GAI-21	AF007257
Roseobacter shippagan	AF100168
Roseobacter algicola	X78315
Antarctic sediment clone Taynaya-15	AF142961
Antarctic sediment clone Burton-9	AF142829
Octadecabacter sp. IC146	AF001377
Paracoccus solventivorans	Y13826
Paracoccus alkenifer	Y13827
Amaricoccus kaplicensis	U88041
Orientia tsutsugamusgi	AF062074
marine plankton clone SAR220	U75257
marine plankton clone SAR203	U75255
Antarctic sediment clone Pendant-24	AF142936
Erythromicrobium ramosum	X72909
Figure 3.4: *Chlamydia/ Verrucomicrobia*	
Endosymbiont of Acanthamoebae UWE1	AF083614
Parachlamydia acanthamoebae	Y07566
Chlamydia trachomatis	AE001347
Antarctic sediment clone Taynaya-24	AF142972
Antarctic sediment clone Clear-2	AF146229
Antarctic sediment clone Clear-29	AF146251
Antarctic sediment clone Burton-29	AF142847
Antarctic sediment clone Ace-12	AF142791
seagrass associated clone HstpL-102	AF159644
Verrucomicrobium spinosum	X90515
Prosthecobacter fusiformis	U60015
Prosthecobacter sp. FC1	U60012
Prosthecobacter sp. FC2	U60013
Prosthecobacter sp. FC3	U60014
soil clone MC17	X64381
soil clone MC31	X64380
freshwater clone LD29	AF009975
Holophaga foetida	X77215
activated sludge clone	Z94005
Antarctic sediment clone Burton-46	AF142864
Amazon soil clone	UEU68683

Figure 3.5: The Firmicutes	
Planococcus mcmeekinii	AF041791
Bacillus subtilis	X60646
soil clone DA134	AJ000983
deep sea clone HTE831	AB010863
Halobacillus sp. MB6-08	U85901
Virgibacillus pantothenticus	D16275
Virgibacillus proomii	AJ012667
Bacillus marismortui	AJ009793
Halobacillus litoralis	X94558
Rubrobacter radiotolerans	X87134
Rubrobacter xylanophilus	X87135
Antarctic quartz stone clone QSSC-137	AF170770
soil clone MC4	X68459
soil clone MC19	X68454
Janibacter sp. QSSC8-6	AF170746
Clostridium botulinum	L37589
Figure 3.6: Unaffiliated clones	
Antarctic sediment clone Clear-32	AF146254
Desulfovibrio gabonensis	U31080
hot spring clone OPB78	AF026989
hot spring clone OPT77	AF026992
Clostridiaceae str.PB	AB020336
Thermoanaerobacter mathranii	Y11279
Dictyoglomus thermophilum	L39875
hot spring clone OPS145	AF027078
hot spring clone OPB46	AF027081
aquifer clone WFeA1-02	AF050576
Rubrobacter xylanophilus	X87135
hot spring clone OPB95	AF027060
hot spring clone OPT3	AF027066
hot spring clone OPS88	AF027062
hot spring clone OPS12	AF027065
hot spring clone OPS150	AF027064
hot spring clone OPB23	AF027068
hot spring clone OPB2	AF027088
hot spring clone OPS152	AF027079
filter biofilm clone Koll 11	AJ224540
Verrucomicrobium spinosum	X90515
Aquifex pyrophilus	M83548
Figure 3.7: Phytolplankton Plastids Genes	
seagrass associated clone HstpL-35	AF159636
Antarctic sediment clone Organic-3	AF142905
planktonic plastid clone OM20	U32670
Odontella sinensis	Z67753
Skeletonema pseudocostatum	X82155
Skeltonema costatum	X82154
ultraplankton plastid clone OCS54	AF001657
planktonic plastid clone OM21	U32671
Emiliania huxleyi	X82156
Ultraplankton plastid clone OCS50	AF001656

Ochrosphaera neapolitana	X80390
Porphyra purpurea	U38804
Glaucosphaera vacuolata	X81903
Zea mays	X86563
Figure 3.8: Phytoplankton Nuclear Genes	
Phaeocystis antarctica SK23	X77481
Phaeocystis antarctica CCMP1374	X77477
Phaeocystis pouchetii	X77475
Phaeocystis globosa	X77476
Emiliania huxleyi	M87327
Coccoid haptophyte CCMP625	U40924
Reticulosphaera socialis	X90992
Chrysochromulina polylepis	AJ004866
Prymnesium calathiferum	U40923
Prymnesium patelliferum	L34670
Guillardia theta	X57162
Figure 3.9: The Acoel Turbellaria	
Haplogonaria syltensis	AF102900
Atriofonta polyvacuola	AF102895
Convoluta roscoffensis	AJ012527
Anaperus biaculeatus	AJ012527
Simplicomorpha gigantorhabditis	AF102894
Philomecynostomum lapillum	AF102897
Amphiscolops sp.	D85099
Convoluta naikaiensis	D83381
Symsagittifera psammophila	AF102893
Paedomecynostomum bruneum	AF102896
Figure 3.10: The Dinoflagellates	
Crypthecodinium cohnii	M64245
Amphidinium belauense	L13719
Alexandrium fundyense	U09048
Prorocentrum micans	M14649
Pfiesteria piscicida	AF077055
Gymnodinium catenatum	AF022193
Pentapharsodinium tyrrhenicum	AF022201
Prorocentrum mexicanum	Y16232
Gyrodinium sp.	AB001438
Gymnodinium mikimotoi	AF022195
Prorocentrum minimum	Y16238
Figure 3.11: The *Cercomonadidae*	
detritus clone LKM45	AJ0130856
Euglypha rotunda	X77692
Cercomonas ATCC50316	U42448
Cercomonas longicauda	AF101052
Cercomonas ATTC50318	U42450
Cercomonas ATTC50319	U42451
Heteromita glabosa	U42447
Lotharella glabosa	AF076169
Pyronema domesticum	U53385