Flavobacteria in the Southern Ocean

Guy Charles Jenvey Abell

B.AppSc, B.Sc (Hons)

Submitted in fulfillment of the requirements for the Degree of Doctor of

Philosophy

Department of Agricultural Science

University of Tasmania (March 2005)

Abstract

The abundance, spatial distribution and diversity of class *Flavobacteria*, a bacterial group with a major role in marine secondary production, was investigated in the Southern Ocean euphotic zone of the ice pack off Eastern Antarctica and along a transect at 140-144°E between latitudes 44.7°S to 63.5°S. Samples were comparatively analysed using 16S rRNA gene-based denaturing gradient gel electrophoresis (DGGE), fluorescent *in situ* hybridisation, real-time PCR and sequence analysis. The results were subsequently compared with direct cultivation approaches.

Surface seawater samples were filter-fractionated into particulate and planktonic fractions and the abundance of particle-associated *Flavobacteria*, ascertained with real-time PCR and DGGE band analysis using *Flavobacteria*-specific primers. *Flavobacteria* abundance was found to be significantly higher in Polar Front Zone (PFZ) and Antarctic Zone (AZ) water samples compared to warmer, nutrient limited Temperate Zone (TZ) and Sub-Antarctic Zone (SAZ) waters. Abundance of particle-associated *Flavobacteria* positively correlated with seawater chlorophyll *a* and nutrient concentrations. The abundance of planktonic *Flavobacteria* populations in the same samples remained relatively static, suggesting increased *Flavobacteria* abundance may relate to enhanced primary production in the PFZ and AZ. This was supported by comparisons of DGGE profiles that demonstrated significant differences occur in the total *Flavobacteria* community structure and 16S rRNA gene diversity between samples from the PFZ and AZ with samples from the TZ and SAZ. This suggests a shift to a different, more psychrophilic *Flavobacteria* community occurs across the Polar Front in the Southern Ocean.

DGGE band sequences revealed a high diversity of class *Flavobacteria* within the Southern Ocean, with 24 genus-level lineages detected. Several of the phylotype clades detected were cosmopolitan in distribution, present in both polar and temperate oceans. Many of the phylotypes clustered in a large, so far uncultivated clade (previously termed "DE cluster 2") widely distributed in seawater but apparently absent from sea-ice. Cosmopolitan phylotype clades occurred throughout the Southern Ocean, while several additional phylotype groups were found only in the colder waters of the PFZ and AZ.

Examination of the cultivable diversity of *Flavobacteria* in Southern Ocean water samples, using a range of growth media, revealed a number of unique phylotypes including three novel genera, some grouping in clades for which only clones are currently available. Several other strains represented novel species belonging to the family *Flavobacteriaceae*, grouping in the genera *Psychroserpens*, *Polaribacter* and *Tenacibaculum*.

A number of seawater microcosms were utilized to examine the colonization of bacteria-free diatom detritus by planktonic bacterial communities over a period of 30 days at 2°C. *Flavobacteria* phylotypes colonizing diatom detritus, determined by DGGE and real-time PCR analysis, were homologous with the dominant phylotypes in the particle-associated fraction of the samples from which they were taken. Colonisation occurred rapidly (<5 days) and comprised a succession of phylotypes, representing a diversity of *Flavobacteria* lineages. The increasing colonization corresponded to an increase in the dissolution of silicic acid compared with the corresponding control microcosms.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for any degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Guy Abell

03/03/2005

Acknowledgements

First and foremost, I would like to thank John Bowman. Without his guidance and enthusiasm this thesis would never have come to be, he has provided support with all aspects of the process, and as a supervisor has been a source of inspiration and often wonder.

I would like to thank all those people who have provided significant technical expertise, including (in no particular order) Sharee McCammon, Matthew Smith, Jenny Skerratt, Andrew Bissett, Andrew Pankowski, Shane Powell, Lyndal Mellefont, Tom Lewis, Carol Nichols, Janelle Brown, Adam Smolenski, Jodie Van de Kamp, Bob Elliot and Tom Ross. Each of you have provided something that was essential to this thesis and as such I am indebted to you. Also sincere thanks to Matt Miller and David Nichols for their assistance, particularly the SCFA analysis.

I am grateful to the members of the School of Agricultural Science, who have provided feedback and support over the course of my studies there, and who have most importantly provided friendship and encouragement.

Thanks are due also to people who have provided field work opportunities and support, including the crew of the RSV *Aurora Australis*, and others with whom I shared voyages.

I would like to extent special thanks to my family and friends who have been there during the good and bad times.

I would like to dedicate this thesis to my namesake and late Grandfather, Guy Abell.

Table of Contents

ABBREVIATIONS

AC – Antarctic Convergence

AD – Antarctic Divergence

ACC – Antarctic Circumpolar Current

AZ – Antarctic Zone

PFZ – Polar Front Zone

SAZ – Sub-Antarctic Zone

TZ – Temperate Zone

CDW – Circumpolar Deep Water

AAIW – Antarctic Intermediate Water.

SIMCO – sea ice microbial communities

rRNA – ribosomal ribonucleic acid

CFB – *Cytophaga-Flavobacterium-Bacteroides*

EPS – exopolysaccharide

FISH – fluorescent in situ hybridisation.

PCR – polymerase chain reaction

DGGE – denaturing gradient gel electrophoresis

DAPI – 4',6-diamidino-2-phenylindole

PBS – phosphate-buffered saline

EDTA –ethylenediaminetetraacetate

SDS – sodium dodecyl sulfate

G+C – guanosine plus cytosine

nMDS – nonmetric multidimensional scaling

ANOSIM – analysis of similarity

FAME – fatty acid methyl esters

GC-MS – gas chromatography-mass spectrometry

T_{OR} – optimal temperature for renaturation

(F)ASW – (filtered) artificial seawater

Publications related to this thesis as of March 2005:

[#]Abell, G.C.J. and Bowman J.P. (2005) Colonization and community dynamics of class *Flavobacteria* on diatom detritus in experimental mesocosms based on Southern Ocean seawater. *FEMS Microbiology Ecology*. (In press 2/3/2005)

*Abell, GCJ & Bowman, JP, 'Ecological and biogeographic relationships of particle associated and planktonic *Flavobacteria* in the Southern Ocean', *FEMS Microbiology Ecology*. 51 (2), 265-277.

Bowman, JP, Nichols, CAM, Smith, MC & Abell, GCJ, 'Extemophiles from Antarctica: biodiversity and biotechnological potential', *Proceedings of the 11th International Symposium on Polar Sciences*, Saegwipo, Jejudo, Republic of Korea (2004)

Abell, GC, Bowman, JP & Bissett, AP, 'Bacterial activity on diatom detritus', *Final Program and Abstracts Book - Micro NZ 2003*, The Edge - Aotea Centre, Auckland, New Zealand, P11.02 (2003)

Abell, GCJ & Bowman, JP, '*Flavobacteria* in the Southern Ocean', *8th Symposium on Aquatic Microbial Ecology SAME-8*, Taormina, Italy, L44 (2002)

Abell GCJ & Bowman JP, "*Flavobacteria* in the Southern Ocean" Australian Society for Microbiology Annual Scientific Meeting 29 Sep-3 Oct 2002, Melbourne, PP12.6.

[#]**Covers research detailed in Chapters 4 and 5.**
***Covers research detailed in Chapters 2 and 3.**

CHAPTER 1. INTRODUCTION

1.1. Microbial ecology of the Southern Ocean

1.1.1. The Southern Ocean marine environment.

The Southern Ocean covers an extensive range of latitude (50-80°S) and comprises approximately 10% of the earth's ocean area, about 36 million km^2 (Laws 1985). The latitude of the Southern Ocean means that the day length is extremely variable during the year, with the winter months characterised by short days with a low angle of incidence of sunlight, while the summer months comprise long days and a high angle of incidence of sunlight. Ocean temperatures in the Southern Ocean range from less than -1°C in Antarctic coastal waters to greater than 5°C at the Polar Front. As a result of the low water temperatures in the Southern Ocean, sea-ice is a distinctive feature of the marine landscape. The composition of Southern Ocean sea-ice is complex with the existence of permanent and seasonal sea-ice, the latter forming during the Antarctic winter and then melting during the austral summer. Between 1973 and 1975 the use of satellite remote sensing to assess the Antarctic sea-ice zone demonstrated minimum sea-ice coverage of 3 x 10^6 km^2 in February and a maximum of 20 x 10^6 km^2 in September-October (Zwally 1983). Icebergs punctuate the sea-ice environment, and it has been suggested they comprise a mass similar to one third of the sea-ice mass at its maximum seasonal extent (Radok 1975). The sea-ice is a significant physical barrier to the ocean, insulating and reducing the incident sunlight reaching the underlying water, and in turn affecting photosynthesis. It also reduces the transfer of heat between the atmosphere and surface waters and reduces the effects of wind on mixing of surface waters. It also provides a

significant habitat for sea-ice microbial communities (SIMCO) with significant exchange of biological material between sea-ice and the underlying water.

The Southern Ocean comprises geographical zones (see Figure 1.1), based primarily on the distribution of physical properties and major ocean current systems (Karl 1992). The major features of the Southern Ocean include the Antarctic Convergence (AC), which includes the Polar Frontal Zone (PFZ) in which the warmer Subantarctic Zone (SAZ) waters meet the colder Antarctic Zone (AZ) waters. In the PFZ water temperature varies from 1 to 2°C in winter to 3 to 5°C in summer. The Antarctic Divergence (AD) lies to the south of the AC and represents a major transitional zone from the predominantly westerly winds to the north and the predominantly easterly winds to the south. The winds that surround the AD result in an upwelling throughout this transitional zone. The Southern Ocean also comprises distinct water masses. The Antarctic Intermediate Water (AAIW), results from mixing at the AC. Nutrient rich upper and lower Circumpolar Deep Water (CDW) which forms through deep water from the Atlantic and Indian oceans mixing with the colder Antarctic surface waters, upwells south of the Polar Front. Antarctic Bottom Water formed in the western Weddell Sea and Ross Sea (Carmack 1977), and along the Adelie Land coast (Rintoul 1998; Orsi et al. 1999) is joined by some of the upwelled CDW that looses buoyancy near the Antarctic coast. The remainder of the CDW gains buoyancy due to warming and freshwater input and is driven north eventually sinking to enter the AAIW. Observations of the Southern Ocean water masses due south of Australia have demonstrated that distinct physical and chemical changes occur as a series of steps across each of the fronts associated with the Antarctic Circumpolar Current (Trull et al. 2001). For the purpose of this thesis, Southern Ocean fronts define four distinct water mass, as reviewed by Deacon (1984): the Temperate Zone (TZ), the SAZ, the PFZ and the AZ

1.1.2. Biogeochemical cycling and trophism.

Studies have demonstrated that bacteria play an important part in biogeochemical processes, particularly in secondary production in which biomass accumulated from photosynthesis (primary production) is eventually decomposed back to inorganic nutrients and thus resupplying the needs of the primary producers (Fenchel, 1988; Cole et al., 1988, reviewed by Azam, 1998; Bidle and Azam 1999, 2001; Brown and Parks 2001; reviewed by Kirchman 2002; Bidle et al. 2002). The intensity of secondary production and thus nutrient resupply determines to a significant degree the potential productivity that can be supported within a habitat (Atlas and Bartha 1998). Understanding the link between bacterial community structure and biogeochemical processes within bacterial assemblages is therefore needed to understand the overall marine foodweb.

Descriptions of carbon and energy transfer as depicted in various foodweb models in the Southern Ocean and other marine environments have become increasingly sophisticated over the last two decades. These models have developed from a simplistic early model only involving phytoplankton and higher trophic levels (Clarke 1985). As the existence and importance of different biological assemblages and processes have become apparent, the need to expand the concept of a Southern Ocean food web to encompass a large range of experimental data is also apparent. The role of bacterial assemblages in such trophic models is significantly more complex than previously thought (Azam 1998) and it is no longer possible to consider bacterial assemblages as innocuous degraders of organic detritus (see Figure 1.2). Rather they are capable of acting in competition with higher trophic levels, and may even play an aggressive role, with the existence of parasitic and predatory species impacting on the higher levels of the food chain. The processes
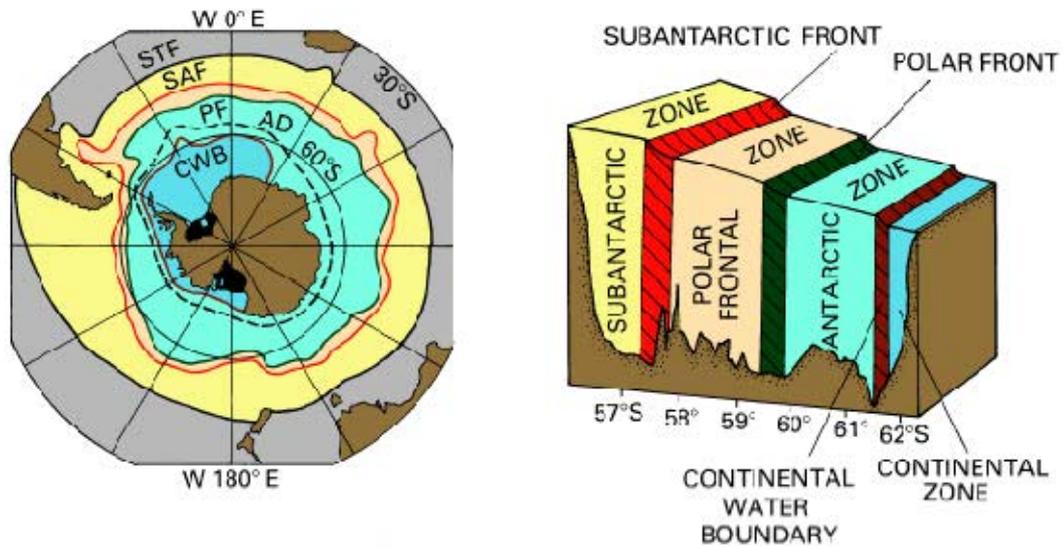
Figure 1.1 Convergences and divergences of the Southern Ocean and a schematic representation of the zonation in the Southern Ocean. STF: Subtropical Front, SAF: Subantarctic Front, PF: (Antarctic) Polar Front, AD: Antarctic Divergence (dashed line), CWB: Continental Water Boundary. The vertical section is derived from data in Drake Passage where the zonation can extend to the bottom; it generally extends down to the level of Circumpolar Water. The dark regions indicate the Weddell and Ross Sea ice shelves. (Taken from Tomczak and Godfrey, 1994)

involved in interaction between trophic levels affect the availability of nutrients, carbon sources, and subsequently the productivity of surface waters and proliferation of higher organisms. Surface water productivity affects the rate of removal of atmospheric CO_2, given that the partial pressure of CO_2 in the surface oceans is in direct equilibrium with CO_2 in the atmosphere. Given that biological processes in the surface waters result in the production of particulate carbon which is either remineralised or buried in the deep-sea sediments, the potential for the oceans to act as either sinks or sources of CO_2 is directly related to the marine food web. Much knowledge is still required to determine the specific roles bacteria play in biogeochemical cycles in the ocean.
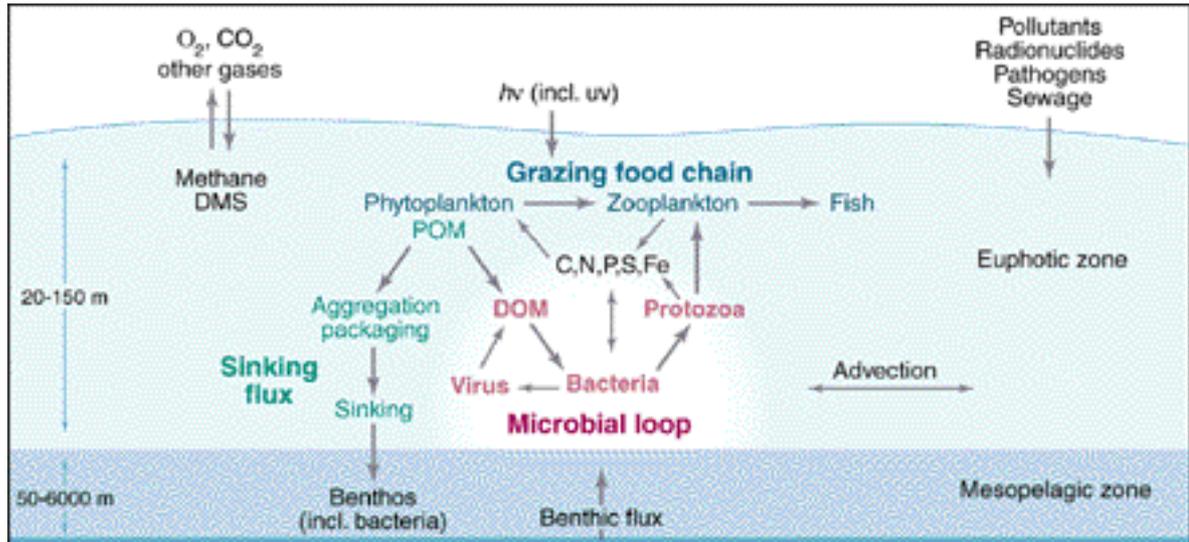
Figure 1.2 The microbial loop: classical version. Modern view of the pelagic food web, emphasizing the microbial loop as a major path for organic matter flux. Competition between the three main flux paths--grazing food chain, microbial loop, and sinking-- significantly affects oceanic carbon cycle and productivity. DOM, dissolved organic matter; DMS, dimethylsulfide. (Taken from Azam, 1998).

1.1.3. Microbial functionality.

The Southern Ocean has been a subject for microbiological research since the early Twentieth Century, and with the improvement of methods so has the picture of life in the Southern Ocean changed. Given the diversity of marine habitats in the Southern Ocean, it is reasonable to expect that there is an equal diversity of microbial communities. The variation in physical environments and niches capable of supporting microbial assemblages is significant within the Southern Ocean. Microbial communities associated with different types of sea-ice, variable salinity and temperature environments, and highly variable nutrient availability would be expected to vary equally significantly. Since the scale of microbial habitats within the Southern Ocean can be considered to encompass very small distances, fractions of millimetres in the case of particulate matter, it can be expected that there will be significant variation in the microbial processes in the Southern Ocean. Such microscale patchiness has been previously suggested in the marine environment (Long and Azam 2001a).

The diversity of microbial populations encountered to date in the Southern Ocean extend across all the Domains of life (Bacteria, Archaea and Eukaryotes) and encompasses a large number of species (see Fig. 1.3), all of which are likely to play an important role in the life cycles of the Southern Ocean. Unlike the study of higher organisms, research into microbial ecology and especially the study of prokaryotic communities is complicated by the morphological simplicity that masks massive taxonomic and metabolic diversity. It is for this reason that initial cultivation-based studies of microbial ecology were limited in their ability to differentiate bacterial communities and the underlying biogeochemical processes (Rappé and Giovannoni 2003).

Chapter 1: Introduction

Initial investigations into the role of bacterioplankton in marine food webs, were thus methodologically limited and as such missed >99% of organisms, and grossly underestimated their metabolism (Azam 1998). Early studies suggested that the heterotrophic bacteria were of little significance beyond the decomposition of organic material and production of inorganic nutrients supporting phytoplankton growth. Studies performed since have suggested that this is definitely not the case, and in fact the role of heterotrophic bacteria in marine food webs is one of great complexity and importance (Kirchman 2000). The role played by bacteria in the marine microbial loop involves relationships of competition, predation and commensalism, across a range of different trophic levels. Bacteria can play a number of different roles within the different trophic levels of the marine food chain, and as such their role is multifaceted, beyond simple recyclers of detritus and regeneration of nutrients. In fact bacteria may be predatory and aggressive in their role in the food chain (Azam 1998). An understanding of the role of bacterial mediated processes in the ocean may allow an understanding of the possible consequences of climate change processes, such as global warming, through the implementation of predictive biogeochemical models. The role of microbes in the fate of organic matter needs to be assimilated with other information about oceanic processes in order to construct such ecosystem models (Azam 1998). All of this knowledge will help demonstrate the link between community structure and function. The abundance and productivity of marine bacteria is likely to be affected by a number of parameters. It is thought that both nutrient availability and the activity of predators may have an effect on bacterial community composition, although which is more important is unknown (Pernthaler et al. 1997). It is likely however, that such factors will not only effect the genotypic, but also the phenotypic composition of bacterial communities (Lebaron et al. 1999).

1.1.4. Distribution of microorganisms.

Small subunit ribosomal RNA (16S rRNA) probe hybridisation experiments have highlighted the stratification of microbial populations within the ocean. Vertical stratification of bacterioplankton communities has been observed in a number of marine environments, with the biggest distinction in microbial community structure occurring at the boundary between the photic and aphotic zones (Giovannoni and Rappé 2000). Archaea have been shown to dominate the deep water (Kirchman 2002; Karner et al. 2001) and in the Southern Ocean are more abundant in surface waters in winter (Murray et al. 1998). More detailed analyses of prokaryotic diversity in Southern Ocean have performed in different Southern Ocean locations including deep sea sites within the Drake passage at the Antarctic Polar Front (Lopez-Garcia et al. 2001), SIMCO (Sea Ice microbial Communities) (Bowman et al. 1997b; Brown and Bowman 2001; Brinkmeyer et al. 2003) and surface seawater (Mergaert et al. 2001). These studies examined the Southern Ocean pelagic zone and sea-ice and included studies of members of a number of bacterial phyla and Archaea. Most bacteria in the Southern Ocean surface waters and ice, however belong to the phylum *Proteobacteria,* in particular members of classes *Alphaproteobacteria* and *Gammaproteobacteria* (Garrity and Holt 2001) and the phylum *Bacteroidetes* (Garrity and Holt 2001), better known as the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group. Other bacterial groups detected in these studies include members of the *Epsilonproteobacteria*, the *Verrucomicrobia*, the *Planctomyces* and the Gram-positives. This has been shown using several very different approaches including cultivation, 16S rRNA gene cloning and fluorescent in situ hybridisation (Bowman et al. 1997b; Murray et al. 1998; Glöckner et al. 1999; Simon et al. 1999; Brown and Bowman 2001; Brinkmeyer et al. 2003).

Chapter 1: Introduction

The distribution of microorganisms within an environment is controlled by factors different to those that constrain larger organisms. The abundance of individuals in microbial species within the world's oceans is so large that dispersal is unlikely to be substantially restricted by geographical and physical barriers. It is this apparent lack of endemism that allows an ubiquitous distribution of microorganisms within the world's oceans. Illustrating this point is the discovery of closely related bacterial species isolated from both the Antarctic and Arctic environments, as well as significant phylogenetic data demonstrating related phylotypes from both environments (Staley and Gosink 1999). It is perhaps the case that microbial species can be found in any environment to which they are physiologically adapted, regardless of its location on the planet. For this reason the distinctive water masses within the Southern Ocean (see section 1.1.) may possess distinctive community structures that are directly related to biogeochemical processes and environmental factors. The Polar Front and the Antarctic Circumpolar Current (ACC) represents such a barrier separating colder waters of the AZ from the warmer waters of the SAZ and TZ (effectively the waters north of the Subtropical Front). Studies suggest the ACC can demarcate different eukaryotic populations, for example dinoflagellates and mesozooplankton (Esper and Zonneveld 2002; Ward et al. 2003). Recent evidence has also shown the ACC can also effect the spatial distribution of prokaryote communities. Selje et al. (2003) found that between the south Atlantic subtropical front and Antarctica, two distinct *Roseobacter* clade (class *Alphaproteobacteria*) 16S rRNA gene phylotypes were detectable, one north and one south of the Polar Front. They suggested that in the two adjacent but different oceanic zones the persistent separation of distinct but closely related phylotypes appear to be maintained by the environmental and biogeochemical properties of the different water masses. It is unclear if this phenomena also extends to

other major bacterial groups such as class *Gammaproteobacteria* or members of the CFB

group.

Figure 1.3. Composite phylogenetic tree displaying relationships among the most widespread SSU rRNA gene clusters from marine prokaryotic plankton. (From Kirchman 2000)

1.1.5. Bacterial association with particulate organic matter.

The marine environment consists of both a liquid phase, supporting free-living planktonic organisms, and particulate organic matter, within which there is a significantly diverse habitat, populated by a range of microorganisms. Particulate matter in the ocean comprises a number of types forming by physical coagulation and zooplankton-mediated aggregation (Kiorboe et al. 1996, Kiorboe 2001). Particulate matter is also associated with sea-ice in the form of various algal assemblages. The microbial ecology of SIMCO has been previously extensively reviewed by Brown and Bowman (2002) and extensive diversity and abundance data is also available (Brinkmeyer et al. 2003). The work presented in this study focuses on pelagic-based particulates rather than those directly associated with SIMCO.

The formation of particles in the pelagic zone is influenced by such factors as the presence of transparent exopolymer particles, derived from dissolved exopolysaccharide (EPS) exuded by phytoplankton and bacteria (Passow and Alldredge 1994). Aggregates can become macroscopic (>0.5 mm), referred to as marine snow. Particulates in the surface waters of the Southern Oceans like other oceanic areas include mostly microaggregates, diatom derived detritus, and other organically originated particulate matter such as zooplankton faecal pellets. Such particles within the ocean provide a diverse range of habitats for colonization by bacteria and protozoa. Marine particulate matter has been found to be enriched in nutrients, trace metals, microbial biomass and productivity, in comparison to the surrounding water (Alldredge and Cohen, 1987; Herndl et al. 1993) and may exhibit distinct oxygen and pH gradients (Alldredge and Silver 1988; Paerl 1994) making them suitable for metabolic processes unsuited to the open water column. The transport of organic matter in the ocean in the form of particulates is

important; particulate matter sinks through the water column, while solutes do not. Thus the fate of organic matter in the water column is determined to a significant degree by the rate of dissolution of sinking aggregates. It is believed that zooplankton faecal pellets as well as marine snow are the major vehicles for vertical transport of organic matter in the ocean (Fowler and Knauer 1986). In turn this vertical flux of organic matter is believed to be a potential cause for the burial of organic matter in the seabed, facilitating its removal from the atmosphere in response to increased global atmospheric CO_2.

Particle-associated bacteria are also able to secrete exopolysaccharide (EPS) slime, enabling the formation of biofilms. Large amounts of EPS are available to be released to the water from sea-ice following the Spiring thaw, where it may significantly contribute to and alter the particle flux of the Southern Ocean (Meiners et al. 2004). Bacteria cells associated in these aggregates, utilise and solubilise the EPS and other macromolecular substrates present resulting in generally larger cells, which exhibit enhanced respiration rates and faster growth rates than their more carbon-limited planktonic counterparts. Particulate-associated bacteria also can reach cell densities 1-2 orders of magnitude higher than the surrounding waters. Production of acylated homoserine lactones for quorum sensing may be responsible for controlling production of enzymes, antibiotics and the formation of biofilms and subsequent dispersal when cells reach high concentrations in particles (Caron et al. 1986; Caron 1987; Smith 1992; Turley and Mackie 1994 Grossart and Simon, 1998; Berman et al. 1999; Grossart and Ploug 2000; Grossart et al. 2001; Gram et al. 2002).

Understanding the dynamics of bacterial colonization of marine aggregates is an important factor in the understanding of the way in which aggregates are solubilised and remineralised. Recent studies have suggested that the way in which aggregates are colonized may be dependent on a number of factors, with the size of the aggregate being

significant in the number of microorganisms colonizing a particle and allowing for a state of equilibrium between the size of the aggregate and the concentration of particle-associated bacteria. Furthermore the bacteria colonizing aggregates may be primarily motile, while aggregates enriched with organic substrates may be colonized faster by tumbling strains (Kiorboe et al. 2002). (Kiorboe et al. 2002) also suggested that there may be a typical residence time on aggregates of about 3 hours, facilitating a rapid exchange between free-living and attached bacteria, consistent with the suggestions of Riemann and Winding (2001) who proposed significant interaction occurs between particle-associated and planktonic bacterial communities in the water column.

Molecular-based studies indicate that the composition of microbial communities dwelling in seawater varies to a certain degree but often contains many distinctive phylogenetic clades e.g. SAR11, SAR86, *Synechococcus* etc. (Rappé and Giovannoni 2003). The majority of studies though have been concerned with the examination of total bacterial communities. Studies specifically examining differences between planktonic and particle-associated microbial communities demonstrated both similarities and differences between attached and free living bacterial assemblages, as well as a higher degree of diversity amongst the free living fraction. (DeLong et al. 1993; Acinas et al. 1999; Hollibaugh et al. 2000). Riemann and Winding (2001), however suggests that the degree to which planktonic and particle-associated communities interact could well be a function of the nature of the particulate matter, and that the degree of interaction considerably varies. They also found that a number of phylotypes were potentially solely present on particles during the course of a phytoplankton bloom. How these bacteria colonize particles during algal blooms or persist in the water column is unknown. Also there is poor understanding of how free living and particle associated bacterial assemblages actually interact in the marine environment. The actual presence of specific planktonic

versus particle-associated phylotypes is also unknown since no underlying trends in community structure have been pinpointed in any study to date. The lack of information appears at least partially due to the fact that comprehensive knowledge of the bacterial species making-up marine particle-associate and planktonic communities is still rather incomplete.

1.2. The *Cytophaga-Flavobacterium-Bacteroides* phylum in the Southern Ocean

The primary focus of the PhD thesis research is to develop a better understanding of the CFB group in the Southern Ocean, including abundance, culture-dependent and – independent diversity, community structure in relation to oceanographic water masses and particulates and finally some aspects of its functionally, namely ability to colonise and degrade phytodetritus. This effort was prompted by much recent accumulated knowledge, which indicates CFB have a very important, if rather under appreciated role in marine ecosystems. The following sections briefly reviews some facts related to marine CFB and their apparent significance in Southern Ocean marine foodwebs.

1.2.1 Cultured CFB group diversity in the marine ecosystem.

The CFB group until recently has had a rather disordered higher-level nomenclature. As of 2004 CFB phylum members are clearly recognized in having certain traits in common, including: 1) Gram-Negative cell wall; 2) binary cell division; 3) a rod-shaped to filamentous morphology; 4) lack of sporulation or resting cell ability (exception *Chitinophaga* and *Sporocytophaga*), 5) non-motile or motile by gliding except for

members of the family *Bacteroidaceae* and relatives (class *Bacteroidetes*) which have peritrichous flagella; 6) an exclusively chemoheterotrophic metabolism, if able to grow anaerobically, carbon sources are always fermented not respired through reductive respiration; 7) cell mass often has a strong pigmentation, usually carotenoid in nature; 8) fatty acids include a high proportion of branch chain fatty acids and 9) possess entirely menaquinone-type respiratory lipoquinones. With ongoing publication of the new edition of the Bergey's Manual of Systematic Bacteriology a guideline (http://bergeysoutline.com) is now in place organizing the various species of the CFB phylum into orders, classes and families based on their phylogenetic relationships. However this may be still subject to some revision. In any case, the CFB group will be eventually reclassified officially as the phylum "*Bacteroidetes*" (Garrity and Holt, 2001).

In respect to the polar marine ecosystem most cultured CFB appear to belong to what is termed class *Flavobacteria* (Cavalier-Smith 2002), which also includes order *Flavobacteriales* and effectively family *Flavobacteriaceae* (Bernardet et al. 1996) and family *Cryomorphaceae* (Bowman et al. 2003a) (Table 1.1, Fig. 1.2). As can be seen in Table 1.1 and Figure 1.2 a large number of marine species concentrate in the family *Flavobacteriaceae* and form a distinct "Marine Clade", the taxonomy of which has been recently reviewed (Bowman 2004). Cultured marine CFB species are also distributed in numerous lineages throughout the CFB phylum (Table 1.1), particularly within family "*Flexibacteriaceae*" (Fig. 1.2). The CFB group, in particular family *Flavobacteriaceae*, is rapidly expanding with more species described every year due to the expanding interest in "biodiscovery". Perhaps not surprisingly most new CFB taxa come from marine ecosystems.

1.2.2. Abundance and functionality of the CFB in the Southern Ocean

On the basis of fluorescent in situ hybridisation (FISH) analyses, the CFB-phylum represents 10-70% of the bacterioplankton biomass in various oceanic pelagic zones (Glöckner et al. 1999), and represent a major component of the particle-associated community in the marine ecosystem (Rath et al. 1998). Studies of the Southern Ocean water column have shown that CFB present can represent a large proportion of the bacterioplankton (25-70%). Heightened abundance of the CFB group has been linked to enhanced levels of primary produced organic matter in surface waters (Simon et al. 1999). Various data collected over the last few years also argues CFB members play an important role in the consumption of DOC, especially the high molecular weight portion (Cottrell and Kirchman 2000; Brown and Bowman 2001; Covert and Moran 2001; Kisand et al. 2002; Schafer et al. 2002, Eiler et al. 2003; Kirchman et al. 2004). The CFB group utilizes a spectrum of organic carbon types ranging from complex polymers such as EPS and proteins to amino acids and monosaccharides. Individual species are often very selective of the carbon source type that they can metabolise. For example, *Cellulophaga* spp. can utilize a wide range of macromolecules including carbohydrate polymers and proteins (Johansen et al. 1999; Bowman 2000), while the species *Cryomorpha ignava* has fastidious growth requirements and is unable to degrade or utilize common substrates of any type (Bowman et al. 2003a). This suggests that populations of different species are likely to be strongly influenced by nutrient concentration, type and quality and thus may be able to compete effectively in a community when preferential substrates and other growth factor concentrations increase.

Table 1.1. CFB found in marine habitats including the Southern Ocean.

Genus – species*	Family	Known marine habitats
Aequorivita – A. antarctica, A. crocea, A. lipolytica, A. sublithincola	Flavobacteriaceae	Southern Ocean seawater, marine sediment, quartz stone subliths
Algibacter – A. lectus	Flavobacteriaceae	Green algal macrophyte , NW Pacific Ocean
Arenibacter – A. latericius, A. certesii, A. troitsiensis	Flavobacteriaceae	Sandy sediments, marine green algal macrophytes, NW Pacific Ocean
Bizionia – B. paragoriae	Flavobacteriaceae	Soft coral, NEW Pacific Ocean
Calefactosor – C. saleffrenus, C. algoritergicola, C. gelidisalsuginis, C. myxarmorum	Flavobacteriaceae	Sea-ice brines, surfaces of Antarctic sea-ice and saline pond associated copepods and amphipods
Cellulophaga – C. lytica, C. fucicola, C. baltica, C. algicola, C. pacifica	Flavobacteriacea	Surfaces of brown algal macrophytes, beach mud, estuarine and brackish coastal waters, seawater
Croceibacter – C. atlanticus	Flavobacteriacea	Seawater, Sargasso Sea
[Cytophaga] marinoflava	Flavobacteriaceae	Seawater, North Sea
[Cytophaga] latercula	Flavobacteriaceae	Marine aquarium, California
Formosa – F. algae	Flavobacteriaceae	Brown algal macrophyte
Gelidibacter – G. algens, G. mesopilus, G. salicanalis, G. gilvus	Flavobacteriaceae	Sea-ice algae and brines, Antarctic quartz stone subliths, Seawater, Mediterranean Sea
Gillisia – G. limnaea, G. hiemivivida, G. illustrilutea, G. sandarakina	Flavobacteriaceae	Antarctic lake cyanobacterial mat; sea-ice algal assemblages
Gramella – G. echinicola	Flavobacteriaceae	Sea urchin, NW Pacific Ocean
Kordia – K. algicida	Flavobacteriaceae	Red-tide algal bloom, Korea
Lacinutrix – L. copepodicola	Flavobacteriaceae	Copepod from Antarctic marine-derived lake
Maribacter – M. sedimenticola, M. aquiviva, M. orientalis, M. ulvicola	Flavobacteriacea	Marine sediment, green algal macrophytes
Mesonia – M. algae	Flavobacteriaceae	Green algal macrophyte , NW Pacific Ocean
Muricauda – M. ruestringensis	Flavobacteriaceae	Intertidal marine sediment, North Sea
Pibocella – P. ponti	Flavobacteriaceae	Green algal macrophytes – NW Pacific Ocean
Polaribacter – P. filamentus, P. irgensii, P. glomeratus, P. franzmannii	Flavobacteriaceae	Antarctic and Arctic sea-ice; Antarctic coastal fjord pycnocline
Psychroflexus – P. torquis, P. gondwanensis, P. tropicus	Flavobacteriaceae	Antarctic sea-ice algal assemblages; Antarctic and Hawaiian hypersaline lakes
Psychroserpens – P. burtonensis	Flavobacteriaceae	Antarctic coastal fjord pycnocline
Robiginitalea – R. biformata	Flavobacteriaceae	Seawater, Sargasso Sea
Salegentibacter – S. salegens, S. holithuriorum, S. mishustinae	Flavobacteriaceae	Antarctic hypersaline lakes; holothurians, NW Pacific Ocean
Subsaxibacter – S. broadyi	Flavobacteriaceae	Antarctica quartz stone subliths
Subsaximicrobium- S. wynnwilliamsii, S. inquilinus	Flavobacteriaceae	Antarctica quartz stone subliths
Tenacibaculum – T. maritimum, T. mesophilum, T. ovolyticum, T. amylolyticum, T. skagerrakense	Flavobacteriaceae	Diseased fish and fish eggs, seawater, sponges and red algal macrophytes (temperate and tropical locations)
Ulvibacter – U. litoralis	Flavobacteriacea	Green algal macrophyte, NW Pacific Ocean
Vitellibacter – V. vladivostokensis	Flavobacteriaceae	Holothurian, NW Pacific Ocean
Winogradskyella – W. epiphytica, W. eximia, W. thalassicola	Flavobacteriaceae	Macrophytes, NW Pacific Ocean
Zobellia – Z. uliginosa, Z. galactanovorans, Z. amurskyensis, Z. laminariae, Z. russellii	Flavobacteriaceae	Marine sediment, macrophytes
Cryomorpha – C. ignava	Cryomorphaceae	Continental shelf sediment, off Antarctica
Brumimicrobium – B. glaciale	Cryomorphaceae	Antarctic sea-ice
Crocinitomix – C. catalasitica	Cryomorphaceae	Coastal sandy sediment off northern Alaska
Anaerophaga – A. thermohalophila	"Anaerophagaceae"	Saline oil well waters
*[Cytophaga] fermentans**	"Anaerophagaceae"	Marine sediment
Marinilabilia – M. salmonicolor	"Anaerophagaceae"	Coastal marine sediment, California
Lewinella – L. cohaerens, L. persica, L. nigricans	"Saprospirceae"	Coastal sandy sediments (temperate to tropical regions)
Algoriphagus – A. ratkowskyi, A. halophila, A. chordae, A. aquimarinus, A. winogradskyi, A. antarcticus	"Flexibacteriaceae"	Antarctic sea-ice, Antarctic lake cyanobacterial mats, tidal flat sediments (Korea), seawater and macrophytes
Aquiflexum – A. balticum	"Flexibacteriaceae"	Seawater, Baltic Sea
Belliella – B. baltica	"Flexibacteriaceae"	Baltic Sea
Cyclobacterium – C. marinum	"Flexibacteriaceae"	Seawater
*[Flexibacter] litoralis**	"Flexibacteriaceae"	Marine aquarium, California
*[Flexibacter]polymorphus**	"Flexibacteriaceae"	*Beggiatoa* mat on a decaying ascidian, Baja California
*[Flexibacter] tractuosus**	"Flexibacteriaceae"	Coastal marine sediment and waters, tropical to temperate sites
Hongiella – H. mannitolivorans, H. ornithinivorans	"Flexibacteriaceae"	Tidal flat sediments, Korea
Microscilla – M. marina	"Flexibacteriaceae"	Marine aquarium, California
Reichenbachia – R. agarperforans	"Flexibacteriaceae"	Seawater, NW Pacific Ocean
Roseivirga – R. ehrenbergii	"Flexibacteriaceae"	Green algal macrophytes, NW Pacific Ocean
Flammeovirga – F. aprica	"Flammeovirgaceae"	Coastal marine sediment and sand (tropical sites)
Persicobacter – P. diffluens	"Flammeovirgaceae"	Intertidal marine sediments, tropical to subtropical sites
Flexithrix - F. dorotheae	"Flammeovirgaceae"	Coastal marine sediment, off India
Rhodothermus – R. marinus	"Crenotrichaceae"	Shallow marine hot springs

*Underlined species are type species; species in bold type are from the Southern Ocean and Antarctica
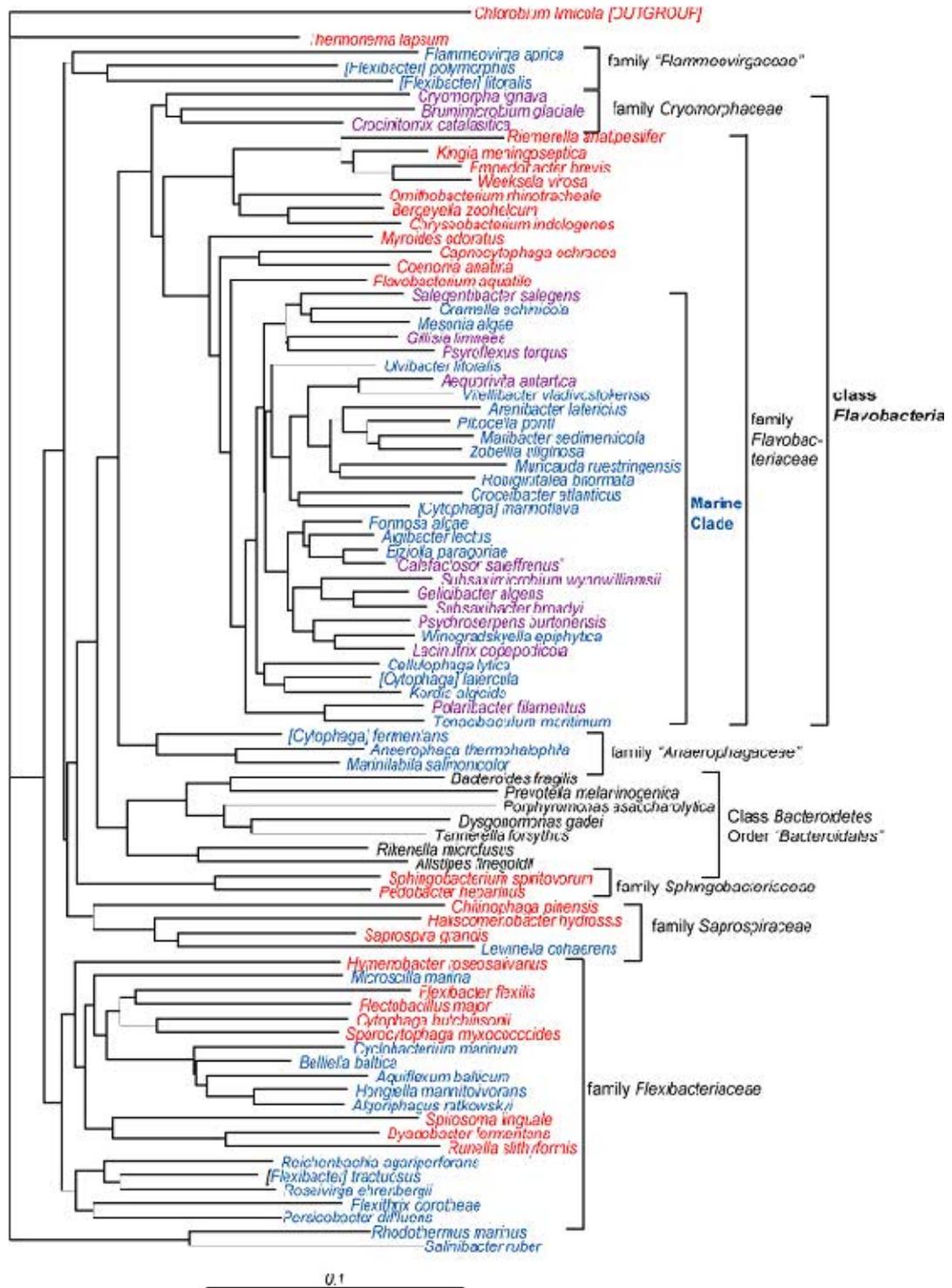
Figure 1.4 16S rRNA gene sequence phylogenetic tree of the CFB phylum showing type species of valid genera (and some misclassified species). Species from marine or saline habitats are shown in blue or purple; species from terrestrial, freshwater and clinical samples are in red, anaerobic species from human and animal intestinal habitats are shown in black. Genera containing predominantly polar species, denoted in purple, concentrate mostly in the "Marine Clade" of the family *Flavobacteriaceae* and in family *Cryomorphaceae* which together collectively make up class *Flavobacteria*.

1.2.3. Culture-independent diversity of CFB in the Southern Ocean

Since the advent of molecular approaches to microbial ecology the use of molecular techniques, PCR-based gene clone libraries and more recently shot gun sequence-based clone libraries (Venter et al. 2004), have provided phylogenetic information about uncultured organisms in the oceanic pelagic zone (reviewed by Rappé and Giovannoni 2003). Clone library gene approaches have allowed a high-resolution examination of the phylogenetic arrangement, structure and variability of pelagic bacterial communities. However, such approaches are labour intensive and costly and so few samples can be investigated at any one time. Being PCR based 16S rRNA gene clone libraries, still the most popular way to assess prokaryotic diversity in samples, may be subject to certain PCR amplification-driven biases, reducing meaningfulness at the quantitative level. Other problems such as generation of chimeric sequences or sequence microvariation due to the PCR/cloning process (von Witzengerode et al. 1997; Speksnijder et al. 2001) are generally less of an issue as they only have a small influence on the overall dataset or can be filtered out. Nevertheless, 16S rRNA gene clone libraries have provided detailed insight into the diversity of CFB in certain Southern Ocean ecosystems including sea-ice (Bowman and Brown 2001; Brinkmeyer et al. 2003) and marine sediments (Bowman and McCuaig 2003). From this data it was evident members of family *Flavobacteriaceae* are a particularly major component of the CFB population overall, and nearly totally dominate oxygenated habitats. CFB diversity and community structure data for the Southern Ocean pelagic zone, including marine particles and the planktonic fraction are not available in as much detail. Culture independent studies of CFB diversity, utilising FISH, in other marine habitats have demonstrated the group to be amongst the most abundant of the heterotrophic bacterioplankton, however few sequences have been recovered from clone libraries of free-living bacterial assemblages (Kirchman,

2001). The association of members of the CFB with marine particles has been demonstrated in a number of studies (Delong, Franks and Alldredge, 1993; Rath et al. 1998). This association of members of the CFB with surfaces, and their ecological significance is likely to lie in their ability to degrade biomacro-molecules and in their surface associated gliding motility (Kirchman, 2000).

1.3. Thesis Project Questions, goals and associated Research Design

The thesis project presented her represents a polyphasic investigation of CFB, with a focus on class *Flavobacteria*, within bacterioplanktonic and particle-associated communities in the Southern Ocean. The rationale for this work as reviewed above is that CFB members represent a large component of Southern Ocean surface water bacterial communities and a likely major players in secondary production. However, we know little about the diversity, community structure and biogeography of CFB in the Southern Ocean. Several questions can be asked related to this lack of knowledge, which the research in this thesis seeks to address.

Thesis Questions:

1. Do factors such as nutrients and primary produced biomass control CFB abundance?

2. Does the ACC and the Polar Front help shape different communities of CFB as found for the *Roseobacter* clade (Selje et al. 2003; section 1.1.4) and are particle-associated and planktonic CFB communities different?

3. How does the diversity compare with other oceanic regions, especially the Arctic Ocean and does this diversity follow trends in the Southern Ocean?

4. Will culture-dependent and independent approaches reveal the same CFB clades and what are the new taxa that can be revealed by traditional culturing?

5. How quickly, to what extent and what types of CFB can colonize particulate organic matter in Southern Ocean seawater and can CFB readily decompose phytodetritus?

Thesis Goals and Research Design Summary

To answer these questions a combination of approaches will be used. The essential investigations of relationships of CFB abundance and spatial distribution will rely on seawater samples collected during cruises of the *RV Aurora Australis*, which are filter fractionated, separating the planktonic fraction from a particle-associated fraction. The two fractions will be then analysed separately by the approaches given below.

Question 1. CFB abundance will be estimated using FISH and the SYBR green real-time procedure testing different filter fractions. In the same samples estimates of chlorophyll *a*, silica, phosphate and nitrate will be measured to determine if they correlate with abundance changes.

Question 2.CFB community structure in filter fractionated samples will be performed using denaturing gradient gel electrophoresis (DGGE). DGGE is a gel-based fingerprinting method in which 16S rRNA genes are separated into a series of bands resulting in a pattern. Differences between different samples on the basis of banding

patterns and band number will be determined using statistical ordination analysis. DGGE will be performed using both CFB-specific and Bacteria –specific PCR primers.

Question 3. CFB 16S rRNA gene diversity will be assessed by sequencing DGGE gel bands as had been done previously for marine sediment samples (Bowman et al. 2003b). From this sequence data, phylogenetic analysis and comparisons with the GenBank nucleotide library database will be used to assess differences in diversity through the Southern Ocean.

Question 4. Isolation of pure cultures of CFB will be performed using various different media containing a range of nutrient levels. The data will be compared to the culture-independent approach covered in Question 3. If decidedly novel CFB taxa are found, for example new genera, these will be characterised and described.

Question 5. Finally, a mesocosm experiment will be performed to observe the colonization of artificially generated detritus from an axenic laboratory culture of *Nitzschia closterium*, a cosmopolitan diatom species found in the Southern Ocean. The process of colonization by the CFB as well as other bacteria will be tracked by FISH, real-time PCR and DGGE (including DGGE and sequence analysis). The decomposition of the phytodetritus will be tracked using silica release as an indicator as the diatom frustules have high silica and organic carbon content and have been shown to be readily decomposed by bacteria (Bidle and Azam 1999, 2001).

CHAPTER 2. Abundance of class *Flavobacteria* in the Southern Ocean

2.1 Summary.

The abundance of members of the CFB phylum and more specifically class *Flavobacteria* in Southern Ocean water samples was performed using FISH and real-time PCR. Samples investigated were collected along a latitudinal transect between 43.7°S to 63.5°S at approximately longitude 142°E. Very low efficiency of binding was encountered for the EUB338 probe in relation to DAPI direct counts (8-32% recovery), even after boosting of intracellular RNA levels using a nutrient/chloramphenicol treatment. Data suggested CFB and *Flavobacteria* abundance was similar and was maximal in the euphotic zone; however abundance highly varied between samples, ranging from 2-50% of the EUB338 probe cell count. *Alphaproteobacteria* and *Gammaproteobacteria* otherwise dominated the bacterioplankton communities in most samples, especially in the highly nutrient limited waters of the SAZ. No correlation in total *Flavobacteria* abundance was found with water temperature, chlorophyll *a* levels or nutrient concentrations along a latitudinal sampling transect. For real-time PCR analyses seawater samples were fractionated into particle-associated and planktonic fractions. Results indicated that planktonic *Flavobacteria* 16S rRNA gene abundance were relatively constant averaging 25% of the total 16S rRNA gene abundance. The relative abundance of particle-associated *Flavobacteria*, however was found to increase along the transect between the SAZ and AZ and positively correlated with chlorophyll *a* and nutrient concentrations suggesting *Flavobacteria* populations were at least partially coupled to primary production.

2.2 Introduction.

Studies of bacterial abundance in environmental samples have previously utilized culture-dependent techniques such as most probable number counting. Culture techniques are limited by their ability to access only the cultivable proportion of the community. Fluorescent in situ hybridisation (FISH) utilizes cellular rRNA to observe microbial communities in a manner that is culture-independent. FISH utilizes fluorophores attached to oligonucleotide DNA probes, which bind to rRNA in bacterial cells and allow for direct detection and enumeration of cells present in a sample through the use of epifluorescent microscopy. FISH has been successfully used to enumerate bacteria in a range of marine samples (Llobet-Brossa *et al.* 1998, Glöckner *et al.* 1999, DeLong *et al*. 1999, Cottrell and Kirchman 2000). The ability of FISH to be applied to an environmental sample, however is dependent on the strength of the fluorescent signal that can be achieved through hybridising oligonucleotide probes onto cellular 16S rRNA molecules. In cells where the level of cellular rRNA is low the fluorescent signal may be undetectable and thus reduce the reliability of quantification. Such situations are often prevalent in cold or oligotrophic environments such as the open ocean. The use of FISH for the analysis of Southern Ocean environments is not well documented (Simon et al. 1999) but the technique has been used successfully in the past to analyze bacterial populations in other cold-water environments (e.g. Wells and Deming 2003, Murray et al. 1998, and Murray et al. 1999). The limitation of FISH means that its application could be reduced to enumeration of bacterial groups in samples of high productivity. Given the relationship between cellular rRNA levels and production, it is accepted that the cells displaying strong signals are likely to represent the active cells in a population, given

the low ribosome content often found in starved cells (Kolter et al., 1993; Roszak and Colwell, 1987).

Real-time PCR represents another approach to quantifying bacterial abundance in environmental samples in a cultivation-independent manner. The method uses the double stranded DNA specific fluorescent dye SYBR Green (Molecular Probes, Eugene, Oregon, USA). Combined with PCR gene targets can be quantified by measuring the fluorescence given off by SYBR green as it binds to double stranded DNA created during each PCR cycle. The technique thus allows the determination of the relative amount of template DNA in an environmental DNA extraction. The technique requires the use of sample standards to generate a standard curve of template concentrations, from which a relative sample template concentration can be then extrapolated. Quantitative PCR technology has yielded a new avenue for the quantification of gene abundance and expression and the possibility of a fast semi-quantitative method for delineating bacterial community structure based on specific and universal PCR primers. The majority of previously described techniques have utilised the 5' nuclease assay for quantification of bacterial 16S rRNA genes. This version of the technique is based on the binding of a fluorogenic probe to PCR products as they are amplified thus giving a quantification of 16S rRNA gene copy number. The method has been successfully applied to the quantification of prokaryotes in environmental samples including seawater (Hermansson and Lindgren 2001; Suzuki et al 2001). Suzuki et al. found that the percentage of the total SSU rDNAs from the domain bacteria, belonging to the members of the *Cytophagales*, decreased with depth and salinity. The SYBR green technique differs in that the need for an internal probe is eliminated; the method is also more easily implemented. The use of SYBR green for direct detection and quantification of DNA or cDNA

amplicons has been successfully in clinical and environmental samples (Rantakokko-Jalava and Jalava, 2001; O'Mahony and Hill, 2002; Malinen et al. 2003; Heuser and Zimmer, 2003; Newby et al. 2003).

For this study specific detection of class *Flavobacteria* in samples, collected along a latitudinal transect, was performed using FISH and real-time PCR. Difficulties found with the use of FISH were to a certain extent overcome using the real-time PCR approach which allowed for relationships in *Flavobacteria* relative abundance to be made along a latitudinal transect of the Southern Ocean.

2.3 Materials and Methods

2.3.1 Seawater sampling.

Seawater samples were obtained during two separate transects across different Southern Ocean zones on the *RV Aurora Australis*. In 2000 samples were mainly collected from stations located in the ice pack off Eastern Antarctica (no. 4-13, Table 2.1, Fig. 2.1). A latitudinal transect of the Southern Ocean was also performed during November 2001 with samples collected from 12 stations (no. 15-26, Table 2.1, Fig. 2.1) between latitudes 44.7°S to 63.5°S along longitude 142 (±2)°E. Water samples were obtained from a depth of 10 m, via an underway water sampler aboard the *RSV Aurora Australis* and were stored for less than 1 h in 25 l sterile carboys before they were processed. Biomass for DNA extraction was collected by filtering 1-5 l of water through a 0.8 μm pore size polycarbonate filter (Millipore) and then a 0.2 μm polycarbonate filter, in succession. Both the 0.8 μm and 0.2 μm filters were immediately stored at −20°C before processing. To minimize the retention of free-living bacteria, the 0.8 μm filters were washed with approximately 1 l of filtered,

autoclaved seawater using gentle filtration. Seawater chlorophyll *a,* nitrate, phosphate and silicate concentrations were measured as previously described (Kopczynska et al. 2001).

2.3.2 Fluorescent in situ hybridisation (FISH)

Internal cellular rRNA levels in various seawater samples were augmented by the addition of an equal volume of a low nutrient seawater medium (Bowman and McCuaig 2003; see Appendix) supplemented with 50 mg l^{-1} chloramphenicol to samples within the filter manifold, followed by incubation for approximately 2 h at *in situ* temperatures (usually 0°C to 10°C). Subsequent microscopic analysis indicated negligible cell division occurred during this incubation. Following incubation, samples were filtered and washed with sterile phosphate-buffered saline solution (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.0). Samples for FISH analysis were prepared in triplicate, with 50 ml of sample filtered through 47 mm 0.2 µm black isopore polycarbonate membrane filters (Millipore). The filters were the fixed in methanol free formalin and stained with oligonucleotide probes as previously described (Glöckner et al. 1999) using hybridization and wash buffers shown in the Appendix. Cy3-labelled oligonucleotide probes (Geneworks, Australia) used included EUB338 (Bacteria; 5'-GCT GCC TCC CGT AGG AGT-3'; Amann et al. 1991), Alf968 (Alphaproteobacteria; 5'-GGT AAG GTT CTG CGC GTT-3' Amann et al. 1997), Gamm42a (*Gammaproteobacteria*; Manz et al. 1992) used in conjunction with unlabelled Beta42a (*Betaproteobacteria*; Manzet al. 1992), CFB319a (CFB group and Flavo558 (class *Flavobacteria*; Weller et al. 2000). Following hybridization, filters were washed and stained for 15 min. at 48°C on a heating block in 5 ml of PBS

containing 2 µg ml^{-1} 4', 6-diamidino-2-phenylindole (DAPI). The filters were dried

and mounted on a glass slide with immersion oil and inspected, with digital pictures

taken using a LDRMBE Leitz microscope (Leica, Heerbruug, Switzerland) fitted with

a DC300F digital camera (Leica), equipped with a 50-watt HBO mercury bulb

(Osram, Regensburg, Germany) and specific filter sets for DAPI and Cy3

fluorophores (Cy3; 531nm excitation and 40nm bandpass, DAPI; 377nm excitation

and 50nm bandpass). Fields were first viewed with the Cy3 filters before switching to

the DAPI filters, in order to minimize photo-bleaching of the Cy3 fluorophore during

DAPI examination. Cell counts for DAPI and each of the probes were determined

from 10-15 random non-overlapping fields (Lebaron et al. 1999) and performed on at

least two different hybridisations of filter sections. A range of bacterial pure cultures

served as positive and negative controls (See Table A.3). A number of samples had

very poor FISH labeling, regardless of probe used; possibly due to extremely low

cellular rRNA levels and so were excluded from the analysis. Analysis of statistical

correlations were performed using the pearson's correlation coefficient in the SPSS

package.

2.3.3 DNA extraction and purification.

Extraction of DNA from filtered samples followed a modification of a method

employed by Fuhrman et al. (1988). Frozen filters were thawed, cut into small strips

with a sterile razorblade, and resuspended in 3 ml of STE buffer (100 mM NaCl, 10

mM Tris HCl; 1 mM disodium EDTA; pH 8.0) in a sterile 15 ml centrifuge tube

(Falcon, USA) and vortexed to detach biological material. To lyse cells, sodium

dodecyl sulphate (SDS, final concentration 1% wt./vol.) was added and the filter was

held at 100°C for 2 min., then cooled on ice. The filter was washed with an additional

1 ml of STE buffer and treated with SDS as before. The combined lysates were

extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and once with

chloroform: isoamyl alcohol (24:1). DNA was precipitated by adding 2 volumes of

cold absolute ethanol and 0.2 volumes of 3 M sodium acetate (pH 8.0) to the aqueous

layer and then storing the tubes overnight at –20°C. The sample was then centrifuged

in a 50 ml centrifuge tube (Nalgene, Rochester, USA) for 30 min. at 15000 g at 4°C.

The pellet was washed with 70% (vol./vol.) ethanol and centrifuged again. Residual

liquid was removed with a pipette and the subsequently air-dried pellet then

resuspended in sterile water. DNA extracts were visualised on 1% (wt./vol.) agarose

gels containing 10 µg ml^{-1} ethidium bromide using UV transillumination to ensure the

extractions gave high quality, high molecular weight DNA (>10kb) for analysis.

2.3.4 Real-time PCR.

Real-Time PCR reactions were prepared using the QuantiTect SYBR Green PCR kit

(Qiagen, USA), in 200 µl flat lid PCR tubes (Axygen Scientific, Union City, USA)

and Rotorgene thermocyler (Corbett Research, Australia). Results were analysed with

Rotorgene software (V. 4.6). Reactions (20 µl volumes) contained 10 µl of 2x

QuantiTect SYBR Green PCR Master Mix, 200 µM of each primer, 0.1-10 ng of

template DNA and UV-treated water. Primers used for amplification and detection of

class *Flavobacteria* 16S rRNA genes included 558f (5'-ATT GGG TTT AAA GGG

TCC-3') (Weller et al. 2000) and 907r (5'-CCG TCA ATT CCT TTG AGT TT-3')

(Lane 1991). Assays were performed using a thermocycling program consisting of an

initial 15 min. 95°C step followed by 35 cycles consisting of 95°C for 30 s, 55°C for

30 s and 72°C for 30 s with fluorescent acquisition, and a further fluorescent acquisition step at 80°C. Total bacterial 16S rRNA gene amounts were estimated with primers 519f (5' CAG CMG CCG CGG TAA TAC 3') (Lane 1991) and 907r. The temperature at which fluorescence analysis was performed was determined by examination of the melting profile of a number of samples, and performed at a temperature at which all primer dimer had melted, but the specific product had not. Amplified products from mixed template samples only contained a single peak indicating that product length variability and guanosine plus cytosine (G+C) content did not have a significant effect on quantification. Positive control standards for the real-time PCR assay included 16S rRNA genes amplified with primers 558f/907r from *Gelidibacter algens* and *Cryomorpha ignava*. The controls were diluted in tenfold series from 1 ng to 0.1 pg and analysed in parallel with seawater DNA samples. Negative controls included samples lacking template DNA and samples lacking primers. Primer concentrations used in the assay were determined by running a dilution series of primer including positive and negative controls (in the case of specific primers) and sample templates. The optimal primer concentration chosen was the minimum primer concentration required to achieve maximum end point fluorescence. All real-time PCR products were examined using agarose gel electrophoresis to ensure products corresponded to the correct size, and to ensure the absence of non-specific product. Experimental samples were diluted to three different concentrations and compared to the standard curve in the same run. Values were corrected for their dilution factor and averaged to calculate the total 16S rRNA amount in the starting material and then compared to the same sample analysed with the *Flavobacteria* specific primers to give a

Table 2.2: Location and temperatures of Southern Ocean samples investigated in this study.

Station Number	Sample Number	Date of collection	Oceanic Region[a]	Latitude (°S)	Longitude (°E)	Water Temp (°C)
2000 Voyage samples:						
4	**5/12**	5/12/00	AZ	66.48	62.55	-1.4
6	**8/12**	8/12/00	AZ	66.20	70.03	-1.4
7	**9/12**	9/12/00	AZ	67.50	77.55	-0.1
8	**11/12**	11/12/00	AZ	68.33	75.55	-0.7
9	**13/12**	13/12/00	AZ	69.17	74.28	0.7
10	**16/12**	16/12/00	AZ	68.05	77.30	1.6
11	**17/12**	17/12/00	AZ	65.07	77.41	0.1
12	**18/12**	18/12/00	AZ	62.06	83.34	0.1
13	**20/12**	20/12/00	AZ	61.21	98.52	0.7
14	**23/12**	23/12/00	PFZ	52.54	124.36	5.6
2001 Latitudinal transect samples:						
15	**G1**	31/10/01	TZ	44.72	145.37	13.6
16	**G2**	1/11/01	TZ	46.17	145.40	13.6
17	**G3**	3/11/01	SAZ	47.00	144.54	11.7
18	**G4**	7/11/01	SAZ	48.47	144.25	10.1
19	**G5**	9/11/01	SAZ	50.24	143.27	7.7
20[b]	**G6**	11/11/01	PFZ	52.50	142.42	5.6
21[b]	**G7**	13/11/01	PFZ	53.45	141.14	3.2
22[b]	**G8**	17/11/01	PFZ	56.00	140.10	2.1
23[b]	**G9**	19/11/01	AZ	58.20	139.45	1.6
24[b]	**G10**	19/11/01	AZ	59.00	139.53	0.1
25	**G11**	21/11/01	AZ	60.50	139.52	0.2
26	**G12**	25/11/01	AZ	63.53	139.56	-1.4

[a]Oceanic region corresponds to specific oceanographic regions in which the sample stations were located as shown in Fig. 2.1.

[b]Stations were also sampled down to 350 m by a Rosette Multi-Bottle Array System (work done in collaboration with J. H. Skerratt).
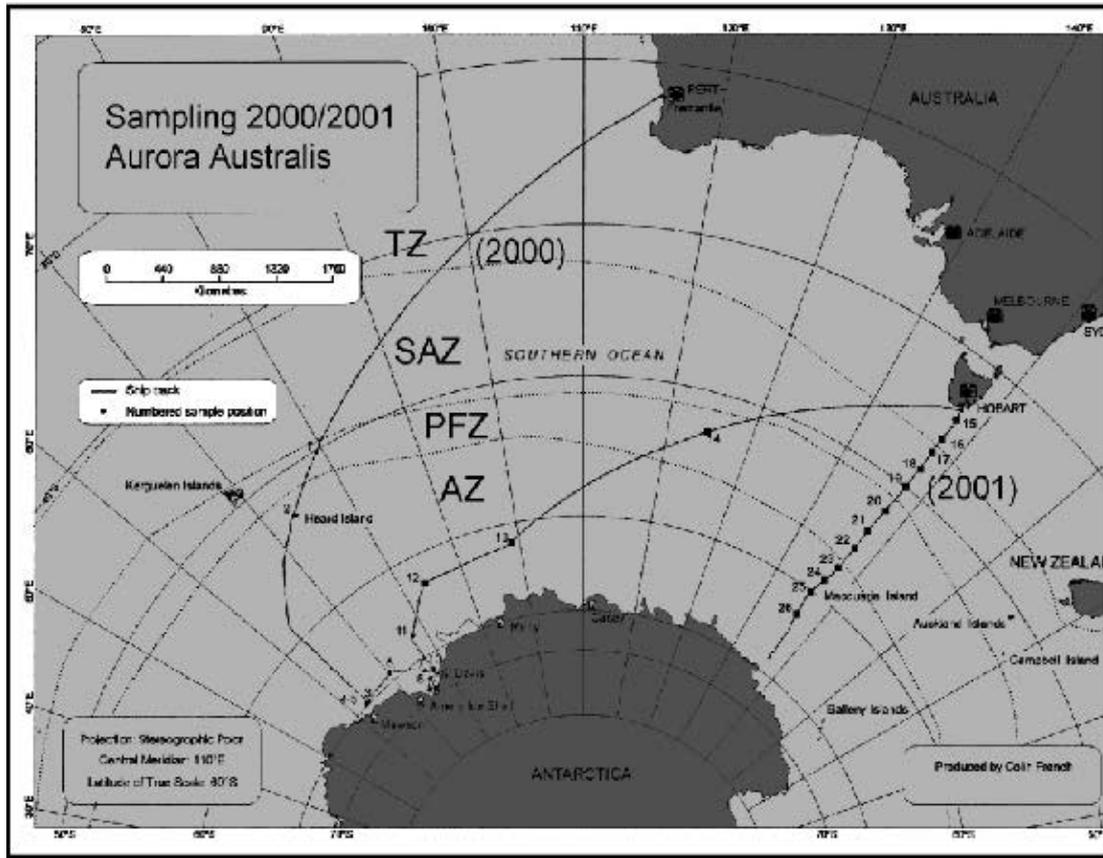
Figure 2.1. Location of seawater sampling stations within the Southern Ocean over two different transects. Stations were assigned to different surface water masses based on hydrographic characteristics as defined by Deacon (1984), including the Temperate Zone (TZ); the Sub-Antarctic Zone (SAZ), located between the Subtropical Convergence and the Polar Front; the Polar Front Zone (PFZ), location of the Antarctic Circumpolar Current; and the Antarctic Zone (AZ).

percent fraction of class *Flavobacteria* 16S rRNA (558f-907r primer set) to total

bacterial 16S rRNA (519F-907r primer set) relative abundance.

2.4. Results

2.4.1. FISH analyses.

For FISH to be successful it requires that there is sufficient cellular 16S rRNA for

hybridisation of enough probe to give a fluorescent signal distinguishable above the

background. Initial studies done in collaboration with J.H. Skerratt indicated bacteria

in samples from the Southern Ocean marine environment often displayed very low

levels of cellular RNA, reflecting the low metabolic rate of the community. This has

been shown to effect hybridization in samples analysed with FISH (Karner and

Fuhrman, 1997). It was found that planktonic bacteria often gave a lower signal than

the particle-associated community reflecting the difference in habitat. Such

differences may include the increased nutrient availability and protection from

predation available to the particle associated fraction. For this reason it was often

difficult to assess the community composition using FISH, and thus only samples that

gave EUB338 labeling of >5% DAPI stained cells were used for quantitative analysis

of community structure. In such cases it is likely that the cells that were detected

represented the metabolically active component of the community. Probes used to

assess the community composition included those specific to the

Gammaproteobacteria, *Alphaproteobacteria*, CFB and class *Flavobacteria*. The

former three groups are widely accepted to dominate the bacterioplankton in surface

waters (Giovannoni and Rappé, 2000; Rappé and Giovannoni 2003) and as such were

chosen to allow comparison of Flavobacterial abundance with the other major

bacterioplankton groups. Preliminary analyses indicated that the Flavo558 probe

consistently bound to an equal or a greater number of cells and produced a brighter

fluorescence
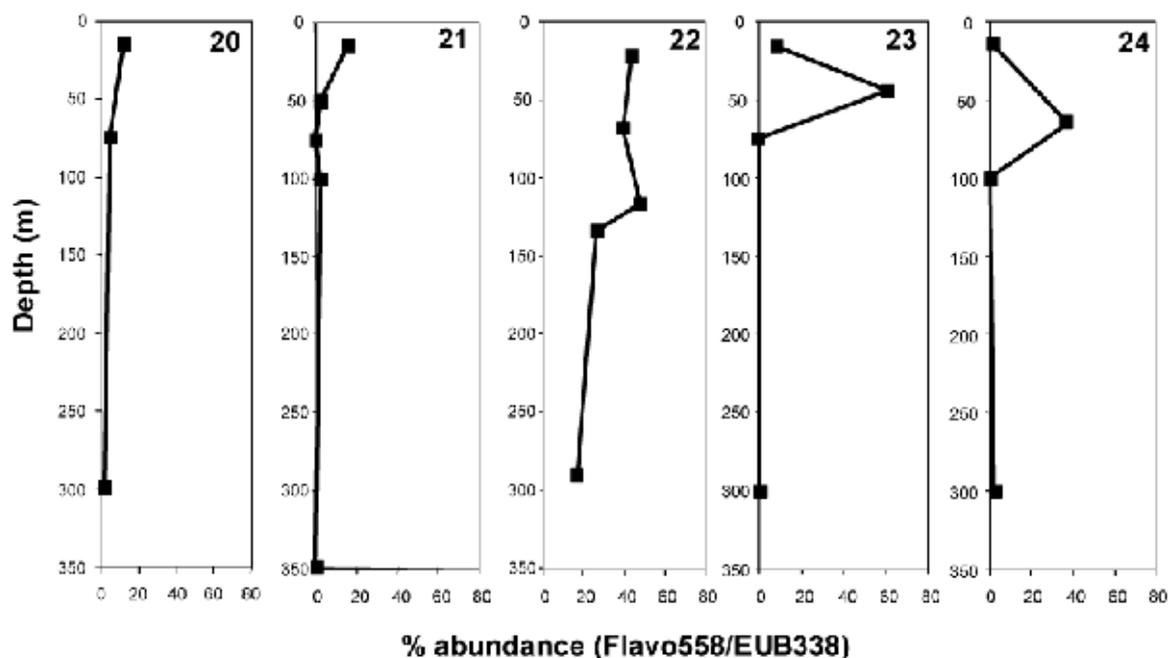
Figure 2.2. The abundance of class *Flavobacteria* in the Southern Ocean pelagic zone

for stations 20 to 24 located in the PFZ and AZ (2001 latitude transect; Table 2.1, Fig.

2.1). This data was obtained in collaboration with J. H. Skerratt.

signal compared to the CFB319a probe suggesting the Flavo558 probe had a greater

hybridization efficiency. Subsequently, all experiments utilized the Flavo558 probe.

Unfortunately, the efficiency of labelling in samples collected during 2000

(sites 4, 6-14, Table 2.1) was extremely poor with only <1% of DAPI stained cells

hybridizing to the EUB338 probe (results not shown), rendering this data unusable.

To overcome this problem pre-boosting of samples with a chloramphenicol/ liquid

nutrient media treatment was instigated. The nutrients increase cellular RNA levels

and thus fluorescence intensity while the chloramphenicol prevents cell division. This

technique has been utilized successfully for seawater samples collected from highly

oligotrophic regions of oceans. (Ouverney and Fuhrman, 1997; MacDonald and

Brozel, 2000). After the treatment samples demonstrated a signal intensity of between

8% and 32% of DAPI stained cells hybridizing with the bacterial probe (Fig. 2.3A).

The recovery levels were still rather low which reduced the comparability of the FISH

data, however the data provided some indications of the abundance and distribution of

different bacterial groups including *Flavobacteria*.

Depth profiles for samples taken at stations 20 to 24 down to 350 m by CTD

rosette samplers indicated class *Flavobacteria* predominated in the Southern Ocean

euphotic zone (Fig. 2.2). At 15-20 m depth the average *Flavobacteria* abundance was

16% (range 2-43%) of the EUB338 cell count. Between depths of 100-350 m, most

samples had abundances less than 5% (Fig. 2.2). At the 10 m depth the abundance of

Flavobacteria and other bacterial groups exhibited significant variation (Fig. 2.3B). In

some samples the EUB338 probe signals were exceeded by the other probes

suggesting hybridization efficiency of the EUB338 varied during the experiments

(Fig. 2.3B), this may also indicate a lack of specificity of the EUB338 probe.

However the EUB338 cell count data correlated with changes in DAPI direct cell

count ($p<0.1\%$). This suggested that the combined Alpha968, Gamm42a and

Flavo558 probed cell populations likely dominated in samples where they exceeded

the EUB338 cell count.

Alphaproteobacteria (16-81% of the EUB338 count) tended to predominate in

the majority of samples. *Flavobacteria* represented the second most abundant group

(2-52% of the EUB count). *Gammaproteobacteria* were also relatively abundant at 1-

42% of the EUB338 count. Microscopic observations revealed *Flavobacteria* and

other bacteria were often strongly associated with particulate matter but also occurred

as planktonic cells as illustrated in Fig. 2.4. The association of *Flavobacteria* and other bacteria in aggregate clumps increased the difficulty in accurately estimating populations. No correlations were found with the FISH-based abundance data with other measured factors for the water samples investigated ($p>0.5$). These included chlorophyll *a* levels and nitrate, phosphate and silica concentrations (Fig. 2.5A).
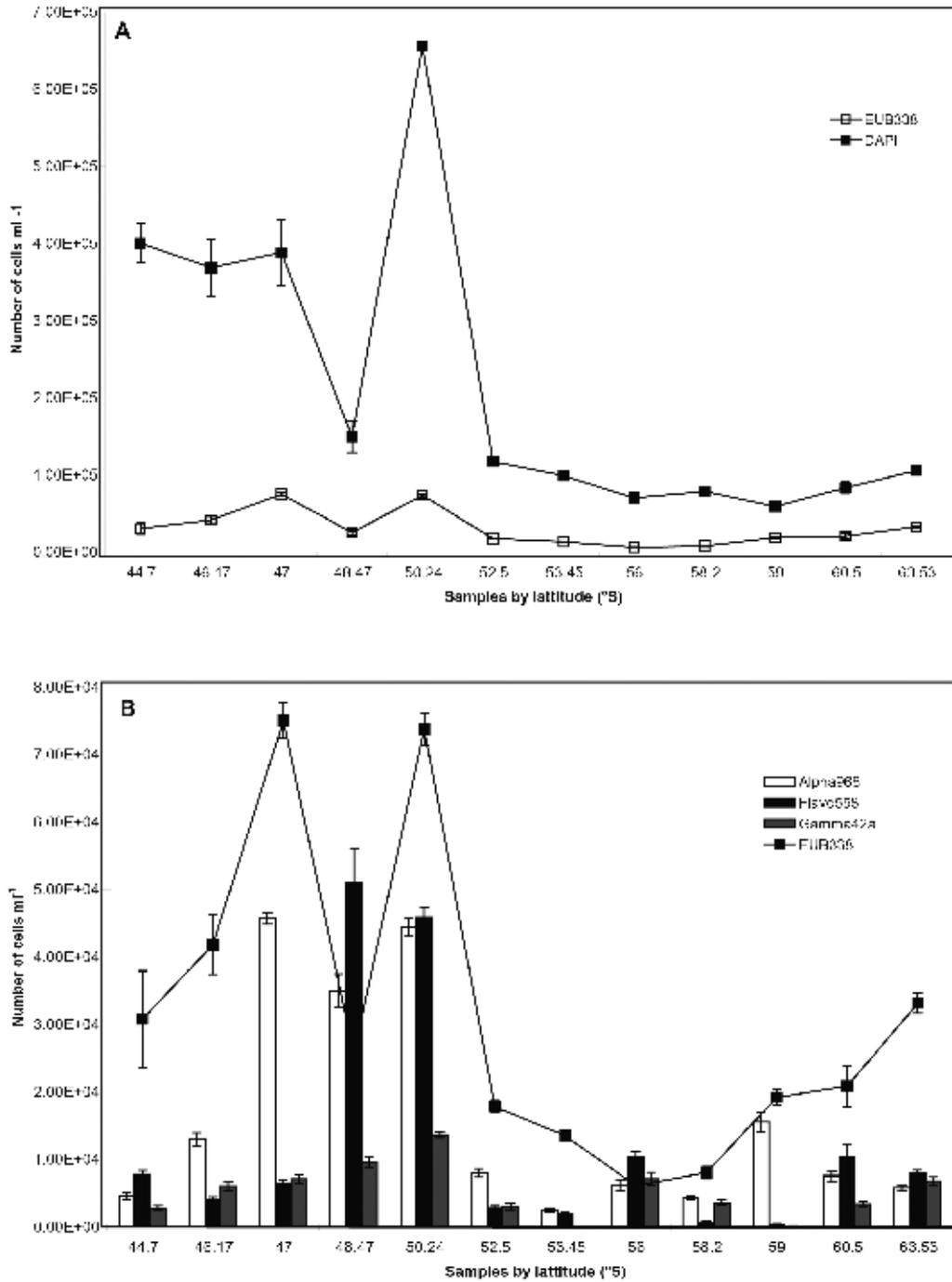
Figure 2.3. FISH data for 2001 latitude transect samples. A. DAPI direct count compared to Eub338 cell count. B. Cell counts for Alpha968, Gamma42a and Flavo558 probes in relation to EUB338**.**
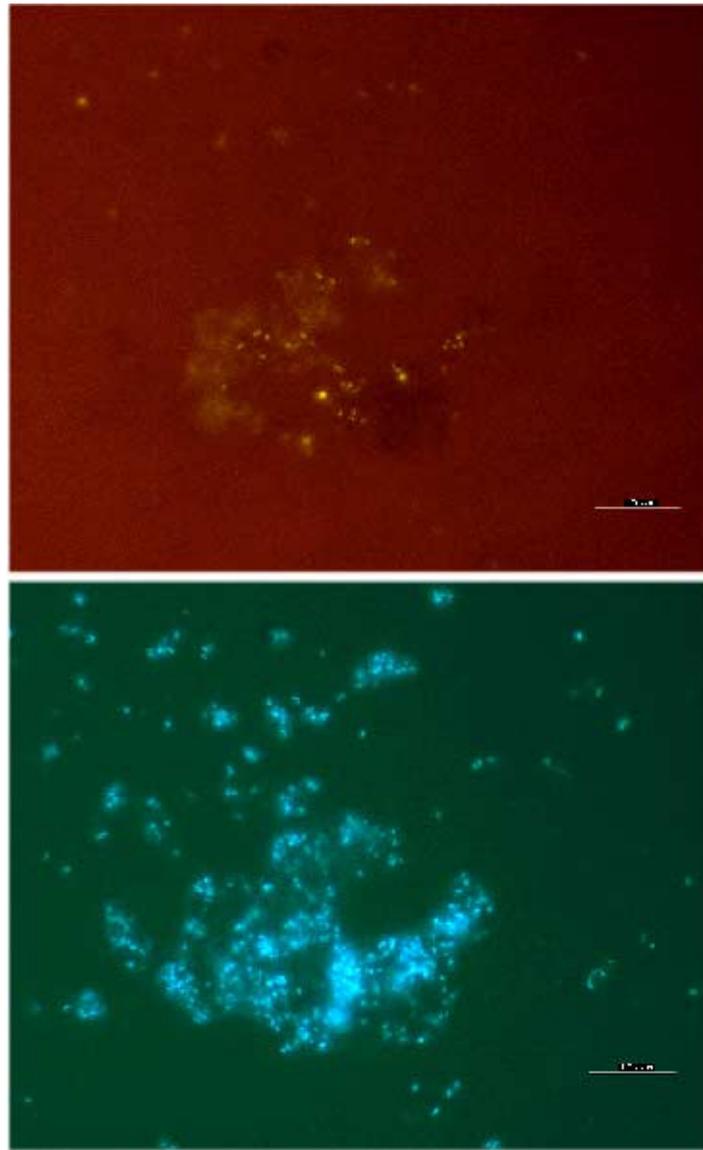
Figure 2.4. Bacterial community composition shown by FISH for 2001 latitude transect samples. A. Flavo558 probe hybridization to the sample from station 17 (47.0°S, 144.54°E) in which *Flavobacteria* represented $1.1. \times 10^3$ cells ml^{-1} Background fluorescence can be seen as the red hue around the image edge, whilst the cells are seen in the centre of the image. B. Sample counterstained with DAPI (6.8×10^5 cells ml^{-1}), scale bars = 10μm.
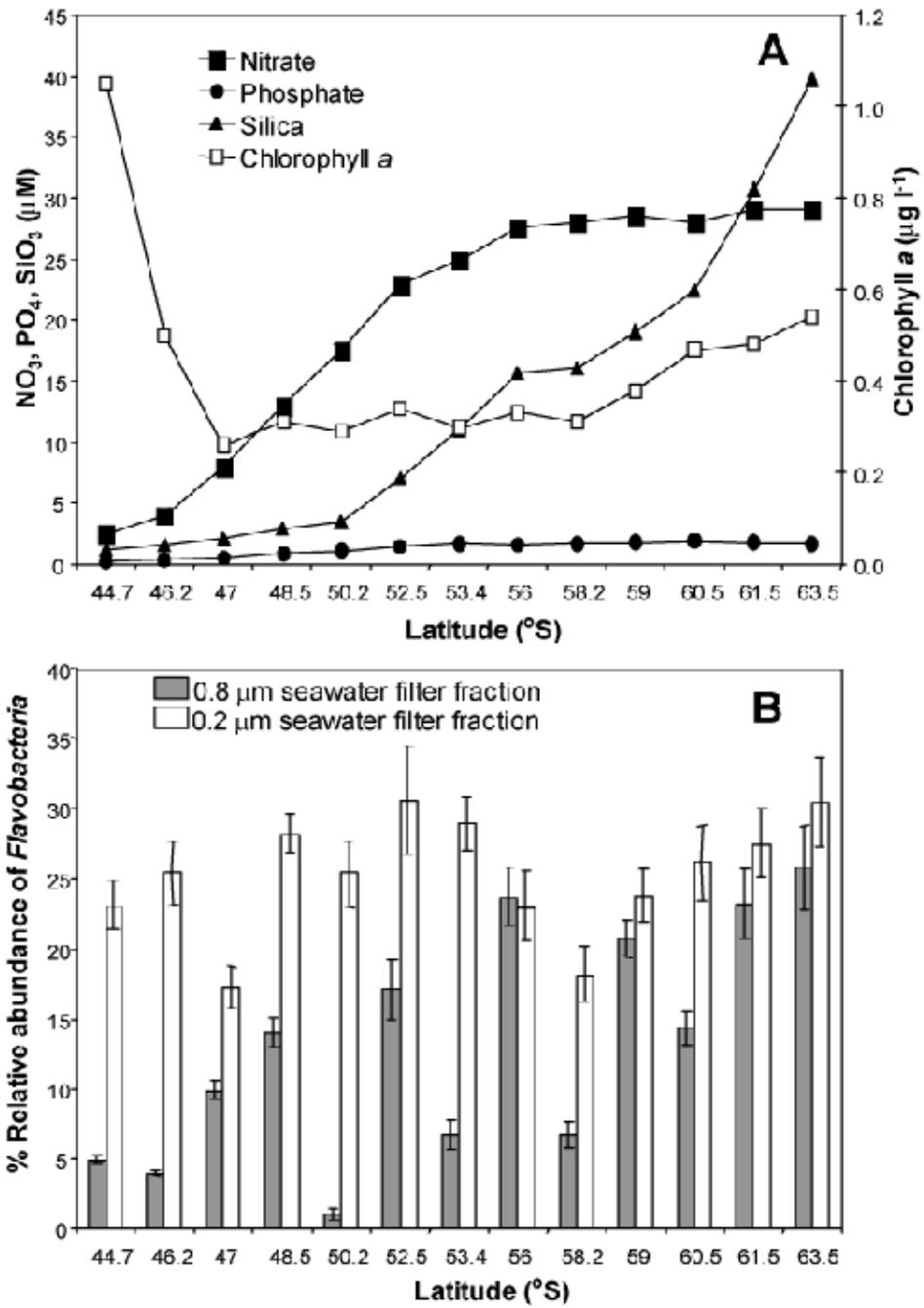
Figure. 2.5. A) Nutrients, chlorophyll *a* and B) relative abundance of class *Flavobacteria* in Southern Ocean seawater filter fractions along a latitudinal transect of the Southern Ocean.

2.4.2. Real-time PCR analyses

Results from the analysis of samples using the real-time PCR assay are shown in Fig. 2.5B) and include the 2001 latitude transect samples which has been fractionated into planktonic and particle containing fractions using successive filtration. No data is available for the 2000 dataset as many of the samples exhibited very poor PCR efficiency possibly due to deterioration of the DNA samples during storage. The planktonic fraction was found to contain a relatively constant *Flavobacteria* relative abundance, averaging 25.0% (± 4.4%, range 17.2-30.6%) of the total 16S rRNA gene abundance (Fig. 2.5B). By comparison particle-associated *Flavobacteria* relative abundance was more variable and constituted from 0.8-25.8% of the total 16S rRNA gene abundance. Along the latitudinal transect the relative abundance of particle-associated *Flavobacteria* increased approximately 2.5-fold between the TZ and SAZ and the AZ (Fig. 2.5B). No correlation was found between *Flavobacteria* FISH and real-time PCR data, indicating the measurement of different populations, which included the active cellular population (FISH) versus the total of active, inactive and dead cellular population (real-time PCR).

2.4.3. Nutrient, Chlorophyll *a* and Abundance Measurements.

Chlorophyll *a* values were >1 µg l^{-1} just south of Tasmania (Fig. 2.5A) and rapidly declined at the Subtropical Convergence (at approx. 46-47°S), this was accompanied by very low nitrate, phosphate and silica levels (Fig. 2.5A). Continuing south nutrient levels and chlorophyll *a* increased in concentration though chlorophyll *a* levels only increased slowly through the PFZ and the AZ. There was a positive

correlation between the increases in particle-associated *Flavobacteria* abundance and chlorophyll *a* (Pearson's correlation coefficient, +0.60, $p<0.05$) along the latitudinal transect. Abundance was also positively correlated with nitrate, phosphate and silica concentration (+0.59-0.70, $p<0.05$) and negatively correlated with water temperature ($p<0.05$). Data and corresponding Pearson's correlation coefficients over the latitudinal transect are included in Tables 2.3 and Table 2.4.

Table 2.3. Data for seawater samples taken on the 2001 latitudinal transect of the Southern Ocean.

Station No./ Region	Temp-erature (^{o}C)	Chloro-phyll (μg l^{-1})	Nutrients (μM)			Flavobacteria			
			Nitrate	Phos-phate	Silica	Relative abudance (%)		No. of clades	No. of DGGE Bands
						0.2 μm	0.8 μm	Both fractions	0.2 μm/ 0.8 μm
15 TZ	13.6	1.05	2.5	0.2	1.25	23.1	4.9	5	17/7
16 TZ	13.6	0.50	4.0	0.3	1.6	25.4	3.9	5	16/11
17 SAZ	11.7	0.25	8.0	0.6	2.1	17.2	9.9	5	15/9
18 SAZ	10.1	0.26	13.0	0.9	2.9	28.2	0.8	5	10/5
19 SAZ	7.7	0.24	17.5	1.1	3.5	25.4	14.0	5	13/0
20 PFZ	5.6	0.29	23.0	1.4	7.1	30.6	17.1	9	22/15
21 PFZ	3.2	0.30	25.0	1.65	11.1	28.9	6.7	8	19/12
22 PFZ	2.1	0.33	27.5	1.60	15.7	23	23.7	12	19/15
23 AZ	1.6	0.34	28.0	1.70	16.0	18.1	6.7	11	18/14
24 AZ	0.1	0.38	28.5	1.80	19.0	23.8	20.7	10	21/14
25 AZ	0.2	0.47	28.0	1.85	22.4	26.1	14.3	13	21/15
26 AZ	-1.4	0.48	29.0	1.70	39.7	30.5	25.8	15	22/18

Table 2.4. Pearson's correlation coefficients for data taken along the 2001 latitudinal transect of the Southern Ocean.

Factor	Temperature	Chlorophyll[a]	Nitrate	Phosphate	Silica	Relative Abundance 0.2 μm	0.8 μm	Clade number	DGGE BAND NUMBER 0.2 μm	0.8 μm
Temperature	-									
Chlorophyll	-0.86**	-								
Nitrate	-0.98**	0.74**	-							
Phosphate	-0.97**	0.76**	0.99**	-						
Silica	-0.87**	0.94**	0.77**	0.75**	-					
Abundance 0.2 μm	-0.22	0.24	0.23	0.23	0.27	-				
Abundance 0.8 μm	-0.63*	0.60*	0.60*	0.59*	0.70*	0.30	-			
Clade number	-0.91**	0.91**	0.85**	0.82**	0.93**	0.20	0.73**	-		
DGGE bands 0.2 μm	-0.69*	0.71**	0.63*	0.61*	0.68*	0.24	0.55	0.77**	-	
DGGE bands 0.8 μm	-0.67*	0.74**	0.62*	0.60*	0.73**	0.15	0.69*	0.83**	0.87**	-

*Significant at $p<0.05$

**Significant at $p<0.01$.

[a]Only data for SAZ, PFZ and AZ samples compared

2.5. Discussion

Abundance data obtained here agreed with previous analyses of Southern Ocean surface water samples, showing that a similar community structure was present, with *Alphaproteobacteria*, *Flavobacteria* and *Gammaproteobacteria* being the predominate taxa (Simon et al. 1999). Simon et al. demonstrated a predominance of CFB in samples from the Polar frontal zone, Antarctic circumpolar current and marginal ice zone, with CFB abundance increasing with latitude, predominating psychrophilic communities. Similar results have also been shown in other open ocean areas (Glöckner et al. 1999; Kirchman 2002, Giovannoni and Rappé 2003; Kirchman et al. 2003; O'Sullivan et al. 2004). A high abundance of CFB group members (up to 70%, detected with the CF319a probe) in Southern Ocean waters (Glöckner et al. 1999; Simon et al. 1999) was found in samples collected from algal blooms, indicative of the strong connection between the CFB and primary production (Brown and Bowman 2001; Kirchman 2002; Horner-Devine et al. 2003). The high abundance values that were encountered in the water column at station 22 (56.0°S, 140.1°E) (Fig. 2.2.) potentially was attributable to an algal bloom, the remnants of which was in the process of sinking through the water column, although there was no direct evidence of this. It was observed, however, that in high chlorophyll, nutrient limited TZ and SAZ waters attached *Flavobacteria* abundance was low (Fig. 2.4B). These samples included a bacterial community that bound poorly to the EUB338 probe (<12% of the DAPI count) suggesting an inactive bacterial population, containing very low rRNA intracellular levels was present. The community also contained *Proteobacteria* (Fig. 2.3) as well as other bacteria, which were not detected

54

with the FISH probes used in this study. These unknown bacteria possibly were picocyanobacteria (*Synechococcus*) which are relatively common in these waters (Kopcynzska et al. 2001) and which compete with heterotrophic bacteria effectively under highly oligotrophic conditions (Rappé and Giovannoni 2003). The low signal intensity encountered with use of the EUB338 probe, could have been attributed to the lack of coverage of the domain bacteria by this probe, however previous studies using this probe in surface waters have demonstrated much higher labeling efficiency, suggesting that it was likely a result of low levels of cellular rRNA. These studies included the Southern Ocean (61%- 96%, Simon et al. 1999) and the Californian coast (>70%, Cottrell and Kirchman, 2000). Future studies could incorporate the use of alternative Eubacterial probes, such as those described by Daims et al. 1999, as well as the use of polynucleotide probes such as those described by Delong et al. 1999 and techniques such as CARD-FISH as described by Pernthaler, Pernthaler and Amann, 2002 to increase hybridization efficency.

Assessment of variation in the distribution of class *Flavobacteria* in Southern Ocean water samples using real-time PCR and FISH demonstrated significant inter-sample variation in the community composition. The two techniques represent different methods for the assessment of the community structure. Both methods demonstrated the presence of *Flavobacteria* in all samples analysed. In the majority of samples analysed *Flavobacteria* represented a numerically significant proportion of the total community analysed. This supports previous studies (Simon et al. 1999), which have suggested the CFB represent a dominant group in surface waters of the Southern Ocean.

Chapter 2: Abundance of Flavobacteria in the Southern Ocean

Like all techniques real-time PCR is subject to methodological biases and involves a number of assumptions. The number of copies of the rRNA gene in the bacterial chromosome has been shown to vary between bacterial species (Klappenbach et al. 2000), and for this reason the method is useful for defining relative abundance but usually not specific bacterial numbers. Biases introduced during the DNA extraction as well as artifacts produced by the PCR amplification of mixed template may cause preferential amplification of certain sequences, thus influencing the outcome of the assay. It is for this reason that as with all molecular techniques used in microbial ecology the results produced should be viewed with a degree of reservation, and the use of other techniques such as microscopic analysis should be incorporated into such studies. The use of SYBR green fluorescent dye for template detection as used here allowed for the quantification of the PCR product rather than copy number, and as such did not allow for size heterogeneity of the 16S rRNA gene between different phylogenetic groups. However, during this study, *Flavobacteria*-specific and Bacteria-specific primers produced negligible size heterogeneity as shown by melting curve data. As 16S rRNA gene copy number varies somewhat in the CFB phylum, typically from 3 to 6 (Barbieri et al. 2002; The Institute for Genomic Research – www.tigr.org), real- time PCR data was reported as a relative abundance which assumed the mean copy number was the same in all samples.

The difficulty in relating FISH abundance data to other factors such as chlorophyll *a* levels necessitated the use of real-time PCR, the caveats of which were previously explained. Nevertheless, the approach provided an insight into the relationship of *Flavobacteria* abundance to other environmental factors. The relation of *Flavobacteria*

to a change in nutrient status of the water column indeed was suggested by the increase of

class *Flavobacteria* relative abundance in the 0.8 μm seawater filter fractions, which

includes cells mostly associated with particulate material. Increases in abundance of

Flavobacteria was observed in relation to chlorophyll *a* and nutrients suggesting that this

segment of the microbial community appears to be coupled to primary production, under

the prevailing oligotrophic conditions of the Southern Ocean. By comparison, no

significant trends were observed in the abundance of *Flavobacteria* in the 0.2 μm

seawater sample fraction, which contains mainly planktonic cells (Fig. 2.5B) and which

may disperse from particulate material as it decomposes and sink (Azam 1998). The

planktonic populations have less access to nutrients and thus not as active, and

consequently more difficult to detect than particle-associated cells (Ouverney and

Fuhrman 1997) (e.g. Fig. 2.4). Bacterial cells associated with particulates, including

Flavobacteria, have been shown to be much more biologically active than cells in

seawater (Ploug et al. 1999).

The Southern Ocean south of the Subtropical Convergence is affected strongly by

iron limitation (de Baar et al. 1990; Timmermans et al. 1998), limiting primary

production. Primary production becomes relatively enhanced in the AZ (Vincent 1988),

due to sea-ice melting distributing atmospherically deposited iron (de Baar et al. 1990)

and creating less surface water mixing (Timmermans et al. 1998) as suggested by the

increases in chlorophyll *a* along the latitudinal transect (Fig. 2.5A). The *Flavobacteria*

are known for a strong association with algae and is often regarded as having an epiphytic

lifestyle (Kirchman 2002), which is relevant to the exclusively chemoheterotrophic nature

of the group. It is reasonable to speculate that the increase in utilizable carbon and

aggregate formation deriving from this primary production potentially provides increased opportunities for the growth of *Flavobacteria*. The high variability of abundance in total populations detectable by FISH (Fig. 2.2 and 2.3) and relative abundance in particles (Fig. 2.5B) thus may derive from localized patchiness of phytoplankton populations occurring in the Southern Ocean due to the effects of advection, iron and silica nutrient limitation (Hence et al. 2003) and possibly also due to competition with other bacteria, in particular *Proteobacteria* (Horner-Devine et al. 2003). More samples are required to be analysed to see if these specific primary productivity: abundance relationships of *Flavobacteria* are robust in the Southern Ocean as well as other oceanic regions.

Chapter 3. Biogeographic relationships of class *Flavobacteria* in the Southern Ocean

Summary 3.1.

The community structure and diversity of class *Flavobacteria* were investigated in the Southern Ocean euphotic zone across a latitudinal transect and in the ice pack off Eastern Antarctica. Surface seawater samples filter-fractionated into 0.8 μm particulate and 0.2 μm planktonic fractions were separately analysed using denaturing gradient gel electrophoresis (DGGE) and DNA sequencing. Data presented in Chapter 2 indicated the abundance of particle-associated *Flavobacteria* positively correlated with seawater chlorophyll *a* and nutrient concentrations, suggesting increased *Flavobacteria* abundance may relate to enhanced primary production in the PFZ and AZ. This finding is further supported by comparisons of DGGE profiles that demonstrated significant differences occur in the total *Flavobacteria* community structure and 16S rRNA gene diversity between samples from the PFZ and AZ with samples from TZ and SAZ. Sequence analysis revealed a broad diversity amongst class *Flavobacteria* in the Southern Ocean with several *Flavobacteria* clades detected in PFZ and AZ waters not detected in TZ and SAZ waters that putatively represent psychrophilic taxa. A total of 24 genus-equivalent lineages were detected and included phylotypes grouping in the family *Flavobacteriaceae* and in the Agg58 clone cluster (family *Cryomorphaceae*). Sequence data included a large, so far uncultivated, cosmopolitan phylogenetic clade ("DE cluster 2") within family *Flavobacteriaceae* that is distributed throughout the Southern Ocean waters but absent from sea-ice. Other bacterial groups found belonged to clades previously shown to be cosmopolitan in ocean surface waters or similar to clades present in Arctic Ocean waters.

3.2. Introduction.

Bacterial assemblages mineralize approximately one-half of the carbon fixed photosynthetically in the marine environment (Fenchel, 1988; Cole et al. 1988). Bacterial biogeochemical cycling thus can significantly determine the potential productivity that can be supported within the marine habitat. Heterotrophic marine bacteria play an important role in pelagic foodwebs (Azam 1998), for example heterotrophic bacteria redisperse silica (Bidle et al. 2003) and can produce iron-binding ligands (Brown and Parks 2001) and thus can significantly affect proliferation of phytoplankton. Fluorescent *in situ* hybridisation (FISH) analyses indicate that within the surface ocean the bacterioplanktonic community includes mostly members of the phylum *Proteobacteria* and the phylum "*Bacteroidetes*", more commonly referred to as the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group (Kirchman 2002).

FISH and clone library studies have consistently indicated CFB group members are abundant in marine aggregates, which encompasses sinking organic detritus from a few microns to several mm in width (Ploug et al. 1999); in the water column (Kirchman 2000, Kirchman et al. 2003; O'Sullivan et al. 2004); sea-ice (Brown and Bowman 2001; Brinkmeyer et al. 2003); surface benthos (Llobet-Brossa et al. 1998; Bowman and McCuaig 2003); and in epibenthic fauna (Webster et al. 2004). In algal blooms CFB group members can make up as much as 70% of the total bacteria (Glöckner et al. 1999; Simon et al. 1999) indicating possibly an opportunistic response to the organic carbon present, which corresponds to their suspected role as major mineralizers of organic matter (Cottrell and Kirchman 2000; Kirchman 2002). Recent mesocosm studies indicated CFB group diversity was highest at the mid-point of a primary productivity gradient, as found for aquatic animals and plants (Mittelbach et al. 2001), while *Alphaproteobacteria* did the opposite (Horner-

Devine et al. 2003). These relationships may relate to shifting balances between competition for colonization sites, optima for nutrient acquisition and predation.

Greater knowledge of the CFB group in seawater would help in improving understanding of marine foodwebs and carbon biogeochemistry in the Southern Ocean, especially if specific associations between taxa and chemical and physical conditions can be established. The CFB group represents a major biomass component in Southern Ocean seawater and sea-ice, typically representing 10-40% of prokaryote cells as measured by FISH, and real-time PCR (Chapter 2). Recent analyses of coastal waters off Plymouth, UK (O'Sullivan et al. 2004) and Delaware, USA (Kirchman et al. 2003) using different CFB sub-group specific probes and PCR primers revealed significant diversity within the phylum at a resolution not usually available if only bacterial universal primers were employed. These studies indicated that a very high proportion of CFB group taxa belong to the class *Flavobacteria*, mostly in family *Flavobacteriaceae* and in various lineages generally represented by the marine snow-associated clone agg58 (DeLong et al. 1993; O'Sullivan et al. 2004). *Flavobacteriaceae*, the clone agg58 group and the recently described family *Cryomorphaceae* (Bowman et al. 2003b) and can be all detected with a specific oligonucleotide probe (Weller et al. 2000).

Spatial distribution patterns and biogeography of CFB in the Southern Ocean euphotic zone were examined in this study using the DGGE approach with communities determined by comparing DGGE band patterns and diversity determined by sequencing DGGE bands. The DGGE method relies on the differing melting characteristics of short fragments of similar sized DNA, allowing the separation of PCR products based on sequence rather than size, as is the case with conventional electrophoresis. DGGE is well documented and is now used routinely to study bacterial community structure (e.g. Fandino et al. 2001; Muyzer et al. 1993; Riemann et al. 2000; Schafer et al. 2001; Van Hannen et al. 1999; Powell et al. 2003;

Bowman et al. 2003a). As each bacterial species in a given sample produces a distinct band or bands in DGGE profiles, analyses can potentially achieve high levels of resolution in moderate to low diversity ecosystems, especially when specific groups are targeted. Limitations and caveats linked to the DGGE technique are well documented (such as gel to gel variation and multiple bands deriving from unique templates), however strategies to ameliorate some of these problems have been successfully applied (Powell et al. 2003) and these were implemented in this study.

The goals of this study were to examine the distribution and diversity of class *Flavobacteria* in the Southern Ocean and to explore relationships between the diversity of the *Flavobacteria* community and various variables including particulate matter, temperature, nutrient availability and oceanographic water masses. The relationship of *Flavobacteria* abundance to these criteria was already investigated in Chapter 2.

3.3 Methods.

3.3.1 Seawater sampling.

Seawater samples were obtained during two separate transects across different Southern Ocean zones as shown in Table 2.1 and Figure 2.1. Details for processing samples are indicated in section 2.3.1.

3.3.2 DNA extraction and purification

Extraction of DNA from filtered samples followed the procedure described in Chapter 2 (section 2.3.3).

3.3.3. DGGE-PCR

DGGE was conducted on all samples indicated in Table 2.1 (Chapter 2). Universal bacteria and class *Flavobacteria* -specific 16S rRNA gene primer sets were used to amplify regions of the 16S rRNA gene, with the amplified products analysed by DGGE (described below). Primers used for amplifying most bacteria in the samples were forward primer 907f (5'-AAA CTC AAA GGA ATT GAC GG-3') (Lane 1991) and GC-clamped reverse primer 1392rc (5'-CGC CCG CCG CGC CCC CGC CCG GCC CGC CGC CCC CGC CCC ACG GGC GGT GTG TAC-3') (Ferris et al. 1996). The *Flavobacteria* specific primer set included 558f (5'-ATT GGG TTT AAA GGG TCC-3') (Weller et al 2000) and 1392rc.

The PCR was performed using Advantage2 Taq (Clontech, Heidelberg, Germany) and 20 mM deoxynucleotide triphosphates, 10 ng genomic DNA and 25 pmol of both primers, in reactions made up to a volume of 50 µl with sterile deionised water. PCR was performed using a touch down protocol on a PTC-200 DNA Engine thermocycler (MJ Research, USA), with 21 0.5°C steps from 65°C to 55°C, with each step including 2 min. 72°C extension and a 1 min. 95°C denaturation steps; and completed with a final 10 min. 72°C extension step. Concentration of genomic DNA added to PCR reactions was varied to optimise the clarity of DGGE gels. In the case of the *Flavobacteria*-specific primer set, this often involved using significantly more template DNA (20-30 ng) in order to get clear banding patterns.

3.3.4 DGGE analysis.

Samples filter fractionated as indicated in section 2.3.1 were analysed by DGGE using bacteria-specific and class *Flavobacteria*-specific primer sets. DGGE was performed using the D-code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA)

using parallel 6% polyacrylamide gels containing a 20% - 55% denaturing gradient, optimized for the *Flavobacteria* primer set and 35% - 75% denaturing gradient, optimized for the universal primer set. Approximately 25 µl of PCR product and 5 µl of gel loading dye were loaded and the gels run at 60°C for 16 h. Gels were stained with $1 \times$ SYBR-gold nucleic acid stain (Molecular Probes, Eugene, USA) and imaged using a UV transilluminator equipped with the MP4+ Instant Camera System (Polaroid, Waltham, USA) with an attached SybrGold filter. In order to reduce variation between individual gel runs duplicate PCR reactions were pooled and DGGE was performed twice for all samples and the banding data pooled (Powell et al. 2003). For sequence analysis, two or more bands that had migrated to the same vertical positions on the gel were excised from the gel using sterile scalpel blades. DNA was eluted by soaking gel slices in 200 µl of sterile water for 30 min and then in 200 µl of STE buffer overnight at 37°C. The DGGE band DNA was then reamplified using 558f/1392rc (*Flavobacteria*) or 907f/1392rc (Bacteria) primers with one µl of the band eluant used in the PCR reaction setup with the Hotstart PCR Kit (Qiagen). PCR was performed with a 15 min. initial 95°C step, followed by 30 cycles of 95°C - 1 min., 55°C - 1 min. and 72°C - 1 min., with a final 4 min. 72°C step. A 10 µl volume of the amplified DNA was re-run on a DGGE gel and compared to the original DGGE band profile from which the band was excised in order to ensure the amplified band corresponded to the original band. If in the correct position, the bands were then eluted as before and then reamplified as indicated above. The secondary amplification DNA product was then purified and sequenced. Sequences were checked between bands migrating to the same gel positions from different samples and it was found these differed by more than 0.1% in 12 cases and included mostly bands obtained by amplification with the bacteria-specific primers. Twofold amplification and extraction of DGGE bands was found to give cleaner sequence data than single extraction, and this was considered to be advantageous, despite the risk of additional PCR error.

3.3.5. Analysis of DGGE banding patterns.

DGGE analysis followed procedures described previously by Powell et al. (2003). DGGE banding patterns based on the *Flavobacteria*-specific primer set showed little variation between replicate gels ($p<0.05$). For banding profile comparisons it was assumed single DGGE bands contained a single taxon and sequencing distinct DGGE bands revealed few examples of highly similar sequences (maximum likelihood distance <0.01) from different band locations within the gel. Bands migrating to the same distance (within $\pm$ 1 mm) in different lanes of the same gel were also assumed to contain the same or very similar sequences (as explained above this was the case). DGGE bands were recorded in a presence/absence matrix by examining results of image analysis utilizing the Diversity Database program (Version 2.2.0, Bio-Rad) and by visual inspection of negative gel images. For the purposes of this study, a band was scored positive if it exceeded minimum threshold intensity relative to the background of the whole gel by at least 15% and corresponded to a band observable to the eye. Samples producing faint DGGE profiles were reanalysed to increase band intensity. Duplicate or triplicate DGGE data for each sample was pooled for the analysis to account for gel-bias (Powell et al. 2003). DGGE banding patterns were analysed with the Primer5 v. 5.2.4 (Plymouth Marine Laboratory, UK) (Clarke and Warwick 2001) program using non-metric multidimensional scaling (nMDS) and one-way univariate analysis of similarity (ANOSIM) as described by Powell et al. (2003). Specific information on the MDS technique has been published by Schiffman and Reynolds (1981).

3.3.6 Sequencing and phylogenetic analysis.

Sequencing of PCR products amplified from DGGE bands was carried out using CEQ DTSC Quick Start kits and the Beckman CEQ2000 automated DNA sequencer (Beckman-Coulter, Berkely, USA). The electrophoretograms were manually checked and sequence data imported into a database using the BioEdit program (Hall 1999). Only sequences of greater than 301 bases in length were included in phylogenetic analyses. For the Bacteria and *Flavobacteria* primer sequences, were 301-347 and 360-686 nucleotides, respectively. Sequences were compared to sequences in the GenBank database using Blast-n searches (http://www.ncbi.nlm.nih.gov/blast). Sequences from this study were then aligned to reference sequences obtained from GenBank, using BioEdit and Clustal W (Thompson et al. 1994). Sequence maximum likelihood distance matrix and neighbor-joining analysis was performed using PHYLIP v. 3.6 (Felsenstein 1993). Bootstrap values (using 1000 replicates) were calculated using the SEQBOOT and CONSENSE programs from the PHYLIP program package. Trees were assembled with full length sequences, then shorter sequences added without changing overall topology. All phylogenetic trees generated used outgroup sequences so are unrooted. Phylogenetic trees were visualized in Treeview (Win 3.2) v. 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html, Roderick Page, University of Glasgow, Scotland).

Distinct DGGE band 16S rRNA gene sequences from this study were deposited under GenBank accession codes AY457085-AY457140, AY496855 -AY496860, AY575779 and AY661611-AY661641. All tables and trees only show distinct phylotypes (Fig. 3.3 and 3.4, Table 3.2 and 3.4, Appendix figures), which were defined in this study as being a collection of highly related sequences with sequence similarities of 99% and greater.

3.4. Results

3.4.1 DGGE banding patterns.

nMDS and ANOSIM analysis of DGGE banding patterns (example Fig. 3.1) was used to examine the differences of class *Flavobacteria* community structure between free-living and particle-containing sample fractions and between different Southern Ocean water masses. The relatively high stress values (the "goodness of fit value" for the nMDS plots) obtained arose from the DGGE band profile heterogeneity and suggested that although the plots (Fig. 3.2) represented potentially useful 2-dimensional representations of the data, too much reliance should not be placed on the detail within the plot (Clarke and Warwick 2001). All samples from the Antarctic Zone (AZ) formed a common group which was distinct from samples from all other Southern Ocean water masses (R=0.321-0.628, $p<0.01$) (Table 3.1). The PFZ was also distinct from the TZ and SAZ samples (R=0.281-0.321, $p<0.05$) (Fig. 3.2A, Table 3.1). No significant difference was found between DGGE patterns for the 0.2 μm and 0.8 μm filter fractions obtained with the *Flavobacteria* specific primers (R=0.043, $p>0.05$) (Fig. 3.2B). The results indicated the same *Flavobacteria* species occur planktonically as well as in and on particulate matter, however species distribution appears different in the waters of the PFZ and AZ compared to the lower latitude samples. No significant differences were found between the profiles of samples from different Southern Ocean zones or from different seawater filter fractions generated with the Bacteria-specific primer set (data not shown).
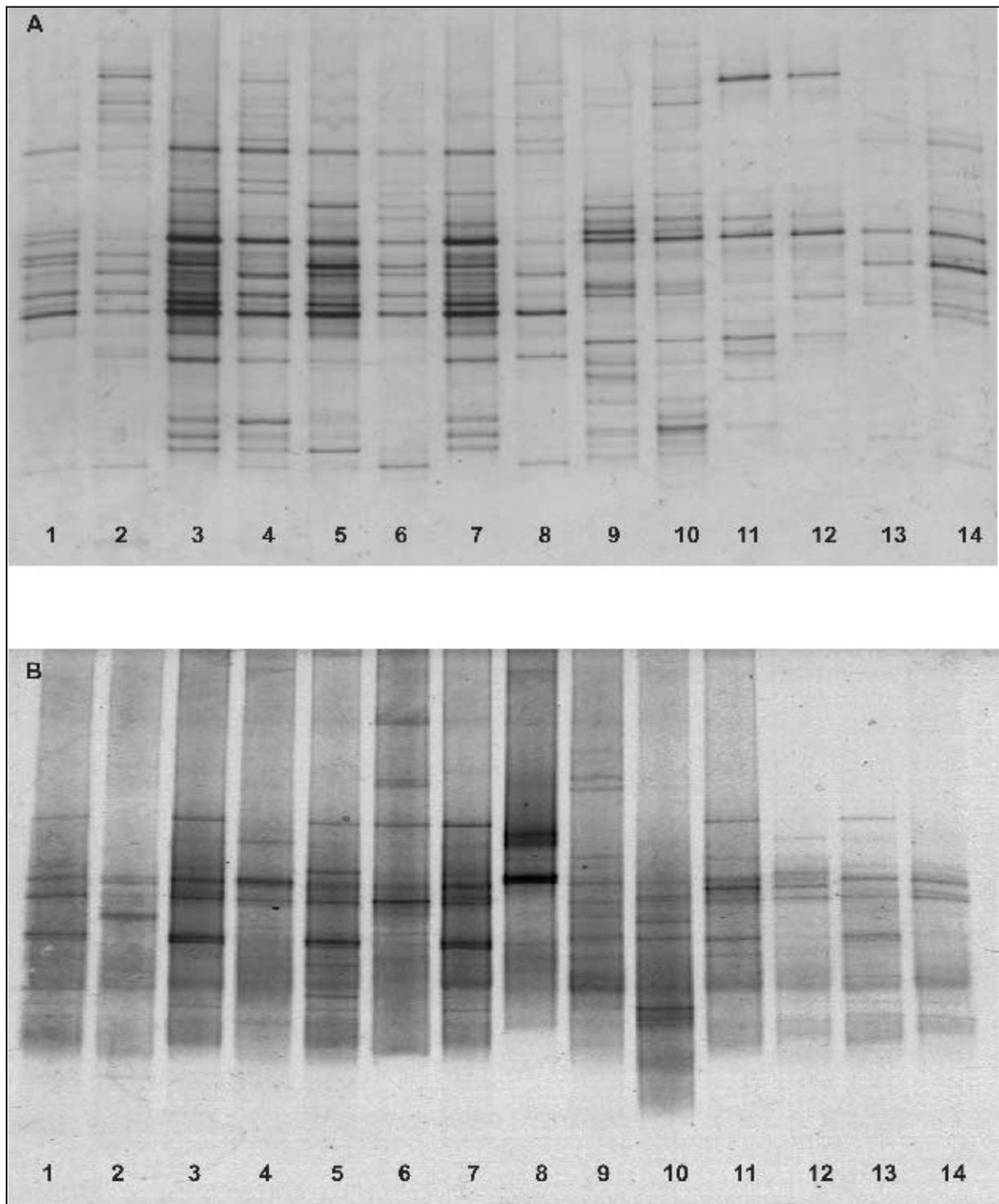
Figure 3.1. An example negative photographic image of a DGGE gel showing A; the *Flavobacteria* community and B; total bacterial community, banding patterns of 0.8 μm (lane numbers 1,3, 5, 7, 9, 11 and 13) and 0.2 μm seawater filter fractions (lane numbers 2, 4, 6, 8, 10, 12 and 14) from different sampling stations. Lanes 1-2, station 4; lanes 3-4, station 6; lanes 5-6, station 8; lanes 7-8, station 12; lanes 9-10, station 16; lanes 11-12, station 21; lanes 13-14, station 26.
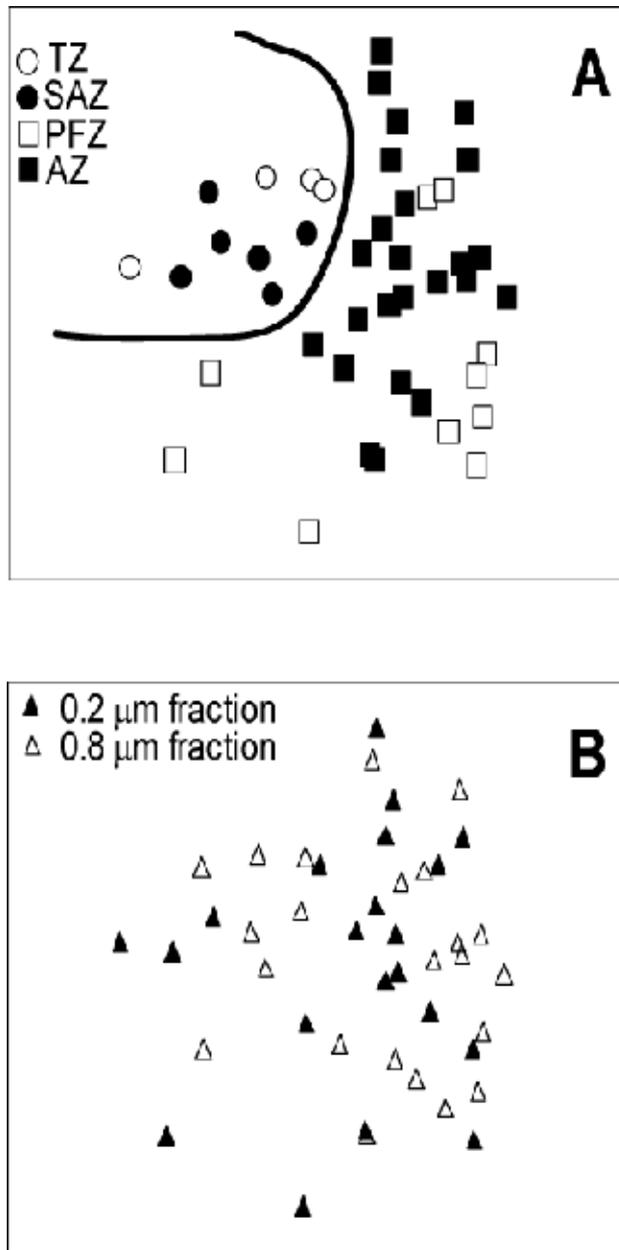
Figure 3.2 A) nMDS plot showing the relative similarities of class *Flavobacteria* community structure based on DGGE banding patterns between oceanic regions. Stress values for both plots were 0.22. B) nMDS plot showing the relative differences between the 0.2 μm and 0.8 μm seawater filter fractions.

Table 3.1. Analysis of Similarities (ANOSIM) of DGGE banding patterns of class *Flavobacteria* between different Southern Ocean zones.

Oceanic Zone	TZ	SAZ	PFZ	AZ
Temperate Zone	-			
Sub-Antarctic Zone	0.375	-		
Polar Front Zone	0.291*	0.281*	-	
Antarctic Zone	0.618**	0.624**	0.321**	-

Significance p<0.05; **p<0.01.

3.4.2. DGGE Band sequence data

Sequences were derived from DGGE-PCR bands to identify the bacterial populations present in the 0.2 and 0.8 μm filter fractions of seawater from 22 sample stations (Fig. 2.1, Table 2.1). 105 gel bands were sequenced from samples analysed using Bacteria-specific primers. Sequences belonging to the CFB phylum comprised 30% of total band sequences and comprised 18 distinct phylotypes. Most band sequences otherwise belonged to *Gammaproteobacteria* (42% of bands) and *Alphaproteobacteria* (15% of bands) (Table 3.2). The remaining bands included sequences clustering in the Planctomycetes (2%), the *Synechococcus-Prochlorococcus* clade (3%), the uncultivated SAR406 clade (1%) and chloroplast/plastid 16S rRNA genes from diatoms and other algae (7%). The non-CFB

Table 3.2. Bacterial diversity (excluding CFB phylum sequences) in Southern Ocean surface seawater samples based on DGGE band sequence data. Similarities calculated over 255-470 bp, Genbank accession numbers for closest relatives are shown on figures A1-A3.

DGGE band Phylotype No.*	GenBank Accession No.	Phylogenetic Group (clade)	Zone	Closest relative Sequence	% Sim-larity	Site of detection or isolation
06030290701	AY661611	Alpha	TZ	NAC1-6 (clone)	98	Atlantic Ocean seawater
21110125	AY661613	Alpha (SAR11)	AZ	"*Pelagibacter ubique*"	100	Pacific Ocean seawater
3121623	AY661615	Alpha (SAR11)	AZ	"*Pelagibacter ubique*"	99	Pacific Ocean seawater
06030290702	AY661612	Alpha (*Roseobacter*)	TZ	HI-4 (clone)	98	Pacific Ocean seawater
3121522	AY661614	Alpha (*Roseobacter*)	AZ	NAC11-3 (clone)	99	Atlantic Ocean seawater
07050290701	AY661616	Gamma (SAR86)	PFZ	OM10 (clone)	97	Atlantic Ocean seawater
07050290729	AY661618	Gamma (SAR86)	AZ	ANT18/2_14 (clone)	98	Southern Ocean seawater
3121218	AY661625	Gamma (SAR86)	AZ	OCS44 (clone)	97	Pacific Ocean seawater
31269	AY661628	Gamma (SAR86)	AZ	Arctic97A18 (DGGE)	99	Arctic Ocean Seawater
6120147	AY661631	Gamma	AZ	*Pseudoalteromonas* sp.	99	Southern Ocean pack ice
2111014	AY661619	Gamma	AZ	*Pseudoalteromonas* sp. *P. distincta*	100	Southern Ocean pack ice; seawater
2111015	AY661620	Gamma	AZ	*Pseudoalteromonas* sp.	100	Southern Ocean pack ice
28050290724	AY661624	Gamma	AZ	*Colwellia psychrerythraea*	99	Southern Ocean sea-ice
22110149	AY661623	Gamma	AZ	Arctic96BD19(DGGE)	99	Arctic Ocean seawater
31258	AY661627	Gamma	AZ	Arct1-36 (clone)	99	Arctic Ocean seawater
21110124	AY661621	Gamma	AZ	Arctic97A6 (DGGE)	100	Arctic Ocean seawater
07050290715	AY661617	Gamma	PFZ	Arctic97A11 (DGGE)	99	Arctic Ocean seawater
22110111	AY661622	Gamma	AZ	Arctic96BD1 (DGGE)	99	Arctic Ocean seawater
6120136a	AY661630	Gamma	AZ	Arctic96B16 (DGGE)	99	Arctic Ocean seawater
3121724	AY661626	Gamma	AZ	ANT18/2_88 (clone)	100	Southern Ocean seawater
6121724	AY661629	Gamma	AZ	ANT18/2_33 (clone)	99	Southern Ocean seawater
06030290714	AY661639	Planctomycetes	TZ	*Pirellula* sp. str. 608	98	Pacific Ocean seawater
07050290722	AY661640	Planctomycetes	PFZ	DBBB-87 (clone)	92	Mediterranean Sea basin
06030290706	AY661637	SAR406	TZ	ZA3312c (clone)	97	Atlantic Ocean seawater
06030290709	AY661638	Cyanobacteria	TZ	*Synechococcus* WH8102	100	Atlantic Ocean seawater
28050290702	AY661641	Cyanobacteria	PFZ	*Synechococcus* WH8016	99	Atlantic Ocean seawater
3121116	AY661635	Chloroplast	AZ	Diatom *Stephanopyxis*	95	Seawater
21110111	AY661633	Chloroplast	AZ	Diatom *Skeletonema*	96	Seawater
21110121	AY661632	Chloroplast	AZ	Diatom (*Haslea* group)	98	Southern Ocean pack ice
6120123	AY661636	Chloroplast	AZ	Diatom (Haslea group)	99	Southern Ocean pack ice
22110122	AY661634	Chloroplast	AZ	Diatom (*Haslea* group)	100	Southern Ocean pack ice

*Phylogenetic trees including the above phylotypes are shown in the Appendix section.

sequences obtained were quite similar to cloned and DGGE band 16S rRNA gene sequences obtained from seawater (Table 3.2) and included representatives of several major seawater bacterial clades (SAR11, SAR86, *Roseobacter* clade, *Synechococcus* subgroup 5.1 etc.) (Table 3.2). Phylogenetic trees for non-CFB sequences are shown in the Appendix (A1-A4).

The *Flavobacteria* specific primer set was clearly able to sample more deeply *Flavobacteria* diversity than the Bacterial-specific primers with 92 DGGE band sequences analysed grouping into 50 phylotypes. All phylotypes grouped in class *Flavobacteria*, including the family *Flavobacteriaceae* (69 sequences, 39 phylotypes) (Fig. 3.3) and the *Cryomorphaceae/*agg58 cluster (23 sequences, 11 phylotypes) (Fig. 3.4). Substantial overlap was found between phylotypes obtained with the two different primer sets. Of the 18 CFB phylotypes detected using universal bacterial primers, 11 identical or near identical (1-2 nucleotide mismatches) equivalent phylotypes were detected using the *Flavobacteria* specific primers. Of the seven phylotypes only detected with Bacterial primers, two phylotypes grouped in the family "*Saprospiraceae*" (Fig. 3.4), which includes filamentous, sheathed, gliding species. These sequences possessed two nucleotide mismatches with the *Flavobacteria* specific primers, which had a highly reliable specificity for class *Flavobacteria* since the phylotypes amplified had either zero or one nucleotide mismatches to the 558f primer.

Flavobacteria phylotypes grouped into a series of yet uncultivated clades or into clades corresponding to known genera, as shown in Table 3.2 and Figures 3.3 and 3.4. Most of these belonged to the family *Flavobacteriaceae* (Fig. 3.3), the remainder clustered within a large clade incorporating the family *Cryomorphaceae*, which contains various polar species

Table 3.3. Class *Flavobacteria* taxonomic groups in the Southern Ocean detected by DGGE, including detection relative to different primer sets utilized and seawater filter fraction as well as incidence of taxonomic groups in the different Southern Ocean zones.

Clades[a]	GenBank accession numbers	Detection of phylotypes with different primer sets and seawater filter fractions				Incidence of taxonomic groups in Southern Ocean zones (combining both seawater filter fractions)		
		Bacterial primers	*Flavobacteria* primers	$0.2 \, \mu m$	$0.8 \, \mu m$	TZ, SAZ	PFZ	AZ
		Number of phylotypes detected out of total number of phylotypes detected for the corresponding clade:				Number of samples containing DGGE band of corresponding clade out of total number of samples		
I	AY457085-457088	2/4	+[b]	+	¼	1/5	1/4	5/13
II	AY457089-457094 AY496855-496856	4/8	6/8	+	+	+	+	+
III	AY457097-457098, AY496855	1/3	+	+	+	+	2/4	7/13
IV	AY457099-457100	-	+	+	+	-	2/4	3/13
V	AY457101-457104	2/4	2/4	2/4	2/4	2/5	3/4	11/13
VI	AY457105-457106	1/2	+	1/2	+	-	2/4	+

73

-	AY457107	-	+	+	+	-	-	1/13
VII	AY457108-457111	2/4	+	1/4	+	-	+	+
Ulvibacter	AY457113-457114	+	+	+	1/2	1/5	2/4	8/13
-	AY457112	-	+	+	+	-	1/4	3/13
VIII	AY457115-457118	-	+	3/4	+	-	3/4	5/13
IX	AY575779	+	-	-	+	-	1/4	6/13
Maribacter	AY457120	-	+	+	+	-	-	1/13
Polaribacter	AY457123-457127	+	+	2/5	+	-	2/4	10/13
Psychroserpens	AY457128-457129	-	+	1/2	+	-	1/4	3/13
Saprospiraceae	AY457138-457139	+	-	+	+	-	-	2/13
AGG58/	AY457131-457137,	3/11	9/11	10/11	9/11	-	2/4	5/13
Cryomorphaceae	AY496858-59							

[a]The phylogenetic position of the taxonomic groups and identity of individual phylotypes are indicated in Fig. 3.3 and Fig. 3.4.

[b]+, all phylotypes detected or all samples were contained at least one DGGE band for the indicated group; -, no phylotypes detected or no samples contained bands of the indicated group.

Figure 3.3. 16S rRNA gene phylogenetic tree of various members of the family *Flavobacteriaceae*, which includes phylotypes detected in the Southern Ocean using PCR-DGGE. Branching points with >60% bootstrap support (1000) replicates have values shown at nodes. The scale bar indicates 0.1 changes per nucleotide. Outgroup was *Rhodothermus marinus*.
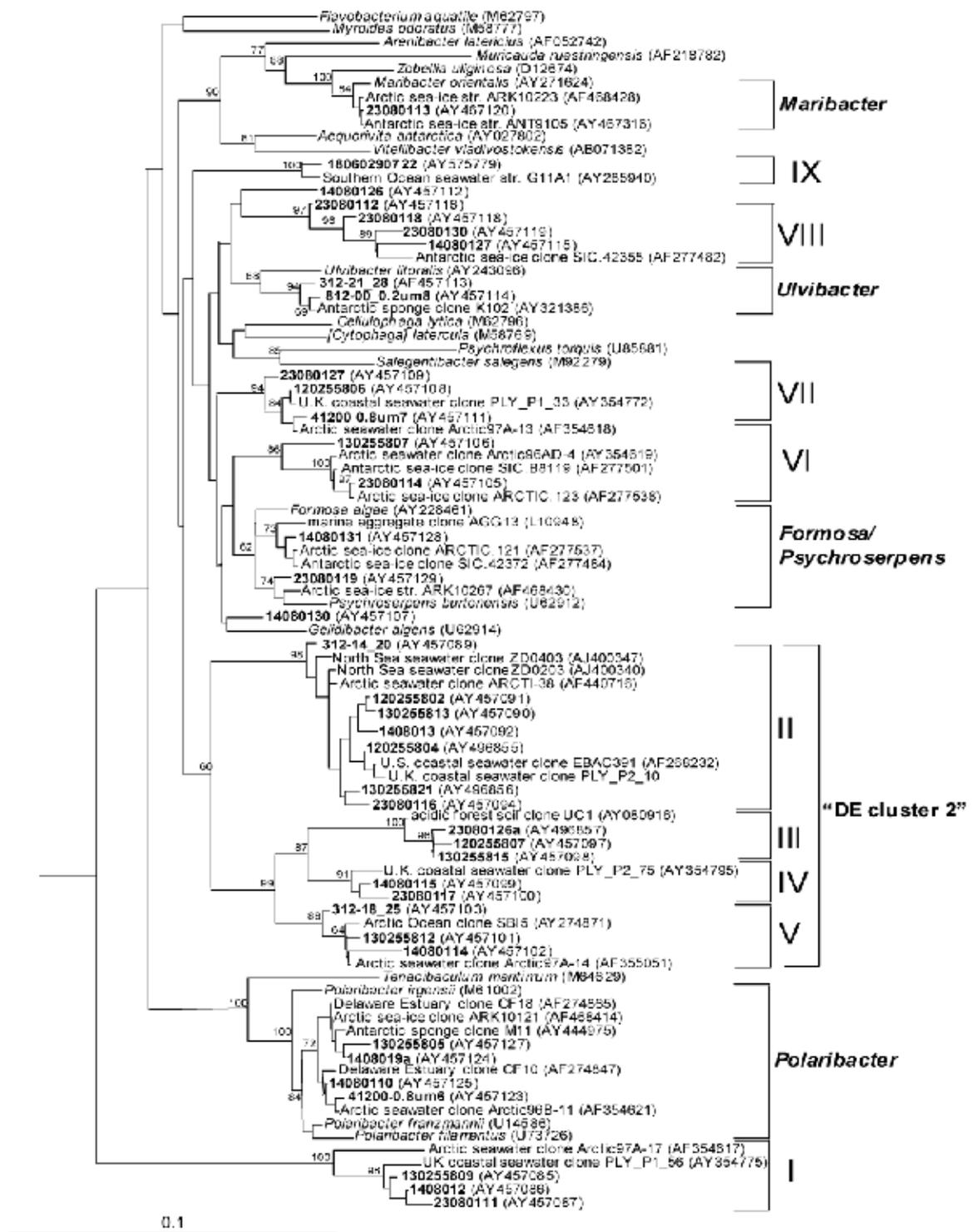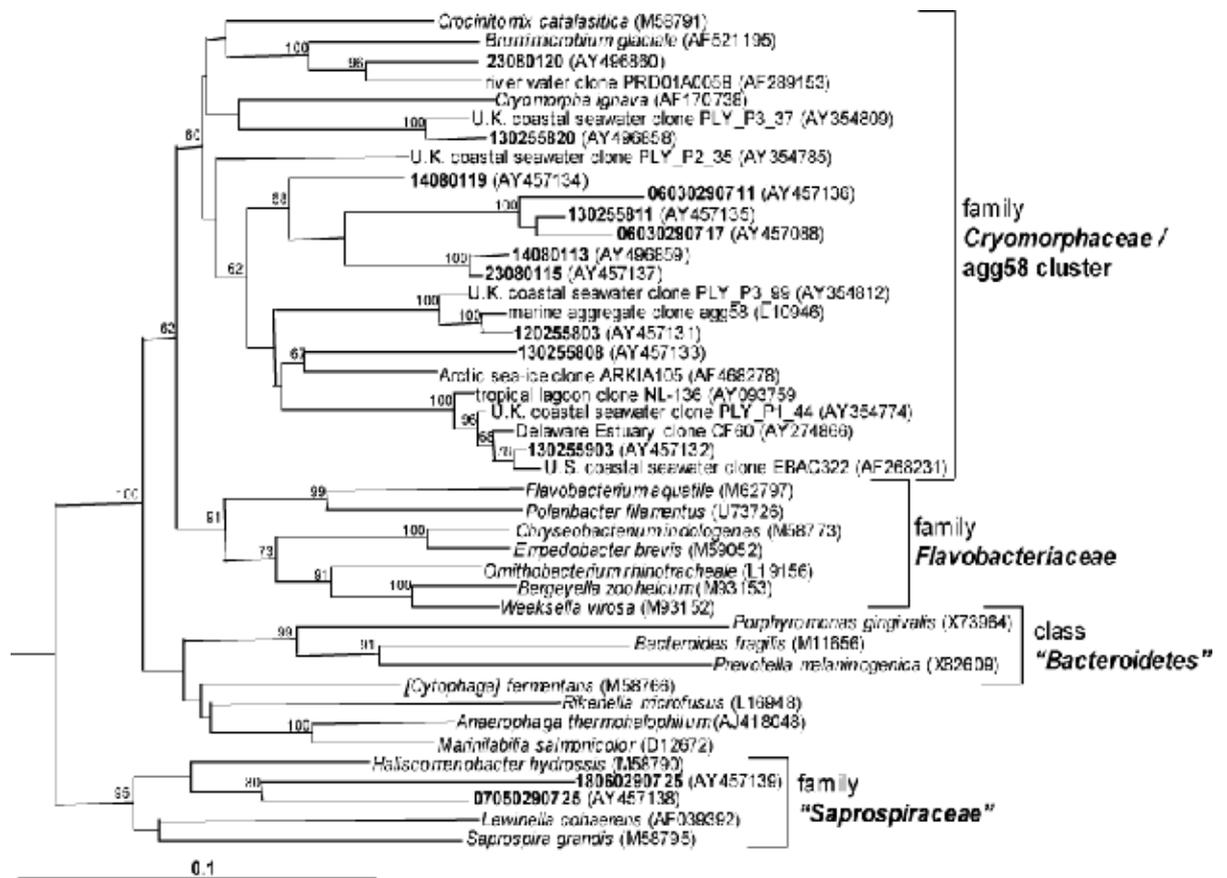
Figure 3.4. 16S rRNA gene phylogenetic tree of various members of the family

Cryomorphaceae/agg58 cluster and the family *Saprospiraceae*, which includes phylotypes

detected in the Southern Ocean using PCR-DGGE. *Rhodothermus marinus* was used as the

outgroup species. Branching points with >60% bootstrap support (1000) replicates have values

shown at nodes. The scale bar indicates 0.1 changes per nucleotide.

(Bowman et al.2003b) and the agg58 clone cluster (Fig. 7), a group associated with algal blooms

and marine aggregates in seawater samples (DeLong et al. 1993; O'Sullivan et al. 2004). The

distribution of individual clades was not found to be affected by what filter fraction was analysed

(Table 3.3), however the clade distribution varied substantially between samples taken from

different parts of the Southern Ocean. Only five different *Flavobacteria* clades were detected in

each of the TZ and SAZ samples (Table 3.3). In the PFZ and AZ samples the number of clades

detected ranged from 8 to 15, depending on the sample. The number of different *Flavobacteria*

clades as well as the mean number of DGGE bands detected in each sample positively correlated

with particle-associated *Flavobacteria* relative abundance, nutrient levels and chlorophyll *a*

concentrations ($p<0.01$ or $p<0.05$). Data and corresponding Pearson's correlation coefficients

over the latitudinal transect are included in Tables A.3 and Table A.4 (Appendix).

3.5. DISCUSSION

A significant gradient in water temperature occurs across the PFZ that acts as an important

physical barrier in the Southern Ocean, reducing water mass mixing and potentially affecting

community distributions (Esper and Zonneveld 2002; Ward et al. 2003; Selje et al. 2003). There

was a temperature differential of about 15°C between the TZ and AZ samples analysed in this

study, that appears significant in shaping the *Flavobacteria* community structure (Fig 3.2A,

Table 3.1). DGGE analyses described a distinct trend in scale, with *Flavobacteria* communities

not resolved at the microscale (Long and Azam 2001) as shown by comparisons of the filter

fractions (Fig. 3.2B), whilst *Flavobacterial* communities demonstrated a still significant but more

constrained heterogeneity in different Southern Ocean water masses (Fig. 3.2A). These findings

are analogous to reports on the distributions of *Roseobacter* phylotypes north and south of the

PFZ (Selje et al. 2003). The predominance of psychrophilic species in Antarctic waters and sea-ice is well established (Bowman et al. 1997b) and the trait occurs amongst members of class *Flavobacteria* (Bowman et al. 1997a; Gosink et al. 1998). Thus it is logical that temperature selection plays an important role in shaping this segment of the microbial community, by selecting for psychrophilic species. These more cold adapted species would not likely compete well in warmer, nutrient poor lower latitude waters and thus could be contributing to differences in the community DGGE profiles. No equivalent differences in DGGE profiles were found using Bacteria-specific primers. This could be due to the large variability and diversity in the structure of the overall bacterial community being sufficient to obscure any community differences. No differences were also found between the particle-associated and planktonic *Flavobacteria* populations indicating the same species were present in the different filter fractions. This similarity may arise from bacterial cells dispersing from particulates as they decompose and sink (Azam 1998) as suggested in Chapter 2 (section 2.5). It is also possible that planktonic bacteria were trapped on the 0.8 µm filter. Conversely, it is also possible that some bacteria attached to small particles passed through the filter were included in the free-living fraction. Results presented in Chapter 5, however suggest that the latter possibility was likely very minimal. Microscopic and PCR based analysis of 0.8 µm filter fractions, performed in Chapter 5, demonstrated very few if any planktonic bacteria associated with the 0.8 µm filters.

No statistically clear differences were found between the TZ, SAZ, PFZ and AZ samples in terms of bacterial 16S rRNA sequence diversity (data not shown) suggesting that the DGGE sequence analysis only included the more abundant species, in addition the overall greater diversity probably tended to obscure differences, if present. The relatively limited number of samples investigated may also have contributed to the lack of differences. However, by focusing on class *Flavobacteria*, relationships could be discerned to some extent between community

structure, diversity and other features of the Southern Ocean. The *Flavobacteria* community 16S rRNA gene diversity was found to be high in the Southern Ocean, however the distribution of phylotypes and clades varied considerably between different water masses (Table 3.3). The incidence of the different *Flavobacteria* clades were found to significantly correlate ($p<0.01$) between the PFZ and AZ samples but not with the TZ and SAZ samples ($p>0.05$), indicating the physical barrier of the Polar Front as well as water temperature and appears to select for different communities of *Flavobacteria* as also shown by DGGE patterns (Fig. 3.2A). TZ and SAZ samples had relatively low *Flavobacteria* 16S rRNA sequence diversity compared with PFZ and SAZ samples with band sequences grouping only in 5 different clades (Table 3.3), including clades I, II, III and V and genus *Ulvibacter* (Fig. 3.3). All of these clades had widespread distribution and are present in PFZ and AZ samples (Table 3.3). Clades II to V, which comprised most of the *Flavobacteria* 16S rRNA gene diversity in the TZ/SAZ (Table 3.3) can be combined together in a larger clade (Fig. 4), equivalent to a clade designated DE cluster 2 (Kirchman et al. 2003), named for clones obtained from the Delaware Estuary, coastal U.S.A. and of the Chukchi Sea, Arctic Ocean. By application of a specific FISH probe DE cluster 2 was found to make up to 10% of total prokaryotes in those samples (Kirchman et al. 2003). The clade is widely distributed in temperate to polar waters as it has been also detected off the coast of England (O'Sullivan et al. 2004), the North Sea (Zubkov et al. 2002), the U.S. west coast (Beja et al. 2000) and the Arctic Ocean (Bano and Hollibaugh 2002) (Fig. 4). Clones derived from either Arctic or Antarctic sea-ice, for which there is now extensive data (Brown and Bowman 2001; Brinkmeyer et al. 2003), however do not cluster in DE cluster 2, suggesting the taxa belonging to this clade are mostly limited to seawater.

The PFZ and AZ samples had 16S rRNA sequence diversity substantially higher than TZ and SAZ samples (Table 3.3). Clades VI, VII, VIII, IX, genus *Polaribacter*, genus

Psychroserpens (Fig. 4), the agg58/*Cryomorphaceae* cluster (Fig. 5) and some more rarely detected phylotypes, detected by DGGE appeared to be restricted to colder waters of the AZ and PFZ (Table 3.3). These clades could thus represent psychrophilic taxa well adapted to cold AZ and PFZ waters. The genera *Polaribacter* and *Psychroserpens* are already known to exclusively contain psychrophilic species (Bowman et al. 1997a; Gosink et al. 1998). These clades and genera, however still may be present in the TZ and SAZ owing to the inherent detection limits of the DGGE-PCR, estimated to be at approximately 0.1% of the total population or approximately 1000 cells ml^{-1} (Casamayor et al. 2000). More sensitive methods and larger sample numbers are required to confirm the complete distributions of these groups between the different Southern Ocean water masses. The use of DGGE for analysis of community composition is effected by a number of limitations, including the effect of multiple heterogeneous 16S rDNA genes within the one bacteria (Dahllof et al. 2000) as well as errors caused by PCR artifacts leading to multiple bands (Muyzer et al. 1998). Despite these limitations and the lack of resolution of non-abundant groups, the technique appears to be well suited to studies such as this, especially when combined with specific primers.

It is possible the difference in *Flavobacteria* 16S rRNA gene diversity and community structure found between the SAZ/TZ and PFZ/AZ samples could be due to the influence of primary production since the number of different *Flavobacteria* clades detected in samples positively correlated to chlorophyll *a* levels, particle-associated *Flavobacteria* abundance and nutrients. Mesocosm studies (Horner-Devine et al. 2003) demonstrated CFB 16S rRNA diversity increased with increased primary productivity (as estimated by measuring chlorophyll *a* fluorescence) to a maximum point whereupon diversity declined in the presence of high productivity levels; levels far above those encountered in samples collected in this study. Horner-Devine et al. utilized a series of 16 mesocosms to examine the relationship between productivity

and bacterial richness, using 16S rDNA based molecular methods as well as microscopic counts. They examined the relationship between productivity and the CFB and proteobacteria groups, in a similar way to which communities were analysed in this study. Here a similar but incomplete relationship was observed, as production in the Southern Ocean regions sampled did not appear to reach levels high enough to hinder further expansion of *Flavobacteria* diversity.

Chapter 4. Culture-dependent survey of Southern Ocean *Flavobacteria.*

4.1 Summary

In order to assess the presence of CFB phylum taxa in Southern Ocean surface waters, bacteria were isolated using media with wide ranges in nutrient content. The isolates were screened using colony pigmentation and morphology followed by partial 16S rRNA gene sequencing to reveal members of the CFB phylum and especially members of family *Flavobacteriaceae*. Twenty-three distinct isolates were found, which on the basis of 16S rRNA gene sequences, grouped within the family *Flavobacteriaceae*. Of these, 12 strains grouped in the genus *Polaribacter* and 6 strains grouped in the genus *Tenacibaculum*. Various strains in these genera appeared to represent novel species on the basis of sequence data. Two psychrophilic orange-pigmented isolates that had a curved rod-like morphology belonged to genus *Psychroserpens* and represented a single novel species, which was designated *Psychroserpens cryoceani* sp. nov. (type strain G512M1). Three isolates represented taxa equivalent to the rank of genera within the family *Flavobacteriaceae,* forming distinct lines of descent and only closely related to environmental clones were also discovered. Phenotypic, chemotaxonomic and genotypic data confirmed these three strains were novel. Strain G812M2 was non-motile, rod-shaped, flexirubin-producing, strictly aerobic, stenohaline, psychrotolerant and saccharolytic. The novel strain has the proposed name *Gelidimarimicrobium roseum* gen. nov., sp. nov. Strain G1A11[T] was a gliding, rod-like, yellow pigmented, strictly aerobic and psychrophilic isolate and has the proposed name *Calefactosor marmoris* gen. nov. sp. nov. Finally, strain GAA0204_net2_3 was a non-motile, yellow-pigmented, psychrotolerant isolate which formed small rod-like cells, tolerated 120 g l^{-1} salinity and

was facultatively anaerobic. The proposed name for this strain was *Maricorpusculum australicum* gen. nov., sp. nov. Non-CFB isolates obtained in the study included members of the *Gammaproteobacteria*, *Alphaproteobacteria*, *Actinobacteria*, and *Firmicutes*. All grouped in or were closely related to established marine genera except for isolate GAA020601 which was only distantly related to the prosthecate genera *Maricaulis* and *Hyphomonas* (similarity 91%) in class *Alphaproteobacteria*.

4.2 Introduction

Members of the CFB phylum are abundant in the Southern Ocean as shown in Chapter 2 and are diverse as shown in Chapter 3. The phylum also constitutes a significant portion of the microbial community globally and has been shown to be able to degrade complex organic molecules such as chitin, agar, xylan and cellulose (Reichenbach, 1992). More recent data suggests that they have an important role in the degradation of DOC in the oceans in particular the higher molecular weight fraction, which includes proteins and polysaccharides (Cottrell and Kirchman 2000; Kirchman, 2002). Relatively recently an interest in the isolation of CFB from natural environments has occurred (driven by curiosity and biotechnology interests) and since 1997 numerous new taxa have been obtained from the marine environment as reviewed in section 1.2.1. CFB from Antarctica and Southern Ocean include several genera such as *Polaribacter*, *Psychroserpens*, *Gelidibacter* and *Aequorivita*. The majority of strains belonging to polar CFB fall into the family *Flavobacteriaceae* grouping in a distinct marine clade (Fig. 1.2). Marine clade members have been isolated from a wide range of habitats, for instance algal and faunal sources have recently been shown to possess a rich diversity of CFB (Table 1.2).

The use of molecular techniques in marine microbial ecology has revealed a number of distinct phylogroups containing only uncultivated organisms. This was shown clearly in Chapter 3 in which several clusters of sequences were found belonging to class *Flavobacteria* that have no close cultivated representative (Fig. 3.3 and 3.4). It is likely that the members of such groups play a key role in the marine ecosystem including the Southern Ocean. Inferring community function is difficult in such cases, given the lack of knowledge of their individual physiologies. Since the understanding of bacterial physiology is important in defining the role of these organisms in the marine ecosystem, there is a need for cultivated representatives of such important phylogenetic lineages. It is hoped that the cultivation and characterization of new phylogroups within the marine environment will lead to a better and more detailed understanding of the role of bacteria in marine processes. The culturing of microorganisms from the environment is an approach that is thought to have only contributed a small fraction of all microorganisms to those that have been discovered. This is highlighted by studies such as that by Suzuki *et al*. (1997), demonstrating the discrepancy between molecular and cultivation based studies. Cultivation of bacteria allows us to investigate the physiology of bacteria in far greater detail than any other approach. It is for this reason that the quest to isolate and cultivate in pure culture organisms that have only previously been described as 'uncultured' continues. As with methodologies for the description of microbial communities, so too there have been advances made in the cultivation of novel bacterial species. Studies such as that performed by Kaeberlein *et al.* (2002) and Connor and Giovannoni (2002) demonstrate the different approaches that are being employed to attempt to cultivate bacteria, and the complexity of growth requirements that are required

by many species of bacteria. Kaeberlein et al. (2002) utilized a diffusion chamber that allowed previously uncultivated bacteria to grow in a simulated natural environment, whilst Connan and Giovannoni (2002) utilized high throughput methods in order to isolate previously uncultivated bacteria.

In this study media with a wide range of nutrient content, from rich (7 g of carbon l^{-1}) to nutrient poor (<0.1 g of carbon l^{-1}) were used to isolate bacteria from the Southern Ocean with a goal to identify novel taxa and to expand the knowledge of bacterial diversity within the Southern Ocean.

4.3 Methods

4.3.1 Isolation and Cultivation.

Water samples (Chapter 2) for bacterial isolation were collected from a depth of 10m either through the underway tap, or through the CTD station aboard the *RSV Aurora Australis*. Samples were plated within a short period of being collected (< 1 hour). Water samples were plated onto solid media containing a variety of nutrient, mineral and vitamin sources. Bacteria were isolated by plating between 10 to 100 µl of seawater samples onto Seawater Nutrient Agar (Bowman et al. 2003); Marine Agar; and SFA agar (Rappé et al. 2002) (formulae are listed in the Appendix) followed by incubation at 4°C for 2 to 12 weeks, after which colonies were typically 1-5mm in diameter. Viable bacterial counts were performed by duplicate plating 10 µl of seawater. Counts were performed after 6 weeks of incubation at 4°C.

Colonies chosen for further analysis were selected based on pigmentation and morphology, transferred to fresh media and incubated at 4°C. Isolated bacterial strains were purified on marine agar to obtain a stable bacterial monoculture. The purified isolates were incubated at 4°C for 3 weeks before analysis. Initial screening involved generation of a partial 16S rRNA gene sequence which was compared to the GenBank nucleotide database (www.ncbi.nlm.nih.gov) and other isolate sequences for the selection of strains for further investigation.

4.3.2 Phenotypic characterization:

Bacteria were cultured on marine agar for inoculation of biochemical and growth test media. Gram-stain, oxidase and catalase tests were performed as described by Smibert and Krieg (1994). Phase contrast microscopy was used to determine cellular morphological features, the presence of gliding morphology (Bowman 2000), and the presence of gas vesicles. Biochemical tests were performed with API-20NE, API20E and API 32 ID AN test strips (BioMerieux-Vitek) using inoculating media supplemented with artificial sea salts (30 g l^{-1}). Hydrolysis of starch, DNA, casein, esculin, tween 80, tributyrin, L-tyrosine, xanthine, uric acid, chitin, carboxymethylcellulose, xylan and dextran used methods described previously (Bowman et al. 1997a). Lecithinase production was tested using marine agar containing 5% (vol/vol) egg yolk emulsion (Oxoid) with clearing zones and opalescence of growth indicative of enzymatic activity. Lipase activity used olive oil as a substrate and was tested according to the procedure of Jette and Ziomek (1994). Oxidative and fermentative acid production from D-glucose

was tested using Leifson's O/F medium (Leifson, 1963). The ability to grow in defined media containing inorganic and organic sources of nitrogen was tested according to Bowman et al. (1997a).

4.3.3 Whole cell fatty acid analysis.

Isolates were grown on marine agar for 3 days at 10°C and then harvested for analysis of whole cell fatty acid profiles. Cells were scraped off plates using a sterile microscope slide, and placed in clean screw cap test tubes. Whole cell lipid analysis was performed as per Bowman *et al*. (1997a). Briefly, the modified one-phase chloroform-methanol-water method was employed for quantitative lipid extraction (White *et al*. 1979). Fatty acids were extracted and then methylated to form fatty acid methyl esters (FAME). FAME samples were then diluted with chloroform containing a C_{23} FAME internal injection standard. Hydroxy fatty acids were converted to their *o*-trymethylsilyl derivatives (Skerratt, *et al*. 1992). FAME samples were analysed by gas chromatography and gas chromatography-mass spectrometry (GC-MS). The locations and configurations of double bonds in monounsaturated FAME were determined by GC-MS of their dimethyldisulfide adducts as described by (White et al. 1979; Nichols et al. 1986).

4.3.4 16S rRNA gene sequencing and analysis.

Genomic DNA was extracted from cells and purified using the Marmur protocol (Marmur and Doty 1962). The 16S rRNA gene from each of the isolates was amplified by PCR using the primers 10f (5'-GAG TTT GAT CCT GGC TCA G -3') as the forward primer and 1520r (5'-AGA AAG GAG GTG ATC CAG CC3') as the reverse primer. The PCR conditions used were similar to those outlined in Bowman et al. (1997b). Each PCR reaction contained each deoxynucleotide at a concentration of 50 μM; 2.5 mM MgCl$_2$; PCR buffer IV (25 mM NH$_4$SO$_4$, 75 mM Tris-HCl pH 9.0, 0.1 % Tween 20); 1 pmol of each primer; 1 unit of thermostable DNA polymerase (Promega); and 1 unit of bovine serine albumin (Promega). Reactions were run in 50 μl volumes with 20-80 ng of genomic DNA added. Thermocycling was performed using an MJ Research DNA engine. The reaction parameters included and initial 5 minute incubation at 95°C followed by 35 cycles of 94°C for 1 min., 52°C for 1 min. and 72°C for 1.5 min., followed by a final incubation of 72°C for 5 min. PCR reactions were visualised using Agarose gel electrophoresis, and purified using the Prep-a-gene kit (Bio-Rad). Sequences of the 16S rRNA gene were generated as per 2.3.7. The sequencing reaction was performed using the 10f, 519f, 907f primers (Chapter 2) and 1520r. Sequence data was viewed and checked using the Bioedit Sequence Alignment Editor (Hall, 1999). The 16S rRNA gene sequence for each strain was compared to the sequences in the Genbank nucleotide database using the BLAST search program of the National Centre for Biotechnology Information website http://www.ncbi.nlm.nih.gov. Sequences were aligned to downloaded reference sequences using the Bioedit sequence alignment editor. Analysis

of the resulting 16S rRNA gene sequence datasets was performed using the PHYLIP

(version 3.6) (Felsenstein, 1993) suite of programs. Evolutionary distances were

determined using the Maximum likelihood algorithm in the program DNADIST and

phylogenetic trees were constructed using the neighbor-joining method using the program

NEIGHBOR. Isolates were deposited in the Australian Collection of Antarctic

Microorganisms (University of Tasmania, Tasmania, Australia). The 16S rRNA gene

sequences generated for members of the *Flavobacteriaceae* have been deposited in the

Genbank nucleotide database under the accession numbers shown in table 3.1.

4.3.5. DNA base composition and DNA:DNA hybridization analysis.

DNA base composition was determined using the thermal denaturation procedure as

described by Sly et al. (1986) using a GBC 916 spectrophotometer fitted with a

thermoprogrammer. DNA base composition values are the mean of three separate

analyses for each strain. *E. coli* was used as a reference strain and has an assumed G+C of

52.0 mol%. DNA:DNA hybridization followed the procedure of Bowman et al. (1998b)

as modified from Huß et al. 1983. DNA samples at approximately 100 μg ml^{-1} were

sonicated to an approximate size of 1 kb, filtered through disposable 0.2 μm filter

cartridges and then dialysed against 2 x SSC buffer (0.3 M NaCl, 0.03 M trisodium

citrate, pH 7.0) at 4°C for 24 h. DNA concentration was then adjusted to approximately

75 μg ml^{-1}, and denatured at 95°C until the increase in absorbance had plateaued.

Hybridisation was then performed at an optimal hybridization temperature (T_{OR})

determined from the following equation:

T_{OR} (°C)= 51 C + 0.47 (mol%G+C),

Where the mol% G+C was the average of the two strains being compared. The DNA samples were tested as 100% hybridization controls (designated sample "A" and sample "B") and as an equal mixtures of DNA samples A and B (designated "AB"). Following denaturation the temperature was reduced to the T_{OR} for 40 min. Experiments were performed 4 or 5 times. DNA hybridisation was then calculated by the decline in absorbance over the 40 min incubation using the following equation:

% hybridization = (4AB – A – B)/2√(A × B) × 100

4.3.6. Microscopic analysis of Isolates.

Isolates were prepared in wet mount on a glass slide and examined using phase crontast microscopy (1000X magnification). Digital pictures were taken using a LDRMBE Leitz microscope (Leica, Heerbuug, Switzerland) fitted with a DC300F gigital camera (Leica).

4.4 Results

The aim of cultivation of bacteria from various regions of the Southern Ocean was to attempt to expand the cultured diversity of members of the CFB phylum. As such the method for selecting isolates for further analysis was aimed at members of this group. Strains falling outside the CFB group, are also included here. Plating of seawater samples on a Marine agar and SNA resulted in mean viable bacterial counts of 1.2 x 10^6 and 2.01

x 10^6 colony forming units (cfu) l^{-1} respectively, with a maximum of 1.3 x 10^7 cfu l^{-1}. Based on data in chapter 2 the mean viable count represented approximately 3% of the mean direct count.

4.4.1 Taxonomy of novel CFB phylum isolates.

As CFB phylum members were the targets of this study the method for screening isolates involved selecting colonies displaying pigmentation, which is a practically universal characteristic of the marine members of the phylum (Bowman 2004). As a result 23 different strains were isolated from Southern Ocean seawater samples, all of which grouped within the family *Flavobacteriaceae* (Table 4.1). Three strains (G11A1, G812M2 and GAA02_04net2_3) appeared to represent novel genera based on 16S rRNA gene sequence data (Fig. 4.1, see also below). Other strains belonged to the genera *Psychroserpens*, *Polaribacter* and *Tenacibaculum* and though closely related to existing species some of these may also represent novel species (Fig. 4.1). Several strains with matching colonial characteristics and practically identical 16S rRNA sequences (Table 4.1) were isolated from multiple samples. Most CFB isolates were obtained throughout the AZ.

For detailed taxonomic characterization time constraints limited the analyses to the most phylogenetically interesting isolates, which included strains G11A1, G812M2 and GAA02_04net2_3, representatives of putative new genera and strains G512M1 and G512M2 isolates of the rarely isolated genus *Psychroserpens*. The following sections detail the specific phenotypic, chemotaxonomic, genotypic and phylogenetic properties of

the new taxa. In summary new taxonomic names are proposed for the novel Southern

Ocean seawater, including three novel genera of the family *Flavobacteriaceae* and a new

species of the genus *Psychroserpens*. The other isolates (Table 4.1) may very well also

include novel species, however their detailed characterization requires much further

work.

4.4.1.1 Characterisation of strain G812M2 as *Gelidimarimicrobium roseum* gen. nov., sp. nov.

Strain G812M2, isolated from a −1.4°C water sample taken at station 4 in the AZ,

grouped closest in terms of 16S rRNA gene sequence to clone ARCTIC.117 detected in

Arctic sea ice (Brown and Bowman, 2001), to a non-extant, psychrophilic sea-ice isolate

IC076 (Bowman *et al*. 1997b) and the algae associated marine bacterium R43 (Patel etal.

2003) (similarity 99.3, 98.8% and 98.1%, respectively). Otherwise, G812M2 was not

phylogenetically close to any described species. *Psychroflexus* spp., *Salegenetibacter*

salegens, Mesonia

**Table 4.1.** Strains isolated from Southern Ocean seawater grouping within the *Flavobacteriaceae*.

Strain:	Sample Station (region)[1]	Water Temp. (°C)	Genus	Colony pigment [2]	16S rRNA gene sequence Genbank accession number
G021102br01	- (AZ)	-	*Polaribacter*	Orange	AY353812
G021102br02	- (AZ)	-	*Polaribacter*	Orange	AY353813
G1112S3 [3]	6 (AZ)	-0.7	*Polaribacter*	Pale Yellow	AY285949
G1112S4A	6 (AZ)	-0.7	*Polaribacter*	Yellow	AY353814
G1112S4B	6 (AZ)	-0.7	*Polaribacter*	Orange	AY353815
G11A1 [3,4]	25 (AZ)	0.5	novel	Clear/Yellow	AY285940
G11A2	25 (AZ)	0.5	*Tenacibaculum*	Yellow	AY353816
G11A3	25 (AZ)	0.5	*Tenacibaculum*	Clear/Yellow	AY353817
G121102s1_3	- (AZ)	-	*Tenacibaculum*	Clear (Gliding)	AY353818
G121102s2_2	- (AZ)	-	*Tenacibaculum*	Clear (Gliding)	AY353819
G121102s2_3	- (AZ)	-	*Tenacibaculum*	Clear Yellow	AY353820
G1612M2	8 (AZ)	1.6	*Polaribacter*	Red/Orange	AY353821
G17M2	9 (AZ)	0.1	*Polaribacter*	Yellow	AY285941
G1A2	15 (TZ)	13.0	*Tenacibaculum*	Yellow	AY285942
G1B2	15 (TZ)	13.0	*Polaribacter*	Clear	AY285943
G512M1 [4]	3 (AZ)	-1.4	*Psychroserpens*	Orange	AY285948
G512M2 [3,4]	3 (AZ)	-1.4	*Psychroserpens*	Orange	AY285947
G812M2 [4]	4 (AZ)	-1.4	novel	Red/Pink	AY298788
G812S3 [3]	4 (AZ)	-1.4	*Polaribacter*	Orange	AY285944
G912S3A	5 (AZ)	-0.1	*Polaribacter*	Yellow/Orange	AY353822
G912S3B	5 (AZ)	-0.1	*Polaribacter*	Yellow	AY285945
G912S4	5 (AZ)	-0.1	*Polaribacter*	Orange	AY285946
GAA02_04net2_3 [4]	- (AZ)	-	novel	Yellow	AY319330

[1]Refer to Fig. 2.1 and Table 2.1 for details of sample stations. Samples with no sample station listed were isolated from plankton tows of the Southern Ocean (AZ).
[2]Pigment for cell mass grown on marine agar.
[3]Strains with near identical 16S rRNA sequences and colonial characteristics were isolated from more than one sample, only a representative strain is shown.
[4]Strains were examined in detailed using polyphasic taxonomic techniques.
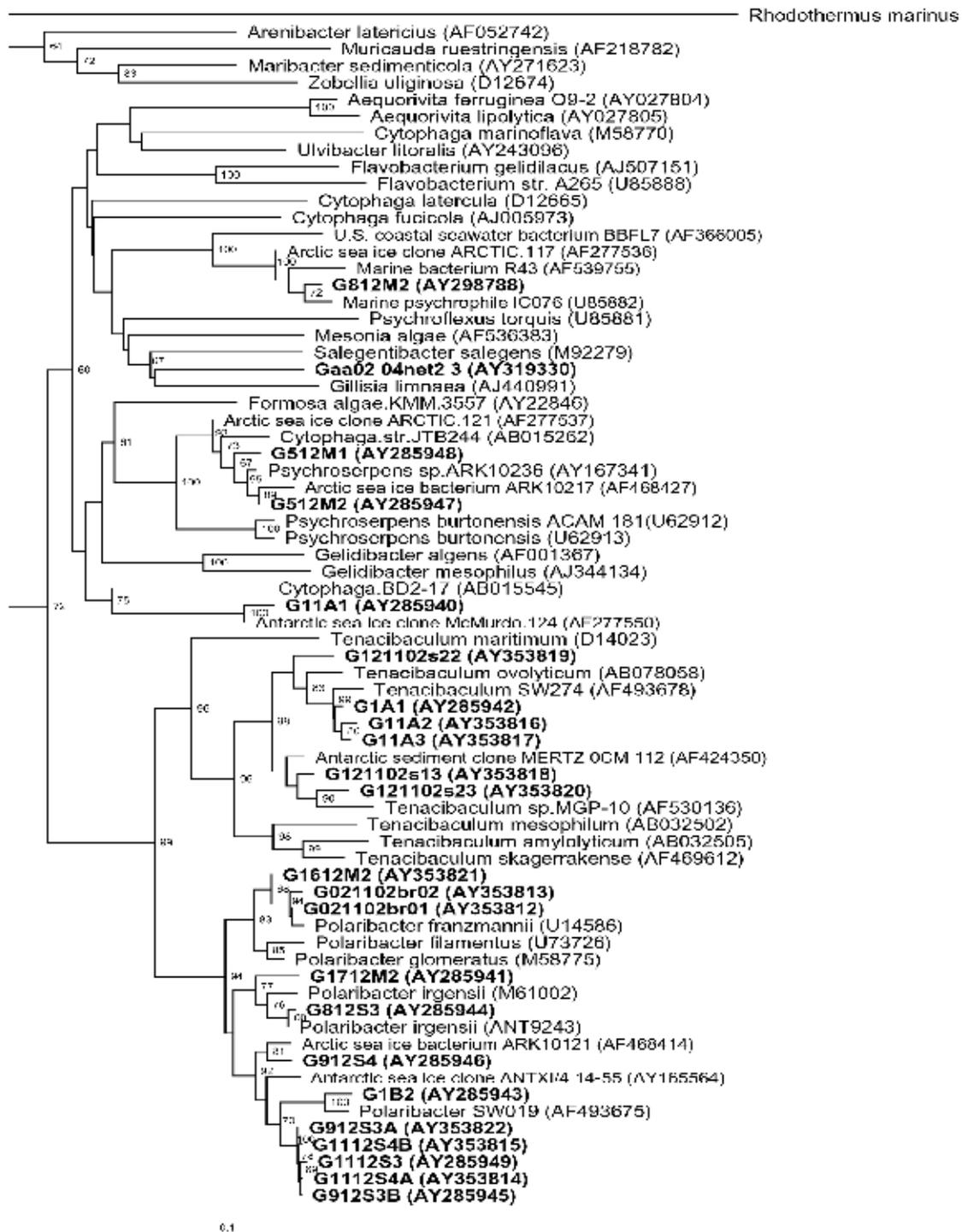
Figure 4.1 16S rRNA gene phylogenetic tree of some members of the *Flavobacteriaceae* and associated marine isolates. *Rhodothermus marinus* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (1000) replicates have values shown at nodes. The scale bar indicates 0.1 changes per nucleotide.

algae, Gillisia spp and *Cytophaga latercula* were the closest related species (similarity 89.1-90.7%) (Fig. 4.1). This phylogenetic distance suggests strongly that strain G812M2 represents a lineage novel at the genus level.

Phenotypic characterization indicates G812M2 is typical of the marine clade of family *Flavobacteriaceae*, being a psychrotolerant, strictly aerobic, Gram-negative, non-motile rod (Fig. 4.2A) growing between 0 to 30°C, possessing an absolute requirement for seawater for growth and a G+C content of 31 mol%. On marine agar, colonies were pink-brown pigmented, older colonies developed a white precipitate. The strain produced flexirubin pigments indicated by a bathochromic shift in the pigmentation to dark brown after the application of a strongly alkaline solution (20% KOH) (Bernardet et al. 2002). It was capable of the degradation of starch, gelatin, casein, esculin, urea, L-tyrosine and tween 80. Other characteristics are listed in Table 4.2 and in the species description (Section 4.6). Fatty acid analysis indicated a predominance of branched chain fatty acids, which included i15:1ω10c, a15:1ω10c, i15:0, a15:0, i16:0, a17:1ω 7c and 16:1ω7c (Table 4.3).

4.4.1.2 Characterisation of strain G11A1 as *Calefactosor marmoris* gen. nov. sp. nov.

Strain G11A1 was isolated from station 25 from seawater at 0.5°C. The 16S rRNA gene sequence data indicated the strain was most similar to Antarctic sea-ice clone McMurdo.124 (Brown and Bowman 2001) (similarity 99.4%) but was only distantly related to other members of the family *Flavobacteriaceae* including *Psychroserpens burtonensis*, *Gelidibacter* spp. and *Formosa algae* (similarity 88.0-89.5%) though

sequence similarity was low, indicating the strain represented a novel genus-equivalent lineage.

G11A1 is a psychrophilic, seawater-requiring, Gram-negative, gliding, rod shaped bacterium growing between 0 to 20°C. Cells were approximately 2μm long in log phase culture (Fig. 4.2B), but in stationary phase culture long filaments (>10 μm) and coccoid morphology were observed. Colonies on marine agar were yellow and demonstrated an irregularly spreading growth margin. Flexirubin pigments were absent. It was capable of gelatin and casein hydrolysis and could degrade tween 80 and L-tyrosine. Other phenotypic details are listed in Table 4.2 and in the species description (section 4.6). The major cellular fatty acids were 16:0, 18:0, i15:1ω10c, i15:0, 16:1ω7c and 3-OH 15:0 (Table 4.3). The DNA base composition was 34 mol%.

4.4.1.3 Characterization of strain GAA0204 net2 3 as *Maricorpusculum australicum* gen. nov. sp. nov.

Strain GAA0204 net2 3 was isolated from the Southern Ocean AZ in 2002 from a plankton net tow sample. The closest relatives were epiphytic species including *Salegentibacter* spp., *Gillisia* spp., *Ulvibacter litoralis* and *Mesonia algae* (similarity 90.3-92.7%). Strain GAA0204 net2 3 was a psychrotolerant, Gram-negative non-motile, rod shaped bacteria (Fig. 4.2C) growing between 0-37°C. Cells in log phase culture were small rods(<1-2 × 0.3-0.4 μm) occasionally forming chains; coccoid or vibrioid-forms were observed in stationary phase culture. On marine agar the colonies were yellow pigmented. Flexirubin pigments were absent. It was capable of gelatin and casein
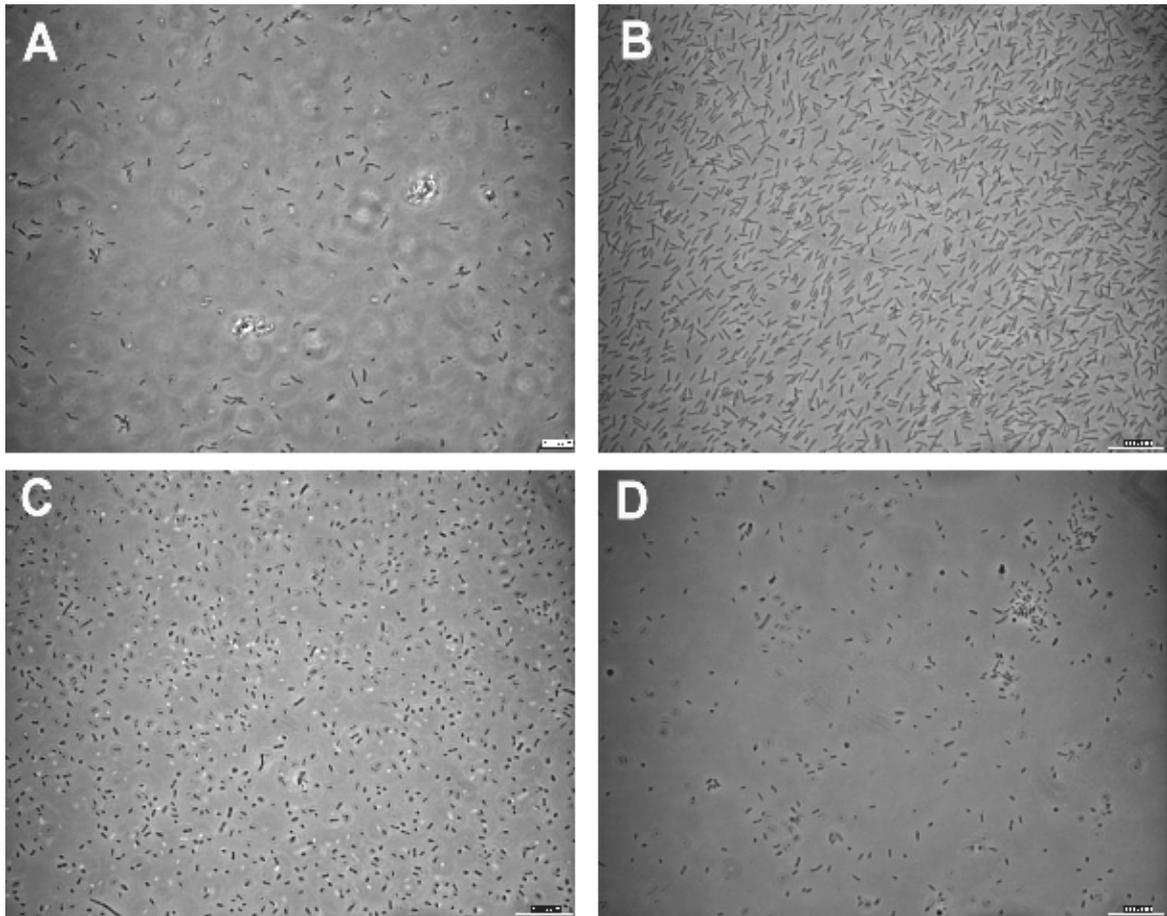
Figure 4.2 Phase contrast micrograph of strains (1000X magnification) A) G812M2, B) G11A1, C) GAA02 04net2 3 and D) G512M1 grown on marine agar for 5 days at 10°C. Bar = 10μm.

Table 4.2. Phenotypic and morphological characterization of novel Southern Ocean strains belonging to family *Flavobacteriaceae.*

Strain	G812M2	G11A1	GAA0204N23	512M1	512M2
Colony texture	butyrous	butyrous	butyrous	viscid	viscid
Colony opacity	opaque	Clear	opaque	opaque	opaque
Pigment	Pink/brown	yellow	yellow	orange	orange
Pigment type carotenoid (C)/flexirubin(F)	F	C	C	C	C
Morphology (log phase/stationary phase)	rod	rod/cocci	rod/cocci, vibrio	rod/cocci	rod/cocci
Gliding motility	-	+	-	-*	-
Growth at 20°C	+	+	+	+w	+w
Growth at 25°C	+	-	+	-	-
Growth at 30°C	+w	-	+	-	-
Growth at 37°C	-	-	+	-	-
NA + 2.5% NaCl	-	-	+	-	-
MA + 0.5 × seasalts (15 psu)	+	+	+	+w	+w
MA + 2 × seasalts (60 psu)	+	+	+	-	-
MA + 3 × seasalts (90 psu)	-	-	+	-	-
MA + 4 × seasalts (120 psu)	-	-	+w	-	-
Starch Hydrolysis	+w	-	-	-	-
Gelatin hydrolysis	+	+	+	-	-
Casein Hydrolysis	+	+	+	+	-
Cytochrome c oxidase	+	+	-	-	-
Christensen's urease test	+	+	-	-	-
α-galactosidase, β-galactosidase	+	-	+	-	-
α-glucosidase, β-glucosidase	+	-	-	-	-
β-N-acetyl-D-glucosaminidase	+	+w	-	-	-
α-fucosidase, β-glucuronidase	+	-	-	-	-
Esculin hydrolysis	+	-	+	+	+
DNAse activity	+w	+w	-	-	-
Tween 80 hydrolysis	+	+	-	+w	+w
L-tyrosine hydrolysis	+	+	+	-	-
Fermentation of D-glucose, NO$_3$ reduction	-	-	+	-	-
Oxidative acid production from D-glucose	+	+w	+w	-	-
Utilisation as sole carbon source:					
D-glucose, casamino acids	+	+	+	-	-
Maltose, caprate, malate	+	-	-	-	-

*+, positive; -, negative; w+, weak activity or growth.. All strains grew at 0-17°C, were Gram negative and produce catalase and alkaline phosphatase. All strains were negative for hydrolysis of agar, carboxymethylcellulose, dextran, xanthine, trybutyrin, uric acid, xylan and chitin; indole production; denitrification; lipase (olive oil), lecithinase (egg yolk), lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase and tryptophan deaminase activity; growth on MacConkey agar and nutrient agar; and the utilization of D-gluconate, adipate, citrate and phenylacetate as sole carbon sources.

hydrolysis, produced a variety of glycosidic enzymes and was capable of reducing nitrate to nitrite and fermenting glucose. The strain utilized glucose and casamino acids as sole carbon sources. It was halotolerant, capable of growth on marine agar supplemented with 120 g l^{-1} sea salts. Other phenotypic details are listed in Table 4.2 and in the species description (section 4.6). The major cellular fatty acids were 16:0, i15:0, a15:0, i16:0, i17:1ω7c and 16:1ω7c (Table 4.3). The mol% G+C was 41 mol%.

4.4.1.4. Characterization of strains G512M1 and G512M2 as *Psychroserpens cryoceani* sp. nov.

Two isolates grouping within the genus *Psychroserpens* were isolated from Station 3 in the Antarctic Zone. On marine agar the strains formed orange pigmented colonies which had a viscid texture, G512M2 more so than G512M1. Both strains had very similar morphology, both were non-motile, gram-negative curved rods in exponential phase cultures (Fig. 4.2D), but demonstrated a coccoid morphology in stationary phase culture. Both were psychrophilic, capable of growth between 0 and 17°C (growth at 20°C was poor in liquid marine broth). The two strains were slow growing and were generally unresponsive in most phenotypic tests (Table 4.2). 16S rRNA gene analysis demonstrated that the two strains were closely related, having a sequence similarity of 98.9% and were most closely related to strain ARK10236, isolated from Arctic pack ice by Brinkmeyer et al. (2003) (similarity 99.2%) and to *Psychroserpens burtonensis* (similarity 96.5%) (Bowman et al. 1997a). DNA base composition of the strains was 30 mol%. The strains shared a DNA hybridization of 77% indicating they belonged to the same species.

Table 4.3. Comparison of whole cell fatty acid profiles for novel Southern Ocean *Flavobacteria* strains.

Fatty acid:	*P. burtonensis*	G512M1	G512M2	G1A11	GAA0 204_net_2-3	G812M2
			% composition:			
14:0	0.3	0.8	1.0	-	1.0	-
15:0	11.9	2.1	5.1	0.2	1.9	1.2
16:0	0.4	0.7	1.5	9.9	5.3	0.2
17:0	0.1	-	-	-	-	-
18:0	-	-	-	9.3	3.9	-
i13:0	0.1	-	-	1.8	-	-
i14:0	0.5	1.8	2.8	1.7	-	-
i14:1ω5c	0.8	1.1	-	-	-	-
i15:1ω10c	17.7	21.7	18.3	13.5	4.4	12.1
a15:1ω10c	9.7	13.2	5.6	-	2.3	21.6
i15:0	10.4	6.0	13.0	35.9	6.3	21.6
a15:0	10.3	9.5	11.6	2.3	13.5	12.1
i16:0	1.1	1.8	0.4	-	5.6	5.6
a16:0	-	-	-	-	-	0.8
i17:1ω7c	2.7	2.0-	5.0	-	5.4	4.6
a17:1ω7c	0.1	1.1	0.9	-	4.6	5.1
i17:0	-	-	--	-	-	1.4
14:1	0.1	-	-	-	-	-
15:1ω6c	16.4	16.8	15.0	8.1	-	-
i16:1ω6c	5.4	3.7	6.4	-	1.3	-
16:1ω7c	1.2	1.0	5.6	6.2	38.5	9.8
16:1ω5c	5.0	2.9	5.6	-	-	0.2
17:1ω6c	1.7	2.0	1.1-	-	-	-
18:1ω9c	-	-	-	-	1.7	-
18:1ω7c	-	-	-	4.1	2.1	-
3-OH15:0	-	-	-	7.0	1.9	1.0
3-OH17:0	-	-	-	0.4	-	-

*Data from Bowman et al. 1997a.

Fatty acid nomenclature: Fatty acids are designated as the total number of carbon atoms: number of double bonds followed by the position of the double bond from the aliphatic end (ω) of the molecule. The prefixes *i* and *a* indicate *iso* and *anteiso* containing fatty acids respectively.

Hybridization values were only 15% to strain ACAM 181, the type strain of *P. burtonensis*. The strains clearly represent a new species as indicated by phenotypic characteristics. *P. burtonesis* differed from G512M1 and G512M2 in having a yellow pigment, helical highly curved cells and inability to hydrolyse esculin amongst other phenotypic traits (Bowman et al. 1997a). The major cellular lipids for the strains were i15:1ω10c, a15:1ω10c, i16:0 and a16:0, similar to that of *P. burtonensis* (Table 4.3). The description of the strains as a new species is presented in section 4.6. Strain G512M1 was designated the type strain.

4.4.2. Other CFB isolates

Six isolates belonged to the marine genus *Tenacibaculum* (Table 4.1). The genus contains yellow pigmented, rod-shaped species that adhere to the surfaces of marine organisms and exhibit gliding motility. All of the strains from this study demonstrated yellow pigmentation when grown on marine agar and all exhibited gliding motility. The isolates formed two clusters both most closely related to the species *Tenacibaculum ovolyticum* (similarity 97.0-98.3%) a pathogenic species isolated from halibut eggs from Norway (Hansen *et al.*, 1992). The strains were distinct from all other described *Tenacibaculum* species (Suzuki et al. 2001) (Fig. 4.1). The first cluster contained isolates G11A2, G11A3 and G1A1, which formed opaque yellow colonies and strain G121102s2_2, which formed translucent yellow colonies. These strains were also very similar to isolate SW274 obtained from coastal seawater (Plymouth, UK) (L. O'Sullivan et al. unpublished). Two strains (G121102s1_3, and G121102s2_3), which formed translucent yellow colonies, formed a second cluster, 97.6-98.3% similar to *T. ovolyticum*. Based on phylogenetic data

it can be hypothesized the strains may represent 2 or 3 novel species. Further comparative analysis with *T. ovolyticum* will be required to confirm this.

The genus *Polaribacter* was well represented in this study with 12 isolates, obtained (Table 4.1). The strains formed 6 distinct species-level lineages. Members of the genus *Polaribacter* are psychrophilic and may form gas vesicles (Gosink et al. 1998). They are thought to be endemic to polar oceans occurring both in seawater and sea-ice. Due to their relatively fastidious nutrient requirements *Polaribacter* spp. may have a strong association with algal assemblages (Gosink *et al*. 1998; Staley & Gosink, 1999). Based on 16S rDNA sequence data, most of the isolates appeared to be novel and distinct from described *Polaribacter* species. Of these, five isolates (G912S3A, G1112S4B, G912S3B, G912S4, G1112S4A and G1112S3) had nearly identical 16S rRNA sequences (similarity 99.5-99.8%) and were most closely related to an Arctic sea ice isolate (strain ARK10121, Brinkmeyer et al. 2003). These isolates exhibited variations in colonial pigmentation and shape on MA despite their high similarity to each other. Another two isolates, strains GB12 and G912S4, were also allied but distinct from the G912S3A strain cluster (similarity 97.5-98.1%) as well as each other (similarity 97.1%). These seven isolates perhaps represent a series of novel *Polaribacter* species, given that the closest described species was *Polaribacter irgensii* (similarity 96.4-97.2%), a species originally isolated from Southern Ocean seawater (Penola Strait, Antarctic Penisula) (Gosink et al. 1998). The remaining isolates were very closely related to *Polaribacter franzmannii* (strains G1612M2, G021102B2 and G021102B1; 100%, 99% and 99% similarity) and to *Polaribacter irgensii* (strains G1712M2 and G812S3, 98% and 99% similarity) and are thus probably members of these species (Fig. 4.1).

4.4.3. Non-CFB Isolates

The majority of CFB isolates obtained in this study from Southern Ocean seawater were members of the *Proteobacteria*. Members of this group are readily cultivated and many strains have been previously isolated from seawater and Southern Ocean marine samples (Bowman et al. 1997b; Mergaert et al. 2001; Brinkmeyer et al. 2003; Rappé & Giovannoni 2003).

4.4.3.1 *Gammaproteobacteria* isolates

Gammaproteobacteria were the most commonly isolated group of organisms from seawater during this study. Strains that demonstrated some degree of pigmentation, one of the criteria used to select for CFB isolates, were selected for identification. As a result 24 pigmented strains affiliated with the *Gammaproteobacteria* were examined. Of these, 12 belonged to the genus *Pseudoalteromonas*, 4 were in genus *Marinobacter*, and 3 isolates each belonged to the genera *Glaciecola* and *Pseudomonas*. Single isolates belonging to the genera *Vibrio*, *Psychrobacter* and *Colwellia* were also found (Table 4.4). Many of the strains were similar, on the basis of 16S DNA, to isolates from polar and marine locations. Strain G4A2 was most closely related to the species *Psychrobacter marincola* and *Psychrobacter submarinus*, aerobic, non-motile, moderately halophilic bacteria, first isolated from Indian Ocean ascidians (Romanenko *et al*. 2002). The strain was also very similar to isolates from Arctic sea-ice (Brinkmeyer et al. 2003). The four strains of *Marinobacter*, G6AA1, G6AA3, GAA020602 and G6AA2, were closely related to the hydrocarbon-degrading species *Marinobacter aquaeolei* and isolates from

Table 4.4 Strains grouping outside the *Bacteroidetes*, demonstrating the closest cultured species, similarity, colony pigmentation when grown on MA, and Genbank Accession number. * numbers in brackets represent the number of bases over which similarity was calculated.

Strain	Closest related cultured species	Similarity (%)*	Colony pigmentation	Accession No.
GAMMAPROTEOBACTERIA:				
G4A2	*Psychrobacter marincola, P. submarinus*	100 (451)	pale pink	AY661574
G6AA1	*Marinobacter aquaeolei*	99 (453)	pale pink	AY661579
G6AA3	*Marinobacter aquaeolei*	99 (451)	pale pink	AY661581
GAA020602	*Marinobacter aquaeolei*	99 (575)	pale pink	AY661589
G6AA2	*Marinobacter aquaeolei*	97 (494)	bright yellow	AY661580
G9103C2A	*Pseudomonas oryzihabitans*	99 (419)	Yellow	AY661586
G9103C3A	*Pseudomonas oryzihabitans*	99 (437)	Yellow	AY661587
G9103C4A	*Pseudomonas oryzihabitans*	99 (423)	Yellow	AY661588
G6B2	*Colwellia psychrerythraea, Thalassomonas viridans*	97-98 (449)	Pale yellow	AY661583
G3A2	*Vibrio lentus, V. splendidus*	99 (448)	brown	AY661573
G1612S4	"*Glaciecola polaris*"	98 (464)	pale pink	AY661570
G12B1	"*Glaciecola gelidimarina*"	97 (622)	bright pink	AY661569
GDEAS204	*Pseudoalteromonas issachenkonii*	100 (572)	Pale yellow	AY661590
G1112M1	*Pseudoalteromonas issachenkonii*	100 (464)	Grey/brown	AY661567
G1712M1	*Pseudoalteromonas issachenkonii*	100 (465)	light brown	AY661571
G4B1	*Pseudoalteromonas issachenkonii*	100 (453)	brown	AY661575
G812M1	*Pseudoalteromonas issachenkonii*	100 (465)	Pale yellow	AY661585
G5BA4	*Pseudoalteromonas issachenkonii*	99 (453)	Pale yellow	AY661577
G5BA5	*Pseudoalteromonas issachenkonii*	99 (452)	Pale yellow	AY661578
G4B2	*Pseudoalteromonas issachenkonii*	98 (455)	Pale yellow	AY661576
G121102S102	*Pseudoalteromonas issachenkonii*	98 (489)	brown	AY661568
G7A2	*Pseudoalteromonas issachenkonii*	99 (452)	Pale yellow	AY661584
G6AB2	*Pseudoalteromonas nigrifaciens*	98 (304)	yellow	AY661582
ALPHAPROTEOBACTERIA:				
G5BA1	*Sphingomonas aerolata*	98 (850)	Orange	AY661593
G5BB2A	*Sphingomonas aerolata*	98 (629)	Pale yellow	AY661595
G4A1	*Sphingomonas paucimobilis*	97 (453)	Yellow	AY661592
GAA0204N2_4	*Erythrobacter citreus*	99 (445)	Yellow	AY661600
GEHC4	*Erythrobacter citreus*	99 (545)	Yellow	AY661604
GAA0204N2_5	*Erythrobacter citreus*	97 (545)	Orange	AY661601
GAA0208B5	*Erythrobacter citreus*	97 (572)	Orange	AY661603
GAA020601	*Maricaulis maris*	91 (631)	Orange	AY661602
G5BA2	*Hyphomonas johnsonii*	99 (831)	Pale yellow	AY661594
G5BB3	*Hyphomonas johnsonii*	99 (629)	Yellow	AY661597
G6B1	*Roseovarius tolerans*	97 (432)	Pale pink	AY661599
G1612S3	*Sulfitobacter brevis*	96 (439)	Yellow	AY661591
G5BB2	*Sulfitobacter pontiacus*	99 (433)	Orange	AY661596
G6AB1	*Sulfitobacter pontiacus*	99 (619)	Brown	AY661598
GRAM-POSITIVES:				
G2B1	*Micrococcus luteus*	99 (255)	Pale yellow	AY661606
G7B2	*Frigoribacterium faeni*	99 (510)	yellow	AY661608
G7B1	*Frigoribacterium faeni*	99 (510)	yellow	AY661609
G7A1	*Frigoribacterium faeni*	99 (510)	yellow	AY661610
G5AB3	*Nocardioides aquiterrae*	97 (255)	Pale orange	AY661605
G5AA3	*Bacillus marisflavi*	100 (255)	Pale orange	AY661607

Arctic Ocean sea-ice, Pacific Ocean seawater and algal cultures (Kaye and Baross, 2000; Hold et al. 2001; Brinkmeyer et al. 2003). The three *Pseudomonas* isolates (strains G9103C2A, G910C3A, G910C4A) were most closely related to the psychrotolerant, soil species *Pseudomonas oryzihabitans*. Interestingly, all three strains were isolated from an aged seawater sample from the AZ stored at 2°C for 2 months, but were not detected in any other samples, suggesting the isolates could represent contaminating species enriched in the stored sample. Isolate G6B2 was closely related to the species *Colwellia psychrerythraea* and *Thalassomonas viridans*. *Colwellia* species appear to be widely distributed in oceanic regions characterised by permanently low temperatures and are associated with sea-ice diatom assemblages at both poles (Bowman et al. 1998a; Junge et al. 2003). *Thalassomonas* species on the other hand appear to prefer warmer waters, isolated originally from the Mediterranean Sea. More sequence and phenotypic data will be required to determine the exact taxonomic relationships of this particular strain. Strain G3A2, isolated from station 17 in the SAZ, was most closely related to *V. splendidus* and *V. lentus*, species found in colder waters unlike other *Vibrio* species which prefer temperate to tropical locations. No *Vibrio* species have ever been isolated from the AZ or Antarctic continental margin before. Strain G121102S102 was most closely related to *Pseudoalteromonas atlantica* at a similarity of 96%, but grouped within the branch containing the genera *Alteromonas* and *Glaciecola*. Further analysis is needed to confirm this strain's genus level relationships. Strain G1612S4 was isolated from the Antarctic Zone during both seasons, and grouped within the *Glaciecola*, closest to the species *Glaciecola polaris*, a psychrotolerant, prosthecate species isolated from both Southern and Arctic Oceans (Van Trappen *et al*. 2004). Strain G12B1, also from the AZ, has an
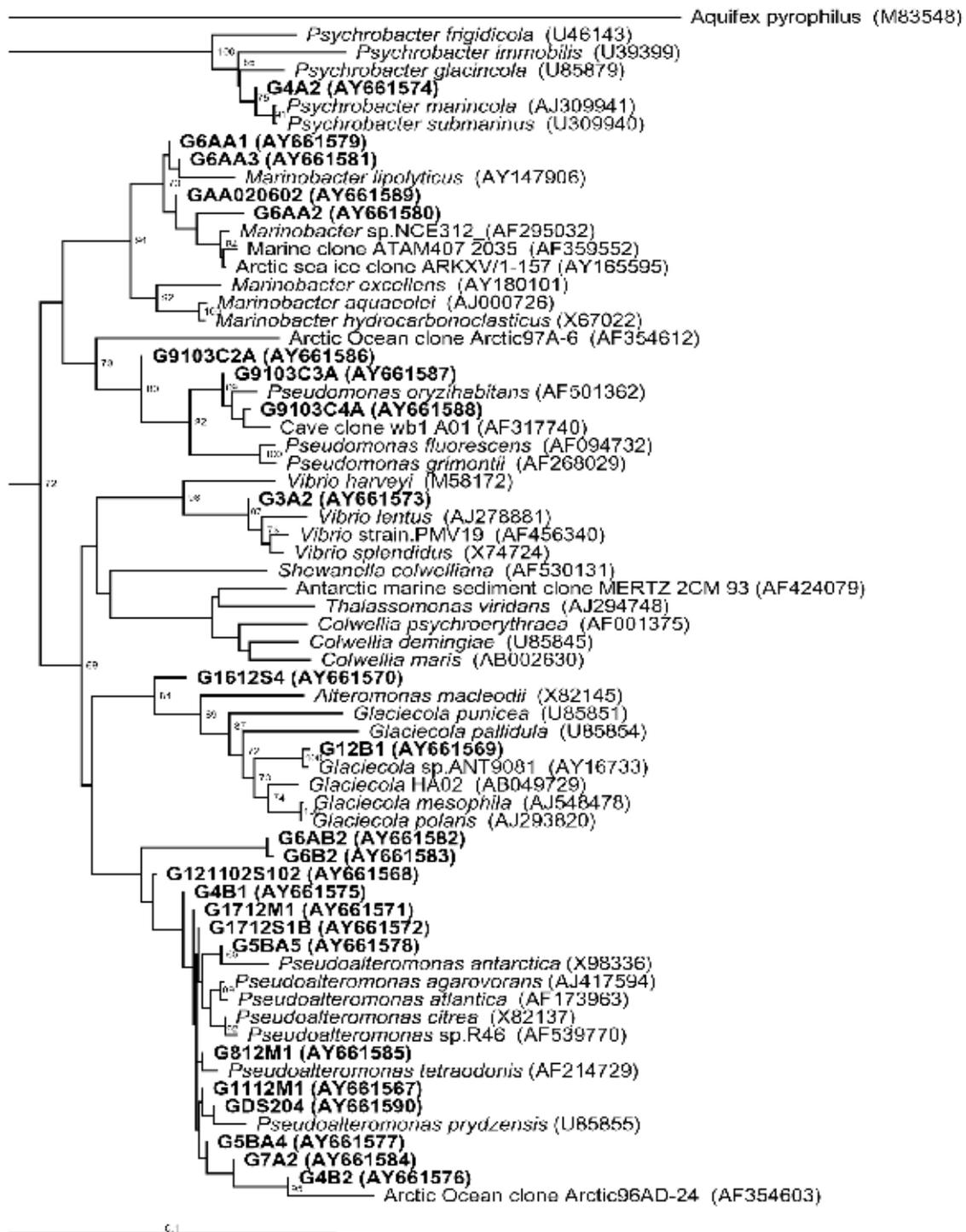
Figure 4.3. Evolutionary distance dendrogram created from 16S rRNA gene sequences of some members of the gamma subclass of the *Proteobacteria* and associated marine isolates. *A pyrophilus* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (1000) replicates have values shown at nodes. The scale bar indicates 0.1 changes per nucleotide.

identical sequence to *Glaciecola* sp.ANT9081, isolated from Weddell Sea pack ice (Brinkmeyer et al., 2003). Despite being pigmented, several isolates clustered amongst non-pigmented *Pseudoalteromonas* species. Members of the genus *Pseudoalteromonas* are readily cultivated from the marine environment, in fact they have been reported to be the most commonly isolated psychrotolerant bacteria from Antarctic sea ice (Bowman et al. 1997b; Bowman 1998). Members of the *Pseudoalteromonas* are halotolerant, motile and require salt for growth. It was not possible to confirm the species identity of any of the *Pseudoalteromonas* isolates from this study without more sequence data, extensive DNA:DNA hybridisation analyses and other definitive analyses (Fig. 4.3).

4.4.3.2 *Alphaproteobacteria* isolates

Members if the *Alphaproteobacteria* have been demonstrated to be present within the free-living and attached communities within the marine environment, and members are amongst the most abundant SSU rRNA gene clones recovered from marine plankton clone libraries (Kirchman, 2000).

Strains isolated in this study that grouped within the *Alphaproteobacteria* are shown in Figure 4.4. Of the 14 isolates investigated 3 isolates appeared to belong to the genus *Sulfitobacter*, one to *Roseovarius*, 2 to *Hyphomonas*, 4 to *Erythrobacter*, and 3 to *Sphingomonas*. Strain GAA020601 was not closely related to any known species.

Several isolates grouped in the family *Sphingomonadaceae* that includes aerobic, carotenoid-containing chemoheterotrophs, cosmopolitan in nature including surface seawater. Isolates G5BA1 and G5BB2a were most closely related to species isolated from

dust and airborne particles (e.g. *S. aerolata*; Busse et al. 2003) and interestingly an isolate from Lake Vostok accretion ice deep below the Antarctica ice sheet (strain M3C203B-B, Christner *et al*. 2001). The two strains were isolated from Station 17 close to the transition from Sub Antarctic Zone to Polar Frontal Zone. Another *Sphingomonas* isolate, strain G4A1, isolated from station 16, and was most closely related to the species *Sphingomonas paucimobilis*. This strain may represent a novel species as it had only 97% similarity with *S. paucimobolis* and related species. Two yellow-pigmented strains, GAA0204NET2-4 and GEHC4, were closely related to the marine species *Erythrobacter citreus* (originally isolated from the Mediterranean Sea, Denner et al. 2002). The former isolate was obtained from a plankton net tow in the Southern Ocean in 2002 and the latter isolated from coastal waters off the Tasman Peninsula, Tasmania. The other orange-pigmented strains, GAA0204NET2-5 and GAA0208BR5, also isolated from Southern Ocean plankton-net tow samples, were related to *E. citreum*. From 16S rRNA gene data it is also likely they could represent a new species. These strains were closely related to an Antarctic pack-ice isolate, strain ANT9115 (Brinkmeyer et al. 2003). Two yellow-pigmented strains, G5BA2 and G5BB3 (isolated from station 19) were closely related to the species *Hyphomonas johnsonii*. *Hyphomonas johnsonii* is a prosthecate, budding, motile, marine bacterium. *Hyphomonas* species have been isolated from a range of marine environments, and have been implicated in biofilm formation and in primary colonisation of submerged surfaces in the marine environment (Weiner *et al*. 2000).

Strain GAA020601, isolated from a Southern Ocean water sample in 2002, was not closely related to any described species. The most closely related isolate was strain SCRIPPS-423, isolated from a culture of the dinoflagellate *Scrippsiella trochoidea* (Hold

et al. 2001) (similarity 94.1%, 630bp). Strain GAA020601 likely represents a novel

genus that groups in a clade also containing the marine prosthecate genera *Maricaulis* and

Hyphomonas although complete 16S rDNA sequence data may influence this grouping.
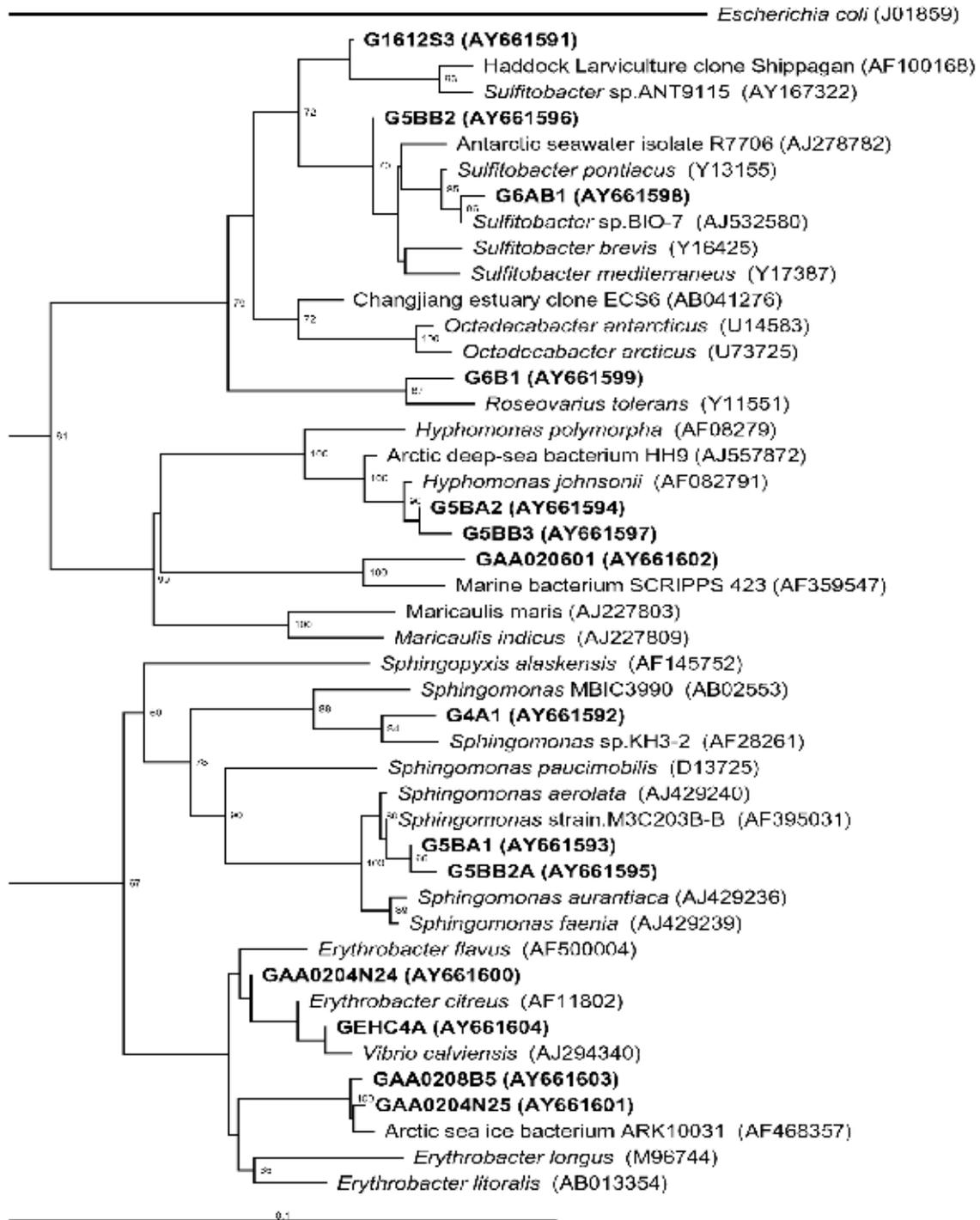
Figure 4.4 Evolutionary distance dendrogram created from 16S rRNA gene sequences of some members of the alpha subclass of the *Proteobacteria* and associated marine isolates. *Escherichia coli* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (1000) replicates have values shown at nodes. The scale bar indicates 0.1 changes per nucleotide.

Several isolates clustered in the *Rhodobacter* cluster which contains many marine, halophilic bacterial species, which are often poorly characterized and with a rather chaotic taxonomy. Strain G6B1 (from station 6), grouped most closely with Antarctic hypersaline lake species *Roseovarius tolerans* (Labrenz et al. 1999). Strain G1612S3 was isolated from station 8 in the Antarctic Zone was closely related to an Antarctic pack-ice isolate ANT9115 and more distantly allied with *Sulfitobacter* spp. (e.g. *S. brevis*, ~96% similarity). Two strains, G5BB2 and G6AB1, both isolated during the 2001 voyage from consecutive samples at 50 and 52 °S, respectively (station 18 and 19) were very similar to *Sulfitobacter pontiacus*, a species from the Black Sea (Sorokin, 1995). Sulfitobacter strains are known to rapidly colonise surfaces in the marine environment (DeLong, Franks and Alldredge, 1993; Acinas et al. 1999).

4.4.3.3 Other Isolates.

The remainder of the bacterial isolates were Gram-positive cocci or rods, with five strains belonging to phylum *Actinobacteria* and 1 strain to the *Firmicutes*. Of the *Actinobacteria*, three strains (G7A1, G7B1 and G7B2 - all from station 21 within the Polar Frontal Zone) were closely related to the species *Frigoribacterium faeni*, a species isolated from airborne dust (Kämpfer *et* al. 2000). *Frigoribacterium faeni* is an aerobic, psychrotolerant bacterium isolated from terrestrial, estuarine and marine environments (e.g. Kisand et al. 2002). Strain G2B1, isolated from Station 14 near the border of the SAZ and the TZ was most closely related to *Micrococcus luteus* a cosmopolitan yellow-pigment coccoid species often isolated from air, soil and water. Strain G2B1 was also very similar to a

North Sea isolate KT1115 (Eilers *et al*. 2000). Strain G5AB3, isolated from Station 17, grouped within the genus *Nocardioides* and was most closely related to groundwater species *Nocardioides aquiterrae* (Yoon *et al*., 2004). It is possible G5AB3 may represent a novel species in genus *Nocardioides*. The single isolate falling into phylum *Firmicutes*, strain G5AA3, had a 16S rRNA gene sequence practically identical to *B. marisflavi* and *B. aquimaris*, orange-pigmented spore-forming species common in coastal marine environments (Yoon *et al*. 2003).
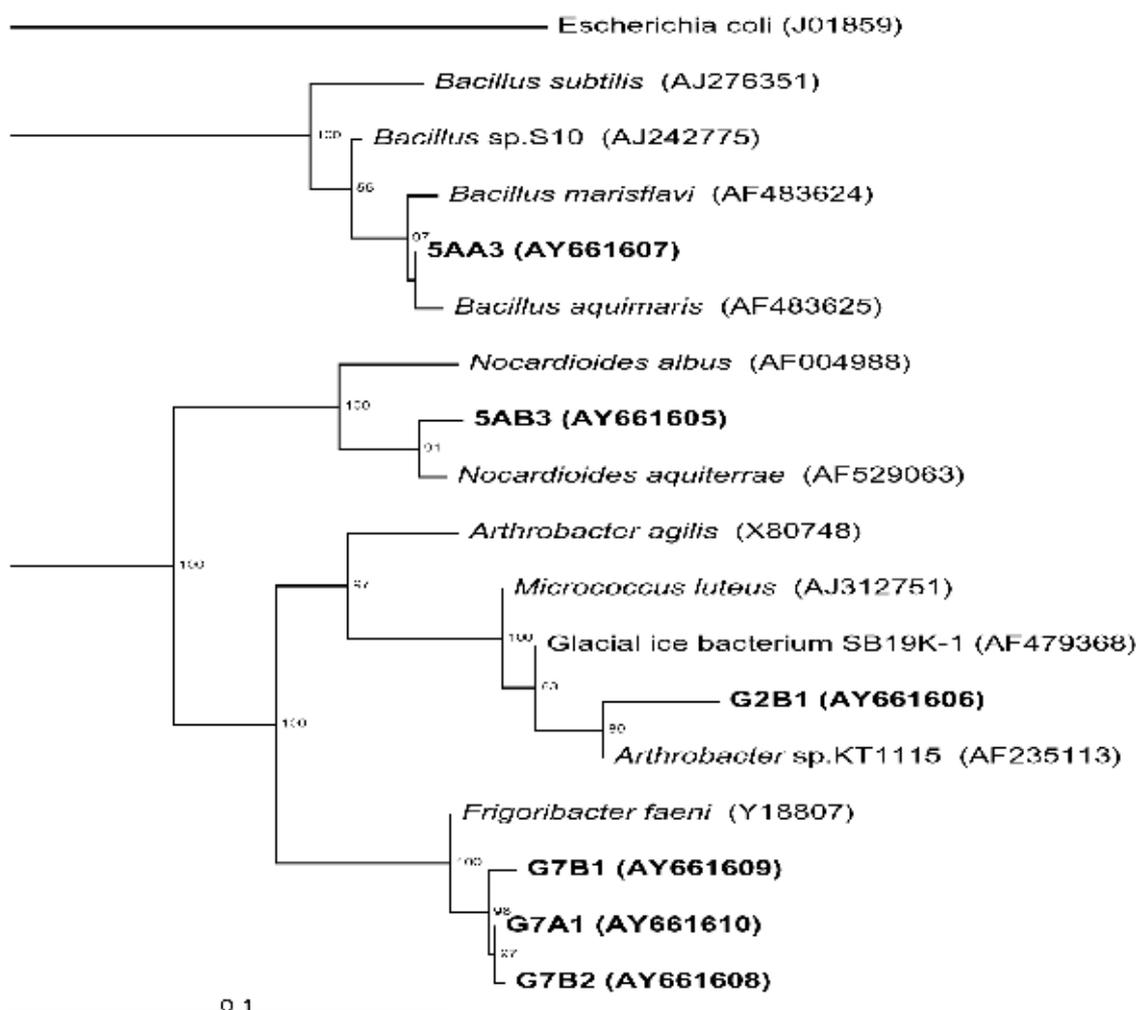
Figure 4.5 Evolutionary distance dendrogram created from 16S rRNA gene sequences of some members of the *Actinobacteria* and the *Bacillus* group and associated marine isolates. *Escherichia coli* was used as the outgroup species for analysis. Branching points with >60% bootstrap support (1000) replicates have values shown at nodes. The scale bar indicates 0.1 changes per nucleotide.

4.5. Discussion.

On the basis of detailed polyphasic taxonomic data strains G11A1 (*Calefactosor marmoris*), GAA0204_Net2_3 (*Maricorpusculum australicum*) and G812M2 (*Gelidimarimicrobium roseum*) clearly represent three separate novel genera and species within the family *Flavobacteriaceae*. The data also demonstrated that strain G512M1 and G512M2 represented a novel species of the genus *Psychroserpens*, designated *P. cryoceani*. Genus and species descriptions are listed at the end of the chapter in section 4.6.

Several other taxa belonging to the genera *Tenacibaculum* and *Polaribacter* also likely represent novel species, however substantial work is required to prove this and was beyond the scope of this thesis to complete. Similarly, many non-CFB taxa also probably represent novel taxa as explained in the results section. Strain GAA020601 for instance is particularly interesting as it forms a novel lineage in the *Alphaproteobacteria* and is only distantly related to described species.

The isolation of bacteria from the Southern Ocean is important for helping define the biological traits of the bacterial species that inhabit such an environment. Such traits are important in helping understand the complexity of Southern Ocean foodwebs, biogeochemical cycles and functionality. By characterizing and describing strains in detail an inventory of biological information (a bioresource in other words) is continually being accumulated and is available for future research. However, the study here was not meant to be any way comprehensive, indeed if anything it reveals the limited scope of both culture-dependent and culture-independent approaches in fully describing bacterial

diversity in an ecosystem, even if the environment is generally ascribed as being only "moderate" in diversity. The majority of strains isolated during this study demonstrated a phylogenetic relationship of greater than 97% with a previously cultivated bacterial strain, with the exception of the *Alphaproteobacterium* strain GAA020601, and the three novel CFB isolates GAA0204N23, G812M2 and G11A1. Culture-independent data obtained from DGGE gel band sequences (Chapter 3) revealed a very different set of CFB taxa. Only a few of the clades detected in the molecular survey in Chapter 3 have isolated representatives isolated here. These included the genera *Polaribacter* and *Psychroserpens* and clade IX which includes strain G1A11 (Fig. 3.3). This obviously indicates that most isolates obtained represented a small component of the total CFB community. While the molecular based survey revealed members of the CFB from across 15 diverse phylogenetic groups, the cultivation based survey was more limited with only strains from 6 groups within the family *Flavobacteriaceae*. They were isolated probably because they are easily cultivable, readily responding to the media used in the experiments. At the same time, the relative ease with which the novel genera were isolated during this study hints at the undiscovered cultivable diversity present in the Southern Ocean, and suggests that there is significant undiscovered diversity of psychrophilic bacteria within the CFB cluster present. The use of both approaches in the end revealed the substantial diversity of CFB in the Southern Ocean and despite the apparent limitations of the efforts described here, to directly reveal this diversity; the data presented is a significant addition to our understanding of the microbial ecology of the Southern Ocean. One commonality between the molecular and cultivation data is the domination of the marine clade of the family *Flavobacteriaceae* in the Southern Ocean.

This suggests the marine clade represents a successful evolutionary radiation in cool to cold marine environments. It is possible that in warmer waters a different biological radiation may occur with different dominant players amongst the CFB. For example, members of the family "*Flexibacteriaceae*" and relatives could be more important as suggested by existing anecdotal evidence (Table 2.1). In the end much further work is needed and different approaches utilized in order to access dominant Southern Ocean CFB clades. This is required so that valuable functional and physiological knowledge can be obtained for the different clades and thus provide a framework for developing more comprehensive knowledge of the microbial ecology of the Southern Ocean.

Future approaches to the cultivation of the novel CFB clades, highlighted by the molecular studies outlined in chapter 3, could incorporate methodologies such as those described by Kaeberlein et al. (2002) and Connor and Giovannoni (2002) as well as the individual cell culture described by Zengler et al. (2002). The use of a range of complex nutrient sources could also be used to isolate members that possess metabolic capacity for the degredation of macromolecules.

4.6. Descriptions of novel taxa.

Description of *Gelidimarimicrobium* gen. nov.

Ge.li.di.ma.ri.mi.cro.bi'um. L. adj. *gelidum* cold; L. neut. n. *mare* the sea; N. L. n. *microbios* small lifeform; N. L. neut. n. *Gelidimarimicrobium* small lifeform of the cold sea. Gram-negative, rod-like cells. Non-motile. Cell mass is pink to brown. Flexirubin pigments are formed. Does not form resting cells or spores. Strictly aerobic. Produces catalase. Chemoheterotrophic. Halophilic. Major fatty acids include i15:1ω10c, a15:1ω10c, i15:0, a15:0, i16:0, a17:1ω 7c and 16:1ω7c. A member of the family *Flavobacteriaceae*, class *Flavobacteria*, phylum "*Bacteroidetes*". The type species is *Gelidimarimicrobium roseum*

Description of *Gelidimarimicrobium roseum* sp. nov.

ro.se'um *roseum*. L. adj. pink. Description is as the genus description plus the following. Colonies are circular, convex, with regular edges and butyrous consistency on marine agar. Older colonies may produce a white precipitate. Growth occurs at 0-30°C. No growth occurs at or above 37°C. Requires sea salts for growth. Grows between 15- 60 psu, with optimal growth occurring at approximately 30-40 psu. Does not grow on nutrient agar, nutrient agar containing 2.5% NaCl or MacConkey's agar. Nutritionally non-exacting and can use inorganic nitrogen sources such as sodium nitrate and ammonium chloride in defined growth media. Utilises D-glucose, maltose, caprate,

malate and casamino acids for growth but not D-gluconate, adipate, citrate or phenylacetate. Oxidase positive. Oxidatively produces acid from D-glucose on Leifson's O/F medium. Hydrolyses gelatin, casein, L-tyrosine, starch, DNA, esculin, urea and tween 80. Does not produces lecithinase or lipase. Produces alkaline phosphatase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-N-acetyl-N-glucosaminidase, α-fucosidase and β-glucuronidase. Does not produce arginine dihydrolase or reduce nitrate. Other phenotypic data are shown in Table 4.2. Mean DNA G+C content is 31 mol%. Isolated from Southern Ocean surface seawater. Type strain is G812M2.

Description of *Calefactosor* gen. nov.

Cal.e.fact.o'sor L. part. adj. *calefactus* (from L. v. *calefacio*) to warm; L. masc. n. *osor* the hater; N. L. masc. n. *Calefactosor* the hater of being warmed. Gram-negative, rod-shaped cells. Possesses gliding motility. Cell mass is golden yellow. Flexirubin pigments are not formed. Does not form resting cells or spores. Strictly aerobic. Produces catalase. Chemoheterotrophic. Halophilic. Major fatty acids include 16:0, 18:0, i15:1ω10c, i15:0, 16:1ω7c and 3-OH 15:0. A member of the family *Flavobacteriaceae*, class *Flavobacteria*, phylum "*Bacteroidetes*". The type species is *Calefactosor marmoris*.

Description of *Calefactesor marmoris* sp. nov.

mar.mor'is. L. n. *marmoris* of the white foamy surface of the sea. Description is as the genus description plus the following. Colonies are circular, convex, translucent, with irregular spreading edges and butyrous consistency on marine agar. Growth occurs at 0-20°C. No growth occurs at or above 25°C. Possesses an absolute requirement for sea salts. Grows between 15- 60 psu, with optimal growth occurring at approximately 30-40 psu. Does not grow on nutrient agar, nutrient agar containing 2.5% (w/v) NaCl or MacConkey's agar. Nutritionally non-exacting and can use inorganic nitrogen sources such as sodium nitrate and ammonium chloride in defined growth media. Oxidase positive. Utilises D-glucose and casamino acids for growth but not maltose, caprate, malate, D-gluconate, adipate, citrate or phenylacetate. Oxidatively produces acid from D-glucose on Leifson's O/F medium. Hydrolyses gelatin, casein, tween 80, L-tyrosine, urea and DNA but not starch or esculin. Does not produce lecithinase or lipase. Produces alkaline phosphatase and weakly β-N-acetyl-D-glucosaminidase. Does not produce arginine dihydrolase or reduce nitrate. Other phenotypic data are shown in Table 4.2. Mean DNA G+C content is 34 mol%. Isolated from Southern Ocean surface seawater. Type strain is G1A11.

Description of *Maricorpusculum* gen. nov.

Mar.i.cor.pus.cu'lum. L. neut. n. *mare* the sea; L. neut. n. *corpusculum* a particle; N. L. neut. n. *Maricorpusculum* sea particle. Gram-negative, rod-like cells. Non-motile. Cell

mass is yellow. Flexirubin pigments are not formed. Does not form resting cells or spores. Facultative anaerobe. Produces catalase. Chemoheterotrophic. Halophilic. Major fatty acids include 16:0, i15:0, a15:0, i16:0, i17:1ω7c and 16:1ω7c. A member of the family *Flavobacteriaceae*, class *Flavobacteria*, phylum "*Bacteroidetes*". The type species is *Maricorpusculum australicum*.

Description of *Maricorpusculum australicum* sp. nov.

aus.tra.li'cum. L. adj. *australicum* southern. Description is as the genus description plus the following. Colonies are circular, convex, with regular edges and butyrous consistency on marine agar. Growth occurs at 0-37°C. No growth occurs at or above 42°C. Requires sodium ions for growth. Grows between 15- 120 psu, with optimal growth occurring at approximately 30-40 psu. Does not grow on nutrient agar or MacConkey's agar. Grows on nutrient agar containing 2.5% NaCl. Nutritionally non-exacting and can use inorganic nitrogen sources such as sodium nitrate and ammonium chloride in defined growth media. Utilises D-glucose and casamino acids for growth but not maltose, caprate, malate, D-gluconate, adipate, citrate or phenylacetate. Oxidase negative. Oxidatively and fermentatively produces acid from D-glucose on Leifson's O/F medium. Hydrolyses gelatin, casein, L-tyrosine and but not starch, DNA, esculin, urea or tween 80. Does not produce lecithinase or lipase. Produces alkaline phosphatase, α-galactosidase and β-galactosidase. Does not produce arginine dihydrolase. Nitrate is reduce to nitrite. Other phenotypic data are shown in Table 4.2. Mean DNA G+C content is 40 mol%. Isolated from Southern Ocean surface seawater. Type strain is Gaa0204_net2_3.

Description of *Psychroserpens cyroceani* sp. nov.

cry.o.cean'I; Gr. neut. n.. *kyros* the cold; L. n. *oceanus* the ocean; M. L. n. *cryoceani* of the cold ocean. Colonies are orange, circular, convex, with regular edges and viscid consistency on marine agar. Flexirubins absent. Cells are non-motile, curved rods and may form coccoidal cells in older cultures. Few if any helical or coiled filamentous cells observed. Spores or other resting stages are not formed. Growth occurs at 0-15°C; weak, unreliable growth occurs at 20°C. No growth occurs at or above 25°C. Requires sea salts for growth. Optimal growth occurs at approximately 30-40 psu. Poor growth with seasalts added at 15 psu, no growth at 60 psu or higher. Does not grow on nutrient agar, nutrient agar containing 2.5% NaCl or MacConkey's agar. Requires yeast extract and vitamins for growth. Cannot use sodium nitrate and ammonium chloride as sole nitrogen sources in defined growth media. Does not utilise D-glucose, maltose, caprate, malate, casamino acids, D-gluconate, adipate, citrate or phenylacetate as sole carbon sources in the presence of yeast extract. Catalase positive. Oxidase negative. Strictly aerobic. Acid is formed neither oxidatively or fermentatively on Leifson's O/F medium or in API 20E or API20NE test strips. Hydrolyses esculin, tween 80 (weakly) and may decompose casein but does not degrade gelatin, L-tyrosine, starch, DNA or urea. Produces alkaline phophatase but does not produce lecithinase, lipase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-N-acetyl-N-glucosaminidase, α-fucosidase or β-glucuronidase. Does not produce arginine dihydrolase or reduce nitrate. Other phenotypic data are shown in Table 4.2. Mean DNA G+C content is 30 mol%. Isolated from Southern Ocean surface seawater. Type strain is G512M1.

Chapter 5: Colonization and activity of *Flavobacteria* on diatom detritus

5.1 Summary.

The ecology of microorganisms responsible for particulate organic matter decomposition and associated secondary production in the Southern Ocean is poorly understood. This study examined the activity of indigenous *Flavobacteria* communities on diatom detritus in three different of seawater microcosms. Seawater was collected from different parts of the Southern Ocean including within the PFZ, in an ice-free area of the AZ, and a site in the AZ ice pack. Detritus from the cosmopolitan marine diatom *Nitzschia closterium* Ehrenberg was resuspended in 25 l of seawater, filtered to remove particulate organic matter, particle-associated bacteria and most eukaryotes, but retaining native planktonic bacterial assemblages. Microcosms were incubated at 2°C for 30 days and samples taken on at 0, 1, 5, 10, 15, 20, 25 and 30 days. Samples were analysed for changes in community composition using denaturing gradient gel electrophoresis (DGGE), real-time PCR and fluorescent in-situ hybridisation (FISH). DGGE banding patterns and FISH images demonstrated a rapid rate of colonization of the phytodetritus. FISH demonstrated that the bacteria colonizing the detritus in all three microcosms were dominated by members of class *Gammaproteobacteria*, although *Alphaproteobacteria* and *Flavobacteria* were also readily detected. Real-time PCR demonstrated that members of class *Flavobacteria* were involved in initial colonization of detrital aggregate, however abundance stayed at approximately the initial levels throughout the experiment. 16S rRNA gene DGGE banding patterns and sequence analysis demonstrated significant

variation in *Flavobacteria* community structure associated with the initial 20 days of the experiment before community stabilization occurred. The community structures between the three microcosms markedly differed and major colonizers were derived from the initial particle-associated *Flavobacteria* community. This study provides an insight into the processes that contribute to the recycling of detrital aggregates in the Southern Ocean, and helps to attribute information about ecological significance of distinct *Flavobacteria* phylotypes.

5.2. Introduction:

The role of marine bacteria in secondary production is an important consideration in describing marine food webs. The link between primary production and bacterial mediated remineralisation can be seen in figure 1.2. It is thought that the proliferation of marine diatoms is in some situations limited by the silica supply, as diatoms have an absolute requirement for silica. In the Southern Ocean, diatoms represent a large proportion of primary producers and thus silica as must regenerated through decomposition of diatomaceous particulate organic matter produced in surface waters. Denaturing gradient gel electrophoresis and 16S rRNA gene sequencing of bacteria colonizing in situ diatom assemblages confirmed previous findings that bacteria associated with aggregates belonged to the CFB, *Gammaproteobacteria* and *Alphaproteobacteria* groups and contributed to silica dissolution from diatom biomass (Bidle et al. 2003). Data shown in chapter 2 (Fig. 2.4A) indicated that, in the Southern Ocean TZ, silica and other nutrients were very low; nearly exhausted due to bursts of new

phytoplankton production in the summer (reported by Kopcynzska et al. 2001). Subsequent new production would only be possible by the decomposition of this biomass since this region is far from continental land masses, and as such nutrient supply is mostly regenerated by bacterial mediated decomposition of existing primary produced biomass. Indeed, bacterial secondary production mineralizes about half of the primary produced biomass in the oceans (Cole et al. 1988). Secondary production is thus a critical mechanism in the maintenance of marine foodwebs. More knowledge is required to understand the ecology of microbially-driven secondary production processes (Paerl and Steppe 2003).

Previous studies aimed at examining bacterial mediated degradation of diatom detritus have demonstrated that marine bacteria accelerate biogenic silica dissolution rates in the sea (Bidle and Azam, 1999). Members of the CFB phylum are often associated with particulate matter in the marine environment (Kirchman 2002), and it has been suggested previously that members of this group play a significant role in the degradation of marine aggregates. Examination of silica cycling through the application of bacterial microcosms has been used to examine specific mechanisms (Bidle and Azam, 2001; Bidle et al. 2003).The use of small scale 'environments' to examine the dynamics of environmental processes have been utilised to describe a number of processes in microbial ecology, including bacterial activity during diatom blooms (Riemann et al. 2000; Prieto et al., 2002.), the effects of variations in nutrient composition on bacterial communities (Lebaron et al., 1999; Pinhassi et al. 1999; Schafer et al. 2001; Joint et al. 2002.) and bacterial motility (Grossart et al. 2001) and to examine viral lysis and the effects of bacterivory on algal blooms (Guixa-Boixereu, Lysnes and Pedros-Alio, 1999).

Chapter 5: Colonization and activity of *Flavobacteria* on diatom detritus

The ability to examine the response of a community to distinct changes such as nutrients, temperature, salinity or grazing allows biologists to reduce the variables and concentrate on specific processes. In this way it is possible to assign specific characteristics or processes to communities or phylogenetic lineages. The use of mesocosms, like many experimental approaches involves certain biases; in the proposed detritus-based microcosms it is likely a "bottle effect" will manifest in which the provided detrital surfaces will result in abnormally enhanced bacterial activity and populations (Heukelekian and Heller 1940). Despite the possible influences of such an effect on the community structures obtained, the microcosm studies provide a unique opportunity to study specific processes through the elimination of complicating variables present in the marine environment, including for example grazing, variations in nutrient availability and temperature.

The study described in this chapter was aimed at examining the response of native Southern Ocean bacterial communities to the addition of diatom detritus. Specifically the goal was to examine the composition and succession of the *Flavobacteria* community colonizing the detritus over the period of incubation. Various questions were asked in relation to this: Will the communities colonizing the detritus derive from major populations already present in the collected seawater samples, especially those already associated with particulates? Will the detritus be colonized by fast growing species rapidly responding to the available nutrients? Data of community composition in this scenario can be compared to culture-independent and culture-dependent surveys performed on Southern Ocean seawater in chapters 3 and 4 where numerous phylotype groups were identified. It was of interest to determine which phylotypes are responding

readily to detrital colonization. In addition, during the experiments a determination of *Flavobacteria* abundance on the added phytodetritus will be made with real-time PCR and FISH. This data provides information on the actual dynamics of colonization by members of the *Flavobacteria* and provides an indicator for whether abundance in particles changes over time in a dynamic fashion or becomes stabilized at some stage. Finally, measurements of dissolved silica was performed to examining the rate of diatom dissolution resulting from bacterial colonization as well as indicating the intensity of bacterial secondary production occurring in the experimental system.

5.3. Methods.

5.3.1 Preparation of diatom detritus:

An axenic strain of *Nitzschia closterium* was obtained from the CSIRO Collection of Living Microalgae and was cultured in silica rich F/2 medium (formula is shown in the Appendix) in continuous light (250 µmol photons m^{-2}.s^{-1}) at 20°C with continuous aeration and mixing. The air stream was filtered using a 0.2 µm pore nylon filter. The final culture was spun down at 1000 g in 200ml Nalgene R centrifuge containers and resuspended in filtered artificial seawater (FASW). The method used to obtain diatom detritus was from Bidle and Azam (2001). This involved subjecting diatom suspensions to 10 cycles of freezing in a dry-ice/ethanol bath and subsequent rapid thawing in a 55 °C waterbath. Following this treatment detritus was washed in FASW using centrifugation (4000 g, 10 min) and finally resuspended in FASW.

5.3.2 Microcosm setup:

The three microcosms were started with particulate free water from three different oceanic regions within the Southern Ocean. The three samples were taken from areas of differing oceanic conditions, locations and temperature as shown in Table 5.1. Native free-living bacterioplankton communities were obtained by filtering three different seawater samples (Table 5.1) onboard the *RV Aurora Australis* through a 0.8 μm capsule filter (Pall Gelman, Ann Arbor, Mich., USA) to remove all particulate matter. The filtrates collected nearly completely filled a 25 l carboy; 10 mg of diatom detritus was added to each of the carboys at time zero (T0), which were stored with gentle aeration at 4°C. Samples for laboratory analyses samples were collected at 0, 2, 5, 10, 15, 20, 25 and 30 days. For DNA extraction, approximately 300 ml of water was sampled from each carboy and filtered through a 0.8 μm polycarbonate millipore filter. The filters were washed by filtering through 50 ml of FASW to remove loosely associated and free living bacteria and then frozen. For FISH, two 50 ml samples were obtained from each carboy and filtered through 0.8 μm filters. Filters were then fixed with 4% formaldehyde for 2 h , then washed with 50:50 ethanol: PBS (Chapter 2), air-dried and stored at room temperature. For each of the three microcosm experiments a control was set up using filtered (0.2 μm) autoclaved seawater, inoculated with diatom detritus and incubated and sampled as per the microcosms.

Table 5.1. Locations of seawater sampling sites used to inoculate bacterial microcosms, including Southern Ocean zone, and water temperature.

Sample	Location	Zone	Temperature
S1	53 54°S, 141 21°E	PFZ	2.4°C
S2	60 44°S, 139 54°E	AZ	0.1°C
S3	63 26°S, 144 05°E	AZ (loose ice pack)	-1.1°C

5.3.3. Dissolved silica analysis:

Samples (3×10 ml) were taken from carboys for silica analysis at 0, 1, 5, 10, 15, 20, 25 and 30 days. The samples were filtered on site with 0.5 μm pore size cellulose nitrate filters and the filtrate was then aliquoted into two 12 ml nutrient analysis tubes, and frozen. Silica analysis was performed using a modification of the method of Graßhoff (1964). Frozen samples were allowed to thaw at room temperature for at least 2 h. A 5 ml aliquot of the sample was placed in an Erlenmeyer flask and mixed thoroughly with 5 ml 0.25 N HCl, 5 ml of 5% (wgt./vol.) ammonium molybdate and 5 ml 1% (wgt./vol.) disodium EDTA. Samples were incubated at room temperature for 5 min. to allow for the formation of a silico-molybdate complex. Following the incubation, the complex was reduced with 10 ml 17% (wgt./vol.) sodium sulphite for 30 min. The absorbance of the solution was measured at 700 nm using spectrophotometry (Smartspec, Bio-Rad

Laboratories, CA, USA). Samples were compared to a standard curve made using

$Na_2SiO_3.5H_2O$, dissolved in water.

### 5.3.4. FISH and 16S rRNA gene-based analyses.

Analysis of samples followed procedures detailed earlier in the thesis including FISH

(section 2.3.2); DNA extraction (section 2.3.3); DGGE based analyses, sequencing and

phylogenetic analysis (sections 3.3.3 to 3.3.6); and real-time PCR analysis (section 2.3.4).

Following optimization, a 40-60% denaturing gradient was used for the DGGE analysis,

as this gave the greatest degree of separation of PCR products.

Chapter 5: Colonization and activity of *Flavobacteria* on diatom detritus

5.4. Results.

5.4.1 Microcosm silica dissolution

Analysis of dissolved silicic acid concentrations in each of the seawater microcosms and the corresponding controls (designated S1, S2 and S3; as indicated in Table 5.1) demonstrated an increase in the silicic acid concentration above the control in all three of the experiments. For all three microcosms the controls reached a final silicic acid concentration of between 1.15 and 1.76 mg l^{-1}, by comparison the microcosms reached final silicic acid concentrations of between 3.2 and 4.0 mg l^{-1}. The rate of increase of silicic acid above that measured in the control microcosm varied between microcosms, with microcosms S1 and S2 demonstrating a significant increase above the control microcosm after 5 days, while microcosm S3 failed to demonstrate a significant increase until 10 days had passed.
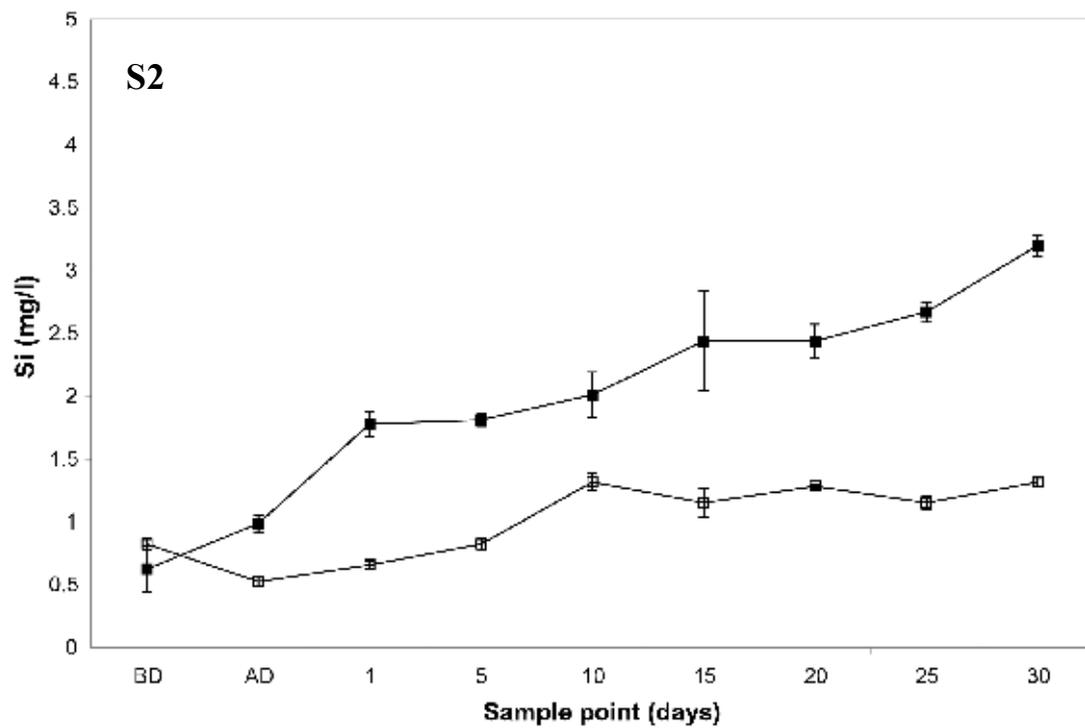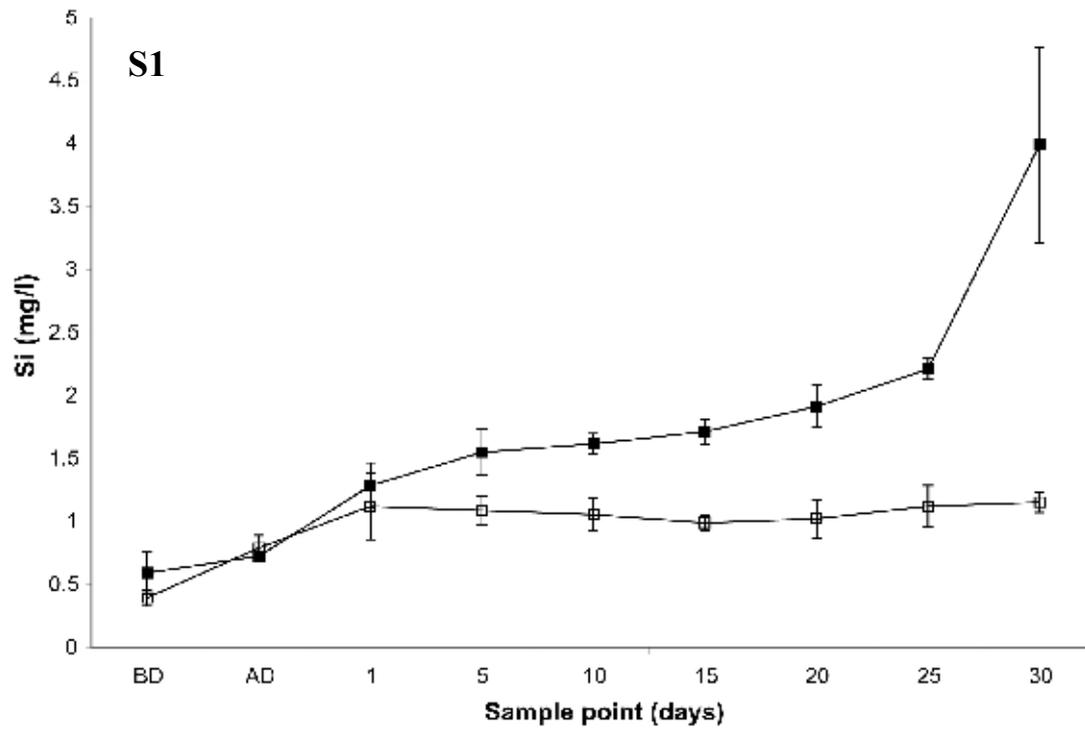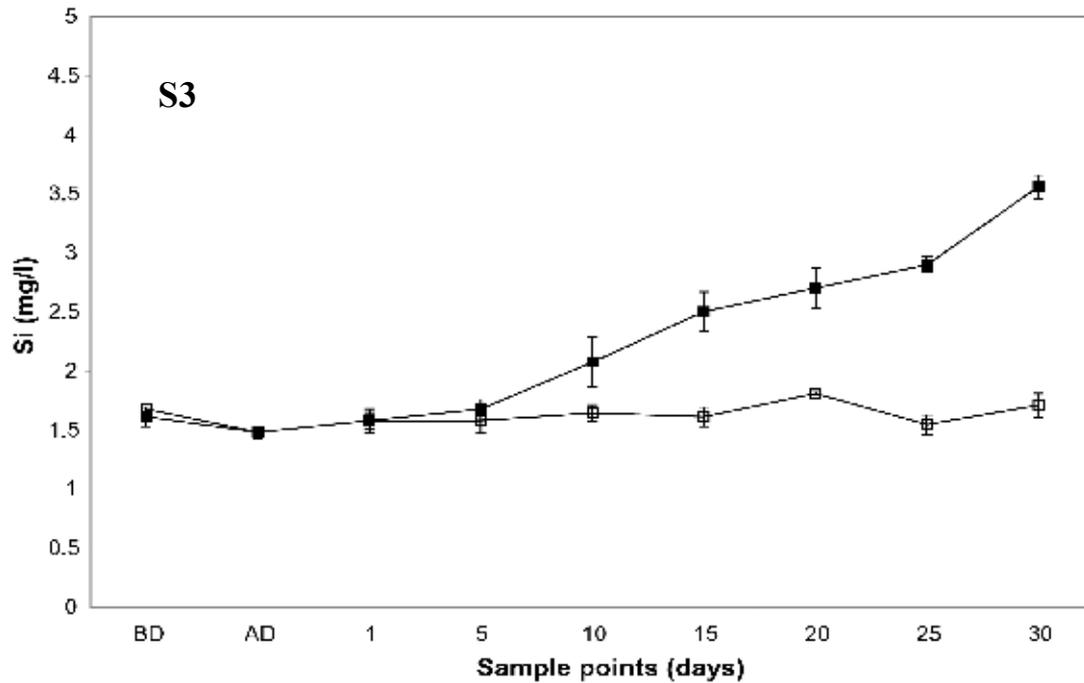
Figure 5.1. Dissolved silicic acid in microcosms S1, S2 and S3 containing native bacterial populations (■), and control microcosms (□) over the 30 days of incubation. Time points "BD" and "AD" represent silicic acid values before (BD) and after (AD) the addition of diatom detritus (at T0), respectively. Error bars demonstrate the standard deviation of results of triplicate water sample analyses taken from each time point.

5.4.2 Quantification of *Flavobacteria* colonizing added diatom detritus.

Analysis of *Flavobacteria* colonization of detritus utilized the real-time PCR technique. The seawater samples used in the microcosm experiments all contained particulates, captured on 0.8 μm filters, that had been colonized by *Flavobacteria* (T0, Fig 5.3). The relative abundance varied 4-fold between the samples, with microcosm S1 possessing a high proportion of *Flavobacteria* in the particulate fraction (a relative abundance of 29%). Colonisation of the added bacteria-free detritus in microcosm S1 was rapidly dominated by members of the *Flavobacteria* reaching a relative abundance of greater than 70% by day 15 before declining to 30-45% between day 15 and day 30. The other two microcosms (S2 and S3) demonstrated much lower *Flavobacteria* abundance in the initial detritus colonization period (<1% relative abundance after 1 day). Over time the abundance increased in proportion to the total bacterial abundance though the extent of this differed considerably between microcosms. Between days 10 and 15 the relative abundance of *Flavobacteria* in microcosms S2 and S3 increased dramatically, corresponding to an increased banding density in the DGGE gels (Fig. 5.2 and 5.3). Likewise, between days 15 and 20, microcosm S1 showed a decrease in *Flavobacteria* abundance corresponding to a change in the community composition as shown by DGGE. For microcosm S2 *Flavobacteria* colonization resulted in abundances, which slightly exceeded the original particulate level, ranging from 7-15% from day 15 to day 30 of the experiment. For microcosm S3 the abundance between day 15 and day 30 only ranged from 1-5%, much lower than the 17% abundance present in the original particulate fraction. Overall, the data suggested that in the case of microcosm S3 the

original *Flavobacteria* population had been excluded or displaced by other varieties of bacteria, however *Flavobacteria* were more successful in terms of colonization in the other microcosms. The colonization patterns also substantially differed suggesting the initial *Flavobacteria* community present responded differently to the added diatom detritus.
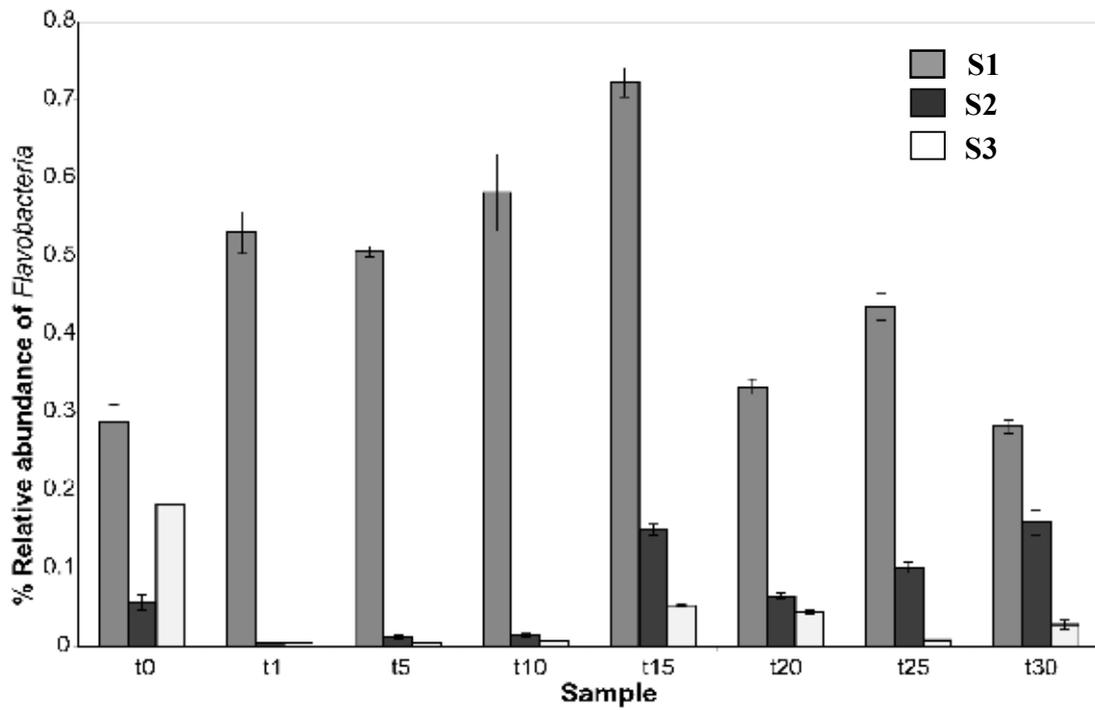
Figure 5.2. Graph of results from real-time PCR analysis of samples from diatom detritus amended seawater microcosms, measuring the relative abundance of class *Flavobacteria* (indicated as a proportion of the total 16S rRNA gene abundance) over the course of the experiment (t0 to t30, days of incubation).

5.4.3 DGGE analysis of *Flavobacteria* colonizing detritus

Examination of DGGE profiles demonstrated that the majority of *Flavobacteria* phylotypes colonizing the diatom detritus particles were present as major communities in the native seawater attached-fraction. This was initially suggested by DGGE bands from microcosm samples corresponding to the same position of bands obtained in profiles of the original particulate-associated communities (Fig. 5.3). This was further confirmed by sequence analysis of the DGGE bands. DGGE band profiles also indicated that the initial particle associated *Flavobacteria* community structures were initially different between the three microcosms (Fig. 5.3).

The DGGE profiles revealed that colonization was rapid. In the S1 microcosm bands were only faintly present after 1 day but became clearly obvious after 5 days incubation. Band intensity varied for some but not other bands in S1 (e.g. T5 compared to T10) suggesting different species in the microcosms changed in population levels substantially during the early to mid-phases of the experiment before eventually establishing a stable community structure after about 25 days of incubation. In the case of microcosms S2 and S3 DGGE bands became only apparent after 10 days incubation and like microcosm S1 attained a stable community structure only after 25 days. These results appear to reflect the real-time PCR data that also indicated populations began to plateau between 20 to 30 days incubation (Figure 5.2).

The DGGE profile patterns obtained for S1 appeared to closely reflect that of the original community over the course of the experiment, however two bands (indicated as numbers 5 and 8 in Fig. 5.3A) was not detected in the original particulate community at

T0, but only became detectable by day 20. In microcosms S2 and S3 by comparison the colonization of the diatom detritus appeared to yield substantially simpler communities by the end of the experiment (Fig. 5.3B and 5.3C). This was especially the case for microcosm S3 where the initial, relatively complex *Flavobacteria* community (~20 visible bands) was reduced to few detectable species (4 or 5 obvious bands; Fig. 5.3C). Based on band intensity these few species were considerably favoured (e.g. DGGE band 1 in microcosm S3) in the microcosms at the expense of initially more abundant species.

5.4.4 Analysis of DGGE band phylotypes

Sequencing of phylotypes detected using DGGE, demonstrated that the members of the *Flavobacteria* colonizing the detritus belonged mostly to cultured genera (Table 5.2, Fig. 5.4), including *Polaribacter*, *Tenacibaculum*, *Maribacter*, *Psychroserpens*, *Aequorivita* and the new genus "*Subsaximicrobium*" (Bowman et al. unpublished). Indeed, many of the phylotypes detected grouped closely with described species or with strains identified in Chapter 4. However, two phylotypes (S104a and S206) demonstrated little homology (<96%, over 663 and 515 bp respectively) with known species or strains.

Microcosm S1 had the greatest diversity of phylotypes colonizing the diatom detritus including representatives of 8 different genera. Except for bands 105 and 108 all were present in the original seawater particulates. Bands 105 and 108 appeared on the diatom detritus only after 20 days incubation and thus must have been present only at very low populations on the original seawater particulates. The colonization of diatom detritus in microcosm 2 was dominated by *Tenacibaculum* species (S201, S202, S203)
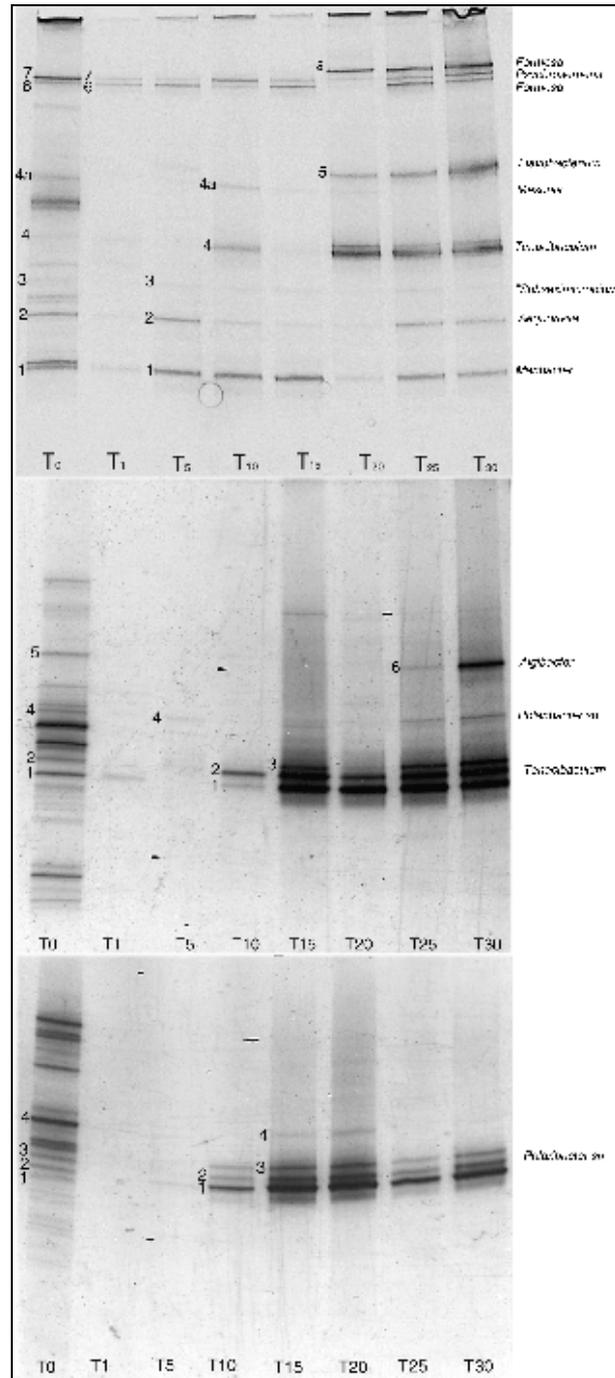
Figure 5.3 DGGE gels of the microcosms experiments demonstrating the succession of *Flavobacteria* phylotypes . Band numbers correspond to phylotypes shown in Table 5.2 and Fig. 5.4. The profile at T0 is derived from the original particulate seawater fraction. Phylogenetic affiliations of bands are shown on the right of the images.

Table 5.2 Phylogenetic affiliation of CFB phylum related bacteria colonizing diatom detritus, detected using DGGE.

| Microcosm	DGGE band no.	Bp	Microcosm detection (days)*	Closest cultured species/strain **	% simil-arity	DGGE Clade/phylo-type***
S1	1	510	1-30	*Maribacter orientalis*	99	*Maribacter*
S1	2	516	1-30	*Aequorivita antarctica*	100	-
S1	3	649	5-30	*Gelidibacter* sp. QSSC5-7 (now "*Subsaximicrobium wynnwilliamsii*")	99	-
S1	4	506	10-30	*Tenacibaculum* sp. strain G1A1	100	-
S1	4a	663	10-30	*Mesonia algae*	96	-
S1	5	508	20-30	*Flavobacterium gelidilacus*	98	-
S1	6	507	1-30	*Formosa algae*	97	14080130
S1	7	507	1-30	*Psychroserpens* sp. strain G512M1	100	*Psychroserpens*
S1	8	549	20-30	*Formosa algae*	96	14080130
S2	1	615	1-30	*Tenacibaculum* sp. strain G121102s2_3	100	-
S2	2	622	10-30	*Tenacibaculum* sp. strain G11A2	100	-
S2	3	537	15-30	*Tenacibaculum* sp. strain G11A2	99	-
S2	4	601	5-30	*Polaribacter* sp. strain G912S3A	99	*Polaribacter*
S2	6	515	25-30	*Algibacter lectus*	94	-
S3	1	501	5-30	*Polaribacter* sp. strain G812S3	100	*Polaribacter*
S3	2	366	10-30	*Polaribacter* sp. strain G1712M2	98	*Polaribacter*
S3	3	458	5-30	*Polaribacter* sp. G1612M2	98	*Polaribacter*
S3	4	579	15-20	*Polaribacter* sp. strain G812S3	100	*Polaribacter*

*Microcosm sampling times over which the DGGE band phylotype was detectable.

**Strains listed as those isolated from Chapter 4.

***DGGE clades (or phylotype) identified in Chapter 3, which are closely related to the microcosm phylotype; a missing entry indicates no equivalent clade or phylotype was observed in the survey presented in Chapter 3.
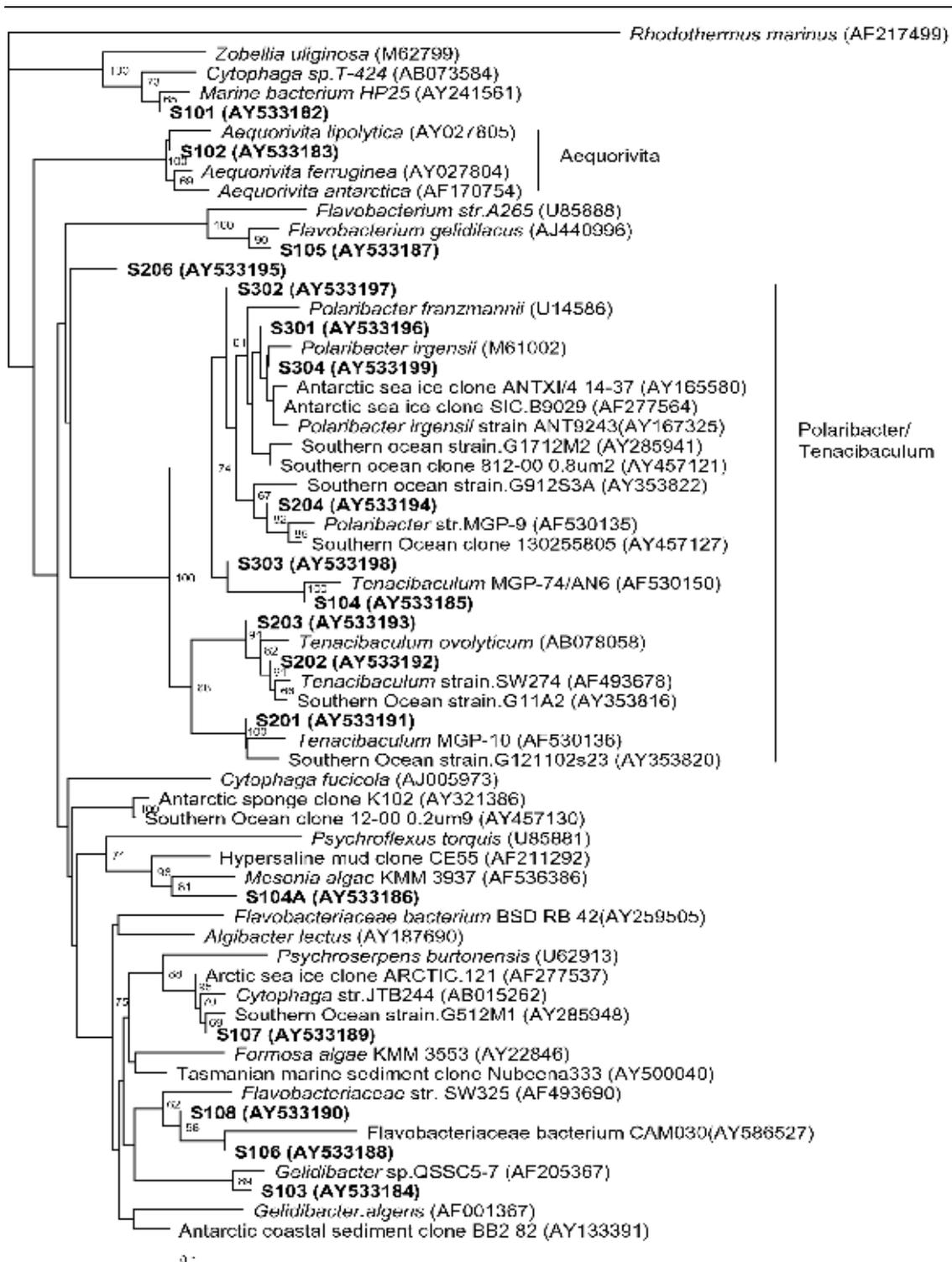
Figure 5.4. 16S rRNA gene phylogenetic tree showing the position of *Flavobacteria* phylotypes colonizing diatom detritus in the microcosm experiments. *R. marinus* was the outgroup sequence. Branching points with >60% bootstrap support (1000 replicates) have values shown at nodes. The scale bar indicates 0.1 changes per nucleotide

and also included a *Polaribacter* sp. (S204). At the 25 d incubation point band S206 appeared and rapidly increased in detectability by day 30. The sequence derived from this band was most closely related to the recently described algal epiphytic species *Algibacter lectus* and *Ulvibacter litoralis*, however clearly represents a novel taxon. Interestingly, band S206 was also prominently present in the original particulate community. In microcosm 3 the *Flavobacteria* that colonized the diatom detritus was dominated completely by *Polaribacter* species. The relative intensities of bands appearing at around day 10 in microcosm 2 and 3 increased in density up to day 30, suggesting that the members of the *Flavobacteriaceae* increased in density on the detritus during this period. This was reflected in the real-time PCR data (Fig. 5.2).

5.4.5 Microscopic examination of detrital particles by FISH.

Examination of the diatom detritus during the course of the microcosm experiments using FISH demonstrated colonization of particulate matter by members of the *Alphaproteobacteria* and *Gammaproteobacteria* as well as members of class *Flavobacteria*. Particulate matter was primarily comprised of aggregated diatom frustules as seen in Figure 3.6. Detrital aggregates formed within the first day and aggregates of significant number of diatoms were detected in all of the samples examined. The use of FISH confirmed the colonization of detritus and demonstrated the presence of members of the *Flavobacteria* attached to the diatom detritus (see Figure 5.5). FISH was not used for quantification of bacterial numbers as early samples demonstrated such strong background fluorescence that counting of bacteria was practically impossible. The
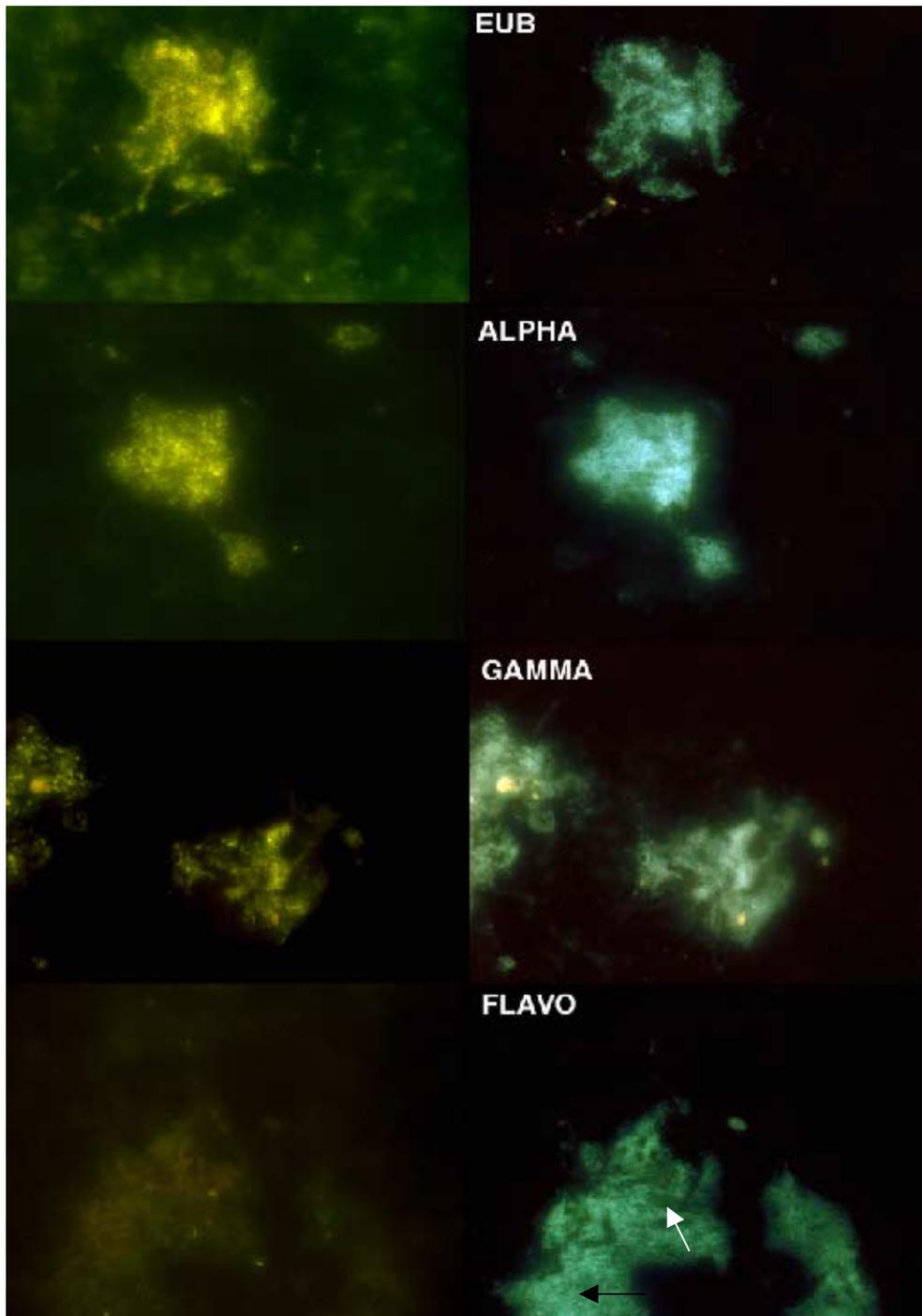
Figure 5.5 Epifluorescent micrographs of detritus particles using FISH to demonstrate colonization by different groups (Magnification 1000X). The left panel demonstrates cells hybridized to the corresponding probe, whilst the right panel demonstrates total (DAPI stained) cells. All samples are from day 20 of microcosm S2. The white arrow demonstrates background detritus, whilst the black arrow demonstrates bacterial cells.

images of later stages of the experiment (such as Figure 5.5) clearly demonstrated the high density of colonizing bacteria attaching to the diatom detritus.

5.5. Discussion.

Examination of dissolved silica in each of the microcosms demonstrated a significant increase in dissolved silica compared to the control flasks over the 30-day sampling period (Fig. 5.1). This increase in dissolved silica, corresponded to an increase in the diversity of bacteria present on the detrital particles, as suggested by the DGGE data in which numerous bands rapidly appeared (Fig. 5.3). Qualitative FISH data indicated that the detrital material eventually became densely populated by bacteria (Fig. 5.5), in particular by members of the *Alphaproteobacteria*, and *Gammaproteobacteria* in microcosms S2 and S3. Real-time data showed that *Flavobacteria* was also present but abundances appear to depend very much on the initial populations present in the seawater samples (Fig. 5.2). The role of members of the class *Flavobacteria* in the colonization and degradation of particulate matter in the marine environment is well documented (Kirchman, 2002). Members of this group have been demonstrated to be commonplace in the fraction of the community attached to particulate matter in the Southern Ocean (Simon et al. 1999) and are highly abundant in SIMCO (Brown and Bowman 2001; Brinkmeyer et al. 2003). Analysis of particulate and planktonic fractions of Southern Ocean samples in chapters 2 and 3 demonstrated the abundance and diversity of *Flavobacteria* appeared to be at last partially coupled to the amount of primary

production that was occurring in the samples and that diversity was enhanced in the more nutrient rich PFZ and AZ waters.

The majority of the *Flavobacteria* phylotypes colonizing the detritus were present in the original particulate fraction of the seawater samples from which the microcosms were based. This suggests that the bacteria represented by these phylotypes are adapted to growth on particulate matter and that colonization of particulate matter was perhaps not completely dependent on the specific nature of the particulate matter. The higher rate at which particulate matter was colonized by members of the *Flavobacteria* in microcosm S1, compared to the other two experiments, could be related to the initial high *Flavobacteria* abundance on the particulate matter in sample S1. The higher proportion of the bacterial community represented by the *Flavobacteria,* as assessed by real-time PCR, could also explain the greater diversity of colonizing phylotypes in microcosm S1 compared to microcosms S2 and S3. Competition from *Proteobacteria* in S1 may have been more muted as they were less predominant. However, in microcosms S2 and S3 *Proteobacteria* may have rapidly outgrown or inhibited many *Flavobacteria* community members preventing their colonization of the detritus; only the most rapidly growing or competitive *Flavobacteria* (perhaps able to release inhibitory substances) were able to become a dominant part of the new detrital community. However, the sudden appearance of DGGE band S206 late in the incubation of microcosm S2 also suggests that conditions in the microcosms may change to favour certain species present at undetectable levels and also suggests that community succession was still occurring in the later stages of the experiment. A longer-term experiment thus may have revealed new phylotypes and obviously longer term experiments need to be run in future. The changes in

Flavobacteria abundance between days 10 and 15 in microcosms S2 and S3, and between days 15 and 20 in microcosm S1, may be an effect of nutrient or substrate limitation and as such may be linked to the succession of *Flavobacteria* demonstrated by DGGE analysis.

A hypothesis can be advanced that the community structures stabilizing at the end of the microcosm experiments to some extent reflected the seawater sampling sites. Microcosm S3 was dominated by *Polaribacter* species. *Polaribacter* incidence in the microcosms from waters collected at lower latitudes appeared to decline, with one DGGE band detected in microcosm S2 and none at all in the relatively more diverse community of microcosm S1. From data presented in this thesis there is good evidence that *Polaribacter* is "endemic" and abundant in the cold waters of the AZ. Data from chapter 3 and 4 strongly point to this fact, with *Polaribacter* phylotypes mostly detected in AZ samples and absent from TZ and SAZ samples (Table 3.3). The genus was also readily isolated from AZ samples (Table 4.1). Overall, this suggests the genus is abundant and active in AZ pack ice zone waters and with an epiphytic nature, Polaribacter species (Gosink et al. 1998; Brown and Bowman 2001) are successful colonizers of primary production biomass in AZ seawater and sea-ice.

Though the particulate fraction was removed from the seawater samples at T0 the filtrate must have contained many of the particle-associated community members. In chapter 3 it was shown that there was substantial similarity in the *Flavobacteria* community between the planktonic and particle-associated communities. It was surmised that bacterial cells disperse from particles as the particles decompose resulting in indistinguishable communities between the fractions of the same samples. Thus the

planktonic population at T0 in the microcosm included species poised to take advantage of the resupply of new detrital material. The different responses observed between the three microcosms again reflected inherent differences in this community. Clearly, dynamic *Flavobacteria* colonization in microcosm S1 was favoured, exactly why is difficult to determine but as discussed above the initial high abundance of *Flavobacteria* may have been a crucial if not the sole factor.

Interestingly, a large number of the phylotypes detected in the three experiments grouped closely with isolates from chapter 4. This suggests that these colonizing species could be reasonably common in the Southern Ocean and may play a significant role in the degradation of particulate matter and in silica cycling. However, none of the clades identified in Chapter 3 containing uncultured *Flavobacteria,* namely clades I-VIII and agg58 cluster members, were detected even though many of these were also detected in particulate fractions of the AZ and other zones of the Southern Ocean. It is notable that many species manifesting DGGE bands present at T0 in the microcosms, especially in microcosms S2 and S3, did not colonize the detritus. Though these bands were not sequenced, due to time constraints, it is suspected that these bands probably represented phylotypes belonging to these uncultured clades since some of them were found in nearly every sample investigated in Chapter 3, in both the particulate and planktonic fractions (especially clades II, III, V, VII; see Table 3.3). This suggests that many *Flavobacteria* present in the original seawaters sample did not colonize the detritus provided. Several reasons can be advanced to explain this, all of which will require new experiments to be performed to assess this problem:

Chapter 5: Colonization and activity of *Flavobacteria* on diatom detritus

1. Many uncultured species could be comparatively slow growing (e.g. Hahn et al. 2004). As a result other fast growing bacteria including various *Flavobacteria* could exclude the uncultured species because they rapidly colonise surfaces on the detrital particles.

2. Secreted substances could directly exclude many uncultured species. Various rapidly colonizing bacteria may produce substances, which inhibit other bacteria thus maximizing their competitiveness and potential dominance. This phenomena has been shown in many instances in the marine ecosystem and various studies show that this trait is particularly common amongst the *Proteobacteria* (e.g. Long and Azam 2001b). A very good example is the species *Pseudoalteromonas tunicata* that produces a wide array of bioactive substances allowing it to exclusively colonize the surfaces of various macrophytes and thus protecting the macrophyte from biofouling (Egan et al. 2002).

3. Nutrients available in the detritus are inappropriate or inaccessible to the uncultured bacteria. It is possible their colonization may only occur in later stages when the fast growing bacteria exhaust common nutrients. Nutrients may also be required from bacteria not yet colonizing the detritus. Kaberlein et al. (2002) found that bacteria appear to produce substances that promote the growth of other bacteria.

4. Finally, a broader explanation is advanced. Uncultured *Flavobacteria* may exist in stable arrangements with various different phytoplankton species under a state of pronounced nutrient limitation and that their populations have slowly accumulated over time to certain sustainable levels. In other words they could represent classic K-type strategists. Since the uncultured populations would likely have initial abundance

advantage compared to the smaller numbers of the fast growing and opportunistic (r-strategist) species they can take effective advantage of the transient appearance of nutrients. Indeed, this exemplifies the concept that the microcosm experiment was affected by a "bottle effect" in which nutrients were released in such high concentrations that r-strategist populations were able to become quickly dominate while K-strategist populations were unable to make any inroads during the short experiment. Perhaps longer-term microcosm experiments performed with diffusion chambers, allowing exchange of seawater and preventing rapid build-up of nutrients and extracellular substances may allow greater colonization by the uncultured *Flavobacteria* clade members.

6.0 Conclusions and Future Directions.

FISH-based analyses previously suggested that the CFB phylum was a major community component in Southern Ocean surfaces waters (Glöckner et al. 1999; Simon et al. 1999). Studies have also shown this group of bacteria is abundant in other oceanic regions and has a significant role in secondary production (Kirchman 2002). The group also has experienced rapid expansion on the taxonomic level and this will continue with the increasing interest in pursuing biodiscovery and biotechnology in marine ecosystems. The basic aim of the PhD research was to uncover the diversity of the CFB in the Southern Ocean and to explore the relationships of community structure on a spatial and environmental level.

Paerl and Steppe (2003) make some very relevant comments in the context of this study: They said -"*As such, it is imperative that we assess the activities and roles of key microbial 'players' along the appropriate environmental scales and gradients catalysing ecological change… We emphasize the importance of assessing ecological change over a range of relevant time scales that vary from minutes to millennia and spatial scales that range from microscale aggregates to ocean basins.*"

In respect to these comments, this thesis demonstrates that the CFB must be a key player in the surface waters of the Southern Ocean, furthermore the ecology of the group is shaped by the Southern Ocean itself. The study was performed over wide spatial scales. Importantly, in order to be able to assess "change" over the long term (e.g. global warming) or assess "impact" (e.g. eutrophication) baseline ecological knowledge needs to be present so that such changes and impacts can be discerned in

the first place. Predictions on the ramifications of such ecosystem alterations can be then made. A benefit of this body of work is that it adds to the baseline knowledge of microbial populations in the Southern Ocean, linking diversity and abundance with various key environmental parameters. Examination of the role of the *Flavobacteria* group using microcosm experiments demonstrated that this group plays a significant role in silica mineralisation. This is further supported by the molecular studies in chapter 3 which demonstrated that the majority of *Flavobacterial* phylotypes uncovered were associated with both the planktonic and attached communities, and as such are likely to be playing a significant role in biogeochemical cycles.

Fundamentally, from the work in this thesis it can be said that 1) CFB members are common in Southern Ocean waters; 2) class *Flavobacteria* lineages predominate; 3) communities are structured between the various Southern Ocean waters masses with the colder PFZ and AZ waters supporting a more diverse community, compared to warmer, nutrient-limited waters of the TZ and SAZ; and finally 4) *Flavobacteria* showing rapid response to living surfaces and nutrients could be identified out of the diversity pool revealed by different approaches.

Problems were encountered using the FISH technique due to the highly oligotrophic nature of the Southern Ocean, however in the thesis the real-time PCR assay was found to be effective for estimating relative abundance in seawater filter fractions (Chapter 2). These analyses clearly demonstrated the *Flavobacteria* represented a significant proportion of the bacterioplankton, especially in the AZ. The partitioning of samples into particle-associated and planktonic fractions also highlighted the interaction that members of the CFB have with particulate matter in the Southern Ocean. Data from the mesocosm experiments indeed suggested the fractionation was very effective in separating planktonic and particle-associated

populations (see Fig. 5.3). DGGE analysis also provided insight into the community relationships at different scales. For example at close scales (particle vs. planktonic) results suggested no community patterns could be discerned as suggested by (Long and Azam 2001a). At broader scales (Southern Ocean zones) diversity patterns became more apparent (Fig. 3.2). Planktonic populations of *Flavobacteria* were relatively constant, however particle-associated *Flavobacteria* abundances were considerably more variable (Fig. 2.5B). The particle-associated abundance, however correlated to some extent with a variety of factors including chlorophyll *a* and inorganic nutrient (nitrate, phosphate and silica) concentrations, as well as clade-level diversity along a latitudinal transect of the Southern Ocean. The transect sampling regime was an interesting strategy as it essentially covered the entire breadth of the oceanographic variation presented by the Southern Ocean. More samples would be useful in proving the robustness of the correlations made in the latitudinal analysis, however the results are compelling enough to indicate potential relationships the CFB have with different nutrient regimes in the Southern Ocean water masses. This links in with strong evidence presented in Chapter 3 that major differences occur between TZ/SAZ and PFZ/AZ *Flavobacteria* communities (Fig. 3.2). The results support analogous biogeographic data for different *Roseobacter* clades in the Southern Ocean (Selje et al. 2003). The cold, more nutrient replete and productive AZ water samples support a richer *Flavobacteria* diversity than the warmer, nutrient-limited waters of the SAZ during the austral summer. DGGE analysis, with the use of specific primers, was also able to highly resolve the biogeography of class *Flavobacteria* in the Southern Ocean, identifying the diversity in AZ waters as well as indicating that many of the clades found are present in other oceanic regions across Earth. This included several uncultured clades (I-VIII, Agg58 cluster, Fig 3.3 and 3.4). AZ/PFZ clades

appear in some ways "endemic", though it is possible that the same species could be present in Arctic Ocean waters. Direct evidence is lacking for this at this time. In any case, the adaptation to an existence in marine environments as well as possessing psychrophilic adaptations is highly evolved in the CFB phylum and appears concentrated in class *Flavobacteria*. This was evident in that most isolates and clones discovered grouped in the marine clade of the family *Flavobacteriaceae* (Fig. 1.2). In the end the use of PCR-DGGE in this study provided an insight into the biogeography of *Flavobacteria* seawater communities at a relatively high resolution over a wide area. This study emphasised the power of the DGGE approach, the use of specific primers could be readily extended to study the abundance and biogeographic relationships of specific *Flavobacteria* clades and other bacterial groups.

The direct isolation of bacterial species using culture media also revealed significant diversity of the *Flavobacteria* but also starkly highlighted the limitations in traditional agar-based isolation techniques (Chapter 4). The cultivation effort however, was able to identify several new genera as well as numerous species of the genera *Psychroserpens*, *Polaribacter* and *Tenacibaculum*. Proposals were made to add some of these new taxa to the bacterial nomenclature and included the description of the genera *Gelidimarimicrobium*, *Calefactosor and Maricorpusculum.* Access to additional novel *Flavobacteria* may be facilitated by the use of high throughput and alternative cultivation techniques (Connon and Giovannoni. 2002; Hahn et al. 2004) using seawater alone as the primary growth media. This is necessary as many *Flavobacteria* clades identified from the molecular surveys (Chapter 3) simply were not readily cultivable on standard organic media such as marine 2216 agar and the more dilute Seawater Nutrient agar. Indeed, it would be useful to use alternative cultivation methods to isolate and describe members of abundant but uncultivated

clades such as the agg58 (O'Sullivan et al. 2004) and DE clusters 2 (Kirchman et al. 2003) which are cosmopolitan in the world's oceans. Other approaches could also be used, for exampled adaptations of the concept advanced by Kaeberlein et al. (2002). In this case, easy to culture but also numerically dominant clades, most obviously genus *Polaribacter,* could be used as a primary culture inoculum to see if uncultured *Flavobacteria* clades are supported by exuded bacterial growth factors either as satellite colonies or supported by supernatant extracts. Alternatively, the same factors could be studied if these organic factors have an opposite, antagonistic effect.

Analysis of the composition of Southern Ocean *Flavobacteria* communities that colonised diatom detritus in a series of mesocosm experiments (Chapter 5) demonstrated a significant difference in diversity between samples (Fig. 5.3 and 5.4). Furthermore, the phylogenetic affiliation of phylotypes detected on the diatom detritus exhibited high levels of similarity with cultivated taxa (Chapter 4). This congruency is in keeping with the idea that bacteria that colonise particulate matter are often easier to isolate as they grow rapidly responding rapidly to nutrient availability, are capable of forming discrete colonies (Kaeberlein et al. 2002), and compete effectively for living spaces (and potentially acting antagonistically in the process). Using these approaches, the hypothesis advanced at the end of chapter 5 is testable; determining whether uncultivated *Flavobacteria* are dominant due to nutrient supply being barely *sustainable*, at levels below which opportunistic species can effectively compete.

The mesocosm experiments demonstrated the differences in the dynamics of colonisation and dissolution of diatom aggregates between the samples investigated and also hinted at differences in the capacity for colonisation and activity of marine particles between oceanic regions. Further samples need to be studied in similar ways

to determine, more accurately, these interactive phenomena. Indeed, the use of mesocosm experiments to examine the role of marine *Flavobacteria* in nutrient cycling, and their activity on a range of marine particles, as well as the interaction between free living and particulate environments represents a useful future avenue of investigation. Information on the rates of colonisation and growth on marine particles will be essential to the understanding of rates of recycling and degradation of aggregates and as well as understanding influence of processes such as quorum sensing, antagonism, predation, chemotaxis and biofilm formation on biogeochemical cycling and secondary productivity.

References:

Acinas, S. G., Anton, J., and Rodriguez-Valera, F. (1999). Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. Applied and Environmental Microbiology **65**(2): 514-522

Alldredge, A. L. and. Cohen, Y. (1987). Can microscale chemical patches persist in the sea? Microelectrode study of marine snow fecal pellets. Science **235**: 689-691.

Alldredge, A. L. and Silver, W.M. (1988). Characteristics, dynamics and significance of marine snow. Progress in Oceanography **20**: 41-82.

Amann, R., Glöckner, F. O., and Neef, A. (1997). Modern methods in subsurface microbiology: in situ identification of microorganisms with nucleic acid probes. FEMS Microbiology Reviews **20:** 191-200.

Amann, R. Springer, N., Ludwig, W., Gortz, H. D. and Schleifer, K. H. (1991). Identification in situ and phylogeny of uncultured bacterial endosymbionts. Nature **351:** 161-164.

Atlas, R. M. and Bartha R. (1998). Microbial Ecology: Fundamentals and Applications, The Benjamin/Cummings Publishing Co. Inc.

Azam, F. (1998). Microbial control of oceanic carbon flux: The plot thickens. Science **280**: 694-696.

Bano, N. and Hollibaugh, J.T. (2002) Phylogenetic composition of bacterioplankton assemblages from the Arctic Ocean. Applied and Environmental Microbiology **68:** 505-518.

Bano, N., S. Ruffin, et al. (2004). Phylogenetic composition of Arctic Ocean archaeal assemblages and comparison with antarctic assemblages. Applied and Environmental Microbiology **70**(2): 781-789.

Barbieri, E., Riccioni, G., Pisano, A., Sisti, D., Zeppa, S. Agostini, D., and Stocchi, V. (2002). Competitive PCR for quantitation of a *Cytophaga-Flexibacter-Bacteroides* phylum bacterium associated with the *Tuber borchii* Vittad. mycelium. Applied and Environmental Microbiology **68:** 6421-6424

Beja, O., Suzuki, M.T., Koonin, E.V., Aravind, L., Hadd, A., Nguyen, L.P., Villacorta, R. Amjadi, M., Garrigues, C., Jovanovich, S.B., Feldman, R.A., and DeLong, E. (2000). Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. Environmental Microbiology **2:** 516-529.

Berman, T., Bechemin, C., and Maestrini, S. Y. (1999). Release of ammonium and urea from dissolved organic nitrogen in aquatic ecosystems. Aquatic Microbial Ecology **16**: 295-302.

Bernardet, J. F., P. Segers, P., Vancanneyt, M., Berthe, F., Kersters, K., and Vandamme, P. (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* Strohl and Tait 1978). International Journal of Systematic Bacteriology **46**: 128-148.

Bernardet, J. F., Nakagawa, Y. and Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. International Journal of Systematic and Evolutionary Microbiology **52**: 1049-1070.

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

Bidle, K. D. and Azam, F. (2001). Bacterial control of silicon regeneration from diatom detritus: Significance of bacterial ectohydrolases and species identity. Limnology and Oceanography **46**(7): 1606-1623.

Bidle, K. D., Manganelli, M. and Azam, F. (2002). Regulation of oceanic silicon and carbon preservation by temperature control on bacteria. Science **298**: 1980-1984.

Bidle, K. D., Brzezinski, M. A., Long, R. A., Jones, J. L., and Azam, F. (2003). Diminished efficiency in the oceanic silica pump caused by bacteria-mediated silica dissolution Limnology and Oceanography **48:** 1855-1868.

Bowman, J.P., McCammon, S.A., Brown, J.L., Nichols, P.D. and McMeekin, T.A. (1997a). *Psychroserpens burtonensis* gen. nov., sp. nov. *and Gelidibacter algens* gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. International Journal of Systematic Bacteriology **47:** 670-77.

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. Applied Environmental Microbiology **63:** 3068-3078.

Bowman, J. P. (1998). *Pseudoalteromonas prydzensis* sp. nov., a psychrotrophic, halotolerant bacterium from Antarctic sea ice. International Journal of Systematic Bacteriology **48:** 1037-1041.

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 (22:6ω3). International Journal of Systematic Bacteriology **48:** 1171-1180.

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. International Journal of Systematic Bacteriology **48:** 1213-122.

Bowman, J. P. (2000). Description of *Cellulophaga algicola* sp. nov., isolated from the surfaces of Antarctic algae, and reclassification of *Cytophaga uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Cellulophaga uliginosa* comb. nov. International Journal of Systematic and Evolutionary Microbiology **50**: 1861-1868.

Bowman, J. P., Mancuso Nichols, C. A., and Gibson, J. A. E. (2003a). *Algoriphagus ratkowskyi* gen. nov., sp. nov., *Brumimicrobium glaciale* gen. nov., sp. nov., *Cryomorpha ignava* gen. nov., sp. nov. and *Crocinitomix catalasitica* gen. nov., sp. nov., novel

flavobacteria isolated from various polar habitats. International Journal of Systematic Evolutionary Microbiology **53**: 1343-1355.

Bowman ,J.P., McCammon, S. A., Gibson, J. A. E., Nichols, P. D. and Robertson, L. (2003b). Microbial metabolic activity and community structure within Antarctic continental shelf sediment. Applied and Environmental Microbiology **69:** 2448-2462.

Bowman, J. P. and McCuaig, R. D. (2003c). Diversity and biogeography of prokaryotes dwelling in Antarctic continental shelf sediment. Applied Environmental Microbiology. **69:** 2463-2484.

Bowman, J. P. (2004). The Marine Clade of the Family *Flavobacteriaceae*: The Genera *Aequorivita*, *Arenibacter*, *Cellulophaga*, *Croceibacter*, *Formosa*, *Gelidibacter*, *Gillisia*, *Maribacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Robiginitalea*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter* and *Zobellia*. In; The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community. M. Dworkin. New York: New York. (http://141.150.157.117:8080/prokPUB/index.htm).

Brinkmeyer, R., Knittel, K., Jurgens, J., Weyland, H., Amann, R., and Helmke, E. (2003). Diversity and Structure of Bacterial Communities in Arctic versus Antarctic Pack Ice. Applied and Environmental Microbiology **69**: 6610-6619.

Brown, G.E. and Parks, G.A.. (2001) Sorption of trace elements on mineral surfaces: Modern perspectives from spectroscopic studies, and comments on sorption in the marine environment. International Geology Review **43:** 963-1073.

Brown, M. V. and Bowman, J. P. (2001). A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). FEMS Microbiology Ecology **35**(3): 267-275.

Brown, M. V. and Bowman, J. P. (2002). Microbial ecology of Southern Ocean sea-ice. In: Recent Advances in Microbiology, V. Asche (ed.). Melbourne: Australian Society for Microbiology, pp. 27-54.

Busse, H. J., Denner, E. B. M., Buczolits, S., Salkinoja-Salonen, M., Bennasar, A., and Kämpfer, P. (2003). *Sphingomonas aurantiaca* sp nov., *Sphingomonas aerolata* sp nov and *Sphingomonas faeni* sp nov., air- and dustborne and Antarctic, orange-pigmented, psychrotolerant bacteria, and emended description of the genus *Sphingomonas.* International Journal of Systematic and Evolutionary Microbiology **53:** 1253-1260.

Carmack, E. C. (1977). Water characteristics of the Southern Ocean south of the Polar Front. A Voyage of Discovery, George Deacon 70th Anniversary Volume (Suppl. to Deep-Sea Res). M. V. Angel (ed.). Oxford, Pergamon Press**:** 15-41.

Caron, D. A., Davis, P. G., Madin, L. P., and Sieburth, J. M. (1986). Enrichment of microbial-populations in macroaggregates (marine snow) from surface waters of the North-Atlantic. Journal of Marine Research **44**: 543-565.

Caron, D. A. (1987). Grazing of attached bacteria by heterotrophic microflagellates. Microbial Ecology **13**: 203-218.

Casamayor, E.O., Schafer, H., Baneras, L., Pedros-Alio, C., and Muyzer, G. (2000) Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: Comparison by microscopy and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology **66**: 499-508.

Cavalier-Smith, T. (2002). The neomuran origin of archaebacteria, the negibacterial root of the universal tree and bacterial megaclassification. International Journal of Systematic and Evolutionary Microbiology **52**: 7-76.

Cho, J.-C. and S. J. Giovannoni (2004). Cultivation and Growth Characteristics of a Diverse Group of Oligotrophic Marine Gammaproteobacteria. Appl. Environ. Microbiol. **70**(1): 432-440.

Christner, B. C., Mosley-Thompson, E., Thompson, L. G., and Reeve, J. N. (2001). Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. Environmental Microbiology **3:** 570-577.

Clarke, A. (1985). Energy flow in the Southern Ocean. Antarctic nutrient cycles and food webs. W. R. Siegfreid, Condy, P.R. & Laws, R.M. (eds.). Berlin, Springer-Verlag**:** 573-580.

Clarke, K.R. and Warwick, R.M. (2001). Change in Marine Communities: an Approach to Statistical Analysis and Interpretation, 2nd ed. Primer-E Ltd., Plymouth, UK. (http://www.pml.ac.uk/primer/).

Cole, J.J, Findlay, S., and Pace, M. L. (1988). Bacterial production in fresh and saltwater ecosystems - a cross-system overview. Marine Ecology Progess Series **43:** 1-10.

Connon, S. A. and Giovannoni, S. J. (2002). High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Applied and Environmental Microbiology **68:** 3878-3885.

Cottrell, M. T. and Kirchman, D.L. (2000). Natural assemblages of marine Proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high- molecular-weight dissolved organic matter. Applied and Environmental Microbiology **66**: 1692-1697.

Covert, J. S. and Moran, M. A. (2001). Molecular characterization of estuarine bacterial communities that use high- and low-molecular weight fractions of dissolved organic carbon Aquatic Microbial Ecology **25:** 127-139.

Deacon, G.E.R. (1984) The Antarctic Circumpolar Ocean. Cambridge University Press, Cambridge.

de Baar, H.J.W., Buma, A.G.J., Nolting, R.F., Cadee, G.C., Jacques, G., and Treguer, P.J. (1990) On iron limitation of the southern-ocean - experimental-observations in the Weddell and Scotia Seas. Marine Ecology Progress Series **65:** 105-122.

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

DeLong, E. F., Taylor, L. T., Marsh, T. L., and Preston, C. M. (1999). Visualization and enumeration of marine planktonic Archaea and Bacteria by using polyribonucleotide probes and fluorescent in situ hybridisation. Applied and Environmental Microbiology **65:** 5554-5563.

Denner, E. B. M., Vybiral, D., Koblizek, M., Kämpfer, P., Busse, H. J., and Velimirov, B. (2002). *Erythrobacter citreus* sp nov., a yellow-pigmented bacterium that lacks bacteriochlorophyll *a*, isolated from the western Mediterranean Sea. International Journal of Systematic and Evolutionary Microbiology **52:** 1655-1661.

Egan, S., James, S., Holmstrom, C., and Kjelleberg S. (2002). Correlation between pigmentation and antifouling compounds produced by *Pseudoalteromonas tunicata* Environmental Microbiology **4:** 433-442.

Eiler, A., Langenheder, S., Bertilsson, S., and Tranvik, L. J. (2003). Heterotrophic bacterial growth efficiency and community structure at different natural organic carbon concentrations. Applied and Environmental Microbiology **69:** 3701-3709.

Eilers, H., Pernthaler, J., Glöckner, F. O., and Amann, R. (2000). Culturability and in situ abundance of pelagic bacteria from the North Sea. Applied and Environmental Microbiology. **66:** 3044-3051.

Esper, O. and Zonneveld, K. A. F. (2002). Distribution of organic-walled dinoflagellate cysts in surface sediments of the Southern Ocean (eastern Atlantic sector) between the Subtropical Front and the Weddell Gyre. Marine Micropalaeontology 46: 177-208.

Fandino, L. B., Riemann, L., Steward, G. F., Long, R. A., and Azam, F. (2001). Variations in bacterial community structure during a dinoflagellate bloom analysed by DGGE and 16S rDNA sequencing. Aquatic Microbial Ecology **23:** 119-130.

Felsenstein, J. (1993). PHYLIP, phylogenetic inference package. (http://evolution.genetics. washington.edu/phylip.html).

Fenchel, T. (1988). Marine plankton food-chains. Annual Review of Ecology and Systematics **19**: 19-38.

Ferris, M. J., Muyzer, G., and Ward, D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Applied and Environmental Microbiology **62:** 340-346.

Fowler, S. W. and Knauer, G.A. (1986). Role of large particles in the transport of elements and organic compounds through the ocean water column. Progress in Oceanography **16**: 147-194.

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

Garrity, G. M. and. Holt, J.G. (2001). A road map to the manual. <u>Bergey's Manual of Systematic Bacteriology</u>, Vol. 1, 2nd ed. D. R. Boone, Castenholtz, R.W., and Garrity, G.M. (eds.). New York, Springer, pp. 119-155.

Giovannoni, S. J., and Rappé, M.S. (2000). Evolution, diversity and molecular ecology of marine prokaryotes. <u>Microbial Ecology of the Oceans</u>. D. L. Kirchman, Wiley and Sons**:** 47-84.

Glöckner, F. O., Fuchs, B.M., and Amann, R. (1999). Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. <u>Applied and Environmental Microbiology</u> **65**: 3721-3726.

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 g*roup and reclassification of *"Flectobacillus glomeratus"* as *Polaribacter glomeratus* comb. nov. <u>International Journal of Systematic Bacteriology</u> **48:** 223-235.

Gram, L., Grossart, H. P., Schlingoff, A., and Kiorboe, T. (2002). Possible quorum sensing in marine snow bacteria: Production of acylated homoserine lactones by *Roseobacter* strains isolated from marine snow. <u>Applied and Environmental Microbiology</u> **68**: 4111-4116.

Graßhoff, K. (1964). On the determination of silica in seawater. <u>Deep Sea Research</u> **11:** 597-604.

Grossart, H. P. and Simon, M. (1998). Bacterial colonization and microbial decomposition of limnetic organic aggregates (lake snow). <u>Aquatic Microbial Ecology</u> **15**: 127-140.

Grossart, H. P. and Ploug, H. (2000). Bacterial production and growth efficiencies: Direct measurements on riverine aggregates. <u>Limnology and Oceanography</u> **45**: 436-445.

Grossart, H. P., Riemann, L., and Azam, F. (2001). Bacterial motility in the sea and its ecological implications. <u>Aquatic Microbial Ecology</u> **25:** 247-258.

Hahn, M. W., Stadler, P., Wu, Q. L., and Pockl, M. (2004). The filtration-acclimatization method for isolation of an important fraction of the not readily cultivable bacteria <u>Journal of Microbiological Methods</u> **57:** 379-390.

Hall, T.A. (1999). A user-friendly biological sequence alignment editor for Windows 95/98/NT. <u>Nucleic Acids Symposium Series</u> **41:** 95-98.

Hansen, G. H., Bergh, O., Michaelsen, J. and Knappskog, D. (1992). *Flexibacter-ovolyticus* sp-nov, a pathogen of eggs and larvae of atlantic halibut, *Hippoglossus-hippoglossus* L. <u>International Journal of Systematic Bacteriology</u> **42:** 451-458.

Hence, I., Zimmermann, R., Beckman, A., and Batman, U.V. (2003) Regional ecosystem dynamics in the ACC: simulations with a three-dimensional ocean-plankton model. <u>Journal of Marine Systems</u> **42:** 31-51.

Hermansson, A. and Lindgren, P. E. (2001). Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. Applied and Environmental Microbiology **67:** 972-976.

Herndl, G. J., Mullerniklas, G., and Frick, J. (1993). Major role of ultraviolet-B in controlling bacterioplankton growth in the surface-layer of the ocean. Nature **361**: 717-719.

Heukelekian, H. and Heller, A. (1940) Relation between food concentration and surface for bacterial growth. Journal of Bacteriology **40:** 547–558.

Heuser, T. and Zimmer, W. (2003). Genus- and isolate-specific real-time PCR quantification of *Erwinia* on leaf surfaces of English oaks (*Quercus robur* L.). Current Microbiology. **47:** 214-219.

Hold, G. L., Smith, E. A., Rappé, M. S., Maas, E. W., Moore, E. R. B., Stroempl, C., Stephen, J. R., Prosser, J. I., Birkbeck, T. H. and Gallacher, S. (2001). Characterisation of bacterial communities associated with toxic and non-toxic dinoflagellates: *Alexandrium* spp. and *Scrippsiella trochoidea*. FEMS Microbiology Ecology **37**: 161-173.

Hollibaugh, J. T., Wong, P.S., and Murrell, M.C. (2000). Similarity of particle-associated and free-living bacterial communities in northern San Francisco Bay, California. Aquatic Microbial Ecology **21**: 103-114.

Horner-Devine, C. M., Leibold, M.A., Smith, V.H., and Bohannan, B.J.M. (2003) Bacterial diversity patterns along a gradient of primary productivity. Ecology Letters **6:** 613-622.

Huß, V. A. R., Festl, H. and Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridisation from renaturation rates. Systematic and Applied Microbiology **4:** 184-192.

Karl, D. M. (1992). Microbial processes in the Southern Oceans. Antarctic Microbiology. New York, Wiley-Liss**,** pp. 1-63.

Jette, J. F. and Ziomek, E. (1994). Determination of lipase activity by a rhodamine-triglyceride-agarose assay. Analytical Biochemistry **219:** 256-260.

Johansen, J. E., Nielsen, P., and Sjoholm, C. (1999). Description of *Cellulophaga baltica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen., nov., sp. nov. and reclassification of *[Cytophaga] lytica* to *Cellulophaga lytica* gen. nov., sp. nov. International Journal of Systematic and Evolutionary Microbiology **49**: 1231-1240.

Joint, I., Henriksen, P., Fonnes, G. A., Bourne, D., Thingstad, T. F., and Riemann, B. (2002). Competition for inorganic nutrients between phytoplankton and bacterioplankton in nutrient manipulated mesocosms. Aquatic Microbial Ecology **29:** 145-159.

Junge, K., Eicken H., and Deming, J. W. (2003). Motility of *Colwellia psychrerythraea* strain 34H at subzero temperatures. Applied and Environmental Microbiology **69:** 4282-4284

Kaeberlein, T., Lewis, K. and Epstein, S. S. (2002). Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science **296:** 1127-1129.

Kämpfer, P., Rainey, F. A., Andersson, M. A, Lassila, E. L. N., Ulrych, U., Busse, H. J., Weiss, N., Mikkola, R., and Salkinoja-Salonen, M. (2000). *Frigoribacterium faeni* gen. nov., sp nov., a novel psychrophilic genus of the family *Microbacteriaceae*. International Journal of Systematic and Evolutionary Microbiology **50:** 355-363.

Karner, M. and Fuhrman, J. A. (1997). Determination of active marine bacterioplankton: A comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Applied and Environmental Microbiology **63:** 1208-1213.

Karner, M. B., DeLong, E. F. and Karl, D. M. (2001). Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature 409: 507-510.

Kaye, J. Z. and Baross, J. A. (2000). High incidence of halotolerant bacteria in Pacific hydrothermal-vent and pelagic environments. FEMS Microbiology Ecology **32:** 249-260.

Kiorboe, T., Hansen, J.L.S., Alldredge, A.L., Jackson, G.A., Passow, U., Dam, H.G., Drapeau, D.T., Waite, A., and Garcia, C.M. (1996). Sedimentation of phytoplankton during a diatom bloom: Rates and mechanics. Journal of Marine Research **54**: 1123-1148.

Kiorboe, T. (2001). Formation and fate of marine snow: small-scale processes with large-scale implications. Scientia Marina **65**: 57-71.

Kiorboe, T., Grossart, H. P., Ploug, H., and Tang, K. (2002). Mechanisms and rates of bacterial colonization of sinking aggregates. Applied and Environmental Microbiology **68**: 3996-4006.

Kirchman, D. L. (ed.). (2000). Microbial Ecology of the Oceans. New York, Wiley-Liss.

Kirchman, D. L. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. FEMS Microbiology Ecology **1317**: 1-10.

Kirchman, D. L., Yu, L.Y., and Cottrell, M.T. (2003) Diversity and abundance of uncultured *Cytophaga*-like bacteria in the Delaware Estuary. Applied and Environmental Microbiology **69:** 6587-6596.

Kirchman, D. L, Dittel, A. I., Findlay, S. E. G., and Fischer, D. (2004). Changes in bacterial activity and community structure in response to dissolved organic matter in the Hudson River, New York. Aquatic Microbial Ecology **35**: 243-257.

Kisand, V., Cuadros, R., and Wikner, J. (2002). Phylogeny of culturable estuarine bacteria catabolizing riverine organic matter in the northern Baltic Sea. Applied and Environmental Microbiology **68:** 379-388.

Klappenbach, J. A., Dubar, J. M. and Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of bacteria. Applied and Environmental Microbiology **66:**1328-1333.

Kolter, R., Siegele, D. A., and Tormo, A. (1993). The stationary phase of the bacterial life cycle. Annual Review of Microbiology **47:** 855-874.

Kopczynska, E. E., Dehairs, F., Elskens, M., and Wright, S. (2001) Phytoplankton and microzooplankton variability between the Subtropical and Polar Fronts south of Australia: Thriving under regenerative and new production in late summer. Journal of Geophysical Research-Oceans **106:** 31597-31609.

Labrenz, M., Collins, M. D., Lawson, P. A., Tindall, B. J., Schumann, P., and Hirsch, P. (1999). *Roseovarius tolerans* gen. nov., sp. nov., a budding bacterium with variable bacteriochlorophyll a production from hypersaline Ekho Lake. International Journal of Systematic Bacteriology **49:** 137-147

Labrenz, M., B. Tindall, et al. (2000). Staleya guttiformis gen. nov., sp. nov. and Sulfitobacter brevis sp. nov., alpha-3-Proteobacteria from hypersaline, heliothermal and meromictic antarctic Ekho Lake. Int J Syst Evol Microbiol **50**(1): 303-313.

Lane, D.J. (1991). 16S/23S rRNA sequencing, In: Nucleic Acid Techniques in Bacterial Systematics. E. Stackebrandt and M. Goodfellow (eds.). New York: John Wiley & Sons, pp. 115-175.

Laws, R. M. (1985). Introduction: Antarctic biology. Antarctic Nutrient Cycles and Food Webs. Berlin, Springer-Verlag, pp. 1-3.

Lebaron, P., Servais, P., Troussellier, M., Courties, C., Vives-Rego, J., Muyzer, G., Bernard, L., Guindulain, T., Schafer, H., and Stackebrandt E (1999). Changes in bacterial community structure in seawater mesocosms differing in their nutrient status. Aquatic Microbial Ecology **19**: 255-267.

Leifson, E. (1963). Determination of carbohydrate metabolism of marine bacteria. Journal of Bacteriology **85:** 1183-1184.

Llobet-Brossa, E., Rossello-Mora, R., and Amann, R. (1998) Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Applied and Environmental Microbiology **64:** 2691-2696.

Long, R. A. and F. Azam (2001a). Microscale patchiness of bacterioplankton assemblage richness in seawater. Aquatic Microbial Ecology **26**: 103-113.

Long, R. A. and Azam, F. (2001b) Antagonistic interactions among marine pelagic bacteria Applied and Environmental Microbiology **67:** 4975-4983.

Lopez-Garcia, P., A. Lopez-Lopez, A., Moreira, D., and Rodruguez-Valera, F. (2001). Diversity of free-living prokaryotes from a deep-sea site at the Antarctic Polar Front. FEMS Microbiology Ecology **36**: 193-202.

MacDonald, R. and Brozel, V. S. (2000). Community analysis of bacterial biofilms in a simulated recirculating cooling-water system by fluorescent in situ hybridisation with rRNA-targeted oligonucleotide probes. Water Research **34:** 2439-2446.

Malinen, E., Kassinen, A., Rinttila, T. and Palva, A. (2003). Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridisation with rDNA-targeted

oligonucleotide probes in quantification of selected faecal bacteria. Microbiology – SGM **149:** 269-277.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K. H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria – problems and solutions. Systematic and Applied Microbiology **15:** 593-600.

Marmur, J. and Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. Journal of Molecular Biology **5:** 109-118.

Meiners, K., Brinkmeyer, R., Granskog, M. A., and Lindfors, A. (2004). Abundance, size distribution and bacterial colonization of exopolymer particles in Antarctic sea ice (Bellingshausen Sea). Aquatic Microbial Ecology **35:** 283-296.

Mergaert, J., Verhelst, A., Cnockaert, M. C., Tan, T. L., and Swings, J. (2001). Characterization of facultative oligotrophic bacteria from polar seas by analysis of their fatty acids and 16S rDNA sequences. Systematic and Applied Microbiology **24:** 98-107.

Mittelbach, G.G., Steiner, C.F., Steiner, S.M., Gross, K.L., Reynolds, H.L., Waide, R.B., Willig, M.R., Dodson, S.I., and Gough, L. (2001) What is the observed relationship between species richness and productivity? Ecology **82:** 2381-2396.

Murray, A. E., C. M. Preston, et al. (1998). Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. Applied and Environmental Microbiology **64**(7): 2585-2595.

Murray, A. E., Wu, K.Y., Moyer, C.L., Karl, D.M. and DeLong, E.F. (1999) Evidence for circumpolar distribution of planktonic Archaea in the Southern Ocean. Aquatic Microbial Ecology **18:** 263-273.

Muyzer, G., Dewaal, E. C., and Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S ribosomal RNA. Applied and Environmental Microbiology **59:** 695-700.

Newby, D. T., Hadfield, T. L., and Roberto, F. F. (2003). Real-time PCR detection of *Brucella abortus*: a comparative study of SYBR green 1,5'-exonuclease, and hybridisation-probe assays. Applied and Environmental Microbiology **69:** 4753-4759.

Nichols, P. D., Guckert, J. B., and White, D. C. (1986). Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulfide adducts. Journal of Microbiological Methods **5:** 49-55.

Orsi, A. H., Johnson, G.C., and Bullister, J. L. (1999). Circulation, mixing, and production of Antarctic Bottom Water. Progress in Oceanography **43**: 55-109.

O'Mahony, J. and Hill, C. (2002) A real-time PCR assay for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* using SYBR Green and the Light Cycler. Journal of Microbiological Methods 51: 283-293.

O'Sullivan, L.A., Fuller, K.E., Thomas, E.M., Turley, C.M., Fry, J.C., and Weightman, A.J. (2004). Distribution and culturability of the uncultivated `AGG58 cluster' of the *Bacteroidetes* phylum in aquatic environments. FEMS Microbiology Ecology **47:** 359-370.

Ouverney, C.C. and J. A. Fuhrman. (1997) Increase in fluorescence intensity of 16S rRNA in situ hybridization in natural samples treated with chloramphenicol. Applied and Environmental Microbiology **63:** 2735-2740.

Paerl, H. W. (1994). Spatial segregation of CO_2 fixation in *Trichodesmium* spp - linkage to N_2 fixation potential. Journal of Phycology **30**: 790-799.

Paerl, H. W. and Steppe, T. F. (2003). Scaling up: the next challenge in environmental microbiology. Environmental Microbiology **5:** 1025-1038.

Passow, U. and Alldredge, A. L. (1994). Distribution, size and bacterial-colonization of transparent exopolymer particles (TEP) in the Ocean. Marine Ecology-Progress Series **113**: 185-198.

Patel, P., M. E. Callow, et al. (2003). Specificity in the settlement - modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha*. Environ Microbiol **5**(5): 338-349.

Pernthaler, J., Alfreider, A., Posch, T., Andreatta, S., and Psenner, R. (1997). In situ classification and image cytometry of pelagic bacteria from a high mountain lake (Gossenkollesee, Austria). Applied and Environmental Microbiology **63**(12): 4778-4783.

Pernthaler, A., Pernthaler, J., and Amann, R. (2002). Fluorescence In Situ Hybridization and Catalyzed Reporter Deposition for the Identification of Marine Bacteria. Applied and Environmental Microbiology **68** (6): 3094-3101.

Pinhassi, J., Azam, F., Hemphala, J., Long, R. A., Martinez, J., Zweifel, U. L., and Hagstrom A. (1999). Coupling between bacterioplankton species composition, population dynamics, and organic matter degradation. Aquatic Microbial Ecology **17:** 13-26

Ploug, H., Grossart, H. P., Azam, F., and Jorgensen, B. B. (1999) Photosynthesis, respiration, and carbon turnover in sinking marine snow from surface waters of Southern California Bight: implications for the carbon cycle in the ocean. Marine Ecology Progess Series **179**: 1-11.

Powell, S.M., Bowman, J.P., Snape, I., and Stark, J.S. (2003). Microbial community variation in pristine and polluted nearshore Antarctic sediments. FEMS Microbiology Ecology **45**: 135-145.

Prieto, L., Ruiz, J., Echevarria, F., Garcia, C. M., Bartual, A., Galvez, J. A., Corzo, A., and Macias, D. (2002). Scales and processes in the aggregation of diatom blooms: high time resolution and wide size range records in a mesocosm study. Deep-Sea Research Part I-Oceanographic Research Papers **49:** 1233-1253.

Radok, U., Streten, N., and Weller, G. (1975). Atmosphere and ice. Oceanus **18**(4): 15-27.

Rantokokko-Jalava, K. and Jalava, J. (2001). Development of conventional and real-time PCR assays for detection of *Legionella* DNA in respiratory specimens. Journal of Clinical Microbiology **39**: 2904-2910.

Rappé, M. S., Connon, S. A., Vergin, K. L., and Giovannoni, S. J. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature **418:** 630-633.

Rappé, M. S. and Giovannoni, S. J. (2003). The uncultured microbial majority. Annual Review of Microbiology **57:** 369-394 2003

Rath, J., Wo, K.Y., Herndl, G.J., and DeLong, E.F. (1998). High phylogenetic diversity in a marine-snow-associated bacterial assemblage. Aquatic Microbial Ecology **14**: 261-269.

Riechenbach, H. (1992). The Order Cytophagales. In: The Prokaryotes, vol. 3. Balloes, A., Trüper, H. G., Dworkin, M., Harden, W., and Schleifer, K. H. (eds.). Berlin: Springer-Verlag, pp. 3631-3675.

Riemann, L., Steward, G. F., and Azam, F. (2000). Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. Applied and Environmental Microbiology **66:** 578-587.

Riemann, L. and A. Winding (2001). Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. Microbial Ecology **42**: 274-285.

Rintoul, S. R. (1998). On the origin and influence of Adelie Land Bottom Water. Ocean, Ice, and Atmosphere : Interactions at the Antarctic Continental Margin. **75:** 151-171.

Romanenko, L. A., Schumann, P., Rohde, M., Mikhailov, V. V., and Stackebrandt, E. (2002). *Psychrobacter submarinus* sp nov and *Psychrobacter marincola* sp nov., psychrophilic halophiles from marine environments. International Journal of Systematic and Evolutionary. Microbiology **52:** 1291-1297.

Roszak, D. B. and Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. Microbiological Reviews **51:** 365-379.

Schafer, H., Bernard, L., Courties, C., Lebaron, P., Servais, P., Pukall, R., Stackebrandt, E., Trousellier, M., Guindulain, T., Vives-Rego, J., and Muyzer, G. (2001). Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: changes in the genetic diversity of bacterial populations. FEMS Microbiology Ecology **34:** 243-253.

Schafer, H., Abbas, B., Witte, H. and Muyzer, G. (2002). Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms. FEMS Microbiology Ecology **42:** 25-35.

Schiffman, S.S. and Reynolds M.L. (1981) Introduction to Multidimensional Scaling. Theory, Methods and Applications. Academic Press, London, UK.

Selje, N., Simon, M., and Brinkhoff, T. (2004). A newly discovered *Roseobacter* cluster in temperate and polar oceans. Nature 427: 445-448.

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

Skerratt, J. H., Nichols, P. D., Bowman, J. P., and Sly, L. I. (1992). Occurrence and significance of long-chain (omega-1)-hydroxy fatty-acids in methane-utilizing bacteria . Organic Geochemistry **18:** 189-194.

Sly, L. I., Blackall, L. L., Kraat, P. C., Tian-Shen, T., and Sangkhobol, V. (1986). The use of second derivative plots for the determination of mol% guanine plus cytosine of DNA by the thermal denaturation method. Journal of Microbiological Methods 5: 139-156.

Smibert, R. M. and Krieg, N. R. (1994). Phenotypic characterization. In: Methods for General and Molecular Bacteriology. Gerhardt, P. (ed.). Washington, D.C.: American Society for Microbiology, Inc., pp. 607-654.

Smith, D. C., Simon, M., Alldredge, A.L., and Azam, F. (1992). Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature **359**: 139-142.

Sorokin, D. Y. (1995). *Sulfitobacter pontiacus* gen. nov., sp. nov.— a new heterotrophic bacterium from the Black Sea, specialized on sulfite oxidation. Mikrobiologiya **64**: 354-365.

Speksnijder, A. G. C. L., Kowalchuk, G. A., De Jong, S., Kline, E., Stephen, J. R., and Laanbroek, H. J. (2001). Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Applied and Environmental Microbiology **67:** 469-472.

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

Suzuki, M. T., Rappé, M. S., Haimberger, Z. W., Winfield, H., Adair, N., Strobel, J., and Giovannoni, S. J. (1997). Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Applied and Environmental Microbiology **63:** 983-989.

Suzuki, M., Nakagawa, Y., Harayama, S., and Yamamoto, S. (2001). Phylogenetic analysis and taxonomic study of marine *Cytophaga*-like bacteria: proposal for *Tenacibaculum* gen. nov with *Tenacibaculum maritimum* comb. nov and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp nov and *Tenacibaculum amylolyticum* sp nov. International Journal of Systematic and Evolutionary Microbiology **51:** 1639-1652.

Suzuki, M. T., Preston, C. M., Chavez, F. P. and DeLong, E. F. (2001). Quantitative mapping of bacterioplankton populations in seawater: field tests across an upwelling plume in Monterey Bay. Aquatic Microbial Ecology **24:** 117-127.

Thompson, J. D., Higgins, D.G., and Gibson, T.J. (1994). Clustal-W - improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22:** 4673-4680.

Timmermans, K.R., van Leeuwen, M.A., de Jong, J.T.M., McKay, R.M.L., Nolting, R.F., Witte, H.J., van Ooyen, J., Swagerman, M.J.W., Kloosterhuis, H., and de Baar, H.J.W. (1998) Iron stress in the Pacific region of the Southern Ocean: evidence from enrichment bioassays. Marine Ecology Progess Series **166:** 27-41.

Tomczak, M. and Godfrey, J.S. (1994) Regional Oceanography: an Introduction. New York, Elsevier Scence.
(Available online: http://www.es.flinders.edu.au/%7Emattom/regoc/pdfversion.html)

Trull, T., Rintoul, S. R., Hadfield, M., and Abraham, E. R. (2001). Circulation and seasonal evolution of polar waters south of Australia: Implications for iron fertilization of the Southern Ocean. Deep-Sea Research Part II-Topical Studies in Oceanography **48**: 2439-2466.

Turley, C. M. and Mackie, P. J. (1994). Biogeochemical significance of attached and free-living bacteria and the flux of particles in the North Atlantic Ocean. Marine Ecology-Progress Series **115**: 191-203.

Van Hannen, E. J., Mooij, W. M., van Agterveld, M. P., Gons, H. J., and Laanbroek, H. J. (1999). Detritus-dependent development of the microbial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. Applied and Environmental Microbiology **65:** 2478-2484.

Van Trappen, S., Tan, T. L., Yang, J., Mergaert, J., and Swings, J. (2004). *Glaciecola polaris* sp. nov., a novel budding and prosthecate bacterium from the Arctic Ocean, and emended description of the genus *Glaciecola***.** International Journal of Systematic and Evolutionary Microbiology **54:** 1765 - 1771.

Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, Al. L., Rusch, D., Eisen, J. A., Wu, D. Y., Paulsen, I., Nelson, K. E., Nelson, W., Fouts, D. E., Levy, S., Knap, A. H., Lomas, M. W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y. H., and Smith, H. O. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. Science **304:** 66-74.

von Wintzingerode, F., Göbel, U. B. and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews **21**: 213-229.

Ward, P., Whitehouse, M., Brandon, M., Shreeve, R., and Woodd-Walker, R. (2003). Mesozooplankton community structure across the Antarctic Circumpolar Current to the north of South Georgia: Southern Ocean. Marine Biology 143: 121-130.

Webster, N.S., Negri, A.P., Munro, M.M.H.G., and Battershill, C.N. (2004) Diverse microbial communities inhabit Antarctic sponges. Environmental Microbiology **6:** 288-300.

Weiner, R. M., Melick, M., O'Neill, K., and Quintero, E. (2000). *Hyphomonas adhaerens* sp nov., *Hyphomonas johnsonii* sp nov and *Hyphomonas rosenbergii* sp nov., marine budding and prosthecate bacteria. International Journal of Systematic and Evolutionary Microbiology **50:** 459-469.

Weller, R., Glöckner, F.O., and Amann, R. (2000) 16S rRNA-targeted oligonucleotide probes for the in situ detection of members of the phylum *Cytophaga- Flavobacterium-Bacteroides*. Systematic and Applied Microbiology **23:** 107-114.

Wells, L. E. and Deming, J. W. (2003). Abundance of bacteria, the *Cytophaga-Flavobacterium* cluster and Archaea in cold oligotrophic waters and nepheloid layers of the Northwest Passage, Canadian Archipelago. Aquatic Microbial Ecology **31:** 19-31.

White, D. C., Davis, W. M., Nickels, J. S., King, J. D. and Robbie, R. J. (1979). Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia **40:** 51-62

Yoon, J. H., Kim, I. G., Kang, K. H., Oh T. K. and Park, Y. H. (2003). *Bacillus marisflavi* sp nov and *Bacillus aquimaris* sp nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. International Journal of Systematic and Evolutionary Microbiology **53:** 1297-1303.

Yoon, J. H., Kim, I. G., Kang, K. H., Oh T. K. and Park, Y. H. (2004). *Nocardioides aquiterrae* sp nov., isolated from groundwater in Korea. International Journal of Systematic and Evolutionary Microbiology **54:** 71-75.

Zengler, K., G. Toledo, et al. (2002). Cultivating the uncultured. PNAS **99**(24): 15681-15686.

Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R., and Burkill, P.H. (2002) A population of the alpha-proteobacteria dominates the bacterioplankton and dimethylsulphoniopropionate uptake after an algal bloom in the North Sea. Deep-Sea Research II, Topical Studies in Oceanography **49:** 3017-3038.

Zwally, H. J., Comiso, J.C., Parkinson, C.L., Campbell, W.J., Carsey, F.D., and Gloersen, P. (1983). Antarctic Sea Ice, 1973-1976. In: Satellite Passive-Microwave Observations. Washington, D.C.: NASA**,** pp. 206.

APPENDIX

CULTURE MEDIA:

Seawater Nutrient Medium

0.05 g Bacteriological Peptone

0.05 g Tryptone

0.05 g Yeast Extract

0.05 g soluble starch

0.02 g Sodium pyruvate

1 ml trace element solution

12 g Agar

1000 ml artificial seawater

The medium is autoclaved (121°C , 15 min), cooled to approximately 50°C. 0.1 ml Solution A, 2.5 ml Solution B and 0.1 ml solution C are added before pouring plates.

Trace metal solution

10 g nitriloacetate

14.45 g $MgSO_4.7H_2O$

3.335 g $CaCl_2.2H_2O$

10 mg $NH_4MoO2.2H_2O$

0.1 g $FeSO_4.7H_2O$

1000 ml distilled water

pH 6.8-7.0

Solution A

0.1 ml 1 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.5). Sterilized by autoclaving (121°C, 15 min.)

Solution B:

20 g D-glucose in 100 ml distilled water, Sterilized by filtration through a 0.2 μm filter

Solution C: vitamin solution

5 mg *p*-aminobenzoate

2 mg folate

2 mg biotin

5 mg nicotinate

5 mg calcium pantothenate

5 mg riboflavin

5 mg thiamine HCl

10 mg pyrodoxine HCl

0.1 mg cyanocobalamin

5 mg of thioctate

100 ml distilled water, pH 7.0. Sterilized by filtration through a 0.2 μm filter.

Marine Agar

5 g Bacteriological Peptone

2.5 g Yeast extract

15 g agar

1000 ml artificial seawater

SFA Media:

1 μM NH$_4$Cl

0.1 μM KH$_2$PO$_4$

12 g Agar

1000 ml artificial seawater

The medium is autoclaved (121°C, 15 min) and the cooled to approximately 55°C. To the media 0.1 ml Solution C (vitamin solution, above) and 1 ml Solution E is added before pouring plates.

Solution E

0.1 g D-glucose

0.1 g D-ribose

0.1 g sodium succinate

0.1 g sodium pyruvate

0.1 g glycerol

0.1 g N-acetyl-D-glucosamine

10 ml distilled water

F/2 Medium

0.075 g $NaNO_3$

0.05 g NaH_2PO_4

0.1 g Na_2SiO_3

1 ml F/2 trace elements

2.31g HEPES

1000 ml aged natural seawater (stored in the dark); autoclaved 121°C 15 min; 0.5 ml

of sterile F/S vitamin solution is added after cooling of the medium.

F/2 trace elements

4.36 g disodium EDTA

3.15 g $FeCl_3.6H_2O$

9. 8 mg $CuSO_4.5H_2O$

2.2 mg $ZnSO4.7H2O$

1 mg $CoCl_2.6H2O$

1.8 mg $MnCl_2.4H_2O$

0.6 mg $NaMoO_4.2H_2O$

1000 ml deionised water

F/2 vitamins (steril-filtered):

0.1 mg biotin

0.1 mg cyanocobalamin

20 mg thiamine HCl

100 ml distilled water (sterilised by filtration using 0.2 μm filters).

Buffers used for fluorescent in situ hybridisation and washing:

Table A.1. Buffers used for FISH hybridisation (Chapter 2)*

Hybridisation Solution	Gamma42a	Alpha968, Flavo558	Eub338
NaCl	1.04 g (0.889M)	1.04 g (0.889M)	1.04g (0.889M)
Tris HCl (0.04 M, pH 7.4)	10 ml (0.02M)	10 ml (0.02M)	10 ml (0.02M)
10% (wgt./vol.) SDS	0.2 ml (0.01%)	0.2 ml (0.01%)	0.2 ml (0.01%)
Deionised formamide	7 ml (35%)	4 ml (20%)	-
Deionised water	2.8 ml	5.8 ml	9.8 ml

*All buffers made up to a total volume of 20ml.

**Table A.2.** Wash solutions used in the FISH protocol (Chapter 2)#.

Wash Solutions	Gamma42a	Alpha968, Flavo558	Eub338
NaCl	0.46 g (0.079M)	1.32 g (0.226M)	5.62 g (0.961M)
Tris HCl (1M, pH 7.4)	2 ml (0.02M)	2 ml (0.02M)	2 ml (0.02M)
EDTA (0.5M) pH 8.0	1 ml (0.005M)	1 ml (0.005M)	1 ml (0.005M)
1% SDS	1 ml (0.01%)	1 ml (0.01%)	1 ml (0.01%)
Milli-Q water	96 ml	96 ml	96 ml

All buffers made up to a total volume of 100ml.

**Table A.3** Control cultures used in FISH analysis (Chapter 2).

| Probe	Positive control	Negative control
EUB338	*Cytophaga lytica*	-
	Escherichia coli *Pseudoalteromonas atlantica* *Sphingomonas* Strain G5BA1	
GAMMA42a	*Pseudoalteromonas atlantica*	*Cytophaga lytica*
ALPHA968	*Sphingomonas* Strain G5BA1	*Cytophaga lytica*
FLAVO558	*Cytophaga lytica*	*Escherichia coli*
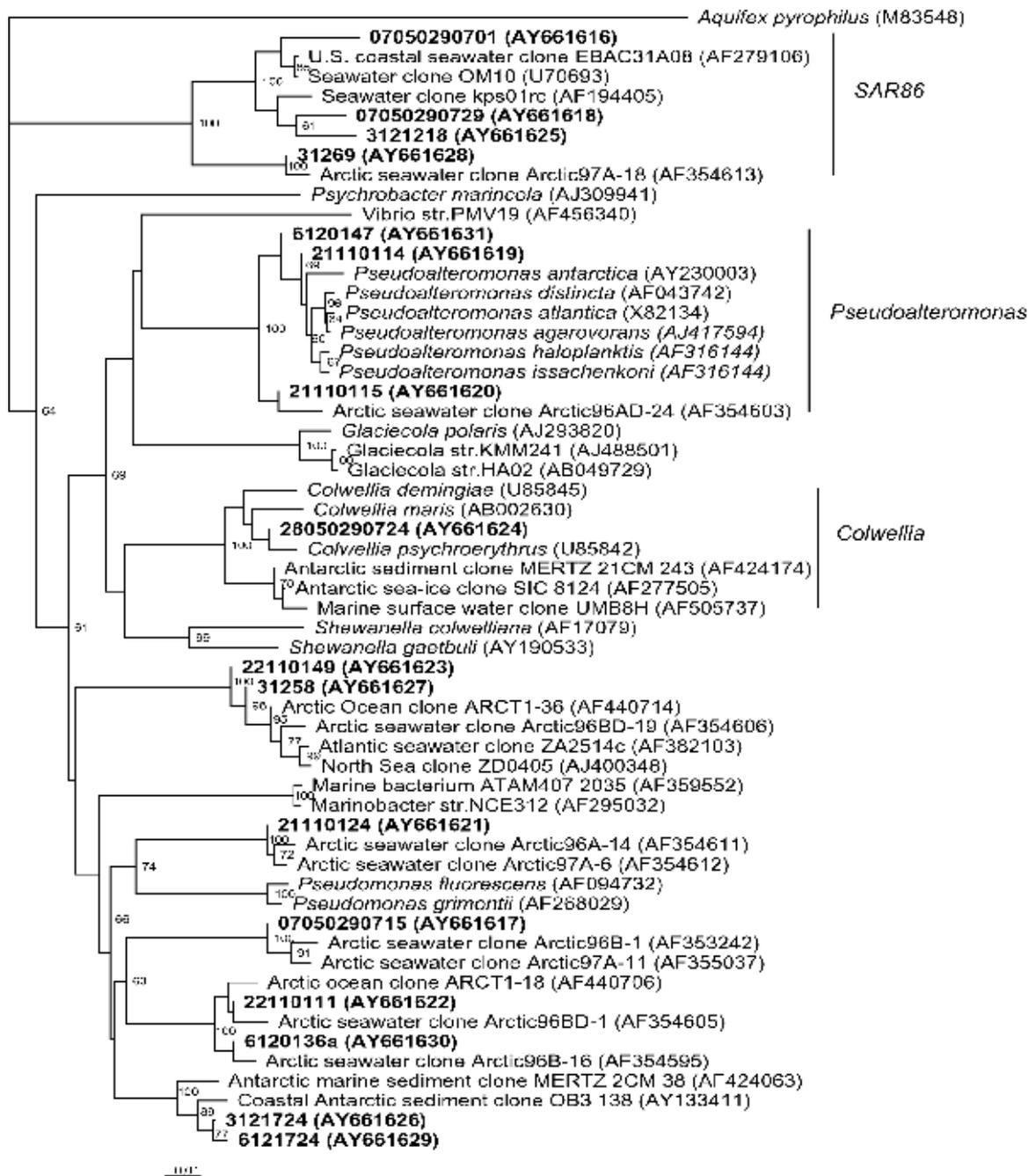
Figure A.1. 16S rRNA gene sequence phylogenetic tree comparing Southern Ocean seawater sample DGGE bands grouping in class *Gammaproteobacteria*. *Aquifex pyrophilus* was used as the outgroup sequence. Branching points with >60% bootstrap support (1000) replicates have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.
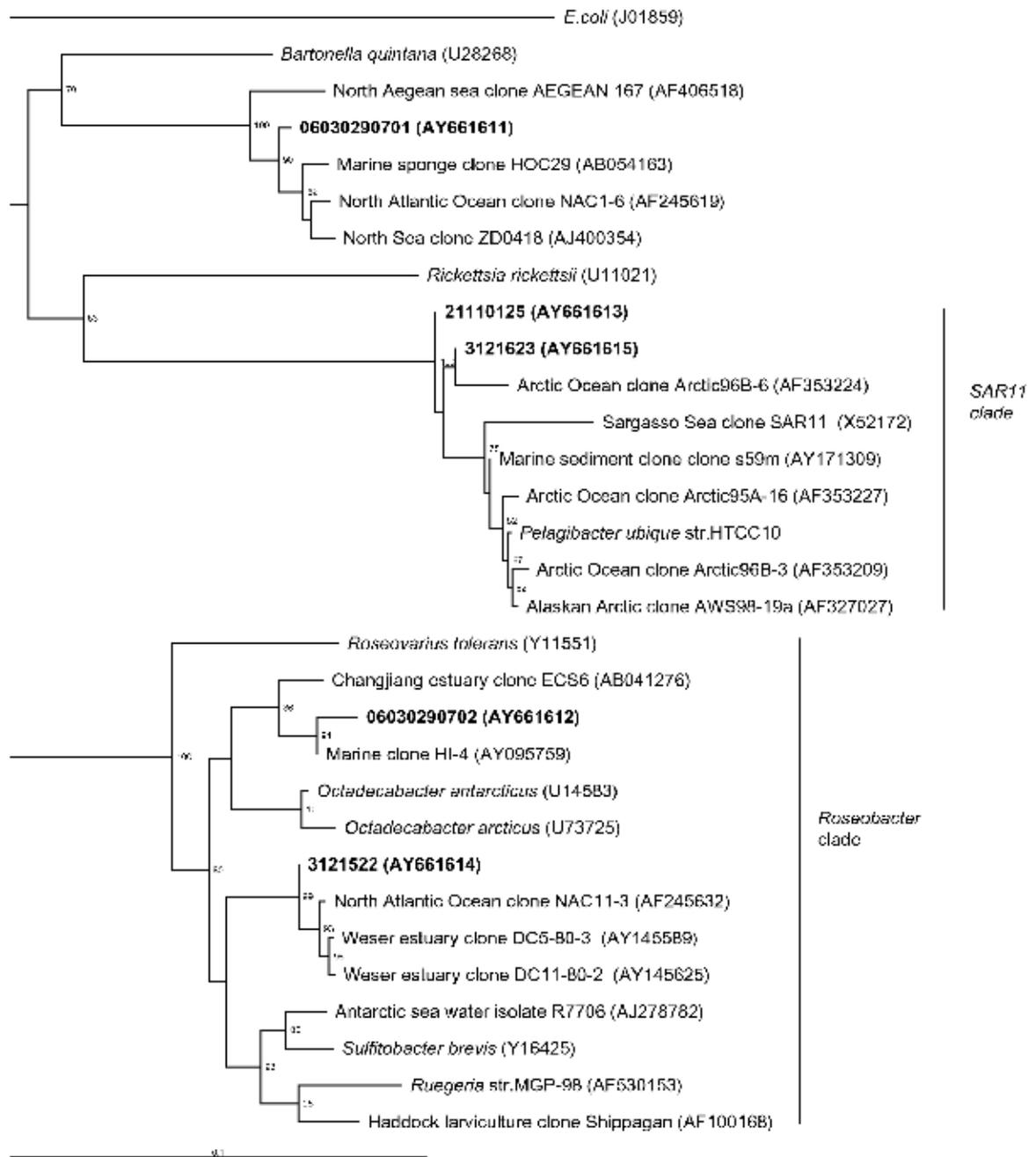
Figure A.2. 16S rRNA gene sequence phylogenetic tree comparing Southern Ocean seawater sample DGGE bands grouping in class *Alphaproteobacteria*. *E. coli* was used as the outgroup sequence. Branching points with >60% bootstrap support (1000) replicates have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.

Figure A.3. 16S rRNA gene sequence phylogenetic tree comparing Southern Ocean seawater sample DGGE bands grouping in the Planctomycetes, SAR 406 clade and the *Cyanobacteria*. *Aquifex pyrophilus* was used as the outgroup sequence. Branching points with >60% bootstrap support (1000) replicates have values shown at nodes. The scale bar indicates 0.01 changes per nucleotide.
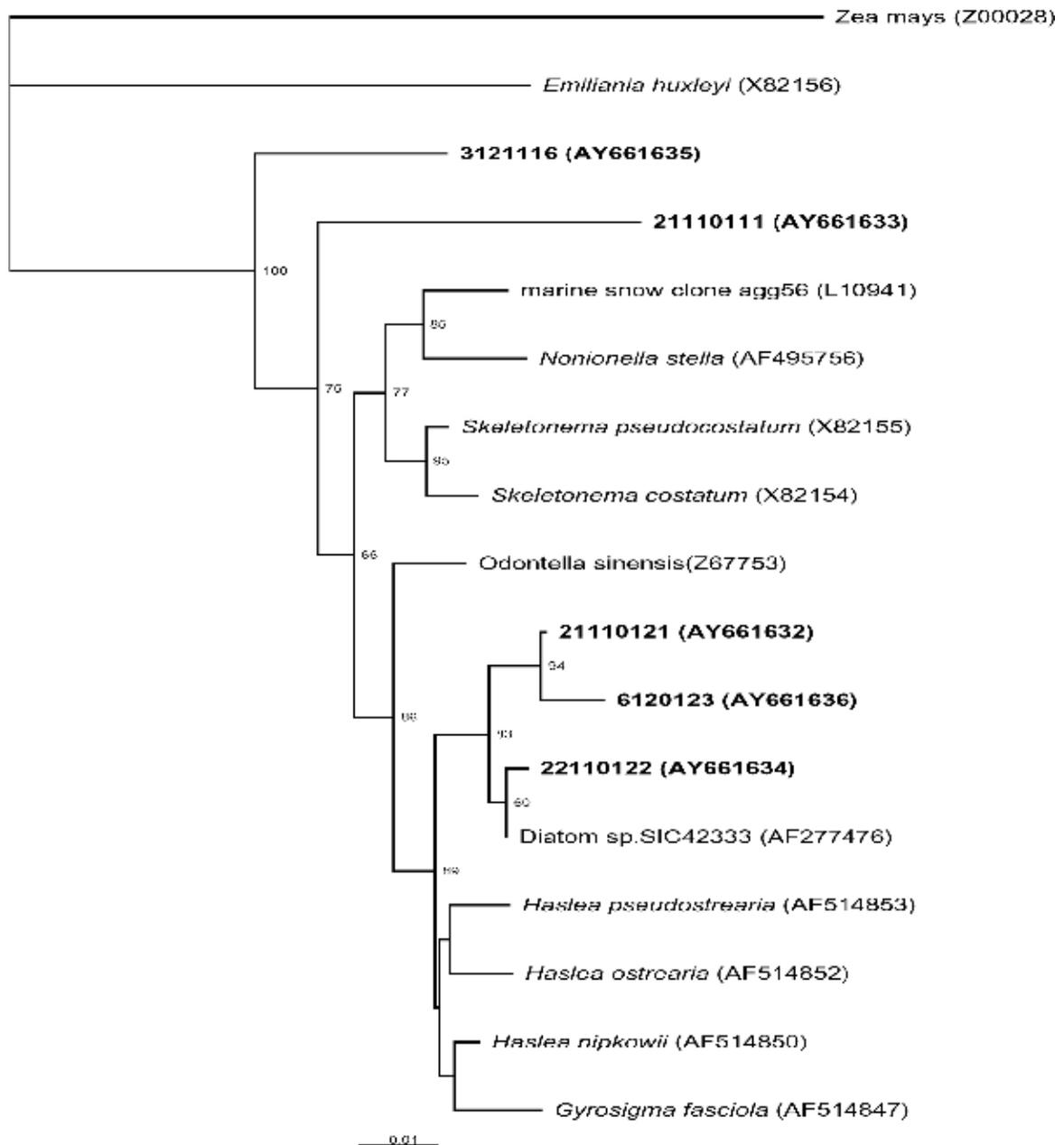
Figure A.4. 16S rRNA gene sequence phylogenetic tree comparing Southern Ocean

seawater sample DGGE bands including 16S rRNA genes of algal and plant

chloroplasts. *Zea mays* was used as the outgroup sequence. Branching points with

>60% bootstrap support (1000) replicates have values shown at nodes. The scale bar

indicates 0.01 changes per nucleotide.
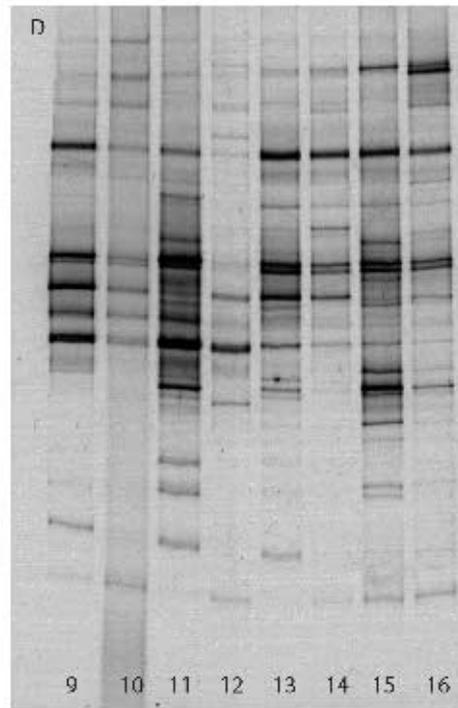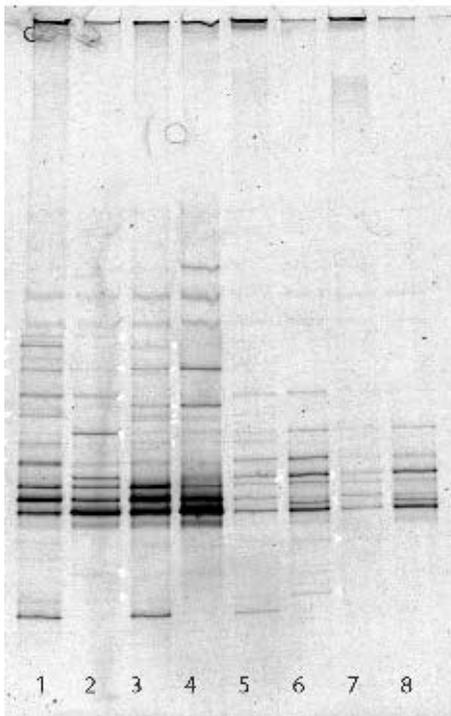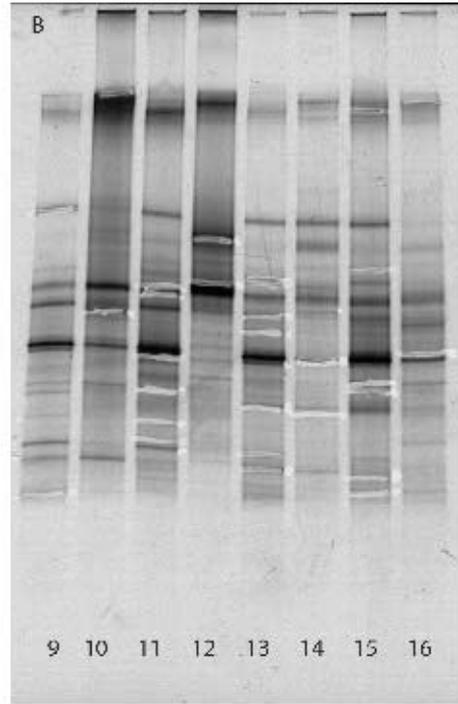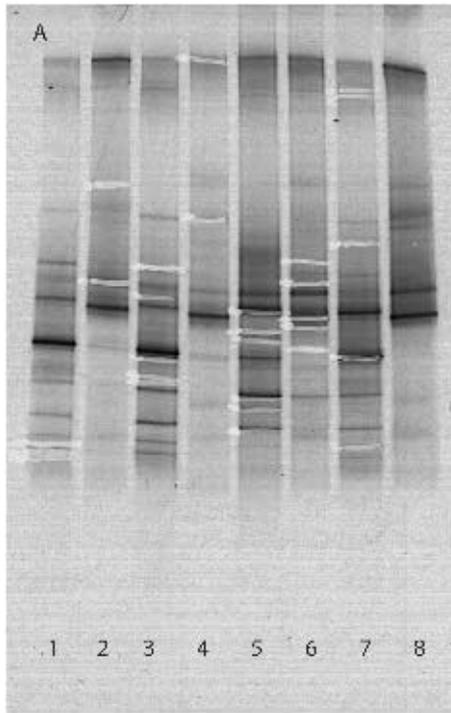
Figure A.5. Negative photographic images of DGGE gels generated using Universal (A & B) and *Flavobacteria* specific primers (C & D). Lanes 1-2 , station 7; lanes 3-4, station 8; lanes 5-6, station 9; lanes 7-8, station 10; lanes 9-10, station 11; lanes 11-12, station 12; lanes 13-14, station 13; lanes 15-16, station 14. Odd numbered lanes correspond to 0.2 μm seawater filter fractions and even numbered lanes correspond to 0.8 μm seawater fractions.
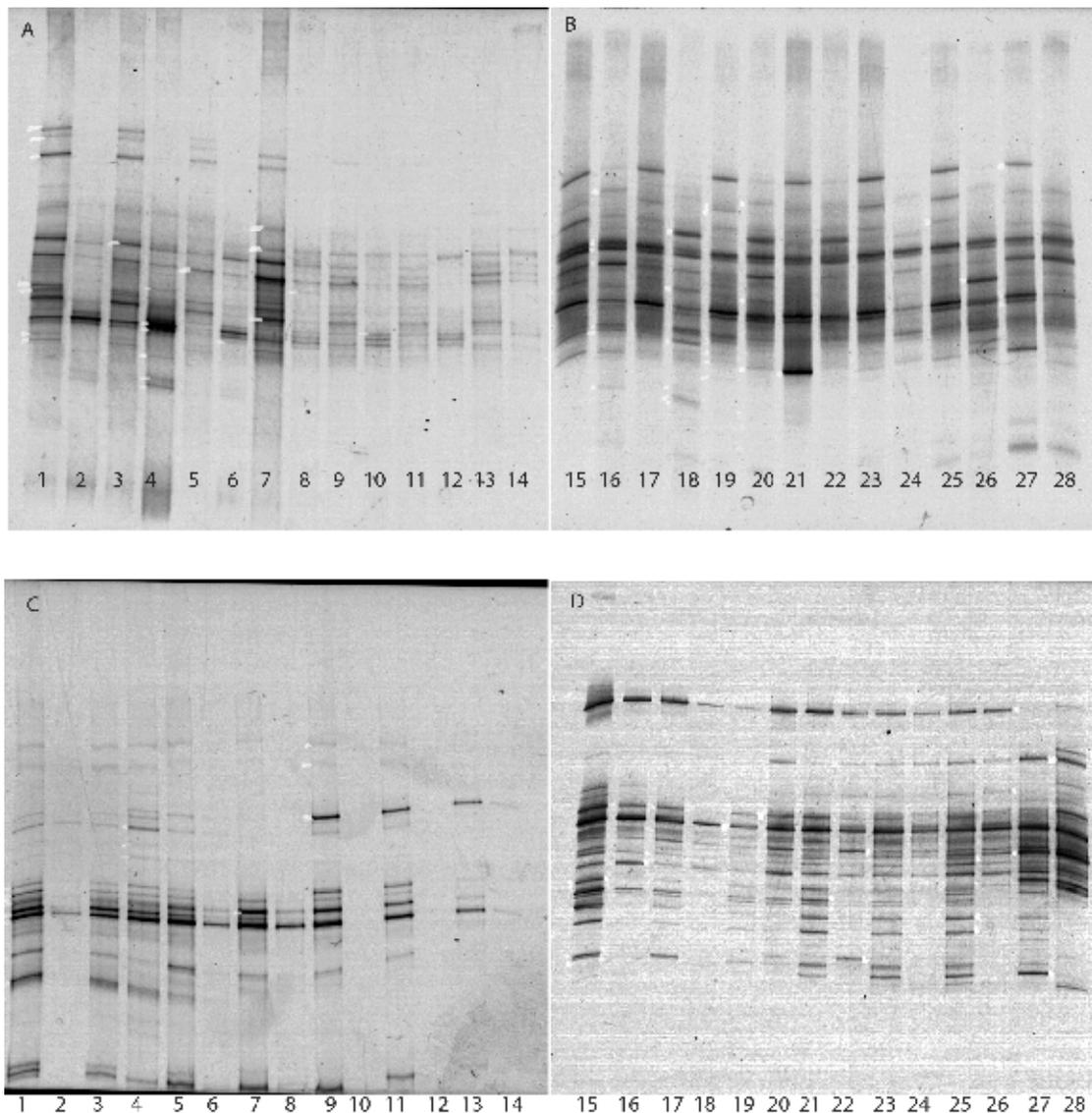
Figure A.6. Negative photographic images of DGGE gels generated using Universal (A & B) and *Flavobacteria* specific primers (C & D). Lanes 1-2 , station 15; lanes 3-4, station 16; lanes 5-6, station 17; lanes 7-8, station 18; lanes 9-10, station 19; lanes 11-12, station 19b; lanes 13-14, station 20; lanes 15-16, station 20b; lanes 17-18, station 21; lanes 19-20, station 22; lanes 21-22, station 23; lanes 23-24, station 24; lanes 25-26, station 25; lanes 27-28, station 26. Odd numbered lanes correspond to 0.2 µm seawater filter fractions and even numbered lanes correspond to 0.8 µm seawater fractions.