Molecular microbial ecology of contaminated marine sediment near Casey Station, Antarctica.

Shane Powell (BSc hons)

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Declaration of originality

This thesis contains no material previously accepted for a degree or diploma by this or any other university, except by way of background information, which is acknowledged in the thesis. To the best of my knowledge this thesis contains no material previously published or written by another person except where acknowledgement is made in the text.

Shane Powell 8/6/04

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The following papers form part of this thesis:

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JP Bowman assisted with the analysis of the clone libraries, particularly the use of the LIBSHUFF program.

JS Stark provided the sediment samples used for the first study and provided guidance with the multivariate statistical analysis.

Abstract

The Antarctic marine environment is a beautiful and productive yet fragile and sensitive ecosystem. In areas close to research stations, there is evidence of the impact of human activities in the form of petroleum and heavy-metal contamination. The process of biodegradation by microbes is a major pathway for the removal of some of these contaminants from the environment. It is therefore important to understand the response of microbial communities to pollution. In this study, molecular techniques were used to investigate the microbial ecology of impacted and non-impacted marine sediments near Casey Station, Antarctica.

Initially, a study of the microbial diversity in an impacted and non-impacted bay showed that the two bays had diverse but not significantly different communities. 16S rRNA gene clone libraries revealed the presence of similar species to those found in other cold marine sediments. However, there was a cluster of clones related to the hydrocarbon-utilising sulfate-reducing genus *Desulfobacula* that was found only in the impacted site. Further investigation using real-time PCR discovered that they were also present in the non-impacted site, but in significantly smaller numbers.

Denaturing gradient gel electrophoresis (DGGE) was used in a nested-design survey of two impacted and two non-impacted locations. There were significant differences both within and between locations. The microbial community structure within impacted locations was more variable than in control locations and correlations with environmental variables showed that pollution was one of a number of factors influencing the microbial communities. In a separate field experiment, a mixture of oils was shown to affect the development of microbial communities.

An *in situ* experiment to investigate the short-term effects of four different oils was conducted at one of the control sites. A polyphasic approach involving

microscopic cell counts, most probable number counts and DGGE was used to analyse the microbial communities. It was found that special Antarctic blend diesel (SAB), a lubricant (Mobil OW40) and the same lubricant after use in a vehicle all had a significant effect on the microbial community when compared to a control treatment. The microbial community in sediment exposed to a biodegradable lubricant (Titan GT1) however was not significantly different to that of the control.

Finally, the biodegradation of nonane, one of the components of SAB, was measured in enrichment cultures developed from sediment exposed to SAB. Sequencing of two DGGE bands from the enrichment culture revealed the presence of *Pseudomonas*- and *Colwellia*-like species.

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1 Introduction

1.1 Introduction

Increasing awareness of the fragility of the environment that we live in has resulted in a desire to understand and protect our ecosystem. The Antarctic is particularly important, both in terms of its unique physical and biological features and also because it is enshrined in international law as a "natural reserve devoted to peace and science". Unfortunately as we have explored the region, we have also polluted it. Most notably there have been incidents of major marine oil spills - the grounding of the Bahia Pariso and the Nella Dan - and reports of extensive terrestrial contamination at research stations such as Casey (Deprez *et al.*, 1999), Amundsen-Scott (Wilkness, 1990) and McMurdo (Tumeo and Wolk, 1994). An understanding of the ecosystem coupled with new technology is required to reduce the impact of the human presence in Antarctica. As microbial communities have a pivotal role in Antarctic ecosystems, it is important that the effect of oil contamination and their response to it is elucidated.

1.2 Microbial ecology of contaminated sites

1.2.1 The role of micro-organisms in contaminated sites

Microbial communities play an important role in contaminated sites, principally by mediating the breakdown of chemical contaminants. Microbes exert an influence on other trophic levels of the ecosystem by incorporating carbon from sources such as hydrocarbons into the food chain; by changing the chemistry of their surroundings through the uptake of other nutrients; and by transforming potentially

toxic compounds. Understanding the response of microbial communities to pollutants allows an estimation of the longevity of the pollutants in the environment and hence an estimation of the longevity of the effects of the pollutants both on the microbial community and other trophic levels of the ecosystem. It also allows the assessment of the relative risks of new sources of contamination. Physical factors such as temperature, water and nutrient availability and physical weathering processes (such as dispersal and evaporation) all influence the way the chemical contaminants interact with the microbial community. Unfortunately, this information is site-specific and the extent of biodegradation can vary between sites - even within the same study area (eg Delille and Delille, 2000). Hence knowledge of the processes occurring at one site is not necessarily indicative of what is happening at another. Extrapolation of observations from temperate to polar regions is particularly tenuous as the biodegradation process is influenced by different factors such as the extreme temperature of polar regions. Information specific to biodegradation in the Antarctic is required in order to best manage Antarctic contaminated sites.

1.2.2 Micro-organisms in cold contaminated sites

Micro-organisms have evolved to live in extremely cold temperatures and diverse microbial populations capable of degrading hydrocarbons have been found in most cold environments. Biodegradation has been observed in alpine (Margesin and Schinner, 1997), sub-Antarctic (Delille and Pelletier, 2002) and Antarctic (Aislabie *et al.*, 1998; Kerry, 1993) soils. It has also been observed in even more extreme environments such as sea-ice (Delille *et al.*, 1997) and glacier cryoconite (Margesin *et al.*, 2002). The bacteria involved appear to be similar to those that carry out biodegradation in more temperate climates, for example *Rhodococcus* (Bej *et al.*,

2000), *Alcanivorax* (Yakimov *et al.*, 1998), *Pseudomonas* and *Sphingomonas* (Aislabie *et al.*, 2000).

Biodegradation and bioremediation have been investigated in experimental oil spills in Antarctica (Kerry 1993; Delille *et al.*, 2002). Similar conclusions were reached in both of these: degradation occurs, albeit slowly; there is an increase in the number of bacteria present at the contaminated site compared to nearby control sites; the proportion of hydrocarbon degrading bacteria increases; and the addition of nutrients or fertiliser in some form increases the extent of biodegradation observed. An early study in the sediment, sea-water and ice of an artificially polluted Arctic marine ecosystem also reached the conclusion that although an increase in the numbers of hydrocarbon-degrading bacteria was observed, biodegradation was slow and hydrocarbons would remain in the ecosystem for some time (Atlas *et al.*, 1978). Equivalent studies have not been carried out in the Antarctic and there is a lack of knowledge on the effects of hydrocarbons on Antarctic marine sediments. Australia's Casey station provides an opportunity to study the microbial ecology of contaminated marine sediments as nearly forty years of human activity in the region has left a substantial legacy of pollution.

1.3 Contaminated sites in Antarctica

1.3.1 History of Casey Station

Casey station is located on the Bailey Peninsula (at approximately 66°17'S 110°32'E) in the windmill islands region of Antarctica (Figure 1.1). It is one of Australia's three continental stations and is the third station to be built in the area. The station is built on the rocky, predominantly ice-free coastline with Law Dome to the east and to the west the chain of islands known as the Windmill Islands. What is

now known as "Old Casey" station was in use from 1969 (after Wilkes station on nearby Clark Peninsula became uninhabitable due to the accumulation of snow and ice) until the current Casey station was opened in 1989 less than a kilometre from "Old Casey". Although today waste is returned to Australia for disposal, in the past waste disposal methods were much the same in Antarctica as in Australia. This involved dumping into uncontained waste disposal sites (tips), open burning of waste and disposal into the marine environment (sea-icing). Accidental spills of chemicals such as fuels and solvents were generally not remediated and continue to contribute to the pollution.

The signing of the Protocol on Environmental Protection to the Antarctic Treaty (Madrid Protocol) in 1991 prompted an initial assessment of the Casey and Old Casey area in the 1993 / 1994 summer. This work identified up to sixteen sites that were categorised as potentially contaminated and four that were classified as contaminated (Deprez *et al.*, 1999). The Thala Valley tip-site at Casey is the most obviously contaminated site but other areas such as the old workshop, the helicopter landing pads and sites where fuel spills were recorded have also been investigated (Deprez *et al.*, 1999). Levels of total petroleum hydrocarbons in some areas are many times higher than the trigger levels for environmental investigation guidelines in Australia. Concentrations of heavy metals are also described as significantly above background levels at some sampling sites.

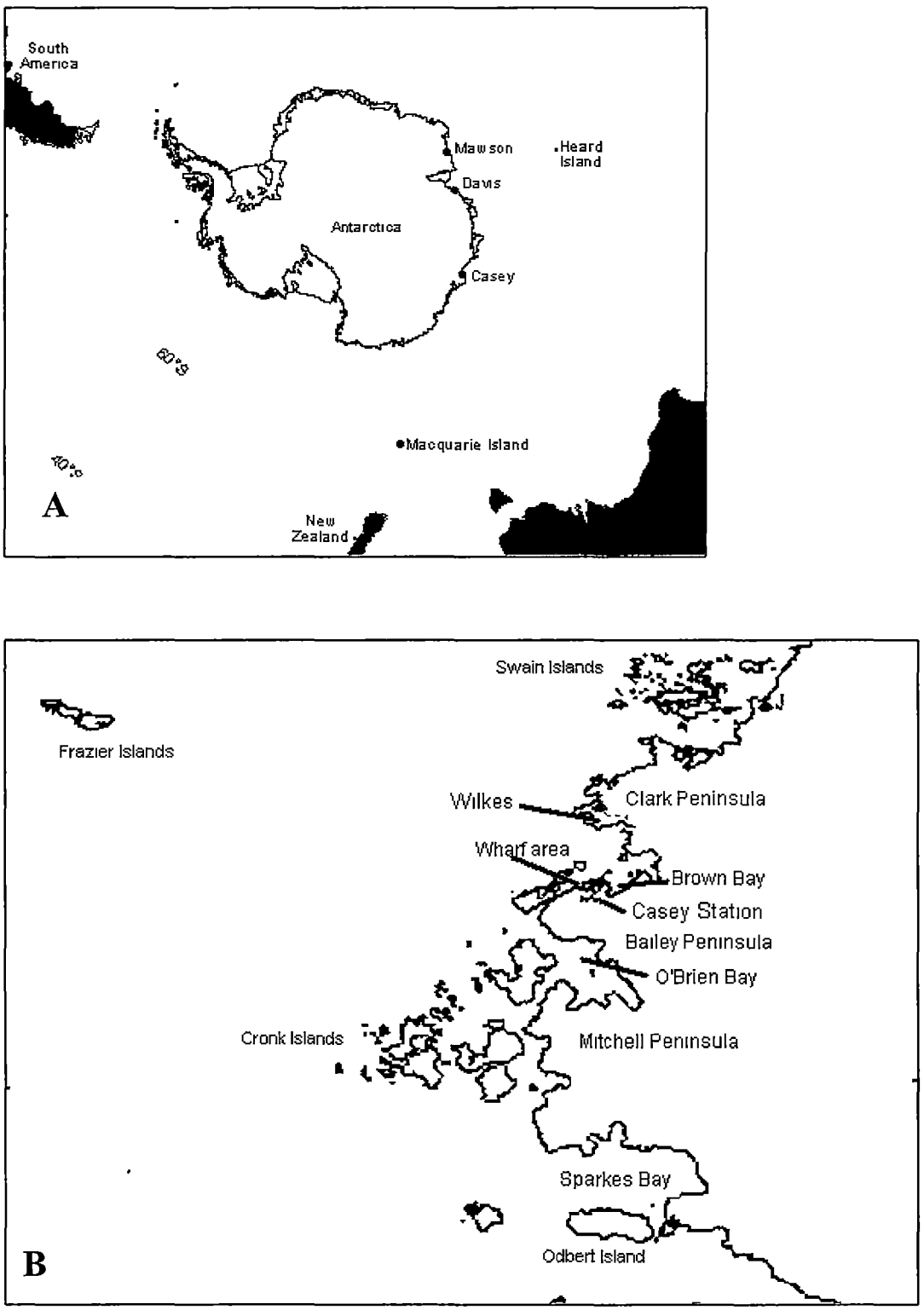

Figure 1.1: Map of Antarctica (A) showing the location of Casey Station; and map of the Windmill Islands region (B) showing location of Casey in relation to sampling areas.

The marine environment surrounding Casey has become contaminated through direct (sea-icing) and indirect (melt streams) sources. During the summer melt, streams run through the Thala Valley tip-site and several hydrocarbon spills carrying contaminants into Brown Bay. Levels of heavy metals and organic carbon measured in Brown Bay, adjacent to Casey station, are higher than those in other bays in the region (see Table 1.1).

Table 1.1 : Range of values for some environmental variables in the marine environment surrounding Casey Station

	Brown Bay	O'Brien Bay	Sparkes Bay	Wharf area
Zinc (mg kg^{-1})	13 - 65	2 - 18	23 - 26	8 - 35
Copper (mg kg^{-1})	5 - 30	1 - 2	2 - 3	2 - 5
Lead (mg kg^{-1})	18 - 85	ND	ND	1 - 6
Iron (mg kg^{-1})	500 - 5300	50 - 230	150 - 220	200 - 400
Manganese (mg kg^{-1})	1 - 4	3 - 5	1 - 3	1 - 3
Arsenic (mg kg^{-1})	5 - 35	1 - 14	4 - 7	3 - 8
Cadmium (mg kg^{-1})	0.5 - 2	0.1 - 2	3 - 4	0.5 - 3
Total organic carbon (g kg^{-1})	15 - 47	10 - 25	29 - 32	14 – 42
Grain size (mean particle diameter μm)	24 -109	108 - 363	34 - 55	30 - 296

Data are summarised from Stark *et al.* (2003a). ND = not detected

1.3.2 Benthic ecology of Antarctic contaminated sites

Studies into the benthic fauna at Casey demonstrated that changes in communities were correlated with heavy metal and hydrocarbon contamination (Stark *et al.*, 2003a). In addition, field recruitment experiments have shown differences in the diatom and benthic infaunal communities that develop in clean sediment as compared to hydrocarbon contaminated sediments (Stark *et al.*, 2003b; Cunningham *et al.*, 2003).

Similar studies have also been carried out on the benthic fauna in Winter Quarters Bay near McMurdo station. Differences were observed in invertebrate communities along a pollution gradient; dramatic changes were seen within a year in communities transplanted from clean to contaminated sediment and a decrease in the survival and burrowing of amphipods in contaminated sediment compared to clean sediment was noted (Lenihan, 1992). It was also found that Antarctic invertebrates responded to contaminated sediment in a similar manner to that usually seen in USA EPA tests (Lenihan *et al.*, 1995).

Trophic levels in benthic ecosystems are known to be closely linked (Kemp, 1990). Bacteria, through their ability to transfer the additional carbon derived from petroleum hydrocarbons into the food chain, seem to be particularly important in the recovery of benthic ecosystems from oil spills (Danovaro, 2000). In addition, as micro-organisms are able to change the chemistry of their surroundings, (for example through uptake of nutrients which affects the redox potential (Schallenberg and Klaff, 1993) and through biodegradation) changes in microbial activity will affect other benthic fauna. Therefore in order to understand how the marine ecosystem is affected by contamination, it is important to understand how microbial communities are affected and how they respond. For these reasons, an investigation of the benthic

microbial communities near Casey was undertaken as part of a multidisciplinary research effort into the impact of human activities at Casey station.

1.4 Methods in microbial ecology

1.4.1 Goals of microbial ecology

When investigating the microbial ecology of contaminated sites, several questions must be addressed. Firstly, whether the contaminants are having any effects on the microbes. Then whether the necessary micro-organisms are present to degrade the contaminants and what other factors influence this process. Smets and Pritchard (2003) describe three criteria provided by the USA National Research Council for showing that intrinsic bioremediation is occurring. These are: demonstration of loss of contaminants from the site; evidence from laboratory studies that indigenous microbes have the potential to degrade the contaminants and evidence that biodegradation is occurring in situ. The first criterion is outside the scope of this work. Instead the focus is on measuring changes in the structure of the microbial population as a result of pollution, primarily oil contamination, and determining whether hydrocarbon biodegradation is possible.

1.4.2 Molecular techniques in microbial ecology

The huge diversity of microbial populations, the difficulty in identifying individuals and the need for pure cultures in order to characterise them, makes microbial ecology challenging. Traditionally, microbiological methods are culture-based. These methods have the advantage of providing information about the identity and characteristics (eg. morphology, growth rate, metabolic capabilities, pathogenicity) of a culture - either pure or mixed. This is often a time-consuming

and labour intensive process as there may be over a hundred different species in a single environmental sample of less than one gram. In addition information generated by this process is often incomplete as many microbes with fastidious growth requirements are uncultivable. The development of molecular methods based on the analysis of DNA from environmental samples has brought new opportunities for studying previously unknown microorganisms as culturing is no longer required.

Any study utilising molecular methods follows the same basic steps (Theron and Cloete, 2000): first the DNA (or RNA) is extracted from the sample, the region of interest is amplified by the polymerase chain reaction (PCR) and then the amplification products are analysed in some way. In some cases this may be the PCR step itself: for example real-time PCR and quantitative PCR are used to measure the levels of particular genes in samples. Alternatively the DNA generated by PCR can be sequenced and compared to databases in order to identify what is present. In hybridisation methods such as Southern blots and slot-blots, probes for specific groups of microbes or particular genes are used to detect whether the target organism is present in the sample. There are also fingerprinting techniques that produce patterns unique to either an organism (eg amplified ribosomal DNA restriction analysis - ARDRA) or a sample (denaturing gradient gel electrophoresis - DGGE).

The initial "flurry of molecular diversity studies" (Amann, 2000) in the nineteen eighties focussed on the use of the 16S rRNA gene as a phylogenetic marker. However, molecular techniques are increasingly being used to study functional genes in the environment such as those involved in denitrification (Gruntzig *et al.*, 2001) and hydrocarbon degradation (Guo *et al.*, 1997; Beller *et al.*, 2002) thus providing information on the activity of microbial populations.

As with any method, those based on DNA or RNA analysis have their drawbacks. Adequate sampling of diverse communities is the first problem. There is the potential for bias and error in each step of the process from the extraction of nucleic acids from a sample, through amplification by PCR to analysis of the PCR products. In addition, phylogenetic-based studies do not provide conclusive evidence about the function or activity of uncultured microbes. Despite this, when coupled with careful experimental or sampling design and rigorous statistical analysis, molecular methods have the potential to reveal the complex relationships between microbes and their environment.

1.4.3 Statistics in molecular microbial ecology

One of the challenges currently facing microbial ecologists is the design of experiments that test environmentally relevant hypotheses in a quantitative, statistically testable manner. Morris *et al.* (2002) reviewed over 2000 papers on microbial diversity published between 1975 and 1999 and noted that there was "very limited use" of statistical procedures and that "very few papers" reported multiple independent tests of hypotheses. With the methods currently in use, it should now be possible to analyse the number of samples necessary for rigorous statistical testing of hypotheses.

Over the last five or so years microbial ecologists have begun to use practices common in other fields of ecology. For example, a range of diversity and evenness indices such as Shannon and Simpson's index have been applied to clone libraries to give some measure of their diversity and structure (Hill *et al.*, 2003; McCaig *et al.*, 1999). This means that conclusions such as "zinc contamination has resulted in a decreased diversity in the soil" can be made more confidently (Hill *et al.*, 2003). New statistical procedures specifically for molecular data are being developed. The

LIBSHUFF program developed by Singleton *et al.* (2001) does a direct comparison of clone library sequence data to determine whether the libraries are significantly different.

Models for estimating the theoretical total diversity of a sample such as Chao-1 estimator (Chao, 1987) are also being used (Bowman and McCuaig, 2003). These methods have recently been reviewed by Hughes *et al.* (2001). More importantly, rarefaction and other ways of estimating how well the population has been sampled are being used more frequently (eg Ravenschlag *et al.*, 1999; Broft *et al.*, 2002; Brinkmeyer *et al.*, 2003). Knowing how well a population is represented by a sample is an important consideration when drawing conclusions from data that may not be representative of the entire population.

When DGGE was introduced into microbial ecology, studies were published in which conclusions were based on differences in banding patterns between a few samples. For example Macnaughton *et al.* (1999b) used duplicate samples from soil microcosms and LaPara *et al.* (2000) used single samples taken from each stage of a bioreactor. In a review of statistical approaches to DGGE Fromin *et al.* (2002) suggested that it was better to use statistical methods to examine changes in overall banding patterns rather than conclude that the appearance or disappearance of a single band was significant. Methods such as principal components analysis (PCA) (Clegg *et al.*, 2003), and cluster analysis (Smit *et al.*, 2001) have proven useful for detecting changes in microbial populations. In comparing the microbial community structure at different stages of a wastewater treatment plant, Boon *et al.*, (2002) used PCA, cluster analysis and non-metric multidimensional scaling (MDS) - all of which led to the same conclusions on differences in the microbial communities at each stage.

Molecular microbial ecology is a rapidly evolving field. In ten years it has progressed from qualitative phylogenetic-based studies to attempting to quantitatively follow microbial processes *in situ*. Improvements in technology and new techniques such as real-time PCR (Malinen *et al.*, 2003), micro-arrays (Zhou, 2003) and stable isotope probing (Radajewski *et al.*, 2003) may offer further insights into the structure and function of microbial communities.

1.5 Research objectives

The work presented in this thesis aimed to investigate the response of Antarctic benthic microbes to hydrocarbon contamination in a quantitative and statistically robust manner. In order to achieve this, the reproducibility and limitations of the molecular methods used were explored and techniques to minimise error were developed.

The first step was to investigate the natural microbial diversity in the near-shore sediments around Casey. The effect of long-term contamination on the microbial population could then be investigated by comparing impacted and non-impacted (control) sites.

Sediment artificially contaminated with diesel and lubricating oil was used in a field experiment to test the hypothesis that the microbial populations in some locations are better adapted to utilising hydrocarbons than others. After eleven weeks *in situ* incubation, hydrocarbon levels were measured and the sediment microbial communities were compared to each other and to control samples from each location.

A variety of different oils are used in the Antarctic each with a different chemical composition and susceptibility to biodegradation. The next stage was therefore to investigate the effects of different oils on benthic microbial

communities. The short-term effects of four different oils were investigated using a polyphasic approach involving microscopy, most probable number counts and DGGE.

Finally, whilst molecular techniques are more than adequate for monitoring changes in microbial community structure, as noted earlier, they cannot provide information about the activity of the microbial community. Perhaps the most important question in the context of microbial ecology of contaminated sites is: does the potential for biodegradation exist at this particular site? To this end an attempt was made to measure biodegradation of diesel in enrichment cultures developed from O'Brien Bay sediment.

2. Microbial diversity of marine sediments near Casey

2.1 Introduction

To study the effects of pollution on benthic microbial communities near Casey, it was first necessary to investigate the natural diversity of microbial populations in the region. Despite the expectation that low temperatures might limit microbial diversity and activity, the overall biomass and composition of microbial populations in cold environments is similar to that of more temperate sediments (Fabiano and Danovaro, 1998; Sahm and Berninger, 1998; Smith *et al.*, 1989).

A variety of culture, biochemical and molecular techniques have been used to examine sediment microbial communities. Smith *et al.* (1989) used phospholipid ester-linked fatty acid analysis to compare sediments from several sites at Anvers Island and McMurdo Sound in the Antarctic. Sulfate reducers were a significant proportion of the bacterial population - up to 4% at Anvers Island and 2% in McMurdo Sound. Sulfate reducing bacteria are also a significant part of Arctic sediments, comprising up to 20% of the population in one study (Ravenschlag *et al.*, 2001) and 43% in another (Ravenschlag, 1999). Delille (1995) used morphological and biochemical properties to classify isolates in a study examining seasonal changes at a subantarctic location. Gram negative bacteria comprised 75% of the population, most of which were pseudomonas-like rods or cytophaga-like isolates. Bacillus, vibrio and micrococcus-like strains were other significant groups. Ruger and Tan (1992) screened isolates from the permanently cold Sierra Leone Abyssal Plain (sediment temperature generally between 2 and 3 °C). Most of the surface sediment isolates belonged to the Vibrio and Alteromonas groups whilst most of the isolates

from 4 cm depth were Bacillus strains. A previously unknown psychrophilic obligately marine Bacillus was also isolated in this study.

Recently, an extensive survey of sediment microbes from the Mertz Glacier Polyna region of the Antarctic was carried out (Bowman *et al.*, 2003). This polyphasic study utilised biochemical, culture and molecular methods. A very active and diverse microbial population was discovered in the sediment. Enzyme assays showed that microbial activity decreased with depth in the sediment core. Molecular methods revealed that the most numerous groups were the Gamma proteobacteria - particularly sulfate reducers, putative sulfur oxidisers, archaea and flavobacteria. In addition, a number of novel psychrophiles were isolated. A total of 57 prokaryote groups were found and many of these appear to be ubiquitous in cold marine sediments.

Molecular techniques such as clone libraries, RNA blots and fluorescent in situ hybridisation (FISH) have become increasingly popular in microbial ecology. 16S rRNA based probes for specific bacterial groups revealed that the dominant groups of bacteria in polar sediments were the cytophaga-flavobacteria, delta and gamma proteobacteria (Ravenschlag *et al.*, 2001; Bowman *et al.*, 2003). Planctomycetes, and sulfur oxidising bacteria were also abundant. These results co-relate with clone library data (Ravenschlag, 1999; Bowman and McCuaig, 2003). Archaea are also readily detected in polar sediments using molecular probing techniques (Bowman *et al.*, 2003; Sahm and Berninger, 1998).

The construction of clone libraries is a well established method in molecular microbiology. It provides high resolution information about the species present in a sample and to a large extent clone libraries are usually presented as a list of species. It is only recently that microbial ecologists have begun to examine them in more detail in order to draw conclusions about the patterns of diversity of microbial

populations thus allowing the data to be more useful in exploring ecological questions. This has led to the use of standard ecological statistics such as the Shannon and Simpsons indices. For example, Hill *et al.* (2003), compared clones from a zinc contaminated soil to those from a control soil. Using various ecological models, they concluded that the zinc contamination had reduced the soil microbial diversity.

Two studies in agricultural soils both concluded that the clone libraries showed high diversity in the microbial populations as only low coverage of the potential diversity was observed (Smit *et al.*, 2001; McCaig *et al.,* 1999). McCaig *et al.* also used similarity coefficients in an attempt to compare their clone libraries. Although the similarity coefficients were low, they concluded that there was no significant difference in the abundance of any particular phylogenetic group between an unimproved and improved pasture. Unfortunately, due to the high diversity of microbial communities, sampling and therefore coverage is usually incomplete. Ecological statistical methods are not ideal for such data. It is also difficult to compare clone libraries to each other directly, an important analysis when investigating environmental changes. Recently a new method for comparing clone library data was developed by Singleton *et al.* (2001). LIBSHUFF is a computer program that can distinguish different clone libraries on the basis of only fifty sequences - although the more diverse the underlying population, the more sequences that are required. This method has already been used by several authors to compare clone libraries (eg. Bowman and McCuaig, 2003; Humayoun *et al.*, 2003; Broft *et al.,* 2002).

Real-time PCR is a relatively new method in microbial ecology that utilises the exponential phase of amplification in PCR to quantitatively measure the gene copy number. By using carefully designed primers for 16S rRNA gene fragments,

specific groups of bacteria in environmental samples can be detected and enumerated. So far it has been used to investigate *Desulfotomaculum* in soil samples with comparable results to cell counts and RNA dot blots (Stubner, 2002), to measure Geobacter species in sediment samples (Stults *et al.*, 2001) and ammonia oxidising beta-proteobacteria in soil (Hermansson and Lindgren 2001). It has also been used with functional gene probes to measure anaerobic hydrocarbon degraders in sediment microcosms (Beller *et al.,* 2002) and nitrate reducers in both sediment and water (Gruntzig *et al.*, 2001). Both these studies concluded that it was an accurate and specific method in comparison with other techniques such as slot-blot hybridisation.

Two molecular techniques were used to explore the microbial diversity in sediments near Casey station. Clone libraries were first constructed from two samples, one from an impacted and one from an unimpacted site. It was hypothesized that if the presence of hydrocarbons and heavy metals had changed the microbial community structure, a significantly different diversity might be seen or groups of sequences might be obviously absent in one of the libraries. Real-time PCR was then used to further investigate one such finding.

2.2 Methods

2.2.1 Site descriptions

Samples were collected from three locations within both Brown and O'Brien Bays (see Figure 1.1). Brown Bay is adjacent to Casey station and is contaminated with both heavy metals and hydrocarbons whilst O'Brien Bay is located approximately 3km to the south of the station and is considered unpolluted. The locations were generally separated by kilometres, except for Brown Bay in which the

locations were separated by approximately three hundred metres. Samples for this survey were collected in a hierarchical nested design. That is, samples were taken from two sites within each location (approximately one hundred metres apart) and from two plots within each site (approximately ten metres apart). Four replicate samples were taken from each plot, one of which was utilised for microbial analysis. The locations were shallow embayments with a range of sediment characteristics (muddy to sandy) and physical attributes (sea-ice cover, depth, aspect etc.).

The samples were collected by diver using a hand-held corer. Samples were taken back to the laboratory and frozen to –20°C within six to eight hours of collection. Sampling locations and procedures are described in greater detail in Stark *et al.*, (2003a).

One Brown Bay and one O'Brien Bay sample were used for the clone library construction but all Brown and O'Brien bay samples were used for the real-time PCR analysis.

2.2.2 DNA extraction

DNA was extracted from sediment samples using a freeze-thaw method based on that described by Rochelle *et al.* (1994). Approximately 1 g or 1 ml of sediment was suspended in 2 ml of lysis buffer (0.15 M NaCl, 0.1 M EDTA, 4% SDS) with 30 mg lysozyme and *ca.* 20 mg polyvinylpolypyrolidone. Samples were heated in a 55°C water bath for 10 minutes and then subject to three rounds of freezing at –80°C for 15 minutes and heating at 55°C for 10 minutes. After the final thaw, samples were extracted with an equal volume of tris-equilibrated phenol followed by extraction with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1). The aqueous phase was removed to a clean tube and 0.7 volumes of isopropanol added. Extracts were incubated for an hour at room temperature,

followed by centrifugation for 30 minutes at 3100 x *g*. Pellets were air-dried and resuspended in 100 µl of sterile milli-Q water overnight at 4°C. Extracts were checked on a 1% agarose gel before the final purification step on Chromaspin columns (Clontech) following the manufacturer's directions. The amount of DNA present in extracts was measured either using the Hoechst fluorometric assay with a Biorad fluorimeter or with a SmartSpec 3000 (Biorad).

2.2.3 Clone library construction and comparison

Two clone libraries were generated using DNA extracted from an O'Brien Bay and a Brown Bay sample. A fragment of the 16 rRNA gene was amplified using Advantage 2 Taq (Clontech) with the supplied 10X buffer and the primers 519f (CAG CMG CCG CGG TAA TAC) and 1392r (ACG GGC GGT GTG GRC). These universal primers are expected to bind to the majority of bacteria and archaea. Each 100 µl reaction mix contained 10 µl of 10X buffer, 2 µl of taq, 1.25 mM of each deoxynucleoside triphosphate, 20 nmol of each primer and 80 ng of template DNA. The following thermal cycling programme was used: initial denaturing at 94°C for 15 minutes; 30 cycles of denaturing at 94°C for 1 minute, annealing at 52°C for 1 minute, extension at 72°C for 1.5 minutes; final extension at 72°C for 10 minutes. The reaction products were purified using the Prep-a-gene kit (Bio-Rad).

The fragment was cloned using the pGEM-T easy vector system (Promega) and transformed into Epicurian coli XL ultracompetent cells (Stratagene) following the manufacturers directions. Transformants were screened using blue – white screening on Luria agar containing Xgal and IPTG. Approximately 250 white colonies from each library were sub-cultured.

Ultraclean mini plasmid preps (MoBio) were used to extract the plasmids from the sub-cultured clones. 3 µl of the extracts were run on a 1% agarose gel

alongside a molecular weight marker in order to verify that the plasmid contained the correct sized insert.

Positive clones were sequenced with the BigDye Terminator Ready Reaction mix sequencing reactions (Applied BioSystems). 7 µl of the plasmid extract was used in a 20 µl reaction with 5 pmol of either the M13f or M13r primer. This generated sequences of approximately 1000 bp. These sequences are deposited under GenBank accession numbers AY133347 to AY133467.

The chimera-check tool of the Ribosomal RNA Database Project (http://www.rdp.cme.msu.edu (Maidak *et al.,* 2001)) was used to check possible chimeric sequences. Sequences were aligned against reference sequences obtained from GenBank (http:/www.ncbi.nlm.nih.gov/blast (Altschul *et al,.* 1990)). DNADIST and NEIGHBOR from the PHYLIP package (Felsenstein, 1995) were used to generate phylogenetic trees. Clone sequences that were more than 98% similar to each other were considered to be the same phylotype (Keswani and Whitman, 2001) for the purposes of calculating diversity statistics. However, all sequences are shown in Figure 2.3.2. Simpson's index ($D = \Sigma p_i^2$) and the Shannon-Wiener index ($H = - \Sigma p_i \ln(p_i)$) were calculated and the Chao-1 estimator (http//www2.biology.ualberta.ca/jbrzusto/rarefact.php) was used to calculate species richness. The method of Singleton *et al.* (2001) was used to compare the similarity of the two libraries directly. This calculation gives a *p* value that is considered to show a significant difference at values less than 0.05.

2.2.4 Real-Time PCR

2.2.4.1 Primer design

Primers were designed to amplify a region of the 16S rRNA gene specific to the group of Brown Bay clones related to *Desulfobacula* shown in Figure 2.2e.

NetPrimer (http://www.premierbiosoft.com/netprimer/netprimer.html) and WebPrimer (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer) were used to assist in the selection of the best primer pair. These were 764F (ACA ATG GTA AAT ACA GAG GGC A) and 943R (GTC GGG TAT AAT CAA CTC TCAT) (approximate *E. coli* positions 1283 and 1462 respectively). The universal primers for the 16S rRNA gene 684F (GTA GCG GTG AAA TGC GTA GA) and 907R (CCG TCA ATT CCT TTG AGT TT) were used to quantify the total amount of bacterial DNA in each sample.

The specificity of the 764F-943R (SRB) pair were initially checked against sequences in GenBank (http:/www.ncbi.nlm.nih.gov/blast, Altschul *et al.*, 1990) using the "find short nearly exact matches" option of the BLAST function. These primers were then used to construct a small clone library (see section 2.2.3) using the same Brown Bay sample as used for the clone library construction. Fifteen clones were sequenced successfully and all clones were closely related to the same *Desulfobacula* group shown in Figure 2.2e.

2.2.4.2 PCR conditions

Real-time PCR was carried out on a Corbett Rotorgene using the Quantitect SYBR Green PCR kit (Quiagen) with 6 pmol of the forward and reverse primer in each 20 µl reaction. 2 µl of template DNA (whether sample or standard) was added to each reaction. Cycling conditions were as follows: hold for 15 minutes at 94°C, followed by 35 cycles of denaturing at 94°C for 20 seconds, annealing for 30 seconds, extension and acquiring signal at 72°C for 45 seconds; hold at 50°C for 1 minute followed by a melt curve from 50 - 95°C. For the specific SRB primer pair the annealing temperature was 53°C and for the universal primer pair, the annealing temperature was 55°C. Sample DNA was diluted so that it fell within the range of

the standard curves. Samples were run in duplicate and analysis was repeated if the duplicates were not within 10 % of each other.

2.2.4.3 Standards

One of the clones from the small clone library described in section 2.2.4.1 was amplified with M13 primers. The reaction was purified using the UltraClean PCR cleanup kit (MoBio) and contained approximately 80 ng DNA per µl. This was diluted in a 10-fold series to create the standards for a five point standard curve that was run in duplicate with every set of samples. Data was only used from runs in which the R value from the standard curve was over 0.99. Three samples, a 1:2, 1:5 and 1:20 dilution of a Brown Bay sample were run several times (in duplicate) in order to check the reproducibility of this method. With the SRB primers, the concentration of target DNA ranged from 0.0002 to 0.003 pg μl^{-1} and the variation (standard error / mean) was between 15 and 20%. For the universal primers the concentration of target DNA ranged from 40 to 300 pg μl^{-1} and the variation was 10%.

2.3 Results

2.3.1 Clone libraries

After discarding sequences that were suspected of being chimeric and sequences that were of poor quality, there were 98 clones from the Brown Bay library and 86 from the O'Brien Bay library. Of these, 66 and 64 respectively were unique phylotypes and fourteen phylotypes were found in both libraries. Many of the other phylotypes were closely related but fell outside of the definition of phylotype used here. This number of unique sequences represents 33% (Brown Bay) and 25% (O'Brien Bay) coverage where coverage is considered to be the proportion

of clones found more than once. The species richness as calculated by the Chao-1 estimator was 304 for Brown Bay and 282 for O'Brien Bay. Both these figures suggest that the microbial diversity of both bays is much higher than detected here. The Simpson diversity index, a measure of the evenness of the species distribution was 0.016 for Brown Bay and 0.011 for O'Brien Bay. Two richness indices were calculated: the Shannon index was 3.96 for Brown Bay and 4.02 for O'Brien Bay and the Margalef index was 14.1 for both Brown Bay and O'Brien Bay. Again this indicates a high microbial diversity in these sediments.

The sequences obtained from the clone libraries were divided into six groups for ease of handling. The most numerous sequences belonged to the delta and gamma proteobacteria followed by the cytophaga - flavobacteria group. The two libraries are compared in Figure 2.1 on the basis of the number of unique phylotypes in each group. Both sites have diverse microbial populations with similar proportions of the phylogenetic groups. The *p* values generated by the method described in Singleton *et al.*, (2001) were 0.063 (when Brown Bay is X and O'Brien Bay is Y) and 0.372 (when O'Brien Bay is X and Brown Bay is Y). Neither of these values indicates a significant difference between the two clone libraries.

When the sequences were aligned onto phylogenetic trees (see Figures 2.2a-f), it is apparent that clusters of very similar phylotypes generally contained representatives from both libraries. However, in both the gamma (Figure 2.2d) and delta (Figure 2.2e) proteobacteria, there were clusters in which there were only Brown Bay clones. In the gamma proteobacteria, these clones were related to the genus *Pseudoalteromonas*. In the delta proteobacteria the cluster of Brown Bay clones were related to *Desulfobacula toluolica* and *Desulfobacula phenolica*, both hydrocarbon oxidising sulfate reducers. The possible presence of this group in O'Brien Bay was investigated further using real-time PCR.

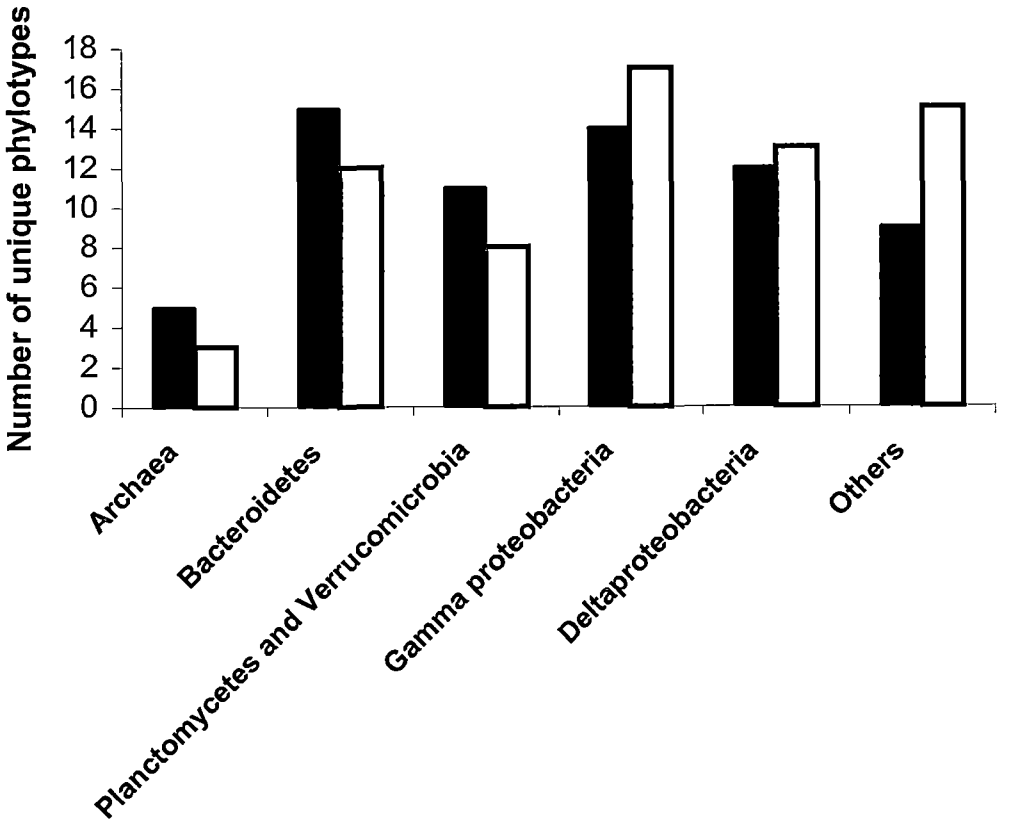

Figure 2.1: Comparison of O'Brien Bay (black) and Brown Bay (white) clone libraries on the basis of the number of unique phylotypes present.

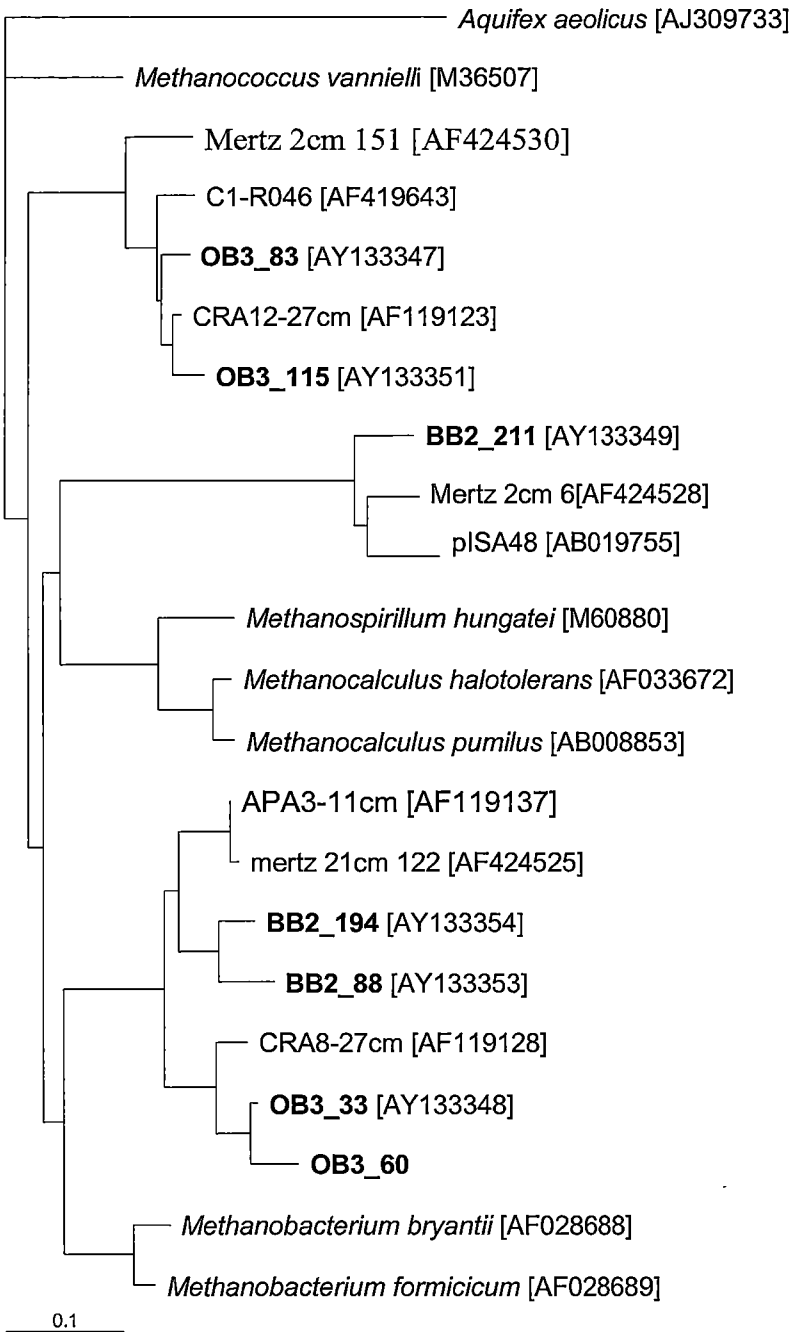

Figure 2.2a: Phylogenetic tree of the Archaea in the O'Brien Bay (numbers beginning with OB3) and Brown Bay (numbers beginning with BB2) clone libraries.

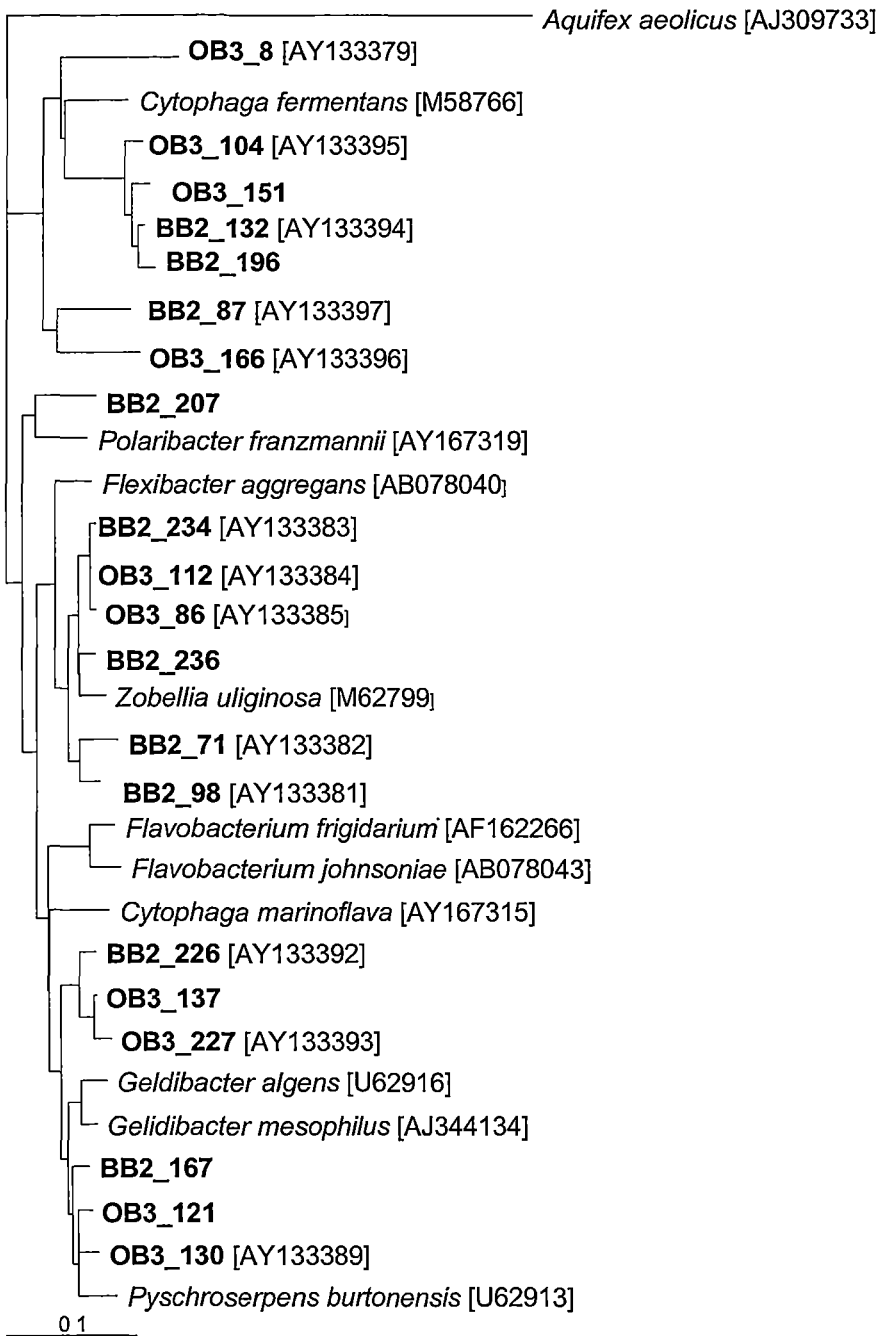

Figure 2.2b: Phylogenetic tree of the Bacteroidetes in the O'Brien Bay (numbers beginning with OB3) and Brown Bay (numbers beginning with BB2) clone libraries.

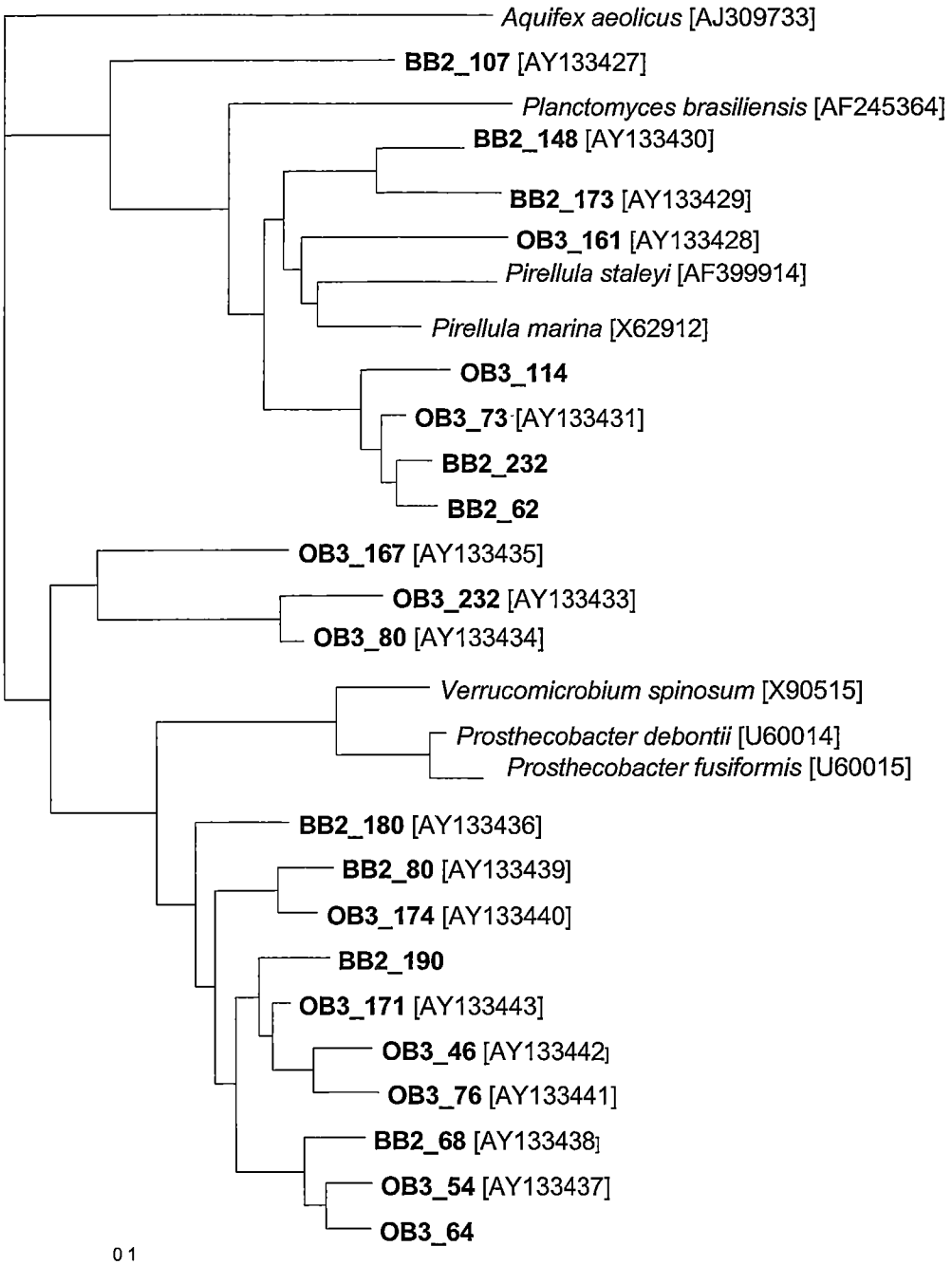

Figure 2.2c: Phylogenetic tree of the Planctomycetes and Verrucomicrobia in the O'Brien Bay (numbers beginning with OB3) and Brown Bay (numbers beginning with BB2) clone libraries.

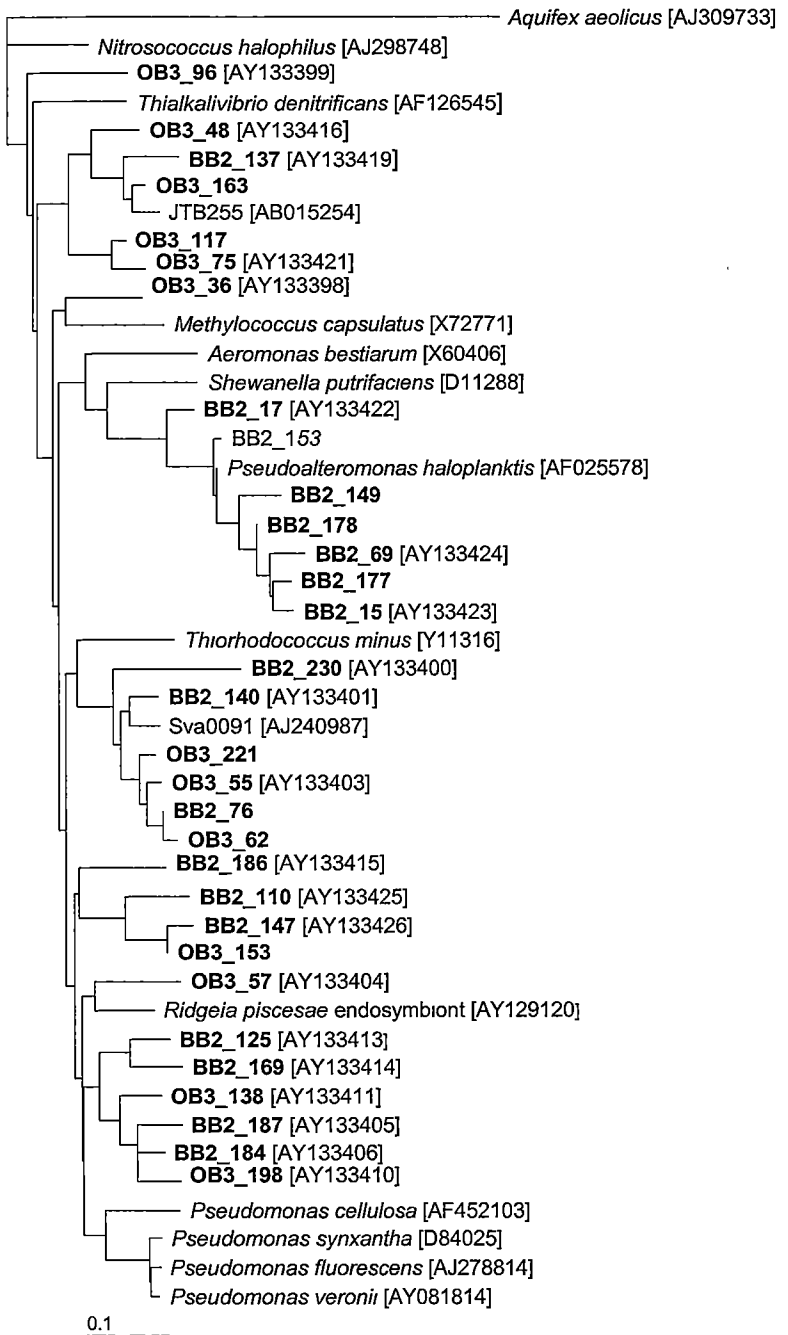

Figure 2.2d: Phylogenetic tree of the Gamma Proteobacteria in the O'Brien Bay (numbers beginning with OB3) and Brown Bay (numbers beginning with BB2) clone libraries.

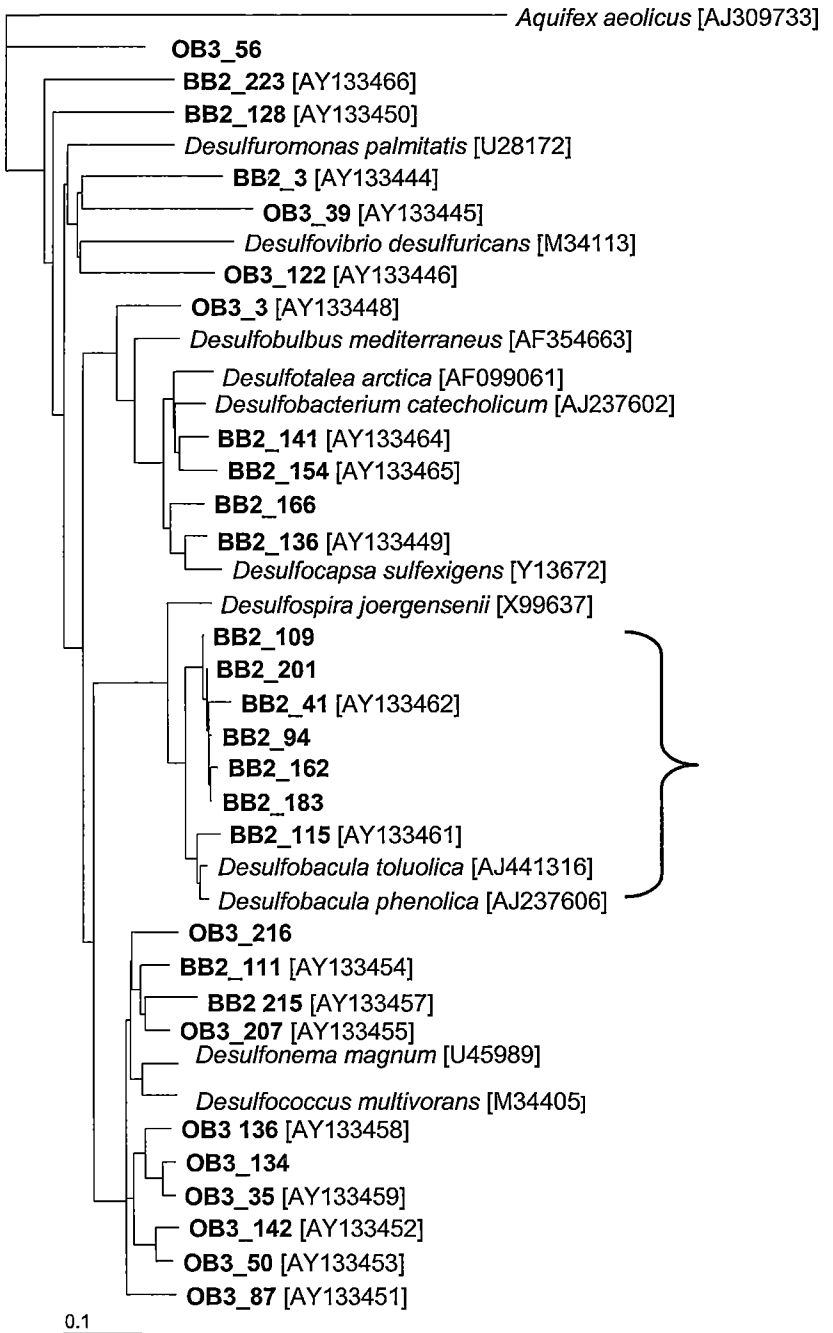

Figure 2.2e: Phylogenetic tree of the Deltabacteria in the O'Brien Bay (numbers beginning with OB3) and Brown Bay (numbers beginning with BB2) clone libraries. The bracket indicates cluster of Brown Bay clones associated with the *Desulfobacula* group.

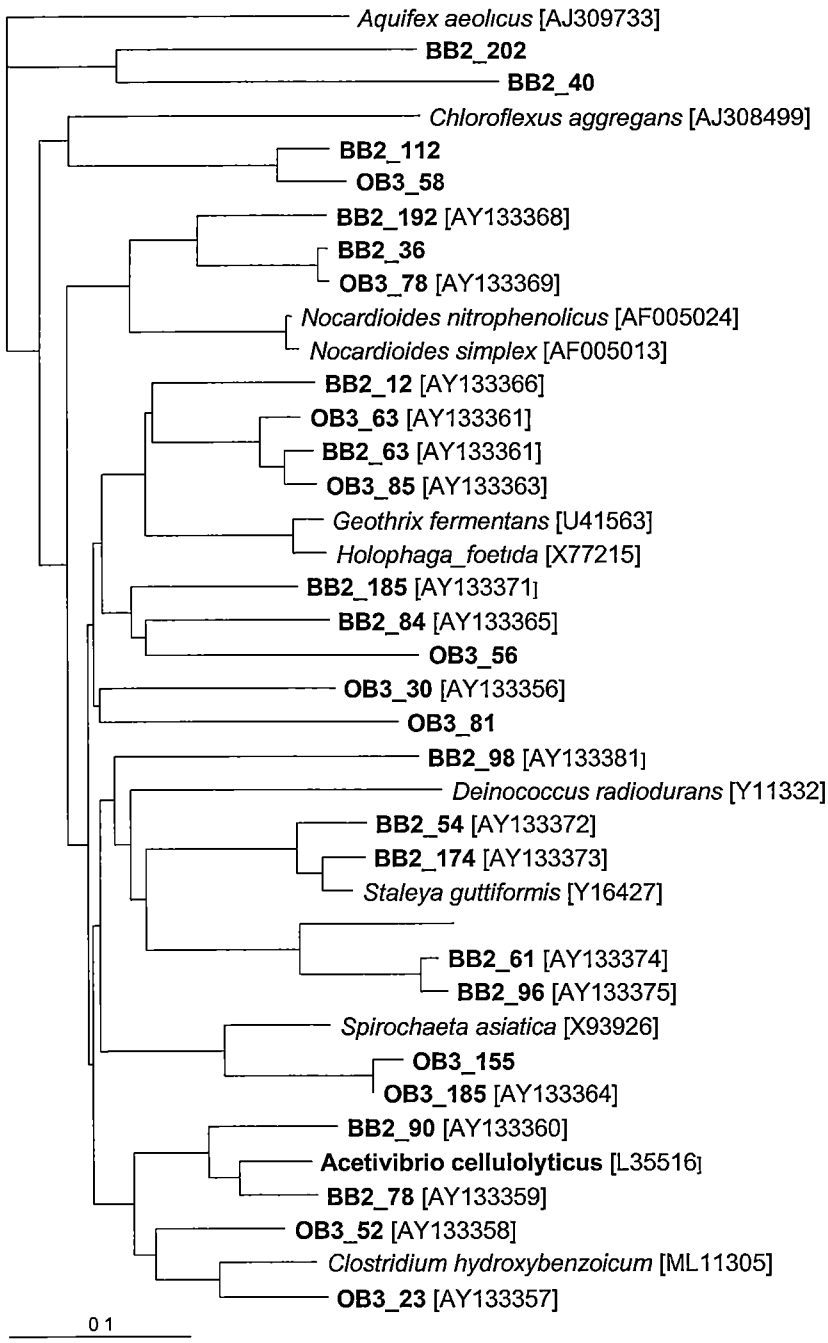

Figure 2.2f: Phylogenetic tree containing all other sequences found in the O'Brien Bay (numbers beginning with OB3) and Brown Bay (numbers beginning with BB2 clones libraries.

2.3.2 Presence of Desulfobacula-type sequences

The amount of SRB 16S rRNA gene DNA, total 16S rRNA gene DNA and the ratio of the two in each sample is shown in Table 2.1. The SRB primers amplified DNA in every sample. The concentration present ranged from 0.03 to 0.8 pg μl^{-1} and the concentration of total 16S DNA ranged from 1900 - 39 000 pg μl^{-1}. The ratio of SRB DNA to total DNA in each sample was used to compare O'Brien Bay and Brown Bay. As the amount of DNA present in each sample was dependent on the amount of sediment used for DNA extraction and on the efficiency of the extraction, comparing the ratio of SRB 16S rRNA gene DNA to total 16S 16S rRNA gene DNA was more useful than just comparing the absolute amounts of SRB 16S rRNA gene DNA. Using the Students t-test, there was a small but significant difference between the two groups ($p = 0.009$).

Table 2.1: Relative amounts of SRB and total 16S rRNA gene DNA (pg μl^{-1}) in O'Brien Bay (OB1 - OB3) and Brown Bay (BB2 - BB4) samples

	OB1				OB2				OB3			
SRB	0.04	0.07	0.04	0.18	0.06	0.02	0.65	0.08	0.31	0.05	0.06	0.80
Total x 1000	4.10	10.1	3.5	24.3	2.0	1.9	18.6	15.8	13.6	4.2	6.2	38.5
Ratio x $1x10^6$	9.2	7.2	10.3	7.6	28.3	8.2	35.2	5.3	23	11.1	9.5	20.8

		BB2				BB3				BB4	
SRB		0.27	0.35	0.25	0.20	0.24	0.27	0.53	0.50	0.05	0.37
Total	x 1000	6.9	12.0	7.0	15.9	5.2	8.2	26.4	20.1	3.3	19.1
Ratio x $1x10^6$		38.8	28.8	36.4	12.9	46.2	33.0	20.0	24.8	14.1	19.5

2.4 Discussion

The two clone libraries described here are the first recorded for near-shore Antarctic marine sediments. However, as they were constructed from only two samples, it is difficult to say how representative they are of the two bays. Clone library generation and analysis is very labour intensive and it is not possible to adequately replicate samples to be sure of characterising all the diversity of a region. The sequences obtained are only a fraction of those present in the sediment, estimated at 33 and 25% coverage for Brown Bay and O'Brien Bay respectively. With such low coverage, it is difficult to draw statistically robust conclusions about the differences or similarities between the libraries although a qualitative comparison is still useful. Both libraries contain a diverse array of sequences as indicated by both the Shannon and Margalef indices. Most of the sequences belong to the gamma proteobacteria, followed by the bacteriodetes group (Figure 2.1). A large number of sequences belong to the delta proteobacteria and a smaller number to the Verrucomicrobia and Planctomycetes. Within the delta proteobacteria and bacteriodetes groups, most of the phylotypes represented on Figures 2.2 are related to cultured organisms. However, in the other groups, particularly the Verrucomicrobia and Archaea, most of the clone clusters are not related to cultured strains. This is a reflection of the difficulties of growing many microorganisms. A substantial proportion are known only by their sequences and nothing is known of their growth requirements or metabolic capabilities.

It was expected that only very large differences in the underlying microbial populations would be reflected in the clone libraries. Since both samples have a similar diversity, both contain the same major phylogenetic groups and a large number of closely related phylotypes (see Figures 2.2) it is likely that they are quite

similar. The method used by Singleton *et al.* (2001) to compare clone libraries also found no significant difference between these two libraries. Other studies comparing marine sediment clone libraries have found similarities between communities from apparently diverse locations. For example, delta proteobacteria, a large group in both the Brown and O'Brien Bay clone libraries, were also a dominant group in clone libraries from lakes in the Vestfold Hills in Eastern Antarctica (Bowman *et al.*, 2000) and Arctic marine sediment (Ravenschlag *et al.*, 1999). Both of these studies also concluded that the diversity of cold marine sediments was surprisingly high. In a study of Antarctic benthic microbial communities, Smith *et al.* (1989) found a significant proportion of the microbial population were sulfate reducing bacteria (4% at Anvers Island and 2% in McMurdo Sound). Stephen *et al.* (1996) recovered several Nitrosospira and Nitrosomonas sequences from marine sediment at a fish farm and a control site. However they could not distinguish whether there were any differences between the polluted and non-polluted sites. Nitrospira-like or nitrosomonas-like sequences were not recovered in this study. This probably reflects the fact that Stephen *et al.* were using targeted primers rather than the universal primers used here.

The most interesting phylogenetic group is the cluster of sequences in the delta proteobacteria that only contains Brown Bay clones (shown in detail in Figure 2.2e). Although the *Desulfobacula* group contains hydrocarbon degrading strains, it is not possible to tell from the 16S rRNA gene sequence alone whether these clones also degrade hydrocarbons. The fact that cultures of these strains are required to determine metabolic capabilities is one of the disadvantages of molecular techniques. Also, because the nature of the construction and sequencing of clone libraries is somewhat random, it is possible that these sequences did occur in O'Brien Bay, but were not sampled as they were less numerous. To this end, primers specific for this

group were designed and real-time PCR was used to probe for the presence of this group in several O'Brien Bay samples.

This group was indeed found in all samples from both Brown and O'Brien Bay (Table 2.1). A significantly greater proportion of all 16S rRNA genes belonged to this group in Brown Bay as compared to O'Brien Bay. However, the significance of this group is still unclear because without cultures, their metabolic capabilities can only be guessed at. They may be more prevalent in Brown Bay because they are able to use the hydrocarbons present in the sediment as a carbon source, or it may be because of other environmental conditions.

The overall results are in keeping with previously observed high microbial diversity in cold marine sediments. The presence of clones related to hydrocarbon degrading sulfate reducers, whilst interesting, is not conclusive proof that biodegradation of hydrocarbons by this group is occurring in Brown Bay. Further investigation of biodegradation in Casey sediments is needed in order to understand the fate of petroleum hydrocarbons in the region.

3. Effect of long-term pollution on benthic microbial communities

3.1 Introduction

It is well known that microbial populations are affected by both heavy metal (Baath, 1989) and hydrocarbon (Aislabie *et al.*, 2001; Delille and Vaillant, 1990) contamination.

A range of hydrocarbons are metabolised by microorganisms: for example alkanes (Engelhardt *et al.*, 2001; Ehrenreich *et al.*, 2000), aromatics (Aislabie *et al.*, 2000) and polycyclic aromatic hydrocarbons (Heitkamp and Cerniglia, 1989). Other hydrocarbons such as hopanes (alicyclic compounds) are more recalcitrant to biodegradation (Atlas, 1981). It has also been suggested that crude oil may have a direct toxic effect on microorganisms (Griffiths *et al.*, 1981). There are numerous studies that have shown changes in microbial populations following petroleum hydrocarbon spills (e.g. Delille and Delille, 2000; Lindstrom *et al.*, 1991). In some cases, the changes occur very quickly. For example Delille *et al.* (2002) describe an experimental oil spill on a sandy subantarctic beach. Within a month the proportion of hydrocarbon degrading bacteria had increased from less than 1% to over 95% of the total microbial population. An even faster response was observed by Griffiths *et al.* (1981) who reported that changes in microbial activity in marine sediment occurred within twelve hours of exposure to crude oil.

Heavy metals also place a selective pressure on microbial populations. In one study Roane and Pepper (2000) found that the addition of cadmium to three soils resulted in a decrease in the number of cultivable microorganisms and also led to the isolation of several cadmium resistant strains. In another study, the addition of a

mixture of metals to soil microcosms resulted in rapid and lasting changes to the original microbial population (Macnaughton *et al.*, 1999b).

As both heavy metal and hydrocarbon contaminants have been present in Brown Bay at Casey for some years, it was hypothesized that the microbial populations would show some signs of adapting to these conditions. Previous work on the benthic fauna in the marine environment surrounding Casey station has shown differences in the infaunal communities that are correlated to the presence of pollutants, the most important of which were heavy metals (Stark *et al.*, 2003a). However, no single pollutant or environmental variable appeared to be important at every location. Infaunal communities in control locations were more diverse than Brown Bay communities and several taxa were not found in any Brown Bay samples. Moreover, some species that are commonly found associated with pollution, such as capitellid polychaetes, were present in Brown Bay but not at control locations.

The aim of the work presented in this chapter was to investigate whether there is any evidence that the microbial populations in the Casey region have changed because of heavy metal and hydrocarbon contamination. To do this, the microbial community structures in pristine and contaminated bays were compared to each other and to patterns of environmental variables such as sediment characteristics and contaminant concentrations. Denaturing gradient gel electrophoresis was used as a fingerprinting technique for community structure. A multivariate statistical approach was taken to analyse the resulting banding patterns and to compare them to environmental variables.

3.2 Methods

3.2.1 Design and sample collection

Samples were collected from the locations within Brown and O'Brien Bays as described in section 2.2.1. Samples were also collected from one location within Sparkes Bay and one location at the Casey Wharf (see Figure 1.1) using the same hierarchical nested design.

3.2.2 DNA extractions

DNA was extracted from frozen sediment samples using the freeze-thaw method described in section 2.2.2.

3.2.3 Denaturing Gradient Gel Electrophoresis

3.2.3.1 PCR

Advantage 2 Taq (Clontech) with the supplied 10X buffer was used for amplification of a fragment of the 16S rRNA gene containing the V3 and V4 regions. Each 50 µl reaction mix contained 5 µl of 10X buffer, 1 µl of taq, 1.25 mM of each deoxynucleoside triphosphate, 20 nmol of each primer and either 20 ng of sample DNA or 1 ng of the standard control DNA mix. The standard DNA mix consisted of 5 ng μl^{-1} each of genomic DNA preparations from four strains grown routinely in our laboratory and chosen because they denatured at a range of different denaturant concentrations. The primers were 907R (CCG TCA ATT CCT TTG AGT TT) and 341F with a GC-clamp (CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG). The touchdown thermal cycling programme consisted of the following steps: initial denaturing step at 94°C for 5 minutes; then 10 cycles of denaturing at 94°C for 1 minute, annealing at 65°C

for 1 minute (decreasing by 1°C each cycle) and extension at 72°C for 3 minutes; followed by 20 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes; final extension at 72°C for 4 minutes and then held at 4°C.

3.2.3.2 Electrophoresis

The DGGE was performed using a D-Code Universal Mutation Detection System (Bio-Rad). Half the volume of the PCR products were run on 6% acrylamide gels with a denaturing gradient of 30 – 65 % (where 100% denaturant is 7 M urea and 40% formamide). Gels were run at 80 V for 16 hours at 60°C in 1 x TAE (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA). Standards were run on either side of the gel and the outside lanes were not used. In order to obtain even heat distribution throughout the tank, the entire tank was placed on a magnetic stirring plate.

Gels were stained in 1:1000 Sybergold (Molecular Probes) in the dark with gentle shaking for approximately twenty minutes. They were then washed once with deionised water and destained with deionised water for twenty minutes before viewing on a UV transilluminator.

3.2.3.3 Image analysis

Photos were scanned in and viewed with the UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from ftp://maxrad6.uthscsa.edu). The best possible banding pattern was obtained by enhancing the contrast and grayscale of the images and in some cases applying a rolling disk background subtraction. This banding pattern was then transformed into a presence/absence matrix for statistical analysis. The standards were used to check for gradient consistency between gels and to assist in comparing the position of bands between gels.

3.2.3.4 Statistical analysis of DGGE banding patterns

A multivariate approach using the Primer5 package (Plymouth Marine Laboratory, U.K.) was used to investigate the DGGE banding patterns. For some analyses, the banding patterns from several runs were pooled and the total presence/absence of bands recorded. Similarity matrices were generated using the Bray-Curtis measure on presence/absence of bands. Non-metric multidimensional scaling plots (MDS) were used to represent the relative similarities between the samples. The stress levels of the MDS plots were generally between 0.1 – 0.2 and cluster analysis (hierarchical agglomerative clustering with group average linkage) was used to check the groupings produced by the MDS procedure. The analysis of similarity (ANOSIM) test (one way) was used to compare groups. ANOSIM R values of 1 indicate that replicates within a location are more similar to each other than to any samples from another location whereas an R value of 0 indicates that there is as much variation within a group as between the two groups being compared. The significance level was also considered, but this was at times affected by the small number of samples in some groups.

3.2.4 Correlation of community structure and environmental variables

The BIOENV procedure in the Primer5 package was used to investigate correlations between environmental variables as reported in Stark *et al.* (2003a) and the microbial community structure described by the DGGE analysis. Total organic carbon (TOC), heavy metals (lead, tin, zinc, copper, iron, antimony, cadmium, chromium, manganese, mercury, nickel and silver) and sediment characteristics (skewness, kurtosis, sorted particle diameter and mean particle diameter) were included in the analyses.

3.3 Results

3.3.1 Evaluation of DGGE reproducibility: PCR and gel effects

The first stage was to evaluate the reproducibility of DGGE banding patterns in the sediment samples. Initially, three samples (one each from Sparkes, Brown and O'Brien bays) were subject to three simultaneous PCR reactions that were then run on the same DGGE gel to provide three PCR replicates for each sample. Each of the replicates was over 95% similar to each other, but the three samples were clearly different to each other. The PCR was repeated on a different day and these reactions run alongside the remaining half reactions from the first round of PCR. Once again, replicates that had been subject to the same round of PCR and run on the same gel were over 95% similar. However for every sample, there were differences between reactions either amplified on different days or run on different gels. The ANOSIM test was used to compare the similarities between each run (Table 3.1). In all three samples, replicates run on the same gel were the most similar whereas those subject to the same PCR reaction but run on different gels, were the most dissimilar. This suggests that differences between gels are the main reason for differences in banding patterns of the same sample.

3.3.2 Microbial community structure

In order to reduce the effects of PCR and gel bias on the banding patterns, all the samples were analysed three times. All samples were amplified in the same round of PCR. This PCR was carried out twice and each sample was run on a total of three gels. That is, one of the rounds of PCR was run on two gels (half the volume of a PCR reaction is loaded onto a gel) and the second round of PCR was run only once. The banding patterns from each run were analysed as described in section

3.2.3.3. The three banding patterns were added together resulting in a matrix where each band was scored from 0 to 3.

The negative image of one of the gels from this analysis is shown in Figure 3.1. The controls in the outside lanes were consistent across all gels used in the analysis. When the results from individual runs were plotted on an MDS, some grouping by site was observed (not shown) although the MDS ordinations were different for each run. Generally, the samples from each location group together although there is some overlap of samples from the different locations in Brown Bay.

The MDS obtained from pooling the banding patterns from each batch together is shown in Figure 3.2 and the ANOSIM R statistic for each pair of locations is given in Table 3.2. Generally, the samples group together within their locations although the Brown Bay locations overlap. O'Brien Bay, Sparkes Bay and Wharf area locations are all distinct (ANOSIM values generally over 0.8). In O'Brien Bay, the two locations from the north side of the bay are more similar to each other (ANOSIM R value of 0.448) than to the location from the south side of the bay (ANOSIM R values of 0.844 and 0.948).

In contrast, the ANOSIM R values for each pair of Brown Bay locations are negative, indicating as much variation within each location as between them. In Figure 3.2 it can be seen that the spread of the Brown Bay locations is greater than the spread of the other locations suggesting a greater heterogeneity in Brown Bay. The two sites within the wharf location are different to each other (ANOSIM R value of 0.8) whereas the two sites within the Sparkes Bay location are more similar (ANOSIM R value of 0.3).

Table 3.1: Comparison of banding patterns for each sample over two PCR amplifications and two DGGE gel runs. A indicates PCR 1/gel 1; B indicates PCR 1/gel 2 and C indicates PCR2/gel 2. ANOSIM R statistics are shown in brackets.

	Sparkes	Brown	O'Brien
Most dissimilar	A,B (0.889)	A,C (1.0)	A,B (1.0)
	A,C (0.556)	A,B (0.704)	A,C (1.0)
Most similar	B,C (0.519)	B,C (0.704)	B,C (0.556)

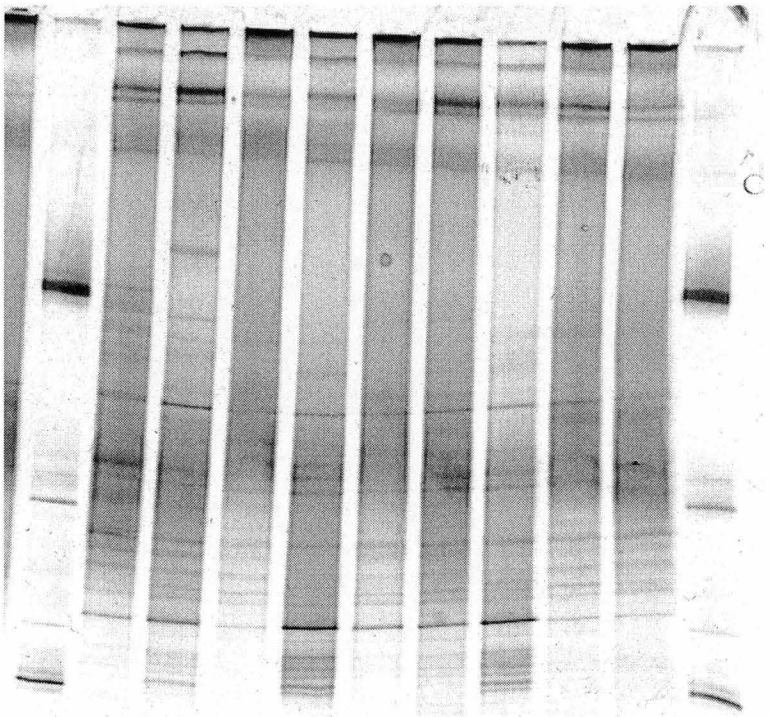

Figure 3.1: Negative image of a photo of a DGGE gel containing nine of the nested samples. The two outside lanes are the standard control DNA mix; lanes 2 and 10 are from Sparkes; Lane 3 is from the Wharf; lanes 4,5,8 and 9 are Brown Bay 3 and lanes 6 and 7 are Brown Bay 4.

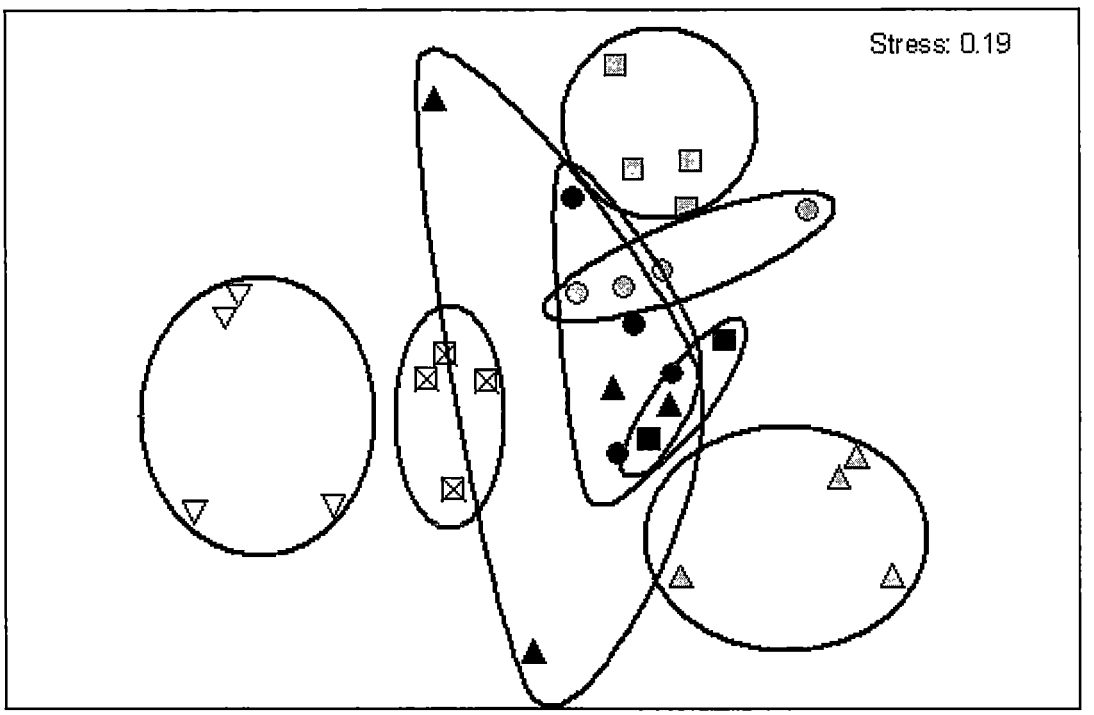

Figure 3.2: MDS showing relative similarities between locations: Brown Bay 2 (▲), 3 (●), 4 (■); O'Brien Bay 1 (◬), 2 (◉), 3 (▣); Sparkes Bay (□) and Wharf (▽). All samples were analysed by DGGE three times, this plot resulted from pooling the three presence/absence matrices.

Table 3.2: ANOSIM values comparing the similarity between pairs of locations based on data pooled from three analyses.

Pairs of Locations	R Statistic	Significance level (%)
Brown2, Brown3	-0.016	54.3
Brown2, Brown4	-0.286	86.7
Brown2, O'Brien1	0.490	2.9
Brown2, O'Brien2	0.255	11.4
Brown2, O'Brien3	0.635	2.9
Brown2, Sparkes	0.406	2.9
Brown2, Wharf	0.875	2.9
Brown3, Brown4	-0.107	73.3
Brown3, O'Brien1	0.839	2.9
Brown3, O'Brien2	0.406	5.7
Brown3, O'Brien3	0.646	2.9
Brown3, Sparkes	0.953	2.9
Brown3, Wharf	0.990	2.9
Brown4, O'Brien1	0.607	6.7
Brown4, O'Brien2	0.804	6.7
Brown4, O'Brien3	0.786	6.7
Brown4, Sparkes	1.000	6.7
Brown4, Wharf	1.000	6.7
O'Brien1, O'Brien2	0.844	2.9
O'Brien1, O'Brien3	0.948	2.9
O'Brien1, Sparkes	0.938	2.9
O'Brien1, Wharf	0.990	2.9
O'Brien2, O'Brien3	0.448	2.9
O'Brien2, Sparkes	0.875	2.9
O'Brien2, Wharf	0.969	2.9
O'Brien3, Sparkes	0.943	2.9
O'Brien3, Wharf	0.969	2.9
Sparkes, Wharf	0.464	2.9

3.3.3 Correlation of community structure to environmental variables

The BIOENV procedure helps define the combinations of environmental variable that best fit the microbial community structure patterns. The environmental data was transformed by several methods including square root, log and fourth root. The square root transform on the environmental data resulted in the best separation of the samples into their locations as seen in MDS plots (Figure 3.3). The correlations from this transform are presented in Table 3.3. However, the same variables appeared in much the same order of importance regardless which of the above transforms was used. From Table 3.3 it can be seen that the highest correlation between environmental variables and the microbial community structure occurs when total organic carbon (TOC), arsenic, iron and manganese are combined ($p = 0.411$). TOC, arsenic, iron and manganese and cadmium consistently appear in the highest correlations. The measures of sediment size and sorting are much less important and rarely appear in the higher correlations for each group of variables.

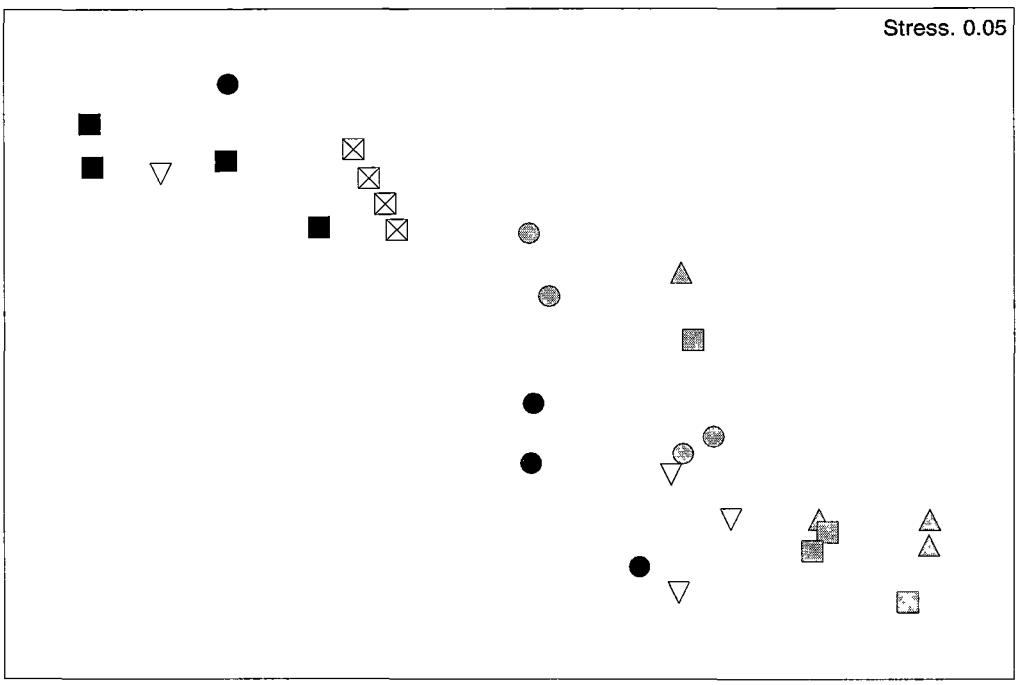

Figure 3.3: MDS showing relative similarities between locations Brown Bay 2 (▲), 3 (●), 4 (■); O'Brien Bay 1 (◬), 2 (◉), 3 (▣); Sparkes Bay (□) and Wharf (▽) based on environmental characteristics reported in Stark *et al.* (2003a).

Table 3.3: Correlations between environmental variables and microbial community structure patterns. All environmental data was subject to a square root transformation. The environmental variables giving the best results when taken *k* at a time are presented. Correlation coefficients are given in brackets; bold indicates the highest overall value of *p*.

k	Combinations of environmental variables				
1	Zn (0.271)	Mn (0.265)	Cd (0.258)	TOC[a] (0.236)	Ni (0.235)
2	TOC, Mn (0.395)	TOC, Cd (0.356)	Zn, Mn (0.345)	TOC, Fe (0.344)	
3	TOC, Fe, Mn (0.405)	TOC, As, Mn (0.401)	TOC, Cr, Mn (0.396)	TOC, Cd, Mn (0.391)	
4	**TOC, As, Fe, Mn (0.411)**	TOC&As&Cd&Mn (0.401)	Zn, TOC, Fe, Mn (0.397)	TOC, particle size[b], Fe, Mn (0.395)	
5	TOC, particle size[b], As, Fe, Mn (0.405)	Zn, TOC, As, Fe, Mn (0.400)	TOC, particle size[b], As, Cd, Mn (0.397)		

a = Total organic carbon; b = mean sorted particle size

3.4 Discussion

3.4.1 Evaluation of DGGE

DGGE banding patterns are known to be influenced by many things: the DNA extraction method used; minor variations in the PCR reaction; the gradient used; gradient and acrylamide variations between gels; the complexity of the microbial community present in a sample and the actual species present. Attempts have been made previously to determine the reproducibility of banding patterns (eg. Murray *et al.*, 1996; Ferrari and Hollibaugh, 1999). However, these mainly compared different rounds of PCR on the same gel. Unfortunately, ecological studies will generate more samples than can be analysed on one gel. In addition, most work attempting to define the sensitivity and reproducibility of DGGE used constructed assemblages (Muyzer *et al.*, 1993; Murray *et al.*, 1996). Whilst this is an effective, controlled approach, these assemblages are much more simple than sediment samples and care should be taken in extrapolating results from DNA mixes to environmental samples. By taking a step-by-step approach, it was possible to show that both gel and PCR effects are important but that differences between gels are slightly more important (Table 3.1). Ferrari and Hollibaugh (1996) found a similar phenomenon. When the same sample was subject to several rounds of PCR and run on the same gel, the patterns were over 90% similar. However, when they repeated a gel, depending on the method used to compare the banding patterns, different samples from the same gel could be more alike than the same sample run on two different gels. They concluded that the 'gel signature' could not be completely removed in the image-processing step. Despite this, the banding patterns generated by repeated analysis of the same sample were more similar to each other than to those from other samples analysed at the same time. That is, at least for the samples

used in this study, the greatest overall influence on banding patterns was differences within the samples themselves. In order to minimise the effect of other factors and allow the sample differences to become the dominant factor, pooling data from multiple runs was found to be an effective solution. These results suggest that analysing a small number of samples only once is not sufficient to determine the relative differences (or similarities) between samples or between sampling locations. Furthermore, without determining the extent of variation in banding patterns caused by gel and PCR effects, banding patterns from different gels should not be compared.

3.4.2 Spatial variation in microbial populations

The ANOSIM tests (Table 3.2) as well as the MDS plot (Figure 3.2) showed that the O'Brien Bay locations were all separate, significantly different groups. Within each location however, the sites and plots are very similar to each other. It is also interesting that the two O'Brien Bay locations that are geographically closer to each other are more similar. This suggests that natural differences in the environment at a scale of kilometres are enough to influence the microbial community structure. On a smaller scale (e.g. hundreds of metres), the communities are much the same, perhaps as a result of homogeneous environmental conditions.

The spatial variation in microbial communities in the polluted Brown Bay locations is very different to the control locations. The Brown Bay locations overlap to a greater extent than the O'Brien Bay locations, but this is probably due to the fact that they are much closer together (300 m apart rather than kilometres apart). The variation within each Brown Bay location is greater than the variation within the control locations. Brown Bay 2, the closest to the tip-site, has two points close together and two outlying points. This is interpreted to be the result of heterogeneous environmental conditions possibly caused by "hot-spots" of

contamination. A sample taken next to a battery fragment for example will have high levels of heavy metals and this will probably influence the microbial community structure.

The Sparkes and Wharf locations both form distinct groups (Figure 3.2). Despite being a control location, Sparkes Bay is slightly more similar to the Brown Bay and Wharf locations than the O'Brien Bay locations. The occurrence of naturally elevated levels of heavy metals in this bay is a possible explanation for this.

Although the locations form distinct groups (in most cases), there is some separation between impacted (Brown Bay and Wharf) locations and non-impacted locations (O'Brien and Sparkes Bays) with Sparkes Bay (which has elevated metal levels) grouping with the impacted locations. The chemistry of the impacted sediments is quite different in each location, Sparkes Bay has some elevated levels of metals, Brown Bay has high levels of heavy metals and some petroleum hydrocarbons and the Wharf location has very high levels of petroleum hydrocarbons. The effect on the microbial communities is not as simple as impacted and non-impacted but perhaps reflects specific components of the pollution and the complexity of heterogeneous environmental conditions.

3.4.3 Correlation of environmental variables with microbial community patterns

In this study TOC levels, in combination with heavy metals, are correlated ($p = 0.4$) with the microbial community structure patterns (Table 3.3). The type and availability of carbon is one of the most important factors influencing the development of a microbial community. Unfortunately data that distinguishes between petroleum hydrocarbons and other organic carbon is not available for these samples. However petroleum hydrocarbons contribute to the total organic carbon

level, which is higher for the impacted locations than non-impacted locations (Table 1.1). Heavy metals also appear to have some influence on the microbial communities, particularly iron, cadmium, manganese, zinc and arsenic. Naturally elevated levels of some of these metals (e.g. cadmium) are found in Sparkes Bay, but others such as iron, zinc and arsenic are far higher at impacted locations (Table 1.1) and are most likely anthropogenic in origin. However, the correlation between these factors and the microbial community structure was not very high. Other factors that have not been measured (for example depth of oxygen penetration into the sediment, hydrocarbon concentrations) may also be just as important.

3.4.4 Conclusions

In this work an attempt was made to define the extent to which gel and PCR biases affected the DGGE banding patterns of sediment microbial communities. In order to obtain reliable results from DGGE, these results suggest that multiple analyses of large numbers of samples should be undertaken and the banding patterns pooled; when large numbers of samples are analysed statistically DGGE can be a very useful tool in microbial ecology.

Sediment microbial communities are very diverse and an array of environmental factors influence patterns of community variability. In this study, variation was observed between control locations as well as between control and impacted locations. However, microbial community structure patterns did correspond to environmental variables, especially those that are anthropogenic in origin.

4. Effect of hydrocarbons on benthic communities

4.1 Introduction

The response of the microbial community to hydrocarbon contamination affects the response of other trophic levels in the benthic ecosystem, as microbes remove hydrocarbons from the sediment and incorporate them into the food chain (Danovaro, 2000). Previously Griffith *et al.* (1981, 1982) found that the addition of petroleum hydrocarbons to sediment increased the number and activity of benthic microbes. More recently changes in microbial community structure upon addition of hydrocarbons have also been observed. For example DGGE and phospholipid analyses showed a significant shift in the microbial community composition during bioremediation of an experimental coastal oil spill (MacNaughton *et al.*, 1999a). However a significant difference between the microbial community structure found in contaminated and control sites is not enough evidence to conclude that specific contaminants have caused these differences.

In Chapter 3 it was concluded that total organic carbon in combination with some heavy metals was, to some extent, correlated with the microbial community structure. A previous survey of the benthic soft-sediment infauna in the Casey region also found that the communities in impacted locations were significantly different to those found in control locations (Stark *et al.*, 2003a). To show that pollutants such as hydrocarbons and heavy metals were the likely cause of these differences, a sediment recruitment experiment was carried out at Casey in the summer of 1998-1999 (Stark *et al.*, 2003b). Sediment was defaunated, artificially contaminated and then deployed in three different locations (Brown, O'Brien and Sparkes Bays - see Figure 1.1) for eleven weeks. The infauna that recruited to each

treatment were then identified and counted. The diatom communities that developed in the sediment were also examined (Cunningham *et al.*, 2003) and the hydrocarbon concentrations were measured in some samples.

It was concluded that the hydrocarbon treatment, a mixture of SAB diesel and lubricant oil, had a significant effect on the recruitment of infauna to the sediment. In Sparkes and Brown Bays there were significantly less crustaceans in the hydrocarbon treatment compared to the controls, although the total number of fauna was greater. Multivariate comparison of the hydrocarbon and control treatments within each location also showed significant differences in the infaunal communities in Sparkes and Brown Bays but not in O'Brien Bay. In addition, the location that the sediment was deployed in was also important. Communities from O'Brien, Sparkes and Brown Bays were all different to each other regardless of the treatment applied to the sediment. Similar conclusions were drawn from the diatom study - that is, both the hydrocarbon treatment and the deployment location had a significant effect on the diatom communities. This was attributed more to changes in abundance of diatom species than to changes in the species present.

Analysis of the hydrocarbon concentrations in samples from each bay indicated that the SAB diesel hydrocarbons had degraded in all locations, but significantly more in Brown Bay which showed high levels of biodegradation (unpublished data). The short-chain *n*-alkanes were barely detectable and in comparison to the pre-deployment samples, the concentrations of the isoprenoids (which are more recalcitrant to biodegradation than alkanes) had decreased. It is possible that the microbial communities in Brown Bay, which has been contaminated for at least twenty years, have adapted to utilising hydrocarbons as a carbon source. Therefore new sources of hydrocarbons in the sediment could be utilised more efficiently in Brown Bay than in other locations.

The observed differences in recruitment of infauna and diatoms and the different extent of biodegradation in the three locations suggest that the microbial communities in each location are different. To investigate this hypothesis and to assess the effect of the hydrocarbon treatment on benthic microbes, the microbial communities in the hydrocarbon and control treatments from Brown, O'Brien and Sparkes Bays were analysed by DGGE.

4.2 Methods

4.2.1 Experimental design and sampling

The experimental design is described in detail in Stark *et al.* (2003b). Briefly, sediment was collected from O'Brien bay, a pristine site near Casey station (see section 2.2.1). To defaunate the sediment it was frozen to -20°C , thawed and sieved through a 500 μm screen. The sediment was allowed to settle and excess water was removed. This sediment was then split into treatments. Nothing further was done to the control treatment. A mixture made up of 50 ml of SAB and 25 ml of synthetic lubricant oil was added to 55 l of sediment to construct the hydrocarbon treatment. It was stirred in and an additional 15 l of seawater was added to the slurry. This was allowed to settle overnight before the excess water was removed. Pre-deployment samples were collected at this time.

The sediment was placed into plastic flowerpots (12 cm deep and 12 cm in diameter) that had 3 holes (8 cm x 8 cm) cut in the side and a 9 cm hole in the bottom. The holes were covered by mesh to retain the sediment but allow water to flow through the pots. Three trays of six pots were deployed in Brown, Sparkes and O'Brien Bays (see figure 1.1) for approximately 11 weeks during the summer. At the end of this time the trays were retrieved by diver and sediment from two pots from

each tray were frozen at -20°C for chemical analysis and DNA extraction. The frozen sediment was divided into surface (< 2 cm) and sub-surface samples. Only the surface samples were analysed in this study.

4.2.2 Analysis of microbial community structure by DGGE

4.2.2.1 Electrophoresis

DNA was extracted from the frozen sediment samples as described in section 2.2.2 with an extra incubation step at 37°C for 20 minutes before the first cycle of freeze-thaw. Two rounds of PCR amplification were carried out as described in section 3.2.3.1. Conditions for the denaturing gradient gel electrophoresis were similar to those described in section 3.2.3.2 with some changes. The denaturing gel was poured (6% acrylamide, 30 – 65 % denaturant) to approximately 2 cm below the bottom of the wells. A stacking gel of 10% acrylamide (0% denaturant) was poured on top. The gels were pre-run at 80V for 30 minutes before the wells were flushed out and half the volume of the PCR product was loaded. Gels were run at 80 V for 16 hours at 60°C in 1 x TAE. After the first 15 minutes, the run was paused whilst the wells were washed out again. Standards were run on either side of the gel and the outside lanes were not used. In order to obtain even heat distribution throughout the tank, the entire tank was placed on a magnetic stirring plate.

Gels were stained in 1:1000 Sybergold (Molecular Probes) in the dark with gentle shaking for approximately twenty minutes. They were then washed once with deionised water and destained with deionised water for twenty minutes before viewing on a UV transilluminator.

Photos were taken with a digital camera and viewed with the UTHSCSA ImageTool program. The best possible banding pattern was obtained by enhancing the contrast and grayscale of the images and in some cases applying a rolling disk

background subtraction. This banding pattern was then transformed into a presence/absence matrix for statistical analysis by scoring each band as present (1) or absent (0). The standards were used to check for gradient consistency between gels and to assist in comparing the position of bands between gels.

4.2.2.2 Statistical analyses

The Primer5 package was used to investigate the banding patterns. The presence / absence data for each sample from three DGGE runs was combined such that for each sample, every band had a score of 0, 1, 2 or 3. A similarity matrix was constructed using the Bray-Curtis measure and a presence/absence transformation of the data. Non-metric multidimensional scaling (MDS) plots were constructed and the ANOSIM procedure (one-way) was used to compare groups.

In section 3.3.1, it was observed that differences between gels contributed significantly to differences in the banding patterns obtained from multiple analyses of the same sample. To overcome this effect, samples were analysed multiple times and the presence / absence data combined. This procedure was also carried out for the current set of samples. In addition, the variation in banding patterns due to gel variability, PCR run variability and sample heterogeneity within a treatment group were explored by looking at the similarity coefficients between pairs of samples. The Bray-Curtis similarity coefficient was calculated on the presence / absence data for each pair of samples where a sample was considered to be a single sample, amplified in a single round of PCR and run once on a gel. The similarity coefficients were also calculated for the same sample amplified within the same round of PCR and run on two different gels and was also calculated for the same sample amplified in different rounds of PCR. The mean and range of the similarity coefficients between different samples in the same treatment group were determined (Table 4.1).

To compare treatment groups whilst excluding PCR and gel-to-gel variability, only samples amplified in the same round of PCR and run on the same gel were compared to each other. The similarity coefficients for each group of comparisons (eg. O'Brien control - O'Brien hydrocarbon) were collated over all the gels and the mean and range determined.

4.3 Results

4.3.1 DGGE reproducibility and variation due to gel and PCR variability

By comparing the similarity of the banding pattern from a single sample subject to either different gel runs or both different PCR and different gel runs, an estimate of the variability due to gel and PCR effects was obtained. For samples for which the same PCR round was run on different gels ($n = 9$), the mean similarity was 54% (range 44 - 86%) and for samples subject to both different PCR rounds and different gel runs ($n = 32$) the mean similarity was 57% (range 22 - 100%). The samples subject to both sources of possible variation had a wider range of similarities than those subject only to one source of variability.

A measure of sample heterogeneity within the same treatment group is shown in Table 4.1. When the similarity coefficients are calculated on samples run in the same round of PCR and on the same gel (column A), the mean similarity is generally higher than when the coefficients are calculated on samples run either in the same round of PCR but on different gels (column B) or in different rounds of PCR and different gels (column C). In nearly every case, as the number of sources of potential variation increase, the mean similarity decreases. It was expected that replicate samples for each same treatment group run on the same gel would be very similar,

but the mean similarity coefficients range from 52 to 89%. It is interesting to note that the homogenized pre-treatment samples had the highest similarity coefficients.

Table 4.1: Mean similarity coefficients (%) calculated between samples from the same treatment group.

	A: Within same gel (same PCR round)		**B: Within PCR round (different gel)**		**C: Different PCR round and gel run**	
Treatment group	*n*	mean (range)	*n*	mean (range)	*n*	mean (range)
Pre-deployment	2	89 (77-100)	4	71 (67-75)	8	55 (40-67)
O'Brien control	4	57 (22-90)	8	71 (50-86)	12	57 (40-67)
O'Brien hydrocarbon	11	56 (22-80)	28	52 (18-89)	17	44 (20-91)
Sparkes control	1	86	4	55 (40-67)	8	51 (40-67)
Sparkes hydrocarbon	10	53 (25-100)	22	56 (36-100)	18	53 (17-75)
Brown control	5	52 (40-67)	11	50 (29-67)	9	54 (33-75)
Brown hydrocarbon	12	73 (43-100)	30	62 (33-91)	30	56 (40-92)

4.3.2 Effect of location and hydrocarbon treatment

Figure 4.1a shows the relative similarity between all the samples on a single MDS. For clarity, the control and hydrocarbon samples are then presented as two separate MDS plots (Figure 4.1b and c). The ANOSIM values describing the strength of the similarities / dissimilarities between these groups and between the control and hydrocarbon samples within each location are given in Table 4.2. It can be seen from Figure 4.1a that there is no strong overall separation between either the different locations or between the control and hydrocarbon treatments.

The mean similarity of different treatment groups was calculated by pair-wise comparison of samples that were run on the same gel. These paired comparisons were ranked by mean similarity and divided into four groups from most similar to most different (see Table 4.3). The most similar comparisons were samples from within the same treatment group whilst the most dissimilar were the comparisons of pre-deployment samples to those deployed in Sparkes and Brown Bays. These rankings generally support the results of the ANOSIM tests shown in Table 4.2 and both statistical analyses detected differences between the pre-deployment samples and some of the post-deployment treatments. The hydrocarbon treatments deployed in Sparkes and Brown Bays were the most significantly different. The implication is that both location and hydrocarbon treatment had an effect on the development of the sediment microbial communities.

The effect of location on the development of the microbial community structure was tested by comparing the control treatments to each other. None of the control treatments at any location are significantly different by the ANOSIM test (Table 4.2: significance levels >10%), possibly because the variation within each location is greater than the variation between locations (Figure 4.1b). The similarity coefficients in Table 4.3 also suggest that the control treatments are not different to

each other. However, comparison of the hydrocarbon treatments to each other (Figure 4.1c) reveals that the samples from Sparkes Bay group closely together and are significantly different from both Brown and O'Brien Bays using the ANOSIM test (Table 4.2: significance levels < 5%). The hydrocarbon treatments in Brown and O'Brien Bays are not significantly different to each other. The comparison of mean similarity coefficients in Table 4.3 also indicate that the hydrocarbon treatments are different from each other, most significantly between Sparkes and O'Brien bays.

The effect of the hydrocarbon treatment on the microbial communities was determined by comparing the control to the hydrocarbon treatment within each location. The ANOSIM values in Table 4.2 show that there is a significant difference between the two treatments in O'Brien Bay but not in either Sparkes or Brown Bay. The ranking of mean similarity coefficients (Table 4.3) also suggests that the difference between the control and hydrocarbon treatments is most significant in O'Brien Bay.

Table 4.2: ANOSIM values comparing similarities of control and hydrocarbon treatments between and within locations. Significant differences are in bold.

Comparison	R statistic	Significance level (%)
Between pre-deployment samples and post-deployment treatments		
O'Brien control	0.287	17
O'Brien hydrocarbon	0.238	17
Sparkes control	0.167	20
Sparkes hydrocarbon	**0.460**	**4**
Brown control	0.324	9
Brown hydrocarbon	**0.500**	**2**
Between control treatments		
O'Brien, Brown	0.266	14
O'Brien, Sparkes	-0.083	57
Brown, Sparkes	-0.083	63
Between hydrocarbon treatments		
O'Brien, Brown	0.043	29
O'Brien, Sparkes	**0.325**	**1**
Brown, Sparkes	**0.289**	**3**
Within location: control versus hydrocarbon treatment		
O'Brien	**0.367**	**2**
Brown	-0.147	84
Sparkes	0.259	11

Table 4.3: Ranking of treatment groups from most to least similar based on mean similarities calculated between samples from the same round of PCR run on the same gel. The mean similarity is given in brackets.

Similar			**Different**
Similarity > 70%	**Similarity 60 - 69%**	**Similarity 50 - 59%**	**Similarity < 50%**
Pre	Brown C - Brown H	O'Brien C	pre - Brown C
(89)	(66)	(57)	(48)
Sparkes C	Sparkes C - Brown C	O'Brien H	O'Brien H - Sparkes H
(86)	(66)	(56)	(47)
Brown H	O'Brien C - Sparkes C	O'Brien C - Brown C	pre - Sparkes C
(73)	(65)	(56)	(46)
	Sparkes C - Sparkes H	pre - O'Brien C	pre - Brown H
	(60)	(55)	(46)
	Pre - O'Brien H	O'Brien H - Brown H	pre - Sparkes H
	(60)	(53)	(41)
		Sparkes H	
		(53)	
		Brown C	
		(52)	
		O'Brien C - O'Brien H	
		(51)	
		Sparkes H - Brown H	
		(51)	

Pre = pre-deployment, C= control treatment, H = hydrocarbon treatment

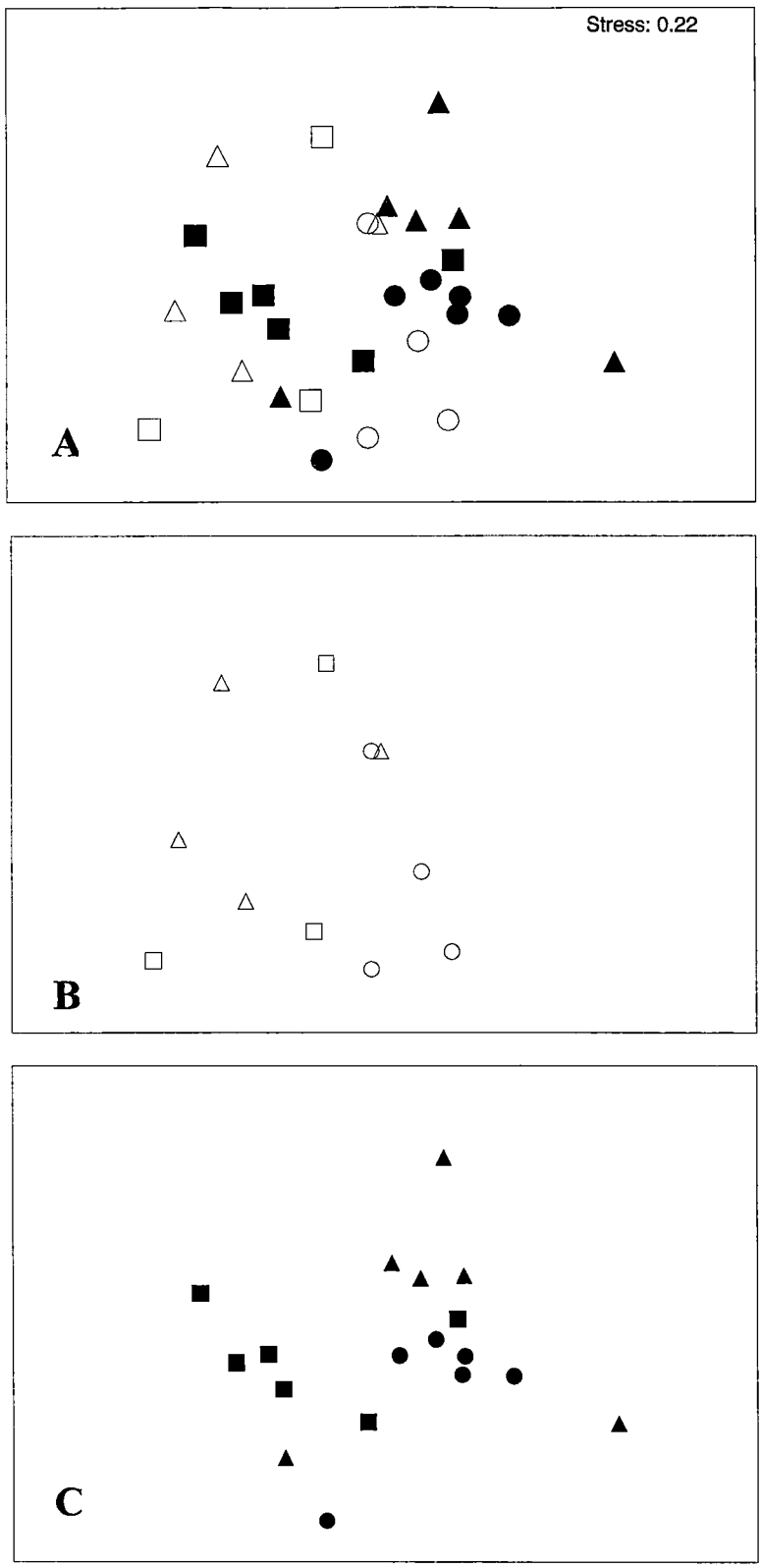

Figure 4.1: MDS showing the relative similarities between the control samples (hollow symbols) and hydrocarbon treatment samples (shaded symbols) deployed in Sparkes (■), Brown (●) and O'Brien Bays (▲). All samples are shown in panel A, for clarity the control (B) and hydrocarbon (C) samples are also presented separately.

4.4 Discussion

4.4.1 Methodological issues

4.4.1.1 Experimental design

The original purpose of this study was to investigate the response of the benthic infauna to hydrocarbon and heavy metal contamination (Stark *et al.*, 2003b). This was done by comparing recruitment of the infauna to each sediment treatment and for this reason it was important to thoroughly defaunate the sediment first - hence the freeze-thaw and sieving. Unfortunately this is not ideal for studying microbial populations as the freeze-thaw treatment would have killed most, but not all, of the sediment microbes. Although all the treatment groups had the same remnant microbial population at the time of deployment, at the end of the eleven weeks *in situ* incubation the microbial communities were a combination of microbes that survived the freeze-thawing and those recruited from the surrounding environment. The differences between the pre-deployment samples and some post-deployment treatment groups suggest that the microbial communities did develop and change over the incubation period. However, because of the remnant microbial population, it is not possible to attribute the response of the microbial communities entirely to differences in the existing microbial populations in the different locations.

4.4.1.2 DGGE variability and diversity of the microbial community

As an evaluation of the DGGE method, it is reassuring that the pre-deployment substrate, originally from O'Brien Bay, changed most when redeployed into Sparkes and Brown Bays. However, the similarity of the banding pattern obtained from the same sample analysed either in different PCR rounds or on different gels was low (54 or 57%). This indicates that for each pair of banding patterns compared, only about half the bands were present in both. It appears that with each round of PCR or

DGGE, bands from different species are detected. This suggests that not only is the diversity in these communities high (as already seen in Chapter 2) but the population is quite evenly distributed. If the communities were dominated by a few species the same bands would always be present.

The high degree of variation in banding patterns between samples from the same treatment group - ranging from 52 to 89% - suggests that there is also a large amount of sample heterogeneity. This range is probably a realistic estimate of small-scale sample variability. Despite this, the same trends are seen in both the ANOSIM values (Table 4.2) and the mean similarity coefficients (Table 4.3).

4.4.2 Effect of location

The effect of the sediment deployment location on the microbial communities is complex. The pre-deployment samples are significantly different to the hydrocarbon treatment from Brown and Sparkes Bay but only marginally different to the Brown Bay control treatment and not significantly different to the O'Brien Bay treatments or the Sparkes Bay control (Tables 4.2, 4.3). As the sediment was originally collected from O'Brien Bay the similarity of the pre-deployment samples to the O'Brien Bay treatments is not surprising. The fact that the communities that developed in Sparkes and Brown Bay are different to the pre-deployment samples suggests that the location of deployment has had an effect. The effect of location should have been most obvious in the comparison of the control treatments to each other. However the control treatments in the different locations are not significantly different to each other (Figure 4.1b, Table 4.2), partially because of the large variation within each location and presumably also because of the large diversity within each sample. In the MDS plot of the hydrocarbon treatments (Figure 4.1c), the samples from Sparkes Bay overlap slightly with the Brown and O'Brien Bay

samples but the ANOSIM test indicates that they are significantly different (Table 4.2). It is likely that the hydrocarbons placed a selective pressure on the microbes, favouring those that are able to utilise hydrocarbons or their degradation products. As the overall microbial population in Sparkes Bay is different to Brown and O'Brien Bays (see section 3.3.2), the component capable of hydrocarbon degradation is probably also different. Similarly, as the overall microbial communities in Brown and O'Brien bays are similar (see section 3.3.2), it is not surprising that similar populations have developed in the hydrocarbon treatments in those two bays. Perhaps this pattern was not observed in the control samples because all the samples started with the same microbial population and diverse communities developed. This diversity resulted in the large within location variation that made differences between locations impossible to distinguish.

4.4.3 Effect of hydrocarbon treatment

O'Brien Bay was the only location for which there was a significant difference between the control and hydrocarbon treatments (Table 4.2, 4.3). This may be due in part to the wide variation in the control samples within each location. Another consideration is that DGGE only detects differences in composition and not absolute abundance. If it had been possible to measure bacterial abundance more differences between the control and hydrocarbon populations within a location might have been observed as other studies in Antarctic soils have found that hydrocarbon contamination can stimulate the numbers of both hydrocarbon degrading and total heterotrophic bacteria (Aislabie *et al.*, 2001; Delille, 2000). Chemical analysis of the hydrocarbon treatments showed that biodegradation had occurred at all three locations, but significantly more so in Brown Bay (unpublished data). This would indicate that the microbial community in Brown Bay was more efficient at

hydrocarbon biodegradation. One explanation for the lack of significant difference between the control and hydrocarbon treatment is that the DGGE is not detecting those species responsible perhaps because the richness and evenness of the populations is too great. However if this were the only explanation there wouldn't be a significant difference in O'Brien Bay either. It is also possible that the biodegradation occurred early in the incubation period and by eleven weeks, the degradable hydrocarbons were depleted. If hydrocarbon biodegradation were no longer a major process the microbial community may have returned to a state that resembled the control treatment. As biodegradation was still occurring in O'Brien Bay, differences between the control and hydrocarbon treatments were still detectable. In Sparkes Bay the control versus hydrocarbon comparison is nearly significant suggesting that it is intermediate to the other two locations.

4.4.4 Comparison with effects on benthic infauna and diatom communities

The hydrocarbon treatment had a different effect on the infaunal (Stark *et al.*, 2003b) and diatom (Cunningham *et al.*, 2003) communities compared to the microbial communities. For the microbial communities the difference between the control and hydrocarbon treatment within a location was only significant at O'Brien Bay. However this difference was also significant in Brown and Sparkes Bay for the infaunal and diatom communities. The difference between control and hydrocarbon treatments was actually less in the recruitment of infauna to O'Brien Bay and this was attributed to the fact that the O'Brien Bay infauna is less stressed by existing pollutants. The diatom communities were most similar in O'Brien and Sparkes Bay whereas the microbial communities are most similar in O'Brien and Brown Bay (see also section 3.3.2).

The basis of the response of microbial communities to hydrocarbon contamination is fundamentally different from that of the diatoms and infauna. Only the microbes are capable of utilising hydrocarbons as a carbon source. Changes in community structure of the diatoms and infauna are most likely due to sensitive species avoiding the contaminated sediment but changes in the microbial community are more likely due to species capable of utilising hydrocarbons becoming dominant. Regardless of the type of response, it is significant that all three (microbes, diatoms and infauna) were affected by the hydrocarbon treatment.

4.4.5 Conclusions

The results of the nested survey presented in Chapter 3 indicated that pollution is one of a number of factors affecting the microbial populations in the Casey region. The results of this chapter provide more evidence that hydrocarbons have an effect on the sediment microbial communities although the exact changes and the mechanisms by which they occur are yet to be elucidated. The location of deployment of the sediment also had an effect on the microbial communities. However the most significant effect was a combination of hydrocarbon treatment and location. The implication of this is that hydrocarbon contamination will have a different effect on the microbial community depending on where it occurs.

Detailed analysis of the variation in the DGGE banding patterns of multiple analyses of the same sample provided an indication of the diversity of the microbial community. The species richness of the communities is not surprising. The fact that different bands appear in each DGGE run implies that the abundance of species is even, that is, that the communities are not dominated by a few species.

Further work on the benthic ecosystem will need to involve a compromise between the requirements for studying the fauna and the microbial population as well

as considering the best way to sample for chemical analyses. A purely molecular approach was taken in this work because the samples had been frozen. With fresh samples, a polyphasic approach involving viable counts, isolation of hydrocarbon degraders and an estimate of total bacterial numbers would be possible to complement a molecular estimate of microbial diversity.

5. Short term effects of four different oils on Antarctic benthic microbial communities

5.1 Introduction

Human activities in the Antarctic are heavily reliant on petroleum and synthetic oil products for transport, power and to heat buildings. Arctic-grade diesel is commonly used for power generation and as a fuel for ships and vehicles. A wide range of lubricating oils are also used in vehicles and heavy machinery. Given the harsh operating conditions and the quantity of oil that is used in the Antarctic, there is a significant risk of spills entering the sensitive marine environment. Large terrestrial spills have been recorded at several scientific stations including Admunsen-Scott base (Wilkness, 1990), Casey station (Deprez *et al.*, 1999), McMurdo station (Tumeo and Wolk, 1994) and near the Alfred Faure base (Delille and Pelletier, 2002). The largest spill in the Antarctic occurred when the *Bahia Parisio* went aground on the Antarctic peninsula spilling 680 000 l of diesel into Arthur Harbour near Palmer Station (Kennicutt, 1990). Marine spills of this type are amongst the most damaging as they cause immediate ecological impacts on many trophic levels (I. Snape pers. comm.). However terrestrial spills can continue to impact on the marine ecosystem for decades after the initial spill event as contaminants continue to leach into adjacent marine environments.

Although fuels are toxic, many of their components are not as persistent in the environment as other oils. Petroleum products consist mostly of hydrocarbons, such as long chained alkanes and aromatic compounds, for which there is extensive evidence of microbial degradation (Atlas, 1981). Other oils such as synthetic lubricants have three main components - alpha olefins, triesters and alkylated

aromatics (including substituted naphthalenes). In addition they contain additives such as antioxidants which are often more recalcitrant to biodegradation. Used lubricant generally has more aromatics than the clean product so it is possible that it will have a different susceptibility to biodegradation and therefore different longevity in marine sediments. Recognising that lubricants are persistent and potentially toxic, recently developed products are being marketed as biodegradable. These products are derived from natural oils such as rapeseed and predominantly contain ester isomers of long chained fatty acids and alcohols. Biodegradation of these esters has been reported as occurring both in pure cultures such as *Micrococcus* sp. (Wright *et al.*,1993) and in microcosms (Sonderkamp *et al.*, 2001). However it is unknown how toxic and biodegradable these oils are in the marine environment when compared with cheaper conventional synthetic lubricants. In cold regions where organisms are potentially more sensitive to the effects of oils, (Poland *et al.*, 2003) any differences could have profound implications for the impacts of oil spills.

To make informed decisions on choice of product and procedures for handling these oils in the Antarctic, comparative information on the effects and longevity of different oils is required. After the initial abiotic weathering processes, degradation by microorganisms is the main pathway for removal of oil contaminants from the environment. Understanding the microbial community response to contamination will assist in understanding the potential effects of oil spills on the marine ecosystem.

Although there is little information relating to oil impacts for Antarctica, the development of the petroleum industry in Alaska in the 1980s resulted in research into the effects of crude oil on Arctic marine ecosystems. Studies by Griffiths and co-workers (1981, 1982) followed the long-term effects of crude oil on microbial communities in subarctic sediments. They measured carbon dioxide production,

glucose uptake, nitrogen fixation, potential denitrification rates and various enzymatic activities and observed considerable differences between control and oil amended sediments (Griffiths *et al.*, 1981). In one study (Griffiths *et al.*, 1982), a reduction in glucose uptake rates was observed within twelve hours of exposure to crude oil suggesting that crude oil had a direct inhibitory effect on benthic microorganisms. After eighteen months *in-situ* incubation, a reduction in glucose uptake was still apparent and it was calculated that the microbial communities would take at least six years to recover. Another *in-situ* investigation was carried out by Haines and Atlas (1982) in the Beaufort Sea. Sediment was artificially contaminated with crude oil and the microbial populations and hydrocarbon concentrations were investigated over a two year period. They found no evidence for either biodegradation or abiotic weathering of hydrocarbons until after a year. A slow increase in the numbers of hydrocarbon degraders eventually resulted in a limited loss of low molecular weight alkanes and aromatic compounds in the following year.

Crude oil spills are currently not a source of pollution in Antarctica. For this reason it was decided to focus this study on diesel and various synthetic oils that are used on Australian research stations. Previous studies in the Casey region have made some progress towards understanding how benthic diatom and infaunal communities respond to petroleum pollution (Cunningham *et al.*, 2003; Stark *et al.*, 2003b). It was found that there were significant differences between the communities that developed in sediment artificially contaminated with a mixture of lubricant and diesel and those that developed in clean sediment. The results of Chapters two and three showed that there were also some differences between the microbial community structure in impacted and non-impacted marine sites near Casey. To build on these studies and specifically determine the effects of different oils on microbial communities, a multi-disciplinary field experiment was initiated in 2001.

Four products were tested *in-situ*: Special Antarctic Blend diesel (SAB); a synthetic lubricant (Mobil OW40); the same lubricant after use in a vehicle and a biodegradable lubricant (Fuchs Titan GT1).

In this chapter the short-term effects of these oils on the benthic microbial communities are presented. The first aim was to confirm that the experimental methodology did not significantly change the microbial community. The microbial response to the pollution after a short (five week) exposure was then examined using a polyphasic approach involving direct counts, most probable number counts and denaturing gradient gel electrophoresis.

5.2 Methods

5.2.1 Sediment collection, treatment, deployment and retrieval

Sediment was collected from O'Brien Bay (66°18'S 110°32'E), a pristine embayment in the Windmill Islands near Casey Station (see Figure 1.1 and section 2.2.1). Sediment was collected into buckets by divers and was pooled once on the surface. After transport back to the station, the sediment was sieved through a 500 μm sieve to defaunate it before dividing into 5 portions of about 35 l each. The sediment was allowed to settle overnight. The overlying water in each portion was reduced to *ca* 7 l and the sediment - water mixture was homogenised to a slurry before addition of one of four treatments. Treatments comprised of either 115 ml of Titan GT1 (biodegradable oil treatment), 115 ml of Mobil OW40 (lubricant treatment), 115 ml of used Mobil OW40 (used lubricant treatment) or 49 ml of a mixture of 97 % special Antarctic blend diesel with 3 % squalene (SAB treatment) added to it. Each sediment treatment was stirred for 5 minutes and then allowed to settle overnight. The next day, prior to deployment in the field, the overlying water

containing excess oils was removed. The control treatment was subject to the same process without the addition of any oil. The ambient temperature was kept at close to 1°C and the salinity remained approximately 34 ppt throughout this process.

The sediment was deployed by divers in open plastic trays (60 x 35 x 10 cm) lined with 300 μm mesh. The trays were placed on the bottom of O'Brien Bay in a randomised block layout. Very little sediment was lost from the trays in this process. After approximately five weeks, four trays from each treatment were retrieved. Divers placed capped cores in the trays and then brought the trays to the surface where the cores were capped at the bottom end and labelled.

5.2.2 Sample handling

Samples were taken from the sediment that was collected prior to sieving and treatment (referred to here as initial samples), from sediment just prior to deployment (pre-deployment samples) and after five weeks in O'Brien Bay (5 week post-deployment samples). The initial and pre-deployment samples were grab samples collected from the bulk sediment - only the post-deployment samples were collected in cores. Samples were kept at 0 °C if used for most probable number counts or preservation for direct counts and immediately frozen at -20°C if kept for DNA extraction. The sediment cores were sliced whilst still frozen and a portion of the top 1 cm was used for DNA extractions.

5.2.3 Total cell counts

Sediment taken from the top 1 cm of a core was preserved in an equal volume of 10% formaldehyde for at least 3 hours at room temperature. An equal volume of phosphate-buffered saline (PBS: 7.54 g l^{-1} NaCl; 0.2 g l^{-1} KH_2PO_4; 1.15 g l^{-1} Na_2HPO_4) was added, mixed by vortexing and then centrifuged at 500 x *g* for 5

minutes. The supernatant was decanted and the PBS wash repeated. The supernatant was again discarded and the preserved sediment was stored in 1:1 PBS : ethanol at -20°C for several months before staining and counting of the cells.

Preserved sediment samples (0.5 ml) were diluted with 1.0 ml of sterile deionised water and the tubes placed on ice. The samples were sonicated for four bursts of 20 seconds each with at least 1 minute on ice between rounds. After sonication the sediment was allowed to settle for 30 minutes. The supernatant was removed to a new tube and 3 ml of water was added to the remaining sediment. Tubes were shaken vigorously and allowed to settle. The supernatant was removed and pooled with the previous supernatant. This process was repeated twice more. Pooled supernatants were centrifuged at 500 x *g* for 5 minutes at 4°C. Supernatants were removed to a new tube and stored at 4°C until stained.

20 µl of the supernatant was diluted 1:45 in sterile deionised water and 100 µl of 50 µg μl^{-1} 4',6'-diamidino-2-phenylindole (DAPI) was added to each supernatant before incubation in the dark for about 30 minutes. This was then filtered through a 0.2 µm black polycarbonate filter (Millipore GTBP). Filter papers were allowed to air dry for a couple of minutes before mounting in a drop of antifade solution (0.1% diphenylamine prepared daily in 1:1 glycerol: PBS).

Slides were counted using a Leica DC 300F epifluorescence microscope with filter cube A. The total number of cells from ten fields of view was recorded for each slide.

5.2.4 Quantifying SAB degrading bacteria

A most probable number method was used to estimate the number of aerobic SAB degrading bacteria. Counts were carried out on samples the day after collection at Casey station. A four tube MPN was performed in sterile 96-well titre trays. The

following marine mineral medium was used: 28.4 g NaCl; 0.1 g $CaCl_2$; 0.5 g $MgSO_4.7H_2O$ and 2.5 g NH_4Cl was dissolved in 900 ml of deionised water and autoclaved. After cooling, 100 ml of a phosphate solution (5.6 g l^{-1} KH_2PO_4 and 47.4 g l^{-1} K_2HPO_4) and 1 ml of a vitamin solution (in mg l^{-1}: biotin 2; folic acid 2; pyridoxine 10; thiamine 5; riboflavin 5; nicotinic acid 5; D-Ca-pantothenate 5; vitamin B12 0.1; p-aminobenzoic acid 5; lipoic acid 5) were added. Three initial 1:10 dilutions were made for each sample in the marine mineral medium. This consisted of adding 0.5 ml of sediment taken from the top cm of the core to 4.5 ml of medium. Serial dilutions in steps of 1:5 were carried out in a total of 200 µl of marine medium. 5 µl of filter sterilised SAB was added to every well and the trays were incubated at 0°C for six weeks. At the end of this time, growth was scored as positive if wells appeared turbid or opaque when held up to the light. The MPN calculator (available from http://members.ync.net/mcuriale/mpn/index.html) was used to calculate the most probable number of bacteria per ml of sediment.

5.2.5 Determination of community structure by DGGE

DNA was extracted from the frozen sediment samples as described in section 2.2.2 with an additional incubation step at 37°C for 20 minutes before the first round of freezing.

Amplification of a fragment of the 16S rRNA gene was carried out using the conditions described in section 3.2.3.1. This first set of DGGE analyses included only two samples from each pre-deployment treatment. In order to increase the statistical power, the DGGE was repeated at a later time with four samples from each pre-deployment treatment. These additional samples were amplified with the following thermal cycling programme: initial denaturing step at 94°C for 5 minutes; then 20 cycles of denaturing at 94°C for 20 seconds, annealing at 65°C for 20

seconds (decreasing by 0.5°C each cycle) and extension at 72°C for 30 seconds; followed by 10 cycles of 94°C for 20 seconds, 55°C for 20 seconds, 72°C for 30 seconds; final extension at 72°C for 4 minutes and then held at 4°C.

The electrophoresis was carried out as described in section 4.2.2 for both sets of analyses.

The DGGE banding patterns were analyzed as described in previous chapters (see sections 3.2.3 and 4.2.2). The Primer5 package was used to produce MDS plots. The ANOSIM test was used to test the significance of differences between groups on both untransformed and presence-absence transformed data. A second multivariate technique, canonical analysis of principle co-ordinates (CAP) as described by Anderson and Willis (2003), was also performed utilizing the CAP programme (available from http://www.stat.auckland.ac.nz/~mja). A discriminant analysis with the Bray-Curtis dissimilarity measure and no transformation options were chosen. The resulting first two constrained axes are presented.

5.3 Results

5.3.1 Effect of process of experimental protocol

Total bacterial numbers and the number of SAB-degrading bacteria are presented in Figures 5.1 and 5.2 respectively. For both methods there were no significant differences (student's t-test: $p > 0.1$) between the initial samples and any of the pre-deployment treatments.

Using the DGGE banding pattern as a measure of the overall community structure, tests for differences between treatment groups is possible using ANOSIM. In the first batch of DGGE analyses, the small number of pre-deployment samples for each treatment resulted in a very limited number of permutations and hence a

minimum possible significance levels of 10%. As there was no significant difference between the initial samples and the pre-deployment control samples, these groups were then pooled (to increase the statistical power of the test) and compared to the pre-deployment treatment samples (Table 5.1). This approach meant that the minimum possible significance was reduced to 4% and none of the treatments were significantly different from this pooled control. It appears that the two day process of collecting, sieving and treating the sediment did not alter the total number of bacteria, number of SAB degrading bacteria or overall microbial community structure by a measurable amount.

5.3.2 Short-term effect of the oils

To determine whether there were any significant changes in the total number of cells or the number of SAB degraders, the post-deployment control group was compared to each post-deployment treatment. For both total and SAB degrading bacteria, there was a significant ($p < 0.05$) increase in the SAB and biodegradable oil (Titan GT1) treatments but not for either the clean or used lubricant (Mobil OW40) (Figures 5.1 and 5.2).

A comparison of the overall community structure in all post-deployment samples is presented in Figure 5.3 as an MDS ordination based on the DGGE banding patterns from the first set of analyses. The corresponding ANOSIM R values are given in Table 5.2. It is quite clear from both the figure and the table that the greatest change in microbial community structure relative to the control occurred in the SAB treatments. The lubricant (Mobil OW40) and used lubricant (used Mobil OW40) treatments are also significantly different to the control when no transformation was applied to the data (based on number of banding occurrences); this difference becomes less significant when a presence-absence transformation is

applied. The biodegradable oil (Titan GT1) treatment however is not statistically different to the control group using either statistical protocol.

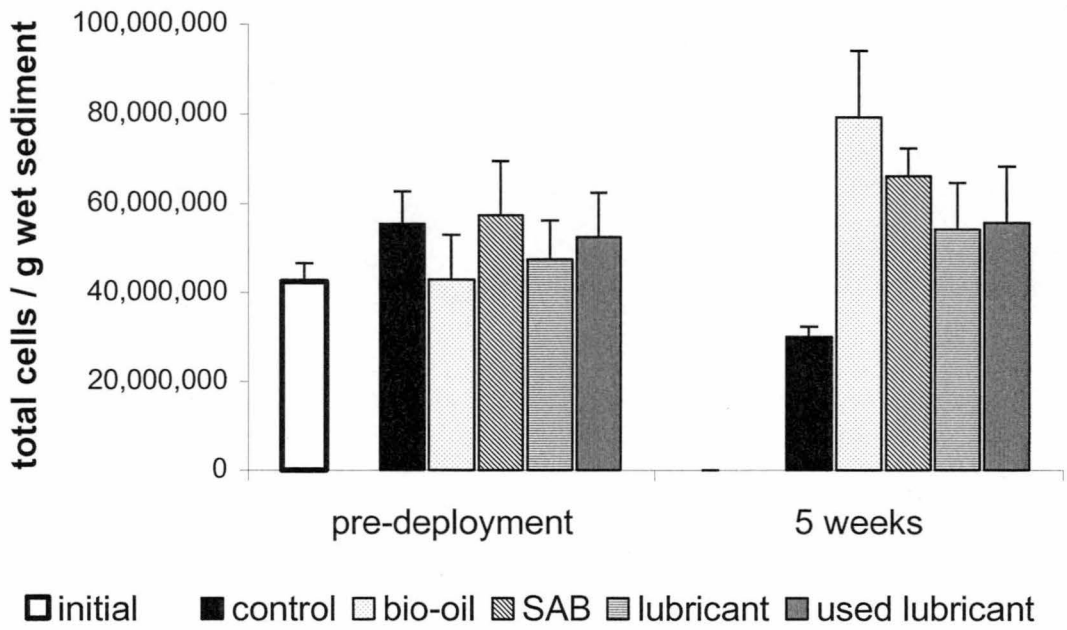

Figure 5.1: Total bacterial cells per g wet sediment. Bio-oil = Titan GT1; SAB = SAB diesel; lubricant = unused Mobil OW40; used lubricant = used Mobil OW40. n = 6 for the initial group; 8 for the pre-deployment groups and 4 for the 5 week [pst-deployment groups. The error bars show standard error. The pre-deployment control is not different to the initial samples nor any pre-deployment treatment. The post-deployment control is significantly different to the bio-oil and SAB treatments.

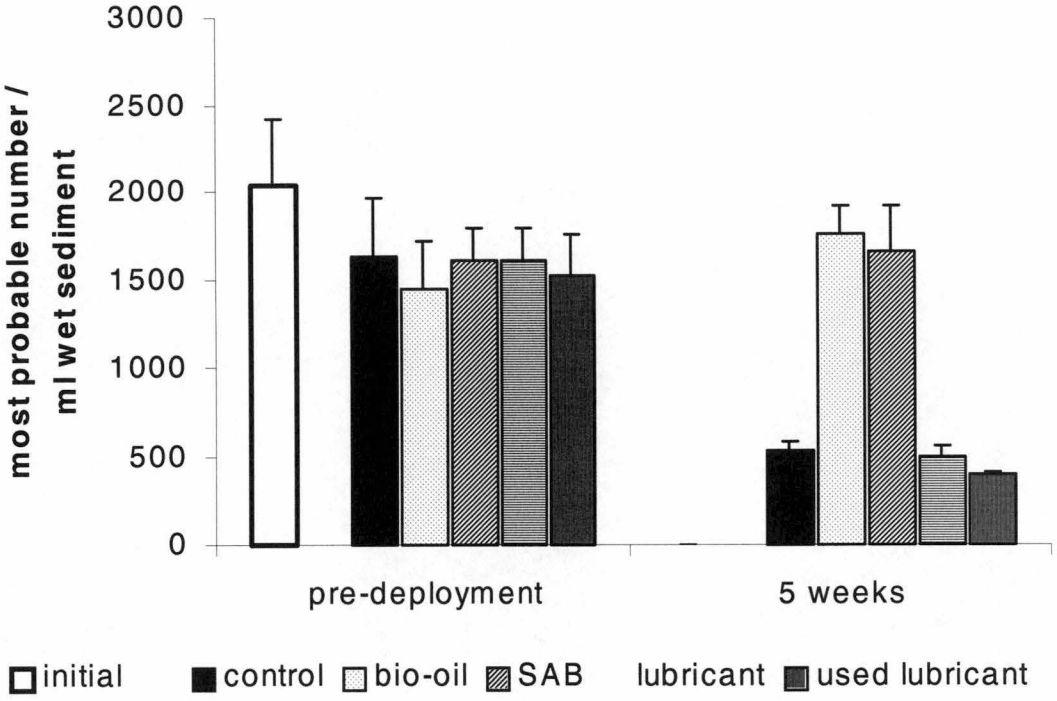

Figure 5.2: Most probable number of SAB degrading bacteria per ml wet sediment. Bio-oil = Titan GT1; SAB = SAB diesel; lubricant = unused Mobil OW40; used lubricant = used Mobil OW40. n = 6 for the initial group; 8 for the pre-deployment samples and 4 for the 5 week post-deployment samples. The error bars show standard error. The pre-deployment control is not different to the initial samples nor any pre-deployment treatment. The post-deployment control is significantly different to the bio-oil and SAB treatments.

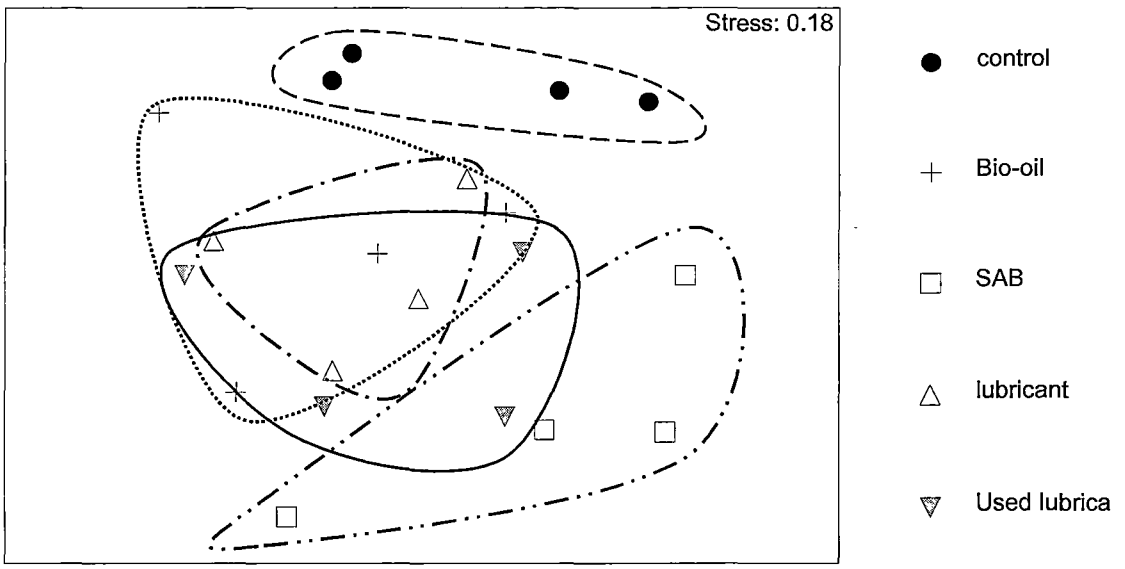

Figure 5.3: MDS showing relative similarities between the different treatments and the control at 5 weeks based on the DGGE banding patterns from the first set of analyses. The corresponding R values (no transformation) are given in Table 5.2. Bio-oil = Titan GT1; SAB = SAB diesel; lubricant = unused Mobil OW40; used lubricant = used Mobil OW40.

Table 5.1: ANOSIM R values and significance levels comparing combined control group (n = 5) to each pre-deployment treatment (n = 2). The minimum possible significance level was 4%.

	no transformation		presence-absence transformation	
Groups	R value	Significance level(%)	R value	Significance level (%)
control, bio-oil (Titan GT1)	0.182	29	0.327	24
control, SAB	-0.227	81	0.036	33
control,lubricant (Mobil OW40)	-0.309	86	0.036	38
control, used (Mobil OW40)	-0.391	90	-0.400	95

Table 5.2: ANOSIM R values comparing each treatment to the control in the post-deployment (5 week) samples (n = 4). The minimum possible significance level was 2.9%. Significant differences are in bold.

	no transformation		presence-absence transformation	
Groups	R Value	Significance level (%)	R Value	Significance level (%)
control, bio-oil (Titan GT1)	0.104	22.9	-0.141	77
control, SAB	0.682	2.9	0.708	2.9
control, lubricant (Mobil OW40)	0.797	2.9	0.432	8.6
control, used (Mobil OW40)	0.417	5.7	0.250	14.3

5.3.3 DGGE issues

In Chapter 3 it was found that between-PCR and between-gel differences had a strong influence on banding patterns and the interpretation of results. To overcome this, all samples were amplified in the same round of PCR and results from multiple gel runs were pooled. This resulted in differences in banding patterns due to real differences between samples being more prominent than differences due to PCR and gel effects. The same approach was successfully used here in the first batch of DGGE analyses.

However, the minimum significance level possible with the number of pre-deployment samples included in the first DGGE analysis was high (10%, reduced to 4% by combining initial and control samples: see section 5.3.1). It was decided to analyse additional samples to increase the number of replicates to four for all groups. When these new banding patterns were plotted as an MDS ordination and analysed with ANOSIM, there appeared to be no significant differences between any of the pre-deployment treatments or between any post-deployment treatment groups. This was unexpected as analysis of the first batch of DGGE had found highly significant differences between post deployment treatments (section 5.3.2).

A second statistical procedure (CAP) that can reveal patterns not seen with an unconstrained plot (such as an MDS) was used to further investigate the post-deployment samples. The plot of the first two constrained axes are shown in Figure 5.4. In this figure it appears that the control samples are different to the other treatments although differences between the treatments are not obvious. When the samples were classified as either control or treatment, a one-dimensional plot was obtained (not shown) and there was a significant difference between the two groups ($p = 0.012$)

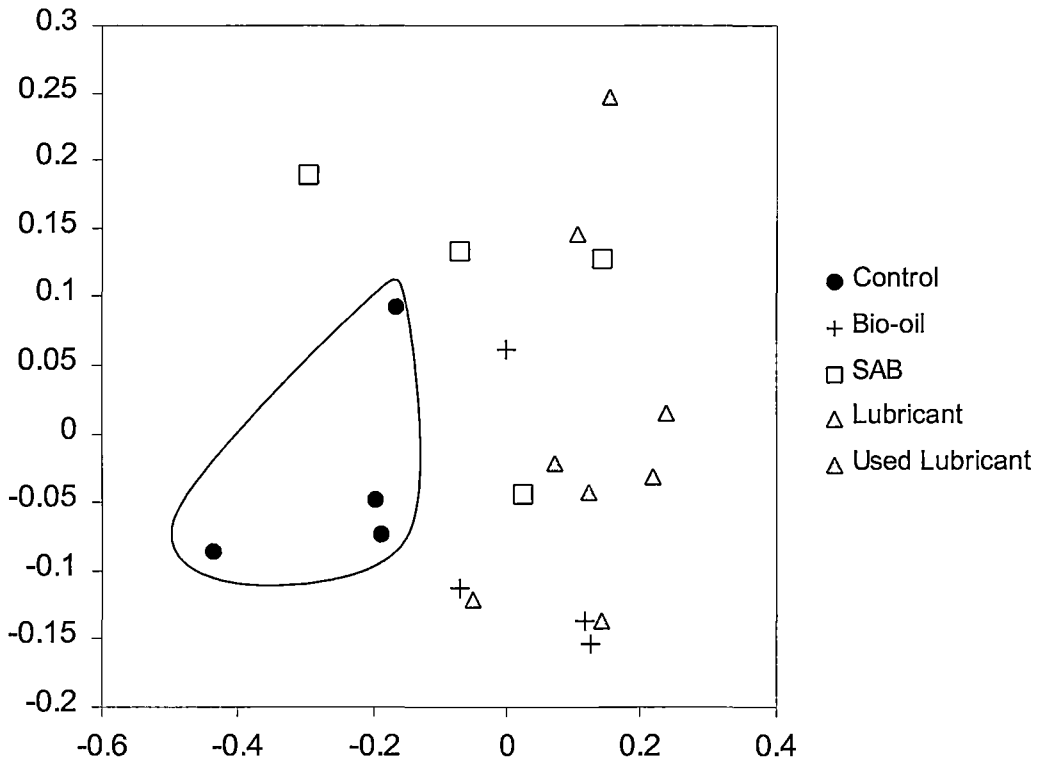

Figure 5.4: Plot of the first two canonical (constrained) axes produced by the CAP program. Bio-oil = Titan GT1; SAB = SAB diesel; lubricant = unused Mobil OW40; used lubricant = used Mobil OW40. The points represent the DGGE banding patterns of the 5 week post-deployment samples from the second round of analyses.

Analysis of a single DGGE run (i.e. each sample amplified and run once on a gel) from the second batch of analyses showed a very strong tendency for samples that were run on the same gel to group together. The differences between gel runs were highly significant (ANOSIM R values over 0.5, significance level 0.1%). As the results from three runs had been combined, this grouping by gel had become obscured because the groups of samples on a particular gel were different each time. Unlike the previous batch, differences in banding patterns due to real differences in the samples had not become more prominent than differences in banding patterns due to gel effects.

Any changes that may have occurred between initial collection and deployment would be even more subtle than the changes seen after deployment for five weeks. As it appeared that the gel signature obscured between treatment differences in this second set of DGGE, any differences that existed between the pre-deployment treatments would probably not have been seen. For this reason it was decided not to include the results from the second batch of DGGE in the analysis of the pre-deployment samples.

5.4 Discussion

5.4.1 Effect of experiment set-up on microbial community structure

To examine biodegradation rates and effects of various oils *in-situ*, a sediment recruitment experiment was designed that initially involved defaunating soft-sediment infauna whilst ideally retaining the original microbiota. In this way impacts on macrofauna are measured by differences in recruitment and recolonisation of the sediment. However changes to the microbial communities are measured in relation to the initial community and to the control at each time point.

For this experiment to accurately reflect what would really happen if these oils were spilt into the marine environment, it was important that the pre-deployment community was as close to the original community (i.e. the initial samples) as possible. A comparison of the initial and pre-deployment samples shows that the total number of bacterial cells (Figure 5.1) and the number of SAB degrading bacteria (Figure 5.2) did not change significantly. The DGGE results (Table 5.1) also show that there is no significant difference between the initial samples and the pre-deployment samples. The microbial communities at the time of deployment, effectively the beginning of the experiment, were essentially the same as that of the naturally occurring microbial community. The protocol for setting up this *in situ* experiment needed to balance the requirements of the macrofaunal and microbial aspects of the study. As it was successful in preserving the microbial communities it may prove useful for other studies that wish to monitor both microbial communities and recruitment of sediment infauna.

5.4.2 Effect of oils after 5 weeks

The differences in the community structure between the control and treatments in the 5 week post deployment samples (Figure 5.3) are to some extent, reflected in the total cell counts and SAB degraders (Figures 5.1 and 5.2).

The SAB appears to have the greatest shift away from the control microbial community in all three measures. As it is known that some components of SAB are readily biodegradable, it is possible that the increase in bacterial numbers is due to the input of carbon from the degradation of the SAB. Previously it has been noted that the oils with the greatest environmental effect are not necessarily those that are most persistent (Haigh, 1995). It is possible that a similar trend is occurring here and the effect seen may be only temporary. Determination of long-term effects is a

further objective of this project and an indication of the longevity of the SAB effects will be obtained when later time points are analysed.

The toxicity and longevity of used lubricants compared to the clean product is not known. Haus *et al.* (2001) linked the chemical and physical properties of mineral oils to their biodegradability and found that as the relative amount of aromatic and polar compounds increased, the biodegradability decreased. However, Eisentraeger *et al.* (2002) that found that neither usage nor the presence of additives such as antioxidants affected the biodegradability of oils. In this study the microbial communities in the clean and used lubricant post-deployment, appear to be similar (Figures 5.1,5.2 and 5.3) In the short-term at least, the toxicity of used Mobil OW40 to microbes is not appreciably different to that of the clean, unused product.

The results of the DGGE (Figure 5.3 and Table 5.2) indicate that the biodegradable oil (Titan GT1) is not significantly different to the controls. This implies that the biodegradable oil is less toxic to the microbial communities than the other treatments. The increase in total cell numbers might indicate that components of this oil are being used as a growth substrate. The fact that the number of SAB degraders has also increased suggests that perhaps these SAB degrading bacteria are also capable of utilising some of the components of the biodegradable oil.

5.4.3 Usefulness of a polyphasic approach

By using a number of methods that target different aspects of the bacterial population, a more thorough understanding of the microbial community response to contamination is obtained than if just one technique is used. Molecular methods are useful because they can provide information on uncultured or difficult to culture microorganisms. DGGE was used to gain an overall picture of changes in the microbial communities without knowing exactly which species were present. Total

cell numbers as estimated by DAPI stained cell counts also provide an overall picture of the microbial communities. However, this method is not very sensitive in that significant changes to a small section of the community may not be detected. The advantage of a method such as most probable number counts is that it provides information on the activity of part of the microbial population. The numbers of SAB degraders was monitored as it is known that biodegradation of diesels does occur in cold regions (see for example Margensin and Schinner, 1998). With the methodology described here, it is unlikely that all the SAB degraders were counted. It is possible that degradation by anaerobic bacteria is also occurring in the sediment and bacteria with fastidious growth requirements may also have been unable to grow under the cultivation conditions employed. It is also unlikely that the SAB degrading bacteria would be able to degrade components of the synthetic lubricant (Mobil OW40) as the chemical composition of the two products is very different. However, it was expected that being able to follow changes in numbers of representative hydrocarbon degraders might indicate which parts of the microbial communities may be responsible for the changes in DGGE banding patterns and overall bacterial numbers. Changes to any active part of the microbial community indicate that that particular treatment is having an impact on the sediment microbes.

DGGE also has its limitations with PCR biases and differences in banding patterns resulting from gel artefacts being two of the most difficult issues to resolve. By running multiple analyses and pooling results these effects can be reduced and it is possible to obtain useful data with this method. In this study the 'gel signature' (Ferrari and Hollibaugh, 1999) became an over-riding issue when additional samples were included and re-analysed. The differences in banding patterns between groups of post-deployment treatments seen in Figure 5.3 became insignificant. In a five week time-span, the changes in the microbial population are potentially very subtle -

perhaps represented by only a few bands out of approximately fifteen. When the gel artefacts are so strong, DGGE is not sensitive enough to detect such changes. Unfortunately, there is currently no available method that will allow the analysis and comparison of overall microbial community structure between a large numbers of environmental samples. Other techniques are available to follow specific species or specific groups of bacteria, for example FISH, RNA hybridisation and real-time PCR (Ravenschlag *et al.*, 2000; Beller *et al.*, 2002).

Despite these problems with the gel signature, a different approach to statistically analysing the data led to the same conclusions. Although differences between the various treatments were not always observed, the control group was always significantly different to the other groups (Figure 5.4). Thus there is confidence that the patterns seen in Figure 5.3 represent community differences in the treatments. In all likelihood, differences exist in the second set of data but are less clearly defined because method variability exceeds the subtle response of the microbial communities.

5.4.4 Conclusions

This comparative study on the effects of different oil treatments on sediment microbial communities is unique. As the pre-deployment microbial communities are not substantially different to the naturally occurring communities, this experimental system represents a reasonable analogue for studying the effects and biodegradation of oils in the Antarctic benthic environment. After only a short *in-situ* incubation it was possible to measure differences in the microbial populations due to the effects of different oil treatments. The SAB diesel appears to have caused the biggest change in microbial community composition. Out of the three lubricants, the biodegradable oil (Titan GT1) has had the least effect and was not statistically different from the

controls. These results provide the first tentative indication that using biodegradable oils might be of genuine environmental benefit in the Antarctic. It is anticipated that this project will continue for up to five years to provide more information on the toxicity and biodegradation of each of the oils.

Whilst the methods used here were useful in showing broad changes to the microbial communities, a more detailed examination of the microbial ecology of these sediments is needed. The dynamics of oil biodegradation are complex and it will be interesting to identify which bacteria are involved, what catabolic pathways they are using and how this is linked to changes in other trophic levels of the benthic ecosystem.

6. Biodegradation of SAB by sediment enrichment cultures

6.1 Introduction

The ability of bacteria to degrade hydrocarbons has been known since the early 1900s (Zobell, 1946). As research into hydrocarbon degradation and bioremediation of petroleum spills progressed, it appeared that if one looked hard enough, it would be possible to find evidence of biodegradation of any hydrocarbon compound. Although many hydrocarbons are readily biodegradable, many are recalcitrant to biodegradation whilst there is no evidence for biodegradation of some compounds. One study into the extent of biodegradation of different classes of diesel components found that n-alkanes were the most completely degraded followed by the aromatics then the cyclic and branched alkanes (Olson *et al.*, 1999). The rate and extent of biodegradation of individual compounds in mixtures of hydrocarbons, such as diesels, depends on the particular compound and on the composition of the mixture (Leahy and Colwell, 1990). Some mixtures appear to stimulate the degradation of compounds that are recalcitrant to biodegradation on their own (Zhang *et al.*, 1998) whilst in other mixtures, toxic compounds prevent biodegradation of otherwise easily degradable compounds.

Biodegradation of diesels has been observed in a variety of cold environments. Previously, diesel biodegradation has been observed in alpine, sub-Antarctic and Antarctic soils (Margesin and Schinner, 1997; Delille and Pelletier, 2002; Kerry, 1993), in Antarctic sea-ice (Delille *et al.*, 1997) and alpine glacier cryoconite (Margesin *et al.*, 2002). Bacteria with the ability to degrade hydrocarbons in cold regions are found in many genera. Several *Pseudomonas* species have been isolated with the ability to degrade a range of hydrocarbons (for examples see van

Beilen *et al.*, 1994). Hydrocarbon utilisers from several genera including *Nocardia*, *Achromobacter*, *Vibrio* and *Pseudomonas* were isolated from Northwestern Atlantic sediments (Mulkins-Phillips and Stewart, 1974). *Rhodococci* capable of degrading hydrocarbons have been isolated from a variety of environments including Antarctic soil (Bej *et al.*, 2000) and Antarctic seawater (Yakimov *et al.*, 1999). Two species, *Alcanivorax borkumensis* and *Oleispira antarctica,* both isolated from permanently cold marine environments, use only a limited number of organic compounds, (preferably *n*-alkanes) as carbon sources (Yakimov *et al.*, 1998, 2003). Anaerobic biodegradation of hydrocarbons, although generally a slower process than aerobic degradation, has been studied in several species of bacteria (for a review see Heider *et al.*, 1999) including some such as *Desulfobacula toluolica* (Rabus *et al.*, 1993) that are found in marine sediment.

Previous work in the Casey region showed that there were differences in the microbial community structure between pristine and contaminated sites (see Chapter 3). Unpublished chemical data from Brown Bay indicates that the petroleum contamination has weathered substantially (I. Snape, pers. com.). In addition, SAB was observed to have a significant effect on the sediment microbial communities found in O'Brien Bay (see Chapter 5). However this does not demonstrate that biodegradation is occurring in the sediments near Casey station. To show that the microbial potential for hydrocarbon biodegradation was present in these benthic microbial communities, degradation of components of SAB in enrichment cultures was measured and an attempt was made to identify some of the bacteria present. Identification of the most easily degraded hydrocarbons and potential hydrocarbon degrading bacteria is the first step to studying the process of hydrocarbon biodegradation *in situ.*

6.2 Methods

6.2.1 Development of enrichment cultures

Sediment that had been amended with SAB (see section 5.2.1) was used to set up a series of enrichment cultures at Casey station. Approximately 50 ml of sediment was added to 50 ml of marine broth (see section 5.2.4) and 500 µl SAB in a 250 ml bottle. This was incubated at 0°C with occasional shaking. After two months the sediment and overlying water were mixed together and 2 ml was used to inoculate 18 ml of marine broth to which 200 µl of SAB had been added. An additional 2 ml was frozen (sample 1). After three weeks incubation at 0°C, this sub-culturing process was repeated and the remainder of the culture was filtered through a sterile 0.22 µm filter, which was then frozen (sample 2). This was repeated twice more at three weekly intervals (samples 3 and 4). At this time the cultures were shipped back to Australia at 4°C. On arrival in Australia, 2 ml was used for another sub-culture and the remainder was frozen (sample 5). This was incubated at 2°C for approximately two months until the microcosms described below were set-up.

6.2.2 SAB biodegradation in microcosms

Autoclaved amber 250 ml bottles with teflon septa had 170 ml of cooled (4°C) medium added to them. 1 ml of inoculum was added to two thirds of the bottles and 250 µl of filter-sterilised SAB was added to all bottles. The SAB contained squalane (2,6,10,15,19,23-hexamethyltetracosane) and adamantane ($C_{10}H_{16}$) that were added as conservative internal standards. Both compounds are highly branched and generally regarded as resistant to biodegradation (Robson and Rowland, 1987; Grice *et al.*, 2000). Bottles were tightly closed and the lids taped on.

The microcosms were incubated on a roller at 4°C. During the set-up process, the enrichment cultures and bottles were kept at 4°C as much as possible.

The enrichment culture used as the inoculum for the microcosms had an OD_{600nm} of 0.003 (approximately 1.6 x 10^6 cells per ml). After the microcosms were inoculated, the remainder of the culture was frozen (sample 6).

Two control and two inoculated bottles were extracted and analysed for hydrocarbons immediately and thereafter at three to four weekly intervals. After 16 weeks, one of the inoculated bottles (sample 7) was used for DNA extraction as described below.

6.2.2 Hydrocarbon analysis

The hydrocarbon analysis was carried out by the Australian Antarctic Division.

Each microcosm bottle was extracted with 10 ml of hexane. The hexane was added, the bottles resealed and vigorously shaken. Extracts were analysed on a Varian 3800 GC-FID with a BP-1 (SGE) column (length: 30 m, inner diameter: 0.32 mm, film: 0.25 μm film). The injector temperature was set at 270°C and 0.6 μl was injected with a 20:1 split. The carrier gas was He at a constant flow rate of 1.7 ml per minute. The temperature program was as follows: 35°C for 2.5 minutes then ramped up by 25°C per minute to 310°C then held for 1.5 minutes. The method was optimised to give small injection discrimination over the range of compounds found in SAB (from C9 to C18). The peak shape was uniform from C9 to C30. To avoid possible bias from GC drift the samples and standards were interspersed and each vial was analysed in duplicate. Peak areas were normalised to adamantane.

A standard consisting of 250 μl of SAB added directly to 10 ml of hexane was extracted and processed with the other samples. This standard represents 100%

recovery of SAB from the microcosms and was used to calculate the percentage recovery.

6.2.3 Microbial community analysis

The microbial community in the enrichment cultures was analysed using DGGE. Bands on the DGGE gel from sample 7 were excised and sequenced to identify bacteria that may be responsible for the observed hydrocarbon losses.

6.2.3.1 DNA extractions

DNA was extracted from sample 1 with the Ultraclean Soil DNA kit (MoBio) following the manufacturer's directions. The heating alternative to the bead-beating step was used.

The filter papers (samples 2, 3 and 4) were extracted using a method adapted from that described in Sei *et al.* (2003). Filter papers were cut into thirds with a sterile scalpel and placed in a 15 ml centrifuge tube. 2 ml of suspension buffer (10 mM tris-HCl, 1 mM EDTA, 350 mM sucrose) and 2 ml of 30% tween-20 were added and the mixture vortexed. Four cycles of freezing at -80°C and thawing at 60°C were carried out. 20 μg of lysozyme was added to each tube and they were then incubated at room temperature for 30 minutes. 4 ml of lysis solution (100 mM tris-HCl, 20 mM EDTA, 300 mM NaCl, 2% SDS) was added and the tubes vortexed. All were incubated at 60°C for 1 hour. The mixtures were extracted with an equal volume of tris-equilibrated phenol. The supernatant was extracted with an equal volume of phenol:chloroform: isoamylalcohol (25:24:1). The supernatant was removed to a clean tube and the DNA was precipitated by addition of 0.1 volumes of 3M sodium acetate and 2 volumes of cold absolute ethanol. After incubation at -20°C for 30 minutes the extracts were centrifuged at 3100 x *g* for 30 minutes at 4°C. The pellet

was washed with ice-cold 70% ethanol and centrifuged for 15 minutes as above. The pellet was air-dried and resuspended in 100 µl of sterile water.

A different procedure was used to extract DNA from the frozen cultures (samples 5, 6 and 7). The cultures were thawed then centrifuged at 3100 x *g* for 30 minutes at 4°C. The pellet was resuspended in 1 ml of saline EDTA. 30 µg of lysozyme and 100 µl of 20 % SDS was added and the tubes were incubated at room temperature for 15 minutes. They were then extracted with an equal volume of phenol:chloroform: isoamylalcohol (25:24:1). The DNA was precipitated with ethanol as described above.

All the crude DNA extracts were further purified using the Ultraclean Soil DNA kits (MoBio) by adding 50 µl of the crude DNA to 25 µl of solution S2 and 150 µl of solution S3 and following the directions from step 13. The final DNA preparation was run on a 1% agarose gel and a single, high molecular weight band was seen, though only very faintly for sample 7.

6.2.3.2 PCR

Some of the samples proved difficult to amplify using the primer 341FC (see section 3.2.3.1). Samples 3,4 and 7 were first amplified with the unclamped primer 341F (CCT ACG GGA GGC AGC AG). In addition, the culture from which sample 7 was taken (see section 6.2.1) was used for direct PCR with the unclamped primer. 2.5 ml of the culture was centrifuged at 3100 x *g* for 30 minutes. The pellet was resuspended in 10 µl of water (sample 7b) and the entire 10 µl was added as template to a PCR reaction. Amplification mix and conditions were as described in section 3.2.3.1 except for the forward primer. 4 µl of the DNA extracts were added as template. The PCR product was purified with the Ultraclean PCR cleanup kit (MoBio) and 4 µl of this was used as the template DNA in a reaction with 341FC for DGGE.

Samples 1,2,5 and 6, the clean PCR products from samples 3,4 and 7 and a DNA extract from one of the post-deployment SAB treatments (see section 5.2.6.1), were amplified as described in section 3.2.3.1.

6.2.3.3 DGGE

The DGGE was performed as described in section 4.4.2. Bands in lanes from samples 7 and 7b were cut out with a sterile scalpel blade (these bands are indicated in Figure 6.2). The gel pieces were washed in 500 µl sterile water for 10 minutes to remove excess denaturant. DNA was eluted from the bands in 200 µl of sterile water at 4°C for thirty minutes before being amplified in the PCR reaction described below.

6.2.3.4 Amplification of DGGE bands

HotStarTaq (Quiagen) mastermix was used to amplify fragments of DNA from the DGGE bands. 5 µl of each eluant was used in a 50 µl reaction containing 25 µl of mastermix and 20 pmol of each primer (341F and 907R). The following thermal cycling programme was used: initial denaturing step at 95°C for 15 minutes; then 25 cycles of denaturing at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute followed by a final extension step at 72°C for 4 minutes and held at 10°C. The products from this reaction were purified with the Ultraclean PCR cleanup kit (MoBio).

6.2.3.5 Sequencing

The CEQ dye terminator cycle sequencing kit (Beckman Coulter) was used to sequence the six DGGE bands shown in Figure 6.2. Approximately 8 ng of the clean PCR product was used as a template with 3.2 µM 341F primer. The thermal cycling program suggested was used (30 cycles of 96°C for 20 sec, 50°C for 20 seconds and 60°C for 4 minutes). Sequencing reactions were purified with an ethanol precipitation following the CEQ kit directions. Samples were resuspended in the provided sample loading solution and transferred to a sample plate. The samples

were run on a Beckman CEQ2000XL automated capillary sequencing system. Sensible sequence data was obtained for two bands and a phylogenetic tree was constructed as described in section 2.2.3.

6.3 Results

6.3.1 Hydrocarbon degradation

Analyses of the hydrocarbons in the samples taken immediately after the microcosms were set-up showed identical patterns (see Figure 6.1a) in the four microcosms (two sterile controls and two inoculated flasks) and the standard (prepared by adding SAB directly to 10 ml of hexane). This confirms that all the microcosms started with the same hydrocarbon content, that there was no loss of volatile components due to evaporation during the set-up process and that the extraction of hydrocarbon from the microcosms was high (99 ± 2%).

After three weeks incubation, comparison of the chromatograms from the sterile controls and inoculated flasks showed that the peak corresponding to nonane (C9 alkane) was smaller in the inoculated flasks (see Figure 6.1b). For the samples taken after 16 weeks, this was converted to percent recovered and is presented in Figure 6.2. Recovery of cyclo-octane is also presented as it has similar physical properties to nonane and any disappearance due to physical processes (eg. sorption to glass or evaporation) would be very similar for the two compounds. The amount of nonane recovered from the inoculated flasks is significantly less ($p < 0.02$) than the amount recovered from the sterile controls. The amount of cyclo-octane recovered however was not significantly different ($p > 0.5$).

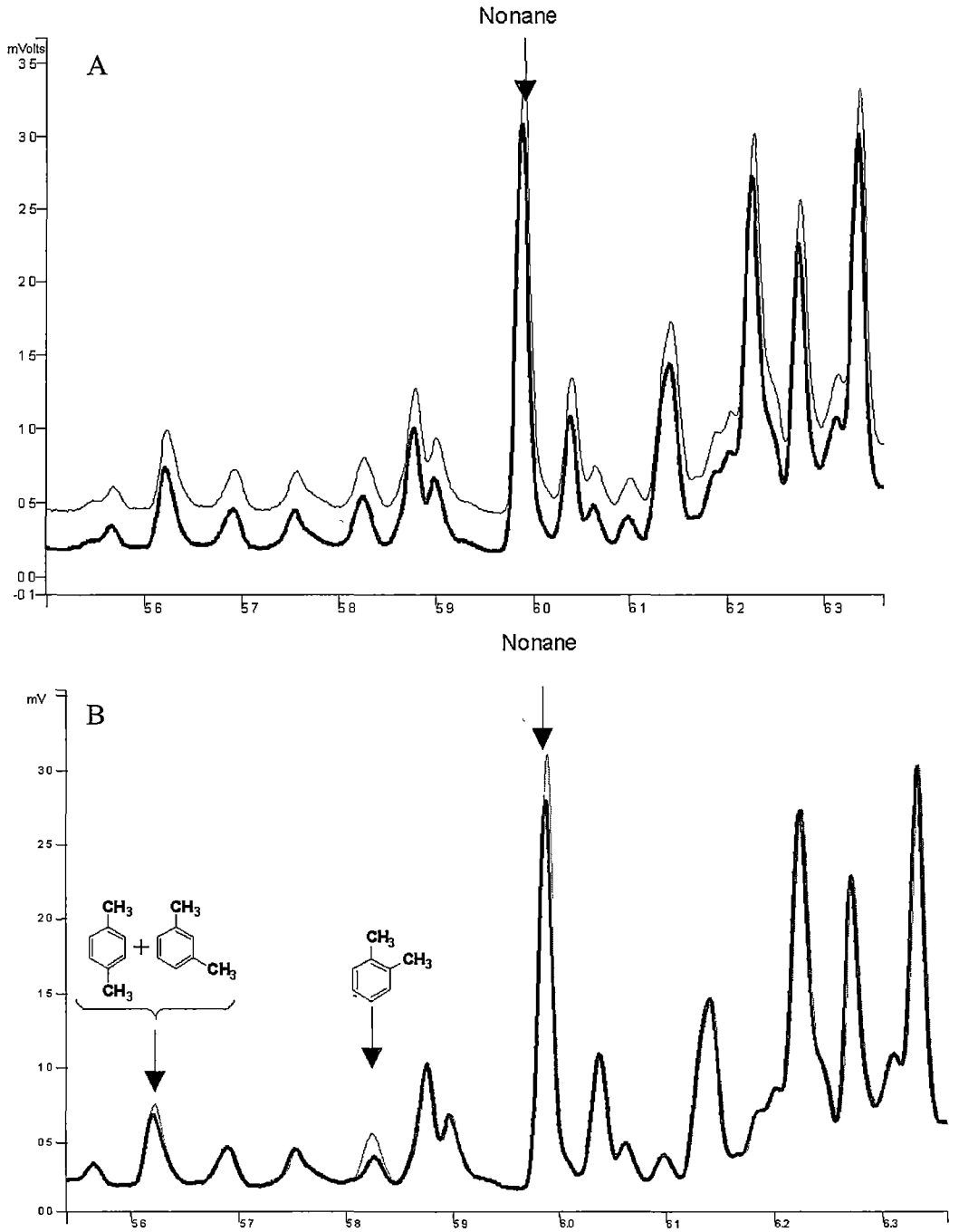

Figure 6.1: GC-FID chromatograms showing the control microcosm (light line) compared to the SAB standard prepared by adding 250 µl of SAB to hexane (dark line panel A) and compared to the inoculated microcosm after sixteen weeks (dark line panel B). In (A) the control chromatogram has been displaced vertically so that the peaks can be clearly seen. The identity of peaks in (B) is based on their disappearance after silica-gel purification of the extracts which retains only the aliphatic components of the SAB.

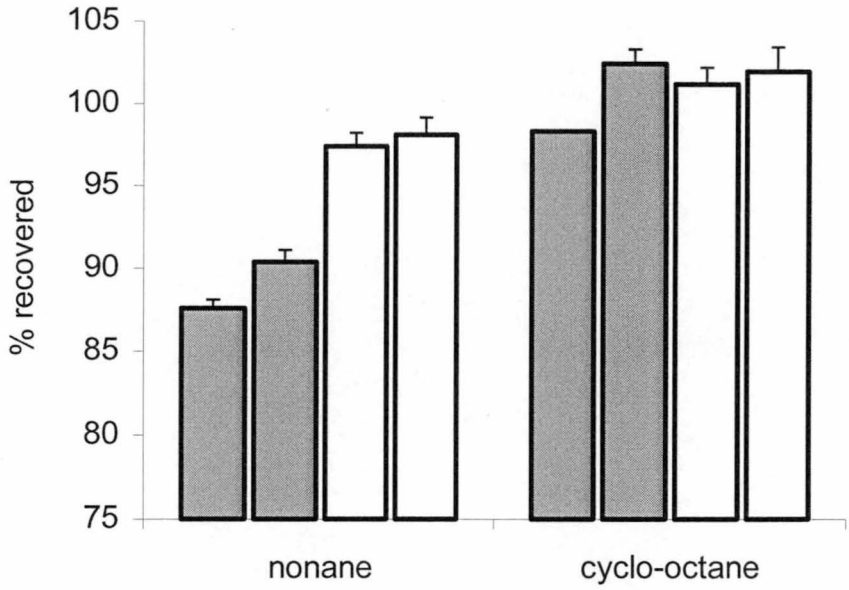

Figure 6.2: Comparison of amount of nonane and cyclo-octane recovered from inoculated (grey) and sterile (white) microcosms. The error bars are the standard deviation obtained from multiple injections of each sample.

6.3.2 Analysis of the microbial community

A photo of the DGGE gel containing samples from the various stages of the enrichment culture is shown in Figure 6.3. From the indicated six excised bands, two sequences were obtained (bands A and B). A BLAST-n search in the GenBank database found the most similar strains were from the Gammaproteobacteria. Band A represents a *Pseudomonas* species, however band B appears to form a novel lineage. The phylogenetic relationship between the sequences obtained from these two bands and other members of the Gammaproteobacteria is shown in Figure 6.4

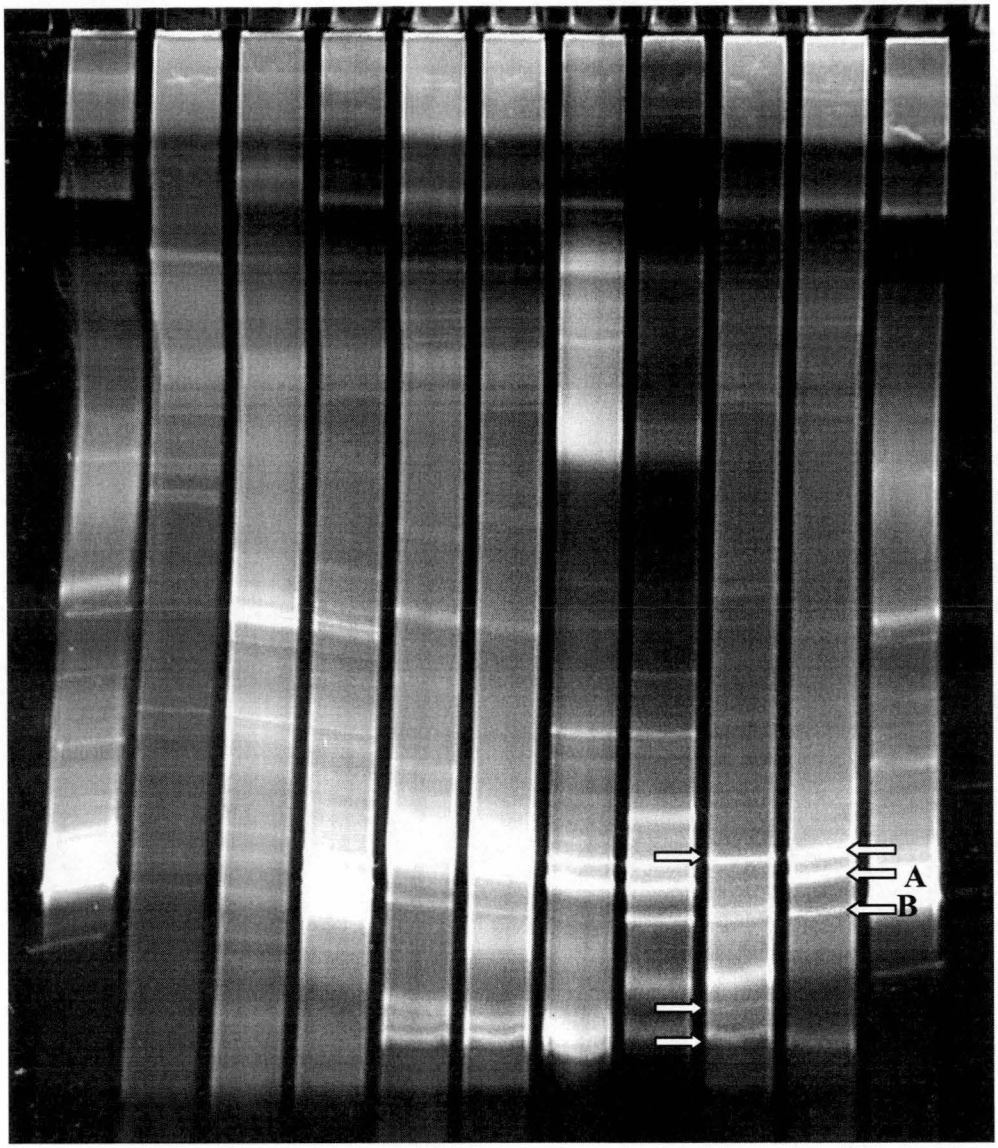

Figure 6.3: Photo of DGGE gel containing samples from the SAB-degrading enrichment cultures. Lanes are (from left to right) control, sediment sample, sample 1, sample 2, sample 3, sample 4, sample 5, sample 6, sample 7, sample 7b, control. The arrows indicate bands that were excised for sequencing; bands A and B were successfully sequenced.

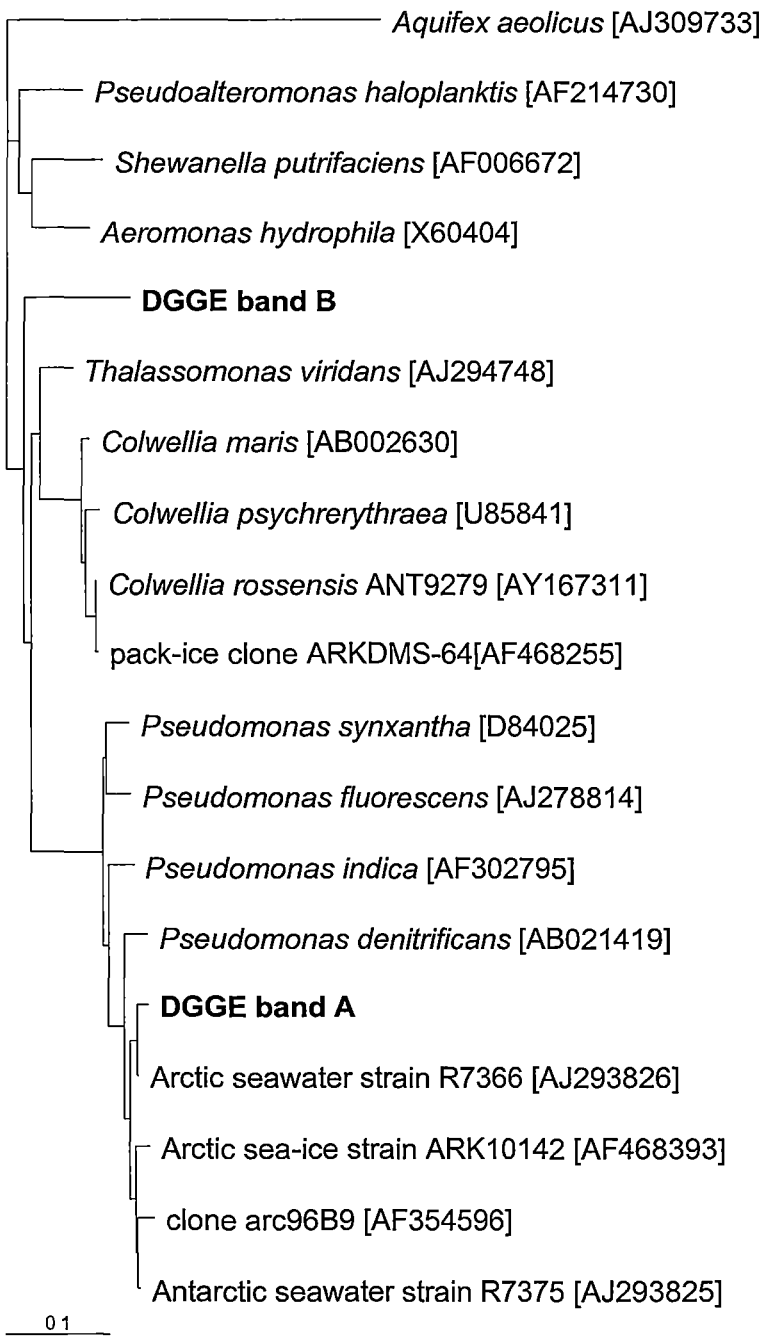

Figure 6.4: Phylogenetic tree showing relationship of DGGE bands obtained from an SAB degrading enrichment culture to members of the Gammaproteobacteria.

6.4 Discussion

After 16 weeks, approximately 90% of the nonane was recovered from the inoculated microcosms compared to 98% from the sterile controls (Figures 6.1 and 6.2). All the cyclo-octane was recovered from both the inoculated and sterile microcosms; this indicates that the observed loss of nonane was due to biodegradation rather than physical processes. Alkanes such as nonane are generally regarded as the most easily degraded hydrocarbons and under aerobic conditions can be oxidised completely to carbon dioxide and water, most often via one of the terminal oxidation pathways (Watkinson and Morgan, 1990).

The limited amount of hydrocarbon biodegradation observed in this study may be due to a number of factors. Low temperatures were probably a major contributor. Alternatively the low numbers of bacteria in the enrichment cultures may have been a significant factor. The OD_{600nm} of the culture used as inoculum showed that initially there were only 9000 cells per ml in the microcosms. The cultures grew very slowly and even after sixteen weeks were not visibly turbid. This may in part be due to the enrichment process. The enrichments were set-up with sediment that had been exposed to SAB but after the second sub-culture no sediment particles remained in the cultures. As many bacteria live attached to sediment particles this may have influenced which species grew in the cultures. There is no doubt that the bacteria present in the microcosms were utilising nonane, but it is possible that species utilising other hydrocarbons were lost through the enrichment process. Using a completely liquid culture was necessary as hydrocarbons sorb to sediment particles making their extraction difficult. The microcosm set-up used here made hydrocarbon extraction more efficient and consistent.

In this experiment, DGGE was used to separate fragments of DNA with different sequences so that they could be sequenced. In Figure 6.3 it can be seen that the brightness of the bands varies from sample to sample. The difference in brightness is partially due to the different extraction procedures used for the different types of samples (sediment, filter paper, frozen cultures). This makes it difficult to compare samples in the one photograph as the short exposure required to be able to see the bands in samples 7 and 7b clearly, was too short to allow the faint bands in the sediment sample to be seen. However, it is obvious that there are fewer bands in the final enrichment cultures compared to the initial stages of enrichment. Of the six excised bands indicated in Figure 6.3, only two resulted in good sequence data. Inspection of the sequence chromatograms of the others revealed poor peaks, probably indicating a low amount of template or overlapping peaks indicating mixed template. This co-migration of bands with different sequences is widely recognized as one of the main limitations of the DGGE technique (Buchholz-Cleven *et al.*, 1997).

The sequences obtained were from the direct PCR amplification of concentrated culture medium. The sequences are only short (about 500 bp) but it is enough to place both within the Gammaproteobacteria. When compared to the sequences available in the GenBank database, band A was most similar to an Arctic seawater isolate (Mergaert *et al.*, 2001) and groups within the genus *Pseudomonas.* Band B was most similar to strains of *Colwellia rossensis* isolated from Antarctic sea-ice (Brinkmeyer *et al.*, 2003). However, it appears to form a novel lineage as it is not closely related to any cultured strains and in fact forms a separate branch to members of the *Colwellia* genus (Figure 6.4). The fact that both these sequences are related to strains isolated from cold water and ice rather than sediment probably reflects how the enrichment process described here selected for free-living rather

than attached bacteria. There seems to have been a combination of three selective pressures on these cultures: SAB as sole carbon source, cold temperature (initially incubated at 0°C) and the seawater salts medium.

In Chapters three and four it was concluded that contamination was one of a number of factors affecting the microbial community structure in the marine sediment near Casey Station. The results presented in this chapter offer the first evidence that the potential for hydrocarbon biodegradation does exist in the near-shore marine sediments. One of the opportunities now is to develop specific gene probes to identify the prevalence of alkane degradation in contaminated sites. Being able to unambiguously observe *in situ* biodegradation is the first step towards defining long-term natural biodegradation rates, which in turn will help to predict the longevity of petroleum spills in the Antarctic marine environment.

7. General Discussion and Conclusions

In 1993 the first paper detailing the use of DGGE in microbial ecology was published by Muyzer *et al.* (1993). At the time it was a promising technique for following changes in microbial communities as each band on the gel was theoretically due to the presence of a different species. It also allowed the processing of multiple samples, which is necessary for large, ecologically relevant studies. Several authors such as Murray *et al.* (1996), Vallaeys (1997) and Schauer *et al.* (2000), explored the limits of detection and reproducibility of the method and although shortcomings were discovered (for example co-migration of different bands) it appeared to be a useful method.

One of the initial steps in this thesis was to explore the reproducibility of DGGE banding patterns (see Chapter 3). Experiments with a few samples found that the banding pattern of a sample varied with PCR and gel run, consistent with other reports in the literature (eg. Ferrari and Hollibaugh, 1999). However, the differences between samples were greater than differences in banding patterns from the same sample. Combining the results of multiple DGGE runs, effectively a process of re-sampling from the extracted DNA, overcame this problem. However, in Chapter 4 further exploration of variability in banding patterns discovered significant variation in the banding patterns from the same sample and in Chapter 5, differences between treatment groups that were significant in one set of analyses were not significant in a later set of analyses. The reasons for this probably originate from the fact that sediment is a complex matrix and sediment communities are very diverse. Obtaining quality DNA from sediment is difficult because of the presence of humic substances and because of differences in the ease of extraction of DNA from different species. The amplification of fragments of DNA by PCR is subject to bias and finally gel-to-

gel variation seemed to be a major source of banding pattern variability. Improvements in technology, such as improved taq polymerase, new DNA dyes and better imaging systems, have assisted in alleviating but not removing some of these problems. Different approaches, such as using specific primers to target smaller groups of micro-organisms, are producing more reliable results (eg. Boon *et al.*, 2002).

Nevertheless, there are two uses for DGGE. It can be used as a means of separating fragments of DNA for sequencing, which provides information on the presence of specific species, or the overall banding pattern can be used as a fingerprint of the community structure. In the latter case a multivariate approach to statistically analysing the banding patterns has proven useful. It can produce a graphical representation of the similarities between individual samples and groups and can offer an estimate of the significance of differences between groups. Procedures are also available that can calculate a correlation between biological variables (such as DGGE banding patterns) and environmental variables.

Other DNA fingerprinting techniques - for example amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (t-RFLP) and ribosomal intergenic spacer analysis (RISA) - are available. However many of the same problems will apply to these techniques: the ability to obtain quality DNA from sediment, the high diversity of microbes present in soils and sediments, sample heterogeneity and the inability to identify bands without further work. New methods such as DNA micro-arrays (Zhou, 2003) are promising and may eventually enable microbial ecologists to follow microbial communities more closely. At the present time these methods are still being developed and tested and not are yet widespread in microbial ecology. The focus of microbial ecology appears to be shifting towards following processes *in situ*, for

which new methodologies such as stable isotope probing (Radajewski *et al.*, 2003) are being developed. Rather than asking "who is out there?" (Amann, 2000) microbial ecologists are now asking "what are they doing?".

In this work, the natural diversity of the microbial populations in two locations was explored using a well-established method : clone library analysis. As expected, a high level of diversity was observed. The two locations, one impacted by pollution the other pristine, shared many similarities. This was also observed in DGGE analysis of multiple samples from these two locations. In Chapter 3, a high degree of spatial variability was observed in Brown Bay whereas the O'Brien Bay samples seemed to cluster geographically. Multivariate analysis correlated samples from these two bays and two others with environmental variables. The best correlations were with TOC, Mn, Fe and As. The correlation however was only 0.4 suggesting that other factors also influence the microbial community structure.

Variability in community structure was also observed in Chapter 4. Within sample variation and variation between samples within the same treatment group was explored. One possible reason for this variation is an even distribution of species abundance, or rather lack of dominance by a few species. Again this highlights the difficulty, and importance, of adequately sampling complex communities. It was observed that hydrocarbon contamination and the location of deployment of the experiment both had an effect on the development of the microbial community structure thus providing more evidence that oil contamination affects microbes.

The field experiment described in Chapter 5 was designed carefully to optimise the amount of data that could be collected for chemical, microbial and infaunal community analysis. Fresh samples allowed a polyphasic approach to describing the microbial population and the three methods used complemented each other. Significant differences were seen in the effects of different oils on the

microbial community structure and it appears that an oil marketed as biodegradable (Titan GT1) has the least effect on the microbial population. Again there were issues with the reproducibility of DGGE results but closer statistical analysis of a second set of data led to the same conclusion.

One of the most important questions in the investigation of the microbial ecology of contaminated sites is whether the potential for biodegradation of the contaminants exists at that site. A microcosm experiment provided evidence that microbes isolated from sediment in the Casey region are capable of hydrocarbon biodegradation.

In summary, five conclusions can be drawn from this work:

i) DGGE with universal primers has a limited usefulness in monitoring sediment microbial communities

ii) multivariate statistical procedures are well suited to analysing DGGE data

iii) Antarctic benthic microbial communities are diverse with an even distribution of species

iv) oil contamination has a significant effect on the benthic microbial communities

v) potential for hydrocarbon biodegradation exists in the marine sediments near Casey

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