Microbial Abundance and Diversity Surrounding Mummified Seals in Miers Valley, Antarctica

A thesis

submitted in partial fulfilment

of

the requirements for the Degree

of

Master of Science in Biological Sciences

at

The University of Waikato

by

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The University of Waikato

Te Whare Wānanga o Waikato

2004

ABSTRACT

The presence of a number of mummified seal carcasses have been reported in the Dry Valleys of Antarctica. The location of seal carcasses were observed up to 240km in-land, and approximately 1500m above sea level. The extremely cold and dry conditions in the Dry Valleys act to mummify the seal remains, preserving these anomalies for an undetermined length of time.

Given the virtual absence of above ground plant biomass in the Dry Valleys, the primary source of soil organic matter sustaining microbial ecosystems was not obvious. It was hypothesised that these large sources of organic contamination, in the form of a seal carcass, may act to enrich the localised environment surrounding the carcasses with carbon and nitrogen. The increased levels of organic carbon and nitrogen would result in increased microbial abundance and diversity.

A total of 100 environmental samples were taken by Professors S.C. Cary, and D.A. Cowan, surrounding, and directly beneath eleven mummified seals located in Miers Valley, the Antarctic Dry Valleys, on Event D023 Terrestrial Microbiology Antarctic New Zealand 2001/2002. Three of these seals (MVS1, MVS3 and MVS13) and the areas surrounding, were sampled extensively, and were the basis of research in this Master of Science thesis.

Microbial diversity was investigated using molecular cloning and sequence analysis, and Denaturing Gradient Gel Electrophoresis, of 16S and 18S rDNA amplicons. A large diversity in bacterial populations present in the environmental samples taken from directly beneath the Miers Valley seal carcasses was observed in comparison to control samples of Miers Valley soils.

The levels of microbial abundance were determined by luciferase-dependent luminometric ATP analysis, and analysis of the total quantities of double stranded

DNA extracted from the environmental samples. Microbial abundance was found to be increased in samples taken in closer proximity to a seal carcass.

Carbon and nitrogen analysis were conducted on all samples, using a fully automated Europa Scientific 20/20 isotope analyser. The level of carbon present in an environmental sample appeared not to influence the abundance of microorganisms. The levels of nitrogen measured were higher in environmental samples collected in close proximity to the seal carcasses. These samples displayed the greatest microbial abundance, and indicated large differences in the bacterial diversity compared to Miers Valley control samples.

On the basis of these results, and ATP data, the hypothesis of this MSc thesis was determined to be correct. Microbial abundance was found to increase with enrichment of nitrogen surrounding MVS1 and MVS13 seal carcasses, and large differences in bacterial diversity were found in environmental samples showing the highest levels of nitrogen enrichment, compared to samples taken further away from the seal, or in Miers Valley control samples.

This thesis effectively described the utilisation of cloning and sequence analysis, and denaturing gradient gel electrophoresis to investigate microbial diversity in Antarctic environmental samples. This study gave a greater understanding in microbial ecology in Miers Valley, Antarctica.

ACKNOWLEDGEMENTS

I would first and foremost like to thank my supervisors Professor Roberta Farrell, Professor Steven Craig Cary, and Professor Roy Daniel for giving me the opportunity to work on this project. I would also like to thank Professor Don Cowan for his assistance in collecting the samples in the field and for his helpful advice, and data throughout my MSc research.

I would like to extend my appreciation also to Thomas Niederberger, and Dr Ron Ronimus at the Thermophile Research Unit for their endless hours of help. Gratitude for greatly needed advice goes also to Dr Ray Cursons at the Genetics Unit of Waikato University, and to Wade Tozer of the Plant Physiology Lab.

I would like to thank all those who I had the pleasure of working with in the Cary Laboratory, College of Marine Studies, University of Delaware. Dr Barbara Campbell, Dr Kathy Koyne, Ray Wheller, and Elizabeth McCliment. I sincerely thank you for the help you gave me, and the kindness you showed me throughout my stay

I would also like to thank everyone I had the distinct pleasure to work with in C2:10. For their technical advice, thank-you Shona Duncan, Arvina Ram, and Dr Joanne Thwaites. To Sandra Wilcocks, thank-you sincerely, for the many hours of help you have given me. With all of you, I have enjoyed many great discussions, and shared many memorable laughs, and have no doubt gained life-long friends.

To my family and friends. It has been a long journey, and one which has many turns still to take. Thank-you for your unlimited support, patience and love.

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LIST OF ABBREVIATIONS

A_{260}/A_{280}	absorbance at 260/280nm
AMS	Accelerator Mass Spectrometry
APS	ammonium persulfate
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BLASTn	BLAST tool that compares a nucleotide query sequence against a nucleotide sequence database.
bp	base pairs
CFU	colony forming units
CTAB	cetyltrimethylammoniumbromide
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribose nucleic acid
ddH_2O	double distilled H_2O
dNTP	deoxy-nucleotide triphosphates (dATP, dCTP, dGTP, and dTTP
EDTA	ethylenediamine tetraacetic acid
µFD	microfarad
fMQ H_2O	0.2 µm filtered Milli-Q H_2O
IPTG	isopropylthio-β-D-galactoside
kb	kilo-base
LB	Luria-Bertani medium
M	molarity
MAF	Ministry of Agriculture and Forestry
Milli-Q	Millipore Corporation
mM	millimolar
MVS	Miers Valley Seal
MVT	Miers Valley transect
µm	micron
µM	micromolar

N	any nucleotide (adnenine, thymine, guanosine or cytosine)
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometer
OD	optical density
OHM	SI unit of electrical resistance
PCR	polymerase chain reaction
pg	pictogram
rDNA	ribosomal DNA
RLU	relative light units
RNA	ribose nucleic acid
rRNA	ribosomal RNA
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SDS	dodecyl sulphate sodium salt
TAE	tris acetic acid EDTA buffer
TE	tris EDTA buffer
TEMED	N,N,N^{I},N^{I}-tetramethylethylenediamine
Tris	trihydroxymethyl amino methane
tRNA	transfer RNA
U	units
UPGMA	unweighted pair group method using arithmetic averages
UV	ultraviolet
V	volts
vol/vol	volume per volume
vol/wt	volume per weight
wt/vol	weight per volume
ww	wet weight
X	one molar concentration
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER ONE

Introduction

1.1 General introduction

Microbes are the most abundant and most diverse forms of life on earth (Whitman et al., 1998). Despite this abundance, little is known about the majority of microorganisms, with only an estimated 0.1-0.5% having been cultivated in the laboratory (Torsvik et al., 1990).

This thesis research aimed to investigate microbial diversity beneath mummified seals found in the Dry Valleys of Antarctica. The presence of Archaea and Eukaryotic organisms was investigated surrounding these sources of organic contamination. The main focus of this research, however, related to bacterial diversity surrounding these anomalies. Microbial diversity within the scope of this study was investigated using molecular analysis of the genes encoding the small subunit ribosomal ribose nucleic acid molecule (16S rRNA).

1.2 Antarctica

A place of great exploration and adventure for the past 200 years, a land referred to as *Terra Australis Incognita,* "the Unknown Southern Land" by Ptolemy, an Egyptian geographer/cartographer nearly 2000 years ago (Heacox, 1998), the continent of Antarctica has held the imagination of scientists and explorers world wide.

Antarctica, shown in Figure 1.1, is the world's fifth-largest continent, spanning an area of 14,245,000 square kilometres. Of this area, approximately 13.72 million square kilometres is covered with ice, with approximately 280,000 square kilometres ice-free. It has the highest mean elevation (2,300 metres), and the lowest temperature recorded (-89.6^{o}C in July 1983) of any continent (Wynn-Williams, 1990).

The biodiversity of Antarctica is much lower than more temperate regions (Campbell and Claridge, 1987). Indeed, the biota of Antarctica contrasts markedly with that at the same latitude in the Northern Hemisphere, where biologically diverse ecosystems with a high biomass also include land-based vertebrate animals (Campbell and Claridge, 1987). Land-based vertebrates are limited to only some areas of the Antarctic, and are far lower in species diversity by comparison to other continents. Vascular plants are scarce, represented by only two species found in the northern part of the Antarctic Peninsula (Campbell and Claridge, 1987). Prokaryotes represent the greatest biomass in the Antarctic ecosystems (Franzmann, 1995). The continent is geographically isolated from other major land masses. This isolation is imposed mainly by the large circumpolar Southern Ocean (Wynn-Williams, 1991).

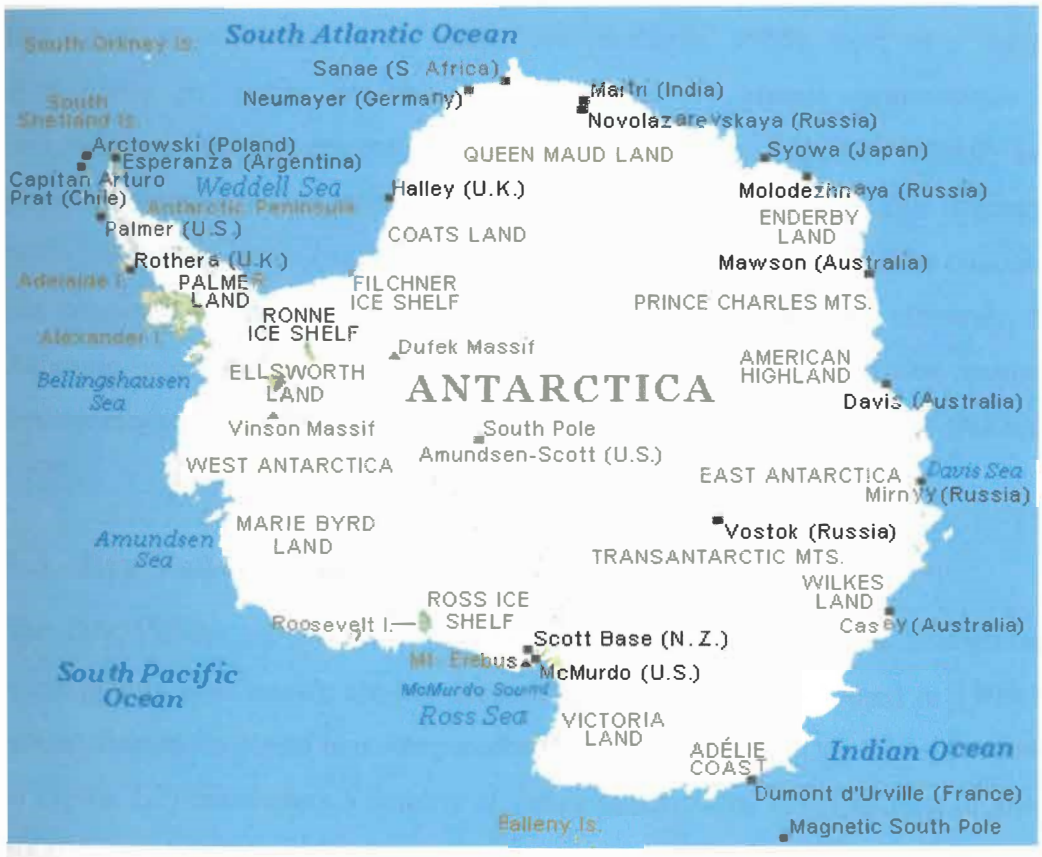


Figure 1.1. Map of the Antarctic continent (Microsoft Encarta Encyclopaedia, 2001).

Other than through human or animal contamination, ocean currents and windborne methods are the only means through which organisms may reach the Antarctic (Wynn-Williams, 1991). The low temperature of the ocean surrounding

the Antarctic and the desiccating conditions faced through airborne dispersal act to limit the migration of all but the hardiest organisms from neighbouring land masses. Given the ability of a number of prokaryotic species to survive in extreme conditions of temperature, pH, salinity and pressure, among other factors, the apparent dominance of the group (Franzmann, 1995) is perchance unremarkable. The wind pattern over Antarctica is influenced largely by the cold mass of ice at its high elevations. The draining of a layer of very cold air over the surface of the continent in the form of down sloping, or katabatic winds, is responsible for the almost constant high winds and blizzard conditions recorded at many coastal stations in Antarctica (Campbell and Claridge, 1987).

It is this continent's isolation from other land masses, its low temperatures, highly productive marine basins, and its comparative freedom from human habitation that spark such interest in its microflora (Sieburth, 1963), both as a unique opportunity to gather baseline data for relatively simple communities in comparatively pristine environments (Ellis-Evans, 1985), and more recently as a potential storehouse for what may be ecologically and economically important biotechnological finds. Isolation and description of microflora from the continent has presented a number of novel findings over the past six decades. However, the Antarctic ecosystem still represents one of the most undescribed extreme environments available to researchers of microbial diversity and ecology (Nichols, 1999).

1.3 Dry Valleys of Antarctica

The Dry Valleys of southern Victoria Land are perhaps the best studied and subsequently well known ice-free areas of the Antarctic. Discovered in 1903 by Robert Falcon Scott and two companions (Heacox, 1998), the Dry Valleys (shown in Figure 1.2) encompass a number of valley systems that cover an area of about 5000 square kilometres and lie between 160^{o} and 164^{o}E, and 76^{o}30' and 78^{o}30'S. The valleys were formed by (typically) east-west oriented glaciers. The glacial flow has since been cut off by the Transantarctic Mountains (Friedmann, 1982), leaving a vast cold, ice-free desert.

The biodiversity of the Antarctic Dry Valleys is minimal in comparison to more temperate regions. The most abundant and widespread invertebrate in the soils of the Antarctic Dry Valleys is the microbial feeding nematode (Burkins, 1996). Its distribution throughout the Valleys is patchy, relating to soil properties such as salinity and pH (Burkins, 1996). The only macroscopic plants that occur on the continental coast of Antarctica or in moist continental oases are mosses and lichens (Vishniac, 1993).

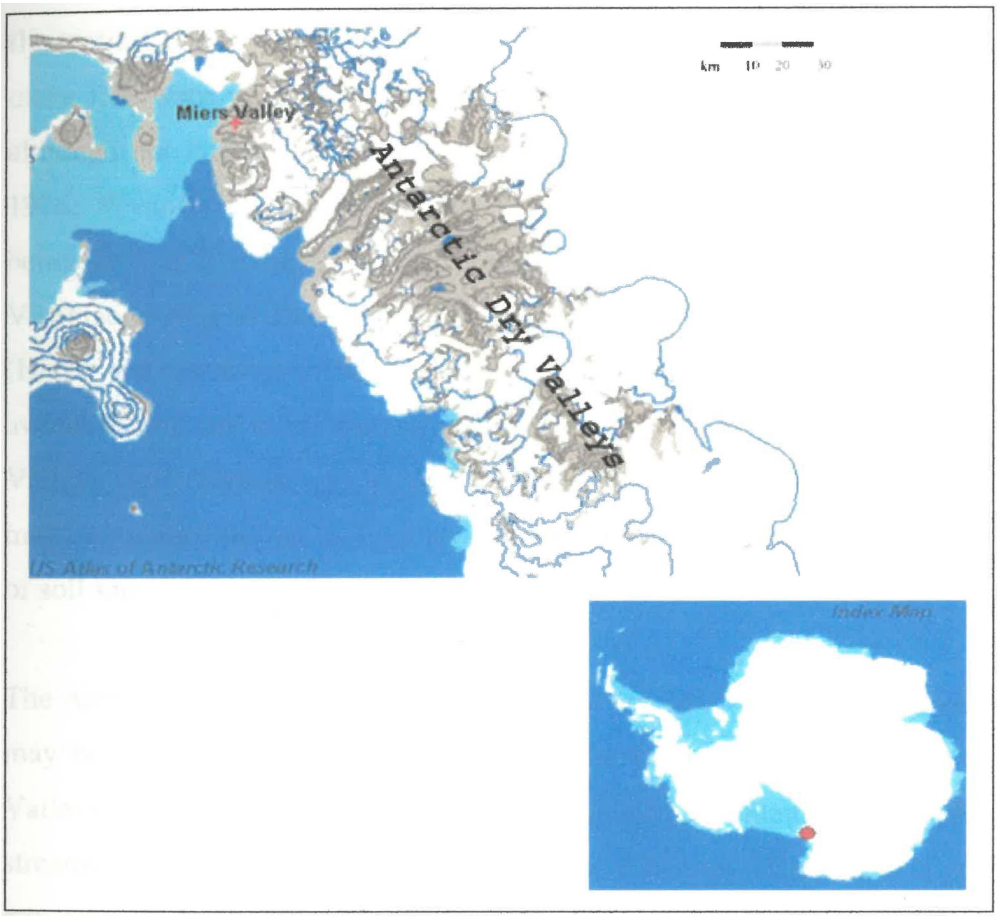


Figure 1.2 Map of Dry Valleys identifying Miers Valley (composed using U.S Atlas of Antarctic Research).

The Dry Valleys have a considerable range of temperatures. During winter, when there is continuous darkness, air temperatures as low as -60°C prevail (McKnight and Tate, 1995; de la Torre, 2003). Alternately, in summer there is continuous light, and air temperatures range from -35°C to +5 °C (McKnight and Tate, 1995; de la Torre, 2003). Most temperature measurements have typically been recorded on the Valley floors near deep permanent lakes (Thompson et al., 1971). It could be argued that thermal perturbation of the lake would make these sites

unrepresentative of the valleys, in particular when compared with areas at higher elevations, implying that mean temperatures calculated on these measurements are not necessarily reflective of how low temperatures in the Dry Valleys can be (McKay, 1998). Surface temperature measurements, taken periodically during successive diurnal cycles, indicated that the temperature of surface mineral soils can fluctuate from -1^{o}C to +16^{o}C (Cowan et al., 2002). Oscillations in temperature of up to 15 ^{o}C due to cloud cover can occur in only minutes (de la Torre, 2003). To survive this environment, organisms must be able to cope with such rapidly fluctuating, extreme temperatures. Low temperatures prevalent in the Dry Valleys make free water scarce during most of the year (Vishniac, 1993). Precipitation, although not infrequent, is scarce and never falls as rain (Uydess and Vishniac, 1976; Wynn-Williams, 1990). The "poorly recorded" snowfall (suggested as being around 5-10cm yr^{-1}) does not accumulate (Harris and Cartwright 1981; Vishniac, 1993) as the potential sublimation rate may approach ca. 50cm yr^{-1} (Harris and Cartwright, 1981). It has been suggested, perhaps, that it is a lack of available moisture rather than any other factor that limits microbial activity in Dry Valleys soils (Uydess and Vishniac, 1976). Water activity in Dry Valleys soils may be reduced further by high levels of salinity found to be present in a number of soil samples taken (Claridge and Campbell, 1977; Cowan et al, 2002).

The Antarctic Dry Valleys are not completely devoid of water. Water availability may be increased during some periods in the year in certain areas of the Dry Valleys. Snow that accumulates on the surrounding mountain tops, supplies melt streams that feed rivers of the region (Wynn-Williams, 1990). For a period of around 6-10 weeks from November to January, glacial meltwater streams flow through many of the valley systems in the Antarctic Dry Valleys (Alger et al. 1995; McKnight and Tate, 1995). Cameron (1969) found that there was an increase in soil moisture from the surface to the depth of ice-cemented permafrost in the Dry Valleys. Further measurements made during the course of their research, however, did not indicate any obvious movement of soil moisture in the vapour phase from permafrost to surface, rendering this source of moisture unavailable to organisms near or at the soil surface. Cameron and his colleagues found that regardless of sample site and depth, the overall average *in situ* moisture content was approximately 3.5%. However, Cameron made note that much higher

and lower values had been obtained for Wright and Taylor Valleys indicating that this level of soil moisture was not consistent for all Dry Valley soils (Cameron, 1969).

1.4 Nutrient availability in Antarctic soils

Most natural soils have limiting abundances of organic and inorganic nutrients. Given the virtual absence of above ground plant biomass in the Antarctic Dry Valleys, the primary source of soil organic matter sustaining soil microbial ecosystems is not obvious (Burkins, 1996). It is generally accepted that the presence of organic matter implies the availability not only of organic carbon but of varying proportions of organic nitrogen and potentially other nutritional requirements (Vishniac, 1993). Hence, it would appear that microbes present in Antarctic soils, and in particular soils of the Dry Valleys region, may be severely limited by a lack of nutrient availability. This opinion was stated by Vishniac (1993) who proposed that organic matter, or perhaps better termed a lack of organic matter, typically inhibited microbial growth in Antarctic soils when water did not. This opinion was not held by everyone. For example Cameron and Conrow (1969) stated that there was no apparent correlation between the numbers and kinds of microorganisms in the Dry Valleys and the exceptionally low levels of organic carbon and nitrogen. Cameron and Conrow (1969), however, proposed that there was a correlation with the degree of maturity of the soil, represented by the degree of structural differentiation, by colour variations and by differences in salt accumulations. Whether Cameron and Conrow's (1969) findings are still relevant, given their work with methodology almost a quarter of a century before Vishniac, could be a matter of debate.

Carbon and nitrogen constitute the two most abundant elements in a prokaryote cell, making up approximately 50% and 12% respectively, of the total dry weight of a typical prokaryotic cell (Madigan et al., 2000). Nitrogen is a major element in proteins, nucleic acids, and is important in many other constituents in a cell. Nitrogen can be found in nature in both organic and inorganic forms. The bulk of available nitrogen in nature is in inorganic form, either as ammonia (NH_3), nitrate (NO_3^-) or N_2 (Madigan et al., 2000).

There have been numerous environmental samples from the Dry Valleys analysed for their chemical properties, in particular the levels of carbon and nitrogen, (Boyd and Boyd, 1963; Vishniac and Hempfling, 1979; Aislabie et al., 2001). A survey of all published physical and chemical analyses of Ross Desert soils revealed not only a diversity of analytical methods, but also substantial variation in results from reportedly the same sites (Wynn-Williams, 1990). Given the small volumes of samples typically used to determine total carbon and total nitrogen, the homogeneity of samples tested with the surrounding soil is a factor that should be considered. Select areas that may have been contaminated with skua guano, or where there was lichen growth, may have been more enriched than surrounding soils. Some of the methods used were perhaps questionable as to their meaningfulness in such a setting. For example, Greenfield (1981) found that loss on ignition analyses (600^{o}C for 24 hours) of samples from Cape Bird indicated an organic matter content of between 2-8% of the total weight of sample. Such high organic matter contents are characteristic of temperate soils. Greenfield hypothesized that some salts, for example carbonate, may have been decomposed at 600^{o}C, resulting in organic carbon determinations that were unreliable, and perhaps indicated values that were higher than those actually present.

On analysis of environmental samples west of, and a traverse north of Coalsack bluff (a rock bluff at the northern limits of Walcott Neve in the Transantarctic Mountains), Cameron (1970) found nearly all samples to be high in NO_3 and reported levels of organic (Kjeldahl) nitrogen of 0.3-0.4 weight percent (wt%). Cameron found organic carbon to be low (0.02-0.05 wt%), stating that these carbon levels were similar to other cold desert soils. Cameron concluded that the extremely low carbon to nitrogen ratios (2-0.1) present in the Antarctic terrestrial environmental samples indicated either highly composed colloidal or microbial organic matter, or relic carbon, such as coal, based on suggested ratios by Horowitz et al. (1969). Cameron noted that carbon particles were found impacted on all of the groups air sample filters, indicating their presence in airborne matter. A later investigation by Cameron (1971), of samples from McKelvey Valley and Pearse Valley, reported levels of 0.001 to 0.006 wt% organic (Kjeldahl) nitrogen, which has a wider range than the samples analysed from north of Coalsack Bluff.

The range of percent weight organic carbon was also broader in McKelvey Valley and Pearse Valley, varying between 0.01 to 0.09 (Cameron, 1971).

Chemical composition of soils may vary with depth. Aislabie et al. (2001) reported water content, pH, ammonia nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), total carbon and total nitrogen for seven samples taken from soil at different depths in Wright Valley, Antarctica. Total carbon and total nitrogen were determined using a Leco PF-2000 analyser. Water content, pH, ammonia nitrogen (NH_4-N) and nitrate nitrogen (NO_3-N) levels given in the data (Table 1.1) were measured following Blakemore et al (1987) methodology.

Table 1.1 (Aislabie et al., 2001) Water content, pH, ammonia nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), total carbon and total nitrogen for seven samples taken from soil at different depths in Wright Valley, Antarctica.

Depth (cm)	Water content (%)	pH	Total carbon (%)	Total nitrogen (%)	NH_4-N (mg/kg)	NO_3-N (mg/kg)
0-2	0.2	7.6	0.03	0.008	1.5	0.0
2-5	1.0	7.7	0.02	0.005	1.2	2.9
5-15	1.9	7.3	0.02	0.052	0.3	129.0
15-48	2.0	7.1	0.02	0.037	0.2	103.8
48-75	3.0	7.2	0.02	0.034	0.0	82.1
75-106	4.5	7.4	0.03	0.031	1.1	86.5
106-125	4.5	7.5	0.03	0.031	0.1	76.8

These results showed very low levels of carbon in the soil (Aislabie et al., 2001). The level of total carbon present appeared stable regardless of depth. Levels of total nitrogen in the soil were also very low. The level of total nitrogen, however, tended to be larger at depths below 5cm than values recorded for the first 5cm of soil from the surface. Inorganic nitrogen in the samples was indicated by the levels of ammonia nitrogen (NH_4-N), and nitrate nitrogen (NO_3-N) given in Table 1.1. These results showed that the levels of ammonia nitrogen varied, showing no apparent trend with increased depth. High levels of nitrate in the soil from Wright Valley were detected. No nitrate nitrogen could be detected, however, from the soil taken up to 2cm from the surface. The highest level of nitrate nitrogen recorded in the sample set was at a depth of 5-15cm, indicating a wide range in the level of nitrate nitrogen in the top 15cm of soil.

The results of Aislabie et al. (2001) appeared to concur with observations made by Cameron et al., (1970, 1971). Both of these Antarctic studies reported high levels of nitrate, and low levels of organic carbon and organic nitrogen in Antarctic terrestrial environmental samples. Wada et al. (1981), found nitrate to be a major component among nitrogenous compounds in soils from Wright Valley and Cape Bird, on Ross Island, Antarctica. They stated that the concentrations of organic nitrogen found (0.34-6.4µg atom N per gram of dry soil), were one to two orders of magnitude lower than those of nitrate, except for those samples collected from a penguin rookery at Cape Bird.

No published data on the chemical composition of Miers Valley terrestrial environmental samples was found. Therefore, no direct comparisons of the chemical compositions of Miers Valley soils to soils collected from Wright Valley (Aislabie et al., 2001; Wada et al., 1981), McKelvey valley, and Pearse Valley (Cameron, 1971), located in the Antarctic Dry Valleys or comparison with soils taken from Coalsack Bluff, in the Transantarctic Mountains.

Although measured levels of organic carbon and organic nitrogen in Dry Valleys soils are regarded as low compared to levels recorded in terrestrial environmental samples from other continents, the levels reported by Aislabie et al. (2001) and Cameron (1970, 1971), may be considered relatively high, given the lack of above ground plant biomass (Cameron, 1970; Burkins, 1996). The measured photosynthetic capacity of Dry Valleys soils may be inadequate to account for observed levels of soil organic matter (Burkins, 1996). It is thought that airborne organic matter may play a crucial role in the dispersion of organic matter throughout the Dry Valleys (Vishniac, 1993). Indeed the highly productive marine basins and ice-covered lakes, harbouring extensive cyanobacterial mats, may well be a source of organic particles carried to the Dry Valleys by wind currents. Parker et al. (1982) estimated that wind may act to remove a total of 2.93×10^4 kg yr^{-1} of particulate material from cyanobacterial mats in three permanently ice-covered lakes in Taylor Valley.

1.5 Isotopic compositions of carbon and nitrogen

Isotopic composition, specifically $\delta^{15}N$ and $\delta^{13}C$, have often been used in scientific research to trace food chains (Peterson and Fry, 1987; Raven, 1987; Handley and Scrimgeour, 1997). The use of isotopic composition to identify sources of carbon and nitrogen, and to trace food chains within a system has often been oversimplified. To accurately interpret $\delta^{15}N$ values in terms of sources and sinks, one must know the source $\delta^{15}N$ values, be able to quantify the fractions occurring between source and sink, and to be most effective, be able to estimate nitrogen fluxes (Handley and Scrimgeour, 1997). An increase in $\delta^{15}N$ with trophic levels of typically 0‰ to 3.5‰ can generally be assumed (Wada et al., 1995). However, surprising $\delta^{15}N$ values are recorded on occasion that do not follow this general rule of enrichment per trophic level (Handley and Scrimgeour, 1997). Since the residual $\delta^{15}N$ of soil is related to both the signature and amount of inputs and outputs, and also to the relative influence of contributing microbial processes, a direct correlation between $\delta^{15}N$ and remaining levels of N in a soil system, is not expected to follow a consistent rule (Handley and Scrimgeour, 1997).

Isotopic compositions were expressed in terms of delta (δ) values, measured in parts per thousand differences from a standard (‰) as calculated by the following equation:

$$\delta X\ (‰) = [(R_{sample}/R_{standard}) - 1] \times 10^3$$ (Peterson and Fry, 1987)

where X was the designated isotope being measured (in this thesis research either ^{13}C or ^{15}N), and R was the corresponding ratio of the isotope ($^{13}C/^{12}C$, $^{15}N/^{14}N$ respectively for carbon and nitrogen). Increases in the values of δX signify increases in the heavy isotope component (Peterson and Fry, 1987; Handley and Scrimgeour, 1997). A fractionation was a change in that ratio (Handley and Scrimgeour, 1997). Positive δ values indicated an enrichment in the heavy isotope in the sample relative to the standard; negative values indicated a depletion (Raven, 1987).

A review of data on the $\delta^{15}N$ of NH_4^+ and NO_3^- in soil for both aquatic and terrestrial environments indicated that inorganic nitrogen was generally positive with respect to the natural abundance of ^{15}N in the atmosphere (Raven, 1987).

Generalised, average $\delta^{15}N$ values for nitrate, listed by Wada et al. (1981), indicated values of 3.2‰ for forest soils; 6.4‰ for cultivated soils due to the input of artificial fertilizers containing NO_3^- (Raven, 1987); 7.0‰ for oceanic waters, and -6.6‰ for rain waters. Representative values of $\delta^{15}N$ distributed throughout the ecosystem were presented in diagrammatic form by Peterson and Fry (1987) (shown in Figure 1.3).

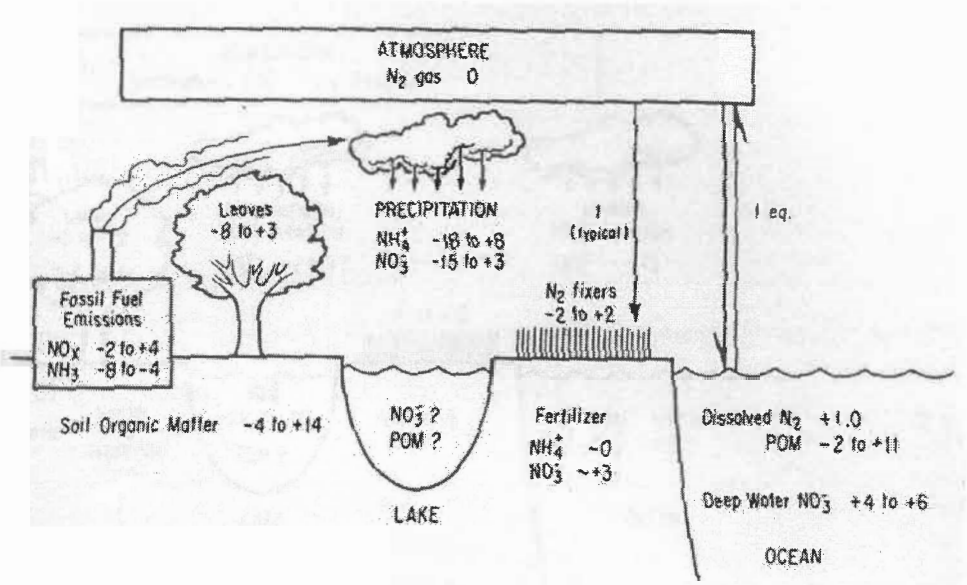


Figure 1.3 The $\delta^{15}N$ distributions in ecosystems (Peterson and Fry, 1987). The double arrow signified an equilibrium isotope fractionation. The number on the single arrow indicates the fractionation occurring during transfers. POM = Particulate Organic Matter.

Delta ^{15}N levels for soil nitrate measured from terrestrial environmental samples taken from Wright Valley and Cape Bird exhibited very low values of -11‰ to -24‰, in comparison to average $\delta^{15}N$ values for nitrate in terrestrial samples (Wada et al., 1981). These values of $\delta^{15}N$ indicated depletion in ^{15}N compared to the level of ^{15}N found in the atmosphere. It has been hypothesised that nitrate in Antarctic soils is mainly derived from atmospheric precipitation which carries NO_χ depleted in ^{15}N, as no other known process can produce nitrogen oxides of such low $\delta^{15}N$ (Wada et al., 1981).

Algal samples collected from Wright Valley and Cape Bird were analysed for levels of $\delta^{15}N$ (Wada et al., 1981). The lowest value of $\delta^{15}N$ given (-49‰), was taken from algae collected from a small saline pond, near the edge of Wright Upper Glacier. It represented one of the lowest $\delta^{15}N$ values ever reported for a terrestrial sample. Algal samples showing high ^{15}N enrichment were also reported

by Wada et al. (1981). A value of $\delta^{15}N$ measuring +30.7‰ was gained from algal felt, and a $\delta^{15}N$ value of +28.6‰ was measured in organic nitrogen collected from the Adelie penguin rookery at Ross Island (Wada et al., 1981). Wada et al. (1981) theorised that the enrichment found in the penguin rookery was due to microbial conversion of uric acid, obtained from the penguins diet of krill and fish, and other organic nitrogen to ammonia. The ammonia evaporated with a high nitrogen isotope fractionation leaving nitrogenous material rich in ^{15}N in soils. Given the cold climate of Antarctica, the slow rate of biogeochemical processes would allow for much greater fractionation of the nitrogen isotope, explaining the high ^{15}N enrichments (Wada et al., 1981).

No research was found indicating the levels of $\delta^{13}C$ present in Antarctic soils. Representative values of $\delta^{13}C$ throughout the ecosystem, however, were presented in diagrammatic form by Peterson and Fry (1987) in Figure 1.4.

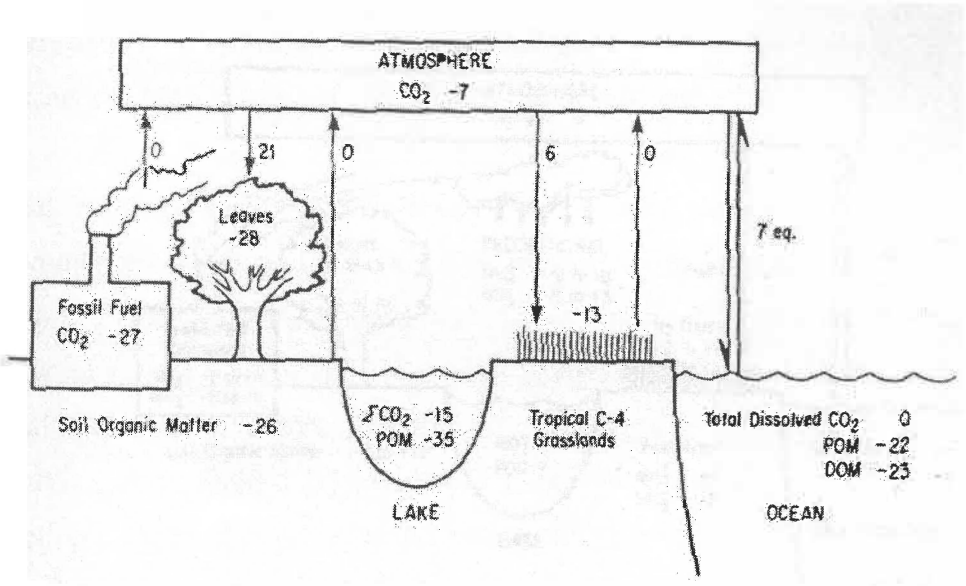


Figure 1.4 The $\delta^{13}C$ distribution in ecosystems (Peterson and Fry, 1987). Single arrows in the diagram indicated CO_2 fluxes. The double arrow signified an equilibrium isotope fractionation. Numbers for pools indicate $\delta^{13}C$ values (‰) and numbers for arrows indicate the fractionation occurring during transfers. POM = Particulate Organic Matter. DOM = Dissolved Organic Matter.

1.6 Mummified seals of the Antarctic

Seals are carnivorous mammals, with a sleek body adapted for swimming and living in cold regions (Heacox, 1998). Seals display impressive agility and speed in the water, on land, however, they appear cumbersome and slow. Their short webbed feet that are modified as flippers, are ill equipped to lug their

disproportionately large bodies across terrain. It is not surprising then to find that seals rarely venture far onshore, except, it seems, in Antarctica (Dort, 1982). In the Antarctic Dry Valleys there have been many sightings of seal carcasses. The extremely arid conditions and cold temperatures desiccate the carcasses, essentially mummifying their remains.

1.6.1 History of observations of Dry Valleys mummified seals

Given the oddity of these mummified creatures, there was surprisingly little literature published. The most comprehensive review of mummified seals in Antarctica was written by Dort (1982). This review gave a comprehensive account of the history of mummified seal sightings, dating back to the early expeditions of Scott and Shackleton. Other attempts have been made to compile data on mummified seals. Péwé et al. (1959) recorded information for 90 seal carcasses seen during the 1957-1958 field season. Barwick and Balham (1967) studied 121 bodies in the Antarctic summers of 1957-1960. Dort, along with other member of the University of Kansas field parties, made observations on 79 seals during the field seasons of 1965-1966, 1966-1967, and 1969-1970 (Dort, 1982).

Seal remains were first discovered when land exploration of the region commenced with Captain Robert F Scott's' first Antarctic expedition 1901–1904 (Dort, 1971). Studying a dead seal, Scott wrote "How it came to be there is beyond guessing. It certainly is a valley of the dead" (Heacox, 1998). Sightings of seal carcasses have been mentioned in a number of publications (Behling and Calkin, 1970; Dort, 1971; Web and Leckie, 1971; Collen, 1978; Kellogg and Kellogg, 1988). It is probable that observant members of almost every field party that has operated in the Dry Valleys area of southern Victoria Land saw at least a few seal carcasses (Dort, 1982). The exact number of seal carcasses present in the Dry Valleys is unknown. Many of the observations were never published, and of those recorded it may be impossible to rule out any repetition, given the lack of detail, and accurate locations of sightings. Dort calculated a minimum of 210 seal bodies from records of sightings in the three main Dry Valleys systems: Victoria, Wright, and Taylor Valley's. Barwick and Balham (1967) reported 88 seals in the Wright Valley as opposed to only 30 sighted seals in the Victoria Valley. The difference in ease with which the two valleys could be entered from the coast was

thought to account for the difference in numbers. Dort estimated an additional 70 reported sightings in the three valley systems, may represent duplications of discoveries. In total it was thought that the remains of approximately 300 seals were present in southern Victoria Land (Dort, 1982). A more recent estimation of the total number of seal carcasses has not been calculated.

Péwé et al. (1959) and Barwick and Balham (1967) noted that most of the carcasses were found at the bottoms of valleys, with many along courses of ephemeral streams. Péwé et al. (1959) also observed several carcasses along the edges of lakes and at the heads of ephemeral streams where the streams issue from the glaciers, or at the heads of stream valleys. Seal remains have been found in inland locations other than the Dry Valleys. Although in comparison with the frequency and number of sightings in southern Victoria Land, these sightings outside of the Dry Valleys are notably rare (Dort, 1982). It is unclear whether this difference in the numbers of seals sighted was due to fewer numbers of seals entering those other areas. Or perhaps it was just that those areas may be less frequented and therefore the opportunity to sight seal carcasses were fewer, presenting a skewed view of inland seal dispersal. Harris and Watkins (1989) reported a carcass at Tvora ($0^{o}5'W$, $72^{o}12'S$), western Dronning Maud Land. The length of the carcass (2.32m), suggesting that it was subadult. The seal was tentatively identified from photographs as being a Crabeater seal (*Lobodon carcinophagus*). The location of the seal was some 240km from the present day ice-shelf, and approximately 1200m above sea level. Given the distance of the seal from the present day ice-shelf it was suggested that perhaps the seal was discarded by a previous expedition that may have carried it as food, for example dog rations. However, the inaccessibility of the location would not only have proven difficult for the seal to traverse, it would also have rendered it a highly impractical area for a campsite, or route for a dog team. Collen (1978) reported finding the remains of four seals during a visit to Black Island, Antarctica. Three were reported to be nearly complete Crabeater seals, one was described as being a small group of disarticulated bones. Collen noted that all four of the seal remains were found lying either in or close to a north-south tending valley, and appeared to have travelled south along these valleys from the Ross Ice-Shelf. The

southernmost carcass was found approximately 40km from the edge of the Ice-Shelf.

Webb and Leckie (1977) reported sightings of seal carcasses of various degrees of preservation in Miers Valley, Antarctic Dry Valleys. They report the sighting of a Crabeater seal carcass on the northeast shoreline of Lake Miers in Miers Valley. The carcass was thought to be a recent arrival as the skin was still soft and pliable. Blood had issued from both eyes, which had been picked out by what was thought to have been skua predation. Webb and Leckie reported staining of the sand beneath the head of the carcass with this blood. A recent New Zealand Antarctic Event (K023) to the area found numerous seal carcasses spread throughout the Valley (Antarctic New Zealand KO23 Science Report, 2002). Web and Leckie also reported the sighting of a fully feathered, but fleshed picked Adélie penguin skeleton on the bank of the Miers Valley stream, approximately 5km up the Valley from the coast. In Southern Victoria land, mummified seals have been found as far as 66km inland from McMurdo Sound, and range up to 1,200m above sea level (Dort, 1982).

Given the fact that Weddell seals (*Leptonychotes weddelli*) are the only long term residents in the Ross Sea region, it is perhaps surprising that 95% of mummified seals in southern Victoria Land that were able to be identified, were Crabeaters (*Lobodon carcinophagus*) (Dort, 1982). Péwé et al. (1959) stated that all except one of the identifiable seals his team discovered were Crabeater seals; the other seal was a Leopard seal (*Hydruga leptonyx*). The Crabeater seal is considered the most abundant marine mammal on earth. Its numbers are estimated to be greater than 15 million (Heacox, 1998). It is normally resident in the pack ice of Antarctica. Small numbers, however, mostly pups and subadults, make their way to McMurdo Sound in the late summer (Stirling et. al., 1971). Crabeater seals are the best equipped of the Antarctic seal species for locomotion on land (Stirling et. al., 1971), which may explain their prevalence in the Dry Valleys.

The lengths of mummified seals that were whole enough to have been measured and recorded, indicated that the majority were pups or subadults. Adult Crabeater seals have an average length of 2.35m, and an average weight of approximately

220kg. The average length for a female Weddell seal (3.3m) is slightly longer than the average length recorded for male Weddell seals (3m). Female Leopard seals are larger on average than male leopard seals, with an average length of females of approximately 3m, and an approximate weight of 370kg. Male Leopard seals on average are 2.8m in length, and weigh 325 kg. Péwé et al. (1959) found that the well preserved carcasses ranged from 1.06m to 2.13m, and had diameters ranging from 0.3m to 0.46m. The degree of preservation varied from seal to seal. Some were reported as being complete, with full bodies, intact pliable skin, and oily fluids exuding from their undersides, and in some cases blood soaking ground beneath the carcass. It can be assumed that these carcasses were recent arrivals, as most carcasses observed were leathery and dry with some specimens reduced only to bone and wind desiccated fragments of tissue (Péwé et al., 1959; Barwick and Balham, 1967; Webb, 1977; Dort, 1982). Barwick and Balham (1967) classified carcasses into seven groups (a – g) according to the degree of erosion they exhibited; (a) being a relatively undamaged complete carcass, with each successive letter indicating more damage to (g), described as minimal remains consisting of seal fragments.

1.6.2 Dating of seals

The age of the mummified seals is intriguing and a topic with differing opinions by some scientists. Visual estimation of age is difficult, as the degree of erosion of specimens is due largely to position of the carcass. Wind movement of sand particles is thought to be the predominant erosion force on seal carcasses (Dort, 1982). Radiocarbon analysis of mummified seal carcasses found in the Dry Valleys of Antarctica have ranged from 615 to 4,600 years (Dort, 1971). Barwick and Balham (1967) reported the approximate ages, determined by carbon dating of two seals. One sample was taken from a complete carcass, that exhibited partial erosion of hair on all exposed surfaces, cracking of skin to expose deeper tissues (due to desiccation), and some erosion of skin to expose the bones of the flippers and the cranium. This sample gave an approximate ^{14}C age of 100 years (refer to Figure 1.5). A second sample taken from minimal remains, consisting of only fragments of bone and tissue gave an approximate ^{14}C age, estimated at 780 years.

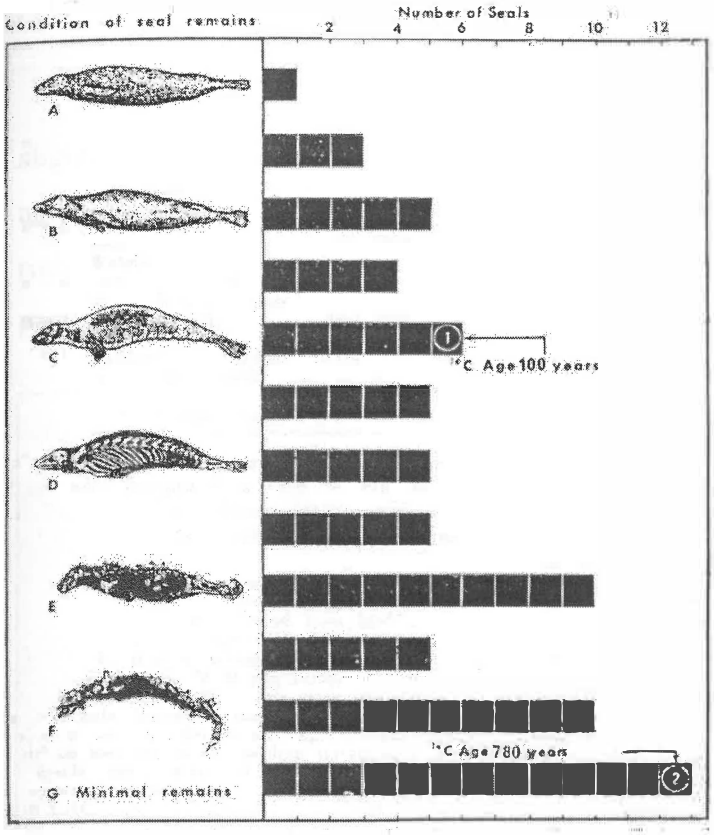


Figure 1.5 Histogram (taken from Barwick and Balham (1967)) showing the numbers of each category of mummified seal remains according to the state of erosion of each carcass. The approximate radiocarbon dates obtained by Barwick and Balham for two carcasses are shown in their respective categories.

Péwé (1962) reported apparent radiocarbon ages of two well preserved carcasses, of 660 ± 300, and 600 to 1720 years. Marini and Blair (1971) sighted an apparent radiocarbon age of 618 ± 100 years from a specimen that was believed to have been dead only a few weeks. Extensive sampling of ocean waters near Antarctica where surface waters are often mixed with, or come directly from, water masses originating at great depth, showed ^{14}C activity that was significantly lower than that accepted as the world standard (Broecker, 1963; Dort, 1982). Radiocarbon ages for organisms living in Antarctic sea water, or situated farther along the food chain, will therefore yield apparent ages that are older than true ages, rendering estimates of Antarctic marine mammals suspect (Dort, 1982).

1.7 Characteristics of seals

1.7.1 Identification of seals

The simplest way to identify a Crabeater seal is to view the teeth (Dort, 1971). Crabeater seals have teeth that have an apical recurved hook and several lateral hooked appendages on the posterior lateral edge (Figure 1.6). When the jaws are opposed, the teeth act rather like a sieve. The incisor teeth are rather forward pointing while the canine teeth are inserted at right angles to the maxilla. Weddell seal teeth are slightly shorter than Crabeater seal teeth, and the former have no lateral appendages, but the teeth look rather like an apical pyramid that is curved

slightly posteriorly. This apical cone is subtended by a broad shoulder on both anterior and posterior lateral edges. The Weddell seal canine teeth are inserted at right angles to the maxilla and the incisors are rather forward pointing (Seppelt, personal communication).

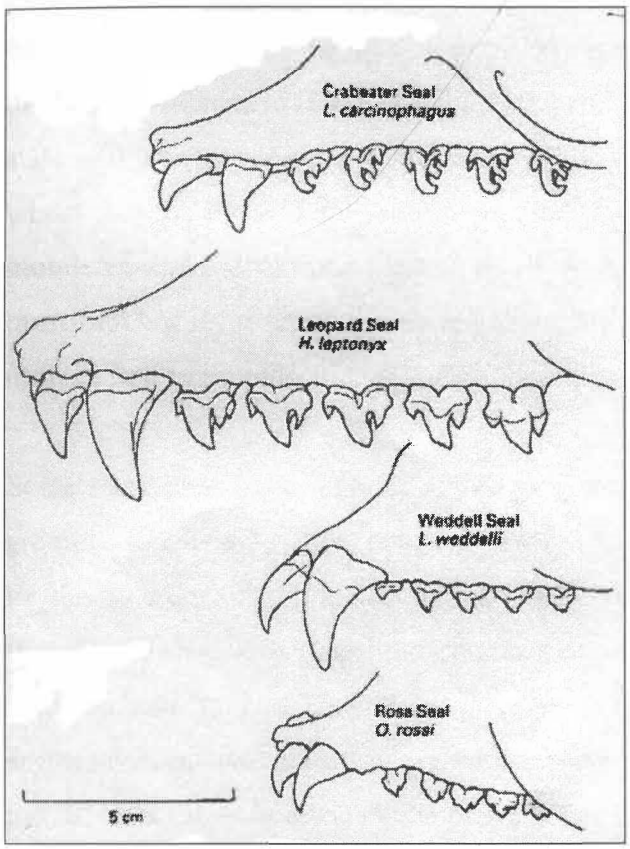


Figure 1.6 Drawings of *Phocoid* teeth, King, 1983.

If teeth can not be visualised, it is possible to estimate the species of seal based on head shape. The nostrum of a Crabeater seal is somewhat longer than a Weddell Seal. This is not a conclusive method of identification, however, and identification based on teeth composition and shape is a more valid method.

1.7.2 Composition of seals

Information as to the composition of seals is important in understanding how these organisms may act as a source of organic carbon and organic nitrogen. Seals store large quantities of lipids as blubber that act as a principal source of metabolic energy (Senanayake and Shahidi, 2002; Best et al., 2003). Blubber is defined as the layer of fatty tissue between the epidermis and the fascia of the underlying muscle (Laws, 1993). The blubber is composed mostly of neutral lipids (98%), and small amounts of polar lipids (Shahidi et al., 1994). Blubber has

three primary functions in marine mammals: energy storage, insulation and buoyancy (Iverson, 2002). Fatty acids contained within seal blubber are stratified (Best et al., 2003). Seal blubber is a source of n-3 polyunsaturated fatty acids (PUFA) (Wanasundara and Shahidi, 1998) which can be more readily found in the inner blubber layer (Best et al., 2003). The most prevalent n-3 PUFA in seal blubber are eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (Senanayake and Shahidi, 2002). When a seal is in a state of net energy loss, as would occur while travelling great distances over land where there is no available source of food, fatty acids stored in the blubber are mobilized and used to provide energy (Best et al, 2003). Fatty acids in the inner most blubber layer are the most metabolically available, and therefore would be utilised first to provide energy during starvation (Best et al, 2003).

Some researches have reported sightings of mummified seals with blood soaked ground surrounding the carcass (Webb and Leckie, 1977; Dort, 1982). Erythrocyte cells and platelet cells in seals contain cholesterol and phospholipids (Fayolle et al., 2000). The lipid compositions of blood cells was found to differ between seal species (Fayolle et al., 2000). Phospholipids and cholesterol in erythrocytes of Antarctic fur seals were measured at 92 $\mu g/10^9$ cells and 337.5 $\mu g/10^9$ cells respectively (Fayolle et al., 2000). This compared to levels measured in human cells (respectively 300 $\mu g/10^9$ cells and 125 $\mu g/10^9$ cells for phospholipids and cholesterol), indicate three times the level of cholesterol in Antarctic Fur seal erythrocyte cells compared to human cells.

1.8 Microbial diversity in the Dry Valleys of Antarctica

Prokaryotes represent the greatest biomass in the Antarctic ecosystems (Franzmann, 1996). There have been numerous investigations into the microbial diversity of Antarctic soils since interest in the area was re-kindled in the 1950's. Interest in exobiology, and the similarities between the Antarctic Dry Valleys soils and climate, and that of Martian terrain, have meant that this area is of particular interest. Antarctic soil microbiology was reviewed by Cameron (1971), Block (1984), Wynn-Williams (1990), Vishniac (1993), and Franzmann (1996). Given the revolution in microbial taxonomy caused by the application of molecular techniques, it is difficult to put much of the work conducted prior to the

mid-1980s into context (Franzmann, 1996). Indeed, when reviewing organisms identified prior to this era, many have been reclassified. Pre-molecular investigations into Dry Valleys microbial diversity were typically determined by culture methods. Pour- and spread-plate techniques often gave viable counts of less than 10 to 100 colony forming units (CFU's) per gram of soil (Cameron and Merek, 1971). Samples from some areas of Antarctica using these techniques showed no microbial growth, indicating sterile soils (Horowitz et al., 1972) or unculturable organisms. Some previous methods used to calculate abundance have been questioned as to their usefulness. It has been argued that much of the data used to calculate abundance of microbes in the Antarctic is relevant only in the context of the problem being investigated (Vishniac, 1993). Line (1988) found that different types of media gave differing viable counts by up to an order of magnitude, but that no one media gave ideal results for any particular soil type. Comparisons of culture-based and microscopic characterisations of organisms isolated from Antarctic soils may not necessarily reflect findings reported from the utilisation of molecular methods. For instance, culture and microscopic investigations into cyanobacterial-dominated endolithic communities indicated a composition made up primarily of two cyanobacteria, *Gloeocapsa* spp. and *Hormathonema* spp. Molecular investigations into the microbial community examining small sub unit (SSU) ribosomal RNA (rRNA) genes amplified from community DNA, indicated only one major and several minor cyanobacterial sequence types, which phylogenetic analysis revealed to be closely related to each other (de la Torre et al., 2003). The dominant cyanobacterial sequence type was identified by Basic Local Alignment Search Tool (BLAST) analysis as being most closely related to the filamentous cyanobacteria *Phormidium* spp., and *Plectonema* spp (de la Torre et al., 2003). These differences in identified organisms using the two different techniques may to some degree reflect the lack of available ribosomal gene DNA (rDNA) nucleotide sequences in public sequence databases. For instance, molecular surveys by de la Torres et al. (2003) failed to find sequences for relatives of *Hormathonema* spp., and found only one rDNA sequence for *Gloeocapsa* spp. These differences in results gained from differing methods must be considered when reviewing microbial diversity. This is particularly important at the present time, while so little is known about Antarctic microbiota (Franzmann, 1996). Within a given time period, and despite the

known biases involved in the production of 16S rDNA clone libraries (von Wintzingerode et al., 1997), 16S rDNA sequences allow the assessment of a broader range of diversity than that obtained by cultivation studies (Brambilla et al., 2001).

Except for studies involving cyanobacteria, Antarctic bacterial strains have typically been identified to genus level only, or are unidentified (Franzmann, 1996). Phylogenetic analysis of organisms isolated from Antarctica indicate a unique diversity, such that many of the organisms isolated that have undergone relatively detailed taxonomic study were identified as new species (Franzmann, 1996). Prokaryotes in Antarctica have been found colonising salt lakes, the anaerobic hypolimnia of lakes, anaerobic sediments, brine channels in ice, thermally heated soils, rocks, as well as the surfaces and digestive tracts of Antarctic organisms (Friedmann, 1982; Franzmann, 1996; Priscu et al, 1998). Sandy, saline soils that are sometimes high in chlorides, nitrates, and sulfates, but are quite low in organic matter (Cameron, 1968), combined with the extreme climatic conditions, apparently provide the Antarctic Dry Valleys with a relatively nominal microbial community. Antarctic soils have been found to contain between 10^4 and 10^9 cell per gram of dry weight (Line, 1988). Recent calculations of biomass present in Antarctic Dry Valleys mineral surface soils determined using ATP gave an estimate of microbial mass ranging from 2.6×10^6 to 4.0×10^8 cells g^{-1} wet weight (ww) (Cowan et al., 2002). These values appeared comparable to the abundance of microorganisms reported by Line (1988), 14 years previously using culture techniques.

Cameron et al. (1968, 1976) listed a number of factors in the Dry Valleys both favourable and unfavourable that influence the population, density, abundance, diversity, complexity and size of Antarctic life forms. It should be noted that his findings were based solely on enrichment culturing techniques, which may show bias towards a particular group of organisms favouring the conditions listed. However, the factors mentioned are indeed likely to have an effect, even if it is not the favourable or unfavourable outcome suggested. Cameron stated that the microbial population dynamic was determined by major factors such as:

(1) Climate and microclimate: in particular, the period of time ın which the temperature was above freezing.
(2) Topography: Cameron's data (1968, 1976) indicated north to south orientation, northern exposure, and gentle north facing slopes were more favourable in terms of abundance and diversity than east to west orientation, southern exposure, and flat or south-facing slopes. Included in this factor was the locality of a population relative to the nearest available water and the period of time with which water was available in unfrozen state. Slow or impeding drainage, generally associated with the presence of glaciers, lakes, streams, snow and ice fields, was found favourable.
(3) Edaphology: Soil containing high levels of salt, high or low pH, and no organic contamination were found to be unfavourable. Reversely soils that did not contain high levels of salt, had a balanced ionic composition, an approximately neutral pH, and showed signs of organic contamination from sources such as seals or skuas, were favourable in terms of microbial abundance and diversity.
(4) Geographic location: Lower Antarctic latitudes and elevations were found more favourable than higher latitudes and elevations.

Antarctic soils are typically aerobic, and anaerobic bacteria have rarely been identified (Cameron, 1971; Line, 1988; Vishniac, 1993). *Clostridium* species were identified from coastal soils (Vishniac, 1993), from soils in the vicinity of Shōwa station (Miwa, 1975), and more recently in a microbial mat sample from Lake Fryxell (Brambilla et al., 2001). Of 568 isolates from Cameron's Antarctic collection, analysed by Johnson et al. (1978) *Corneybacterium* (23%) and corneyform-related bacteria (56%), such as *Arthrobacter, Brevibacterium, and Cellulomonas* were predominant. Species of *Bacillus*, *Micrococcus*, *Nocardia*, and *Streptomyces*, *Actinebacter, Flavobacterium, and Pseudomonas* were also reported. These isolates were gained from samples taken primarily around McMurdo Station, and Victoria Land Dry Valleys region.

Hydrocarbon-degrading bacteria have been isolated from Antarctic soils. These hydrocarbon degraders have predominantly been members of the Nocardiaceae family, gram-positive, rod shaped bacteria, which have been found widely distributed in Antarctic soils (Aislabie, 1997). Vishniac (1993) summarised

reports of a number of genera identified by scientists from Antarctic soils (Table 1.2). These organisms were typically identified using culture techniques.

Table 1.2 Antarctic soil bacterial isolates compiled by Vishniac (1993)

Genera	Reference	
Acinetobacter	Johnson et al., 1978	identified as *Achromobacter*
Alcaligenes	Ni, 1986	
Azotobacter	Boyd and Boyd, 1962, 1963a; Cameron, 1971	
Beijerinckia	Ni, 1986	
Corynebacterium	Johnson et al., 1978	
Deinococcus	Hirsch et al.., 1985; Siebert and Hirsch, 1988	
Flavobacterium	Johnson et al., 1978	
Janthinobacterium	Wynn-Williams, 1983a	identified as *Chromobacterium*
Micrococcus	Johnson et al., 1978	
Moraxella	Line, 1988	
Mycobacterium	Meyer et al., 1967; Cameron, 1971	
Nitrobacter	Cameron, 1971	
Nocardia	Johnson et al., 1978	
Oerskovia	Line, 1988	
Planococcus	Miller et al., 1983; Shivaji et al., 1988	
Plesiomonas	Ni, 1986	
Pseudomonas	Johnson et al., 1978	
Rhodococcus	Cameron, 1971	identified as *Jensenia* and *Mycococcus*
Staphylococcus	Johnson et al., 1978	
Streptomyces	Johnson et al., 1978	
Xanthomonas	Ni, 1986	

Cryptoendolithic microorganisms were reported in the Dry Valleys colonizing the pore spaces of exposed sandstone rocks 1 to 5mm beneath the surface, forming stratified microbial communities (Friedmann and Ocampo, 1976; Johnston and Vestal, 1991; de la Torre, 2003). This Cryptoendolithic environment offers protection from oscillating temperatures, harsh drying winds, and was found to retain liquid water from snowmelt internally for several days (Friedmann, 1982; de la Torre et al., 2003). In the Dry Valleys two types of endolithic organisms were reported, chasmoendoliths that lived in rock fissures and cracks, and crytoendoliths that inhabited structural cavities in porous rocks (Friedmann, 1982). These microbial communities were usually dominated by either lichen or cyanobacteria which were thought to provide nutrients to the microbial community present through photosynthesis (Johnston and Vestal, 1991; de la Torre et al., 2003). As these organisms do not themselves actively penetrate the rock substrate by solubilisation, they can inhabit only those sandstone rocks that have either a porous structure, or are weathered and permeated by fissures

(Friedmann, 1982). Lichen-dominated communities were the most prevalent in the Dry Valleys (de la Torre et al., 2003). Culture independent analysis of phylogenetic compositions of two cryptoendolithic communities, one microscopically determined to be dominated by lichen, the other microscopically determined to be dominated by cyanobacteria, were investigated by de la Torre et al. (2003). Examination of small subunit (SSU) rRNA genes amplified from the apparent lichen-dominated community DNA yielded some interesting results. de la Torre and his colleagues (2003) reported three phylotypes that appeared to dominate clone libraries consisting of a total of 672 clones from this community. These three phylotypes, representative of fungi (29%), chloroplasts (22%) and green algae (22%), accounted for more than 70% of the clones. The fourth most abundant phylotype from this lichen community accounted for approximately 6% of clones in the universal libraries. BLAST analysis identified this group as belonging to bacterial phylogenetic order *Cytophagales*. Four phylotypes representing 4% of clones were identified belonging to the class *Actinobacteria*. Six α-proteobacteria accounted for approximately 5% of clones. Two single clones were observed relating to γ-*Proteobacteria* and the *Planctomycetales*. Three clones did not group with specific bacterial taxa, and possibly represented currently unrecognized phylogenetic groups. On examination of SSU rRNA genes amplified from the apparent cyanobacterium-dominated group, 26 from 480 clones were identified as unique phylotypes using RFLP analysis (de la Torre et al., 2003). These clones represented five clone libraries consisting of rRNA genes amplified using two universal, two bacterial specific, and one cyanobacterium specific primer sets. de la Torre (2003) reported that three phylotypes, cyanobacterium (30%), *α-proteobacteria* (31%), *Deinococcus* (26%), accounted for over 87% of the clones in the libraries, regardless of the primer combinations used for PCR amplification. Other phylotypes identified in the clone libraries included representatives of *Actinobacteria,* green nonsulfur bacteria, *Acidobacteria,* and *cytophagales.* Each phylotype from the cyanobacterium-dominated community accounted for less than 2% of the total number of clones. de la Torre (2003) reported that several of the bacterial groups including *Actinobacteria* and *Proteobacteria* were represented by several distantly related sequence types. de la Torre et al. (2003) noted that there were twelve distinct actinobacterial phylotypes belonging to the class *Actinobacteria* representing over

6% of the clones. These results perhaps indicated a broad diversity and potentially important roles of actinobacteria in the cyanobacterium dominated community.

The phylogenetic compositions of culture independent analysis of cryptoendolithic microbial communities (de la Torre et al., 2003) were indicative of other culture independent research into microbial biodiversity in the Antarctic Dry Valleys (Priscu et al., 1998; Gordon et al., 2000; Brambilla et al., 2001; Christner et al., 2001; Sjöling and Cowan, 2003). A significant proportion of culture independent research into microbial diversity of the Dry Valleys has centred around lakes, and meltwater streams (Priscu et al., 1998; Gordon et al., 2000; Brambilla et al., 2001; Christner et al., 2001; Sjöling and Cowan, 2003) Although the research representative of this thesis investigated microbial diversity of soil microorganisms where there was no indication of meltwater streams, or the direct presence of any lakes (Cary and Cowan, personal communication), the lack of knowledge of Antarctic microbiota, and a number of recent investigations into 16S rDNA diversity of a number of Antarctic Lake isolates, mean that it is worth reviewing recent findings in this area. Phylogenetic analysis of 16S rDNA sequence data of Antarctic Lake isolates indicated the presence of a number of Classes of bacteria that have been isolated from Dry Valleys Soils (Johnson et al., 1978; Vishniac, 1993; de la Torre et al., 2003).

Photosynthetically-based ecosystems were found in many areas of the Dry Valleys where there was the presence of available water (McKnight and Tate, 1995). Water in the form of glacial meltwater, seasonal meltwater in lakes, and small moist oasis in glaciers, enables the survival and activity of algal mats of filamentous cyanobacteria, chlorophytes, and mosses for short periods in the summer (McKnight and Tate, 1995; Priscu et al., 1998; Christner et al., 2003).

Both aerobic and anaerobic prokaryotes have been found in some Antarctic lakes. Windborne organisms carried by strong katabatic winds get deposited on the ice surface and migrate downward during summer (Brambilla et al., 2001). Assessment of more than 350 16S rDNA cloned inserts, from a mat sample taken from Lake Fryxell, revealed a rich spectrum of bacterial diversity (Brambilla et al., 2001). Brambilla and colleagues (2001) reported representative sequences

belonging to the classes *Proteobacteria*, *Actinobacteria,* Order *Verrucomicrobiales,* sub phylum *Clostridium/Bacillus* and Phylum *Cytophyga-Flavobacterium-Bacterioides.* Assessment of 173 16S rDNA clones from libraries made from samples taken of glacial meltwater lake sediment on Bratina Island, indicated seven major lineages of the domain bacteria: α-*Proteobacteria*, γ-*Proteobacteria*, δ-*Proteobacteria, Cytophyga-Flavobacterium-Bacterioides*, the *Spirochaetaceae*, and *Actinobacteria* (Sjöling and Cowan, 2003).

Cryoconite holes found in glaciers in the Antarctic Dry Valleys were found to contain the presence of diverse species of both Bacteria and Eukarya (Christner et al., 2003). These holes are formed when particulates lodged on the surface of a glacier are warmed by solar irradiation and melt into the underlying ice. Cryoconite holes contain liquid water and particulates from melted glacial ice (Christner et al., 2003). According to Christner and his group, photosynthesis by algae and cyanobacteria during the polar summer can provide nutrients for complex community development. Sequencing of 16S rDNA clones by Christner's group revealed the presence eight bacterial lineages (*Acidobacterium, Actinobacteria, Cyanobacteria, Cytophagales, Gemminmonas, Planctomycetes, Proteobacteria, and Verrucomicrobia).* These organisms are similar to those identified in permanent ice that covers Lake Bonney in the Dry Valleys region, and showed similarities to bacteria identified by 16S rDNA sequencing of samples taken in maritime meltwater pond sediments from Bratina Island, and samples taken from Lake Fryxell (Priscu et al., 1998; Brambilla et al., 2001; Sjöling and Cowan, 2003). This similarity in bacterial species, Christner believed, supported their hypothesis that cryoconite holes were most likely seeded by particulates from the local environment and indeed supported Brambilla's statement, that organisms from surrounding glaciers and land are deposited by wind onto the surface of the lake ice, and migrate downward during the Antarctic summer.

Common to all of the culture independent assessments of microbial diversity from the various areas listed previously (Priscu et al., 1998; Brambilla et al., 2001; Christner et al., 2001; de la Torre et al., 2003; Sjöling and Cowan, 2003) is the abundance of bacterial Classes *Actinobacteria, Proteobacteria,* and *Cytophaga-*

Flavobacterium-Bacterioides. *Actinobacteria* correspond to the high G+C group of Gram-positive bacteria as their DNA usually contains large amounts of the bases guanine and cytosine (Madigan et al., 2003). All *Actinobacteria* are aerobic, and are common inhabitants of soil and plant materials (Madigan et al., 2003). *Proteobacteria* represent the majority of known Gram-negative bacteria (Madigan et al., 2003). *Proteobacteria* show extreme metabolic diversity. Most are anaerobic and have been reported in a wide range of environments (Madigan et al., 2003). The *Proteobacteria* are divided into five major groups labelled with the Greek letters alpha through to epsilon (Madigan et al., 2003). *Cytophaga-Flavobacterium-Bacterioides* represent a diverse range of both obligatory aerobic and obligatory anaerobic Gram-negative bacteria (Madigan et al., 2003). *Flavobacterium* and *Cytophaga* are widely distributed in soil and aquatic habitats (both marine and freshwater). Some species of *Flavobacterium* have been shown to be psychrophilic or physchrotolerant (Madigan et al., 2003). Interestingly given their isolation from Antarctic environments, *Flavobacterium* typically utilise glucose and very few other carbon compounds as an energy source (Madigan et al., 2003). *Cytophaga* bacteria are obligately aerobic (Madigan et al., 2003). Many have been shown to digest polysaccharides such as cellulose or chitin, while some *Cytophaga* species are known fish pathogens. *Bacteroides* are obligatory anaerobic, nonsporing species that are saccharolytic (Madigan et al., 2003). *Bacteroides* have been isolated from the intestinal tracts of humans and other mammals, and measurements have shown 10^{10}-10^{11} cells in human feces. They can synthesize sphingolipids such as shpingomyelin, cerebrosides and ganglosides, common in mammalian tissues, especially in the brain and other nervous tissues (Madigan et al., 2003).

Soil moisture levels in the Antarctic Dry Valleys were found to vary not only between locations (as discussed in section 1.4), but also varied with depth at a single location. Cameron (1969) found that as relative humidity increased in Victoria, King-David, and Wheeler Valleys, the abundance and diversity of microflora increased. Cameron et al. (1968, 1970) found that for three out of every four sites they investigated, subsurface microflora was more abundant than surface microflora, with viable bacteria tending to be most abundant at the permafrost level. Using culture methods, Cameron (1968) found most of the

bacterial isolates to be *Bacillus* spp., soil diptheroids, *Micrococcus* spp., and *Mycoccus* spp. Algae found present in samples included *Oscillatoria* spp., *Microcoleus* spp., *Schizothrix* spp., *Anacystis* spp., and *Coccochloris* spp. Cameron (1968) also reported the presence of fungi, including ascomycetous molds and some yeasts.

Epiflourescence microscopy with stain dipheynlamidino (DAPI staining) of cells from environmental samples taken at different depths from Wright Valley, indicated that CFU counts above 100 per gram of dry weight soil, were only present in environmental samples taken from the top 5cm of soil (Aislabie et al., 2001). These results appeared to conflict with Cameron's (1968, 1970) earlier findings, where microbial abundance was reported to increase with depth.

The presence of culturable filamentous fungi were detected in environmental samples taken between the depths of 2cm and 75cm from the soil surface by Aislabie et al. (2001). Only three samples were represented in this range, and perhaps a greater number of samples taken between these depths would better represent the levels of filamentous fungi present throughout a vertical gradient in soil. No filamentous fungi were detected in environmental samples taken from the top 2cm of soil. Numbers of detectable filamentous fungi between 2-75cm, were minimal (<10). Despite the lack of any apparent filamentous fungi in surface soils, numbers of detectable filamentous fungi did not increase with depth, therefore, it can not be concluded that these findings support Cameron's results (1968, 1970) of increasing microbial abundance and diversity with depth.

Fungal biodiversity in Antarctic soils was found to increase with the availability of water (Vishniac, 1996). Dry Valleys soils have a lower abundance of fungi than other areas of Antarctica such as the sub Antarctic Islands (Vishniac, 1993). Calculated values of fungal abundance in the Dry Valleys indicated approximately one microcolonly per gram of soil (Vishniac, 1993). Various fungal species listed in Table 1.3 were isolated from Victoria Land, in which the Dry Valleys is located.

Table 1.3 Fungal species isolated from Victoria Land, Antarctica

Species Isolated	Reference
Acremonium charticola	Onofri et al., 1994
Alternaria citri	Baublis et al., 1991
Aspergillus sp.	Baublis et al., 1991; Onofri et al., 1994
Beauveria sp.	Mercantini et al., 1993
Geomyces sp.	Onofri et al., 1994
Helminthosporium anomalum	Cameron et al., 1974
Malbranchea sp.	Mercantini et al., 1993
Penicillim sp.	Baublis et al., 1991; Greenfield 1981
Pestalotia sp.	Onofri et al., 1994
Philaphora sp.	Cameron et al., 1974; Greenfield, 1981
Rhizopus	Cameron et al., 1974
Trichoderma sp.	Cameron et al., 1974; Greenfield, 1981
Trichophyton sp.	Mercantini et al., 1993
Verticillium sp.	Line, 1988

Archaea have been described from the Antarctic environment (Franzmann, 1996; Cavicchioli et al., 2000; Purdy et al., 2003). The discovery of Archaea in Antarctica did not occur until 1988, before which the knowledge of Antarctic prokaryotic diversity concerned only bacteria (Franzmann, 1996). Based on molecular probe and sequencing studies, it is apparent that Archaea are numerically abundant in a diverse range of low temperature environments (Cavicchioli et al., 2000). However, very few free-living low-temperature-adapted Archaea have been cultivated (Franzmann, 1996; Cavicchioli et al., 2000). Recent investigations into microbial diversity in various locations in Antarctica have indicated their presence in a number of Antarctic environments. Franzmann and his associates have been responsible for the cultivation of six Archaea from the Antarctic environment. These include three Euryarchaeota from the lakes in the Vestfold Hills region of Antarctica, two methanogens *Methanococcoides burtonii* and *Methanococcoides frigidum* isolated from Ace Lake, and one halophile, *Halorubrum lacusprofundii* (Franzmann, 1988, 1992, 1996, 1997). Archaeal diversity was investigated by Sjöling and Cowan (2003) in glacial meltwater lake sediment, from Bratina Island, Antarctica. Assessment of 17 partial 16S rDNA archaeal specific clones using restriction analysis, resulted in seven archaeal OTUs (operational taxonomic units) detected, all belonging to the group *Crenarchaeota*. The distribution, abundance and activity of methanogenic Archaea were investigated for Lake Heywood and Shallow Bay, Signy Island, Antarctica, by Purdy and colleagues (2003). Purdy reported that oligonucleotide

probing of RNA extracted directly from sediment indicated that Archaea represented 34% of the total prokaryotic signal in Lake Heywood, but represented only 0.2% of the total prokaryotic signal in RNA extracted from Shallow Bay sediments. Analysis of the archaeal libraries from Lake Heywood and Shallow Bay, showed five distinct groups of Archaea. Analysis of 72 clones from a library generated using Archaea specific 16S rDNA primers revealed the presence of two different phylotaxa of Archaea in a mat sample from Lake Fryxell, Dry Valleys, Antarctica (Brambilla et al., 2001). Recent culture independent analysis of cryptoendolithic communities dominated by lichen or cyanobacteria, utilised archaeon-specific PCR primers in an attempt to enrich for archaeal rRNA (de la Torre et al., 2003). No archaeal sequences were identified from any clones analysed. While these results confirm the presence of Archaea in the Antarctic environment, all indicate low archaeal diversity.

Archaea comprise a significant fraction of total prokaryote cell abundance in the marine waters of the Antarctic Peninsula and indeed may form a significant proportion of Southern Ocean picoplankton (DeLong et al., 1994; Church et al., 2003). Recent studies have found that abundance and species composition of Archaea vary both temporally and spatially in the Antarctic marine environment (Church et al., 2003).

In the Antarctic Dry Valleys the yeast populations generally composed less than 1% of the total enumerable heterotrophic microbial population(Atlas, 1978). The yeast populations did, however, compose a large percentage of the total enumerable fungal population (Atlas, 1978). Antarctic fungi have been described as robust, flexible survivors equally at home in polar and temperate environments (Baublis, 1991).

Epilithic lichens can be found scattered on the floors and at the mouths of walls and higher elevated hanging valleys (Cameron et al., 1976). They, however, only inhabit sites or strips between the 80% relative humidity line and the snowline (Wilson, 1970). The Queen Maud Mountains is the farthest south lichens have been found (Cameron et al., 1976).

Although numerous microorganisms have been isolated from Dry Valleys soils, critical studies indicated that most, if not all of these isolates were derived from atmospheric contaminants carried in by winds and that they survive in frozen soil without actively growing (Friedmann, 1982; Vishniac, 1996). Cameron et al., (1976) noted that the presence in samples of algae and diatoms, where there was no apparent availability of water could be misconstrued as active endemicity. Cameron claimed that the harsh dry climate acting to freeze dry organic mass, combined with strong winds as well as dispersal by man and birds, are responsible for distributing the biomass from more productive areas into areas where conditions did not favour productivity. The task of identifying indigenous microbes from immigrants is a difficult one. Cameron and Merek (1971) stated that the probability that an organism was indigenous would be increased if it could be demonstrated that growth occurred following wetting of the soil, and that the microorganisms would not grow in the nearest less-harsh environment. Characterising a species as indigenous ultimately requires evidence of growth *in situ*, or of demonstration that it is a new species (Vishniac, 1996). Both molecular and enrichment culture based-methods used to study microbial diversity would not discriminate between non-active soil residents, and those organisms actively metabolising and growing in the soil. Various methods of *in situ* analysis of microbial activity have been tried by scientists in the Dry Valleys. Vishniac and Mainzer (1973) implanted sterile glass microscope slides in soil at various locations in Wright Valley and the Asgard Range. They theorised that only actively multiplying bacteria that came in contact with the slides would adhere to the glass and form microcolonies. The slides were implanted in soil ranging in elevation from 100m to 2200m, where diurnal soil temperatures were recorded as ranging from -5^{o}C to +5^{o}C. After electron microscopic examination of the slides, Vishniac and Mainzer (1973) reported the presence of large aggregates of bacteria, and also the presence of algae, including diatoms.

Adenosine triphosphate (ATP) analysis has long been used to measure soil microbial activity (Paul and Johnson, 1977). ATP can only be detected in living microbes (Meighan et al., 1997). Applications of ATP determination in microbiology are based on capturing the micro-organisms, releasing the ATP from within the cell, and measuring the amount of bioluminescence generated (Celsis

Technical information). With the use of sensitive light equipment, bioluminescence can be quantified as relative luminosity units (RLUs) and used to give an indication of levels of microorganisms in soil (Meighan et al., 1997). Recently in Antarctic research, Cowan and his colleagues have used bioluminescent ATP detection as a means of determining microbial biomass (Cowan et al., 2002) and as an indication of microbial activity *in situ* in soil (Don Cowan, personal communication). Using a Portable Hygiene Monitor from Celsis Instruments (Cambridge, U.K) i*n situ* analysis of environmental samples taken from an area underneath a source of organic carbon was conducted. Analysis of RLUs gained, indicated increased levels of microbial activity in the environmental samples taken from beneath the organic carbon contaminant when compared to *in situ* soil samples analysed further away from the contamination source (Don Cowan, personel communication). Caution must be exercised when interpreting ATP result, as ATP values may reflect the degree of metabolic activity rather than the level of microbial biomass in a sample (Atkinson et al., 2000). ATP analysis is not a method of identifying microorganisms, and although it provides information as to the level of microbial activity and microbial abundance, it must still be used in combination with culturing and molecular techniques to identify the microbes present in a sample. Combined with portable PCR systems (ESML, U.S.A.), the use of portable ATP bioluminescence assays allows microbial activity and molecular analysis to be conducted simultaneously on *in situ* samples. However, identification of a microorganism using molecular techniques in an area of high microbial activity does not conclusively prove active endemicity of that organism.

1.9 Analysis of microbial diversity using 16S rDNA

For the past 17 years, microbiologists have relied upon DNA sequence information for microbial identification based primarily on the genes encoding the 16S rRNA (Klappenbach et al., 2001). The 16S rRNA molecule has highly conserved regions between organisms (Kreig, 1994; Klappenbach et al., 2001) and differences in 16S rDNA sequence between bacterial species have been exploited as an indicator of diversity (Muyzer et al., 1993). Molecular methods used to analyse microbial diversity typically rely on polymerase chain reaction (PCR) amplification of 16S rRNA genes from complex samples followed by one or a combination of the following:

- Cloning and sequencing of amplicons
- Separation of amplicons according to chemical composition using temperature or denaturing gradient gel electrophoresis
- TRFLP (Terminal Restriction Fragment Length Polymorphism) which separates amplicons based on size following a restriction endonuclease digestion (Klappenbach et al., 2001).

Cloning and sequencing of 16S ribosomal deoxyribose nucleic acid (rDNA) amplicons, and denaturing gradient gel electrophoresis were methods utilised in the course of this thesis research to investigate the microbial diversity of environmental samples taken surrounding mummified seal carcasses in Miers Valley, Dry Valleys, Antarctica.

1.9.1 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE), is used for analysis of 16S rDNA gene segments in order to profile complex microbial communities and to infer the phylogenetic affiliation of the community members (Muyzer et al., 1993; Ferris et al. 1996; Øvreås et al., 1997; Cary S. C. 2001; Ibekwe et al., 2001; Gray et al., 2002). The technique uses a PCR amplified segment of a 16S rRNA gene using conserved primers, where one of the primers used has a length sequence rich in pyrimidine bases cytosine and guanosine (GC clamp) attached at the 5' end. This GC clamp imparts melting stability to the PCR products in a denaturing gradient gel. The resulting products which are essentially all the same size, are separated into discrete bands based on sequence differences during electrophoresis through a denaturing gel that contains an increasing linear denaturing gradient (Ferris et al., 1996).

DGGE constitutes a molecular approach to analyzing the genetic diversity of complex microbial populations (Muyzer et al., 1993). Sequence data can be gained directly from DGGE gel fragments by isolation and PCR amplification of DNA bands of interest (Ferris et al., 1996; Ferris and Ward, 1997; Teske et al., 1996). DGGE as a technique has an advantage over cloning and sequencing as a method of microbial analysis in that if provides an immediate display of the constituents of a population in both a qualitative and semi-quantitative way (Muyzer et al, 1993). Assumptions made on the number of organisms present in a

population, based on DGGE gel analysis, however, must be made carefully. It is possible that an organism may have multiple operons with different 16S rRNA and rDNA sequences (Lima and Correia, 2000; Klappenbach et al., 2001), or that molecular methods produced artefacts such as heteroduplex bands which do not correspond to any real organism (Ferris and Ward, 1997). Therefore, a particular species of organism may be represented by more than one band, and some bands present may not represent unique sequence types present in a community.

1.9.2 Cloning and sequence analysis

Cloning and sequence analysis of selected clones has been applied previously to assess diversity of PCR amplified 16S rDNA (Giovannoni et al., 1990; Priscu et al., 1998; Brambilla et al., 2001; Christner et al., 2003). One major drawback of this method is that species which constitute a low percentage of the population are not readily detectable in this way, even with analysis involving large numbers of clones (Muyzer et al., 1993). It is important to consider that the relative ratios of rRNA genes in clone libraries do not necessarily correspond to the abundances of organisms represented in the original ecosystem (de la Torre et al., 2003). It is, therefore, inferred that cloning and sequence analysis is a qualitative rather than a quantitative method of microbial community analysis (Muyzer et al., 1993). Factors such as bias involved in PCR amplification or cloning (Suzuki and Giovannoni, 1996), or different numbers of rRNA operons in different organisms (Farrelly, et al., 1995; Klappenbach et al., 2000), can influence the proportions of rRNA phylotypes in clone libraries (de la Torre et al., 2003). There is evidence to suggest that bias and selection in PCR, however, is more likely to result in the overrepresentation of minor components rather than the loss of diversity (Suzuki and Giovannoni, 1996; Purdy et al., 2003). Research conducted by Farrelly et al. (1995) found that the number of *rrn* genes per equimolar amounts of DNA from three organisms investigated, directly correlated to the ratio of PCR products obtained. Therefore, organisms that have a higher number of 16S rRNA genes will be more represented in PCR amplicons than organisms that may be present in and equal amount, but have fewer copies of the gene.

Nucleotide sequences gained from the sequencing of PCR amplicons are compared to known sequences registered in various public databases. Sequence

analysis using public nucleotide databases for comparing Antarctic microorganisms, is limited by our vastly incomplete knowledge of Antarctic and non-Antarctic microbes (Franzmann, 1996). A lack of comparable sequences has resulted in the majority of Antarctic microorganisms that have undergone taxonomic studies being classified as new species (Franzmann, 1996). As our knowledge of prokaryote and eukaryote diversity increases, this problem will reduce.

1.10 Hypothesis

In a harsh, nutrient-deprived environment such as the Antarctic Dry Valleys, a large localised enrichment in the form of a seal carcass offers a significant potential to effect the local microbial community. Such a large mass on a microscopic scale would offer an abundance of carbohydrates and lipids in the form of decomposed muscle tissue, blood, and oily bodily fluids. Although most mummified seals on discovery are desiccated, recently deceased seals in the Dry Valleys have been observed with blood and oily bodily fluids being excreted (Dort, 1982). Such a large object in comparison to microenvironments in the soil may offer not only nutrients but also physical shelter for some heterotrophic species, for example from predominant wind directions. Past research involving localised enrichments of specific areas and their affects on microbial communities showed both increases in microbial abundances, and the inverse effect. Indeed, the dynamics of a population is thought to almost always change, with some species in a population better able to utilise the enrichment material than others. The hypothesis of this thesis research was that a large mass of organic carbon and organic nitrogen as provided by a mummified seal in Miers Valley, an area lacking any other obvious forms of organic matter such as plant biomass, acted to enrich the nominal microbial communities present in the soil directly beneath and surrounding seal carcasses. We predicted that there would be an apparent increase in microbial abundance, and a difference in the diversity of microorganisms present in soil where seal matter increased the levels of carbon and nitrogen, compared to microbial communities present in Miers Valley control samples.

1.11 Aims of this research

- To observe soil microbial diversity in transect environmental samples surrounding Miers Valley mummified seals, and in environmental samples taken from a vertical sampling transect from the Miers Valley floor to a high saddle at the top of the Marshall Valley to be used as controls.
- To compare microbial diversity between environmental samples within a sampling transect, and between sampling transects, in relation to the levels of carbon and nitrogen, taking into consideration the distance and direction of the sample from the seal.

1.12 Execution of research

- Environmental samples were collected on Event K023 Terrestrial Microbiology Antarctica New Zealand 2001/2002. The Event personnel included the following members:

  Professor D.A. Cowan, (University of the Western Cape, South Africa)

  Professor S. C. Cary (University of Delaware, USA)

  Miss S. Whiting (University College London, UK)

  Miss S. Hawkins (Waikato University, NZ)

- The MSc thesis research (Lisa Robson) was conducted in the following locations :

  November 15, 2002 – March 15, 2003:

  College of Marine Studies, University of Delaware, U.S.A.

  March 15, 2003 – February 28, 2004:

  Department of Biological Science, School of Science and Technology, University of Waikato, New Zealand.

CHAPTER TWO

Materials and Methods

2.1 Antarctic sampling

All environmental samples used in this thesis were collected by Professor D.A. Cowan and Professor S.C. Cary on Event K023 Terrestrial Microbiology Antarctica New Zealand 2001/ 2002. Environmental samples were collected in the Miers Valley region of the Antarctic Dry Valleys. The extensiveness of sampling varied from seal to seal based on the seals accessibility, and the suitability for sampling of the location where the seal was found. As a control, environmental samples were taken in a vertical sampling transect from the Miers Valley floor, to a high saddle at the top of the Marshall Valley (Figure 2.1).

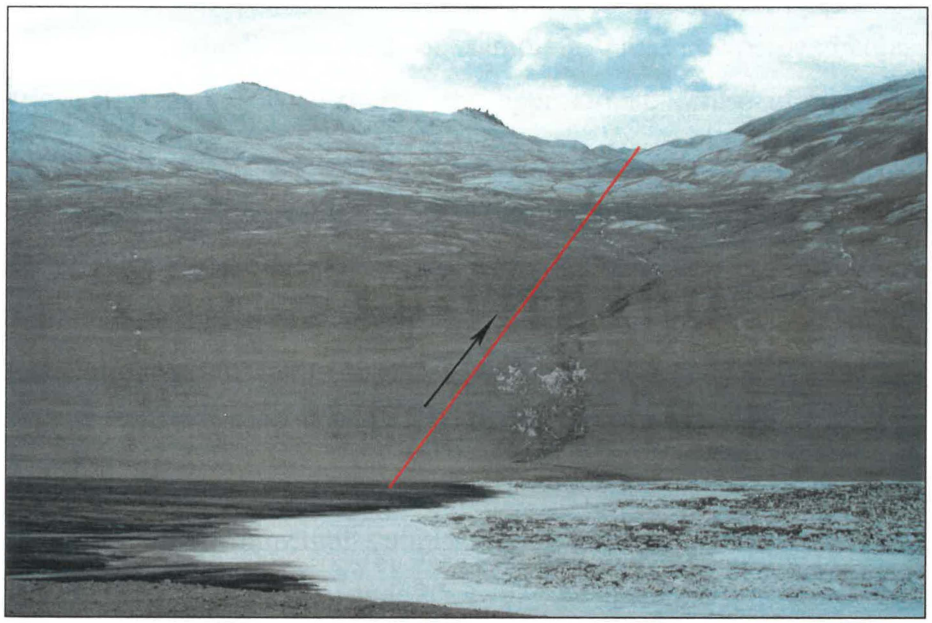


Figure 2.1 Miers Valley vertical transect. Photograph taken by D. Cowan.

Environmental samples taken along the control transect, were collected where there were no obvious signs of organic contamination. The control transect environmental samples were taken at elevations ranging from 554 to 2689ft (169

to 820m). A description of the GPS coordinates and altitudes of the control transect environmental samples is given in Table 2.1.

Table 2.1 Geographic location (GPS coordinates) and altitude of environmental samples taken from the control transect in Miers Valley, the Antarctic Dry Valleys on Antarctic Event K023.

Sample Designation	GPS Coordinates		Altitude (ft)
MVT1	78' 05.679	163' 48.271	554
MVT2	78' 05.670	163' 48.285	553
MVT3	78' 05.582	163' 48.324	582
MVT4	78' 05.541	163' 48.310	601
MVT5	78' 05.480	163' 48.370	662
MVT6	78' 05.378	163' 48.462	768
MVT7	78' 05.324	163' 48.520	860
MVT8	78' 05.184	163' 48.690	1094
MVT9	78' 04.904	163' 48.853	1400
MVT10	78' 04.685	163' 49.178	1698
MVT11	78' 04.503	163' 49.297	2001
MVT12	78' 03.968	163' 52.083	2689

All environmental samples were taken aseptically by scooping soil material into sterile 15ml or 50ml greiner bro-one tubes using a sterile spatula. The transect sampling method consisted of sampling along a perpendicular axis, with transecting points occurring at the approximate centre of the seal carcass, as depicted in Figures 2.2, 2.6, and 2.8. Seals one, three and thirteen (MVS1, MVS3, MVS13, respectively) were selected for more comprehensive sampling as they were located on relatively level soil surfaces. A total of 40 environmental samples and one tissue sample, were collected for MVS1 both horizontally and vertically. Vertical samples were collected at the soil surface level, 5cm below the surface, and 10cm below the soil surface as depicted in Figures 2.2, 2.3, 2.4 and 2.5. Not all horizontal points on the transect were sampled at all three depths. All subsurface environmental samples collected from MVS1 sampling transect are depicted in Figures 2.4 and 2.5, with the exception of A6-2 and B1-3. The environmental sample designated A6-2 was taken 10cm below the surface of the soil, at the horizontal location marked as A6-1 in Figure 2.2. The environmental sample labelled B1-3 was mislabelled, and it was unclear what depth, or horizontal location along the B transect line it was collected.

Surface environmental samples were labelled XY or XY-1, where X was the transect letter, and Y was the position along the transect. Environmental samples taken 5cm below the surface of the soil were labelled XY-1*. Environmental samples taken 10cm below the surface of the soil were labelled XY-2.

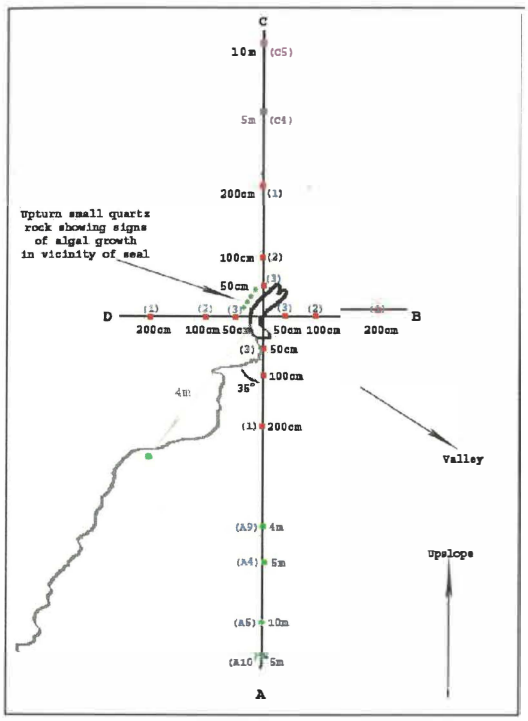


Figure 2.2 MVS1 sampling profile.

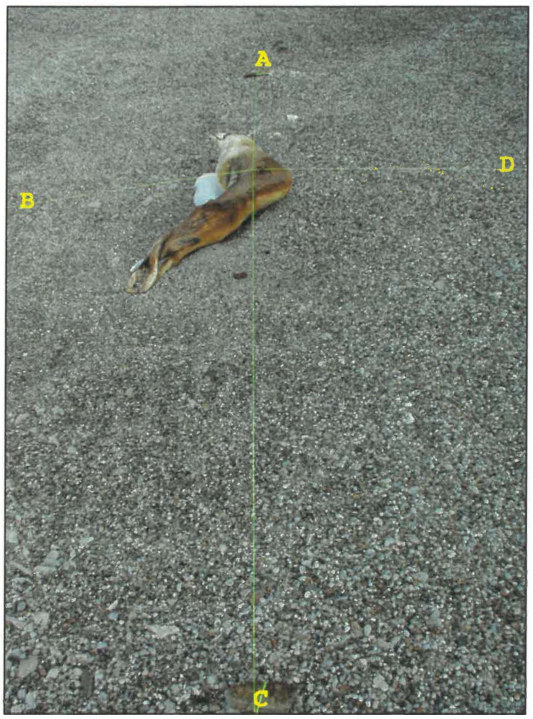


Figure 2.3 Photographic image of MVS1 sampling transect. Photograph taken by Professor S.C. Cary.

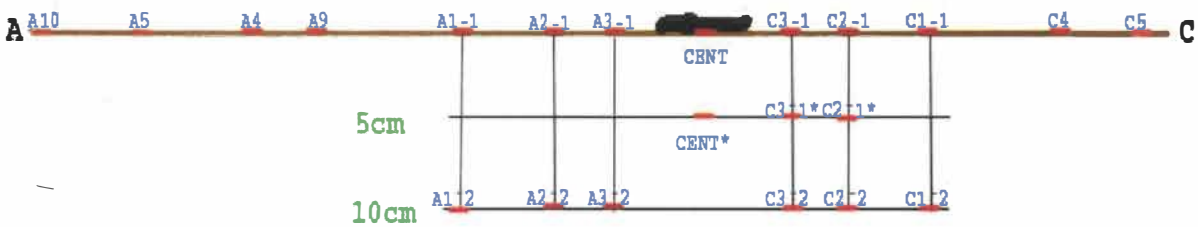


Figure 2.4 Cross-sectional view of MVS1 sampling transect showing horizontal and vertical sampling along transect lines A and C. Note: Samples A6-1and A6-2 are not shown in this profile as they were not actually present on the A transect line.

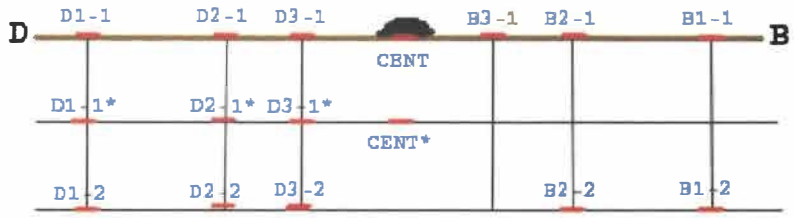


Figure 2.5 Cross-sectional view of MVS1 sampling transect showing horizontal and vertical sampling along transect line B and C.

A number of seal carcasses present in Miers Valley were located on steep rocky slopes and were considered unsuitable for grid sampling. A total of 12 seals were selected for environmental sampling of soil material surrounding the seal carcasses. A description of the GPS coordinates, as well as the altitudes, that the seal carcasses were positioned at are described in Table 2.2.

Table 2.2 Geographic locations (GPS coordinates) and altitudes of soil samples taken surrounding mummified seals in Miers Valley, the Antarctic Dry Valleys on Antarctic Event K023.

Seal	GPS Coordinates of Seal Location		Altitude (ft)
MVS1	78° 04.762	163° 49.256	1584
MVS2	78° 04.307	163° 50.313	1953
MVS3	78° 04.115	163° 51.140	2175
MVS4	78° 04.032	163° 51.636	2342
MVS5	78° 04.000	163° 51.856	2506
MVS6	78° 03.999	163° 51.903	2525
MVS7	78° 03.981	163° 51.939	2537
MVS9	78° 03.993	163° 51.913	2575
MVS10	78° 03.993	163° 51.913	2575
MVS11	78° 04.000	163° 51.988	2595
MVS12	78° 03.995	163° 52.023	2639
MVS13	78° 04.017	163° 51.530	2196

A profile of MVS3 sampling transect is depicted in Figures 2.6 and 2.7. A total of 13 environmental samples and one tissue sample were collected from MVS3 sampling transect.

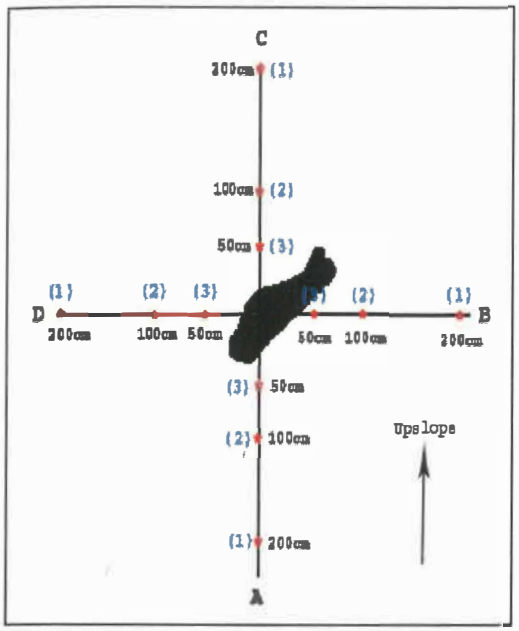


Figure 2.6 MVS3 sampling profile.

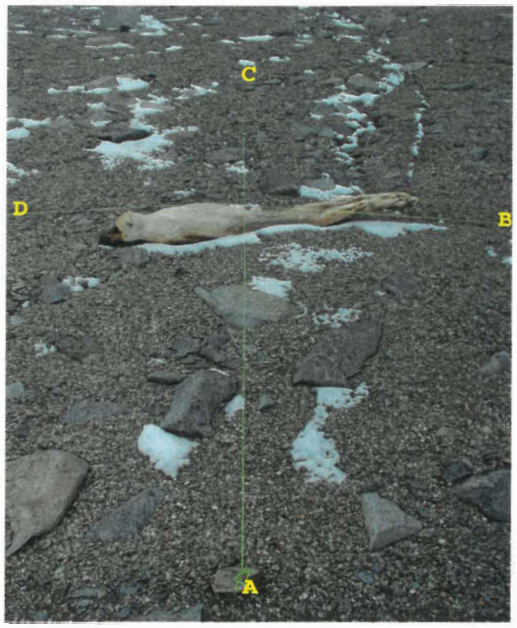


Figure 2.7 Photographic image of MVS3 sampling profile. Photogragh taken by Professor S.C. Cary.

A profile of MVS13 sampling transect is depicted by Figures 2.8 and 2.9. A total of 13 environmental samples were collected from MVS13 sampling transect. Sample A1, however, was misplaced and not used for microbial analysis.

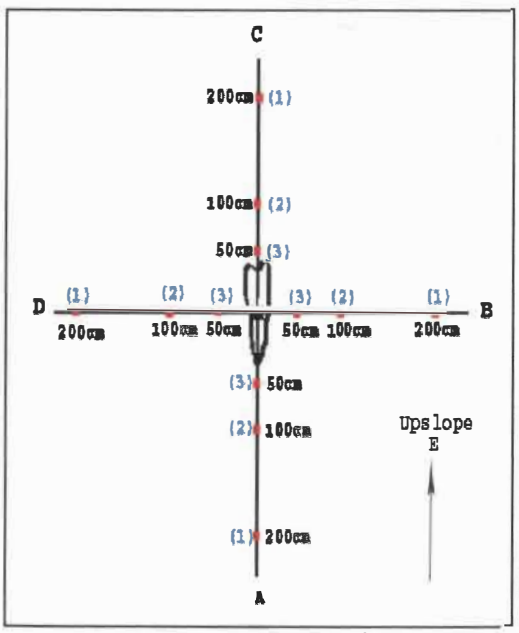


Figure 2.8 MVS13 sampling profile.

Figure 2.9 Photographic image of MVS13 sampling profile. Photograph taken by Professor S.C. Cary.

Samples were stored at approximately -10 °C to 0 °C while in Antarctica. During transportation, samples were placed on ice, however, temperatures may have varied from -10 °C to 20 °C. Upon receipt in Hamilton, New Zealand, the samples were stored at the University of Waikato at –20°C before being sub-sampled under aseptic conditions for future isotope analysis. These sub-samples remained at –20°C at the Department of Biological Sciences, University of Waikato, Hamilton New Zealand. The original samples were then transported on ice to the College of Marine Studies, University of Delaware, Lewes, DE, U.S.A and kept there at a constant temperature of –70°C until future use.

All distance measurements recorded during sampling were measured in feet. For the purpose of this MSc thesis research, distance measurements will be converted to metres.

2.2 Identification of seals

The seals designated MVS1, MVS3, and MVS13 were tentatively identified by photographs sent to Professor Rod Seppelt, Australian Antarctic Division, Tasmania, Australia.

2.3 DNA extraction

2.3.1 Optimisation of DNA extraction

Three different well established methods of DNA extraction were trialled for DNA extraction of the environmental samples: cetyltrimethylammoniumbromide (CTAB) (Jones and Walker, 1963), Bead-Beating (Miller et al., 1999), Qiagen QIAmp DNA Stool Mini Kit. These methods were undertaken on four environmental samples taken in a transect surrounding seal designated MVS4 in Miers Valley. All chemicals were sourced from APS Ajax Finechem unless otherwise specified.

2.3.1.a. CTAB DNA extraction protocol (Jones and Walker, 1963)

Approximately 1.5g of environmental sample were placed into a sterile 50ml greiner bro-one tube. Three to four ml of CTAB buffer (2% (w/v) CTAB; 1.4M NaCl; 0.2% (v/v) 2-mercaptoethanol; 20mM ethylenediamine tetraacetic acid (EDTA); 100mM Tris-HCl pH7.4) were then mixed and gently vortexed with the

sample. The mixture was then heated to 65°C and maintained at 65°C while shaking at 100 revolutions per minute (rpm) for 20 minutes. The sample was centrifuged at 1000rpm for 60 seconds at room temperature. All centrifugation was done using an Eppendorf Centrifuge 5415D, Hamburg, Germany, unless otherwise stated. The supernatant (3ml) was then transferred to a sterile 15ml greiner bro-one tube. To the supernatant a 3ml mixture of chloroform : isoamyl alcohol was added at a ratio of 24:1 respectively. The liquid was vortexed well, and placed on a rocking bed at room temperature. After 20 minutes of rocking the sample was centrifuged for seven minutes at 12,000rpm in order to separate the phases. The top aqueous phase (0.6ml) was transferred to a new tube, being careful to ensure none of the sediment or organic phase was transferred also. To this 0.6ml sample, 0.3ml of 5M NaCl and 0.6ml of ice-cold isopropanol were added and mixed well. The liquid was centrifuged at 12,000rpm for 15 minutes after which the liquid was decanted and discarded. The precipitated DNA was then washed with 150µl of 70% ethanol, centrifuged for five minutes at 12,000rpm, and the supernatant discarded. This was repeated once more being careful to remove as much excess liquid as possible. The remaining pellet was left to air dry for approximately 10-20 minutes, and then re-suspended in 20µl of LoTE buffer (3mmol/L Tris; 0.2mmol/L EDTA; pH 7.5). Extracted DNA was stored at -70°C until future use.

2.3.1.b Qiagen QIAmp DNA Stool Kit

A modified version of the protocol for isolation of DNA from faecal stools for pathogen detection was used; the protocol was derived from pages 16-19 of the Qiagen Mini Kit Handbook 08/2000. All chemicals and microcentrifuge tubes used for this protocol were supplied with the Qiagen Kit (California, U.S.A). Half a gram of environmental sample was weighed and placed on ice in a 2ml microcentrifuge tube. To the microcentrifuge tube, 1.4ml of ASL buffer was added. The tube was vortexed continuously for one minute then placed in a 95°C water-bath for five minutes. The sample was vortexed for a further 15 seconds, and then centrifuged for one minute at full speed (13,200rpm). The supernatant was removed and placed into a new microcentrifuge tube. To this 1 InhibitEX tablet was added and the sample vortexed immediately for one minute continuously. After mixing the sample was left at room temperature for one

minute to allow inhibitors to adsorb to the inhibitEX matrix. The sample was centrifuged for a further three minutes at full speed to pellet all inhibitors bound to InhibitEX. The 400µl of supernatant was removed and placed in a new 1.5ml microcentrifuge tube. To this 15µl of Proteinase K was added followed by 400µl of Buffer AL. The solution was vortexed for 15 seconds and incubated for 10 minutes in a 70°C water bath. Four hundred microlitres of 100% ethanol was added to the lysate and the solution was mixed by vortexing. This mixed lysate was then applied to a QIAamp spin column and centrifuged at full speed for 1 minute. The filtrate was discarded and 500µl of Buffer AW1 was added to the spin column. The column was centrifuged at full speed for 3 minutes after which the filtrate was again disgarded. The spin coumn was transferred to an unused, sterile 1.5ml microcentrifuge tube and 100µl of Buffer AE added directly onto the membrane. The column was incubated at room temperature for 1 minute then centrifuged for 1 minute at full speed to elute DNA. The DNA was stored at -70°C until further use.

2.3.1.c PSC – B method for DNA extraction from environmental samples

PSC – B (phosphate, SDS, chloroform – bead beater) method for DNA extraction from environmental samples (Miller et al., 1999) was used with slight modifications. The environmental sample (0.5g) was transferred to a 2ml, screw capped, conical bottomed polypropylene tube containing 0.5g each of 0.1mm and 3.0mm silica-zirconium beads. To this 300µl of phosphate buffer (100mM NaH_2PO_4), 300µl of SDS lysis buffer (100mM NaCl; 500mM Tris pH 8.0; 10% SDS) and 300µl of chloroform : isoamyl alcohol (24:1) were added. The vials were shaken in FastPrep® FP120 Cell Disrupter (BIO101 Thermo Savant, USA) at 4.5m/s for 45 seconds and centrifuged at full speed (13,200rpm) for 5 minutes to pellet debris. The supernatant was transferred to new microfuge tubes, and 7M ammonium acetate was added to make a final concentration of 2.5M, mixed gently, then spun for a further 5 minutes at maximum speed. The supernatant was transferred into a new microfuge tube and 0.54 volumes of isopropanol added. The samples were incubated for 15 minutes at room temperature, and centrifuged for 15 minutes at 13,200rpm. The supernatant was removed, and the pellets washed with 1ml of 70% ethanol. The pellets were air-dried and re-suspended in 20µl of 0.2µm filtered, double distilled H_2O. Extracted DNA was stored at -70°C.

2.3.1.d Revised PSC – B method for DNA extraction from environmental samples

The protocol for PSC-B DNA extraction from environmental samples was moderately revised in order to reduce shearing of DNA. The same protocol as described in Section 2.3.1.c was followed, however, the speed of the FastPrep® FP120 Cell Disrupter (BIO101 Thermo Savant, USA) was reduced to 4.0m/s for only 30 seconds. Three hundred microlitres of chloroform : isoamyl alcohol (24:1) was added after the sample had been shaken in the FastPrep® rather than before, and the solution was vortexed for 15 seconds. The samples were centrifuged for an additional five minutes at 13,200rpm before the supernatant was transferred to a new, sterile microfuge tube.

2.4 DNA quantitation

The amount of DNA extracted from samples was quantified spectrophotometrically. All spetrophotometry was conducted using a Beckman DU640B spectrophotometer, U.S.A, unless otherwise stated. An aliquot, 5µl, of original sample was diluted into 45µl filtered Milli-Q water (fMQ H_2O). For quantitation of double stranded DNA, an optical density (OD) of 1, corresponded to approximately 50ng/µl at 260 nm. The quantity of double stranded DNA present in the sample was calculated by equation as follows:

$(OD_{260} - OD_{320})$ x (dilution factor x 50)

Quantitation was measured in ng/µl. The purity of the DNA was estimated from the ratio of $(OD_{260} - OD_{320})$ / $(OD_{280} - OD_{320})$, (Sambrook et al., 2001). All samples were diluted to a 10ng/µl concentration.

2.5 DNA amplification

All samples and were amplified using PCR (Saiki et al., 1988) with the following universal bacterial specific,16S rDNA primers as follows:

338Fgc – 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC TCC TAC GGG AGG CAG CAG 3' (Muyzer et al., 1993)

519RC – 5' ATT ACC GCG GCT GCT GG 3' (Muyzer et al, 1993)

All primers used in this thesis research, are listed in Appendix 1. PCR was performed in a total volume of 25µl with the following components: 1X PCR Buffer (SIGMA), 0.2mM dNTPs (SIGMA), 2mM $MgCl_2$, 0.2µm of each primer, 0.2µl Jump Start Taq (SIGMA), and 10ng of template DNA. The reaction mixture was denatured for two minutes at 94°C, followed by 20 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 65 °C for 20 cycles, decreasing in annealing temperature by 0.5 °C each cycle, and extension of primers for one minute at 72 °C. A further denaturation for 30 seconds at 94 °C was carried out for eight cycles, followed by eight cycles of annealing for 30 seconds at 56 °C, and elongation of primers for one minute for a further 8 cycles. A final incubation at 72 °C for 5 minutes was carried out, the samples were then cooled to 4 °C and held. PCR was achieved by PTC-100 Programmable Thermal Controler (MJ Research, Inc.) when working at the College of Marine Studies, University of Delaware, U.S.A., and Eppendorf Mastercycler Gradient when working at the Department of Biological Sciences, University of Waikato, New Zealand.

2.6 Electrophoresis

PCR products were analyzed by agarose gel electrophoresis through 1% (wt/vol) SeaKem agarose gel (BioWhittaker Molecular Applications, USA) prepared with 0.5X TBE buffer (0.0445M Tris base; 0.0445M Boric Acid; 1mM EDTA pH 8.0). The PCR (5µl) product was mixed with 1µl of 6X loading buffer (0.25% bromophenol blue, 40% glycerol in water) and was electrophoresed for 30 minutes at 100 volts. Gels were stained in 0.5X TBE with 0.5µg/ml ethidium bromide for 10-15 minutes, and destained in 0.5X TBE for 5-10 minutes. The fragment of bacterial 16S rDNA migrated at a distance, estimated from the migration of molecular size markers, of 220bp.

2.7 Denaturing gradient gel electrophoresis

All samples were run on a denaturing gradient gel electrophoresis (DGGE) to view species diversity within each sample population. All DGGE was performed using a DCode™ Universal Mutation Detection System (BIORAD). Gels were poured and cast using a Gradient Delivery System (Model 475, BIORAD)

according to Section 4 of the BIORAD Instruction Guide. Stock solutions of 100% (20ml 40% Acrylamide/Bisacrylamide 37:5:1, 2ml 50X TAE; 40ml deionized formamide; 42g Urea; up to 100ml deionized H_2O) and 0% denaturant (20ml 40% Acrylamide/Bisacrylamide 37:5:1; 2ml 50X TAE; 78ml deionized H_2O), was made prior to use and stored in brown glass containers at 4°C. Before preparing a gel, the 100% denaturant stock was placed at 60°C to ensure all crystals were fully dissolved. Solutions of high and low gradient were prepared as required (see Table 2.3) and kept on ice. One hundred microlitres of DCode dye solution was added to the high gradient solution to allow any impurities in the gradient to be easily seen when pouring. The high and low solutions were kept on ice, and 144.4µl of freshly made 10% ammonium persulfate (APS) solution (BIORAD) was added, with 14.4µl of N,N,N^{I},N^{I}-tetramethylethylenediamine (TEMED) (BIORAD). The gradient solutions were swirled gently to mix, and the DGGE gel immediately poured.

All DGGE gels were made with an 8% acrylamide concentration, and run initially with a 35–65% denaturing gradient. All gels were loaded with a maximum allowable volume of PCR product (20µl), along with an equal volume of 2X DGGE loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol) for six hours at a constant temperature of 60°C, and 130 volts in 7L of 1X TAE. Gradient concentrations were calculated using Table 2.3.

Table 2.3 Volumes to use when making up low and high denaturant solutions

% Denaturant	ml 0% solution	ml 100% solution
20	12.8	3.2
25	12	4
30	11.2	4.8
35	10.4	5.6
40	9.6	6.4
55	7.2	8.8
60	6.4	9.6
65	5.6	10.4
70	4.8	11.2
75	4	12

The DGGE was stained in 1µg/ml ethidium bromide ddH_2O solution for 20 minutes, then rinsed in ddH_2O for a further 20 minutes. Gels were visualised using Fisher Biotech Viewing platform (College of Marine Studies, University of

Delaware), or TFX-35M GIBCO BRL UV Transilluminator and viewed with Scion Image, Beta 4.0.2, Scion Corporation© (Department of Biological Sciences, University of Waikato).

2.7.1 Concentration of PCR product for higher resolution DGGE

In an attempt to concentrate the 16S rDNA amplicons analysed by DGGE, 92µl of PCR product was ethanol precipitated using sodium acetate (3M). One tenth the volume of PCR product was added to the 3M sodium acetate stock. To this, 2.5 times the volume of molecular grade absolute ethanol was added. The solution was left at room temperature for 15 minutes, and then spun down at 13,000rpm for 20 minutes at room temperature. The supernatant was removed, and the pelleted DNA was rinsed twice using 1ml 70% ethanol, centrifuging for four minutes at 13,000rpm each time to ensure the DNA remained pelleted at the bottom of the microfuge tube. The DNA was air dried until all excess ethanol had evaporated, and then resuspended in 20µl of 0.2µm filter sterilised ddH_2O. The concentrated 16S rDNA amplicons were then used directly for DGGE analysis, or stored at -70^oC until needed.

2.7.2 Isolation of bands of interest

The bands of interest on the DGGE gels were stabbed to isolate DNA for sequencing. Ethidium stained bands were stabbed using a 20µl aerosol-barrier pipette tip, being careful not to expose the gel to UV for too long to prevent significant denaturation of DNA within the gel to occur. Each filter tip containing a small fragment of the gel was placed into a separate microfuge tube containing 20µl of filtered ddH_2O. The stabbed DNA was mixed into the water by fitting a pipettor onto the tip and pumping up and down three times. The microfuge tubes containing the mixed DNA and water were incubated overnight at 4^oC. Ten microlitres of this water was used as template for a second round of PCR amplification using the same primers and PCR conditions as before but substituting the extra volume of template for the same volume of water, and ensuring a total volume of PCR product of no less than 50µl.

To verify that the stabbed-band amplicons were indeed single products, 20ul of PCR product was again submitted to DGGE with the remainder of the PCR product being stored at -70^{o}C. Single products appeared as single bands.

2.7.3 Sequence analysis of isolated bands

Once the band of interest was verified as being a single product, the remainder of the PCR product was ethanol precipitated using ammonium acetate (7.5 M), to concentrate amplified DNA and potentially remove low molecular weight material, such as primers and dNTPs. The volume of PCR product was measured, and half of this volume of ammonium acetate was added to the amplified DNA. Molecular grade, absolute ethanol at an amount of 2.5 times the volume of PCR product was then added, and the solution mixed well. The precipitate solution was then incubated at -70^{o}C for a minimum of 40 minutes, after which the solution was centrifuged at 13,000rpm for 20 minutes at room temperature. The supernatant was removed, and the pelleted DNA was rinsed twice using 1ml 70% ethanol, centrifuging for four minutes at 13,000rpm each time to ensure the DNA remained pelleted at the bottom of the microfuge tube. The DNA was air dried until all excess ethanol had evaporated, and then resuspended in 15µl of 0.2µm filter sterilised ddH_2O. The presence of DNA was confirmed by electrophoresing 5µl of resuspended DNA as described in Section 2.6. The amplified DNA was sequenced by the Waikato DNA Sequencing Facility, University of Waikato using forward primer as follows:

338F - 5' TCC TAC GGG AGG CAG CAG 3' (Muyzer et al., 1993)

2.7.4 Optimisation of DGGE

Efficiency in terms of DNA bands on the DGGE gels visualised by two different stains, SYBR Green stain (Molecular Probes, Oregon, U.S.A) and ethidium bromide stain (Life Technologies, New York, U.S.A), were evaluated. Fifty microlitre of PCR reactions containing Miers Valley Seal Sample Set 3 (MVS3) were set up as described previously. Two 8% acrylamide gels were poured with a 30 – 65% gradient. Equal volumes of PCR product (20µl of each sample on both gels) were loaded with equal volumes of 2X loading buffer so both gels were

replica of one another. These were run for six hours at 60°C and 130V (standard conditions as described in Section 2.7).

The SYBR Green stain was made in 200ml of 1X TAE pH 8.19. 20µl of 10,000X stock of SYBR Green stain was added to give a final concentration of 1X. The ethidium bromide stain had a final concentration of 1µg/ml in ddH_20. Both gels were stained in the respective stains for 20 minutes, and then rinsed for a further 20 minutes. The gel stained with SYBR Green was rinsed in 1X TAE, and the ethidium bromide stained gel was rinsed in ddH_20. Care was taken not to touch the gel with SYBR staining, as any fingerprints (even with gloves) would fluoresce. A photograph was taken immediately of both gels using Fisher Biotech Viewing platform (College of Marine Studies, University of Delaware). The SYBR stained gel was then wrapped in clear cellophane and photographed using STORM digital gel box (Amersham Biosciences, New Jersey, U.S.A.). The ethidium bromide gel could not be photographed in this manner as the camera did not contain the correct filter.

2.8 Analysis of DGGE images

DGGE images were analysed using GelCompar© Applied Maths BVBA, Version 4.1. Gels were normalised using a standard reference lane run with each DGGE. If the reference lane was not present, or not visible, as was the case for a number of gels, a reference lane was attached using ADOBE photoshop elements 2.0, aligning the reference lane according to replica runs of a sample in another gel where the reference lane was present. Automatic band detection was used with a minimal profiling % of 6.00, and a minimal area % of 0.50. The default settings of 1.00 and 0.50 were used for position tolerance and optimisation, respectively. Comparison of lanes within each gel and between gels was achieved using the clustering bands, band-based similarity coefficient. The similarity between two tracks was calculated using the Dice Coefficient (1945), and the unweighted pair group method using arithmetic average (UPGMA) was the clustering method selected.

2.9 Archaeal diversity in samples

The presence of archaeal species in Miers Valley samples was investigated using PCR amplification methods. Primers specific for archaeal 16S rDNA genes were used as follows:

Arc1F -5' CCAGGCCCTACGGGGCGCA 3' (Øvreåas et al., 1997)

Arc2R – 5' GTGTGCAAGGAGCAGGGAC 3' (Ron Ronimus, personal communication)

PCR was conducted using DNA extracted from three environmental samples MVS1cent-1, MVS3cent, MVS13cent, and transect control environmental samples: MVT4, MVT7, MVT10, using the following protocol. PCR was performed in a total volume of 25µl with the following components: 1X PCR Buffer (SIGMA), 0.2mM dNTPs (SIGMA), 2mM $MgCl_2$, 0.2µm of each primer, 2 units of Promega Taq Polymerase, and 5ng of template DNA. The reaction mixture was denatured for five minutes at 94°C, followed by 22 cycles of denaturation for 30 seconds at 94°C, annealing for one minute at 65 °C for 22 cycles, decreasing in annealing temperature by 0.5 °C each cycle, and extension of primers for pme minute at 72 °C. A further denaturation for 30 seconds at 94 °C, followed by annealing for 1 minute at 55 °C, and elongation of primers was done for one minute for a further 14 cycles. A final incubation at 72 °C for 5 minutes was carried out, and the samples were then cooled to 4 °C and held at 4 °C.

Twenty microlitres of each MVS13cent and MVT10 archaeal PCR product were run on a 2% agarose gel, and stained using a 1µg/ml concentration of ethidium bromide in 0.5X TBE. The desired bands were cut out of the gel using a sterile scalpel blade, and removed from the agarose using a Spin Prep™ Gel DNA Kit (Novagen) following the manufacturers' instructions. Three microlitres of each of the 50µl product was used in a 50µl PCR reaction set up as above, replacing template DNA with the product purified from the agarose gel, and using archaeal DGGE primers as follows:

Arc3R – 5' CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CTG TGC AAG GAG CAG GGA CG 3' (Ron Ronimus, personal communication).

Arc 931F – 5' AGGAATTGGCGGGGGAGCAC (Jackson et al., 2001).

The reaction mixture was denatured for five minutes at 94°C, followed by 22 cycles of denaturation for 30 seconds at 94°C, annealing for one minute at 60 °C for 22 cycles, and extension of primers for one minute at 72 °C. A further denaturation for 30 seconds at 94 °C, along with annealing for 30 seconds at 60 °C, and elongation of primers was done for one minute for 14 cycles. A final incubation at 72 °C for 5 minutes was carried out, the samples were then cooled to 4 °C and held at 4 °C.

DGGE was undertaken after positive amplification of MVT10 and MVS13 samples using primers 154R and 155F, by running 4µl of product with 1µl of 6X loading buffer on a 1% agarose gel for 30 minutes at 100 volts. The DGGE was prepared as described in Section 2.7, using a 30-50% gradient.

To identify archaeal organisms present in the samples, the individual bands on the DGGE were stabbed using the protocol above in an attempt to gain sequence data on the organisms present. To verify that the stabbed-band amplicons were indeed single products they were once again submitted to DGGE analysis, using a 30-50% gradient.

The presence of Archaea in genomic DNA extracted from environmental samples collected from MVS13 sampling transect, and environmental control transect samples, were investigated using PCR amplification. Universal 16S rDNA primer 77R and primer 149F specific for archaeal 16S rDNA were used as follows:

77 1522R 5'- AAGGAGGTGATCCARCCGCA -3' (Johnson, 1994).

149 5F 5'- CCGGTTGATCCTGCCGG -3' (Reysenbach et al., 2000).

The same protocol was used as for the archaeal PCR as described earlier in this section.

2.10 Eukaryotic diversity in samples

The presence of eukaryotic species in Miers Valley samples was investigated using PCR amplification methods. Primers universal for eukaryotic 18S rDNA genes were used and are as follows:

Euk A: 5'- AACCTGGTTGATCCTGCCAGT -3' (Medlin et al., 1988)

Euk B: 5'- GATCC(AT)TCTGCAGGTTCACCTAC -3' (Medlin et al., 1988)

PCR was conducted initially on four genomic DNA samples in total. The PCRs were performed in a total volume of 25µl with the following components: 1X PCR Buffer (SIGMA), 0.2mM dNTPs (SIGMA), 2mM $MgCl_2$, 0.2µm of each primer, 0.2µl Promega Taq Polymerase, and 5ng of template DNA. The reaction mixture was denatured for two minutes at 94^{o}C, followed by 28 cycles of denaturation for 30 seconds at 94^{o}C, annealing for 30 seconds at 56 ^{o}C for 28 cycles, and extension of primers for two minutes at 72 ^{o}C. A final incubation at 72 ^{o}C for five minutes was carried out, the samples were then cooled to 4 ^{o}C and held at 4 ^{o}C. A PCR reaction using the same protocol as above, was made using MVS1, MVS3, MVS13 centre samples along with the remainder samples in MVS13 sample set, using universal eukaryotic DGGE primers as follows:

EukAGC: 5' - CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCC CAACCTGGTTGATCCTGCCAGT 3' (Medlin et al., 1988)

Euk 516 (R) : 5' – CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCC CCACCAGACTTGCCCTCC 3' (Inacio et al., 1988)

The PCR product was run on a 1% Seakeem agarose gel, and stained in 1µg/ml ethidium bromide solution in 0.5X TBE.

2.11 PCR amplification of 16S rDNA used in clone libraries

All samples used for the construction of clone libraries were amplified using PCR with bacterial 16S rDNA specific primers as follows:

Eub A (R): 5' AAG GAG GTG ATC CA(ACGT) CC(AG) CA 3' (Medlin et al., 1988)

Eub B (F) 5' AGA GTT TGA TC(AC) TGG CTC AG 3' (Medlin et al., 1988)

The PCRs were performed in a total volume of 50µl with the following components: 1X PCR Buffer (SIGMA), 0.2mM dNTPs (SIGMA), 2mM $MgCl_2$, 0.2µm of each primer, 0.2µl Promega Taq Polymerase, and 4ng of template DNA. The reaction mixture was denatured for two minutes at 94°C, followed by 28 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 56°C for 28 cycles, and extension of primers for two minutes at 72°C. A final incubation at 72°C for five minutes was carried out, the samples were then cooled to 4°C and held at 4°C.

2.12 Production of clone libraries MVS1, MVS3 and M VS13

Three environmental samples, MVS1, MVS3 and MVS13, were selected for the construction of clone libraries at the College of Maine Studies, University of Delaware. These environmental samples were each sampled from directly beneath the seals of the three main sample sets (MVS1, MVS3, MVS13). DGGE analyses, and DNA quantitation of these samples, both indicate that these samples contained the greatest abundance of organisms. The segment of 16S rDNA was amplified as described in Section 2.10. The amplified gene was transformed into TOPO vector 4.0 using a TOPO TA Cloning® Kit for sequencing using One Shot® Chemical Transformation, according to the manufacturers instructions (Invitrogen™, U.S.A). No purification of the PCR product was required as sharp, clean DNA bands were observed. Colonies were incubated at 37°C, shaking at 200rpm in 250µl of SOC medium (composition in Appendix 2) provided in the kit. 10-50µl from each transformation, along with 20µl of SOC medium (to ensure even spreading) was then spread on a pre-warmed LB plate (composition in

Appendix 2) containing ampicillin at a concentration of 100ug/ml. Colonies were then grown overnight at 37°C. Ninety-six single round colonies were then picked from each library using sterile toothpicks, and inoculated into 96 individual wells of a round bottomed microtitre plate, with each well containing 170µl of LB glycerol medium with 100ug/ml ampicillin. Colonies in the microtitre plates were then incubated once more at 37°C overnight without shaking. Five microlitres of each well were then sampled to replicate the plates in three new 96 well microtitre plates containing 170µl of LB glycerol medium with 100ug/ml. The replica plates were then also incubated at 37°C overnight without shaking. All 96 well plates containing clone libraries were stored at -70°C.

A PCR reaction amplifying genomic DNA, extracted from five random samples from each well was set up using M13F and M13R primers to determine whether or not the transformants all contained vectors with the right insert size. The M13F and M13R primers were as follows:

M13F 5' –GTAAAACGACGGCCAG- 3' (Invitrogen, Topo TA Cloning Version N)

M13R 5' –CAGGAAACAGCTATGAC- 3' (Invitrogen, Topo TA Cloning Version N)

The replica plate containing MVS1 clone library was sent on dry ice to Amersham Biosciences (San Diego, U.S.A) for sequencing.

Clone libraries MVS3 and MVS13 underwent restriction endonuclease digestion analysis using restriction enzymes *Hin*P1I and *Rsa*I. Replicate organisms were identified, and removed. Sequencing of the remaining MVS3 clones was conducted by Agencourt Bioscience Corporation, U.S.A. MVS13 clones have yet to be sequenced.

2.13 Production of clone library MVT10

A clone library of one of the control transect environmental sample was prepared as control to compare soil microbiology in the area from which it had been taken that had not been contaminated from any obvious external source such as seals or

skua guano. This clone library was produced at the Department of Biological Sciences, University of Waikato, New Zealand. The work was completed under ERMA approval number GMO03/UOW003 in the PC2 Level Transitional Containment Facility, C2:08.

2.13.1 Preparation of electrocompetent cells

Electrocompetent *Escherichia coli* cells were prepared using a method from, Sambrook and Russell, (2001). These cells were frozen using liquid nitrogen and stored at -70°C until future use.

2.13.2 Clean up of PCR product

The PCR amplification reaction described previously (Section 2.10), using genomic DNA extracted from environmental sample MVT10, produced a relatively sharp band, hence purification consisted only of a SpinPrep™ PCR Clean-Up (Novagen) to remove DNA polymerases, dNTPs, salts, and >99% of primers so they did not interfere with cloning. The clean up was conducted according to manufacturer instructions (novatech@novagen.com). The purified PCR product was quantitated spectrophotometrically and estimated to be around 10ng/µl (Waikato DNA Sequencing Facility, University of Waikato).

2.13.3 Ligation reaction

The ligation reaction was made according to the volumes of reagents as given in Table 2.4, using pGEM®-T Easy Vector Systems (Promega Corporation, Wisconsin, U.S.A.).

Table 2.4 Ligation reagents and volumes.

5X Rapid Ligation Buffer, T4 DNA Ligase	2µl
pGEM®-T Easy Vector (50ng)	1µl
PCR product	6µl
T4 DNA Ligase (3 Weiss units/µl)	1µl

Due to the low concentration of purified PCR product (approximately 6 ng/µl), the maximum amount of 6µl of PCR product was added. The reaction was incubated overnight at 4°C.

2.13.4 Electroporation

Electroporation was conducted ın the University of Waikato PC2 Level Transitional / Containment Facility, C2:08, using a BIORAD Gene Pulser™, and a BIORAD Pulse Controller. The machine was set to 250µFD capacitance, and 200 OHMS. The Pulser was then set to 1.8 for the 0.1cm electrodes being used. Two microlitres of ligation reaction were transferred to 50µl of elcectrocompetent cells that had been thawed slowly on ice. This was then placed in a 0.1cm Gene Pulser® Cuvette (BIORAD) that had been chilled on ice. Care was taken during this process to ensure no air bubbles had formed in the viscous cells, as this could potentially have caused arcing, which would have destroyed the sample. The cuvette was dried and the electrical pulse applied. 950µl of pre-warmed SOC medium (described in Appendix 2) was added to the transformed cells, and incubated with for two hours at 37°C shaking at 200rpm. A 200µl volume of the transformed cells were then plated onto pre-warmed LB medium plates (described in Appendix 2) containing 100ug/mg of ampicillin. IPTG/X-Gal was not added to the plates as suggested in the reference used (Promega Technical Manual No. 042), as this had previously been shown not to be effective for these electrocompetent cells, probably due to the selection of a colony not containing the F' episome required for blue/white colour screening (Thomas Niederberger, personal communication).

2.13.5 Colony picking and plasmid isolation

A single round colony from cloned plates was inoculated into 10mL of sterile LB media containing 100µg/mL of ampicillin using sterile toothpicks, and incubated at 37°C for approximately 16 hours with constant shaking at 100rpm. This mixture was then centrifuged for five minutes at 3700rpm at a temperature of 10°C. The supernatant was poured off, and pellet resuspended in 0.2ml of solution one (50mM glucose; 25mM Tris pH 8.0; 10mM EDTA). After vortexing to mix, 400µl of fresh solution two (1% SDS; 0.2M NaOH) was added. The vials were mixed immediately by inversion and placed on ice for 2-3 minutes. This was carefully timed to avoid degradation of the plasmid. To this 300µl of solution three (60ml of 5M potassium acetate; 11.5ml of glacial acetic acid; 28.5ml of H_2O) was mixed by vortexing for 10 seconds. After incubation on ice for at least five minutes the tubes were spun at 8500 rpm for 12 minutes at 10 °C using a

Beckman J2-21M, Induction Drive Centrifuge, (U.S.A.) 0.75µl of supernatant was transferred to a new microfuge tube, to which an equal volume of isopropyl alcohol was added. The tubes were mixed by inversion and incubated at room temperature for at least five minutes. The tubes were spun at full speed (13,200rpm) for seven minutes at room temperature, then washed twice with 1ml of 70% ethanol, centrifuging briefly in between washes to ensure the pellet remained at the bottom. The pellets were air dried in an inverted position, then resuspended in 100µl of TE (10mM Tris, 1mM EDTA pH 8.0) with 1µl of 10mg/ml RNAse added to each. The plasmid preparations were stored at 4°C.

2.13.6 Restriction endonuclease analysis

All plasmid preparations were checked for correct insert size (approximately 1500bp) by restriction endonuclease digestion with restriction enzyme *Eco*RI, which cuts DNA at the palindrome 5'G▼AATTC3' (New England BioLabs). 8µl of the plasmid preparation was added to a solution containing 2µl 10X NE Buffer *Eco*RI (New England BioLabs), 0.5µl of *Eco*RI restriction enzyme, and 9.5µl of filtered MQ H_2O. The digest solution was then incubated at 37°C for two hours after which 10µl of SDS loading buffer (was added) (0.25% bromophenol blue, 40% glycerol in water; 1% SDS). The solution containing SDS was then incubated at 65 °C for 15 minutes, before being loaded onto a 2% agarose gel, and run at 140V for two hours. The gel was stained as before and viewed and photographed using TFX-35M GIBCO BRL UV Transilluminator, with Scion Image, Beta 4.0.2, Scion Corporation©.

In order to reduce redundancy of cloned sequences two further digests using restriction enzymes *Hin*P1I which cuts DNA at the palindrome 5' G▼CGC3', and *Rsa*I which cuts DNA at the palindrome 5'GT▼AC 3', were conducted on those samples shown to contain the correct insert. These digests were set up as before, the differences being with *Hin*P1I (New England BioLabs) the buffer used was 10X NE Buffer2, and with *Rsa*I (New England BioLabs) the buffer used was 10X NE Buffer1.

2.14 Isotopic abundance in environmental samples

Abundance of stable isotopes $\delta^{13}C$ and $\delta^{15}N$ present in environmental samples from MVS1, MVS3, MVS13, and MVT sampling transects, was determined at the University of Waikato Stable Isotope Unit using a fully automated Europa Scientific 20/20 isotope analyser (Cheshire, U.K.). All environmental samples were ground using a mortar and pestle to a fine powder, then UV treated for 24 hours in a Heraeus, Hera Safe biological safety cabinet, to sterilize the soils in compliance with MAF requests. In between samples, mortar and pestles were washed with Virkon detergent, rinsed and then baked at 60°C. Initially between 49mg and 56mg of each sample were weighed into tin cups using a precision microbalance. For re-analysis of some samples, weights lower than this were required. Exact weights were recorded as isotopic fractionation between product and residual materials resulting from yields that are not accurately quantitative may result in a false apparent isotopic composition of the samples (Peterson and Fry, 1987). The samples were measured for $\delta^{13}C$ and $\delta^{15}N$ and estimates (if possible) of %C and %N were given. Urea standards were analysed every 12 samples. Internal reference checks were also done at these regular intervals.

2.15 Radiocarbon dating of seal sample

Approximately 0.89g of mummified tissue and bone from Miers Valley Seal 3 (MVS3) were sent to the University of Waikato ^{14}C Laboratory for dating by the Accelerator Mass Spectrometry (AMS) technique.

Treatment at the University of Waikato ^{14}C Laboratory included a physical pre-treatment of cleaning, grinding and removal of visible contaminants. A chemical pre-treatment then followed, involving decalcification in 2% HCl, followed by rinsing and drying, then gelatinisation at pH3 with HCl at 90°C for four hours, followed once more with a rinse and dry. Isotopic fractionation of ^{14}C and approximate age calculated using the AMS technique.

CHAPTER THREE

Results

3.1 Identification of seals

MVS13 and MVS3 were tentatively identified as Weddell seals, where as MVS1 was identified as a Crabeater seal (Professor Rod Seppelt, personal communication (as described in Section 2.2).

The photographic images (taken by Professor S.C. Cary) of the three seals were not optimal for identification purposes as they did not show a full lateral view of the head, or teeth. The identity of the three seals was therefore an estimate only, and further photographs would be needed to conclusively identify the carcasses.

3.2 Optimisation of DNA extraction

Three different methods of DNA extraction were trialled for DNA extraction of the Antarctic environmental samples; as stated in Section 2.3.1. The quantity of double stranded DNA gained from each sample varied according to which method was used (Table 3.1).

Table 3.1 Quantity of DNA extracted from Miers Valley Seal 4 (MVS4) environmental samples.

Extraction Method	Sample	Quantity of DNA (ng/µl)	OD260/OD280 ratio
CTAB	4.1	56	2.2
	4.2	161	1.6
	4.3	21	2.8
	4.4	none detected	
Bead-beating	4.1	106	2.1
	4.2	132	2.01
	4.3	4	1.7
	4.4	6	2.5
QIAmp DNA Stool Kit	4.1	10	2.3
	4.2	14	1.8
	4.3	7	2.6
	4.4	7	2.3

DGGE analysis of 16S rDNA amplicons from genomic DNA extracted using the three methods from MVS4 environmental sample 4.1, indicated that bead-beating

had the most abundant DNA, and by ethidium bromide staining of the acylamide gel the most intense staining of bands (depicted in Figure 3.1). A faint band was present in the sample extracted using the commercial QIAmp DNA Stool Kit (indicated by a red arrow in Figure 3.1) that did not appear to be present in either of the CTAB extraction or the bead-beating extraction method samples. This perhaps indicated that different extraction methods may select for DNA representing different organisms. Given the clear visual difference illustrated by DGGE among the three methods used to extract DNA from sample 4.1 as shown in Figure 3.1, and the lack of available sample for further extraction trials, the bead-beating method for isolation of DNA from environmental samples was chosen.

Initial electrophoresis of the extracted DNA showed a large degree of shearing in the samples, preventing larger segments of the 16S rDNA gene from being amplified. This shearing was reduced by modifying the bead-beating process marginally. Modifications included reducing the amount of shaking in the FastPrep® FP 120 Cell Disrupter, and adding the chloroform after the shaking process. As no experiment was conducted to test the percent efficiency using a known quantity of DNA, the efficiency of genomic DNA extraction using the beat-beating method was not known.

Figure 3.1 DGGE gel of MVS4 transect environmental sample 4.1. Bacterial 16S rDNA extracted using bead-beating, CTAB and QIAmp DNA Stool Mini Kit methods of DNA extraction.

3.3 Quantity and quality of extracted DNA

The amount of genomic DNA extracted from Miers Valley environmental samples was quantified spectrophotometrically as described in Section 2.4. The quantities of extracted genomic DNA shown in Table 3.2 varied in range between no DNA detected and 536 ng/µl.

All blank extractions (DNA extractions using only reagents and no environmental sample) were also quantified spectrophotometrically (data not shown).

Repeat extractions using the bead-beater method (Miller et al., 1999) were conducted on those environmental samples where no double stranded DNA was detected from extractions. The quantities specified in Table 3.2, depict the highest volume with the exception of MVS1-centre samples where all quantities of double stranded DNA extracted from this environmental sample were listed.

An OD_{260}/OD_{280} ratio between 1.8 and 2.0 is optimal for double stranded DNA (Sambrook and Russell, 2001). As a result of the low quantities of double stranded DNA in the extractions, this ratio was found to vary widely, and based on PCR amplification and DGGE results, was not found to affect molecular analysis.

Table 3.2 Quantity and quality of genomic DNA extracted from Miers Valley environmental samples.

Transect	Sample	Quantity of DNA (ng/µl)	OD_{260} /OD_{280} ratio
MVS1	A1-1	82.75	2.35
	A1-2	none detected	
	A2-1	235.25	2.14
	A2-2	none detected	
	A3-1	536.10	2.08
	A3-2	none detected	
	A4	127.30	2.02
	A5	155.95	2.27
	A6-1	118.55	2.19
	A6-2	none detected	
	A9	125.00	2.14
	A10	9.10	1.70
	B1-1	63.25	2.19
	B1-2	none detected	
	B1-3	none detected	
	B2-1	55.00	2.00
	B2-2	none detected	
	B3-1	none detected	
	C1-1	22.65	2.24
	C1-2	none detected	
	C2-1*	5.00	
	C2-1	5.00	5.00
	C2-2	none detected	
	C3-1*	17.00	
	C3-1	10.85	2.49
	C3-2	none detected	
	C4	259.85	2.10
	C5	none detected	
	D1-1*	20.00	
	D1-1	none detected	
	D1-2	none detected	
	D2-1*	none detected	
	D2-1	22.85	1.70
	D2-2	none detected	
	D3-1*	none detected	
	D3-1	43.60	2.14
	D3-2	none detected	
	Centre-1*	45.00	
	Centre-1(1)	382.05	2.02
	Centre-1(2)	133.00	2.13
	Centre-1(3)	259.65	1.69
	Centre-1(4)	435.60	2.05
	Centre-1(5)	67.70	2.20
	Centre-1(6)	154.60	2.18
	Centre-1(7)	258.45	56.65
	Centre-1(8)	56.65	2.31
	Centre-1(9)	405.40	2.05

Transect	Sample	Quantity of DNA (ng/µl)	OD_{260} /OD_{280} ratio
MVS3	A1	70.95	2.00
	A2	28.60	2.00
	A3	31.65	1.90
	B1	21.30	2.30
	B2	42.15	1.70
	B3	198.35	2.00
	C1	40.60	1.90
	C2	46.05	2.06
	C3	36.10	2.05
	D1	128.30	2.00
	D2	73.05	1.95
	D3	78.80	2.03
	Centre	134.70	2.15
MVS13	A2	20.70	1.99
	A3	21.20	2.63
	B1	50.10	2.20
	B2	13.00	1.80
	B3	8.90	1.84
	C1	32.05	2.34
	C2	14.60	2.04
	C3	34.40	2.08
	D1	144.75	2.05
	D2	48.60	1.94
	D3	41.75	2.04
	Centre	278.45	2.04
MVT	1	none detected	
	2	7.80	1.69
	3	7.35	1.80
	4	15.20	2.22
	5	8.75	2.69
	6	232.00	2.04
	7	11.20	1.27
	8	203.45	2.03
	9	231.30	2.05
	10	12.40	2.58
	11	7.20	4.11
	12	87.00	2.13

3.3.1 Optimisation of denaturing gradient gel electrophoresis methodology

Various factors were experimented and changed in order to optimise the DGGE process. Initially, DGGE gels showed a lot of amplicons not being clearly separated at the top of the wells. The gradient was changed from 35-65%, to 30-60% to try to compensate for this; however, the presence of bands near the bottom of the 30-60% gel indicated that some bands possibly had run off the bottom of the gel.

DGGE was established using PCR amplified 16S rDNA of genomic DNA extracted from three environmental samples from MVS3 sampling transect (shown in Figure 3.2). A gradient of 0-100% was used in an attempt to establish an ideal gradient for clear separation of bands without loss of DNA off the end of the gel. In order to get sharper, more focused bands, the length of time used to run a gel was trialled. Three lanes were loaded with 40µl of PCR product and loading buffer. One hour later the same three samples (from the same PCR reaction), were loaded, again with 40µl of PCR product and loading buffer. At two hours from the initial start time, the same loading on another three lanes was done again. The gel was run in total for six hours. All three samples produced the same banding pattern when loaded at the three different times. However, those that were left to electrophorese the longest, (six hours), produced much sharper bands, suggesting that a longer running time would be optimal. The DNA bands migrated approximately two thirds the total length of the acrylamide gel. Ultimately, a specific gradient that could be used for all gels was not decided upon, as some amplicons (presumably indicative it was presumed of microbial populations) had bands that tended to migrate further than others. All gels were run with a gradient ranging from 25-30% as the low solution, and 60-70% as the high solution. Differences in the length of separation between bands were accounted for by running a standard artificial colony with every gel.

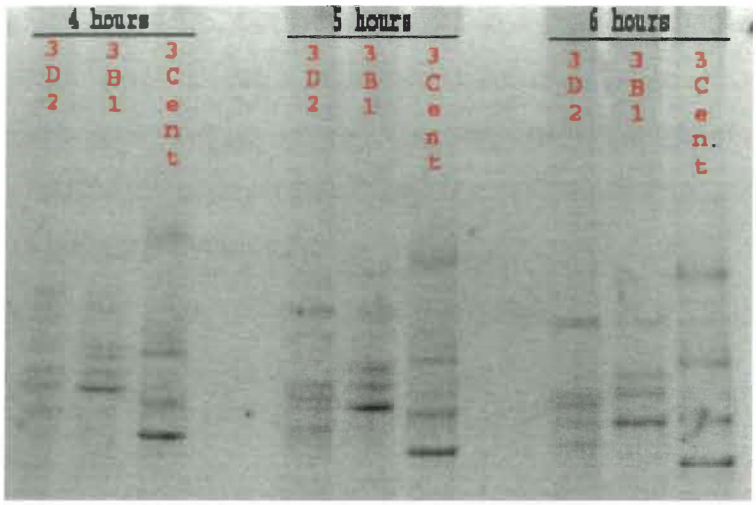


Figure 3.2 Bacterial DGGE gel of samples D1, B1 and centre from MVS3 sample transect. Three PCR products were loaded three times each with one hour intervals between loading.

A gel was trialled running at lower voltage overnight. This, however, produced pinched bands, resulting in a poor image (data not shown).

The results of earlier bacterial DGGE experiments showed a very large number of faint bands. This indicated that the samples contained high diversity with very few clearly dominant species. Such high diversity and the close proximity of bands to one another would prove hard to isolate bands for sequencing. A number of methods to optimise PCR amplification were tried in order to increase the concentration of DNA loaded onto the gel as follows:

1. The concentration of $MgCl_2$ was experimented with, however the original concentration used proved to be the most effective (data not shown).
2. The concentration of DNA template used in each reaction was increased from 10ng/μl to 30ng/μl in a 50μl reaction.
3. The number of cycles in the PCR amplification was increased from 28 cycles to 31.

Differences as a result of the change in template concentration and increased number of total PCR cycles were visually determined by DGGE (Figure 3.3). Difference in the number and strength of bands present were determined for four samples of genomic DNA extracted from MVS1 centre environmental sample. Samples A and C were PCR amplified with 10ng concentration of template DNA, and samples C and D were PCR amplified with a concentration of template DNA of 30ng. The PCR amplification of A and B environmental samples involved 28

complete cycles, and environmental samples C and D were amplified with a total of 31 complete cycles. Environmental sample D visualised on the bacterial DGGE showed the darkest and clearest bands. As a result all further PCR amplifications for DGGE analysis used a 30ng concentration of DNA, and all were amplified for 31 complete cycles.

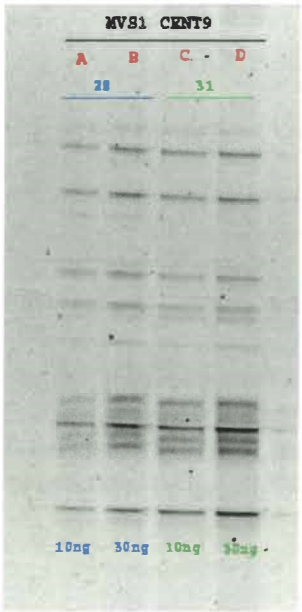


Figure 3.3 DGGE gel of MVS1 centre environmental sample. Environmental samples A and C, were PCR amplified with 10ng concentration of template DNA, and environmental samples C and D were PCR amplified with a concentration of template DNA of 30ng. The PCR amplification of A and B environmental samples involved 28 complete cycles, environmental samples C and D were amplified with a total of 31 complete cycles.

SYBR Green and ethidium bromide stains used to visualise bands were compared, and various methods of photographing DGGE gels were trialled in order to get a better image (as described in Section 2.7.4). The image taken using the STORM digital gel box of the SYBR Green stained DGGE gel was significantly skewed, caused by wrapping the gel in cellophane. This resulted in an image where the viewed lanes were no longer parallel and could not be compared (data not shown).

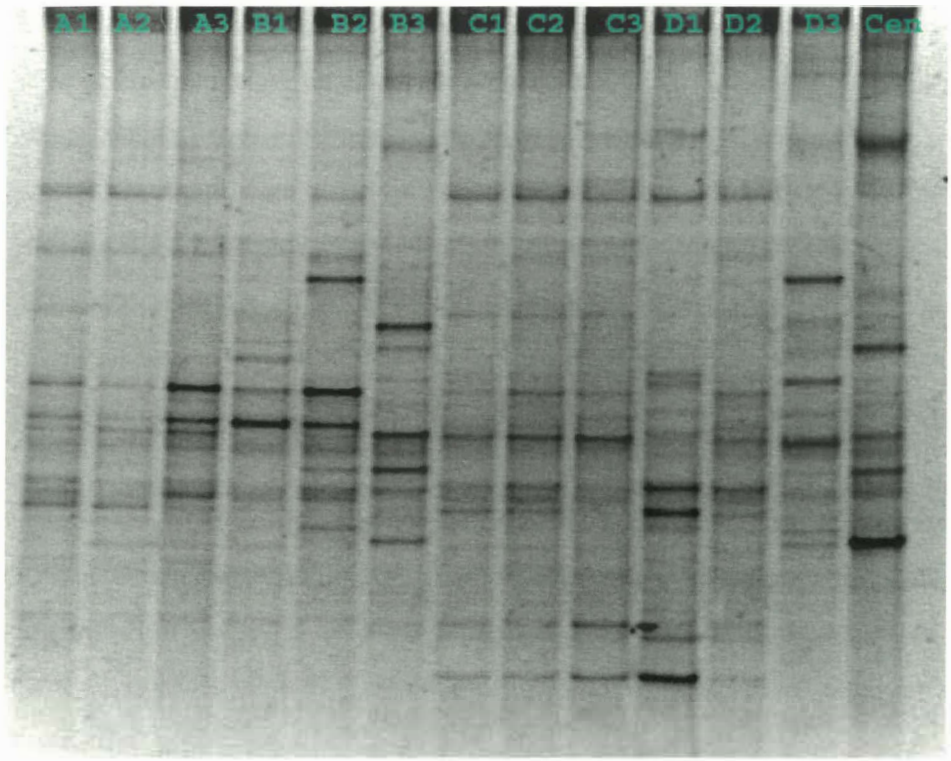


Figure 3.4 SYBR Green stained DGGE gel of bacteria present in MVS3 environmental samples (photograph taken using the Fisher Biotech Viewing platform).

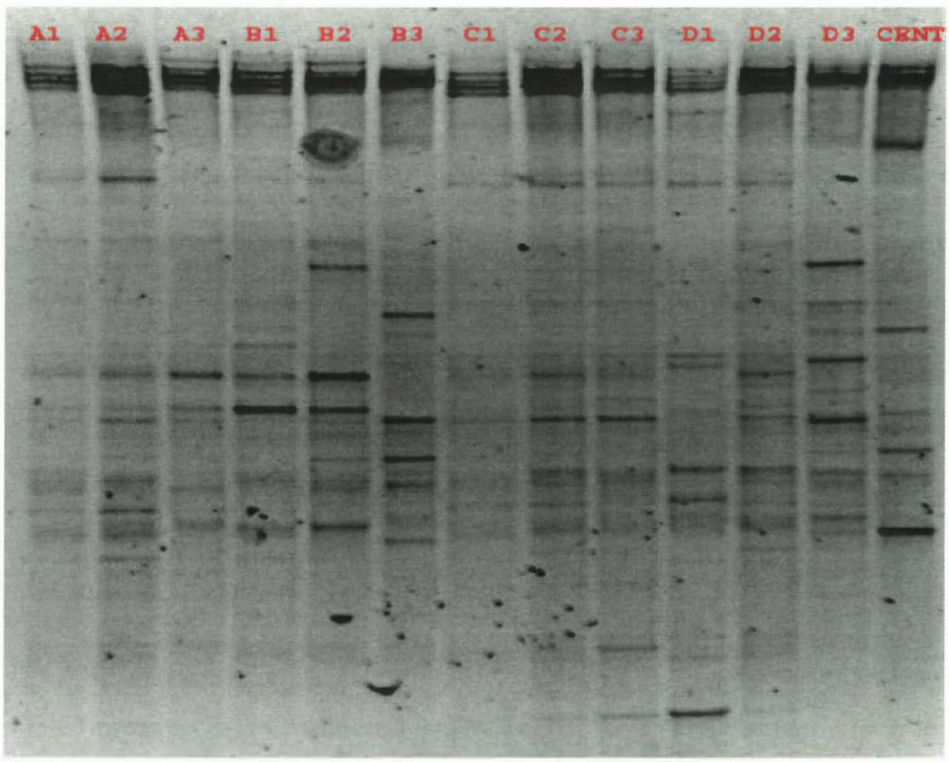


Figure 3.5 Ethidum bromide stained DGGE gel of MVS3 bacterial populations present in environmental samples (photograph taken using the Fisher Biotech Viewing platform).

Visually some differences were observed between bands photographed using the Fischer Biotech viewing platform shown in Figures 3.4 and 3.5. With the SYBR Green stained gel, the bands were visually clearer. PCR product A1 had more pronounced and more readily identifiable bands in the SYBR Green stained gel as compared to the bands in the ethidium bromide stained gel. There appeared to be more imperfections in the ethidium bromide stained gel with what looked like an ethidium bromide stain in B2, and the presence of large amounts of dust and filter paper which optimally should have been washed away to gain a better image.

Based on visual comparison of the two MVS3 DGGE gels stained using either SYBR Green or ethidium bromide, and taking into account the cost and time consumption involved with each method, ethidium bromide appeared to be the more favourable method. Subsequently all DGGE gels were stained with ethidium bromide.

Further research was conducted, comparing the similarity between SYBR Green stained DGGE gel and ethidium bromide stained DGGE gel using GelCompar software to attempt to quantify the differences in banding patterns observed between the two staining methods (see Section 3.4.1).

3.3.2 Results of developing DGGE methodology on analysing biodiversity

Given the diversity contained within the Miers Valley environmental samples, visual analysis was difficult. The high number of bands present in some environmental samples, meant human error and bias could potentially skew observations.

3.3.2.a GelCompar analysis

All DGGE gels in this research were analysed using GelCompar© Applied Maths BVBA, version 4.1, Comparative Analysis of Electrophoresis Patterns. GelCompar is a PC directed software package that allows the normalisation, grouping, and identification of electrophoresis patterns. Normalisation of gels was accomplished through the alignment of specific reference tracks in a gel. A track represented a single lane in a DGGE. A track in DGGE analysis, therefore, represented 16S rDNA amplicons from an environmental sample, indicative, it

was presumed, of a microbial population. The specific reference tracks were used for the alignment of all gels in a database. The alignment of reference tracks was done by aligning their corresponding bands, and by subsequent interpolation of the intermediate values. Non-reference tracks were aligned gradually according to their closest neighbouring reference tracks. Gels within a database were compared using a band-based similarity coefficient method of cluster analysis. Bands were automatically detected using a minimal profile percent setting of 6.00, and minimal area percent of 0.60. These values were higher than the default settings, as use of the default settings (minimal profiling percent 5.00 and minimal area percent 0.5) lead to the detection of bands in some gels, that could not be visually confirmed. The similarity between two tracks was calculated using the *Dice* (1945) coefficient[3] (S_D). Based on the calculated values of similarity, a dendogram was constructed, representing a hierarchic representation of linkage levels between pairs of individual or groups, using the UPGMA clustering algorithm. The similarity values calculated by the *Dice* coefficient were presented at the top of the dendogram.

DGGE were normalised using a standard reference lane, constructed using DNA extracted from eight bacterial isolates. These isolates were individually PCR amplified with the same bacterial specific primers used to PCR amplify the Miers Valley environmental samples for DGGE analysis. PCR product for each sample was then combined giving a final volume that could be used on approximately 20 DGGE gels.

3.3.2.b GelCompar analysis of SYBR green and ethidium bromide stained DGGE gels

Differences in the bands detected by the two staining methods were indicated by values of similarity and groupings of related samples between tracks, from the two gels. Similarity values calculated using GelCompar software were presented by a dendogram (Figures 3.6a and 3.7). Differences in the lengths of branches on the dendogram indicated differences in the similarity of band identification and placement between tracks.

Differences in the bands detected by the two staining methods (SYBR Green and ethidium bromide) were indicated by values of similarity and groupings of related samples between tracks, from the two gels. Similarity values were calculated using GelCompar software and presented by dendograms (Figures 3.6a and 3.7). A track represented a single lane in a gel using the GelCompar system. A track in DGGE analysis, therefore, represented 16S rDNA amplicons from an environmental sample, indicative, it was presumed, of a microbial population. Differences in the lengths of branches on the dendogram indicated differences in the similarity of band identification and placement between tracks. Similarity was calculated by GelCompar using a band-based similarity coefficient method of cluster analysis.

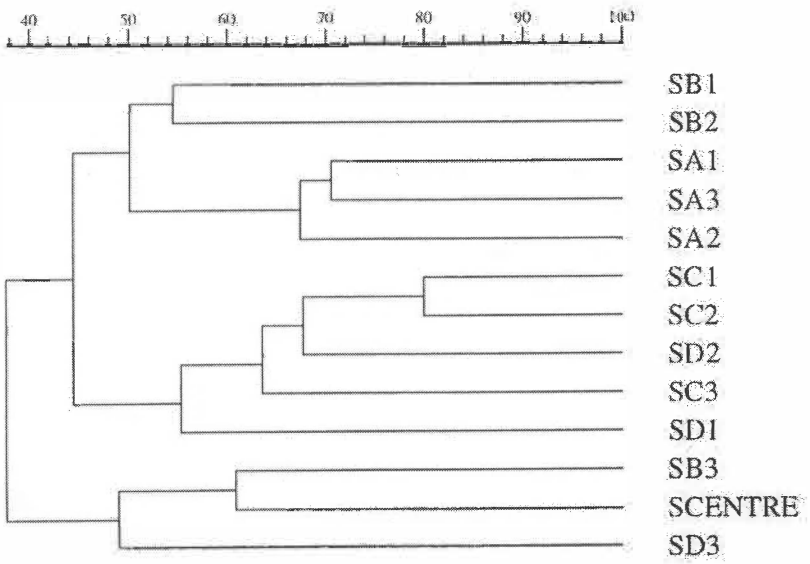


Figure 3.6a Dendogram showing band comparison of MVS3 bacterial population present in environmental samples analysed by DGGE and stained with SYBR Green.

In the dendogram representing the SYBR Green stained DGGE gel (Figure 3.6), tracks representing MVS3 environmental samples B3, Centre and D3 shared the greatest similarity with one another and were indicated to share only 38% similarity with the remainder tracks as highlighted in Figure 3.6b.

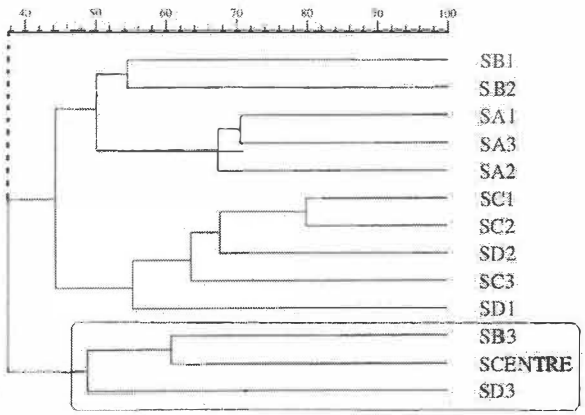


Figure 3.6b Dendogram highlighting similarity of tracks representing SB3, SCENTRE, and SD3 environmental samples analysed by DGGE and stained using SYBR Green.

Tracks representing environmental samples taken on the A direction transect line in the SYBR Green stained DGGE grouped together as being the most similar. So too did the tracks representing environmental samples taken in the B direction transect line, with the exception of B3. Tracks representing environmental samples taken in the C and D directions along the sampling transect appeared to be less different to one another in terms of similarity when compared to tracks representing environmental samples from A and B transect lines. Track D1 was identified as being most similar to track C3, and track D2 was identified as being most similar to tracks C1 and C2.

GelCompar analysis of the ethidium bromide stained DGGE gel, depicted in the dendogram shown in Figure 3.7, indicated different values of similarity between tracks, and different groupings of most similar tracks representing the same PCR products as those stained by SYBR Green stain. Track B3 appeared to be distinct from the remainder samples on the gel, sharing only 22% similarity with any of the other tracks. The track representing MVS3 centre sample, showed the greatest similarity with tracks D3 and D1. Track A2 was identified as being most similar to tracks C2 and D2, rather than track A1, as observed in the GelCompar analysis of the SYBR Green stained gel.

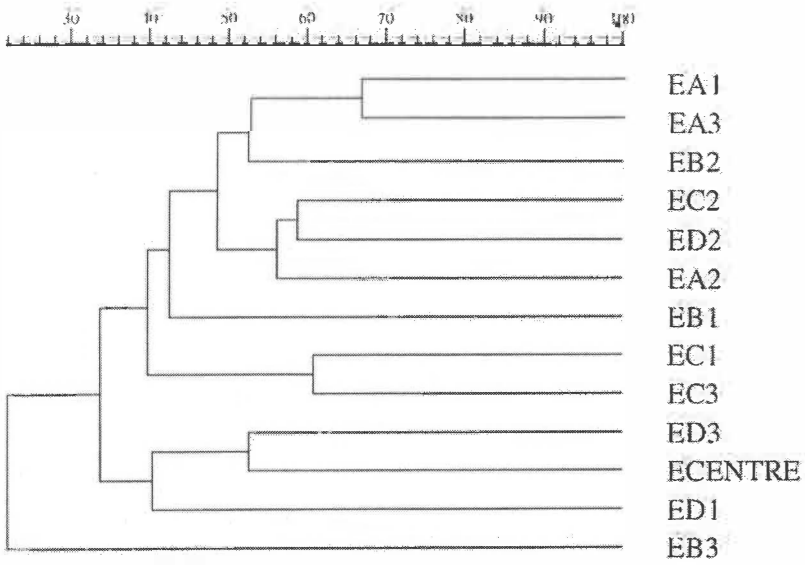


Figure 3.7 Dendogram showing band comparison analysis of MVS3 environmental samples visualised by ethidium bromide stained DGGE gel.

Analysis of the SYBR Green stained DGGE gel using the GelCompar software (Figure 3.6), showed diversity between tracks of up to 62%. No track on the SYBR Green stained DGGE gel was more than 80% similar to another track. Analysis of the ethidium bromide stained DGGE gel using the GelCompar system (Figure 3.7) showed diversity between tracks of up to 78%. No tracks on the ethidium bromide stained DGGE were more than 74% similar.

To directly compare the differences between bands detected using the two different staining methods, both gels (shown in Figures 3.4, 3.5) were analysed together by band comparison using GelCompar software (Figure 3.8). Tracks representing environmental samples from the SYBR Green stained DGGE were denoted with a 'S'. Tracks representing environmental samples from the ethidium bromide stained DGGE were denoted with an 'E'.

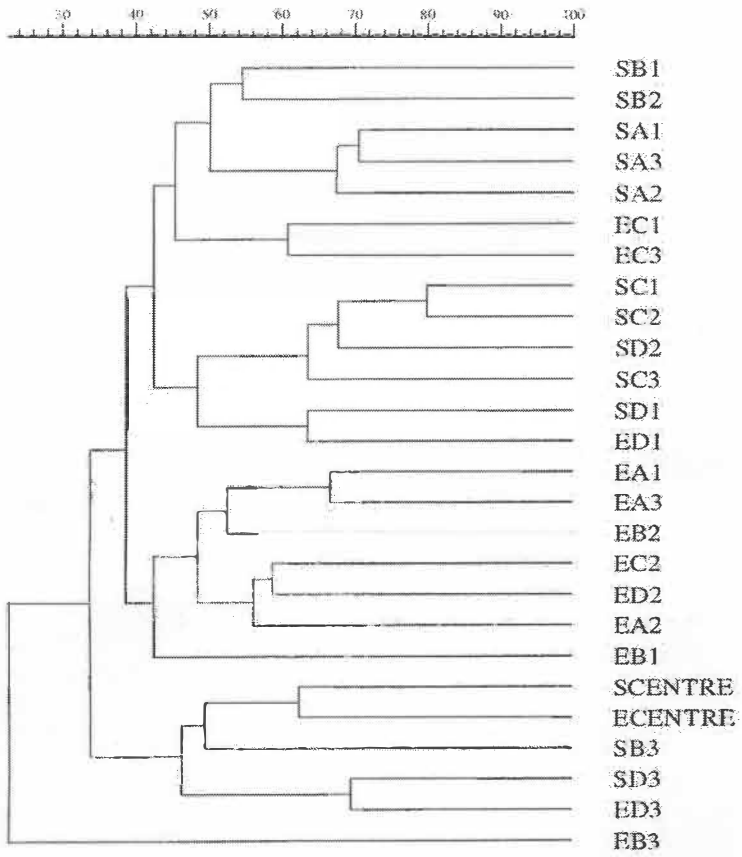


Figure 3.8 Dendogram showing similarity between replica MVS3 environmental samples visualised using DGGE and stained with either SYBR Green or ethidium bromide.

Track EB3 (shown in Figure 3.8), was shown to be distinct from the remainder tracks from both gels, sharing only 24% similarity. Given the lack of similarity between track EB3 and other tracks in the dendograms (shown in Figures 3.7 and 3.8), in particular the track representing a replica sample SB3 stained with SYBR Green, one could suggest some other factor was involved. An example could be loading error, which may account for the difference. Tracks D1, D3 and centre from both gels were identified as being the most similar to one another, however they shared only 64%, 70%, and 62% similarity respectively. The tracks representing MVS3 centre environmental samples from both gels matched more closely to tracks SB3 and tracks representing environmental sample D3 from both gels.

Overall tracks representing SYBR Green stained PCR products tended to show more similarity by band detection and comparison with other SYBR Green stained PCR products, and tracks representing ethidium bromide stained PCR

products tended to show more similarity to other tracks representing ethidium bromide stained PCR products.

Given the extra handling care and expense of SYBR Green stain, ethidium bromide proved to be the more satisfactory stain and bands were visualised with the transilluminator system.

Bacterial DGGE gels of the Antarctic environmental samples produced a multitude of bands indicating high bacterial diversity present in the sample population (Figures 3.7, 3.9, 3.10, 3.15, 3.16, 3.17, 3.20, 3.22, 3.24, 3.26, 3.28). A large number of these bands were extremely faint, and presented a problem during analysis. For environmental samples from the control transect and MVS13 sampling transect, the quantities of genomic DNA extracted were minimal, and DGGE gels of these environmental samples produced such faint bands as to be determined unusable for analysis. In an attempt to further improve DGGE gel clarity, 92µl of 16S rDNA PCR product from environmental samples collected from the MVS13 sampling transect were concentrated by ethanol precipitation. The concentrated PCR product was resuspended in 15-20µl of filtered ddH_2O for at least 20 minutes, before an equal volume of 2X loading buffer was added and the sample analysed by DGGE.

The difference in band detection, using the standard settings applied to GelCompar for all of the DGGE analysis, was determined by comparing two replica bacterial DGGE gels of MVS13 sampling transect; one using PCR product without any further concentration shown in Figure 3.9, while the other used PCR product that had been concentrated approximately 4.5 times by ethanol precipitation depicted in Figure 3.10. Both gels were run under standard conditions for temperature, time, gradient, and the same genomic DNA samples extracted from MVS13 environmental samples were used in PCR for both gels.

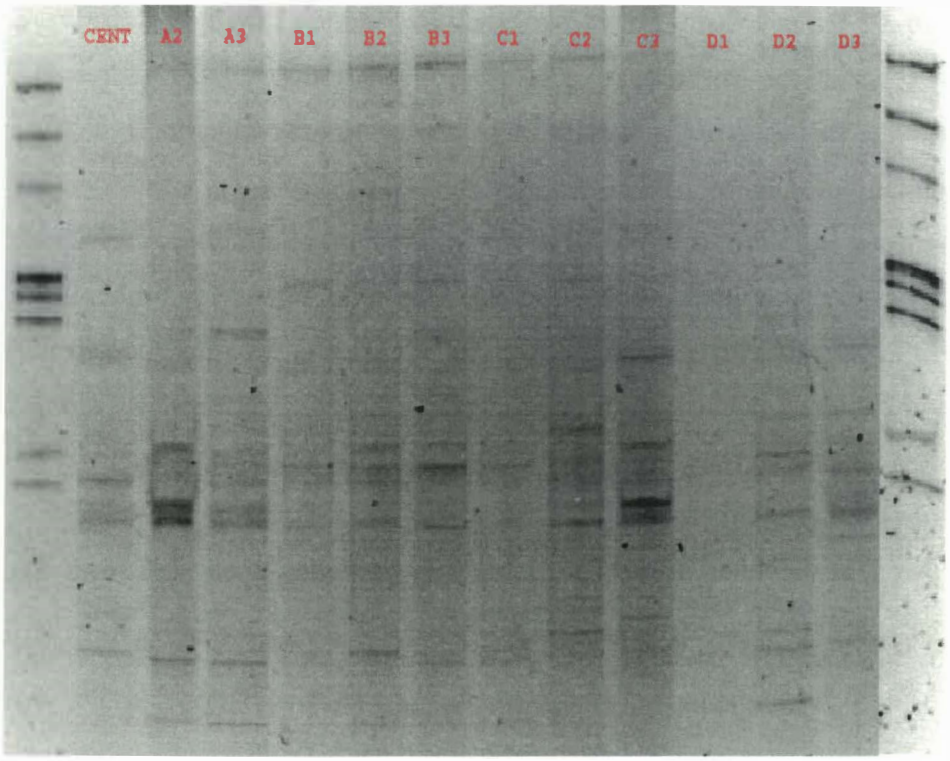


Figure 3.9 DGGE gel image of MVS13 bacterial diversity.

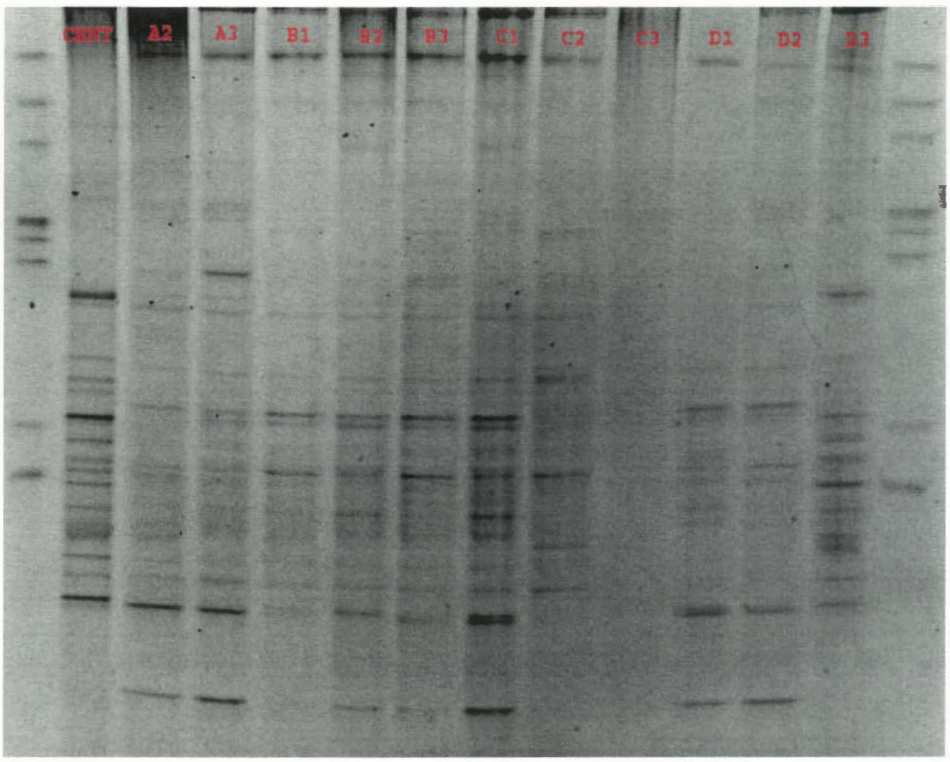


Figure 3.10 DGGE gel image of MVS13 bacterial diversity using concentrated PCR product.

By visual analysis of the two gels as shown in Figures 3.9 and 3.10, the gel using concentrate PCR product appeared to have considerably more bands present for each environmental sample population. This was clearly evident when comparing the two centre sample lanes on both gels, the centre sample of concentrated PCR product had at least seventeen apparent bands present, while the centre sample on the standard gel had only seven apparent bands. In both gels, there was a sample that did not amplify, sample D1 for the DGGE gel using standard PCR product, sample C3 for DGGE gel using concentrated PCR product.

Both gels were analysed using GelCompar software. Similarity between lanes was determined by band comparison and depicted in the dendogram shown in Figure 3.11.

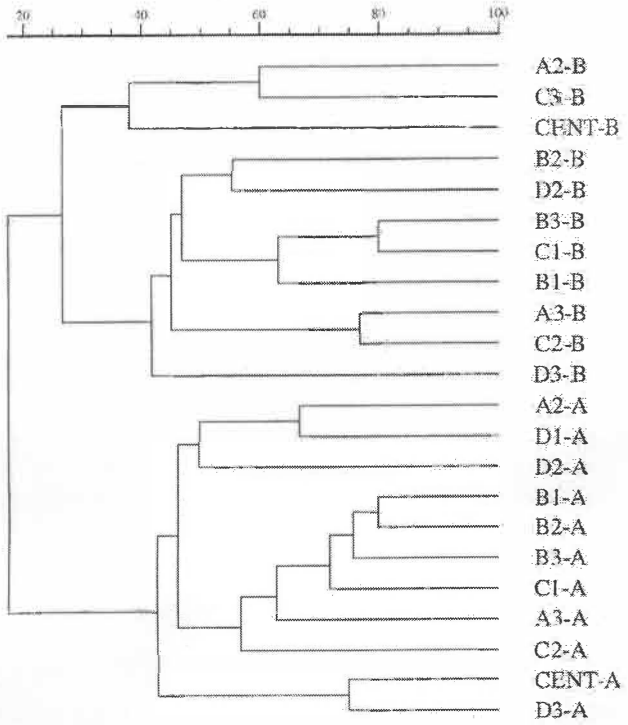


Figure 3.11 Dendogram showing similarity between replica MVS13 environmental samples. Environmental samples denoted -A, were concentrated by sodium acetate ethanol precipitation, and had approximately 4.5 times the concentration of PCR amplified DNA than samples –B.

The two gels showed no similarity according to this method of analysis as tracks representing environmental samples from both gels grouped into one of either two groups according to concentration of PCR product used in the DGGE. The two groups showed only 18% similarity between them. The tracks within each group appeared to be grouped differently, also. For example, the track representing the

centre sample in gel B showed the most similarity with tracks A2 and C3 from group B, whereas the track representing the centre sample from group A showed the most similarity with track D3 in group A. The absence of a different sample from each group (D1 for group B, and C3 for group A) may account for some of differences in the intra-group matching.

The results indicated that gels with varying concentrations of PCR product loaded appeared not to be comparable using this method. Therefore, bacterial DGGE gels of Miers Valley environmental samples using different concentrations of PCR product, could not be compared using GelCompar software with the settings and analysis matricies used.

3.4 Reproducibility of DGGE for analysis of bacterial diversity

The reproducibility of DGGE used to analyse bacterial diversity in environmental samples collected from Miers Valley was tested. When considering the small amount of soil being used to extract DNA (0.5g), the method of DNA extraction used, and the comparative lack of abundance of soil microbes when compared to other continents (Campbell and Claridge, 1987), the potential for hotspotting, (selecting for small pockets of microbial communities on some but not all occasions) was considered to be significant. To test whether hotspotting occurred, nine consecutive DNA extractions were carried out on a single environmental sample, the centre sample taken from the soil surface in the MVS1 sampling transect. As with all other DNA extractions, a blank extraction, containing only the reagents used in the extraction process (without any sample) was carried out. These extracted DNA samples were PCR amplified, and electrophoresed on an agarose gel to test for the presence of a 250bp band (the approximate size of the region selected for by primers 338F and 519R used in PCR amplification). The nine MVS1 centre samples all containing a band of approximately 250bp in length were subjected to DGGE to compare diversity (shown in Figure 3.12). No band was present in the blank extraction sample, indicating the extraction reagents did not contain any PCR amplifiable contaminants. All nine MVS1 extracted DNA samples produced an identical banding pattern, indicating that the DNA results were in fact reproducible and no hotspotting was observed in these samples with ethidium bromide stain. The bands varied marginally in strength, indicating that

the strength of a band was not necessarily an accurate indication of its dominance in a population.

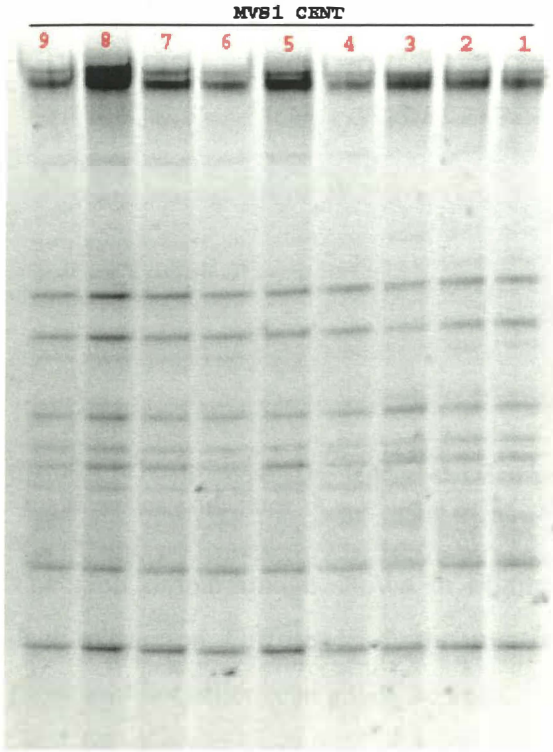


Figure 3.12 DGGE gel of replica DNA extractions from MVS1 centre environmental sample.

Given the visually apparent, nearly identical alignment of bands for each DNA extraction in Figure 3.12, analysis of the DGGE gels using GelCompar software under standard conditions used to analyse all gels provided the opportunity to test the accuracy of the band detection, and band comparison matricies.

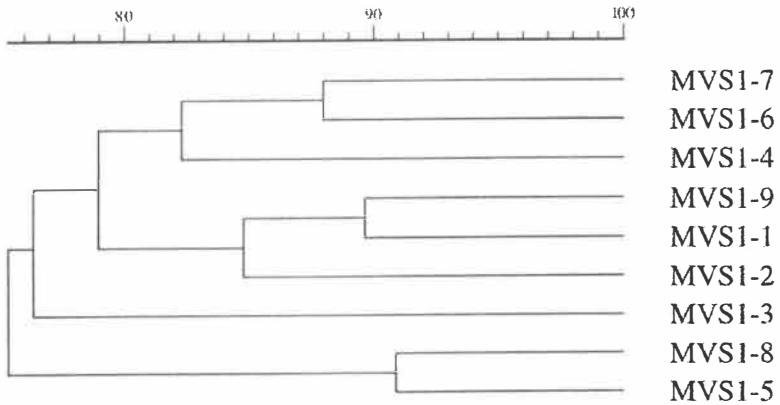


Figure 3.13 Dendogram showing similarity between nine replica DNA extractions of MVS1 centre environmental sample.

Comparison of tracks representing replica MVS1 centre extractions analysed using DGGE, indicated diversity between the nine extractions of up to 28%

(Figure 3.13). Tracks representing extraction 8 and extraction 5 showed the greatest similarity at 92%. From the results it was concluded that DGGE gel banding patterns that may appear visually identical for band stabbing purposes, on analysis using GelCompar, may show up to 28% diversity.

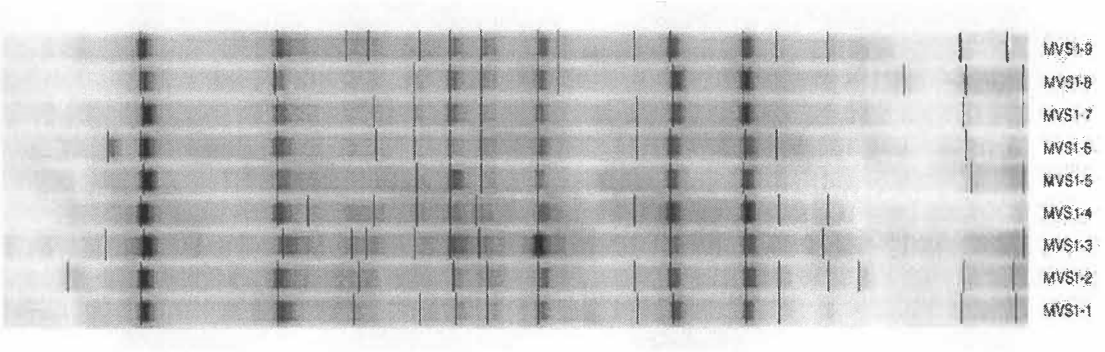


Figure 3.14 GelCompar 2D gel image showing band detection of DGGE comparing MVS1 centre sample replica extractions.

The GelCompar image shown in Figure 3.14, indicated the bands detected for each extraction. Differences in the number and position of bands detected in some lanes and not others can easily be seen.

These results indicated that the intensity of the stained band present was a significant factor in band detection and subsequent band comparison analysis when using GelCompar. Based on these results a standard error of up to 28% may be expected for all sample comparisons using this method of analysis. Therefore, values of similarity between tracks analysed by GelCompar were used as an indication of calculated similarity between environmental sample populations, but the values themselves were not considered quantitatively accurate.

3.5 Bacterial diversity in samples

Three DGGE gels comparing bacterial specific 16S rDNA amplicons from MVS1 surface and subsurface environmental samples were compared (Figures 3.15, 3.16, 3.17). Environmental samples taken 5cm and 10cm below the soil surface are indicated on Figures 3.15, 3.16 and 3.17 by pink rectangles and yellow rectangles respectively.

3.5.1 MVS1

MVS1 environmental samples were collected according to the profile shown in Chapter 2, Figure 2.2. MVS1 was located at an altitude of 483 m, between the Miers Valley floor, and a high saddle at the top of Marshall Valley.

The method of sampling used for MVS1 environmental samples consisted of sampling along a perpendicular axis with transecting points occurring at the approximate centre of the seal carcass. Each line of transect originating from the seal outward was designated a letter A-D. The transect line in the theorised down-slope direction was designated transect line A. Slope of the transect sampling site was not calculated. The direction of transect line A, therefore, represented an estimate of slope only.

Visual analysis of the three DGGE gels of MVS1 sampling transect shown in Figures 3.15, 3.16 and 3.17, indicated the presence of bands (labelled as B1 and B2 in Figure 3.16, and C1 in Figure 3.17) present only in environmental samples collected from the soil surface. A band that was apparent only in environmental samples taken at 5 cm and 10 cm below the soil surface, labelled as A2 in Figure 3.15, B4 in Figure 3.17, and C2 in Figure 3.17, was also indicated.

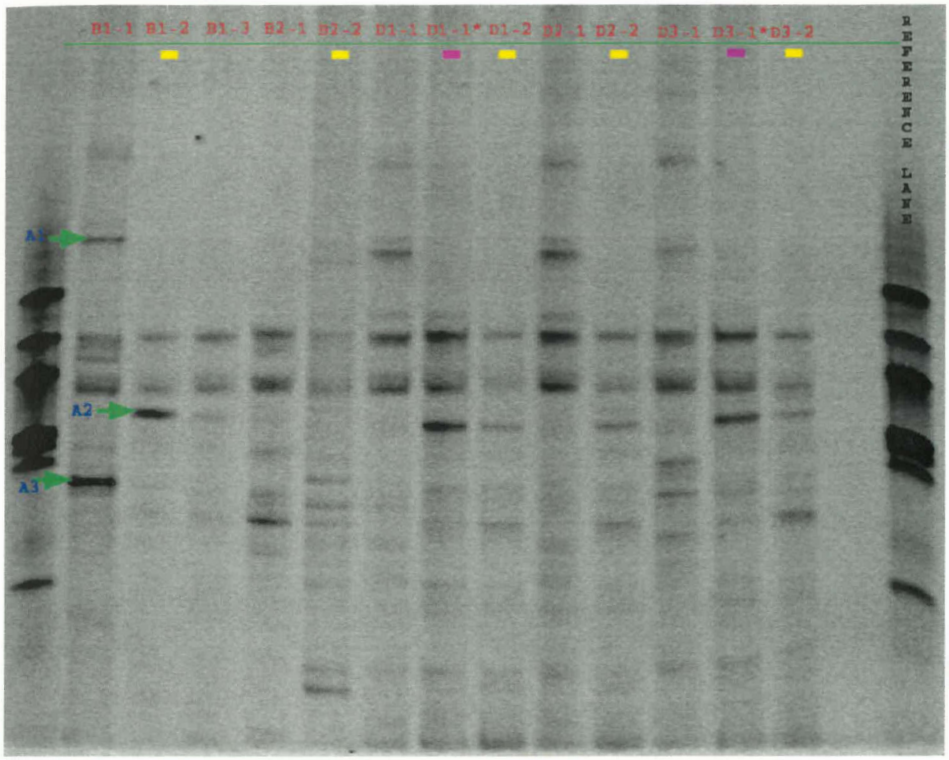


Figure 3.15 DGGE gel of MVS1 surface and subsurface environmental samples taken from B and D transect lines.

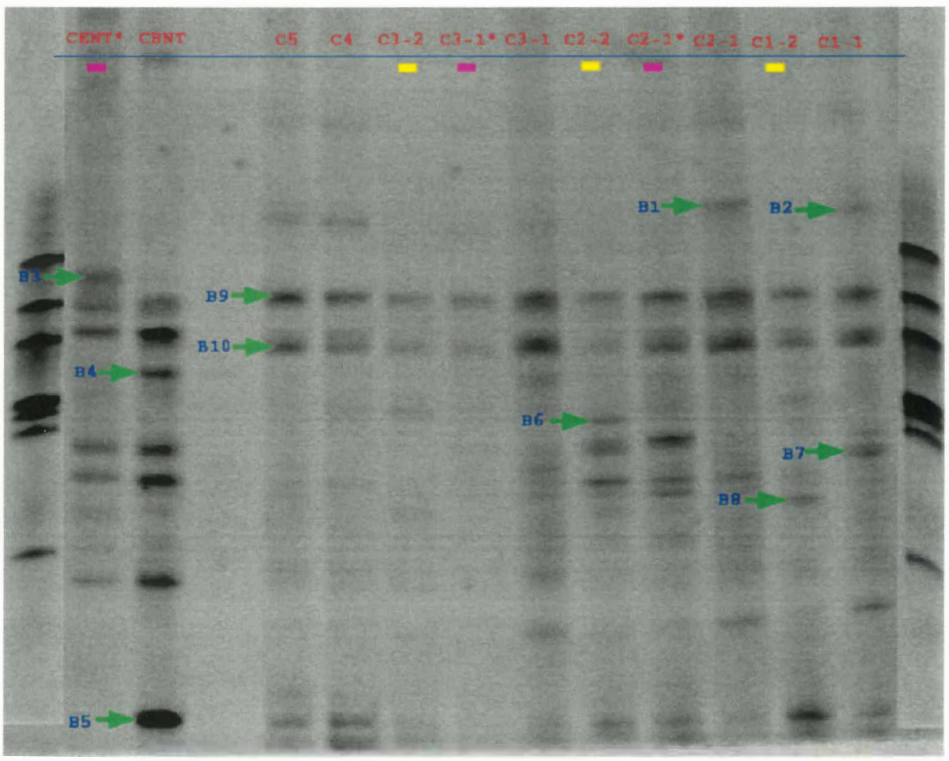


Figure 3.16 DGGE gel of MVS1 surface and subsurface environmental samples taken from the C transect line, and directly beneath the seal carcass.

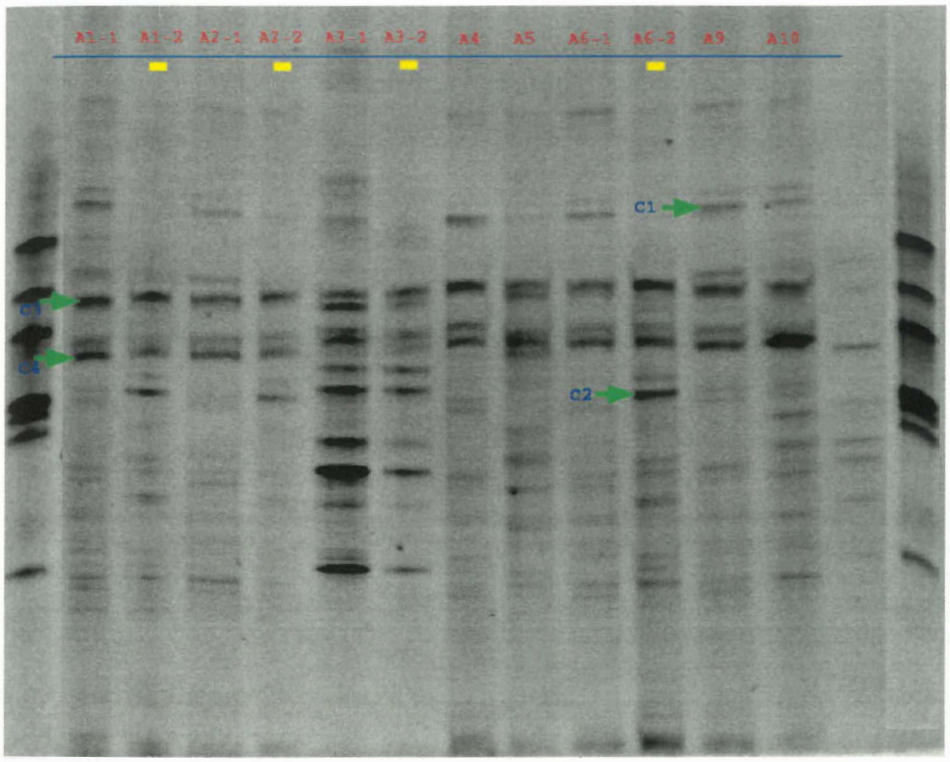


Figure 3.17 DGGE gel of MVS1 surface and subsurface environmental samples taken from the A transect line.

Bands of interest (highlighted by green arrows on Figures 3.15, 3.16, 3.17) were stabbed to isolate the DNA band, and PCR amplified.

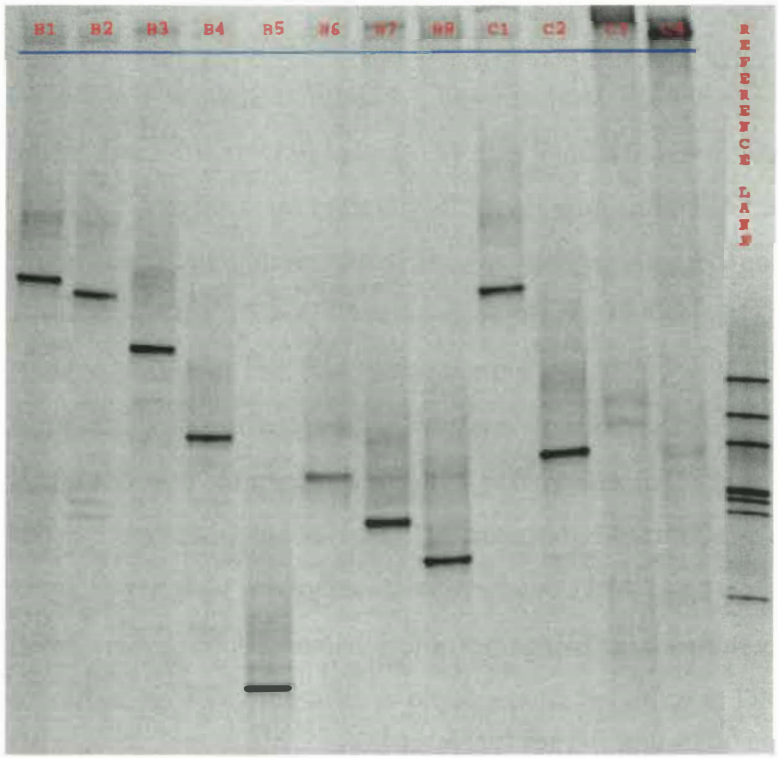


Figure 3.18 DGGE gel of MVS1 bacterial bands of interest after subsequent PCR.

Positive amplicons were analysed using DGGE to determine if they were single isolates (Figure 3.18). Band stabs A1, A2, A3, B9, and B10 did not PCR amplify, and subsequently do not appear on Figure 3.18. Amplicons of isolated bands determined by DGGE analysis to be single isolates were sequenced. The results of BLASTn analysis of DNA isolate sequences are presented in Table 3.3.

Table 3.3 Results of BLAST search on nucleotide sequences of isolated bands.

Isolate Designation	Sequence Alignment			Nearest phylogenetic neighbor (GenBank accession number)	Class
	n^a	% identity[b]	Bit Score		
B1	151	92	123	Uncultured bacterium clone D121 (AY274130)	Unknown
B3	150	89	99.6	Uncultured earthworm intestine bacterium (AY154548)	Unknown
B4	163	100	50.1	Uncultured actinomycete GT33 (AF256021)	*Actinobacteria*
B5	140	96	204	*Brevibacterium linens* WS3030 (AY017083)	*Actinobacteria*
B7	136	96	196	Uncultured bacterium clone G9 (AY268276)	Unknown
B8	149	95	176	uncultured green non-sulfur bacterium (UGR582210)	*Chloroflexi*
C1	140	95	155	*Brevibacterium linens* WS3030 (AY017083)	*Actinobacteria*
C2	161	98	240	Uncultured gamma proteobacterium (AY129793)	*γ-proteobacteria*

Note: n^a in Table 3.3 is the length of 16S rDNA in nucleotides used for alignment and phylogenetic analysis. Percent identity[b] represents the percent identity of the nearest known phylogenetic neighbours, determined by BLASTn analysis. The bit score represents the normalised sum of scores for each letter-to-letter and letter-null position in an alignment. The higher the score indicted, the better the alignment.

The quality of sequences gained from the isolated bands were generally fair or poor. Only sequences for DNA band isolates B5 and C2 were regarded as good when rated for overall quality by the Waikato DNA Sequencing Facility. The sequence lengths were typically short, ranging from 136 bp to 163 bp. The nearest phylogenetic neighbour identified for both isolates B5 and C1 was identified as *Brevibacterium linens.* The band representing isolate C1 was found only in surface samples. The band representing isolate B5 was observed in a number of samples, both surface and subsurface. It appeared to be very prominent in the surface centre sample, but was not detected in the centre environmental sample taken 5 cm below the surface. The uncultured bacterium clone D121, identified as being the nearest phylogenetic neighbor to DNA band isolate B5 was gained from heavy metal contaminated mine tailings. The uncultured bacterium clone G9, identified as being the nearest phylogenetic neighbor to DNA band isolate B7 was gained from shower curtain biofilms. The uncultured gamma proteobacterium,

identified by BLASTn analysis as being the nearest phylogenetic neighbor to DNA band isolate C2, was gained from samples taken in a nutrient limited cave environment.

A dendogram produced using GelCompar software, indicating similarity between tracks representing MVS1 environmental samples is shown in Figure 3.19. Analysis of tracks representing the three bacterial DGGE gels of PCR product amplified from MVS1 transect environmental samples (Figure 3.19), indicated a diversity in banding patterns of up to 74%. Environmental samples taken 5 cm below the soil surface are indicated by pink boxes in the dendogram (Figure 3.19). Environmental samples taken 10 cm below the soil surface are indicated by yellow boxes in the dendogram (Figure 3.19).

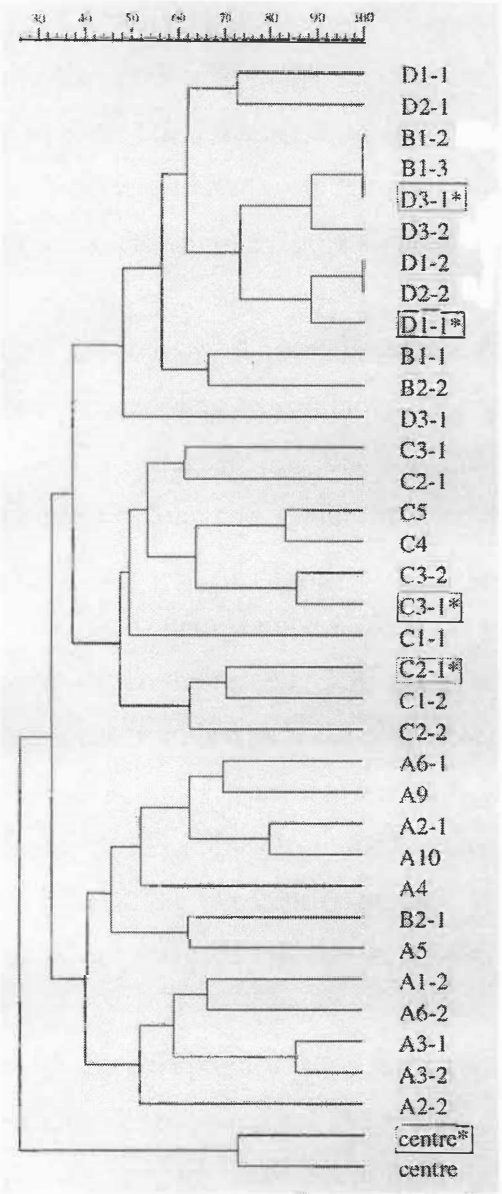


Figure 3.19 Dendogram showing similarity based on band comparison between MVS1 surface and subsurface environmental samples analysed using DGGE.

Tracks representing surface and subsurface centre samples, matched most closely to one another, and were distinct from the remainder of the tracks analysed; sharing only 28% similarity. These environmental samples were collected from directly beneath the seal carcass, and therefore had the closest proximity to the carcass.

Track D3-1* representing an environmental sample taken 5cm below the soil surface and 50cm from the approximate centre of the carcass, showed an identical banding pattern to tracks B1-2 and B1-3, both taken at 200cm from the approximate centre of the carcass along the B transect line.

Track D1-2 representing MVS1 environmental sample taken 10cm below the soil surface, 200cm from the approximate centre of the carcass along transect line D, and track D2-2 representing MVS1 environmental sample taken 10cm below the soil surface, 100cm from the approximate centre of the carcass along transect line D, were shown to be 100% similar.

The dendogram, (shown in Figure 3.19), appeared to separate tracks into four groups according to similarity. One group contained tracks representing samples from the A transect line; one contained tracks representing samples taken from the C transect line; one group represented tracks taken from both B and D transect lines; and one consisted only of the two tracks representing the surface and subsurface centre samples. With the exception of the centre samples, which were analysed on the same DGGE gel as all C transect samples, this grouping reflected the manner with which samples were analysed on each acrylamide denaturing gel (i.e. all samples from the A transect line were analysed on a single DGGE gel together, all samples from the C transect line were analysed on a single DGGE gel along with the two centre samples, and finally all samples from B and D transect line were analysed together on a DGGE gel).

With the exception of the tracks centre and centre* representing subsurface and surface centre samples, and tracks A3-2 and A3-1 representing subsurface and surface samples taken 50cm from the approximate centre of the seal carcass, no other tracks representing surface and subsurface samples taken at the same horizontal location on a transect line matched the most closely to one another. Tracks C3-1* and C3-2 representing MVS1 environmental samples taken 5cm and 10cm below the surface respectively, 50cm from the approximate centre of the seal carcass; were the most similar to one another (84%). Tracks D1-1 and D2-1 representing environmental samples taken from the soil surface 200cm and 100cm, respectively, from the approximate centre of the carcass along transect line D showed the closest similarity to one another (73%).

The results indicated that there was no apparent gradient of similarity between tracks representing samples along a transect line. Tracks representing MVS1 environmental samples taken from the transect line A (the estimated direction of

down-slope), did not show any greater similarity to tracks representing samples taken from directly beneath the seal carcass.

3.5.2 MVS3

The bacterial diversity of environmental samples collected from MVS3 sampling transect was investigated using DGGE analysis (Figure 3.20). MVS3 was located at an altitude of 663m, positioned on a platform approximately 61m above the east side of snowy lake. Environmental sampling surrounding the carcass of MVS3 consisted of samples taken from the soil surface only, with environmental samples taken along a perpendicular axis with transecting points occurring at the approximate centre of the seal carcass shown in Chapter 2, Figure 2.6. Transect line A indicated the estimated direction down-slope.

Visual analysis of ethidium bromide stained bands from MVS3 environmental samples viewed by DGGE (Figure 3.20), indicated that the centre sample taken from directly beneath the carcass of MVS3 did not have the highest number of bands present when compared with other environmental samples on the gel. Based on the assumption that individual bands in a DGGE gel represented a particular bacterial species, MVS3 centre sample, therefore did not contain as much bacterial diversity compared to other environmental samples in the sampling transect. All of the DNA bands viewed in MVS3 centre sample were present and had migrated at the same location along the denaturing gradient in at least one of the other environmental samples analysed from MVS3 sampling transect.

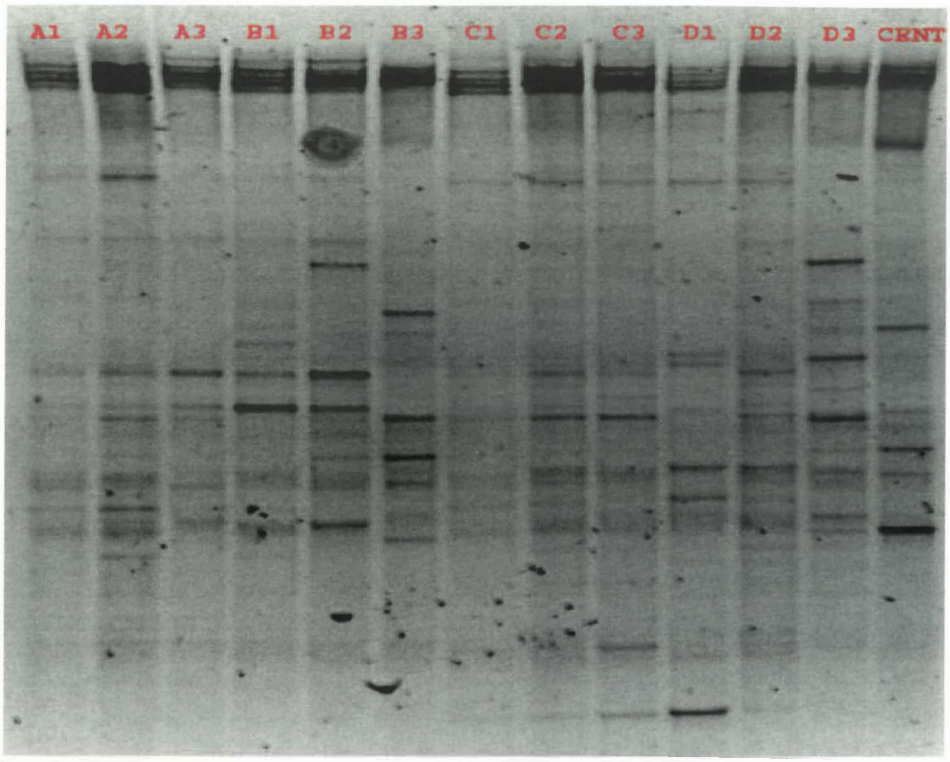


Figure 3.20 DGGE gel of MVS3 transect environmental samples.

Analysis of the ethidium bromide stained DGGE gel loaded with PCR product that had not been concentrated, showed diversity between the twelve tracks representing MVS3 environmental samples, of up to 78%.

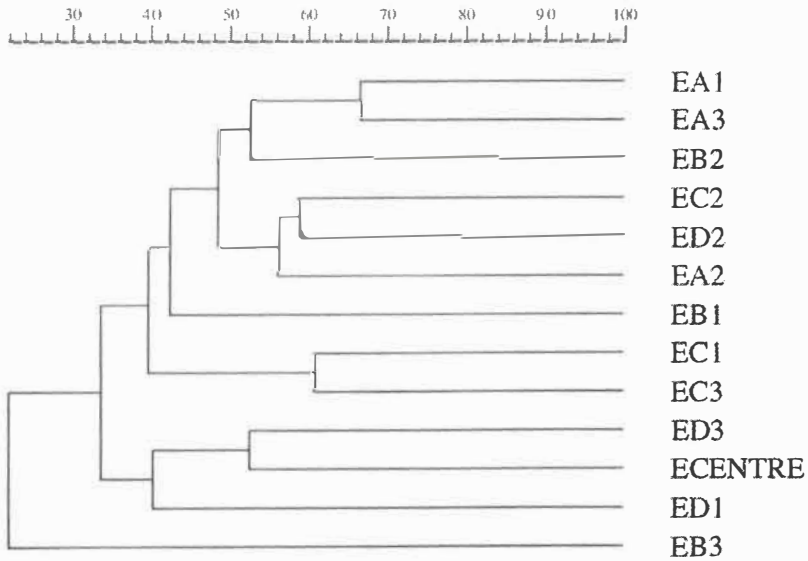


Figure 3.21 Dendogram showing similarity based on band comparison between MVS3 environmental samples analysed by DGGE.

Track B3 representing MVS3 environmental sample taken 50cm from the approximate centre of the seal carcass, appeared to be distinct from the remainder tracks analysed, sharing only 22% similarity with any other tracks representing MVS3 environmental samples. The track representing MVS3 centre sample showed the greatest similarity to tracks D3 representing MVS3 environmental sample taken 50cm from the approximate centre of the carcass along transect line D and D1 representing MVS3 environmental sample taken 200cm from the approximate centre of the seal carcass. Overall, it appeared that tracks representing 16S rDNA amplicons from MVS3 environmental samples showed no apparent pattern in terms of horizontal distance or direction from the seal carcass other than the clustering of tracks C2, D2, and A2, which all represent environmental samples taken 100cm from the approximate centre of the seal carcass on transect lines C, D, and A respectively. No tracks of MVS13 transect samples appeared to be more than 74% similar.

3.5.3 MVS13

The bacterial diversity of environmental samples collected from MVS13 sampling transect was investigated using DGGE (Figure 3.22). Seal 13 was located at an altitude of 670m. The sampling method used was the same as that used for MVS3 sampling transect, with environmental samples only collected from the soil surface, and the estimated down-slope direction labelled as transect line A.

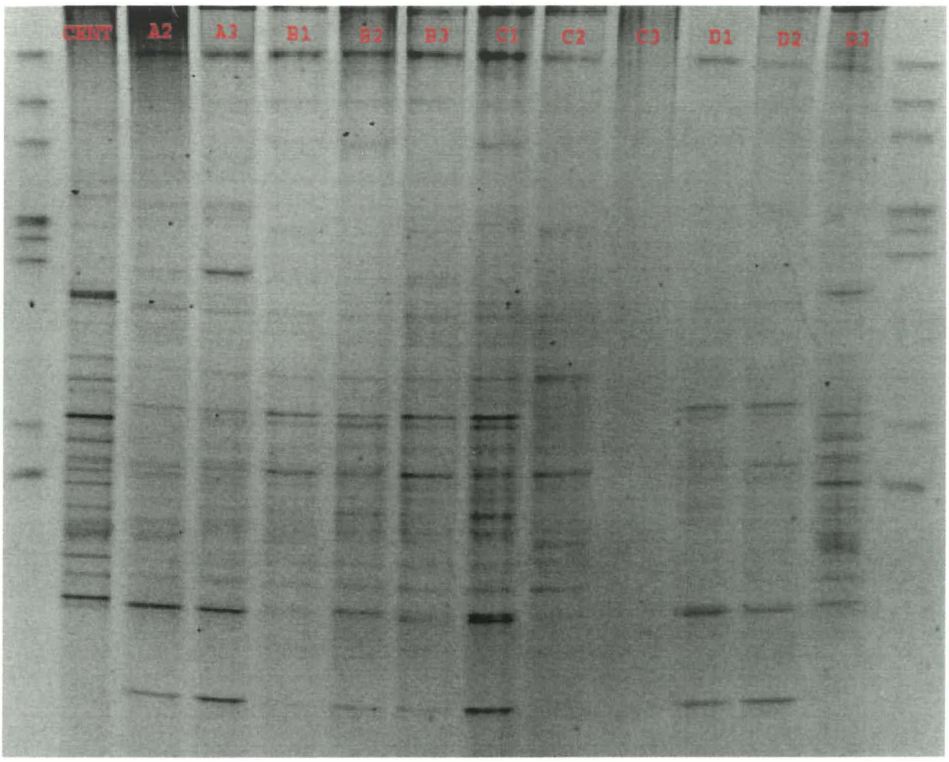


Figure 3.22 DGGE gel of MVS13 transect environmental samples.

Visual analysis of MVS13 ethidium bromide stained DGGE gel, indicated that the cent sample, representing the environmental sample taken from directly beneath the carcass of seal 3, appeared to show the greatest similarity to environmental sample D3, collected 50 cm along the D transect line from the approximate centre of the seal carcass. Isolation of DNA bands of interest from the DGGE gel were unsuccessful, therefore, the species represented by the visualised bands could not be discerned from DNA sequence analysis. Similarity between MVS3 environmental samples analysed by DGGE in Figure 3.22 was determined using GelCompar software (Figure 3.23).

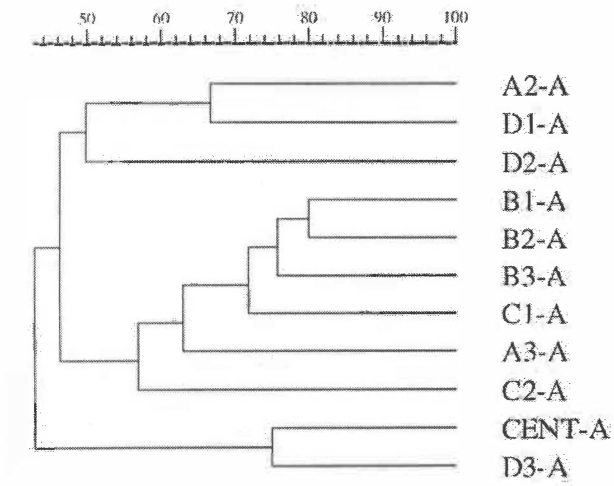


Figure 3.23 Dendogram showing band comparison analysis of environmental samples from MVS13 sampling transect analysed by DGGE.

Dendogram analysis of tracks representing MVS13 environmental samples indicated a diversity between of up to 59%. The track representing MVS13 centre sample was most similar (74%) to track D3 representing MVS3 environmental sample taken 50cm from the approximate centre of the seal carcass. These two tracks appeared distinct from the rest of the tracks in the gel, with only 44% similarity between banding patterns. Tracks representing MVS3 environmental samples taken on the B direction transect line showed the most similarity with one another. Diversity between all tracks was no greater than 44%.

Environmental samples taken from Miers Valley control transect (MVT) were taken at a variety of altitudes increasing in altitude at varying intervals from MVT1-MVT12 (with the exception of MVT1 and MVT2) as listed in Chapter 2, Table 2.1. The distance between MVT environmental samples was not indicated in the sampling notes. MVT environmental samples were taken as controls for microbial diversity present in the soil at varying altitudes and hence were taken from areas that did not show any signs of organic contamination, such as seal remains or skua guano. No MVT environmental samples were reported as being near to any obvious lichen growth.

A DGGE gel of concentrated bacterial 16S rDNA amplicons from environmental samples taken from MVT control sampling transect is shown in Figure 3.24. A DGGE gel of bacterial 16S rDNA amplicons that were not concentrated when

viewed showed the presence of a number of extremely faint DNA bands that appeared too faint for analysis purposes.

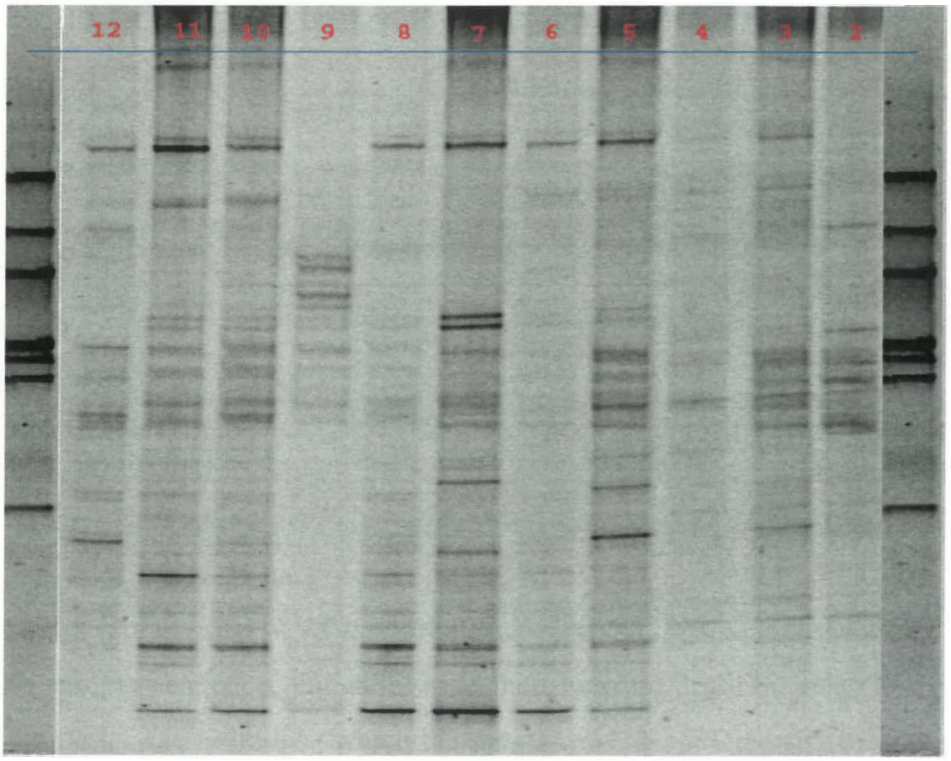


Figure 3.24 DGGE gel of Miers Valley control transect (MVT) environmental samples.

Visual analysis of MVT DGGE gel indicated a large diversity of bacterial species present in the environmental samples. MVT9 environmental sample appeared to have a bacterial population distinct from other populations when compared to the other MVT environmental samples, based on the fewer number of DNA bands viewed and their position along the denaturing gradient. Similarity between samples was calculated using GelCompar software as depicted in Figure 3.25.

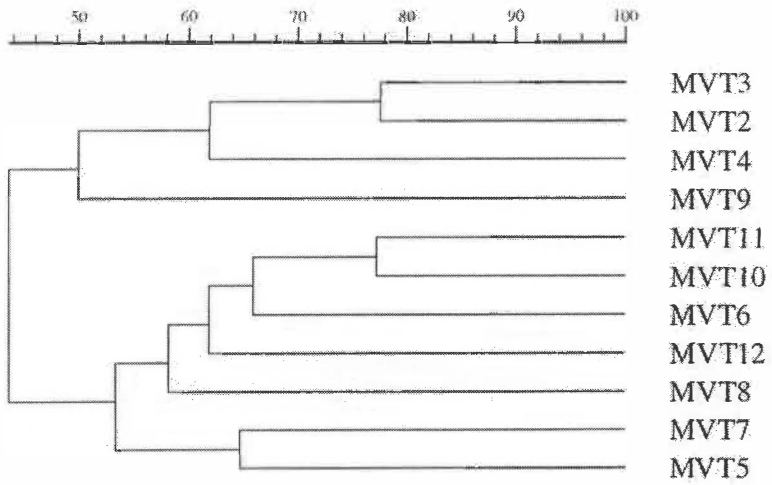


Figure 3.25 Dendogram showing band comparison similarity between control transect environmental samples.

Tracks representing MVT environmental samples MVT2 and MVT3 showed the most similarity to each other, as did MVT10 and MVT11; however, no other tracks representing MVT environmental samples showed any similarity according to sample sequence, indicating that there was no apparent relationship between similarity and altitude at which the sample was taken. Diversity between all tracks representing the transect samples was no greater than 44%. The highest value of similarity between two tracks was 78% for tracks representing environmental samples MVT3 and MVT2, and for tracks representing environmental samples MVT10 and MVT11.

Of the 13 seals studied in the Miers Valley area, eight of these seals (MVS1, MVS3, MVS4, MVS7, MVS9, MVS10, MVS11, and MVS13) had environmental samples collected from soil directly beneath the seal specimens. As these environmental samples were taken from soil in the closest proximity possible to the seals, it was hypothesised that they would display the greatest effect of seal enrichment on microbial diversity and abundance.

DNA was extracted from all of these centre environmental samples, and the sample bacterial population was visualised using DGGE of PCR amplified, bacterial 16S rDNA for all samples with the exception of MVS13 (due to its unavailability at the time) (Figure 3.26).

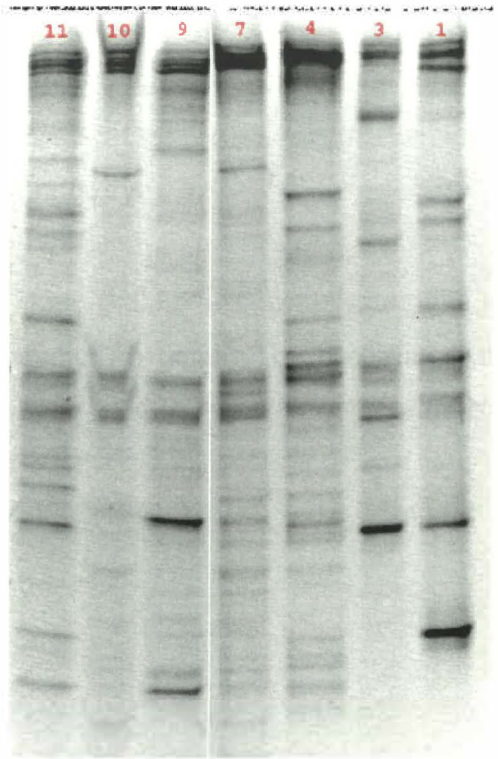


Figure 3.26 DGGE gel of Miers Valley seal centre environmental samples.

Visual analysis of the DGGE gel comparing the eight centre samples, indicated widely varying bacterial diversity present in the eight environmental samples. Based on the number of bands present, MVS4, MVS7, and MVS11 centre samples all appeared to have a greater bacterial diversity than MVS1 centre and MVS3 surface centre samples. Similarity between the eight centre samples was calculated using GelCompar software (Figure 3.27).

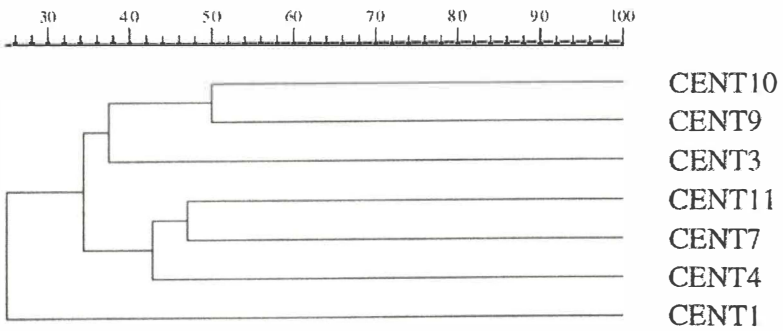


Figure 3.27 Dendogram showing similarity by means of band comparison between environmental samples taken directly beneath mummified seals in Miers Valley, Antarctic Dry Valleys.

From the dendogram (shown in Figure 3.27) of Miers Valley centre samples, it appeared that the track representing the sample taken from directly beneath the

carcass of Miers Valley Seal one (MVS1) shared the least similarity in terms of band comparison (26%) to all of the other centre samples viewed. No two tracks representing MVS centre environmental samples shared more than 50% similarity.

Three seal environmental sampling transects (MVS1, MVS3, and MVS13), were studied in depth. The bacterial diversity of these three samples was compared using DGGE gels of PCR amplified bacterial 16S rDNA (Figure 3.28).

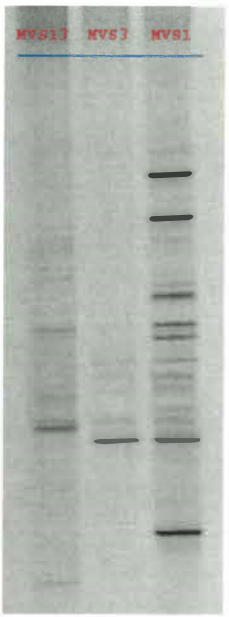


Figure 3.28 DGGE gel of centre envrionmental samples from MVS13, MVS3, and MVS1 seal sample transects.

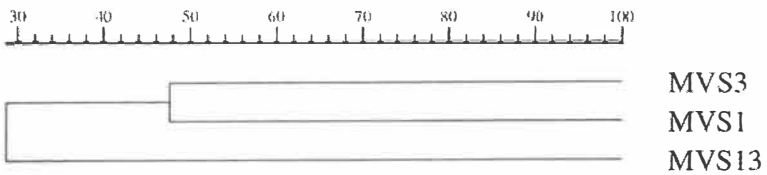


Figure 3.29 Dendogram showing band comparisons as an indication of population similarity between MVS13, MVS3, and MVS1 centre environmental samples.

Visual analysis of the DGGE gel shown in Figure 3.28 indicated that bacterial diversity was much greater in MVS1 centre than either the MVS3 or the MVS13 centre samples. Similarity of the three environmental centre samples was calculated using GelCompar software (Figure 3.29). The dendogram shown in Figure 3.29 indicated that tracks representing MVS3 centre and MVS1 centre

environmental samples shared the greatest similarity (48%). The track representing MVS13 centre sample was the least similar of the three, indicating only 29% the other two tracks.

The similarity in terms of banding patterns between the three MVS1 DGGE gels of surface and subsurface environmental samples, and with the DGGE of all control transect samples was accomplished. The four DGGE gels were aligned using a common reference lane, and compared using GelCompar software (Figure 3.30).

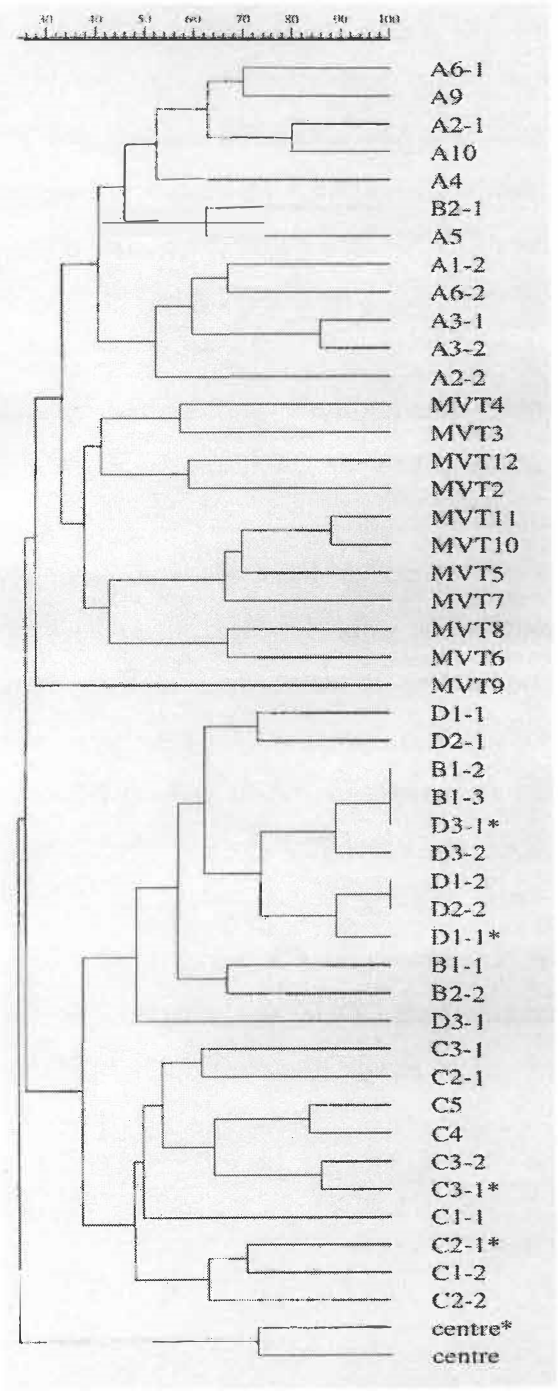


Figure 3.30 Dendogram showing similarity between four DGGE analysing surface and subsurface environmental samples from MVS1 sampling transect and MVT control transect.

All tracks representing environmental samples appeared to share at least 27% similarity among one another. Tracks representing surface and subsurface MVS1 centre samples appeared distinct from all other tracks, sharing only 27% similarity. Tracks representing control transect environmental samples appeared to group together on the dendogram, so did tracks representing samples taken along the A transect line of MVS1 sampling transect, with the exception of the track

representing sample B2-1, which was taken along the B transect line of MVS1 sampling transect. According to the dendogram shown in Figure 3.30, tracks representing environmental samples taken along the A transect line shared more similarity with tracks representing control transect environmental samples (34%) than tracks representing other MVS1 environmental samples taken from transect lines B, C and D (28%).

Tracks representing environmental samples taken from the control transect (shown in Figure 3.30) showed a different grouping of similarity compared to previous analyses of the samples (Figure 3.25). This may reflect the use of a different database used to create the comparisons. Each entry into another database resulted in re-loading and aligning all gel images. The same gel images were used in each database, and alignment was based on the same standard reference lanes. All standard settings for band detection and band comparison were used. The differences between the groupings of similarity for the two dendograms (Figures 3.30 and 3.25) can not be explained.

Similarity in terms of band comparison was calculated for DGGE gels of control transect samples and MVS3 environmental transect samples using GelCompar software, and is shown in Figure 3.31.

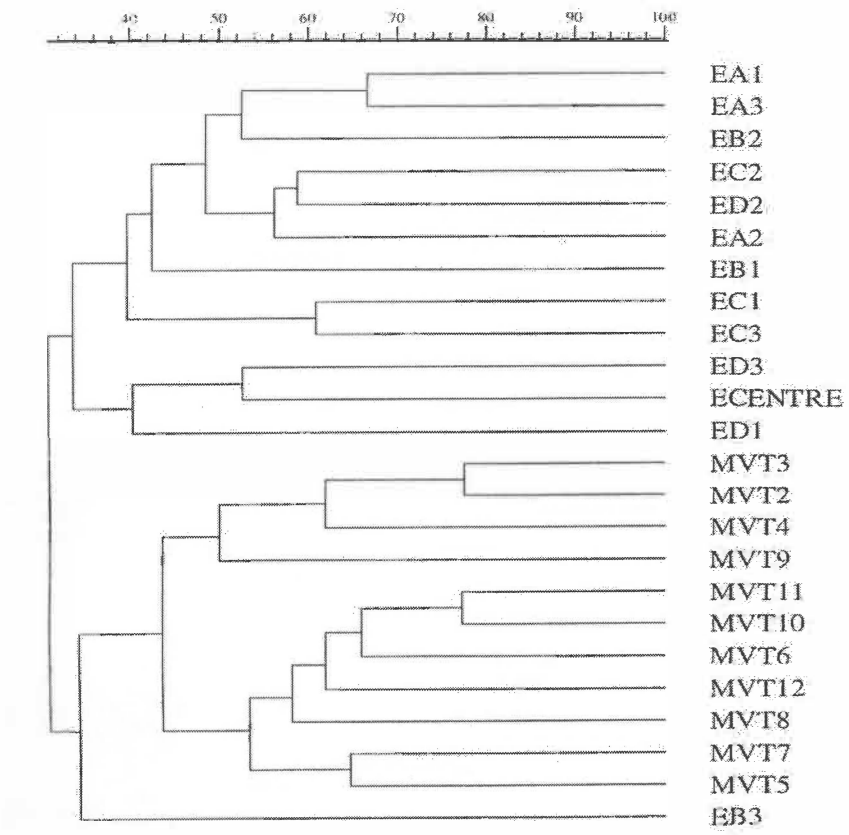


Figure 3.31 Dendogram showing similarity between MVT environmental samples and MVS3 envrionmental samples analysed by DGGE using GelCompar software.

All tracks representing environmental samples in the dendogram (shown in Figure 3.31), appeared to group into two distinct groups based on sampling transect, all tracks representing MVT environmental samples were grouped together, and all tracks representing MVS3 environmental samples were grouped together, with the exception of the track EB3 representing MVS3 environmental sample collected 50cm from the approximate centre of the seal carcass along the B transect line. The two groups shared 31% similarity. The lowest values of similarity within each group, was 34%.

The similarity between bands present in two bacterial DGGE gels of environmental samples from MVS13 sampling transect and MVT control transect, was calculated using GelCompar software (shown in Figure 3.32).

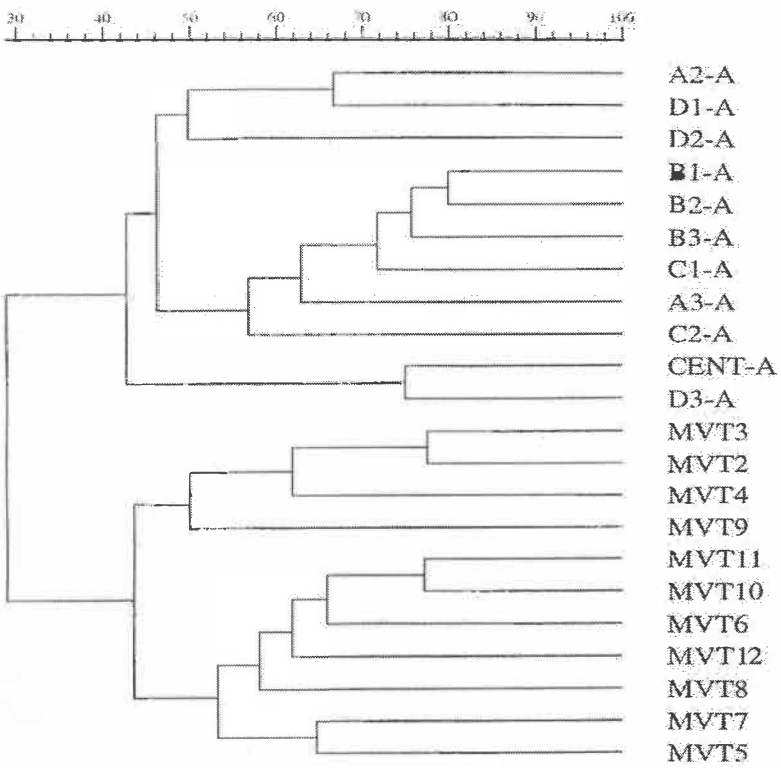


Figure 3.32 Dendogram of band comparison between of MVT environmental samples and MVS13 environmental samples analysed by DGGE.

All tracks representing environmental samples appeared to group only with tracks representing environmental samples collected from the same sampling transect. The similarity between the two groups of tracks representing environmental samples from MVT control sampling transect and MVS13 sampling transect was approximately 29%.

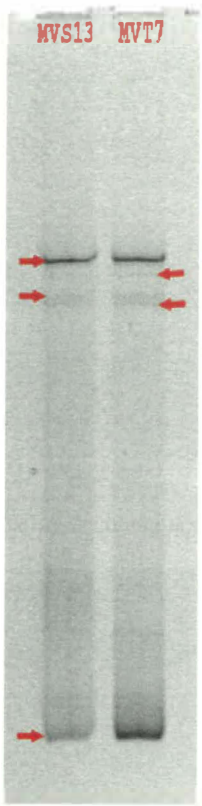
3.6 Archaeal diversity in samples

Archaeal diversity was examined using primers specific for Archaeal 16S rDNA in environmental samples (as described in Appendix 2) from MVS1, MVS3, MVS13 and MVT sampling transects. Archaeal diversity in centre environmental samples from MVS1, MVS3 and MVS13 sampling transects, and MVT4, MVT7 and MVT10 transect control samples were examined. A positive amplification was demonstrated of MVS13 centre environmental sample and MVT7 environmental sample was obtained, but no amplification occurred in the other four samples used (data not shown). This possibly indicated that Archaea were not present in the environmental samples, or that the concentration present was too minimal to amplify. It was considered that perhaps increasing the amount of DNA

template in the PCR reaction would increase the number of samples with a positive amplification. Alternately, a PCR inhibitor may have been present in those samples that did not amplify. However, given the consistent PCR amplification of bacterial 16S rDNA this is unlikely.

Another PCR reaction was performed using only centre samples MVS1, MVS3, MVS13, following the same protocol but increasing the amount of sample DNA template to 15ng (triple the previous amount used). Once again the same result was observed with positive amplification for sample MVS13 only (data not shown).

After submitting the positively amplified MVS13 centre sample, and MVT7 sample to DGGE, and staining with ethidium bromide to view the banding patterns (Figure 3.33), both samples appeared to be identical. This indicated that species diversity in the two samples was identical. Band stabs of the five bands present in each sample were done in an attempt to gain sequence data to identify the organisms present in the sample. A DGGE gel of the PCR amplified stabs should show only a single band for each PCR product representing the band stabbed. Of the five bands stabbed, one did not amplify with PCR at all (stab A). Stab B and stab C both had the same pattern of four out of five of the original bands in the population samples. Stab D and stab E had a replica pattern of the MVS13 and MVT7 population sample DGGE gel. As no single species could be isolated from the sample population using this method, no sequence data was gained to identify potential archaeal organisms DNA present.



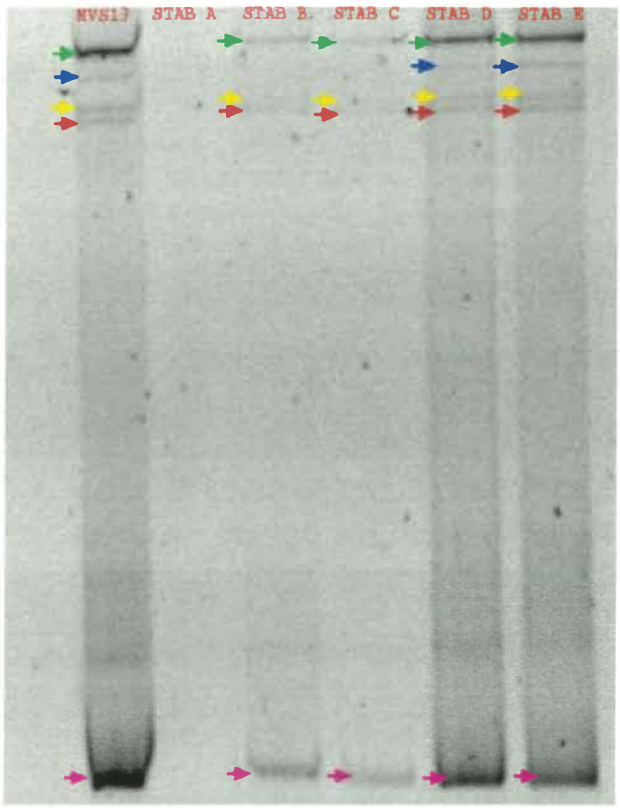


Figure 3.33 (left) Archaeal DGGE gel of MVS13 centre environmental sample and MVT7 transect control environmenal sample.

Figure 3.34 (right) Archaeal DGGE gel of MVS13 band stabs. Isolated species are indicated by single bands. None of the band stabs appeared to isolate any single species.

A profile of Archaea present in all Miers Valley Seal 13 transect samples, and all transect control samples was attempted using PCR amplified products of 16S rDNA. In the MVS13 sample set two samples, A2 and D2 showed positive amplification with each containing a band migrating approximately 1500bp in size. MVS13 centre sample did not show positive amplification using primers 77 1522R and 149 as it did with primers Arc1F and Arc2R. The faint appearance of a high molecular weight contaminant was detected in environmental samples A2, B2 and C3. A high molecular weight contaminant was also present in transect control samples MVT7 and MVT11. Very faint bands of approximately 1500 bp were detected in MVT5, MVT11, and MVT12 samples. As with MVS13 centre sample, no positive amplification of a band the correct size was observed in MVT7 using primers 77 and 149, whereas a positive amplification was seen using primers 150 and 151 which select for a smaller region (approximately 1000bp) of the 16S gene.

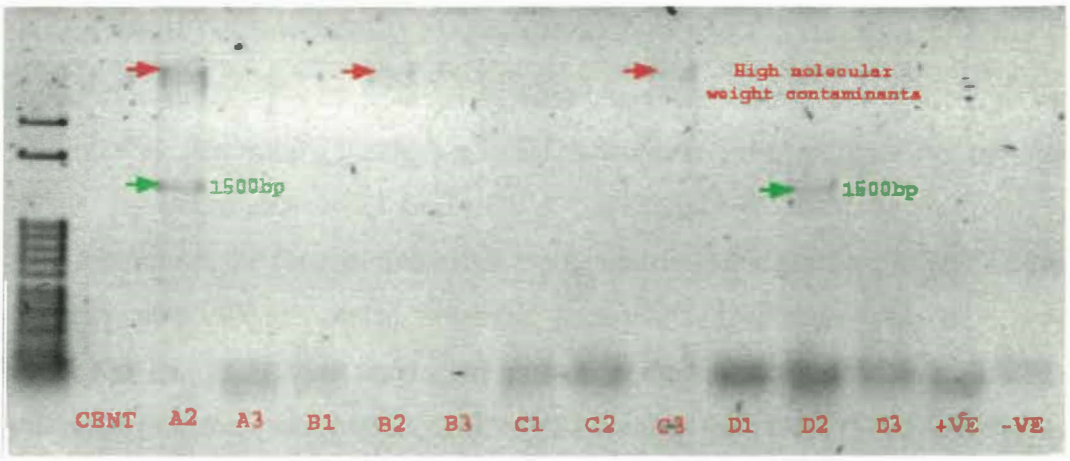


Figure 3.35 1% agarose gel electrophoresis of 16S rDNA from MVS13 transect, PCR amplified using archaeal specific primers.

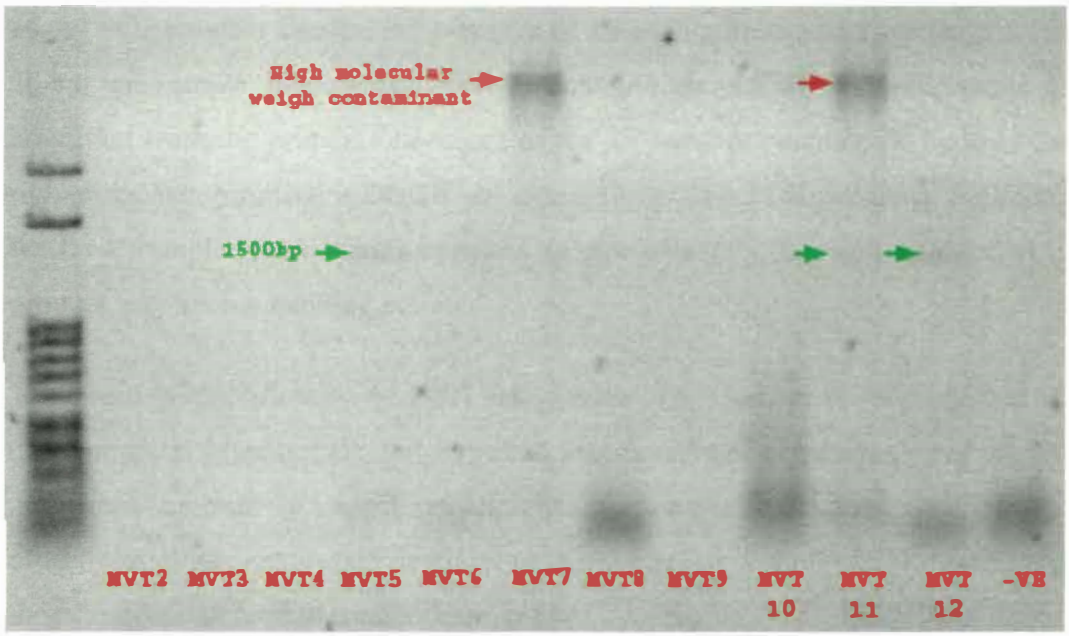


Figure 3.36 1% agarose gel electrophoresis of 16S rDNA from MVT control transect, PCR amplified using archaeal specific primers.

The period of time in which all DNA extractions were conducted, thermophillic Archaeal species were being worked with in the College of Marine Studies, University of Delaware. Given that not all environmental samples PCR amplified using Archaeal primers, the possibility that the samples had been contaminated with external Archaeal species, can not be ruled out.

3.7 Eukaryotic diversity in samples

Eukaryotic diversity was assessed in environmental samples taken from Miers Valley was determined using universal eukaryotic primers, specific for 18S rDNA. By electrophoresis of amplicons it was established that there was positive amplification in the form of two bands from environmental samples MVS1 centre, MVS3 centre, MVS13 centre, however, there was only a barely detectible band from MVT6. PCR was conducted on environmental samples from MVS13 sampling transect and MVS1 and MVS3 centre samples (15 environmental samples in total), using universal eukaryotic primers containing a GC clamp that selected for a shorter region of 18S rDNA (approximately 500bp). Electrophoresis of 4µl PCR product showed the presence of a very high molecular weight band in all but one sample; this was considered a contaminant as it was not expected to be amplified from the primer. Only three of the 15 samples contained a band of the right size. When run on a DGGE gel, these 18S rDNA PCR products, including the three samples containing amplicons at approximately the right size, failed to produce any visible banding patterns.

The positive amplification of PCR using eukaryotic specific primers viewed by electophoresis (data not shown) indicated that eukaryotic organisms were present in at least some of the sample populations. Further work will need to be done in order to establish eukaryotic diversity within the Miers Valley seal transects, and the Miers Valley control transect samples.

3.8 Restriction endonuclease analysis of MVT10 clone library

Plasmids containing the expected and correct sized DNA insert of approximately 1500bp, were identified by restriction analysis of MVT 10 clones as described in Section 2.13.6. Plasmids with the inserted fragment of DNA produced two or more bands equal in total to approximately 1500bp (the approximate size of the 16S rDNA gene) in some samples, and a single 1500bp band present in other samples (data not shown).

To reduce redundancy of sequence analysis of the clones, samples found to contain the correct sized DNA inserts were subjected to further restriction analysis using two tetrameric restriction enzymes *Hin*P1I , and *Rsa*I (data not shown).

Restriction enzyme *Hin*P1I was found to cut the vector multiple times resulting in gels containing large numbers of bands. A high degree of diversity present in the clone library was demonstrated by restriction analysis. Given this diversity the decision was made to sequence all 96 clones of the MVT clone library.

3.9 16S rDNA sequence analysis

Sequence analysis was conducted on clone libraries produced from 16S rDNA PCR product, amplified from genomic DNA extracted from MVS1 centre environmental sample, and MVT10 control transect environmental sample. MVS1 centre environmental sample was taken from surface soil, directly beneath the carcass, Miers Valley seal one. Clone libraries were produced from 16S rDNA PCR product amplified from genomic DNA extracted from MVS3, and MVS13 centre samples also (and these will be sequenced post MSc thesis research).

The bacterial isolates from MVS1 clone library, indicated by BLASTn analysis of clone sequences are depicted in Table 3.4.

Table 3.4 Bacterial isolates from MVS1 clone library.

Isolate Designation	Sequence Alignment			Nearest phylogenetic neighbor (GenBank accession number)
	n[a]	% identity[b]	Bit Score	
A01.X	764	95	1106	*B.linens* (BL16SRN3)
A01.Y	760	97	1227	*Brevibacterium antarcticum* (BAN577724)
A02.X	632	97	1072	*Arthrobacter rhombi* (ARY15885)
A02.Y	617	97	1076	*Actinobacterium* EC5 (AY337600.2)
A03.X	534	97	880	Agricultural soil bacterium (ASO252656)
A03.Y	626	98	1112	Arctic sea ice bacterium (AF468359)
A04.X	521	97	902	*Paracoccus alcaliphilus* (AY014177)
A04.Y	786	94	1084	*Paracoccus alcaliphilus* (AY014177)
A05.X	702	97	1241	*Brevibacterium linens* (BLI315491)
A05.Y	665	96	1114	*Brevibacterium celer* (AY228463)
A06.X	629	98	874	*Psychrobacter luti* (PGE430828)
A06.Y	678	98	1199	*B.globisporus* (BG16SRRN)
A07.X	635	97	1100	Blackwater bioreactor bacterium (AF394173.1)
A07.Y	734	96	1124	*Planococcus sp.* 'SOS Orange' (AF242541)
A08.X	641	98	1148	Blackwater bioreactor bacterium (AF394173.1)
A08.Y	693	94	1059	*Planococcus maritimus* (AY428552.1)
A09.X	594	95	741	*Psychrobacter sp.* (AY382586.1)
A09.Y	788	97	1277	*Psychrobacter frigidicola* (AJ609556.1)
A10.Y	719	98	1199	*Brachybacterium sp.* (AF513397)
A11.Y	656	88	464	Uncultured soil bacterium (USO390469)
A12.Y	658	92	714	Uncultured Brevibacterium (AY080985.1)

Continuation of Table 3.4.

Isolate Designation	Sequenc Alignment			Nearest phylogenetic neighbor (GenBank accession number)
	n^a	% identity[b]	Bit Score	
B01.X	743	97	1134	*Psychrobacter luti* (PGE430828)
B01.Y	663	97	1110	*Psychrobacter luti* (PGE430828)
B02.X	707	92	955	*Brevibacterium celer* (AY228463)
B02.Y	623	98	1138	*Arthrobacter sp.* (ART495808)
B03.X	727	97	1154	*Planococcus sp.* (AF242541)
B03.Y	602	97	1086	Blackwater bioreactor bacterium (AF394173)
B04.X	642	97	1118	Blackwater bioreactor bacterium (AF394173)
BO4.Y	611	98	1068	*Planococcus sp.* (AF242541)
B05.X	658	99	1211	*Arthrobacter sp.* (ART495808)
BO5.Y	671	97	1176	*Arthrobacter sulfureus* (AB046358)
BO6.X	647	98	1183	*B.globisporus* (BG16SRRN)
BO6.Y	641	98	1199	*B.globisporus* (BG16SRRN)
B07.X	650	97	1092	*Brevibacterium linens* (BLI315491)
B07.Y	579	94	805	*Brevibacterium linens* (BLI315491)
B08.X	662	97	1088	Blackwater bioreactor bacterium (AF394173)
BO8.Y	639	98	1136	*Planococcus sp.* (AF242541)
BO9.X	444	89	385	*Flavobacterium xinjiangense* (AF433173)
B09.Y	692	97	1253	*Psychrobacter luti* (PGE430828)
B10.X	546	95	757	*Arthrobacter sp.* (ART495808)
B10.Y	810	96	1181	*Arthrobacter sulfureus* (AB046358)
B11.X	328	96	228	*Brevibacterium sp.* (AY275507)
B11.Y	717	97	1191	*Brevibacterium linens* (BLI315491)
B12.X	694	97	1035	*Arthrobacter sp.* (AF170748)
B12.Y	606	97	916	*Arthrobacter rhombi* (ARY15885)
CO1.X	539	98	957	*Planococcus southpolaris* (PSO314747)
C01.Y	349	96	500	*Planococcus psychrotoleratus* (AF324659)
C02.X	641	91	688	Uncultured Cytophagales (AY250875)
C02.Y	557	95	543	Uncultured Cytophagales bacterium (AY250875)
C03.X	646	94	783	*Arthrobacter sp.* (ART495808)
C03.Y	649	96	1043	*Arthrobacter sulfureus* (AB046358)
C04.X	644	97	1068	*Arthrobacter psychrolactophilus* (AF134183)
C04.Y	588	96	854	Naphthalene-utilizing bacterium (AF531478)
C05.X	616	98	1072	*B.globisporus* (BG16SRRN)
C05.Y	559	97	835	*B.globisporus* (BG16SRRN)
C06.X	875	97	1243	*B. psychrophilus* (BP16SRRB)
C06.Y	514	95	745	*B.globisporus* (BG16SRRN)
C07.X	751	96	1174	*Brevibacterium linens* (BLI315491)
C08.X	728	97	1168	*Psychrobacter luti* (PGE430828)
C09.X	279	93	365	*Planococcus southpolaris* (PSO314747)
C10.X	661	95	835	*Arthrobacter sp* (AF170748)
C11.X	544	98	934	*Psychrobacter luti* (PGE430828)
C12.X	808	96	1126	Blackwater bioreactor bacterium (AF394173)
D01.Y	684	98	1126	Bacterium UMB13F (AF505743)
D02.X	804	94	870	*Lysobacter antibioticus* (AB019582)
D02.Y	601	92	640	Uncultured Stenotrophomonas (AY080994)
D03.X	361	91	236	*Arthrobacter sp.* (AF170748)
D04.X	415	90	428	Uncultured Cytophagales (AY250875)
D05.X	730	95	914	*Brevibacterium linens* (BLI315491)
D07.X	423	91	410	*Planococcus southpolaris* (PSO314747)
D09.X	692	97	944	*Psychrobacter luti* (PGE430828)
D10.X	385	94	505	*Planococcus sp.* (AF242541)
D12.X	329	90	359	Uncultured Brevibacterium sp. (AY080985)

Continuation of Table 3.4.

Isolate Designation	Sequenc Alignment			Nearest phylogenetic neighbor (GenBank accession number)
	n^a	% identity[b]	Bit Score	
E01.X	248	91	252	Uncultured antarctic soil bacterium (AY167310)
E01.Y	452	94	515	*Psychrobacter luti* (PGE430828)
E02.X	447	94	466	*Psychrobacter jeotgali* (AF441202)
E02.Y	363	95	541	Marine psychrotrophic bacterium (MPS308373)
E03.X	687	97	1148	*Psychrobacter luti* (PGE430828)
E04.X	333	93	458	*B.globisporus* (BG16SRRN)
E04.Y	550	97	934	*B.globisporus* (BG16SRRN)
E06.X	644	97	1120	*Arthrobacter rhombi* (ARY15885)
EO6.Y	525	97	914	Blackwater bioreactor bacterium (AF394169)
E07.X	623	96	1011	Uncultured Cytophagales bacterium (AY250875)
E07.Y	523	92	613	Uncultured bacterium clone (AY100545)
E08.X	248	96	198	*Arthrobacter sulfureus* (AY158033)
E08.Y	491	93	599	Bacterium SG-3 (AF548381)
E09.X	137	96	210	*Arthrobacter sulfureus* (AY158033)
E10.X	646	96	1084	Blackwater bioreactor bacterium (AF394173)
E11.X	731	97	1142	*Psychrobacter luti* (PGE430828)
E12.Y	539	95	666	*Brevibacterium linens* (BLI315491)
F01.Y	780	97	1231	*Psychrobacter luti* (PGE430828)
F02.X	617	98	1118	*Planococcus sp.* (AF242541)
F02.Y	779	97	1185	Blackwater bioreactor bacterium (AF394173)
F03.X	737	98	1178	*B.globisporus* (BG16SRRN)
F03.Y	647	96	991	*B.globisporus* (BG16SRRN)
F04.X	377	91	391	Uncultured bacterium clone (AY218564)
F04.Y	774	98	1176	Uncultured bacterium clone KD1-64 (AY218564)
F06.X	629	98	1098	*Arthrobacter sp.* (AB039736)
F06.Y	641	97	1068	*Arthrobacter psychrolactophilus* (AF134183)
F07.X	580	95	827	*Psychrobacter luti* (PGE430828)
F07.Y	744	97	1005	*Psychrobacter luti* (PGE430828)
F08.X	639	98	1166	*Arthrobacter sp.* (ART495808)
F08.Y	712	97	1259	*Arthrobacter sulfureus* (AB046358)
F09.X	491	97	860	*Planococcus sp.* (AF242541)
F09.Y	736	97	1255	Blackwater bioreactor bacterium (AF394173)
F10.X	595	97	1045	*Brevibacterium linens* (BLI315491)
F10.Y	710	95	1007	*Brevibacterium linens* (BLI315491)
F11.X	664	96	1086	*Brevibacterium linens* (BLI315491)
F11.Y	683	94	975	*Brevibacterium linens* (BLI315491)
F12.X	481	93	579	*Brevibacterium linens* (BLI315491)
F12.Y	533	92	702	*Brevibacterium celer* (AY228463)
G01.X	394	93	478	*Psychrobacter pacificensis* (AB016056)
G01.Y	499	97	852	*Psychrobacter luti* (PGE430828)
G02.X	386	96	472	*Carnobacterium funditum* (S86170)
G02.Y	664	96	1029	*Carnobacterium funditum* (S86170)
G03.Y	731	97	1217	Jellyfish-degrading bacterium (AB101583)
G04.X	640	98	1170	*B.globisporus* (BG16SRRN)
G04.Y	674	97	1152	*B.globisporus* (BG16SRRN)
G05.X	675	95	829	Uncultured bacterium clone KD1-64 (AY218564)
G05.Y	732	97	1174	Uncultured bacterium clone KD1-64 (AY218564)
G06.X	786	96	1128	Blackwater bioreactor bacterium (AF394173)
G06.Y	658	97	1128	*Planococcus sp.* (AF242541)
G07.X	539	98	979	*Flavobacterium micromati* (FMI557888)
G07.Y	545	91	618	Uncultured eubacterium (AF270946)
G08.X	541	96	890	*Arthrobacter psychrolactophilus* (AF134179)
G08.Y	671	96	1082	Naphthalene-utilizing bacterium (AF531478)

Continuation of Table 3.4.

Isolate Designation	Sequenc Alignment			Nearest phylogenetic neighbor (GenBank accession number)
	n^a	% identity[b]	Bit Score	
G09.X	609	97	932	*B.linens* NCDO 739 (BL16SRN3)
G09.Y	718	97	1203	*Brevibacterium linens* (BLI315491)
G10.X	388	95	593	*Psychrobacter aff. glacinola* (PAF297439)
G10.Y	636	96	971	*Psychrobacter luti* (PGE430828)
G11.X	800	93	775	Uncultured soil bacterium (USO390469)
G11.Y	613	89	480	Uncultured soil bacterium (USO390469)
G12.X	683	97	1162	Blackwater bioreactor bacterium (AF394173)
G12.Y	734	97	1092	*Planococcus sp.* (AF242541)
H01.X	650	92	743	*Brevibacterium celer* KMM 3637 (AY228463)
H01.Y	714	97	1144	*Brevibacterium linens* (BLI315491)
H02.X	706	97	1080	Antarctic bacterium R-9183 (UBA441006)
H02.Y	816	98	1251	*Arthrobacter sp.* (ART495808)
H04.X	781	98	1275	*B.globisporus* (BG16SRRN)
H04.Y	635	99	1189	*B.globisporus* (BG16SRRN)
H05.X	709	98	1223	*Psychrobacter luti* (PGE430828)
H05.Y	773	98	1160	Bacterium UMB13F (AF505743)
H06.X	574	92	718	*Flavobacterium xinjiangense* (AF433173)
H06.Y	850	97	1257	*Flavobacterium degerlachei* (FDE557886)
H07.X	800	97	1203	Antarctic bacterium R-8963 (UBA440998)
H07.Y	822	93	813	*Flavobacterium xinjiangense* (AF433173)
H08.X	649	99	825	*Brevibacterium linens* (AY017083)
H08.Y	669	97	1142	*B.linens* NCDO 739 (BL16SRN3)
H09.X	490	95	704	Uncultured Arctic sea ice bacterium (AY165598)
H09.Y	801	98	1296	*Psychrobacter luti* (PGE430828)
H10.X	726	95	1061	*B.linens* NCDO 739 (BL16SRN3)
H10.Y	827	95	1253	*Brevibacterium linens* (BLI315491)
H11.X	662	98	1086	*Arthrobacter sp.* (AF170748)
H11.Y	683	98	1176	*Arthrobacter rhombi* (ARY15885)

Note: n^a represented the length of 16S rDNA in nucleotides used for alignment and phylogenetic analysis. Percent identity[b] represented the percent identity of the nearest known phylogenetic neighbours, determined by BLASTn analysis. The Bit Score was the normalised sum of scores for each letter-to-letter and letter-null position in an alignment. The higher the bit score indicted, the better the alignment.

A total of 96 colonies were picked for sequence analysis of cloned inserts from MVS1 clone library. Cloned inserts were sequenced in both the forward and reverse direction (bacterial isolates designated .X in Table 3.4 represented forward sequences, and bacterial isolates designated .Y represented reverse sequences). Of the 192 sets of sequence data, 155 were subjected to BLASTn analysis shown in Table 3.4. None of the cloned sequences for MVS1 were indicated as being identical to any of the known 16S rDNA sequences in the nucleotide database with BLASTn analysis. The bacterial clone sequences contained within the MVS1 library showed amongst themselves to have greater than 99% similarity, but less than 88% similarity to already known sequences present in the database.

Six Classes of bacteria were identified from BLASTn analysis of sequenced clone inserts from MVS1 clone library including *Flavobacterium, Sphingobacteria, α-proteobacteria, γ-proteobacteria, Bacilli,* and *Actinobacteria* (Figure 3.37). Seventeen out of the 96 recombinant cloned inserts were identified as being DNA of the Genus *Arthrobacter*. The *Brevibacterium* Genus was identified in 14 out of the 96 cloned inserts. Members of the Genus *Psychobacter* represented 17 out of the 96 cloned inserts. Fourteen of the 96 cloned inserts were identified as being members of the Genus *Planococcus.*

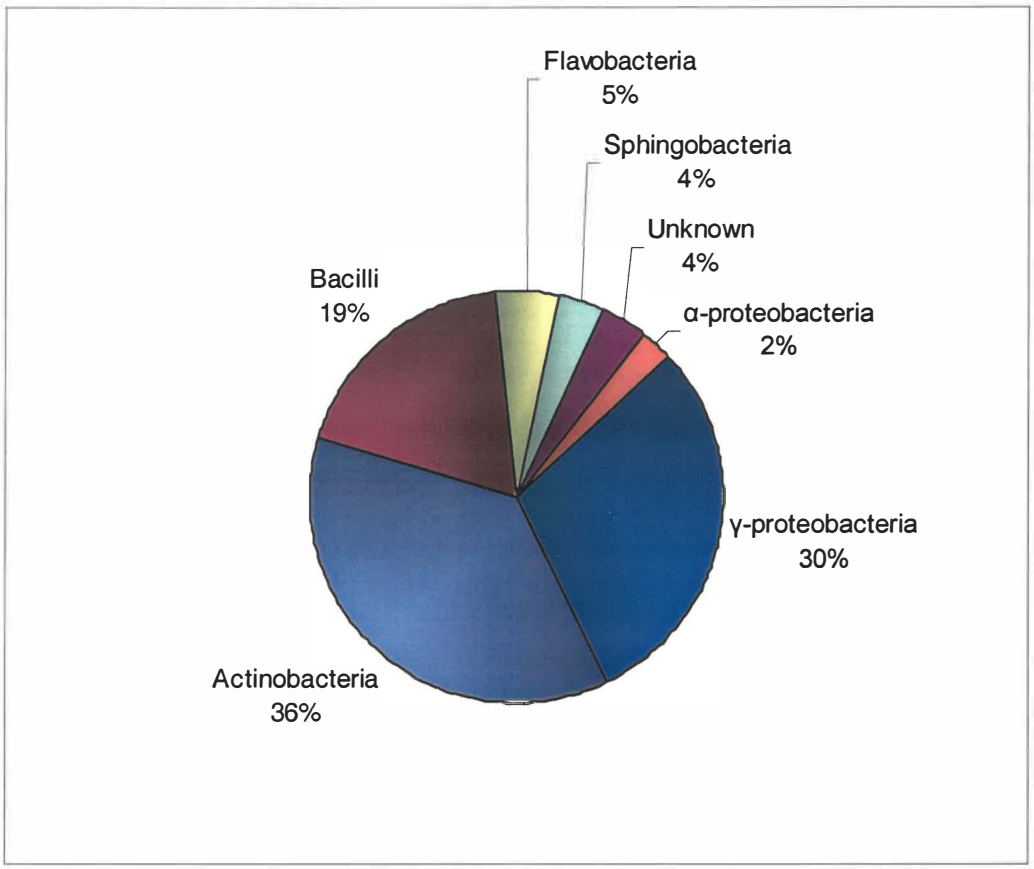


Figure 3.37 Diagram showing the relative abundance of organisms in each specified Class determined by BLASTn analysis of sequenced 16S rDNA clones, present in MVS1 clone library.

A total of 96 colonies containing cloned inserts were picked for sequence analysis from MVT10 clone library. Of these 96 colonies, 67 were successfully sequenced. These 67 sets of sequence data were subjected to BLASTn analysis (shown in Table 3.5). None of the cloned sequences for MVT10 were indicated as being identical to any of the known 16S rDNA sequences in the nucleotide database with BLASTn analysis. The bacterial clone sequences contained within the

MVT10 library showed amongst themselves to have greater than 96% similarity, but less than 86% similarity to already known sequences present in the database.

Table 3.5 Bacterial isolates from MVT10 clone library.

Isolate Designation	Sequence Alignment			Nearest phylogenetic neighbor (GenBank accession number)
	n[a]	% identity[b]	Bit Score	
B115	767	96	975	Uncultured bacterium clone CR99-2-73 (AF429259)
B103	766	97	1245	Uncultured bacterium clone (AY274163)
B106	662	90	676	Uncultured Verrucomicrobia bacterium (AF431595)
B107	554	92	648	Uncultured Acidobacteria bacterium (AY214862)
B109	705	88	688	Cytophaga sp. 16S rRNA gene, strain JTB250 (AB015264)
B111	657	90	504	Agricultural soil bacterium (ASO252665)
B113	694	92	753	uncultured bacterium (AJ583204)
B101	661	95	880	Uncultivated soil bacterium (AF013515)
B117	810	92	585	Uncultured Acidobacteria bacterium (AY193006)
B120	710	96	1112	*Hongia koreensis* (ASP16S161)
B123	738	88	379	*Chloroflexus aurantiacus* (CAU308500)
B124	674	95	938	Uncultured bacterium clone (AY375088)
B125	610	96	910	Uncultured diatom clone (AF418973)
B127	373	98	543	Uncultured CFB group bacterium (AF445698)
B129	820	92	1039	Uncultured bacterium clone (AY274135)
B132	459	94	567	*Pseudonocardia saturnea* (PSA252829)
B65	787	91	852	Uncultured bacterium (AF234151)
B66	396	91	365	Uncultured bacterium (AF234151)
B67	558	94	624	Uncultured bacterium clone (AY218661)
B68	730	95	880	*Pseudonocardia saturnea* (PSA252829)
B69	555	91	502	Rhizosphere soil bacterium (RSO252601)
B71	615	90	521	*Rubrobacter radiotolerans* (RRU65647)
B72	550	95	759	Bacterium Ellin404 (AF432234)
B75	862	91	959	Uncultured bacterium (AY218602)
B76	233	98	281	Unidentified bacterium (AY344421)
B78	647	95	981	Uncultured bacterium (AF443581)
B79	683	95	880	*Pseudonocardia saturnea* (PSA252829)
B80	704	97	1130	Uncultured bacterium (AY274144)
B81	815	94	920	Uncultivated soil bacterium clone (AF013550)
B82	762	89	779	Agricultural soil bacterium (ASO252665)
B83	691	94	896	Uncultured soil bacterium (AF423245)
B84	658	93	833	Uncultured soil bacterium (AF507687)
B85	607	99	862	Uncultured Antarctic cyanobacterium (AY151722)
B86	225	98	297	*Pseudonocardia petroleophila* (PPE252828)
B88	736	96	1130	Bacterium Ellin5294 (AY234645)
B89	693	93	914	Uncultured bacterium (AF234151)
B90	802	90	539	Uncultivated soil bacterium (AF013522)
B91	751	95	1114	Unidentified bacterium (AY345493)
B93	761	97	1144	MTBE-degrading bacterium (AF176594)
B94	560	94	777	*Rubrobacter radiotolerans* (RRU65647)
B95	694	96	285	*Hongia koreensis* (ASP16S161)
B96	992	96	90	Uncultured bacterium (AY212618)
B97	786	89	464	Uncultured bacterium (AF234122)
B99	772	86	547	Uncultivated soil bacterium (AF013546)
B7	642	94	894	Uncultured gold mine bacterium (AF337827)

Continuation of Table 3.5.

Isolate Designation	Sequence Alignment			Nearest phylogenetic neighbor (GenBank accession number)
	n[a]	% identity[b]	Bit Score	
B27	732	94	1009	Uncultured bacterium clone (AY274135)
B29	778	93	817	unidentified bacteria (UBA536440)
B30	732	97	821	Uncultured bacterium clone (AY364069)
B33	875	94	1233	Uncultured bacterium clone (AY218639)
B38	802	95	1185	Bacterium Ellin325 (AF498707)
B4	626	94	854	*Pseudonocardia saturnea* (PSA252829)
B45	612	94	876	*Actinobispora yunnanensis* (AYU252822)
B48	665	95	979	Uncultured gold mine bacterium (AF337865)
B49	875	93	957	Rhizosphere soil bacterium (RSO252670)
B50	762	97	1142	Uncultured bacterium (AY439192)
B56	852	94	1116	Uncultured soil bacterium clone (AF423245)
B57	790	99	860	Uncultured Antarctic cyanobacterium (AY151722)
B58	810	94	1080	Uncultured bacterium (AY100601)
B60	683	90	805	Bacterial species (BSPZ95730)
B61	751	96	1023	Uncultivated soil bacterium (AF013522)
B62	688	96	932	Uncultured bacterium (AF403186)
B2	703	95	741	Potato plant root (PPL252709)
B28	664	96	1017	Uncultured diatom clone (AF418973)
B41	839	95	1033	Bacterium Ellin5301 (AY234652)
B127	793	96	1170	Uncultured CFB group bacterium (AF445698)
B76	662	92	404	Uncultured bacterium (AF328189)
B86	459	91	412	*Pseudonocardia saturnea* (PSA252829)

n[a] represented the length of 16S rDNA in nucleotides used for alignment and phylogenetic analysis. Percent identity[b] represented the percent identity of the nearest known phylogenetic neighbours, determined by BLASTn analysis. The bit score was the normalised sum of scores for each letter-to-letter and letter-null position in an alignment. The higher the Bit Score indicted, the better the alignment

Five Classes of bacteria were identified from BLASTn analysis of sequenced clone inserts from MVT10 clone library, including; *β-proteobacteria, Shpingobacteria, Verrucomicrobia, Chloroflexi,* and *Actinobacteria* as indicated in Figure 3.38. Approximately 64% of the total number of sequenced clone inserts was identified as an unknown bacterium. This percentage value was much higher than that observed in MVS1 clone library (4%). Two of the cloned inserts of MVT10 clone library were identified as being representative of cyanobacterium, no *Cyanobacteria* were identified in MVS1 clone library. No bacterial isolates from MVT10 clone library were identified as being most similar to any of the four genera (*Brevibacterium, Arthrobacter, Planococcus,* and *Psychrobacter*) identified as being dominant in MVS1 bacterial isolates.

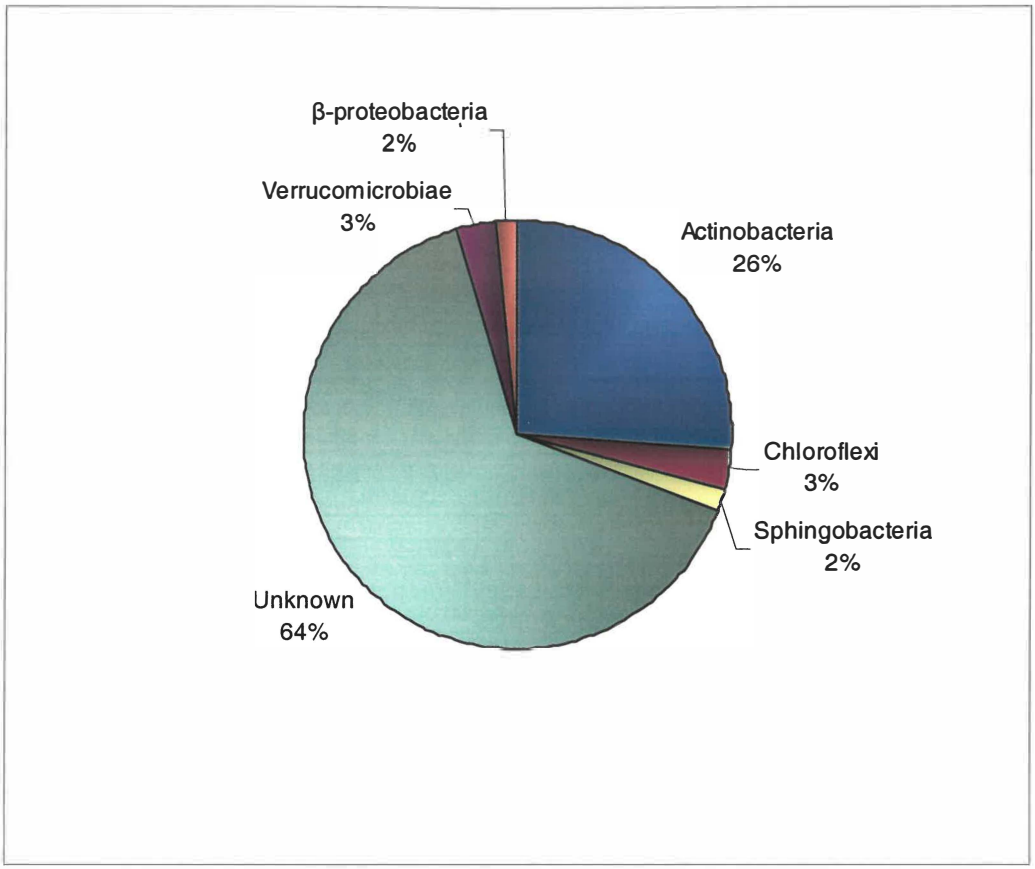


Figure 3.38 Diagram showing the relative abundance of organisms in each specified Class determined by BLASTn analysis of sequenced 16S rDNA clones present in MVT10 clone library.

3.10 Carbon and nitrogen analysis of Miers Valley environmental samples

Abundance of stable isotopes $\delta^{15}N$ and $\delta^{13}C$ present in environmental samples taken from MVS1, MVS3, MVS13 and four transect samples (MVT3, MVT5, MVT6, and MVT8) from MVT control sampling transects were measured. Values for total carbon and nitrogen, both organic and inorganic, were also measured by the University of Waikato Stable Isotope Unit. Instrument error is about ±1 delta. Between sample delta error is higher as it is a combination of machine error plus sample heterogeneity.

Nitrogen analysis of environmental samples taken from MVS1, MVS3, MVS13 and MVT are listed in Tables 3.6, 3.7, 3.8, 3.9. The tables include the designated name of the sample; the weight of the sample analysed in mg; the percentage of N in the volume of sample measured; the total amount of nitrogen measured in the

sample in µg; the level of $\delta^{15}N$ present in the measured sample; and the percentage of atom present in the volume of environmental sample measured.

3.10.1 Nitrogen analysis

Table 3.6 Nitrogen analysis of environmental samples collected from MVS1 sampling transect.

Sample	Weight (mg)	%N	Micro g N	Delta 15N
Centre 1	55.47	0.08	44.7	18.21
Centre 1-5cm	55.38	0.02	13	N level too low
A1	54.93	0.01	3.8	N level too low
A1-10cm	55.02	0.00	2	N level too low
A2	55.99	0.01	4.5	N level too low
A2-10cm	55.49	0.00	2	N level too low
A3	55.77	0.04	19.6	N level too low
A3-10cm	55.48	0.00	2	N level too low
A4	55.79	0.00	2.2	N level too low
A5	55.45	0.01	6.7	N level too low
A6	55.23	0.00	2.1	N level too low
A6-10cm	55.11	0.01	3	N level too low
A9	55.42	0.01	3.5	N level too low
A10	54.78	0.01	4.5	N level too low
B1	55.28	0.00	2.5	N level too low
B1-10cm	55.87	0.00	1	N level too low
B1-3	55.1	0.00	1	N level too low
B2	55.41	0.01	3.8	N level too low
B2-10cm	54.98	0.01	7	N level too low
B3	55.98	0.02	8.7	N level too low
C1	55.94	0.00	1.5	N level too low
C1-10cm	55.32	0.00	2	N level too low
C2	55.58	0.00	2.3	N level too low
C2-10cm	55.19	0.01	5	N level too low
C3	55.04	0.01	4.7	N level too low
C3-10cm	55.11	0.00	3	N level too low
C4	55.82	0.01	4.0	N level too low
C5	55.45	0.00	1.9	N level too low
D1	55.91	0.00	1.7	N level too low
D1-10cm	55.12	0.00	2	N level too low
D2	55.37	0.01	3.5	N level too low
D2-10cm	55.76	0.00	2	N level too low
D3	55.28	0.01	4.9	N level too low
D3-10cm	55.24	0.00	2	N level too low

There appeared to be no obvious trend in micrograms of nitrogen in surface and subsurface samples, as some samples taken at the same vertical location appeared to have increased levels, while some were lower in value. The only detectable level of nitrogen, the only value above 30µg, (the minimum level of N per sample) was the MVS1 surface centre environmental sample. All other samples in

MVS1 sample set appeared to be too low in $\delta^{15}N$ and total nitrogen content to get an accurate measurement using this method.

Table 3.7 Nitrogen analysis of environmental samples collected from MVS3 sampling transect.

Sample	Weight (mg)	%N	Micro g N	Delta 15N
Centre 3	55.01	0.02	9.8	N level too low
A1	55.72	0.00	2.0	N level too low
A2	55.9	0.00	2.4	N level too low
A3	55.58	0.01	5.1	N level too low
B1	55.86	0.00	2.1	N level too low
B2	55.98	0.00	2.7	N level too low
B3	55.95	0.03	15.5	N level too low
C1	55.33	0.01	4.7	N level too low
C2	55.33	0.00	2.2	N level too low
C3	55.58	0.01	3.8	N level too low
D1	55.03	0.01	3.3	N level too low
D2	54.86	0.01	4.5	N level too low
D3	55.62	0.02	9.1	N level too low

None of the samples in this sample set had levels of nitrogen significant to gain accurate measurements using this method.

Table 3.8 Nitrogen analysis of environmental samples collected from MVS13 sampling transect.

Sample	Weight (mg)	%N	Micro g N	Delta 15N
Center 13	55.43	0.06	35	18.45
A2	55.63	0.01	5	N level too low
A3	55.29	0.01	4	N level too low
B1	55.58	0.00	2	N level too low
B2	55.33	0.00	2	N level too low
B3	55.37	0.00	2	N level too low
C1	55.84	0.00	1	N level too low
C2	55.73	0.01	3	N level too low
C3	55.64	0.06	31	15.98
D1	55.88	0.00	2	N level too low
D2	55.44	0.00	2	N level too low
D3	55.68	0.19	105	10.35

Three samples in MVS13 sample set appeared to have detectable levels of nitrogen using this method of stable isotope analysis, Centre-13, C3, D3, as opposed to only one sample in MVS1 sample set, and none in MVS3. Sample D3 had the highest level of total nitrogen, both organic and inorganic, with a %N level of 0.19, more than three times the level of C3 and Centre-13. However, sample centre-13 had the highest level of $\delta^{15}N$, with a value of 18.45. Interestingly, sample D3 had the lowest level of $\delta^{15}N$ in of the three measurable samples.

Table 3.9 Nitrogen analysis of environmental samples collected from MVT sampling transect.

Sample	Weight (mg)	%N	Micro g N	Delta 15N
MVT3	54.95	0.00	2	N level too low
MVT6	55.35	0.01	3	N level too low
MVT8	55.2	0.01	5	N level too low
MVT5	55.11	0.00	3	N level too low

None of the samples in this sample set had levels of Nitrogen high enough to gain accurate measurements using this method.

3.10.2 Carbon analysis

Table 3.10 Initial carbon analysis of environmental samples taken from the soil surface in MVS1 sampling transect.

Sample	Weight (mg)	%C	Micro g	comment	Delta 13C	Atom%
		(Approx only)				
Centre 1	55.47	1.56	867		-9.00	1.101
Centre 1-5cm	55.38	1.14	632		-2.60	1.108
A1	54.93	0.50	273	small	-4.43	1.106
A1-10cm	55.02	8.26	4545	too big	-0.88	1.110
A2	55.99	0.43	243	small	-4.33	1.106
A2-10cm	55.49	8.55	4745	too big	-0.53	1.111
A3	55.77	0.90	500		-4.66	1.106
A3-10cm	55.48	8.14	4515	too big	-0.39	1.111
A4	55.79	0.46	259	small	-1.96	1.109
A5	55.45	0.66	367		-4.63	1.106
A6	55.23	0.54	299	marginal	-1.83	1.109
A6-10cm	55.11	7.94	4374	too big	-0.23	1.111
A9	55.42	0.60	334		-4.17	1.107
A10	54.78	0.79	431		-1.99	1.109
B1	55.28	0.54	299	marginal	-2.24	1.109
B1-10cm	55.87	1.85	1035	too big	-1.39	1.110
B1-3	55.1	0.71	389		-0.37	1.111
B2	55.41	5.40	2990		-1.04	1.110
B2-10cm	54.98	0.44	241	small	-3.75	1.107
B3	55.98	4.36	2439		-0.67	1.110
C1	55.94	1.07	599		-0.57	1.111
C1-10cm	55.32	5.46	3018	too big	-0.75	1.110
C2	55.58	0.58	323		-1.35	1.110
C2-10cm	55.19	1.47	810		-1.41	1.110
C3	55.04	0.62	343		-3.41	1.107
C3-10cm	55.11	4.89	2697	too big	-0.88	1.110
C4	55.82	0.52	289	marginal	-4.72	1.106
C5	55.45	0.75	416		-2.94	1.108
D1	55.91	0.45	249	small	-3.36	1.108
D1-10cm	55.12	8.43	4648	too big	-0.45	1.111
D2	55.37	0.53	295	marginal	-4.86	1.106
D2-10cm	55.76	8.31	4631	too big	-0.20	1.111
D3	55.28	0.52	287	small	-7.02	1.104
D3-10cm	55.24	7.81	4316	too big	-0.40	1.111

The minimum level of carbon required per sample for Delta ^{13}C is 300µg. Ten of the samples in this set measured had less than this value, despite the fact that a maximum amount of sample was used in the measurements. All, however, had values higher than 200µg. The range of carbon level required for an accurate %C determination, using the standards measured for this run, was 1400-1900µg. Many of the samples had levels of carbon higher than 1900µg. For these samples, an approximate %C value could only be given. Samples were re-weighed and re-measured using a higher reference to get a more accurate %C value.

Table 3.11 Re-analysis of environmental samples taken from the soil surface of MVS1 sampling transect

Sample	Weight (mg)	%C	Micro g	Delta 13C	Atom % 13C
Center 1	55.59	1.39	771	-8.90	1.101
Centre 1-5cm	55.38	1.14	632	-2.60	1.108
A1	55.48	0.63	351	-3.37	1.108
A1-10cm	6.28	9.84	618	-0.30	
A2	55.2	0.35	196	-3.67	1.107
A2-10cm	5.56	10.91	606	-0.71	
A3	55.04	0.80	440	-4.38	1.106
A3-10cm	5.8	10.35	600	-0.34	
A4	55.61	0.54	299	-3.79	1.107
A5	55.19	0.40	222	-1.16	1.110
A6	55.82	0.44	247	-1.49	1.110
A6-10cm	5.95	9.53	567	-0.23	
A9	55.21	0.52	288	-3.32	1.108
A10	55.29	0.67	370	-1.49	1.110
B1	55.16	0.44	242	-1.83	1.109
B1-10cm	25.44	1.85	472	-1.08	
B1-3	39.15	0.70	273	-0.40	
B2	24.86	5.59	1389	-0.82	1.110
B2-10cm	62.84	0.41	260	-3.81	
B3	29.3	4.47	1309	-0.25	1.111
C1	55	0.94	518	-0.65	1.111
C1-10cm	8.76	6.58	576	-0.01	
C2	55.01	0.48	262	-0.77	1.110
C2-10cm	32.14	1.51	485	-1.08	
C3	55.63	0.57	318	-2.91	1.108
C3-10cm	9.56	5.70	545	-0.24	
C4	55.48	0.42	233	-4.23	1.107
C5	55.38	0.68	379	-3.49	1.107
D1	55.19	0.36	201	-3.16	1.108
D1-10cm	5.6	10.47	586	-0.14	
D2	55.01	0.39	217	-1.50	1.110
D2-10cm	5.77	10.45	603	-0.38	
D3	55.29	0.38	212	-4.10	1.107
D3-10cm	6.11	9.66	590	-0.61	

The percentage of total carbon present in MVS1 surface and subsurface samples was presented graphically (as shown in Figures 3.39 and 3.40) according to position in the sampling transect.

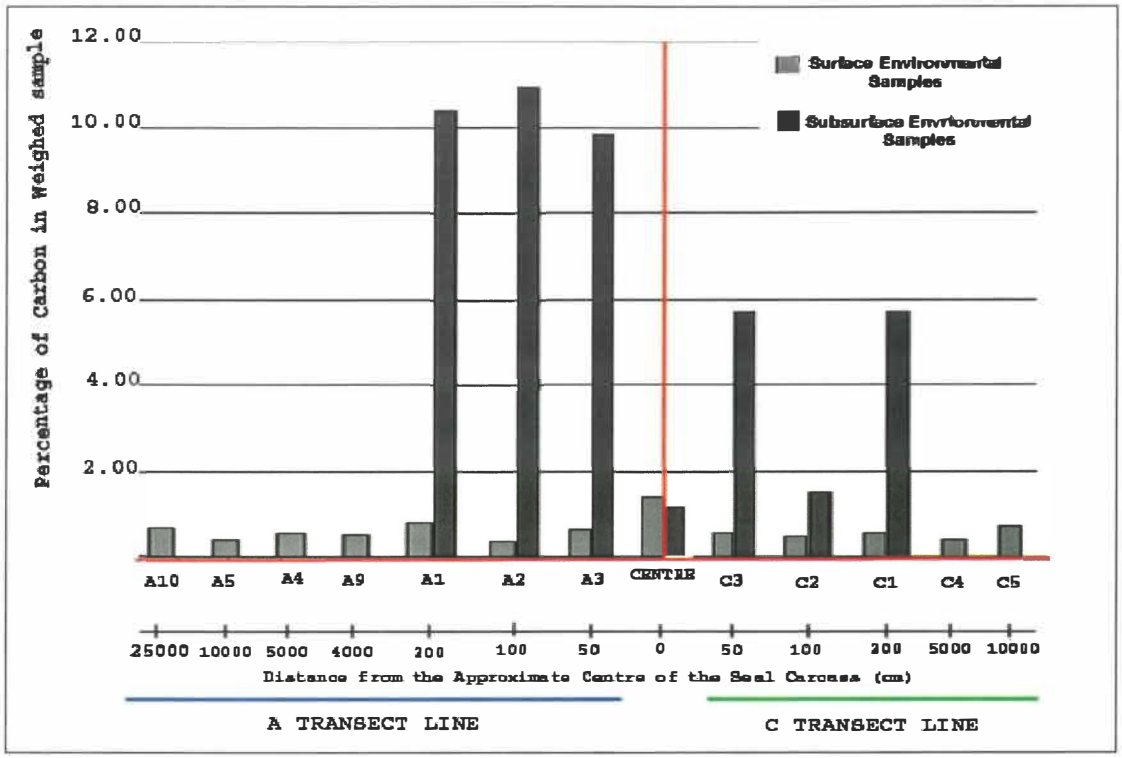


Figure 3.39 Histogram showing the percentages of total carbon in surface and subsurface environmental samples collected from MVS1 sampling transect lines A and C.

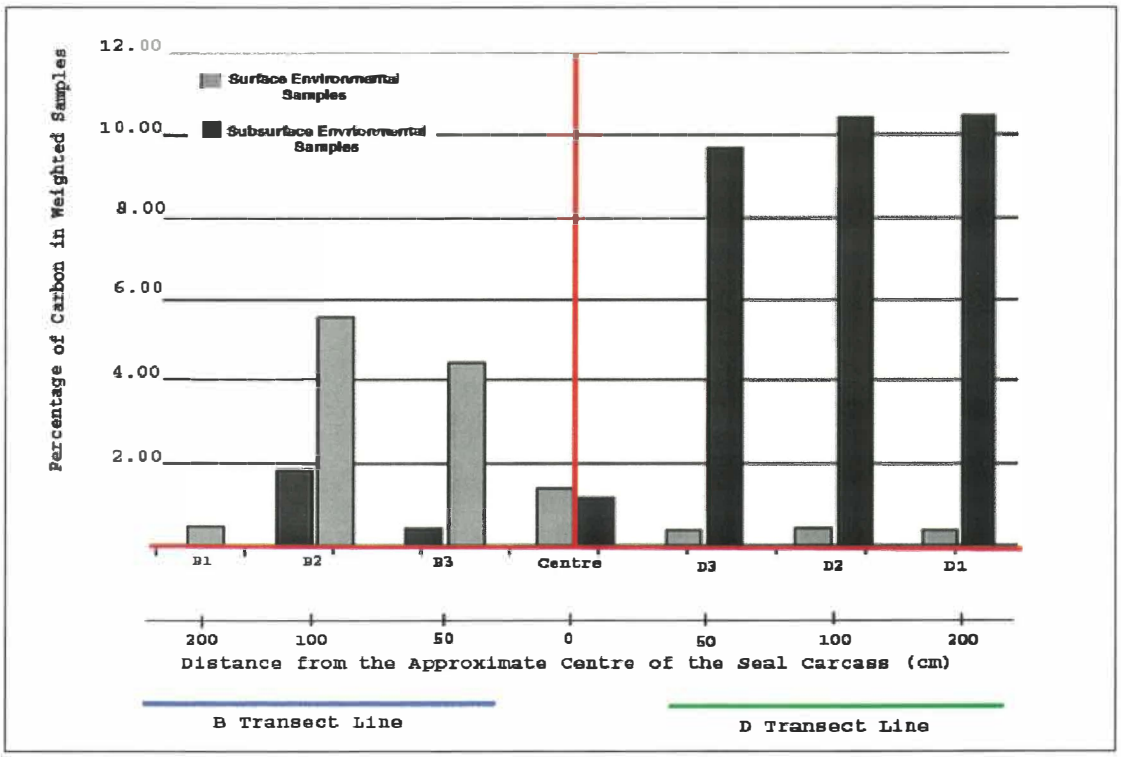


Figure 3.40 Histogram showing the percentages of total carbon in surface and subsurface environmental samples collected from MVS1 sampling transect lines B and D.

Lower volumes of sample were measured for those samples that had levels of carbon too high for %C determination. These volumes were calculated by the University of Waikato Stable Isotope Unit to give accurate results. Typically, on re-analysis, those samples where lower volumes (less than 55mg) were used, the %C values appeared to be higher. There appeared to be very large differences (up to 31 times greater) in the levels of total carbon given by %C determinations, in subsurface samples compared to samples taken from the surface of the transect. The percentage of total carbon in the samples did not appear to increase with closer proximity of the sample to the MVS1 carcass. Subsurface samples measured were typically taken at a depth of 10cm, with the exception of Centre 1-5cm, which was taken at a depth of 5cm. Delta ^{13}C values measured, were generally larger for surface rather than subsurface samples.

Table 3.12 Initial carbon analysis of environmental samples collected from MVS3 sampling transect.

Sample	Weight (mg)	%C	Micro g	Delta 13C	Atom%
Centre 3	55.01	1.40	769	-2.61	1.108
A1	55.72	0.72	404	-0.51	1.111
A2	55.9	0.57	319	-2.08	1.109
A3	55.58	1.11	617	-2.00	1.109
B1	55.86	0.69	386	-0.09	1.111
B2	55.98	0.92	516	-2.53	1.108
B3	55.95	1.00	558	-3.93	1.107
C1	55.33	0.87	479	-1.46	1.110
C2	55.33	1.31	724	-0.08	1.111
C3	55.58	0.98	547	-1.39	1.110
D1	55.03	0.90	493	-1.25	1.110
D2	54.86	0.75	409	-2.70	1.108
D3	55.62	0.91	507	-4.91	1.106

All samples in this MVS3 environmental sample set had values below 1400µg, which was the minimum required to gain an accurate determination of %C. However, all had values above 300µg, which was the minimum level required per sample for $\delta^{13}C$ analysis. The samples were re-weighed, and re-analysed using lower references for a more accurate %C determination.

Table 3.13 Re-analysis of carbon in environmental samples collected from MVS3 sampling transect.

Sample	Weight (mg)	%C	Micro g	Delta 13C	Atom % 13C
Center 3	55.12	1.27	701	-2.52	1.108
A1	55.53	0.64	356	-0.69	1.110
A2	55.77	0.47	261	-1.20	1.110
A3	55.12	0.98	538	-1.92	1.109
B1	55.04	0.63	349	-0.46	1.111
B2	55.09	0.82	449	-2.77	1.108
B3	55.2	0.88	484	-4.37	1.106
C1	55.06	0.78	429	-1.04	1.110
C2	55.46	1.24	687	-0.25	1.111
C3	55.39	0.86	474	-1.29	1.110
D1	55.63	0.78	434	-1.31	1.110
D2	55.19	0.63	350	-2.56	1.108
D3	54.98	0.75	414	-4.69	1.106

The percentage of total carbon was presented graphically (shown in Figures 3.41, 3.42) for MVS3 environmental samples according to position of the sample in the transect.

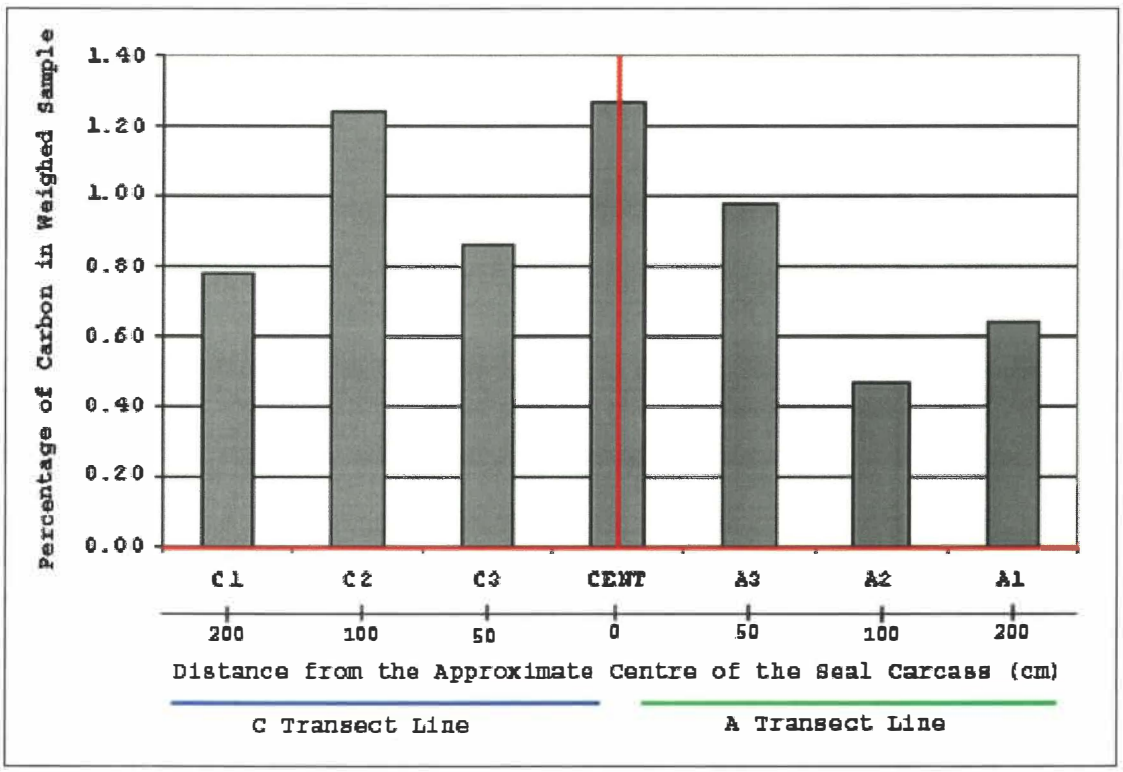


Figure 3.41 Histogram showing the percentages of total carbon in environmental samples collected from MVS3 sampling transect lines C and A.

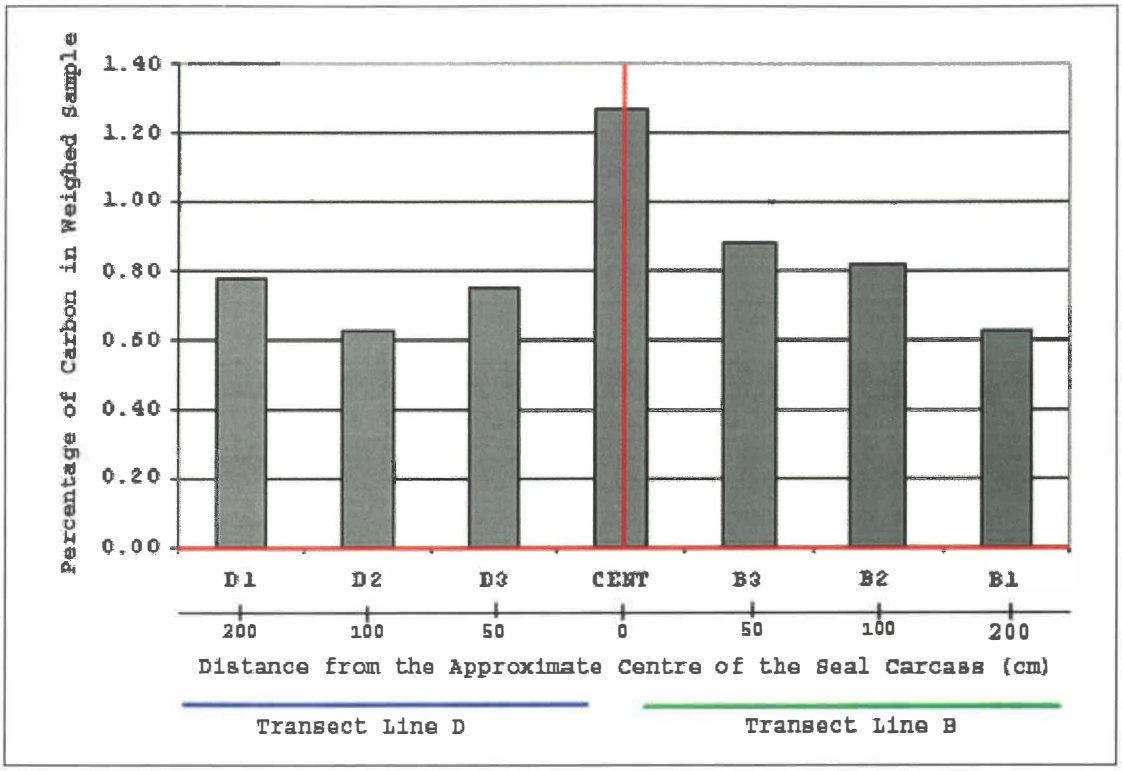


Figure 3.42 Histogram showing the percentages of total carbon in environmental samples collected from MVS3 sampling transect lines D and B.

The highest value of total carbon, 1.27%, (both organic and inorganic) given as a percentage of sample weighed in MVS3 transect samples, was measured in the environmental sample taken from directly underneath the seal. There appeared to be no trend in the level of carbon measured versus the proximity of the sample to MVS3 carcass. The lowest value of the stable isotope $\delta^{13}C$ (-4.69), was in sample D3, with a low value of -4.37 found in sample B3. The %C values for all of the samples after re-analysis using a lower reference, are all lower in value.

Table 3.14 Carbon analysis of environmental samples taken from MVS13 sampling transect.

Sample	Weight (mg)	%C	Micro g	Delta 13C	Atom%
Center 13	55.43	0.91	503	-6.27	1.104
A2	55.63	0.74	410	-1.12	1.110
A3	55.29	0.75	412	-1.57	1.110
B1	55.58	0.74	410	-1.56	1.110
B2	55.33	0.56	310	1.02	1.112
B3	55.37	0.70	388	-0.60	1.111
C1	55.84	0.46	258	0.38	1.112
C2	55.73	0.87	486	-1.21	1.110
C3	55.64	1.34	745	-15.55	1.094
D1	55.88	0.60	335	-0.36	1.111
D2	55.44	0.46	253	-1.14	1.110
D3	55.68	1.65	918	-24.81	1.084

The percentage of total carbon was presented graphically (shown in Figures 3.43 and 3.44) for MVS13 environmental samples according to position of the sample in the transect.

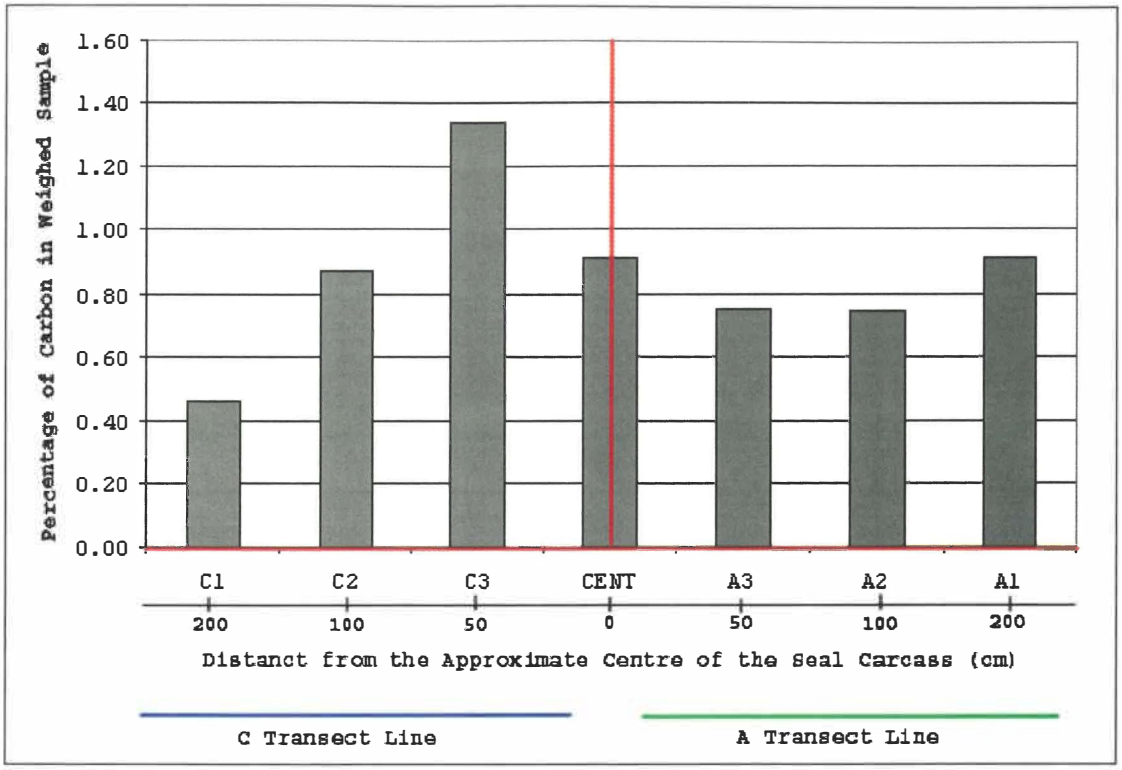


Figure 3.43 Histogram showing the percentages of total carbon in environmental samples collected from MVS13 sampling transect lines C and A.

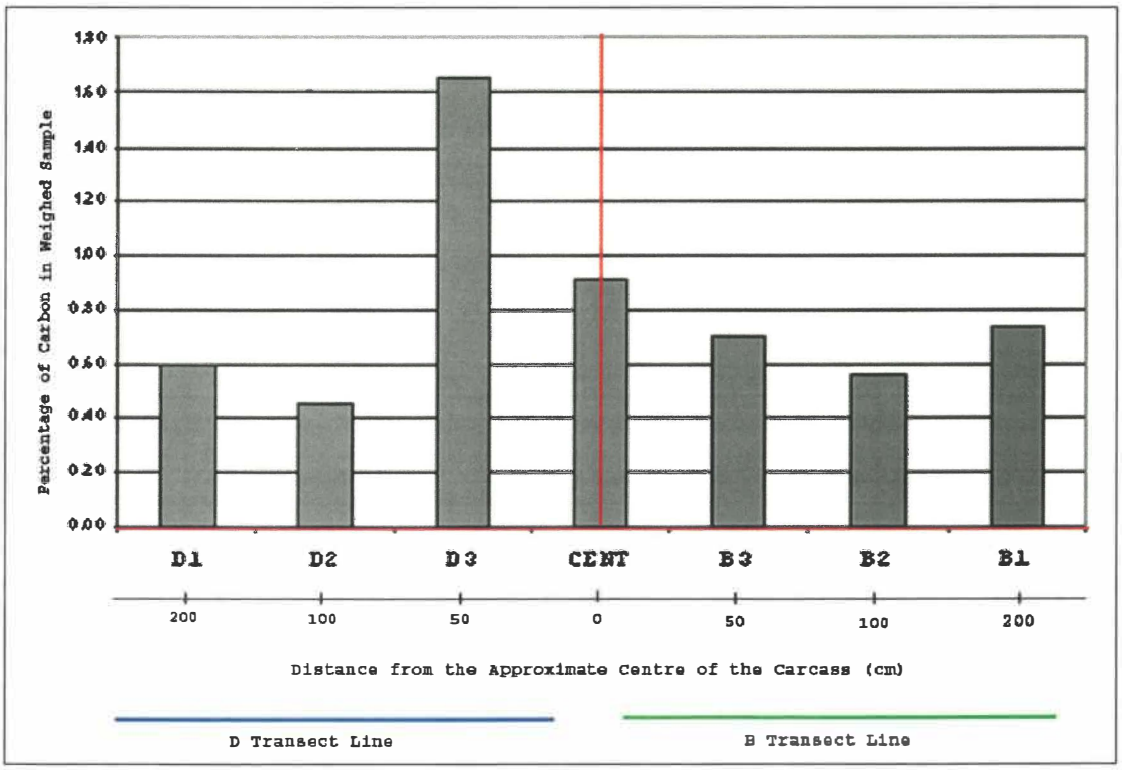


Figure 3.44 Histogram showing the percentages of total carbon in environmental samples collected from MVS13 sampling transect lines D and B.

Samples D3, C3, collected 50cm from the approximate centre of MVS13 seal carcass and the centre sample, collected from underneath the carcass, had the highest values of total carbon in the sample set, with values of 1.65, 1.34, and 0.91, respectively. There appeared to be no trend relating the levels of carbon measured in MVS13 environmental samples, and the proximity of the sample to the seal carcass.

Samples D3 and C3 appeared to have low $\delta^{13}C$ values in comparison with the three sample sets delta ^{13}C results.

Table 3.15 Carbon analysis of environmental samples taken from MVT sampling transect.

Sample	Weight (mg)	%C	Micro g C	Delta 13C
MVT3	54.95	0.39	216	-0.57
MVT6	55.35	0.47	262	-2.99
MVT8	55.2	0.62	341	-1.52
MVT5	55.11	0.44	241	-1.23

The percentage of total carbon was presented graphically (shown in Figure 3.45) for the analysed MVT environmental samples.

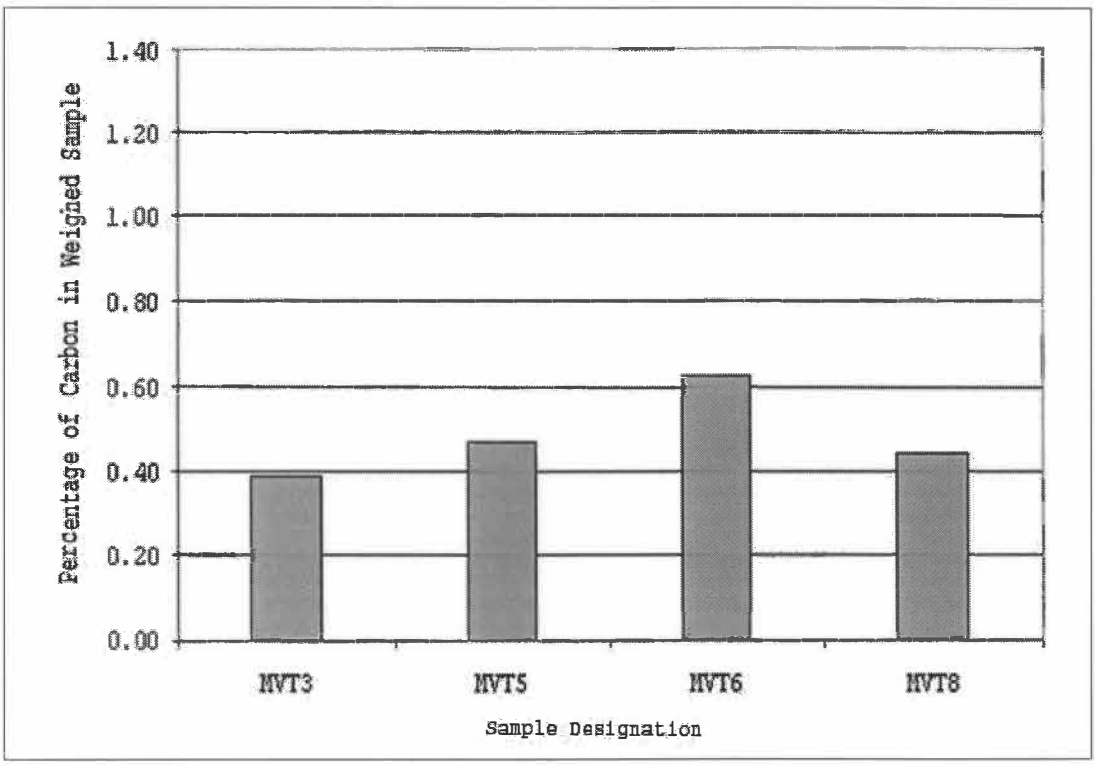


Figure 3.45 Histogram showing the percentages of total carbon in selected MVT environmental samples.

The percentage of total carbon measured in the four MVT samples ranged from 0.39-0.62%. The transect control samples measured had an average %C value of 0.48. The $\delta^{13}C$ values ranged from -0.57 to -2.99, with a difference of 2.42.

3.11 Radiocarbon dating of seal sample

Radiocarbon dating using Accelerator Mass Spectrometry (AMS) technique was conducted by the University of Waikato 14C Dating Laboratory, on MVS3 seal tissue sample in an attempt to determine its approximate age. MVS3 (shown in Figure 2.8) was a well preserved seal, tentatively identified as a *Leptonychotes weddelli* (Weddell seal) (Rod Seppelt, personal communication).

For AMS samples less than 10,000 years old the standard error is typically ±60 years.

Table 3.16 Radiocarbon analysis of Miers Valley Seal 3 tissue.

$\delta^{14}C$	-56.4 ± 4.2 ‰
$\delta^{13}C$	-24.6 ± 0.2 ‰
$D^{14}C$	-61.0 ± 4.5 ‰
% Modern	93.9 ± 0.4 %
Result	**505 39 BP**

The gelatin stable isotope results gained from MVS3 seal tissue sample were $\delta^{15}N$: 9.20 and $\delta^{13}C$: -22.29 (as depicted in table 3.16). These results were unusual for a marine vertebrate. A calibrated date range was not included as it was not known what reservoir correction should be used for Antarctic animals. It is likely to be quite large, however, and is likely to have a correction of greater than 400 years since this is the average correction for the worlds oceans. An even larger correction would likely suggest that the bone was <100 years old (Dr Fiona Petchey, personnel communication). The result was given in *% Modern* as per Stuiver and Polach, 1977, indicating that the conventional age of the seal tissue sample is younger than 200 yr BP. This is based on the Libby half-life of 5568 yr with correction for isotopic fractionation applied.

CHAPTER FOUR

Discussion, Conclusions and Future Recommendations

This thesis investigated the microbial diversity present in environmental samples taken surrounding mummified seal carcasses, located in the Miers Valley area of the Antarctic Dry Valleys. Given the harsh and barren environment of the Dry Valleys, it was hypothesised that these carcasses would provide a source of organic carbon and organic nitrogen (and possibly other nutrients that were not tested for), resulting in an increased abundance and a difference in the diversity of heterotrophic microorganisms present in the environment directly affected by the seal carcasses.

4.1 Sampling of Miers Valley environmental samples

The mummified seals located in Miers Valley in the Antarctic Dry Valleys were sampled on Event K023 Terrestrial Microbiology Antarctica New Zealand 2001/2002, by Professors S.C. Cary, and D.A. Cowan. Miers Valley is located just south of Marshall Valley and west of Koettlitz Glacier, on the coast of Victoria Land. The valley is ice-free except for Miers Glacier in the upper western part and Lake Miers near the centre. Mummified seals have been reported previously from this area (Webb and Leckie, 1977). Given the preservation of seal carcasses observed in the Dry Valleys (Dort, 1982), the seals sampled on Event K023 by Cary and Cowan may represent the same seals reported by Webb and Leckie (1977).

A total of eleven seals were sampled, located at altitudes ranging from 483-804m. The areas surrounding three of these seals were sampled extensively, due to their location on relatively level sandy ground suitable for transect sampling. These three sampling transects surrounding the mummified seal carcasses (MVS1, MVS3 and MVS13) were the focus of microbial biodiversity studies in this thesis

research. Miers Valley Seal one (MVS1) was located at an altitude of 483m. From the photographic image taken by S.C. Cary (shown in Chapter 2, Figure 2.3), MVS1 appeared to be well preserved, with skin tissue still intact over most areas of the carcass, and cracking occurring only in some parts of exposed skin surface. MVS1 was tentatively identified from the photographic image as *Lobodon carcinophagus* (Crabeater Seal) (Rod Seppelt, personal communication) Thirty-nine environmental samples in total were collected from MVS1 sampling transect, taken from the surface (0-2cm) and subsurface (5cm and 10cm) of soils (as shown in Section 2.1.

MVS3 was located at an altitude of 663m. A total of thirteen environmental samples were collected from the top 2cm of soil surface. Based on the photographic image taken by S.C. Cary (shown in Chapter 2, Figure 2.7), MVS3 appeared the most preserved of the three seals, with no visible cracking of the skin surface. MVS3 was tentatively identified from the photographic image as *Leptonychotes weddelli* (Weddell Seal) (Rod Seppelt, personal communication)

MVS13 consisted of thirteen environmental samples, taken from the top 2cm of soil from the surface, located at an altitude of 670m. Based on the photographic image taken by S.C. Cary (Chapter 2, Figure 2.9), MVS13 was tentatively identified as a Weddell Seal (Rod Seppelt, personal communication). From the photographic image, MVS13 appeared to be the least well preserved carcass of the three, with much of the skin and flesh eroded, exposing large sections of bone.

Based on the state of preservation of the three seal carcasses (MVS1, MVS3, and MVS13) it is probable that MVS13 was present in the Dry Valleys the longest. This is not certain, however, as the approximate age determined by ^{14}C dating was estimated for MVS3 seal carcass only, and the rate of erosion of mummified seals has been demonstrated to vary with location, due mainly to differences in exposure to wind movement of sand particles (Dort, 1982).

The age of the MVS3 carcass was estimated to be less than 100 years old, based on results gained from radiocarbon dating using AMS technique. This estimated age was not conclusive, as the standard error for AMS samples less than 10,000

years old is typically ±60 years (Fiona Petchly, personal communication). No calibrated date range was included in the radiocarbon dating analysis, as it was not known what reservoir correction should be used for Antarctic animals. Radiocarbon ages for organisms living in Antarctic sea water, or situated farther along the food chain, yield apparent ages that are older than true ages due to significantly lower levels of ^{14}C activity in ocean waters near Antarctica (Broecker, 1963; Dort, 1982). As an example, an apparent radiocarbon age of 618 ±100 years was sighted from a seal carcass in Antarctica that was believed to have been dead for only a few weeks (Marini and Blair, 1971). Given the frozen, dried, well preserved state of a number of mummified seals observed in the Antarctic Dry Valleys (Péwé et al., 1959; Barwick and Balham, 1967; Dort, 1971; Webb and Leckie, 1977; Dort, 1982), it is perhaps possible that these carcasses have been there for greater than 100 years (Dort, 1982). However, as previous expeditions to Miers Valley (Webb and Leckie, 1977) did not photograph or state the GPS coordinates of observed seal carcass, and the rate of erosion is so variable (Dort, 1982) an estimate of age is not given.

Microbial populations surrounding mummified seals were compared with microbial populations present in Miers Valley transect control samples (MVT). These environmental samples were taken at varying altitudes and locations along an axis from the Miers Valley floor, to a high saddle at the top of the Marshall Valley (shown in Chapter 2, Figure 2.1). The approximate distances between MVT environmental samples was not indicated in sampling notes, therefore the distances between environmental samples in relation to other MVT samples, and Miers Valley seals were not known. It was stated in sampling notes that MVT environmental samples were taken from locations where there were no obvious signs of organic contamination in the form of skua guano or seal remains, and no incidence of lichen growth near MVT environmental samples was reported. As no photographic images of MVT sample sites were taken, the assumption that microbial populations present in MVT environmental samples were not influenced by organic contamination, can not be substantiated.

4.2 Microbial abundance in Miers Valley environmental samples

The hypothesis of this thesis research stated that microbial abundance would be greater in environmental samples that were enriched with organic carbon and organic nitrogen from the carcasses of selected mummified seals, compared to environmental samples collected from Miers Valley that were not enriched by organic contamination.

No direct quantitation of microorganisms was conducted on environmental samples from Miers Valley in this thesis research. Microbial abundance in environmental samples was compared based on the quantities of double stranded DNA extracted (shown in Chapter 3, Figure 3.2) and *In situ* ATP analysis, on environmental samples from MVS1, MVS13, and MVT sampling transects, conducted by Professor D.A. Cowan on Event K023 (shown in Appendix 3).

4.2.1 ATP analysis

ATP analysis (determined by luciferase-dependent luminometric analysis) was used to measure microbial abundance (Cowan et al., 2002). Two values of relative luminosity units (RLU's) were given for each environmental sample assayed. The RLU values indicated for some samples appeared to vary largely (data given in Appendix 3). All comparisons made using the ATP data in this thesis used the highest RLU value recorded for each environmental sample. RLU values for MVT environmental control samples, ranged from 806 in MVT1 to 27691 in MVT11. Only two environmental samples from MVS1 and MVS13 sampling transects had RLU values higher than 27691 recorded for MVT11. These were MVS1 cent environmental sample (42839) collected from the soil surface, and MVS1 environmental sample D3-1 (30789), taken 50cm from the approximate centre of the seal carcass, along transect line D. These environmental samples also indicated some of the highest levels of carbon and nitrogen in the Miers Valley environmental samples analysed (discussed in section 4.5).

As no photographic images of MVT transect environmental samples were taken, it is not known whether the high RLU values recorded for MVT9 (25973) and MVT11 (27691) were influenced by organic contamination, or whether the levels of microbial abundance and activity were naturally higher in these sample areas.

MVS1 transect environmental samples, taken in closer proximity to the seal carcass, typically indicated higher RLU values in comparison to environmental samples taken at a greater distance from the seal carcass. A number of exceptions were noted, however, for example MVS1 environmental sample A10, taken 25m from the approximated centre of the seal carcass, in the estimated down-slope direction, indicated a value of 21962 RLUs. MVS13 environmental samples did not show the same trend, this was possibly due to the extremely dry soil observed for the D3 and centre environmental samples. Moisture content of the samples was not measured because only small volumes (typically under 50 grams) of sample were collected. Therefore, the exact level of moisture in these samples compared to other samples in the transect was not known.

4.2.2 Extraction of DNA from Miers Valley environmental samples

The quantity of double stranded DNA extracted from Miers Valley environmental samples varied widely from no DNA detected to 536.10ng/µl (as shown in Chapter 3, Figure 3.2). The highest quantities of DNA extracted from seal transect environmental samples were in surface soils taken from directly beneath a seal carcass, or in close proximity (50cm) to the centre of a carcass. The quantities of double stranded DNA extracted from MVT environmental samples ranged from none detected to 232ng/µl. Three environmental samples, MVT6, MVT8 and MVT9 indicated high quantities of double stranded DNA compared to the majority of Miers Valley environmental samples (232ng/µl, 203.45 ng/µl, and 231.30ng/µl respectively).

4.2.3 Efficiency of the PSC-B method of DNA extraction as a method of assessing microbial diversity

The quantities of genomic DNA extracted from Miers Valley environmental samples varied from none detectable by spectrophotometric analysis to 536.10ng/µl, as shown in Chapter 3, Table 3.2. Nine repeat DNA extractions of MVS1-cent sample, taken from the top 2cm of soil directly beneath the carcass of MVS1, ranged in the quantity of double stranded DNA extracted from 56.65ng/µl to 435.60 ng/µl.

The apparent consistency of bacterial species determined by DGGE analysis, given the wide variance in quantities of double stranded genomic DNA from environmental samples, perhaps indicated that microorganisms were distributed relatively evenly throughout the sample site, and that the method of DNA extraction was highly variable as to its efficiency. The percent efficiency of the PSC-B method for DNA extraction from environmental samples using a known concentration of DNA was not tested. It was, therefore not known if the variation in the quantities of double stranded DNA extracted were the result of pocketing of microbial communities, or whether variation was due to methodology. Therefore, the quantities of DNA extracted using the PSC-B method were not accurate determinants of microbial abundance. Rather, DNA quantities were used in this thesis to corroborate trends shown in ATP data.

4.2.4 Microbial abundance in subsurface environmental samples versus surface environmental samples

MVS1 sampling transect was the only sampling transect from Miers Valley that contained both surface and subsurface environmental samples. Surface samples were taken from the top 2cm of soil, subsurface samples were taken at 5cm and 10cm below the surface from some horizontal locations on the transect.

Previous investigations into the microbial diversity of environmental samples from Victoria, King-David, and Wheeler Valleys in the Dry Valleys indicated that microbial abundance was greater in subsurface environmental samples due to the increased soil moisture levels (Cameron et al. 1968, 1970). A more recent investigation into culturable bacteria from environmental samples taken at different depths in Wright Valley, indicated the highest numbers of culturable bacteria per gram of dry weight soil were in samples taken from the top 5 cm of soil from the surface (Aislabie et al., 2001). The quantities of total DNA extracted from surface and subsurface samples in Miers Valley differed markedly. For example, the quantities of DNA extracted from surface samples in MVS1 transect A ranged from 9.1ng/µl to 536.1ng/ul. However, no A transect subsurface samples contained any detectable quantities of double stranded DNA using the same method of quantitation. DNA was present in amplifiable quantities in some of the subsurface samples, as 16S rDNA was successfully amplified in some subsurface samples. Therefore, based on the quantities of DNA extracted,

microbial abundance was greater in surface soils from Miers Valley. These results did not conclude with Cameron et al. (1968, 1970) findings.

In situ ATP analysis conducted by Professor Don Cowan on MVS1 and MVS13 transect samples (Appendix 3) typically showed higher levels of RLUs in surface samples compared to subsurface samples. The highest RLU value measured (42839) was in MVS1 centre sample, taken from the top 2cm of soil, directly beneath the seal carcass. *In situ* ATP analysis of the sample collected 5cm below the MVS1 centre sample indicated an RLU value of 10385, approximately four times lower. These ATP results further conflicted with Cameron et al (1968, 1970) results, and indicated that microbial abundance of samples used in this research was higher in surface samples compared to samples taken 5cm and 10cm below the soil surface.

Both Aislabie et al. (2001) and Cameron et al. (1968, 1970) utilised culture techniques to quantify microbial abundance in Dry Valley soils. Previous research into Antarctic microbial abundance indicated that different types of media gave differing viable counts of microorganisms by up to an order of magnitude. Therefore, conflicting levels of microbial abundance observed in surface and subsurface samples by Aislabie et al. (2001) and Cameron (1968, 1970), may be the result of the different media used. ATP analysis was shown in previous research to reflect the level of microbial activity, rather than microbial abundance (Atkinson et al., 2000). However, given the generally higher quantities of DNA extracted from environmental samples high in ATP, this method appeared to be a valid technique for estimating trends in microbial abundance. ATP analysis in this thesis research did not attempt to quantify the level of microbial abundance in samples. Future research will involve the quantification of microbial abundance from ATP data and microscopy techniques.

4.3 Microbial diversity detected by DGGE analysis

DGGE was used in this thesis to view microbial diversity of eukaryotic and prokaryotic organisms, and as a method of isolation for further sequence analysis of DNA bands of interest. The hypothesis of this thesis research stated that microbial diversity would differ in environmental samples where seal matter had increased the levels of carbon and nitrogen present, compared to microbial

communities present in Miers Valley control samples. Assuming the hypothesis is correct the environmental samples taken in closer proximity to the seals, would therefore display the highest microbial diversity. Diversity in DGGE gel analysis was determined by the number of bands present. Anaylsis was based on the assumption that 16S rDNA amplicons from different species of organisms will migrate to different positions in a denaturing gradient gel, due to differences in nucleotide sequence (Muyzer et al., 1993). Banding patterns in DGGE gels are not always indicative of microbial diversity within a sample population, examples of this are discussed in Sections 1.9.1 and 4.4.

4.3.1 Optimisation of the DGGE methodology

Bacterial diversity of PCR amplified 16S rDNA segments was viewed usıng DGGE for environmental samples taken from the Miers Valley sampling transects MVS1, MVS3, MVS13, and control transect MVT. Various methods were trialled to optimise DGGE in terms of band abundance, density and separation of bands. Given the bacterial diversity contained within the environmental samples, and the apparent differences between sample transects (such as the range of gradient needed to encompass all bands in the population), optimisation was not sample specific and was applied to standard methods used for all DGGE.

Optimisation methods included increasing the concentration of DNA template used in PCR amplification from 10ng in a 50µl reaction, to 30ng in a 50µl PCR reaction, and increasing the total number of PCR amplification cycles from 28 cycles to 31. These changes were found to increase the strength of bands visualised by DGGE, but did not appear to influence the number of bands able to be visualised (shown in Chapter 3, Figure 3.4).

An optimal gradient of denaturant within DGGE acrylamide gels was not determined. For DGGE analysis of bacterial diversity, a gradient of 30-65% was typically used. DGGE for the purpose of band isolation (Chapter 3, Figures 3.16, 3.17, 3.18) was found to be more successful with a decreased gradient range of 30-55%.

Two staining methods used to visualise DGGE gels were trialled and compared; SYBR Green and ethidium bromide. Other methods of visualising bands of DNA in acylamide gels are available, such as silver staining which can be used in conjunction with ethidium staining, however, this method was not tried. Both the SYBR Green stained (Chapter 3, Figure 3.5) and ethidium stained (Chapter 3, Figure 3.7) images, taken using a Fischer Biotech viewing platform, appeared to be relatively clear. Upon visual analysis, it appeared that the SYBR Green stained gel had more prominent bands than replica samples stained using ethidium bromide. Although the difference in band intensity could possibly be attributed to other factors such as loading error, the overall difference in clarity between gels rendered this highly unlikely. The sensitivity of SYBR Green for detecting double stranded DNA in agarose and polyacrylamide gels, was reported as being at least twenty-five times more sensitive than results achieved with ethidium bromide (Molecular Probes Product Information). A number of impurities could be seen in the photographic image of the ethidium stained gel. These impurities appeared to be dust, most likely obtained from filter paper used in the transfer of the gel. The blotch present in sample lane B2 was most likely caused by incomplete diffusion of concentrated ethidium bromide in the stain mix. Such imperfections, thus, were the result of bad technique. Thorough mixing of freshly made staining solution and washing of gels using ddH_2O were measures that were adapted to alleviate these problems. The decision as to which gel staining method was more suitable for the purposes of this thesis, was based solely on visual images as analysis of the two gels using GelCompar software was conducted at a later date. After direct comparison of the two methods using MVS3 environmental samples, the difference in sensitivity of the SYBR green method, in comparison to ethidium bromide was deemed not great enough to warrant the increased cost and handling difficulties involved with the use of SYBR Green stain. Subsequently all gels were stained with ethidium bromide.

Individual analysis using GelCompar software of the two DGGE gels of MVS3 sampling transect stained with SYBR Green and ethidium bromide indicated different groupings of similarity in comparison to one another. These differences observed with GelCompar analysis were not anticipated upon visual analysis, and highlighted the need for consistency with staining techniques used for all gels

during the course of this research. For example, the track representing environmental sample B3 shared 58% similarity with the track representing MVS3 centre sample in the SYBR Green stained gel. However, in analysis of the DGGE gel ethidium bromide stained, the track representing sample B3 appeared separate from all other tracks, sharing only 22% similarity with any tracks. Only the tracks representing environmental samples A1 and A3 showed the closest similarity to one another in the analysis of both types of stained gels, differing in the values of similarity given by approximately 3%. Direct comparison of the two DGGE gels using GelCompar analysis showed only three tracks representing MVS3 environmental samples centre, D1, and D3 as the only replica samples stained using the two methods that aligned most closely with one another. These samples, however, aligned with only 62%, 64%, and 70% similarity, respectively. Such large differences between the gels highlighted with GelCompar analysis were not anticipated upon visual analysis.

Ethanol precipitation of PCR product used in DGGE proved to be a successful method of enhancing band abundance and density. A clear difference in the number of bands able to be visualised was observed when comparing two DGGE of bacterial amplicons amplified from MVS13 transect environmental samples, one without concentrate DNA (Chapter 3, Figure 3.10), the other using PCR product that had been concentrated approximately 4.5 times (Chapter 3, Figure 3.11). The use of concentrated PCR product in DGGE analysis of bacterial diversity, however, was found to inhibit isolation of DNA from a single band of interest (data not shown). Successful isolation of specific bands was only achieved using PCR product that had not been concentrated by ethanol precipitation. Concentrated PCR product was therefore only used for those sample sets that failed to produce visually comparable DGGE using amplicons that had not been concentrated, such as MVS13 and MVT. GelCompar analysis two DGGE gels containing amplicons at different concentrations (as shown in Chapter 3, Figures 3.9 and 3.10) showed them as being two separate groups, with very little similarity between tracks representing environmental samples originating from the same transect point. These results indicated the need for consistency regarding the concentration of amplicons used in DGGE analysis, as significant differences in

concentration, such as those demonstrated, resulted in DGGE that was incomparable using these methods.

4.3.2 DGGE analysis of MVS and MVT bacterial 16S rDNA in environmental samples

All environmental samples from MVS1, MVS3, MVS13, and MVT sampling transects where DNA was successfully extracted were analysed by DGGE to view bacterial diversity within the sample populations. Single bands in a DGGE were generally ascribed to a single species in a population (Muyzer et. al. 1993). However, exceptions have been known to occur where one species may be represented by more than a single band (Klappenbach et al., 2001). The presence of organisms represented by multiple bands can only be determined through sequence analysis of isolated band DNA. This was a limitation in this thesis research, as isolation of DNA from a single band in DGGE typically proved unsuccessful. Sequence data of DNA band isolates was gained for only a few samples from MVS1 environmental sampling transect and (shown in Chapter 3, Table 3.3).

All DNA bands that were isolated for nucleotide sequence analysis were selected by visual analysis of DGGE. Bands of particular interest were those present only where it was thought that contamination by the seal carcass would have a direct affect, such as the centre samples. No DNA bands were successfully isolated from MVS13 or MVS3 samples.

Comparison between DGGE gels of MVS1 environmental samples indicated a difference in diversity according to whether or not the sample was taken from the surface or subsurface. DGGE gels of the MVS1 transect revealed a band present only in surface samples (Chapter 3, Figure 3.16, band A1; Figure 3.17, band B1, B2; Figure 3.18, band C1). BLASTn analysis of isolate C1 (Chapter 3, Table 3.2) identified the nearest phylogenetic neighbour as being *Brevibacterium linens*. BLASTn analysis of the nucleotide sequence of B5 DNA isolate, (a predominant band found at the soil surface directly beneath MVS1 seal carcass but not found in the soil sample taken 5cm below the soil surface, directly beneath the seal carcass) also indicated the nearest phylogenetic neighbor as being *Brevibacterium linens*.

DNA from band isolates B5 and C1 migrated at very different positions along the denaturing gradient. DGGE analysis of the DNA isolates with a common reference lane indicated that they were positioned at approximately the same position on the gel as the original band-stabs, and each was represented by a single band. Therefore, the likelihood of contamination due to the selection of more than one band was minimal. The presence of more than one band in DGGE analysis for a single species has been noted previously, and may be due to different numbers of rRNA operons present in an organism that display a certain degree of intra-genomic sequence diversity (Klappenbach et al, 2000; 2001). Polymorphisms in the 16S rRNA genes of five isolate cultures of *Brevibacterium linens* were reported by Lima and Correia (2000). Lima and Correia reported that genome sizes for the five cultures of *Brevibacterium linens* ranged from 3.2 and 3.9 Mbp and that at least four rRNA operons were present on the genome of the *B. linens* strains that were studied. PCR amplicons, generated through simultaneous amplification of all 16S rRNA gene copies on a genome, were used in DGGE analysis. Hence, the presence of more than one band representing different strains of *Brevibacterium linens*, or different rRNA operons within a strain was not implausible. *Brevibacterium linens* was identified by BLASTn analysis of clones isolated from MVS1 centre sample (as shown in Section 3.10), further confirming its presence in the MVS1 sampling transect.

A band indicated as A2 (Chapter 3, Figure 3.16), B4 (Chapter 3, Figure 3.17) and C2 (Chapter 3, Figure 3.18) was apparent only in subsurface samples. BLASTn analysis of sequence data for band DNA isolates B4 and C2 (both found to be at the same position along the denaturing gradient), identified the nearest phylogenetic neighbours as being two separate classes of organism (*Actinobacteria* and *γ-proteobacteria* respectively). The overall quality attributed by the University of Waikato Sequencing Facility of the B4 nucleotide sequence was reported as being poor, with a value of 77, and a read length of 27. The overall quality for C2 isolate sequence data was described as good with a value of 93 and a read length of 159. Given the poor quality of sequence data gained for the DNA isolate B4, the assumption that the DNA of band isolates B4 and C2 represented more than one species, can not be concluded. Future work will

include the repeat isolation of band DNA to determine the nearest phylogenetic neighbour of bands more conclusively.

GelCompar analysis of the three bacterial DGGE (shown in Chapter 3, Figure 3.19) containing MVS1 transect samples indicated 2 groups of samples {B1-2, B1-3, D3-1*} and {D1-2, D2-2} that were 100% similar within their groupings. This degree of similarity was not displayed for any other samples visualised using DGGE. Both groups consisted of surface and subsurface samples taken at the same horizontal location on the transect with the exception of sample D3-1*. The similarity of this sample to the two B1 surface and subsurface samples was perhaps even more remarkable when considering the positions of the samples on the transect, and that most of the MVS1 samples analysed using GelCompar tended to group according to the transect line they were sampled from. One could argue, based on the similarity displayed between B and D transect samples that these groupings of similarity reflected the manner with which the three DGGE gels were analysed , rather than transect line. This argument was given further weight by the lack of any obvious relationship between horizontal position of a sample and similarity between samples.

A lack of any apparent gradient of similarity along transects was also apparent for MVS1 sampling transect, and MVS13 transect with the exception of samples B1-A, B2-A, and B3-A. The centre samples from MVS3 and MVS13, both showed the greatest similarity to sample D3 from their respective transects. MVS13 had the highest degree of similarity between all environmental samples in the transect. No sample in the MVS13 sampling transect was less than 44% similar to any other sample in the transect.

MVS1 environmental sample B1-3 appeared to be mislabelled. No explanation explaining what depth this sample was collected was presented in the sampling notes, and no other samples were labelled this way. Therefore, it was not known which position and depth along MVS1 transect it was taken. The sample was included in analysis, however, for completeness.

DGGE analysis of bacterial diversity present in the transect control samples indicated a reasonably high degree of similarity, determined both visually, and upon analysis using GelCompar software. The eleven samples viewed appeared to have similar banding patterns, however, there was a large difference in the density of bands present between samples. Visual comparison of the MVT transect samples indicated much more similarity between MVT samples, despite the greater distances between samples and the differences in elevation, when compared with the bacterial DGGE of the three seal transects.

Comparison between the DGGE gel of MVT transect samples, with DGGE gels of each of the three MVS transects indicated that samples within the MVT transect shared greater similarity with each other, than any of the MVS environment samples. The validity of the control transect as a control for microbial diversity in the Miers Valley area could perhaps be questioned. Cameron et al. (1968, 1976) listed a number of factors both favourable and unfavourable that influence the population, density, abundance, diversity, complexity and size of life forms in the Dry Valleys. Among these factors, Cameron (1968, 1976) listed topography, edaphology, and geographic location as being important. Cameron (1968, 1976) stated that orientation of the area, salinity and pH were all factors that directly influence a microbial community. As pH, salinity and ionic composition were not tested for in the Miers Valley samples, any differences between sampling transects which may account for the differences in microbial communities observed, are not known.

The centre samples from MVS transects were thought to be most affected by the seal carcasses due to their close proximity. The soil surrounding seals MVS1, MVS3 and MVS13 were analysed extensively in this thesis research. A DGGE comparing samples taken directly beneath all seal carcasses sampled in Miers Valley (with the exception of MVS13) indicated that bacterial diversity was greater in MVS4, MVS7 and MVS11 samples compared to MVS1 and MVS3 (as shown in Chapter 3, Figure 3.26). Comparison between MVS1, MVS3 and MVS13 centre samples (as shown in Chapter 3, Figure 3.28) indicated that the greatest diversity was contained within MVS1 centre sample. MVS3 centre sample indicated very little diversity by comparison, but was more similar by

GelCompar analysis to MVS1cent than MVS13cent (shown in Chapter 3, Figure 3.29).

4.3.3 Reproducibility of DGGE as a method for comparing Antarctic bacterial diversity

No literature was found in the course of this research that had applied DGGE techniques to Antarctic environmental samples. Given the lack of any obvious primary source of organic matter in the form of plants, in the Antarctic Dry Valleys, the sources of organic nutrients sustaining these communities are likely localised as the result of point source contamination. These localised enrichments may result, potentially, in small pockets of microbial communities distributed throughout the soil. Given the small amount of soil used to extract DNA (0.5g) the potential for "hotspotting" (selecting for small pockets of microbial communities on some but not all occasions) was considered. The selection of variably distributed and sized microbial colonies could present a skewed view of microbial diversity within an area. To determine if hotspotting was a factor influencing DGGE analysis, nine replica DNA extractions of MVS1 centre sample were analysed using bacterial DGGE (Chapter 3, Figure 3.13). Visual analysis of the DGGE indicated that all extractions of MVS1 centre sample presented an identical banding pattern in terms of band presence, but nominal variations in band strength were seen. As DGGE in the scope of this study was used for qualitative rather than quantitative analysis, hotspotting appeared not to be a factor in DGGE analysis of that sample.

A number of other factors were found to influence the reproducibility of DGGE including

- The length of time an acrylamide gel was left to set; too short and the gel would not set properly, resulting in the loss of PCR product; too long and the gel would start to dry out, resulting in fading of DNA bands in the centre of the DGGE when visualised (data not shown). This was possibly caused by the slow leaching of acrylamide from the centre outward in the gel.
- Crystallisation of urea crystals in the 100% denaturant solution.

The most significant factor influencing DGGE reproducibility was imperfections that occurred in the denaturing gradient when a DGGE gel was poured too fast (data not shown). This resulted in the migration of amplicons to different positions on a gel than would otherwise occur, seriously affecting latter analysis. This may provide an explanation for alignment difficulties between gels, and may explain differences in BLASTn analysis of nucleotide sequences from band DNA isolates such as those seen between band stabs B4 and C2 (Chapter 3, Figures 3.17, 3.18 respectively).

Research conducted by Watanabe et al. (2001), indicated that the primers utilised in this research may not select for all bacterial groups evenly. Sequences of a number of newly recognised groups of bacteria are diverse and include mismatches to the universal bacterial primers used, likely reducing amplification efficiency. It is likely then that these groups would hardly appear in DGGE fingerprints (Watanabe et al., 2001), indicating that DGGE analysis may not be representative of all bacterial species present in the Antarctic soil samples.

4.3.4 Archaeal and Eukaryotic diversity in Miers Valley environmental samples

DGGE analysis of Eukaryotic populations present in Miers Valley environmental samples was unsuccessful. Positive PCR amplification using universal eukaryotic primers in MVS1, MVS3, MVS13 centre samples and MVT6 indicated the presence of eukaryotic DNA in these samples. Further investigation into eukaryotic diversity in MVS13 transect environmental samples and surface centre samples from MVS1 and MVS3 sampling transects was hindered by the presence of a high molecular weight contaminant present in eukaryotic primer 519 Fgc (described in Appendix 1) which contained a GC clamp for DGGE analysis.

Diversity, derived from the use of eukaryotic primers, was unable to be viewed due to the failure of PCR products to produce any visible banding patterns in a DGGE gel. As no sequence data was obtained, it was unknown if the eukaryotic DNA PCR amplified was of seal origin, or from eukaryotic microorganisms present in the environmental samples, or a combination of both.

Future research involving repeat DGGE analysis, using uncontaminated reagents is needed to investigate the diversity of Eukaryotic DNA present in Miers Valley environmental samples.

DGGE analysis of Miers Valley environmental samples using primers specific for Archaeal 16S rDNA indicated low diversity of Archaeal DNA in two Miers Valley environmental samples (MVS13 and MVT7)

Analysis of archaeal DNA, using archaeal 16S rDNA specific primers in MVS13 and MVT sampling transects was hindered by the presence of a high molecular weight contaminant, as depicted in Chapter 3, Figures 3.35 and 3.36.

Isolation of DNA bands from MVS13 environmental centre samples to identify archaeal 16S rDNA present was unsuccessful, as all bands stabbed failed to be single isolates.

4.3.5 Validity and reproducibility of DGGE using the GelCompar system for analysis

The visually identical banding pattern shown in the DGGE containing bacterial 16S rDNA amplicons from replica extractions of MVS1 centre sample (Chapter 3, Figure 3.13), provided an opportunity to determine the approximate accuracy of GelCompar analysis. All nine replica extractions contained the same concentration of template DNA in the PCR reactions. It was therefore surprising that GelCompar analysis indicated up to a 28% difference in the similarity between banding patterns in the nine tracks. The highest similarity any two tracks displayed was 92%. Using automatic band detection, and band comparison, it could therefore be concluded that GelCompar analysis, using the parameters specified, had a standard error of up to 28%.

The diversity observed by GelCompar analysis, of the nine replica extractions was attributed to the level of band detection utilised to automatically detect and compare bands for analysis. The differences in the number of bands detected in each track were shown in Chapter 3, Figure 3.14. The nine replica extractions appeared visually to have an identical banding pattern, but showed variation in the

densities of bands present at the same positions along the gradient for different extractions. The sensitivity of band selection, determined by minimal profiling percent and minimal area percent, was decreased from the default setting of 5.00 and 0.50 respectively, to values of 6.00 and 0.60. The default values were found to detect bands that were not visible to the naked eye, and where imperfections occurred in the DGGE gels or image. The decreased sensitivity of band selection, as a result, may not have selected for the fainter bands present and hence a certain degree of error was involved in analytical applications.

A number of problems aligning gels in a database using the standard reference lane were encountered. A lack of accurate alignment may account for the grouping in similarity of samples according to DGGE gels for MVS1 transect samples, and indeed may account for the lack of similarity determined between transect control samples and the three seal transect samples. Problems encountered during alignment were not only due to variance in the visibility of reference lanes and hence the need for the insertion of a reference lane onto the photographic images of some DGGE; DGGE images that varied in focus, and in particular zoom also proved difficult to align. As mentioned in Section 4.2.3, variances in the denaturing gradient that occurred while pouring an acrylamide gel may resulted in the melting of DNA strands at variable locations between DGGE, causing alignment problems.

The similarity between tracks was calculated using the *Dice*, band-based similarity coefficient, using the following equation:

$$\frac{2\eta_{AB}}{\eta_A + \eta_B}$$

Where $2\eta_{AB}$ represented the number of bands common for both A and B, η_A represented the total number of bands in A, and η_B represented the total number of bands in B. Similarity was therefore calculated in a pairwise manner, and the total number of tracks being compared had no effect on the similarity values presented. However, the fewer the numbers of bands detected in each track, the higher the values of similarity indicated. Given the large number of DNA bands viewed in

Miers Valley environmental samples, this may have misrepresented similarity values indicated between tracks. This may have resulted in the grouping samples with very few apparent bands, possibly as a result of an error in methodology, as being more similar. An example of this may be seen in DGGE analysis of MVS1, MVS3, and MVS13 environmental centre samples shown in Chapter 3, Figure 3.28 and 3.29. Very few bands were visually apparent in the MVS3 centre sample, however, the track representing MVS1 centre sample was indicated as being more similar to MVS3 than MVS13, despite visual analysis which seemed to contradict these findings.

The method of referencing DGGE using a standard reference lane, proved to be unreliable. On occasion reference lanes could not be visualised. Perhaps continual freeze thaw of the reference combined PCR product was a factor and aliquots of reference solution should be used in the future. Given the large number of DGGE conducted over the course of this research, multiple analysis of each sample was generally conducted. Gel banding patterns of replica samples where the reference lane was easily detected were aligned visually, and a reference lane was added manually using Adobe Photoshop Elements 2.0, to enable GelCompar analysis.

4.3.6 Summary of microbial diversity in Miers Valley environmental samples determined by DGGE analysis

DGGE analysis of bacterial populations present in the environmental samples supported the proposed hypothesis, that bacterial diversity was different in areas likely to be more affected by seal organic matter. Bacterial diversity was determined by the number of apparent bands represented in a sample. In MVS1 seal transect, the bacterial diversity present in the surface and subsurface environmental samples taken from directly beneath the seal carcass, indicated little similarity with other samples in the transect. In MVS3 and MVS13 transects, the centre samples displayed the closest similarity to environmental samples taken 50cm from the centre of the seal carcass, representing the next closes sampling distance to the seal in the transect.

As band isolation was successful only for a few samples from the MVS1 sampling transect, further identification of bands from other sampling transects is needed.

Given the apparent bacterial diversity contained in the centre samples of seals other than MVS1, MVS3 and MVS13, sampled in Miers Valley, future research should perhaps be extended to include a greater number of seal transects.

Eukaryotic and Archaeal DNA were detected in some Miers Valley environmental samples. However, problems in the PCR amplification of eukaryotic and archaeal SSU ribosomal DNA meant that analysis was unsuccessful.

4.3.7 Analysis of sequence data from MVS1 and MVT10 cloned 16S rDNA

A total of 384 colonies containing cloned inserts were picked for sequence analysis from four sample clone libraries (MVS1, MVS3, MVS13, and MVT10). The genomic DNA used to produce the 16S rDNA insert fragments for the three seal clone libraries (MVS1, MVS3 and MVS13) was gained from transect centre samples taken from the top 2cm of soil directly beneath the seal carcasses. These centre samples contained a high abundance of microorganisms compared to other samples in the transects, based on *in situ* ATP analysis (Appendix 1) and quantitation of total genomic DNA extracted.

The genomic DNA used to produce the 16S rDNA insert fragments for the clone library MVT10, was gained from control transect sample MVT10. Bacterial DGGE analysis of this sample (Figure 3.24) indicated a relatively diverse bacterial population.

Clone libraries MVS3 and MVS13 underwent restriction endonuclease analysis by members of the Cary Laboratory, College of Marine Studies, University of Delaware, using restriction enzymes HinP1I and *Rsa*I. Replicate organisms were identified, and removed. Cloned inserts from MVS3 and MVS13 libraries have yet to be sequenced.

A total of 192 cloned inserts were partially sequenced. Ninety six of these, the cloned inserts gained from MVS1 clone library, were sequenced in both the forward and reverse direction. Sequence data containing less than 200 base pairs were removed resulting in a total of 152 sequences for individual clone inserts remaining.

Comparative 16S rDNA analysis showed that none of the cloned insert sequences for MVS1 and MVT10 libraries were identical to any of the known 16S rDNA sequences in the nucleotide databases (BLASTn) (Chapter 3, Tables 3.4 and 3.5). Most of the bacterial isolate sequences contained within the MVS1 and MVT10 clone libraries showed greater than 86% similarity, but less than 99% similarity to already known sequences present in the databases, indicating the presence of a number of new species. Based on complete 16S rDNA sequences, a binary similarity value of 97.5% indicated the presence of two distinct species (Stackebrandt and Geobel, 1994). This value has recently been questioned, however, based on observed differences in 16S rRNA genes of some single species of Archaea (Amann et al., 2000). Therefore, the use of a 97.5% limit for species separation should be treated with caution (Sjöling and Cowan, 2003).

Comparison of BLASTn analysis of nucleotide sequences from MVS1 and MVT10 cloned inserts, indicated very little similarity in bacterial diversity between the two environmental samples. Approximately 64% of the total number of sequenced clone inserts were identified by BLASTn analysis as being unknown bacteria, compared to only 4% of cloned inserts from MVS1 clone library.

Four dominant genera of organisms were identified in bacterial cloned inserts from MVS1 library; *Brevibacterium, Arthrobacter, Planococcus,* and *Psychrobacter*. No bacterial cloned inserts from MVT10 library, however, were identified by BLASTn analysis as being members of these genera.

The *Brevibacterium* genus was identified in 14 out of 95 clones in the MVS1 clone library. *Brevibacterium* have been isolated from Antarctic soils previously (Johnson et al., 1978). *Brevibacterium* are *Corneybacterium*, which include members of the *Arthrobacter* and *Cellulomonas* genera. *Corneybacterium* were reported as making up 23% of bacterial isolates identified by Johnson et al. (1978) from Cameron's Antarctic collection. Eleven of the 14 clones identified as being of the *Brevibacterium* genus, were *Brevibacterium linens* species. *B. linens* has been isolated from the surface of several types of cheese (Lima and Correlia, 2000). Due to their importance in cheese ripening, many strains classified as *B. linens* have been deposited in culture collections, or reported in papers (Lima and

Correlia, 2000). To date, 132 nucleotide sequences are present for *B. linens* in the GenBank database. *B. linens* was demonstrated to tolerate high salt concentrations (8-20%), desiccation for extended periods, carbohydrate starvation, and was shown to be capable of growth at a broad pH range (Boyaval et al., 1985). *B. linens* organisms display high proteolytic activity, varied lipolytic activity and aminopeptidase activity. It is, therefore, likely that they would be able to utilise lipids present in seal blubber, and may be a reason for their relatively high occurrence directly beneath the carcass of MVS1.

Brevibacterium linens is phenotypically similar to *Arthrobacter globiformis* (JGI, 2004), both of which are members of the Class *Actinobacteria*, and represented 36% of the cloned inserts in the MVS1clone library. Seventeen out of 95 clones isolated from MVS1 clone library were identified as belonging to the *Arthrobacter* genus. Organisms of the genus *Arthrobacter* are Gram-positive, aerobic bacteria (Madigan et al., 2003) that have been isolated previously in Antarctic soils (Johnson et al, 1978; Lonhienne et al., 2001), *Arthrobacter* species have been found to be abundant in soils throughout the world (Felske et al., 2000). *Arthrobacter* species have been demonstrated to survive periods of desiccation as long as six months, and nutrient starvation for very long periods (Boyaval et al., 1985; Madigan et al., 2000), which perhaps explains their survival in the harsh Antarctic environment.

Members of the phylum proteobacteria were represented by the classes α, and γ-proteobacteria, and constituted 32% of bacterial isolates from the MVS1 clone library. Genus of *γ-proteobacteria* identified in the library included *Psychrobacter* genus which represented 17 out of 96 clones in the MVS1 library. At least four species of *Psychrobacter* have been isolated from Antarctic soils (Bozal et al., 2003) and Antarctic sea ice (Bowman et al., 1997). BLASTn analysis of 12 of the 154 nucleotide sequences indicated a blackwater bioreactor bacterium, a member of the *γ-proteobacteria,* as the nearest phylogenetic neighbor. The blackwater bioreactor bacterium was identified as a member of the *Xanthomonadaceae* family (Morgan et al, 2002), of which other members have been isolated from Antarctic soils (Ni, 1986).

Members of the class *Bacilli* represented 19% of all bacterial isolates from MVS1 library. Members of the *Planococcus* and *Bacillus* genera, constituted the majority of organisms represented in the MVS1 library in this class. Both Genera have been previously identified from Antarctic environmental samples (Johnson et al., 1978; Bowman et al., 1997; Sheridan and Brenchley, 2000).

This study was unlikely to have exhausted the diversity available in the clone libraries, as there were some cloned 16S rDNA genes that were encountered only once.

4.4 Summary of microbial diversity determined by analysis of 16S rDNA

The relative ratios of rRNA genes in clone libraries, and the strength of bands in DGGE, do not necessarily correspond to the abundance of DNA representing organisms in the environmental samples. Factors such as differential efficiencies of PCR amplification or cloning (Suzuki and Giovannoni, 1996), or different numbers of rRNA operons in different organisms (Klappenbach et al, 2000) can influence the proportions of rRNA phylotypes in clone libraries (de la Torre et al, 2003). Organisms represented may be extremely easy to lyse, or more accessible to DNA extraction. Alternately, some species present in the environmental samples may be difficult to lyse, and using the PSC-B method of DNA extraction may not be represented. Therefore, the methods of analysing microbial diversity used in this thesis are qualitative rather than quantitative techniques.

Based on BLASTn sequence analysis of the DNA isolated from bands in DGGE gels of MVS1 environmental samples, and bacterial organisms identified from sequence analysis of MVS1 cloned inserts, the two methods appeared comparable. *Brevibacterium linens* was identified in both the MVS1 clone library and DGGE analysis of MVS1 environmental samples. *Brevibacterium linens* has been shown to have four rRNA operons present on its genome (Lima and Correia, 2000) which may explain the prevalence of DNA from this species in DGGE and cloning analysis.

Future research will include phylogenetic analysis and comparisons between sequence data of cloned inserts from the four clone libraries; MVS1, MVS3, MVS13 and MVT.

4.5 Carbon and nitrogen levels in Miers Valley environmental samples

To test the proposed hypothesis that the mummified seal carcasses provided a source of organic carbon and organic nitrogen enrichment to surrounding soils, the percentage of total carbon and total nitrogen both organic and inorganic, and the levels of stable isotopes $\delta^{15}N$ and $\delta^{13}C$ were measured for the Miers Valley environmental samples.

4.5.1 Isotopic composition of carbon and nitrogen

Isotopic composition, specifically $\delta^{15}N$ and $\delta^{13}C$, have often been used in scientific research to trace food chains (Peterson and Fry, 1987; Raven, 1987; Handley and Scrimgeour, 1997). To interpret $\delta^{15}N$ in terms of sources and sinks, one must know the source $\delta^{15}N$ values, be able to quantify the fractions occurring between source and sink, and to be most effective, be able to estimate nitrogen fluxes (Handley and Scrimgeour, 1997). A value of $\delta^{13}C$ (-24.6 ± 0.2‰) for MVS3 seal tissue was given in ^{14}C dating of the sample. However, no value of $\delta^{15}N$ was measured for MVS3 seal tissue, and no values of $\delta^{15}N$ and $\delta^{13}C$ were measured for MVS1 and MVS13 seal tissue. This lack of data prevented any conclusive source to sink (tracer) relationships to be identified. However, the stable isotopic values given in the data could arguably be used as indicators of source when samples directly beneath the seal, and in close proximity to the seal, were compared with samples further away, and with control samples completely isolated from the site.

Four samples, from a total of 63 Miers Valley environmental samples analysed, had measurable values for $\delta^{15}N$, as depicted in Table 4.1. These samples were all collected from either directly beneath a seal carcass, or were the closest samples in proximity to the seal in the transect (50cm).

Table 4.1 Measurable values of $\delta^{15}N$ measured for Miers Valley environmental samples

Sample	Distance From the Approximate Centre of the Seal	$\delta^{15}N$ (‰)	%N
MVS1cent (surface)	0 cm	18.21	0.08
MVS13cent	0 cm	18.45	0.06
MVS13 - C3	50 cm	15.98	0.06
MVS13 - D3	50 cm	10.35	0.19

Those environmental samples where measurable levels of $\delta^{15}N$ were present appeared to be very enriched in $\delta^{15}N$ compared to average values recorded for forest soils (3.2‰) (Wada et al., 1981). Environmental samples high in ^{15}N have been isolated from algal felt in Antarctica (30.7‰), and in soil collected from a penguin rookery at Ross Island (28.6‰) (Wada et al., 1981). Wada et al. (1981) hypothesised that the enrichment found in the penguin rookery was due to microbial conversion of uric acid, obtained from the penguins diet of krill and fish, and other organic nitrogen to ammonia. The ammonia then evaporated with a high nitrogen isotope fractionation leaving nitrogenous material rich in ^{15}N. Given the cold climate of Antarctica Wada et al. (1981) stated that the slow rate of biogeochemical processes would allow for much greater fractionation of the nitrogen isotope, explaining the high ^{15}N enrichments. Antarctic seals have a similar diet to penguins, consisting of a high amount of krill and fish. A slow rate of microbial breakdown of organic nitrogen in the soil, and a high degree of fractionation is plausible in this case, and may well explain the high levels of enrichment observed underneath two of the three seals sampled, and in two of the four transect samples taken 50cm from the approximated centre of MVS13.

No measurable values of $\delta^{15}N$ were recorded for any of the MVS1 subsurface environmental samples.

The four environmental samples where measurable levels of $\delta^{15}N$ were detected also contained the most depleted values of $\delta^{13}C$ (Table 4.2).

Table 4.2 Four environmental samples with the most apparent depletion of $\delta^{13}C$

Sample	Distance From the Approximate Centre of the Seal	δ13C (‰)	%C
MVS1cent (surface)	0 cm	-8.90	1.39
MVS13cent	0 cm	-6.27	0.91
MVS13 - C3	50 cm	-15.55	1.34
MVS13 - D3	50 cm	-24.81	1.65

Environmental samples D3 and C3 had more depleted levels of $\delta^{13}C$ than the centre samples from MVS1 and MVS13 sampling transects, (-24.81‰ and -15.55‰ respectively). The level of $\delta^{13}C$ in MVS13 environmental sample D3 (-24.81‰) was comparable to representative values of soil organic matter (-26‰) described by Peterson and Fry (1987). The level of $\delta^{13}C$ measured in MVS13 transect environmental sample D3 (-24.81‰) was also similar to the level of $\delta^{13}C$ measured in MVS3 tissue. As no isotopic analysis was conducted on MVS13 tissue, and the level of $\delta^{13}C$ measured directly beneath MVS13 appeared much more enriched in $\delta^{13}C$ by comparison, it could not be concluded that MVS13 was the source of carbon enrichment.

Environmental samples from MVS3 sampling transect were all enriched for $\delta^{13}C$. Levels of $\delta^{13}C$ in MVS3 transect environmental samples ranged from -0.25‰ to -4.69‰. The MVS3 transect environmental sample most depleted in $\delta^{13}C$ was D3, with a value of -4.69‰.

The levels of $\delta^{13}C$ measured in four MVT transect environmental samples (MVT3, MVT5, MVT6 and MVT8), ranged from -0.57‰ to -2.99‰. Therefore, it appeared that many of the environmental samples taken from seal transects displayed $\delta^{13}C$ levels comparative with the range of $\delta^{13}C$ observed in MVT control samples.

Analysis of MVS1 surface and subsurface transect environmental samples, typically showed more enrichment of $\delta^{13}C$ in surface environmental samples compared to subsurface environmental samples.

Although neither transect line C3 or D3 was in the estimated direction of downstream (designated transect line A), one possible explanation for the high levels of enrichment in carbon and nitrogen compared to other Miers Valley

environmental samples is that they may lie in the predominant wind directions. In such a windy environment (due to the katabatic down-flow of air from the Transantarctic Mountains) wind-flow of seal particles in a predominant direction may act to enrich certain areas with more seal organic matter than others.

Further sampling to measure the levels of stable isotopes $\delta^{15}N$ and $\delta^{13}C$ in seal tissue of MVS1, MVS3 and MVS13, and a digestion of environmental samples in order to concentrate $\delta^{15}N$ to measurable levels, would be required to accurately trace the enrichment of the seal carcasses in the surrounding environment.

4.5.2 Total carbon analysis

Values of total carbon and nitrogen have been reported previously for Antarctic Dry Valleys soils (Boyd and Boyd, 1962; Cameron et al, 1968, 1970; Vishniac and Hempfling, 1979; Aislabie et al. 2001).

The percentage of total carbon in MVS1 environmental samples appeared to be significantly higher in samples taken 10cm below the soil surface, compared to samples collected from the first 2cm of soil from the surface. Analysis of environmental samples collected in a vertical sampling profile in Wright Valley, showed little variation in the percentage of total carbon present (0.02% - 0.03%) at different depths in the environmental samples (Aislabie et al., 2001). Baldock and Skjemstad (1999) stated that the highest concentration of soil organic matter was typically found at the soil surface, and a sampling depth of 10cm was recommended for determining %N and %C values for agricultural purposes.

The levels of total carbon in MVS1 surface and subsurface environmental samples were depicted in Figure 4.1. As no subsurface samples were collected from the MVT sampling transect, it is not known if the higher levels of carbon in subsurface samples were present normally in Miers Valley soils, or whether it was the result of carbon enrichment by the seal carcass. As the level of carbon present in subsurface samples did not appear to increase with closer sample proximity to the seal carcass, it is likely that the level of total carbon found in Miers Valley soils were typically higher.

The total carbon values reported by Aislabie et al. (2001) (0.2-0.3%) were minimal compared to those gained from the seal transects (0.35% - 10.91%) and were much lower than those found in the MVT environmental samples (0.39% - 0.62%). This possibly indicated that either Miers Valley contained soil that was much more enriched in carbon than Wright Valley, or, perhaps, that the analytical methods used to measure the percentage of total carbon were incompatible.

4.5.3 Total nitrogen analysis

Given that the nitrogen analysis of Miers Valley environmental samples did not indicate the proportions of inorganic and organic nitrogen (for example NH_4^+ and NO_3^-), comparisons between the levels of nitrogen in Miers Valley soils, and the levels reported for other Dry Valley locations are limited.

The highest value for total %N taken from Miers Valley seal transect samples was 0.08%. This was taken from directly beneath MVS1 (Centre). Interestingly, none of the subsurface samples taken in MVS1 transect showed any large increases in total nitrogen content when compared to surface samples taken at the same location. Two samples (A6-10, B2-10) showed an increase of 0.01, however with only two decimal places given, it can't be determined if this was a significant increase compared to surface samples A6 and B2 whose values were both 0.00. Aislabie et al. (2001) reported percentage of total nitrogen values ranging from 0.008% - 0.052%, with the lowest values of 0.008% and 0.005% taken from two samples in the top 5cm of soil from the surface. The largest value of 0.052 % was taken from 5-15cm below the surface.

Aislabie et al. (2001) results indicated that greater enrichment of nitrogen could be found 5cm below the soil surface. In the seal transect analysis, it can be concluded that this was not the case. From total nitrogen content, indicated by the %N figures, it was evident that the highest levels of nitrogen were present directly beneath the seals, or in those transect samples taken closest to the seals (i.e. A3, B3, C3, D3). Samples taken further away from the seals in the transect showed values similar to those found in the control samples (0.00 % - 0.01 %).

Values above 0.01 in all Miers Valley environmental samples were only observed in samples taken from directly beneath, or in close proximity to a seal carcass.

Soil $\delta^{15}N$ was not easily measured as there were not methods compatible with high sample throughput for analysing the $\delta^{15}N$ of separate forms of N in soils (Handley and Scrimgeour, 1997). This research would have benefited from measurements discriminating organic and inorganic forms of nitrogen.

4.5.4 Summary of carbon and nitrogen analysis

Surface environmental samples taken from directly beneath the three seal carcasses were enriched with carbon and nitrogen when compared to other environmental samples in the transects, and MVT environmental samples. However, based on analysis of Figures 3.39-3.44, there appeared to be no significant trend of increasing levels of carbon with closer proximity to a seal carcass. As $\delta^{13}C$ and $\delta^{15}N$ data was not sufficient to conclusively identify enrichment of environmental samples by seal organic matter, the direct enrichment of organic carbon from the mummified seals can not be proven. However, based on the highest values of total carbon and nitrogen, and $\delta^{13}C$ and $\delta^{15}N$, detected in samples taken from directly beneath MVS1 and MVS13 and in samples taken in the closest proximity to MVS13 carcass (C3 and D3), the enrichment of environmental samples surrounding the mummified seal can not be disproven.

It is important to realise that the majority of nitrogen and carbon entering the Dry Valleys ecosystem was likely to be derived from point sources, and hence would not be evenly distributed. Select areas that could have been contaminated with skua guano, or where there was lichen growth, may be more enriched than surrounding soils. Although this thesis research looked at a theorised point source of carbon and nitrogen enrichment, in the form of a mummified seal, other point sources of enrichment along the transect may still occur. Hence, there is a possibility that some Miers Valley environmental samples, even those from directly beneath the seal, may have other sources of enrichment, that may have potentially skewed the results.

4.6 Overall conclusions

In conclusion, this study addressed the hypothesis that mummified seal carcasses present in Miers Valley, the Antarctic Dry Valleys provided a source of organic carbon and nitrogen resulting in increased microbial abundance and a difference in microbial diversity observed in soils surrounding and directly beneath the carcasses.

An aim of this MSc thesis research was to observe soil microbial diversity in environmental samples surrounding mummified seals in Miers Valley, and compare these with samples collected from Miers Valley soils, where no obvious signs of organic contamination were detected.

DGGE analysis of bacteria present in MVT transect environmental samples displayed a high degree of diversity. Comparisons of bacterial populations present in MVT samples, with seal transect samples, indicated very little similarity. Cloning and sequence analysis of MVS1 centre, and MVT10 environmental samples, indicated a large difference between the diversity of cloned inserts identified in the two libraries. Six classes of bacteria were identified by BLASTn analysis of MVS1 cloned inserts. Four genera of bacterium (*Brevibacterium, Arthrobacter, Planococcus,* and *Psychrobacter*) were dominant in MVS1 cloned inserts. All of these genera have been described previously in Antarctic soils. The majority of cloned inserts from MVT10 were identified by BLASTn analysis as unknown bacteria. None of the genera of bacteria identified by BLASTn analysis of MVS1 cloned inserts were identified in sequence analysis of MVT10 sequenced cloned inserts.

This MSc thesis research also aimed to compare microbial diversity between environmental samples within a sampling transect, and between sampling transects, in relation to the levels of carbon and nitrogen measured, taking into consideration the distance and direction of the sample from the seal.

DGGE analysis of bacterial populations present within seal transects displayed no gradient of similarity with proximity of the sample to the seal carcass. In MVS1

seal transect, the bacterial diversity present in the surface and subsurface environmental samples taken from directly beneath the seal carcass, indicated little similarity with other samples in the transect. In MVS3 and MVS13 transects, the centre samples displayed the closest similarity to environmental samples taken 50cm from the centre of the seal carcass, representing the next closest sampling distance to the seal in the transect.

There appeared to be an enrichment of carbon and nitrogen in environmental samples taken surrounding MVS1 and MVS13 seal carcasses. Less enrichment was observed in environmental samples surrounding MVS3 carcass.

The highest levels of total carbon were measured in MVS1 subsurface samples. Based on *in situ* ATP analysis, and the quantities of genomic DNA extracted, the abundance of microorganisms, appeared to be greater in surface environmental samples compared to subsurface samples. Bacterial diversity, determined by DGGE analysis indicated a difference in the diversity of bacterial DNA present in surface and subsurface samples. Based on these findings the level of carbon present in an environmental sample appeared not to influence the abundance of microorganisms, but may have influenced the diversity of bacteria present.

The levels of nitrogen in Miers Valley environmental samples were typically low. Samples showing the highest levels of nitrogen were all collected from surface environmental samples, taken directly underneath a seal carcass, or in the closest proximity to a carcass along a transect line. These samples contained the highest quantities of double stranded DNA extracted. ATP analysis of MVS1 transect samples, indicated a higher abundance of microorganisms with closer proximity to the seal carcass, in comparison to transect samples taken at a greater distance. Bacterial diversity determined by DGGE analysis, did not appear to increase with closer proximity to the seal. Environmental samples taken closer to the seal carcasses appeared to have higher levels of nitrogen, and a greater abundance of microorganisms compared to samples taken further away from the seal carcasses.

The hypothesis of this thesis research, that microbial abundance would be greater and a difference in the diversity of bacteria would be observed in environmental

samples enriched with seal organic matter, was determined to be correct. Microbial abundance was found to increase with enrichment of nitrogen surrounding MVS1 and MVS13 seal carcasses. Molecular analysis of 16S rDNA indicated large differences in bacterial populations observed using sequencing and cloning, and DGGE techniques.

This thesis effectively described the utilisation of cloning and sequence analysis, and denaturing gradient gel electrophoresis to investigate microbial diversity in Antarctic environmental samples. This study gave a greater understanding in microbial ecology in Miers Valley Antarctica.

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Appendix 1

List of primers used in thesis research:

Primer name	Nucleotide Sequence	Reference
338Fgc	5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC TCC TAC GGG AGG CAG CAG 3'	(Muyzer et al., 1993)
519RC	5' ATT ACC GCG GCT GCT GG 3'	(Muyzer et al., 1993)
338F	5' TCC TAC GGG AGG CAG CAG 3'	(Muyzer et al., 1993)
Arc1F	5'-CCAGGCCCTACGGGGCGCA-3'	(Øvreås et al., 1997)
Arc2R	GTGTGCAAGGAGCAGGGAC	(Ron Ronimus, personal communication)
Arc3R	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CTG TGC AAG GAG CAG GGA CG	(Ron Ronimus, personal communication)
(Arc 931f)	AGGAATTGGCGGGGGAGCAC	(Jackson et al., 2001)
77 1522R	5'- AAGGAGGTGATCCARCCGCA -3'	(Johnson, 1994)
149 5F	5'- CCGGTTGATCCTGCCGG -3'	(Reysenbach et al., 2000)
Euk A	5'- AACCTGGTTGATCCTGCCAGT -3'	(Medlin et al, 1988)
Euk B	5'- GATCC(AT)TCTGCAGGTTCACCTAC -3'	(Medlin et al, 1988)
EukAGC	5'CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCG CCCCAACCTGGTTGATCCTGCCAGT 3'	(Medlin et al, 1988)
Euk 516 (R)	5'CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCG CCCCACCAGACTTGCCCTCC 3'	(Inacio et al, 2003)
Eub A (R)	5' AAG GAG GTG ATC CA(ACGT) CC(AG) CA 3'	(Medlin et al, 1988)
Eub B (F)	5' AGA GTT TGA TC(AC) TGG CTC AG 3'	(Medlin et al, 1988)
M13 F	5' –GTAAAACGACGGCCAG- 3'	(Invitrogen Topo TA Cloning, Version N)
M13 R	5' –CAGGAAACAGCTATGAC- 3'	(Invitrogen Topo TA Cloning, Version N)

Appendix 2

List of Media Used

SOC Medium

Per litre:	
Tryptone (Becton Dickinson, Cockeysville, U.S.A.)	20g
Yeast extract (Becton Dickinson, Cockeysville, U.S.A.)	5g
NaCl	0.5g
Volume made up to 1L with deionized H_2O	

20ml of sterile 1 M solution of glucose was added after the media had been autoclaved.

LB (Luria-Bertani) Medium

Per litre:	
Tryptone	10g
Yeast extract	5g
NaCl	10g
Volume made up to 1L with deionized H_2O	

Approximately 0.2ml of 5 N NaOH was added to adjust the pH to 7.0. Media was seterilized by autoclaving.

For LB plates, 15g of agar was added to the medium before autoclaving. Agar was allowed to cool to approximately 60°C and 20 mL was poured aseptically into 90 mm Petri plates.

Appendix 3

ATP Analysis of MVS transects.

Transect	Sample	RLU 1	RLU 2	Transect	Sample	RLU 1	RLU 2
MVS1	A1-1	5409	7169	**MVS13**	A1	7778	10887
	A1-2	1371	921		A2	3856	4781
	A2-1	20284	22546		A3	15975	19707
	A2-2	6144	2767		B1	2917	3546
	A3-1	12067	18093		B2	3298	3718
	A3-2	964	2258		B3	3506	3460
	A4	8304	9079		C1	1050	2635
	A5	4703	5948		C2	3457	6311
	A6	6122	4098		C3	9650	11198
	A9	11652	13356		D1	7826	5293
	A10	21962	12232		D2	21635	14803
	B1-1	7519	6911		D3	5252	3617
	B1-2	717	646		Centre	2640	2129
	B1-3	179	127				
	B2-1	6413	8293				
	B2-2	1071	832				
	B3-1	21290	23852	**MVT**	1		806
	C1-1	6399	9872			1016	1299
	C1-2	542	796		2	1415	5030
	C2-1	14562	9755		3	3203	2627
	C2-1*	1710	1486		4	2816	3892
	C2-2	-	-		5	6239	7349
	C3-1	11070	11835		6	12277	17323
	C3-1*	4743	9845		7	8107	15969
	C3-2	724	685		8	14580	19686
	C4	12639	12657		9	14419	25973
	C5	1133	268		10	3185	2972
	D1-1	3361	5917		11	30034	27691
	D1-1*	648	1098		12	2955	1211
	D1-2	397	416				
	D2-1	12249	9706				
	D2-1*	602	621				
	D2-2	-	-				
	D3-1	15386	30789				
	D3-1*	1218	1626				
	D3-2	-	-				
	Centre	42839	2037				
	Centre*	8452	10385				