Characterisation of microbial communities associated with hypolithic environments in Antarctic Dry Valley soils

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Abstract

The Eastern Antarctic Dry Valley region is a polar desert, where conditions of extreme aridity, high temperature fluctuations and high irradiation levels make it one of the most extreme environments on earth. Despite the harsh environment, the soils in this region yield a wide range of bacterial and eukaryotic phylotypes in greater abundance than previously believed. In the Dry Valleys, highly localized niche communities colonise the underside of translucent quartz rocks and present macroscopic growth.

Short- and long-term environmental monitoring of hypolithic habitats indicate that, although the temperature in these microenvironments are comparable to that of the open soil at the same depth, the overlying rock provides some thermal buffering. *In situ* monitoring of soil humidity suggests that the protection of the hypolithic microenvironment from the highly desiccating atmosphere increases the potential water availability in this microhabitat. The photosynthetic organisms in these communities seem to thrive even though the transmission of incident light through the overlying quartz rock is approximately 2.3% of the incident light. The availability of light is the key environmental factor leading to the establishment of primary autotrophs and the development of macroscopic communities beneath translucent rocks as opposed to non-translucent rocks.

Hypolithic communities in the Miers Valley were classified into three distinct groups on the basis of gross morphology: cyanobacteria-dominated (Type I), moss-dominated (Type II) and lichenised (Type III) communities. Previously, these communities have not been characterised by molecular methods. In this study, culture-independent, direct DNA extraction methods were used to access the metagenome of different hypolithic communities and associated control sites. PCR-DGGE community fingerprint analysis, based on SSU rRNA genes and ITS sequences, showed a high degree of bacterial diversity and lower archaeal and eukaryote diversity in all Dry Valley soil habitats. Furthermore, although some phylotypes are shared, not all hypolithic communities are the same. SSU rRNA gene and ITS sequence clone libraries were also constructed to further compare the metagenomes of the hypolithic communities and to accurately classify phylotypes. A hypothesis for hypolithic community development in the Dry Valleys of Antarctica has been proposed.

Declaration

I declare that *Characterisation of microbial communities associated with hypolithic environments in Antarctic Dry Valley soils* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Nuraan Khan 26 November 2008

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List of Abbreviations

%RH	relative humidity (%)
$(NH_4)_2SO_4$	ammonium sulphate
℃	degrees Celsius
µg	microgram
µl	microlitre
µM	micromolar
µmol m^{-2} s^{-1}	micromols per square meter per second
ARDRA	amplified ribosomal DNA restriction analysis
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cfu	colony forming units
cm	centimetre
CTAB	cetyl trimethyl ammonium bromide
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E	evenness
EDTA	ethylene diamine tetraacetic acid
g C g^{-1} soil	grams of carbon per gram of soil
g C m^2	grams of carbon per square metre
H’	Shannon diversity index
HCl	hydrochloric acid
hrs	hours
IPTG	isopropyl β-D-thiogalactosidase
ITS	internal transcribed spacer
kb	kilobases
KCl	potassium chloride
km	kilometre
l	litre
LB	Luria Bertoni medium
LB-amp	Luria Bertoni medium containing ampicillin
m	metre
M	molar

MDS	multi-dimensional scaling
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minutes
ml	millilitre
mm	millimetre
mM	millimolar
$MnCl_2$	manganese chloride
NaCl	sodium chloride
NaH_2PO_4	sodium dihydrogen orthophosphate
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
OD_{600}	optical density at 600nm
PCR	polymerase chain reaction
PIPES	1,4-Piperazinebis (ethanesulfonic acid)
rpm	revolutions per minute
rRNA	ribosomal oxyribonucleic acid
s	seconds
S	species richness
SDS	sodium dodecyl sulphate
SSU	small subunit
TAE	tris acetic acid EDTA
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

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Chapter 1: Literature Review

1.1 Antarctica and the Dry Valleys

1.1.1 Location

The Antarctic continent offers some of the coldest, most arid environments on Earth (Onofri *et al*., 2004). However, climatic conditions are not homogenous across the continent and various climatic regions may be distinguished. Of particular interest are the McMurdo Dry Valleys (or Ross Desert) situated in South Victoria Land (Figure 1.1). This is the most extensive ice-free desert in Antarctica (Friedmann & Ocampo, 1976; Onofri *et al*., 2004), comprising an area of roughly 4800 km^2 (less than 2% of the total land area of the continent) (Vishniac, 1993; Cowan & Ah Tow, 2004).

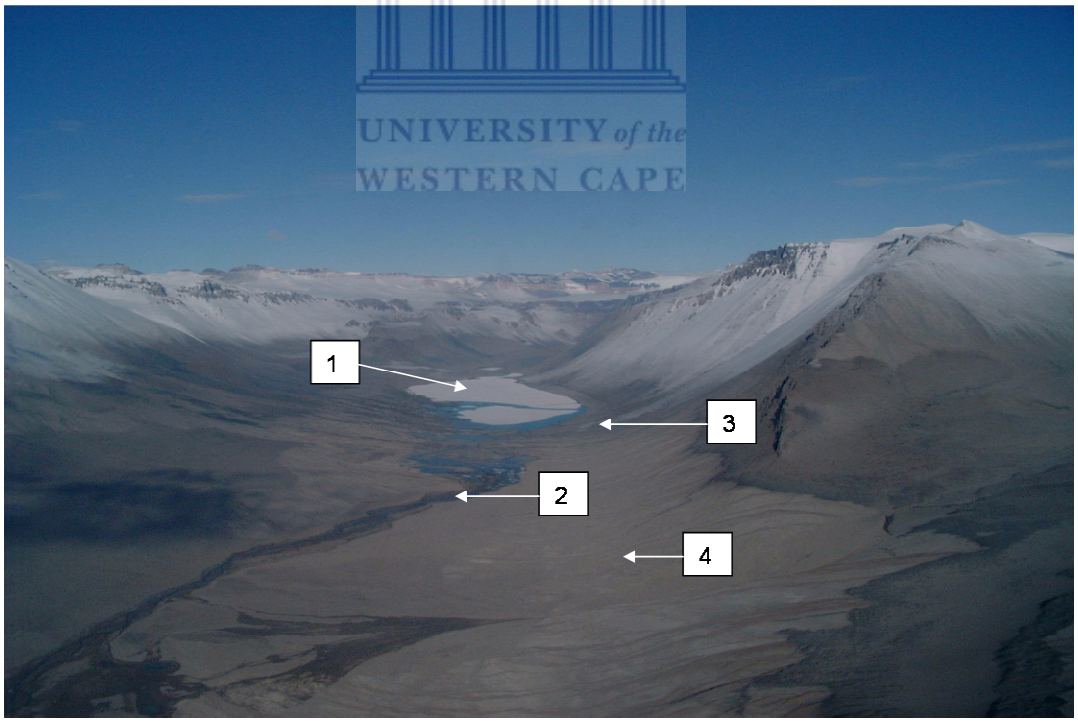

Figure 1.1: The lower Wright Valley, McMurdo Dry Valleys, Eastern Antarctica. This valley incorporates all the typical terrestrial microbial habitats of the Dry Valleys including lakes [1], lake-derived streams [2], glacial meltwater streams [3] and desert soils [4] (Picture courtesy of D. A. Cowan).

These deglaciated terrestrial environments provide a unique combination of extreme climatic conditions including extremely low temperatures, wide temperature fluctuations and low water availability. Dry katabatic winds of up to 100 km hr^{-1} descend from the Antarctic ice plateau into the valleys and contribute to the maintenance of desert conditions (Friedmann, 1982; de la Torre *et al.*, 2003). These deserts consist of exposed rock and soil, with no consistent ice or snow cover although lakes are commonly present on the valley floors (Figure 1.1) (Friedmann, 1982).

1.1.2 Temperature

The mean annual temperature of the Ross Desert ranges from -20ºC to -25°C (Wynn-Williams, 1988; Ascaso & Wierzchos, 2002), although summer and winter temperatures vary markedly. The air temperature fluctuates between -20°C and -50°C in the winter season, but lower values have been recorded (down to -60°C) (Friedmann, 1982; Wharton et al.; 1986; de la Torre et al., 2003; Onofri et al., 2004). Depending on the weather and the geographical position, the air temperature during summer has been found to fluctuate between -35°C and 3°C, but higher values have been recorded (de la Torre et al., 2003).

The surface ground temperatures can vary significantly from air temperature. While the mean summer temperature hovers around 0°C, the ground temperature during periods of sunlight averages 15°C. Extreme fluctuations in surface temperature are common and depend on direct sunlight and cloud cover. In summer, temperature oscillations of up to 15ºC due to variations in cloud cover are common and occur in the order of minutes (de la Torre *et al.*, 2003). Furthermore, continuous summer sunshine has been known to increase the internal temperature of rocks up to 10ºC above the air temperature (de la Torre *et al.*, 2003). Observations of temperature fluctuations from -15°C to 27.5°C within 3 hours have been reported for Ross Desert soils (Cameron, 1974).

1.1.3 Water

The Dry Valley mineral soils receive little precipitation and are constantly exposed to a desiccating atmosphere, rendering them the most barren soils in the ice-free Antarctic regions. Winter humidity levels in the region are extremely low (<10% relative humidity), with the upper soil layer of the mineral soils typically containing 0.5% to 2% w/w water (Cowan & Ah Tow, 2004). Average moisture contents ranging from 0.2% to 3.9% w/w water during the austral summer were recorded in Taylor Valley soils (Connell *et al*., 2006). Although a cemented permafrost layer can be found mere centimetres below the surface, there is a steep desiccation gradient within mineral soil profiles with a supersaturated permafrost layer and extremely arid upper soil horizon (Figure 1.2) (Cowan & Ah Tow, 2004). Due to the highly desiccating atmosphere, insufficient liquid water from the melted permafrost layer becomes available for growth of microorganisms in the upper soil horizon (Figure1.2) (Wynn-Williams, 1988).

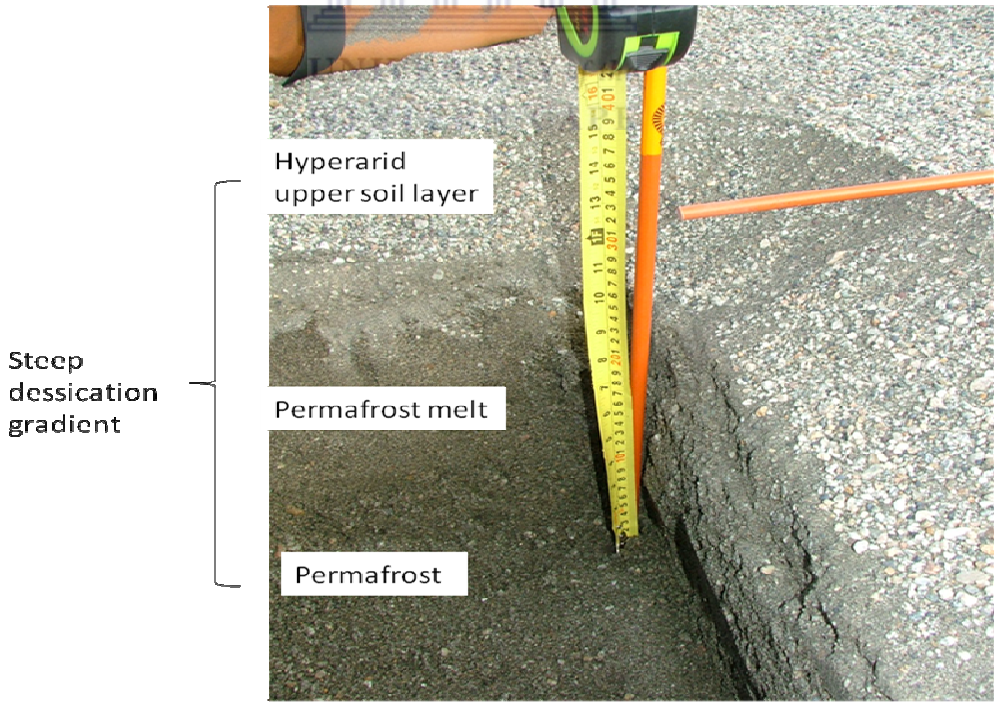

Figure 1.2: Depth profile of typical Dry Valley mineral soils indicating a supersaturated permafrost layer, permafrost melt layer and hyperarid surface soils (Picture by Cowan & Khan, Miers Valley, 2008).

Snow is the only form of precipitation in the region with a mean annual precipitation of 15 g cm^{-2} $year^{-1}$ (Cowan & Ah Tow, 2004). The infrequent snowfall mostly sublimes due to the extremely low atmospheric humidity or is blown away by the high katabatic winds (Friedmann, 1982; Cowan & Ah Tow, 2004; Onofri *et al.*, 2004). Therefore, little moisture in the form of precipitation penetrates the upper soil horizon. The low precipitation, in combination with low atmospheric humidity, results in highly arid soils.

1.1.4 Mineral salts and organic content

Biological water availability in desiccated Antarctic soils may be further reduced due to high soil salinity levels (Claridge & Campbell, 1977; Vishniac, 1993). While high salt contents are generally considered unfavourable for growth (Cameron, 1971), bacterial growth of some species from Ross Desert soils were observed in media containing 15% (Horowitz *et al.*, 1972) and 20% NaCl (James *et al.*, 1990). High concentrations of sodium, calcium, magnesium, chloride and sulphate originating from the substratum as well as from sea spray blown inland accumulate in the dry mineral soils (Wada *et al.*, 1981; Wynn-Williams, 1990). Although unevenly distributed on a microscale, high soil salinity is evident in all Ross Desert soils with salinity decreasing with altitude (Vishniac, 1993).

The organic content of Dry Valley soils is very low, generally constituting <0.1% of dry soil weight (Campbell *et al.*, 1998) with an average of 0.064 ± 0.035% total organic carbon (Matsumoto *et al.*, 1983). Much of the organic content in these soils is presumed to derive from the past primary production and glacial legacy in the region, physical abrasion of rocks (exfoliation of endolithic communities), erosion of the exposed sediments of ancient lake beds, and marine- and lake-derived matter (Nienow & Friedmann, 1993; Moorhead *et al.*, 1999; Burkins *et al.*, 2000). Other studies have shown that some contribution to the existing organic pools is due to primary production in existing soil and, to a greater extent, lithic (rock) communities (Moorhead *et al.*, 1999). Distribution of organic matter is thought to be aided by strong katabatic winds (Moorhead *et al.*, 1999; Cowan & Ah Tow, 2004).

Organic carbon concentrations of soils in the Taylor Valley increased with decreased distance from the Ross Sea coastline with the 0.01 g C g^{-1} soil average for inland sites increasing to 0.05 g C g^{-1} soil for coastal sites (Burkins *et al.*, 2000; Burkins *et al.*, 2001). The average organic carbon concentration for the region was determined to be 0.03 g C g^{-1} soil with a range of 37 g C m^{-2} near the Taylor Glacier to almost 260 g C m^{-2} near the Ross Sea (Burkins *et al.*, 2001). The mean residence time of organic carbon in the Dry Valleys was calculated to be 23 years (Burkins *et al.*, 2001) which is extremely low considering most of the organic content of these soils were thought to be a legacy of lake sediments deposited during the last glacial maximum (Barrett *et al.*, 2005). This may indicate that the simple food webs in the Dry Valley ecosystem may be actively cycling the recently fixed carbon in addition to legacy sources of organic matter (Barrett *et al.*, 2005).

Carbon dioxide (CO_2) efflux from soil is the result of biological respiration and/or georespiration (the inorganic equilibration of soil carbonate in the presence of water vapour) (Oyama & Berdahl, 1977; Burkins *et al.*, 2001). Due to the low biological activity in Antarctic desert soils, the physical controls on CO_2 flux from soil are of similar magnitude to biological contributions (Parsons et al., 2004). While higher biological activities in other regions likely mask the relatively small contribution to CO_2 flux by georespiration, the physical input becomes significant and must be taken into account in low productivity environments (Parsons et al., 2004). The study by Burkins *et al.* (2001), which eliminated the effects of georespiration, found the CO_2 efflux of the Taylor Valley soils to be 0.1±0.08 µmol CO_2 m^{-2} s^{-1}. This is very low in comparison to hot deserts and the Arctic tundra (Raich & Schlesinger, 1992). Assuming maximum productivity for 60 days, the Dry Valley soils could release 6.5±5 g C m^{-2} y^{-1}, still one of the lowest soil carbon respiration figures reported for terrestrial ecosystems (Burkins *et al.*, 2001).

1.1.5 UV irradiation

Antarctica experiences high periodic incident UV irradiation with long periods of complete darkness (Wynn-Williams, 1990; Cowan & Ah Tow, 2004; Onofri *et al.*, 2004). The effects of stratospheric ozone depletion and increased UV-B irradiation is most pronounced in Antarctica where over 50% of the ozone column can be depleted during spring. This in turn leads to increased UV-B levels which increases the environmental stress inflicted on living organisms in the region (Madronich *et al.*, 1998). It is thought that Dry Valley mineral soil communities may not be directly affected by the increase in UV radiation as these communities avoid the exposed soil surface and preferably colonise the interior or underside of rocks (Cockell *et al.*, 2002; Cowan & Ah Tow, 2004).

1.1.6 Life

The Antarctic Dry Valleys are considered to be some of the coldest, most arid deserts found on Earth (Cowan & Ah Tow, 2004). They present what is arguably one of the more hostile environments for the survival of life (Ascaso & Wierzchos, 2002; Connell *et al.*, 2006). The terrestrial Dry Valley systems were once thought to be sterile (Boyd *et al.*, 1970). This view can no longer be supported as modern molecular techniques have shown very large and diverse populations of microorganisms to be present in the soil. For example, using various techniques including ATP, lipid and DNA quantification, Cowan *et al.* (2002) demonstrated that the mineralised soils of the Dry Valleys may contain between 10^6 and 10^8 prokaryote cells per gram of soil. The unique, harsh environmental conditions experienced in the Dry Valleys, especially the rapid temperature fluctuations and extreme aridity, mimic those postulated for early Mars and this region has long served as a model for exobiological studies (Andersen *et al.*, 1990; McKay, 1993).

1.2 Antarctic soil microbiology and microbial diversity

Increasing environmental stress is often linked to decreasing biological diversity (Lawley *et al*., 2004). Although higher eukaryotes are found in the Antarctic Peninsula (the northern latitudes), microbial biomass and diversity in the Dry Valleys are significantly lower than more temperate climes (Cowan & Ah Tow, 2004). The extreme environment is thought to result in fairly simple ecosystems supporting simplified food webs (Wall & Virginia, 1999).

A study by Yergeau *et al.* (2007a) showed that prokaryote community structure was largely determined by location and/or associated location-dependent environmental conditions, whereas microbial abundance was associated with nutrient input and climate buffering due to the presence of vegetation cover. There is a decreased bacterial abundance with increased latitude due to a decrease in vegetation cover. In the case of fungal diversity, this was only found to be true if a regional-scale comparison between maritime and continental Antarctica was made (Lawley *et al*., 2004). This could indicate a high level of site isolation and possibly endemism in the Antarctic, which is different to studies based on other continents (Lawley *et al*., 2004).

Although the soils of the Dry Valleys support a potentially diverse microbial community, there has not been a systematic survey of bacterial diversity in the region (Wynn-Williams, 1996). Until recently, all studies focusing on the distribution, abundance and diversity of microorganisms in Antarctic Dry Valley habitats were culture-dependent, which is now accepted as an inappropriate method for determining the true diversity of microbial habitats.

Culture-based studies have shown that while chromogenic (i.e. pigmented) bacteria inhabit the soil surface layer, non-pigmented bacteria dominate below the soil surface (Cameron *et al*., 1970; Aislabie *et al*., 2006). Desiccated surface soils have been shown to contain fewer culturable bacteria than the moist sub-surface soils above the

permafrost (Aislabie *et al.*, 2006). The numbers of bacteria in Dry Valley soils, based on culture-dependent studies, ranged from 0 to 10^7 colony forming units (CFU) per gram of soil (Boyd *et al.*, 1966; Cameron *et al.*, 1970). Total biomass values based on culture-dependent techniques are questionable as it is widely accepted that >90% of microorganisms may not be isolated by traditional culture methods (Amann *et al.*, 1995; Kirk *et al.*, 2004; Aguilera *et al.*, 2006). Culture-independent methods increase microbial biomass estimates up to four orders of magnitude higher than previously reported by culture-dependent methods (Cowan *et al.*, 2002).

Culture-based studies have also shown that although some endemic bacterial species have been isolated from the Dry Valleys, the majority of species isolated are cosmopolitan in nature (Cowan & Ah Tow, 2004). Phylogenetic methods of bacterial community analysis in the Miers Valley have revealed that up to 50% of the sequences retrieved correspond to uncultured bacteria (Smith *et al.*, 2006). The study by Smith *et al.* (2006) showed a diverse range of prokaryote phylotypes and identified eight broad phylotypic groups: *Cyanobacteria*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia*, *α-Proteobacteria*, *β-Proteobacteria*, *Chloroflexi* and *Bacteroidetes*. The study also showed that, while many major prokaryote groups such as *Acidobacteria* were not identified using culture-dependent methods, they appeared to be relatively common on the basis of phylogenetic analysis. This is further substantiated by Aislabie *et al.* (2006) who found that although *Acidobacteria* were one of the dominant groups in the clone libraries, they were not identified by culture-dependent methods. Phylogenetic studies of Dry Valley mineral soils have not commonly yielded anaerobic bacterial or archaeal 16S rRNA gene signals (Smith *et al.*, 2006).

Both culture-dependent and -independent methods have been used to assess the diversity of bacteria in soils from the Wright Valley and Marble Point in the McMurdo region (Aislabie *et al.*, 2006). 16S rRNA gene clone libraries identified 27 dominant phylotypes, with 11 of the 27 phylotypes belonging to the group *Bacteroidetes*, 8

belonging to the *Actinobacteria* and the rest distributed among the *Proteobacteria*, *Thermus-Deinococcus*, *Acidobacteria*, *Firmicutes* and *Cyanobacteria*. Although both Gram-positive and -negative rods and coccibacilli were isolated, the cultured bacteria only represented 8 of the 27 dominant phylotypes identified by molecular methods. The study showed that while some phylotypes, such as those belonging to the *Proteobacteria* were prevalent in all sites sampled, others were prevalent in specific sites only (Aislabie *et al.*, 2006).

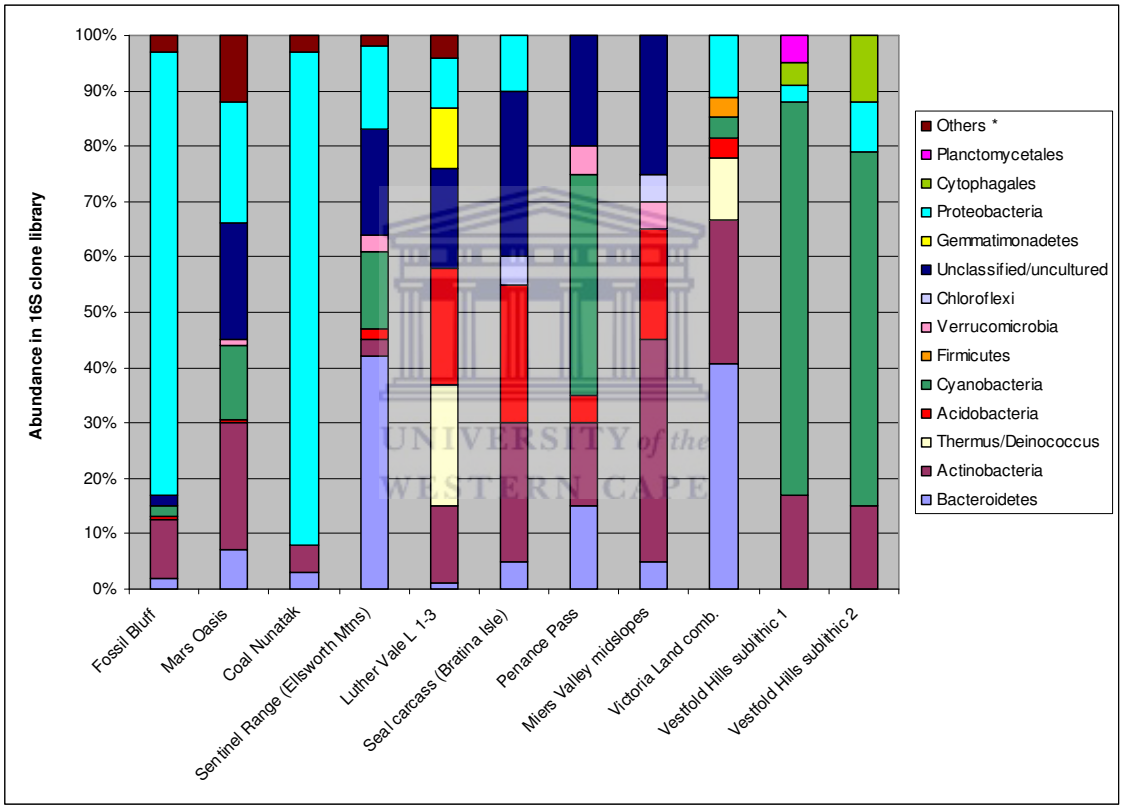

Figure 1.3: A snapshot of prokaryotic diversity across a range of Antarctic arid soil habitats using culture-independent techniques. For Mars Oasis, Fossil Bluff, Coal Nunatak and Sentinel Range, 'Others' category includes Chloroflexi, Firmicutes, Fusobacteria, Gemmatimonadetes, Planctomycetes and the candidate phyla OP10 and TM7. For Luther Vale, 'Others' category includes Chloroflexi, Firmicutes, Bacteroidetes, Verrucomicrobia, Nitrospira and Cyanobacteria.

Figure 1.3 presents a snapshot of the prokaryote diversity detected over a range of arid Antarctic soil habitats. The data collected from recent literature on the subject

indicates the range and diversity of organisms found in dessicated soils of Antarctica as well as the differences in microbial diversity in different regions and habitats on the continent (Figure 1.3). Briefly, features of these habitats were:

- Fossil Bluff, Mars Oasis, Coal Nunatak and the Sentinel Range (Ellsworth Mountains): bare frost-sorted soil at locations across the Antarctic Peninsula (Yergeau *et al.*, 2007b).
- Luther Vale L 1-3: a low productivity, low moisture soil in a dry cirque below Luther Peak, Admiralty Range, Northern Victoria Land (Niederberger *et al.*, 2008).
- Seal carcass (Bratina Island), Penance Pass and Miers Valley mid-slopes: three discreet desiccated mineral soils in the Dry Valleys, Ross Dependency, Eastern Antarctica (Smith *et al.*, 2006).
- Victoria Land comb.: combined data from low moisture soils at Marble Point and in the Wright Valley (Aislabie *et al.*, 2006).
- Vestfold Hills sublithic 1 and sublithic 2: two discreet quartz stone sublithic communities (Smith *et al.*, 2000).

Early culture-dependent studies supported conclusions that bacteria from Antarctic soils may be entirely cosmopolitan in distribution, as the bacteria detected were all assigned to known genera such as *Arthrobacter, Corynebacterium* and *Micrococcus* (Baker & Smith, 1972). However, more recent culture-independent studies have shown that a significant number of uncultured bacterial phylotypes (with BLAST identities <95%) are detected in 16S rRNA gene clone libraries, indicating a large pool of novel, possibly endemic taxa that await identification (Smith *et al.*, 2006).

1.2.1 Cyanobacteria

The diversity of cyanobacteria in Antarctica has been more extensively studied, although knowledge concerning distribution patterns and relative abundance is still lacking (Adams *et al.*, 2006). Most studies of cyanobacteria in the Antarctic have

focused on lake systems and meltwater ponds or streams where macroscopic growth may be observed (Taton *et al*., 2003, 2006; de los Rios *et al*., 2004; Jungblut *et al*., 2005). Advanced molecular techniques have revealed higher cyanobacterial diversity (including possible endemic species) than previously known (Taton *et al*., 2003; Wood *et al*., 2008). In comparison, few studies have been conducted on the diversity and distribution of cyanobacteria in soils (Cavacini, 2001; Wood *et al*., 2008).

Wood *et al.* (2008) identified species in Miers Valley soils belonging to the orders *Nostocales, Chroococcales* and *Oscillatoriales*. The study also indicated the presence of a number of as yet uncharacterised cyanobacteria, as many 16S rRNA gene sequences from the clone libraries had low similarity to any database entries. Smith *et al.* (2006) suggested that cyanobacterial populations in the soils of the Miers Valley were free-living species rather than derived from cryptoendolithic populations, as suitable rock structures to support cryptoendolithic growth were absent in the sampling region. Moreover, the cyanobacterial phylotypes found (*Nostoc, Phormidium* and *Oscillatoria)* differed from those typically associated with cryptoendoliths.

It has also been suggested that cyanobacteria and algae found in Dry Valley mineral soils are derived from lake and pond mats that are distributed by wind (Broady, 1996; Moorhead *et al*., 2003; Aislabie *et al*., 2006). A recent study by Wood *et al.* (2008) combined the use of automated ribosomal intergenic spacer analysis (ARISA) and 16S rRNA gene clone library techniques and concluded that soils in the Miers Valley contained multiple cyanobacterial phylotypes, a significant proportion of which were also found in the nearby Miers Lake and hydroterrestrial cyanobacterial mats. This indicates that lakes and ponds play a significant role in contributing to the cyanobacterial diversity of Dry Valley soils. Cyanobacterial mats represent an important carbon source in soils with low organic matter content and may play a vital role in ecosystem functioning (Adams *et al*., 2006).

Cyanobacterial distribution in the Dry Valleys is non-homogeneous and the presence of these organisms is thought to be related to water availability as they are more abundant in moist soils. Smith *et al.* (2006) also suggested that methods for determining water availability that are based on soil water content may be an insufficient determinant for the presence of cyanobacteria and that atmospheric humidity may play a larger role in meeting water requirements of cyanobacteria. This proposal has been substantiated by Wood *et al.* (2008) who did not find an increase in cyanobacterial diversity with increased soil water content. Although cyanobacteria are the most abundant and vital primary producers in the region, the environmental factors that promote the growth of cyanobacteria in Antarctic soils are still largely unknown.

1.2.2 Fungi

Yeast, yeast-like and filamentous fungi are widely dispersed in the Antarctic, but in low abundance (Vishniac, 1996). They have been found in cryptoendolithic (Vishniac, 1996), marine (Fell, 1974), and air and soil samples from various locations in the Antarctic (Sun *et al*., 1978). Endemic fungi have been found growing on historic huts (Arenz *et al*., 2006) and a diversity of cosmopolitan fungi have been isolated from Dry Valley lakes and areas affected by human activity. Although fungi are known to be important in soil ecosystems, the distribution, importance and function of fungi in the McMurdo Dry Valleys has not yet been established.

Connell *et al.* (2006) investigated the distribution and abundance of fungi in soils of different moisture content in the Taylor Valley (site average moisture contents ranging from 0.2% to 3.9%). The study showed that the distribution and abundance of filamentous and non-filamentous (yeast and yeast-like) fungi were affected by different parameters. While the abundance of filamentous fungi showed a significant positive correlation with increased distance from marine and glacial water sources, carbon content, moisture content and soil pH (pH 7.2 to 10.5), no such correlation could be made for non-filamentous fungi. In contrast, yeast and yeast-like fungi were

more evenly distributed throughout the Valley and showed a significant correlation to lower soil salinity and lower elevation. Interestingly, the study also found the distribution of nematodes to be similar to that of filamentous fungi. Both cosmopolitan and endemic fungal species were identified, although the true diversity of fungi in this region is still unknown due to the dependence of this study on culturing methods.

1.3 Communities, ecosystems and trophic structure

Due to the extreme environmental conditions, the Dry Valleys have no vascular plants or large land animals. Since plant roots are absent, carbon cannot be moved to the depths of the soil easily, thus the dominant food web is a decomposition-based one limited to the near surface environment (Virginia & Wall, 1999). Community structure and dynamics are controlled primarily by resource availability and environmental conditions (Moorhead *et al.*, 1999). There are strong 'bottom up' controls on ecosystem structures that are imposed by the limited availability of resources and prevailing conditions (Moorhead *et al.*, 1999). Nutrient cycles and trophic interactions are limited to microbial populations and micro-invertebrates (Vishniac, 1996). The microbial ecosystem is driven by abiotic rather than biotic factors (Connell *et al.*, 2006). It is believed that the community structure of arid Antarctic soils would be relatively simple compared to the complexity of soil communities in temperate regions (Wall & Virginia, 1999; Connell *et al.*, 2006). These "simple" communities would therefore present an ideal opportunity to discover and define interactions between soil microorganisms and the environment that may apply to other, more complex situations.

A food web constructed for the Antarctic Dry Valleys was much simpler than one generated for a contrasting hot desert in Mexico (Wall & Virginia, 1999). Algae and cyanobacteria formed the basis of the food web (i.e. the bottom of the food chain) for the Antarctic Dry Valley system (Wall & Virginia, 1999). These contribute to the organic matter and allow for the growth of bacteria and fungi. The system contained a single microbivore (*Scottnema lindsaye* feeding on bacteria and yeast), one

bacterivore (*Plectus antarcticus* feeding on bacteria) and a single omnivore-predator (*Eudorylaimus antarcticus* feeding on algae, bacteria, fungi, nematodes and other small fauna). No higher invertebrates were detected (Wall & Virginia, 1999). The trophic structure that exists in the Dry Valleys is the simplest found on Earth, as there are no other soil systems known where nematodes represent the top of the food chain (Virginia & Wall, 1999). The diversity of organisms (nematodes) in the upper trophic levels is extremely low in comparison with other environments, with little or no functional diversity. In consequence, the system is highly susceptible to disturbance (Virginia & Wall, 1999; Wall & Virginia, 1999). The lower trophic levels (cyanobacteria, bacteria and fungi) are significantly more diverse, indicating greater functional diversity and stability (Smith *et al.*, 2006; Wood *et al.*, 2008).

In the Antarctic Dry Valleys, most biological communities are protected from the severe climatic conditions through their development in niche microenvironments such as beneath the surface of translucent rocks. The following section relates to lithobiontic microorganisms in particular as this study focuses on the micro-climatology and -biology of the hypolithic habitat.

1.4 Lithobiontic microorganisms

Lithic or lithobiontic microorganisms are typically associated with a rock or stone substrate. Endolithic organisms are prevalent in the Antarctic polar desert where extreme environmental conditions exist. These organisms inhabit the interstitial spaces in crystalline or porous rocks (cryptoendoliths) or cracks and fissures in rock strata (chasmoendoliths) (Golubic *et al.*, 1981; Friedmann, 1982; Cowan & Ah Tow, 2004). The presence of endolithic organisms in the region is ubiquitous where suitable substrates are available for colonisation. As endolithic communities are photosynthesis-dependent, colonisation requires some degree of translucence of the rock substrate (Nienow & Friedmann, 1993). Hypolithic communities, in particular, colonise the rock-soil interface on the base and along the sides of translucent rocks

or stones that are partially buried in the soil (Nienow & Friedmann, 1993; Cockell & Stokes, 2004; Smith *et al.*, 2000).

The study of lithobiontic organisms and their relationship to their ecological niche in the Dry Valleys began in 1976, when Friedmann and Ocampo observed the presence of cyanobacteria within Beacon sandstone. Interest in lithobiontic organisms in desert environments is growing due to the ability of these organisms to withstand a combination of harsh environmental conditions.

The Antarctic austral summer (mid-November to mid-February) presents the most optimal climate conditions for biological processes, as the sun is above the horizon for 24hrs a day (i.e. maximum solar radiation) and the mean air temperature is at its highest (Nienow & Friedmann, 1993). UV irradiation, although necessary for certain processes such as photosynthesis, may have other, potentially lethal effects on organisms. UV irradiation causes damage to biological compounds such as DNA through direct absorption and UV-induced generation of excited oxygen states (Vincent & Quesada, 1994). Although all microorganisms possess repair processes to mend the damage caused by UV irradiation, lithobiontic organisms are further protected by their ecological niche, the overlying rock strata (Cockell *et al.*, 2002).

The availability of liquid water in the Ross Desert is generally believed to be the limiting factor in microbial growth, especially during the short austral summer period when temperature and light conditions are favourable for photosynthesis (Cowan & Ah Tow, 2004). Although snowfall is infrequent in the region, snowmelt provides liquid water which can be retained internally by rocks for several days for use by endolithic organisms (de la Torre *et al.*, 2003).

Estimates based on nanoclimate data suggest that endoliths are able to metabolise for less than 1000 hrs year^{-1} (Friedmann *et al.*, 1987). Most of the inorganic nutrients

that are required by endoliths are thought to be available in the rock substrate (Friedmann, 1982). Heterotrophic bacteria and fungi are often constituents of endolithic communities. They utilise the metabolites produced by the phototrophic component, as well as dead cells, as a carbon source (Hughes & Lawley, 2003). Endolithic communities are important as they play a role in global processes such as weathering of rocks and nutrient-cycling (de la Torre *et al.*, 2003). These communities have been suggested to be the main primary producers in the extreme terrestrial environment of the Dry Valleys (de la Torre *et al.*, 2003). However, not all Valleys in the McMurdo Dry Valley region have the necessary rock substrate for the development of endolithic communities. The Miers Valley for example, lacks the sandstone or marble rock strata that are known to harbour endolithic communities (Nienow & Friedmann, 1993). Hypolithic communities are believed to be the main primary producers in the Miers Valley.

Antarctic endolithic communities have been characterised by microscopy and culture-dependent methods (e.g. Friedmann *et al.*, 1988) and, more recently, by the use of molecular techniques (e.g. de la Torre *et al.*, 2003). Two classes of cryptoendolithic communities have been described; cyanobacteria-dominated and lichen-dominated, based on the dominant organism (Friedmann, 1982).

The dominance of cyanobacteria in cryptoendoliths has been shown microscopically, with fungi and bacteria also present (Ascaso & Wierzchos, 2002). Several strains of cyanobacteria isolated from cyanobacterial cryptoendoliths were identified as species of *Chroococcidiopsis* or a related genus (Friedmann, 1982). Unidentified colourless bacteria, visible with a light microscope, are often associated with cryptoendolithic lichens (Friedmann, 1982). Although microscopic analysis of cyanobacterial cryptoendoliths indicated the presence of cyanobacteria morphologically similar to *Gloeocapsa* sp. and *Hormathonema* sp., 16S rRNA gene sequence analysis revealed only one principal cyanobacterium present; a phylotype most closely related to *Plectonema* sp. strain F3 (de la Torre *et al.*, 2003). The study also revealed two other

abundant microorganisms: a member of the *Thermus-Deinococcus* phylogenetic group and a representative of the α-*Proteobacteria*. Due to the presence of these organisms in similar numbers to the principal cyanobacterium in the clone library, it is suggested they may have equivalent levels of abundance in the environment and may be involved in a tightly regulated syntrophic relationship (de la Torre *et al.*, 2003). Although the clone libraries in the study were dominated by a few organisms, the less prevalent clones represented a high degree of diversity. For example, a total of 16 *Actinobacteria* phylotypes were identified and several sequences were encountered only once or were not similar to any known phylogenetic group.

According to microscopy- and morphology-based identification, the cyanobacteria-dominated endoliths found in carbonate rocks from Goldman Glacier were dominated by *Synechocystis*-like cells (de los Rios *et al.*, 2004). The endoliths sampled from granites from Mont Falconer and samples from Commonwealth Glacier were also cyanobacteria-dominated. The closest morphological match of the dominant cyanobacterium at Mont Falconer was *Cyanothece* while *Gloeocapsa*-like cells in close proximity to a filamentous cyanobacterium were found in Commonwealth Glacier samples (de los Rios *et al.*, 2004).

Lichen-dominated cryptoendoliths are most prevalent in the Antarctic Dry Valleys (Friedmann, 1982; de la Torre *et al.*, 2003). These communities are typically arranged in distinct zones: an upper black zone of approximately 1 mm thick, a middle white zone of approximately 2–4 mm thick and a lower green zone (Friedmann, 1982). Below the green zone, colourless fungal hyphae penetrate further into the rock. All the zones were formed by filamentous fungi and unicellular green algae, which form the lichen association (Friedmann, 1982).

Over 70% of the clones in the rRNA gene clone libraries of an Antarctic lichen-dominated cryptoendolithic community consisted of three phylotypes: fungi (29% of clones) identified as the ascomycete fungus *Texosporium sancti-jacobi*; chloroplasts

(22% of clones) most probably derived from the green alga *Trebouxia jamesii*; and green algae (22% of clones) identified as *Trebouxia jamesii* (de la Torre *et al.*, 2003). Although various associated photobionts (algae) were identified by microscopic methods, molecular methods revealed only one dominant green alga (de la Torre *et al.*, 2003). The authors suggest that the various photobionts previously identified could be morphological variants of the same organism (as is the case with some fungal associations) or may not be present in sufficient numbers to be detected by molecular methods (de la Torre *et al.*, 2003). The absence of these organisms in the clone library could also be due to the inherent problems encountered with DNA extraction and PCR-based methods (see Sections 1.6.1 and 1.6.2).

The principal fungal phylotype identified in lichen-dominated cryptoendoliths, *T. sancti-jacobi* and the principal green alga, *T. jamesii*, are known components of lichen associations (Friedl & Rokitta, 1997) and are presumed to constitute the dominant lichens in the community (de la Torre *et al.*, 2003). The fourth most abundant phylotype, sharing only 95% identity to a bacterial *Cytophagales* sp., accounted for 6% of the clones. Other less prevalent clones in the library clustered with *Actinobacteria*, α-*Proteobacteria*, γ-*Proteobacteria* and *Planctomycetales*, while three sequences did not consistently group with any specific bacterial taxon (de la Torre *et al.*, 2003).

Heterotrophic bacteria are frequently found in intimate association with the dominant phototrophic microorganisms in endolithic communities (de la Torre *et al.*, 2003). According to de la Torre *et al.* (2003) this is an indication that these bacteria substantially contribute to primary productivity. However, this close relationship could also indicate the level of nutrient cycling that occurs in the niche environment.

1.5 Hypolithic microorganisms

Sublithic or hypolithic microbial communities are referred to as hypolithons and have been found to comprise mainly cyanobacteria and unicellular algae (Thomas, 2005). Broady (1981) described these communities as consisting of distinctly coloured bands of growth on the bottom of translucent rocks while the study by Smith *et al.* (2000) confined the description of hypolithons to the thin (0.2 mm to 0.5 mm) bright green biofilm that adhered very tightly to the stone surface. Hypolithic communities may also appear as a layer of growth attached to the underside of the rock (Figure 1.4).

Hypolithic communities have been found in both hot (Schlesinger *et al.*, 2003; Warren-Rhodes *et al.*, 2006) and cold (Smith *et al.*, 2000; Cockell & Stokes, 2004; Wood *et al.*, 2008) deserts, where similar conditions of extreme temperature fluctuations and aridity exist. Although lithic communities from a variety of substrates have been described, including quartz (Schlesinger *et al.*, 2003), flint (Berner & Evenari, 1978), limestone (Cockell & Stokes, 2004), gypsum (Hughes & Lawley, 2003) and sandstone (Friedmann & Ocampo, 1976), most hypolithic communities described to date have been discovered under one of the most common translucent rocks, quartz (Figure 1.4). As hypolithic communities are primarily photosynthetic, sufficient translucence of the overlying rock to allow for adequate light penetration is essential (Cockell & Stokes, 2004).

Figure 1.4: Typical hypolithic community attached to the underside of translucent quartz in the Miers Valley (Picture by Cowan & Khan, 2008) (Note: The quartz rock was loosened from surrounding soil in order to improve visualisation of the attached community). Quartz rock colonised by hypolithons are characteristically embedded in the mineralised soil.

In the extreme conditions of the Dry Valley region, the hypolithic habitat acts as a sheltered environment for photosynthetic microorganisms, where a favourable microenvironment for microbial life is created. The overlying rock acts as a buffer, protecting the habitat from the elements of the macroenvironment such as intense UV irradiation and scouring by the strong katabatic winds (Friedmann, 1982).

In 2004, Cockell and Stokes investigated the colonisation of the underside of non-translucent dolomite rocks in the polygonal terrains at Mars Oasis on Alexander Island in the Antarctic Peninsula. They showed that while 100% colonisation occurred on the edges of the polygons, only 5% colonisation occurred within the polygons (Cockell & Stokes, 2004). It was then proposed that periglacial activity resulting in rock sorting improved the light penetration around the edges of rocks thus explaining the widespread colonisation of the underside of non-translucent rocks (Cockell & Stokes, 2004). However, hypolithic communities are not exclusively associated with polygon stone fields, as they have also been found in other Dry Valley environments (personal observation). Hypolithic growth in the Vestfold Hills was found to cover 66

cm^2 m^{-2} and contribute a total biomass of 0.85 mg chlorophyll m^{-2} (Broady, 1981). This represents a significant carbon addition to the overall ecosystem.

1.5.1 The hypolithic microenvironment

Insufficient water availability in the arid Dry Valleys is thought to limit biological growth in the region (Wynn-Williams, 1988). In the hypolithic microhabitat, the overlying rock is believed to trap moisture, providing hypolithons with a source of liquid water, a scarce resource in the arid Dry Valleys (Cockell & Stokes, 2004). Broady (1981) found that soils directly beneath hypolithic rocks consistently had higher water contents than the surface layers of the adjacent soil. Furthermore, quartz stones that lay close to the surface (i.e. not embedded in the soil) showed similar water content beneath the rock compared to that of the adjacent soils and had no visible colonisation (Broady, 1981).

The moisture content of the basic (pH 7.4 to 7.7) soils beneath hypolithic rocks sampled from areas of the Vestfold Hills, was 6% to 14% w/w water, whereas the adjacent soils contained 0.5% to 2% soil moisture (Smith *et al.*, 2000). It has also been proposed that water vapour or liquid water diffuses upward through capillary action from the melting ice-permafrost boundary and is trapped by the overlying rock, thereby providing an accessible water source for the hypolithic community (Cowan & Ah Tow, 2004).

As in the polar deserts of Antarctica, hypolithic cyanobacteria are key photoautotrophs and primary producers in hyperarid, hot deserts such as the Atacama Desert. Temperature, soil pH, habitat availability (i.e. abundance of suitable translucent stones), soil toxicity and inadequate carbon influx were eliminated as factors limiting growth of hypolithic communities in the hot desert environment (Warren-Rhodes *et al.*, 2006). It was instead proposed that liquid water availability was the factor limiting growth of photosynthetic microbes. This was further

substantiated by Pointing *et al.* (2007) who found that the percentage of quartz rocks colonised by hypolithic communities decreased as aridity increased.

Moreover, an analysis of net carbon exchange rate data (Schlesinger *et al.*, 2003) also showed that hot desert hypolithic communities are sensitive to variations in moisture availability. Warren-Rhodes *et al.* (2006) suggest that the critical dry limit of photosynthesis and primary production in hot deserts is a mean annual rainfall of less than approx. 5 mm yr^{-1} and/or long periods of zero precipitation (in the time-scale of decades). Schlesinger *et al.* (2003) propose that the major advantage hypolithons derive from colonising the underside of quartz rocks is the ability of the rock to transmit growth sustaining light to the moist microenvironment.

As expected, the temperature experienced on rock and soil surfaces is closely linked to ambient air temperature and solar radiation (Nienow & Friedmann, 1993). During winter, when sunlight is absent, rock surface temperatures are on average 1-2°C lower than ambient air temperature. In summer however, when sunlight is constant, the surface temperatures increase significantly above ambient air temperature (Nienow & Friedmann, 1993). Furthermore, wide temperature fluctuations occur on rock surfaces; for example, temperature oscillation over a range of 7.7°C within 42 minutes were recorded with the temperature shifting from positive to negative figures 14 times within that time period (Friedmann, 1982). This may be a significant factor in the preferential colonisation by microorganisms of the underside of rocks. The temperature regime in the hypolithic habitat resembles those described for endolithic habitats (Nienow & Friedmann, 1993), where temperatures are more stable and fluctuations are greatly reduced by the presence of the overlying rock.

The “greenhouse” effect can be described as an increase in temperature due to the trapping of solar energy by, in this case, the overlying rock. Analysis of the physical characteristics of quartz hypolithic habitats in a hot desert showed a greenhouse effect during daylight hours. However, this may not have a substantial effect on

photosynthesis as this phenomenon is diminished during cool and relatively wet periods in the Atacama which are conducive to photosynthesis (Schlesinger *et al.*, 2003). Moreover, despite the potential for "greenhouse" effects, the hypolithic microenvironment was found to be cooler than non-translucent rocks in adjacent desert pavement due to the reflectivity of white quartz (Schlesinger *et al.*, 2003).

Hypolithic communities are similar to endoliths as the primary producers present use light transmitted through the overlying rock to photosynthesise in an environment where the irradiance levels are less than 0.1% of the incident light (Schlesinger *et al.*, 2003; Thomas, 2005). In the Southern Mojave Desert, hypolithic communities were found to have colonised all 295 quartz stones sampled, with growth under stones up to 25 mm thick that transmit 0.08% incident light (Schlesinger *et al.*, 2003).

Quartz rocks reflect 40% to 50% of incident light, with the proportion of reflected light decreasing with increasing wavelength (Schlesinger *et al.*, 2003). Although light transmission was found to be relatively constant over the spectrum, the thickness of the rock plays a role in transmission of higher wavelengths. For example, the transmission of incident light with a wavelength of 650 nm through 12 mm thick quartz was approximately 2.8%, while transmission of the same wavelength through 25 mm thick quartz was 0.08% (Schlesinger *et al.*, 2003). In the Vestfold Hills, Antarctica, Broady (1981) found that translucent quartz from 13 mm to 80 mm thick transmit 2.7% to 0.6% of the incident light.

It has been proposed that the lower depth limit of growth of the hypolithic community under the translucent rock is determined by light transmission. Microbial colonisation beneath large rocks is confined to the periphery as insufficient light is transmitted to the base of the rock to support photosynthesis (Broady, 1981; Schlesinger *et al.*, 2003). Variation in light transmittance through the rock also leads to zonation (Figure 1.5) (Nienow & Friedmann, 1993), as with endolithic communities. For example, in some hypolithic communities, green- or orange-pigmented green algae were found

nearer the soil surface while cyanobacteria were found deeper under the rock (Figure 1.5Figure 1.5) (Nienow & Friedmann, 1993).

Figure 1.5: Zonation visible in a typical cyanobacteria-dominated hypolithic community with orange-pigmented organisms around periphery and green cyanobacteria directly underneath rock (Picture courtesy of D. A. Cowan).

The net carbon exchange rate (NCER) of photoautotrophic hypolithic microorganisms in the Southern Mojave Desert was found to increase as a result of an increase in the flux of photosynthetically active radiation (PAR) (NCER of 1.7 – 2.7 µmol m^{-2} s^{-1} at a PAR flux of 200 – 400 µmol m^{-2} s^{-1}) (Schlesinger *et al.*, 2003). Despite differences in the thickness of the quartz or potentially different species composition of the hypolithic communities examined, the incident quantum-use efficiency was 0.019 (Schlesinger *et al.*, 2003). This was based on CO_2 uptake over a PAR range of 0 to 50 µmol m^{-2} s^{-1} and illustrated by the initial slope of the photosynthetic light response curve (r^2 = 0.90) (Schlesinger *et al.*, 2003). Although low, this efficiency is sufficient for development of hypolithic communities in the extreme environment.

As with other low productivity environments, although the total biomass of hypolithic communities may be low, their relative contribution to the carbon and nitrogen turnover rates may be significant. The productivity of hypolithons (in terms of carbon assimilation) in the Arctic was estimated at a mean (±standard deviation) productivity

of about 0.8 ± 0.3 g m^{-2} yr^{-1} (Cockell & Stokes, 2004). If one considers that the estimated mean productivity from plants, lichen and bryophytes in the region combined is 1.0 ± 0.4 g m^{-2} yr^{-1} (Bliss *et al.*, 1984), then hypolithon productivity almost doubles the productivity estimates for the polar desert environment (Cockell & Stokes, 2004). Although these rates of productivity are extremely low when compared to those of temperate grasslands and prairies (approximately 1 kg m^{-2} yr^{-1}) (Thomas, 2005), hypolithon production is a vital source of nutrition for grazing nematodes and protozoans. Hypolithic photoautotrophs can therefore be viewed as the basis for the survival of an entire ecosystem in the extreme cold, dry environment of the Antarctic Dry Valleys (Freckman & Virginia, 1997).

1.5.2 Hypolithic communities

Phylogenetic analysis of 31 unique 16S rRNA gene sequences from Atacama hypolithic communities revealed 26 cyanobacterial sequences, possibly belonging to six novel cyanobacterial groups (Schlesinger *et al.*, 2003). The study also found one clone closely related to *Chroococcidiopsis* and two groups related to *Mycrocystis aeruginosa*, while other clones represented other heterotrophic bacteria (Schlesinger *et al.*, 2003). Fungi belonging to the genera *Cryptococcus* and *Mycosphaerella* were also identified. The study showed the hot desert hypolithic communities to be diverse, complex and dominated by phototrophic organisms (Schlesinger *et al.*, 2003).

Antarctic hypolithic communities are similar in that they harbour a diverse, complex community dominated by phototrophic organisms. Direct metagenomic analysis of the diversity of 16S rRNA genes from 2 hypolithic communities showed the dominance of cyanobacteria (71% – 76% of clones) (Smith *et al.*, 2000). ARISA profiles of hypolithic communities sampled in the Miers Valley suggested the presence of multiple cyanobacterial species within each hypolithic community (Wood *et al.*, 2008).

The unicellular (*Chroococcidiopsis*) and filamentous (*Plectonema*) cyanobacteria dominate hypolithic communities in the low organic content mineral soils typical of the Dry Valleys (Nienow & Friedmann, 1993). While filamentous cyanobacteria are more prevalent in slightly wetter soils, whereas unicellular cyanobacteria and *Desmococcus* spp. (a pleurococcoid green alga) are more common in drier soils (Nienow & Friedmann, 1993).

Light microscopy of sublithic material and suspensions from the Vestfold Hills revealed the presence of both oscillatorian cyanobacteria (with morphology typical of the *Lyngbya/Phormidium/Plectonema* group) and coccoidal cells morphologically similar to *Chroococcidiopsis*, *Desmococcus*-like algae, *Navicula*-like diatoms, some mites and nematodes (Smith *et al.*, 2000). Cyanobacterial isolations revealed two dominant filamentous morphotypes, both of which grew between 2ºC and 20ºC, yet displayed no growth at 25ºC (therefore classified as psychrophilic organisms) and both belonging to the *Phormidium* subgroup according to 16S rRNA gene sequence analysis (Smith *et al.*, 2000). Further cyanobacteria-enriched isolations yielded the presence of *Chroococcidiopsis*-like and *Synechococcus*-like cells and an isolate most closely related to *Lyngbya* sp. strain PCC7419 (Smith *et al.*, 2000).

Hypolithic communities were found to be highly diverse in terms of the number of different ARISA fragment lengths (AFL's) identified (51 different AFL's) and the average number of AFL's per sample (12.1 AFL's per sample) (Wood *et al.*, 2008). Although cyanobacterial libraries derived from hypolithic communities were not constructed in the study by Wood *et al.* (2008), identification of some ARISA fragments could be inferred from other Dry Valley clone libraries. For example, an AFL of 767 bp was found in all soil samples from the Dry Valleys (including hypolithic community samples), and has been identified as an uncultured cyanobacterium (GenBank accession number DQ181681) with 96.5% similarity to *Leptolyngbya frigida* (Wood *et al.*, 2008). Similarly, an AFL of 477 bp found in hypolithic samples was identified as *Nostoc edaphicum*.

Culture-based methods showed that heterotrophic bacterial isolates from Vestfold Hills hypolithic communities fell into 3 groups, namely the *Proteobacteria* (α and γ subdivisions), the order *Cytophagales* and the class *Actinobacteria*, with many isolates also representing novel taxa at the species level (Smith *et al.*, 2000). While 20% to 40% of the viable isolates from sublithic samples displayed psychrophilic tolerance, the remainder were mostly non-halophilic and psychrotolerant (Smith *et al.*, 2000). Although some species such as *Pseudomonas, Psychrobacter, Stenophromonas, Arthrobacter, Achromobacter, Micrococcus, Rhodococcus, Janibacter* and *Gelidibacter* were found in all samples, others were more erratically distributed and not found in all hypolithic communities (Smith *et al.*, 2000).

Although hypolithic community 16S rRNA gene clone libraries were dominated by cyanobacteria, other clones clustered (at varying levels of incidence) with: α-proteobacteria, γ-proteobacteria, the order *Cytophagales*, the order *Planctomycetales* and the class *Actinobacteria* (second most abundant, 9% of all clones) (Smith *et al.*, 2000). A single eukaryote clone clustered with the class *Ulvophyceae* while no archaeal clones were detected (Smith *et al.*, 2000).

Metagenomic and culture-dependent data have shown that many identified organisms clustered with marine taxa (previously only detected in marine environments) as their closest relatives (Smith *et al.*, 2000). Furthermore, a relatively high proportion of psychrophilic and halophilic bacteria typical of marine environments were detected. It was therefore proposed that wind could transport organisms from marine to terrestrial environments where they eventually colonise hypolithic environments, seen as a 'refuge' from the harsh terrestrial landscape (Smith *et al.*, 2000). Multivariate analysis of Miers Valley hypolithic communities confirmed that each discrete hypolithic habitat harboured a unique community while analysis of similarities (ANOSIM) data showed that hypolithic cyanobacterial diversity was not significantly different from that of lake or hydroterrestrial cyanobacterial mats (Wood *et al.*, 2008). This indicates that these

mats may contribute significantly to the terrestrial cyanobacterial populations and that wind could play a role in dispersal.

The soils underlying the hypolithic communities contained most of the heterotrophic bacteria isolated from the sublithic growth attached to the rock, but lacked cyanobacteria, α-proteobacteria and flavobacteria (Smith *et al.*, 2000). These groups appear to be exclusively restricted to the sublithic growth adhered to the rock. It was suggested that adherence to the overlying rock offered the best protection for psychrophilic microorganisms since psychrophiles were absent in soil below the rock (Smith *et al.*, 2000). The favourable microenvironmental conditions created by the presence of the buffering rock therefore appear to decrease as depth below the rock increases.

While epifluorescent DAPI (4'-6-diamidino-2-phenylindol stain) counts (which do not include cyanobacteria) for hypolithic communities were similar to underlying soils (mean 1.1 x 10^9 cells g^{-1} dry weight and mean 0.5 x 10^9 cells g^{-1} dry weight respectively), viable cell counts of the sublithic material proved several magnitudes higher than that for underlying soils (mean 2.1 x 107 cfu g^{-1} and mean 2.3 x 104 cfu g^{-1} respectively) (Smith *et al.*, 2000). These data support the view that hypolithic microorganisms play a significant role in the functioning of the polar desert ecosystem.

Dry Valley hypolithic communities are viable, diverse and complex and are sheltered from the harsh environmental conditions by the overlying rock. The communities are dominated by cyanobacteria but also contain heterotrophic bacteria and some fungi. As these communities account for a significant proportion of the productivity in the Dry Valleys, further research is required to characterise these communities and elucidate their role in the ecosystem processes of the region.

1.6 Metagenomics and methods for community analysis

In the past, microscopy and laboratory culturing methods were used to characterise endolithic communities (Siebert *et al.*, 1996; Ascaso & Wierzchos, 2002; de los Rios *et al.*, 2004). As it is widely accepted that most microorganisms cannot be cultured under standard laboratory conditions, these methods do not give the true composition of the original community (de la Torre *et al.*, 2003; Aguilera *et al.*, 2006). Conventional microscopy techniques do not reveal all microorganisms present in soil as many microorganisms bind to sediment particles and cannot be detected (Muyzer *et al.*, 1993). Furthermore, due to their small size, similarity and lack of distinctive taxonomic features, the identification of bacteria and small phototrophic and heterotrophic eukaryotes based on morphological characteristics is difficult and unreliable (Aguilera *et al.*, 2006).

Diversity studies are important, not only for the development of fundamental scientific knowledge, but also to understand the links between diversity and community structure. Furthermore, these studies shed light on community function, how changes in diversity relate to changing conditions due to environmental or anthropogenic influence and what the consequences of these changes in diversity may mean. There is some disagreement in the scientific community regarding the importance of taxonomic or genetic diversity if functional diversity is maintained; i.e. is taxonomic diversity important if all ecosystem processes can be carried out by fewer taxonomic groups? It seems prudent however, given the limitations of methods to study diversity, to assume that functional redundancy (more than one taxonomic group carrying out the same function) does not exist and taxonomic diversity is important to maintain (Kirk *et al.*, 2004). For example, although arbuscular mycorrhizal fungi (AMF) were once thought to be functionally redundant because of a lack of host-specificity, this has since been shown to be untrue and AMF do provide benefits to different plant hosts (Kirk *et al.*, 2004).

Generally, even if an organism may be functionally redundant in one respect, it may not be redundant in other functions and may have different tolerances and susceptibilities to abiotic and biotic stresses (Kirk *et al.*, 2004). It is widely believed that the more diverse a population, the more resilient to stress and more capable it is of adapting to changes in environmental conditions (Kirk *et al.*, 2004). Diversity studies are therefore important to provide insights into the effects of stresses on microbial communities and the resiliency of communities that may be vital for ecosystem processes.

Although microorganisms are estimated to contribute one third of the earth's biomass (Whitman *et al.*, 1998) and are known to play vital roles in major environmental processes such as nutrient cycling and influence other ecosystem processes, our knowledge of their functions, processes and even diversity is decidedly limited (von Mering *et al.*, 2007). Reliable and accurate methods for the study of soil microbial diversity are necessary in order to address the questions of the current soil community diversity and structure and how changes in environmental conditions may influence ecosystem functions.

It is widely accepted that approximately 99% of the natural microbial population cannot be cultivated using standard laboratory methods (Amann *et al.*, 1995; Torsvik *et al.*, 1998; Handelsman, 2004; Kirk *et al.*, 2004; Aguilera *et al.*, 2006). While it could be argued that the 1% that is culturable is representative of the entire population and 99% of organisms are simply in a different physiological state that resists culturing, it is more likely that 99% are phenotypically and genetically different and only a minority (1%) is cultivated (Rondon *et al.*, 1999). Due to the limitations of culture-based techniques, various methods have been developed to identify and study entire microbial populations. The development and increased use of culture-independent methods have demonstrated that the phylogenetic diversity of most (if not all) microbial biotopes have been grossly underestimated, including those present in the Dry Valleys of Antarctica.

Metagenomics is a relatively fast-growing field that employs culture-independent methods (Figure 1.6) to assess collective microbial genomes (termed the 'metagenome') extracted from a particular habitat/environmental sample (Handelsman *et al.*, 1998). Due to its lack of dependence on culturing techniques, metagenomics is a powerful tool for determining microbial diversity and has been widely used to characterise the microbial communities in various habitats including soil systems (Muyzer *et al.*, 1993; Duarte *et al.*, 1998; Yeates & Gillings, 1998; Krsek & Wellington, 1999; Niemi *et al.*, 2001; Aguilera *et al.*, 2006).

An integration of metagenomics and classical microscopic and culture-based methods can enhance microbial ecology studies (Riesenfeld *et al.*, 2004; Aguilera *et al.*, 2006). For example, some species identified by cultivation or microscopy were not detected by molecular analysis of the same sample (Aguilera *et al.*, 2006). Conversely, molecular methods have been found to reliably differentiate between species that were not previously distinguished due to their similar morphologies (Aguilera *et al.*, 2006) or the inability to culture all organisms (Dunbar *et al.*, 1999). Metagenomic analysis may improve efforts to culture previously uncultured microorganisms by providing clues regarding the physiology of previously unculturable organisms, such as novel substrate preferences (Liles *et al.*, 2003; Riesenfeld *et al.*, 2004).

The validity of using molecular techniques for the analysis of microbial communities is dependent on the extraction of representative nucleic acids from the entire community (Figure 1.6) (Yeates & Gillings, 1998; Miller *et al.*, 1999). Furthermore, the routine application of these techniques for extensive analysis of microbial diversity requires the methods employed to be both rapid and inexpensive (Yeates & Gillings, 1998).

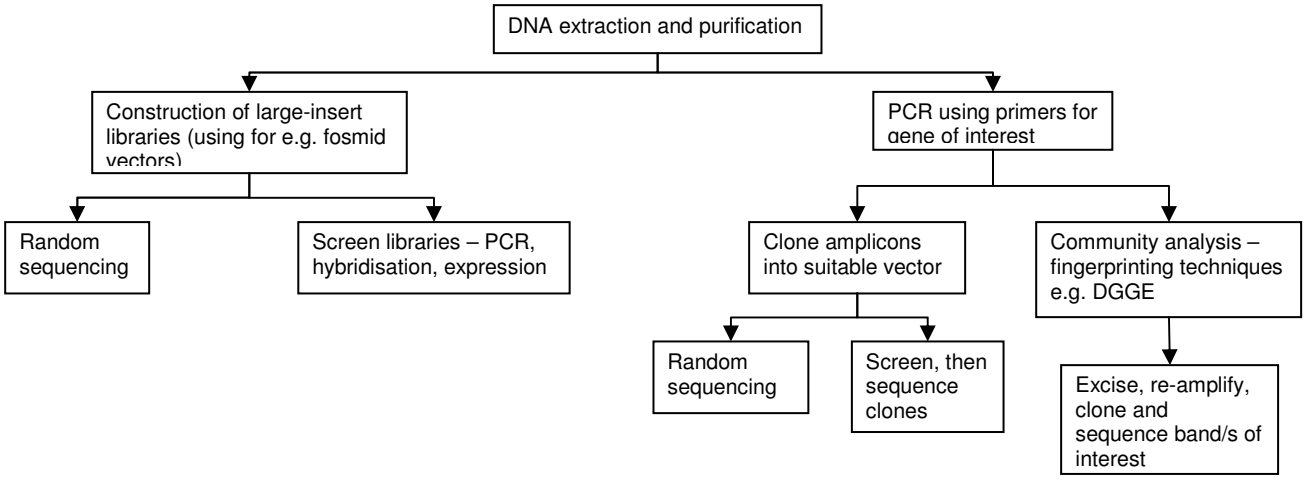

Figure 1.6: Simplified flow diagram indicating common methods used for metagenomic analysis.

Metagenomic analysis of soil systems has developed more slowly than that of aquatic environments, as soil is a more complex substrate. Aside from the inefficiencies of DNA extraction steps that result in inadequate cell lysis, soil contains many compounds that bind DNA or inhibit enzymatic reactions for restriction analysis, cloning and PCR (Zhou *et al.*, 1996; Miller *et al.*, 1999; Riesenfeld *et al.*, 2004). DNA adsorption to soil particles is directly related to soil composition and DNA recovery is lowest with clay-rich soils even if large amounts of DNA are present (Frostegard *et al.*, 1999). Elimination of humic substances from DNA extracts is also vital (Niemi *et al.*, 2001) as these compounds interfere with downstream PCR and cloning reactions (Smalla *et al.*, 1993; Tebbe & Vahjen, 1993). Proteins are also inhibitors of PCR and must be removed (Krsek & Wellington, 1999). However, significant advances in DNA extraction methods that eliminate or radically decrease impurities and inhibitors have led to a surge in the study of soil microbial communities (Tsai & Olsen, 1992; Tebbe & Vahjen, 1993; Jackson *et al.*, 1997).

1.6.1 Metagenomic DNA extraction

The extraction of high quality metagenomic DNA (in terms of molecular weight and fragmentation/shearing) is influenced by differences in cell wall structure of different organisms, the adhesion behaviour of microorganisms to sediment particles and the

physical and chemical properties of the soil (Niemi *et al.*, 2001). For example, Gram positive organisms, fungi and spores require a more harsh extraction method than Gram negative organisms. With mixed populations, the ideal extraction method would be able to disrupt structurally different cells and propagules to produce maximum yields of high molecular weight DNA with minimum shear and free from inhibitors i.e. maximum purity (Yeates & Gillings, 1998; Krsek & Wellington, 1999; Niemi *et al.*, 2001). Large fragments are required for direct cloning in order to minimise the number of clones to be screened (Miller *et al.*, 1999). Furthermore, highly fragmented DNA may prevent amplification of the desired sequence and may increase the possibility of obtaining chimeric PCR products and the consequent detection of artefact 'species' (Liesack *et al.*, 1991; van Wintzgerode *et al.*, 1997).

The extraction of DNA from microbial cells and propagules can be accomplished *ex situ* (where cells are recovered from the soil matrix before lysis) (Krsek & Wellington, 1999) or *in situ* (direct lysis within the soil matrix) (Miller *et al.*, 1999). Direct lysis is most popular as it produces higher DNA yields than *ex situ* methods with equivalent DNA fragment size from a greater range of organisms (Duarte *et al.*, 1998; Krsek & Wellington, 1999). Furthermore, direct lysis often results in purer DNA extracts than *ex situ* methods (Krsek & Wellington, 1999).

Although numerous DNA extraction methods have been described (Picard *et al.*, 1992; Smalla *et al.*, 1993; Zhou *et al.*, 1996; Yeates & Gillings, 1998; Miller *et al.*, 1999), all these methods incorporate one or more of three basic elements: physical disruption, chemical lysis and enzymatic lysis (Miller *et al.*, 1999; Ma & Michailides, 2007). The most popular methods for physical disruption are freeze-thawing and bead mill homogenisation (Miller *et al.*, 1999). Chemical lysis procedures differ widely, although most include detergents such as sodium dodecyl-sulphate (SDS) (Kuske *et al.*, 1998; Yeates & Gillings, 1998) and/or NaCl (Zhou *et al.*, 1996) and various buffers in the lysis mixtures (Yeates & Gillings, 1998; Miller *et al.*, 1999). SDS, which disrupts the hydrophobic layers of cell membranes, is the most widely used chemical lysis

method (Zhou *et al.*, 1996; Ma & Michailides, 2007). Some modifications to basic chemical lysis methods include the use of high temperatures (Kuske *et al.*, 1998), phenol or chloroform extraction steps (Miller *et al.*, 1999) and incorporation of chelating agents such as EDTA (Yeates & Gillings, 1998) to inhibit nucleases. Lysozyme digestion (Tsai & Olson, 1991) is the most widely used enzymatic lysis procedure while proteinase K digestion (Zhou *et al.*, 1996) is also popular.

The extraction method employed has a significant effect on DNA yield (Miller *et al.*, 1999). Bead mill homogenisation (or bead-beating) involves the vigorous shaking of samples in the presence of glass, ceramic or silica beads (Yeates & Gillings, 1998; Aguilera *et al.*, 2006) or quartz sand which results in ballistic disintegration of cells in the sample (Yeates & Gillings, 1998). Bead-beating has been proven to be superior to freeze-thawing for physical cell lysis (Smalla *et al.*, 1993; Miller *et al.*, 1999; Aguilera *et al.*, 2006). The improvement in cell lysis does not fully account for the improvement in DNA yield (Miller *et al.*, 1999; Aguilera *et al.*, 2006) but is rather attributed to the vigorous shaking employed in this method, which is thought to dissociate DNA attached to cell debris and soil particles (Miller *et al.*, 1999; Aguilera *et al.*, 2006).

Commercial extraction kits which combine mechanical (bead-beating) and chemical (detergent) lysis methods were also found to produce high molecular weight DNA extracts that yield the broadest and most intense banding patterns with denaturing gradient gel electrophoresis (DGGE) using universal bacterial primers (Niemi *et al.*, 2001) and universal eukaryotic primers (Aguilera *et al.*, 2006). Shearing of DNA may be avoided by decreasing homogenisation times and lower bead-beating speeds (Miller *et al.*, 1999). Moreover, extraction methods that include a bead-beating step have been shown to extract DNA from cells and tissues that are notoriously difficult to lyse such as *Bacillus* spp., insect tissue (Borneman *et al.*, 1996) and other eukaryotes (Yeates & Gillings, 1998).

The inclusion of organic solvents such as phenol or chloroform in the lysis mixture also significantly increases DNA yield (Miller *et al.*, 1999). Furthermore, a phenol/chloroform step was also shown to be more effective than the use of polyvinylpolypyrrolidone (PVPP) spin columns for purification of extracts (Krsek & Wellington, 1999). While effective for removing humic compounds, the use of PVPP [unlike the use of cetyl trimethyl ammonium bromide (CTAB)] can result in a loss of DNA (Zhou *et al.*, 1996). The addition of a lysozyme pre-treatment step was found to decrease DNA yield while the inclusion of Chelex 100 (a chelating agent) increased co-extraction of humic acids (Miller *et al.*, 1999). This is inappropriate, as even nanogram quantities of humic acids present in DNA extracts can inhibit downstream processes (Tsai & Olson, 1992).

The inhibitory effect of humic substances on *Taq* polymerase used in PCR varies, depending on the source of the DNA polymerase (Tebbe & Vahjan, 1993). Differences in DNA polymerase activity in the presence of humic substances should be taken into account (van Wintzgerode *et al.*, 1997), especially for amplification from soil samples. PCR primers also vary in their sensitivity to the presence of impurities in DNA extracts (Krsek & Wellington, 1999). In order to reduce the impact of inhibitors, DNA purification methods can be employed to remove/reduce inhibitory substances (Zhou *et al.*, 1996; Niemi *et al.*, 2001), additives such as bovine serum albumin (BSA) can be added to the PCR mix to reduce the inhibitory effects of contaminants (Borneman *et al.*, 1996) or a combination of DNA purification and PCR additives can be used (Berthelet *et al.*, 1996).

While dilution of DNA extracts reduces the inhibitory effects of contaminants on PCR, it also results in significant changes in the composition of 16S rRNA gene clone libraries constructed from environmental samples (Chandler *et al.*, 1997). These changes are thought to be due to random fluctuations in primer efficiency when template DNA is at very low concentrations (in the low picogram range) or due to molecular sampling errors (Chandler *et al.*, 1997). It is suggested that sampling error

decreases in significance with higher template concentrations (approaching the nanogram range) and that several template dilutions be used to ensure maximum diversity in clone libraries (Chandler *et al.*, 1997).

1.6.2 PCR amplification of phylogenetic marker genes

The choice of primers and number of amplification cycles used in PCR with multiple templates (such as metagenomic DNA extracts) may introduce bias, where end-product concentrations are disproportionate to the initial template concentrations (Suzuki & Giovannoni, 1996). Some DNA sequences tend to be relatively easily amplified regardless of their abundance (Aguilera *et al.*, 2006). This PCR bias is thought to be due to the progressive inhibition of the formation of template-to-primer hybrids by the preferential re-annealing of gene products (Suzuki & Giovannoni, 1996). However, this bias is thought to be small for environments of high diversity as it is unlikely that the amplification of any gene would produce amplicons in an inhibiting concentration (Suzuki & Giovannoni, 1996). Increased specificity and hybridisation efficiency of primers also decreases biased amplification (van Wintzgerode *et al.*, 1997). 'Universal' primers used for diversity studies should therefore be tested against the database to ensure the best possible primer efficiency and specificity.

The GC content of template DNA also results in differential amplification during PCR as genes with higher GC content dissociate less efficiently (Reysenbach *et al.*, 1992). This results in preferential amplification of templates with lower GC content (Reysenbach *et al.*, 1992). This effect may be reduced by the addition of a denaturant such as acetamide to the PCR mix in order to enhance denaturation of high GC templates (Reysenbach *et al.*, 1992). Hot start PCR and higher denaturation and annealing temperatures also serve to increase the specificity of annealing and decrease preferential denaturation of the template (Chandler *et al.*, 1997).

Small subunit rRNA (SSU rRNA) (16S rRNA/18S rRNA) genes and internal transcribed spacer (ITS) regions are the most commonly used phylogenetic markers for diversity studies as:

- they are present in the genomes of all known organisms (Kirk *et al.*, 2004);
- they have well defined regions for taxonomic classification due to their conservative nature (Lane *et al.*, 1985); and
- they allow for the prediction of evolutionary relationships (Lane *et al.*, 1985; Pace, 1999).

Furthermore, these defined regions are not subject to horizontal gene transfer and a large (and rapidly growing) sequence database is available (Kirk *et al.*, 2004). In addition, incorrect identification of bacteria was found to be far less common when SSU rRNA gene sequences were used for identification in comparison to identification by morphology, Gram stain, enzyme activity and substrate utilisation (Biovin-Jahns *et al.*, 1995). Incorrect identification of SSU rRNA gene sequences is more often than not caused by a lack of rRNA gene sequences of close relatives in the database (Biovin-Jahns *et al.*, 1995).

Genome size and the copy number of the SSU rRNA genes affect the amount of product obtained in PCR amplification of SSU rRNA genes (Farrelly *et al.*, 1995). It has been argued that since genome size and copy number of SSU rRNA genes for uncultured microorganisms is unknown, the number of species in SSU rRNA gene clone libraries from environmental samples cannot be determined (Farrelly *et al.*, 1995). This fact notwithstanding, metagenomic analysis still allows for the identification of many previously unknown microorganisms (Borneman *et al.*, 1996).

SSU rRNA gene sequences can be used to construct phylogenetic trees based on sequence identity. The use of phylogenetic trees based on marker gene sequences in molecular studies is essential as they help to convey relatedness between phylotypes

in a visual map (Hugenholtz *et al.*, 1998). Many culture-independent microbial diversity studies based on 16S rRNA gene diversity have shown unexpected levels of species richness and complexity in the uncultured microbial population (Ward *et al.*, 1990; Riesenfeld *et al.*, 2004).

With the use of cloning-sequencing methods, extensive analysis of a large number of clones is required to obtain any qualitative information about the population composition and species that constitute a very low percentage of the total population are not readily detected (Muyzer *et al.*, 1993). Furthermore, large numbers of different clones with identical inserts would be analysed in order to reach a plateau on the rarefaction curve (i.e. number of different inserts detected remains the same with an increase in the number of clones analysed). The expense of sequencing different clones with identical inserts may be somewhat avoided by restriction enzyme analysis of inserts. However, this method is both time-consuming and allows for the detection of only a small fraction of sequence differences (Muyzer *et al.*, 1993). Multiple restriction digests could be used to increase detection of sequence variations.

1.6.3 Community fingerprinting

The classical cloning-sequencing strategy can potentially provide an exhaustive description of microbial communities (Dunbar *et al.*, 2002) but sequencing of thousands of clones is impractical and costly. Therefore, alternative methods of assessing entire communities and changes in communities under various conditions are necessary. Various metagenomic fingerprinting methods that allow for the rapid assessment and visualisation of entire microbial communities have been developed. These fingerprinting techniques require PCR amplification of phylogenetic marker genes from metagenomic DNA extracts and separation of resultant amplicons by various methods (Kirk *et al.*, 2004).

The most common method of separation of these PCR-amplified fragments is by electrophoresis (Fromin *et al.*, 2002). These methods include: denaturing gradient gel electrophoresis (DGGE;) (Muyzer *et al.*, 1993), temperature gradient gel electrophoresis (TGGE;) (Heuer *et al.*, 1999), single strand conformation polymorphism (SSCP;) (Lee *et al.*, 1996), restriction fragment length polymorphism (RFLP and terminal-RFLP) also known as amplified ribosomal DNA restriction analysis (ARDRA;) (Tiedje *et al.*, 1999) and ribosomal intergenic spacer analysis (RISA and automated RISA;) (Fisher & Triplett, 1999).

All these techniques separate the amplified fragments based on sequence differences which affect the migration properties of the sequence in an electric field. Differential migration of amplified phylogenetic marker genes with different sequences (therefore theoretically representing different organisms) form banding patterns unique to the community, providing a phylogenetic 'fingerprint'. Changes in populations over time or due to changes in the environment can be rapidly assessed by changes in the community fingerprint. Furthermore, microbial diversity between habitats on all spatial scales can be compared.

The field of microbial ecology has benefited from these fingerprinting methods as it allows for the simultaneous assessment of a number of samples (Ferrari & Hollibaugh, 1999) and requires approximately 1 g soil samples to produce reproducible fingerprints (Ranjard *et al.*, 2003). The use of fingerprinting software allows for the rapid quantification and visualisation of similarities/differences in microbial diversity. The calculation of similarity indices and the construction of dendrograms based on the community banding patterns indicate the degree of similarity between different communities. Assuming each band presented in a fingerprint profile represents a different organism, similarity matrices can be used to calculate diversity indices such as the Shannon-Weaver index to compare biodiversity between habitats.

1.6.4 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis is a popular community fingerprinting method. Although it was originally developed for the detection of point mutations in DNA, this technique was adapted to the study of microbial genetic diversity (Muyzer *et al.*, 1993). The method is relatively simple: marker genes from metagenomic DNA extracts are amplified using primers modified by addition of a 35–40 bp GC-clamp attached to the 5'-end of the forward primer to ensure that part of the DNA remains double-stranded (Muyzer *et al.*, 1993; Kirk *et al.*, 2004), effectively acting as a 'hook' halting migration within the gel. Amplicons (of the same length but different sequences) are then electrophoresed through a polyacrylamide gel with an increasing linear concentration gradient of chemical denaturants (formamide and urea) (Muyzer *et al.*, 1993; Kirk *et al.*, 2004). Double-stranded DNA melts in sequence specific domains and migrates differentially through the gel, allowing for separation based on differences in sequence (Muyzer *et al.*, 1993; Muyzer, 1999). The mobility of the fragments decreases as they are denatured (Muyzer *et al.*, 1993).

The incorporation of a GC-clamp ensures that almost 100% of all possible sequence variations can be detected by differential migration on DGGE gels (Myers *et al.*, 1985; Sheffield *et al.*, 1989). Muyzer *et al.* (1993) found that the formation of stable, partially melted fragments did not occur without the incorporation of the GC-clamp. Instead, amplicons completely melted into two single strands with different mobility. It was also determined that a 40 bp GC-clamp was more successful than a 30 bp one in the formation of stable, partially melted fragments (Muyzer *et al.*, 1993). Furthermore, the GC-clamp was only effective at increasing stability when it was incorporated on the 5' primer and not the 3'primer (Muyzer *et al.*, 1993).

Upstream methods for PCR-DGGE (DNA extraction and purification) have distinct effects on the fingerprinting profiles produced (Krsek & Wellington, 1999; Niemi *et al.*, 2001; Aguilera *et al.*, 2006). Although theoretically DGGE can separate amplicons with single base-pair differences (Miller *et al.*, 1999), this is dependent on the

resolution of the gel. While most studies have targeted rRNA genes for DGGE-based diversity studies, other functional gene targets, for example *nifH*, are increasingly being used to provide diversity information on specific groups of organisms competent in a defined function (Zehr *et al.*, 2003).

DGGE allows for the simultaneous analysis of multiple samples and is a relatively inexpensive method (Kirk *et al.*, 2004; Aguilera *et al.*, 2006). It is also rapid, reliable and reproducible (Kirk *et al.*, 2004) given that the DNA extraction methods employed remain constant. As multiple samples can be analyzed concurrently, DGGE also allows for detection of differences or changes in microbial populations within a single habitat or between habitats (Ferris & Ward, 1997; Diez *et al.*, 2001; Gelsomino & Cacco, 2005). The pioneer study by Muyzer *et al.* (1993) showed that microorganisms can be detected by DGGE even when they constitute less than 1% of the total population.

The community level fingerprints derived from DGGE banding patterns have been used for diversity studies based on the number of bands present, their intensity (Kirk *et al.*, 2004) and their migration position. The banding pattern or fingerprint provides a profile of the total sample population where the relative intensity of each band most likely represents the relative abundance of the particular species in the population (Muyzer *et al.*, 1993). DGGE is a less time-consuming and laborious method than classical cloning-sequencing and immediately displays the constituents of a mixed community in both a qualitative and semi-quantitative manner (Muyzer *et al.*, 1993).

DGGE fingerprints can also be analysed for specific organisms or groups of organisms by hybridisation with species- or group-specific oligonucleotide probes (Liesack & Stackebrandt, 1992). Furthermore, bands of interest in DGGE profiles may be excised, re-amplified and sequenced directly for further identification, eliminating the need to first clone the PCR product (Muyzer *et al.*, 1993; Aguilera *et al.*, 2006). Previous studies comparing the phylogenetic trees obtained from partial SSU rRNA

gene sequences from bands excised from DGGE gels and full-length SSU rRNA gene sequences showed that phylogenetic assignments were similar and that this method can be used to establish groups of organisms even though some of the more deeply branching orders of the groups may differ (Lane *et al.*, 1985; Schmidt *et al.*, 1991). Therefore, although the sequences obtained from bands excised from DGGE gels are short (up to approx. 500 bp) the sequences are highly reliable for taxonomic identification at least up to the genus level (Aguilera *et al.*, 2006).

As it is based on DNA extraction and PCR methods, DGGE is subject to limitations based on variable DNA extraction and purification efficiency (Theron & Cloete, 2000; Niemi *et al.*, 2001) and PCR biases (van Wintzgerode *et al.*, 1997). Furthermore, different DNA sequences may have the same melting behaviour and therefore similar mobility in the denaturing gel resulting in a single band representing more than one sequence (Gelsomino *et al.*, 1999). Although co-migration of bands with different sequences but similar melting properties can occur, this problem may be avoided by increasing the resolution of the DGGE profiles by using narrower denaturation gradients (Muyzer *et al.*, 1993). However, minor bands may still be masked if their concentration is less than the detection limit of the staining solution (Aguilera *et al.*, 2006). This problem may be solved to some extent by the use of more sensitive staining methods such as silver staining (Aguilera *et al.*, 2006).

Many organisms contain multiple 16S rRNA genes, resulting in multiple bands on DGGE representing a single species (Gelsomino *et al.*, 1999; Aguilera *et al.*, 2006). The inaccuracy introduced by multiple bands with different mobility but the same identity may also be due to the formation of heteroduplex molecules during PCR (Aguilera *et al.*, 2006). In addition, relative band intensity may not necessarily correspond to relative species abundance. For example, when examining eukaryotic communities in acid environments, Aguilera *et al.* (2006) found that neither DGGE band intensity nor the number of clones retrieved per species reliably corresponded to abundance established through microscopic methods.

Direct DNA extraction methods employing bead-beating in combination with SDS treatment was found to be one of the best physico-chemical lysis methods for obtaining whole community DNA (Miller *et al*., 1999) for community profiling as it reveals the highest diversity as well as produces the most stable, reproducible genetic profiles (Kozdroj & van Elsas, 2000). Metagenomic DNA extraction and community profiling combined with cloning and sequencing may be used to characterise and compare niche communities in the Antarctic Dry Valleys.

1.7 Aims and Objectives

Microbial diversity and abundance in Antarctic terrestrial habitats has proven much greater than was previously anticipated. The major phyla found in soil habitats worldwide are represented, along with microclimate-specific phyla associated with desert soils and the physico-chemical features these habitats present. Hypolithons in the Miers Valley may represent the only stable communities in the area as they contain the elements for energy capture and turnover of primary nutrients and are physically protected from strong katabatic winds (Smith *et al*., 2006). Antarctic Dry Valley hypolithic communities have not been extensively characterised using molecular methods of analysis. Molecular analysis of the diversity of these communities, in terms of composition, structure and richness is necessary in order to determine their evolution in their discrete environment. Furthermore, the assessment of microbial diversity is the first step toward determining the modulation of hypolithic communities by environmental factors and elucidating ecological roles of the community members.

The main aims of this project are:

- Characterisation of the physico-chemical aspects affecting the hypolithic habitat on terrestrial Antarctica in order to determine the driving forces for the establishment and growth of hypolithic communities .
- Characterisation of terrestrial Antarctic hypolithic communities in terms of

microbial diversity.

- Comparison of the diversity of microorganisms between discrete hypolithic communities and between hypolithic and other terrestrial Antarctic environments in order to determine if all hypolithic communities are similar to each other or other soil environments.

Chapter 2: Materials and Methods

This study used field observations to characterise the physico-chemical properties of the hypolithic environment in Antarctic Dry Valleys. Metagenomic techniques including DGGE fingerprinting and cloning-sequencing methods were used to characterise hypolithic communities.

2.1 Sample Collection

All hypolithic community samples were collected from the Miers Valley, Eastern Antarctica during the summer season in December 2005, January 2006 and January 2008. Distribution of suitable translucent quartz rock was not homogenous throughout the Miers Valley, but instead was concentrated in a fairly narrow band to the north and north-west of Lake Miers (78°05.01'-78°05.921'S, 163°49.496'-163°48.149'E). All hypolithic communities for this study were collected from this zone.

Both the quartz rock and the associated community were recovered using aseptic techniques. Hypolithic community samples consisted of the microbial community adhering to the rock and the top 1 cm of soil beneath the rock. The samples were collected using a steel spatula that was swabbed with alcohol between each collection. All samples were placed in sterile polyethylene Whirl-Pak bags. The quartz rocks were also collected and placed in separate Whirl-Pak bags.

For each hypolithic community sample collected, two different control samples were also collected at similar soil depths: soil from under a nearby non-translucent rock and an open soil control. The non-translucent rock controls consisted of the top 1–2 cm soil beneath an appropriate rock located within 1 m^2 of the hypolithon collected. Open soil controls consisted of the top 1–2 cm of uncovered soil collected from four points (all within 1 m^2 of the hypolithon collected) and thoroughly mixed. Sub-samples of all collections were placed in separate Whirl-Pak bags for carbon/nitrogen

determination. All samples were stored at <0°C in the field and during transport, then at -80°C until required.

2.2 Characterisation of physico-chemical properties of the hypolithic microenvironment

2.2.1 Temperature and humidity

Air and soil temperature in the field was measured in degrees Celsius (°C) using a JENWAY 230 temperature meter and probe. Soil temperatures at sites where CO_2 flux was also measured were monitored using the LI-COR 8100 soil temperature probe. A Digitron 2020R humidity meter and probe was used to measure both air and soil relative humidity (% RH) at field sampling sites.

Long-term temperature and humidity data was collected by using micro-data loggers at appropriate sites. Both temperature and % RH data were logged using the Thermochron/Hygrochron iButtons (model DS1921G, Embedded Data Systems) at 2 min intervals for 2 days in January 2006 and at 5 min intervals for 7 days during the January 2008 expedition. Temperature and % RH conditions were also logged at 4 hour intervals over a 12-month period from January to December 2006.

2.2.2 Light

Incident light and light transmitted through colonised quartz rocks were measured in the field using a LI-COR LI-190SA quantum sensor and light meter. The quantum sensor used measures photosynthetically active radiation (PAR) in the 400 nm to 700 nm range in µmol m^{-2} s^{-1} (i.e. µmol of photons m^{-2} s^{-1}).

Long-term monitoring of incident light over 7 days during the January 2008 sampling expedition was carried out using the LI-COR quantum sensor (LI-190SA) mounted on the levelling fixture (LI-COR 2003S). Data was captured with the LI-1400 datalogger.

The instrument was configured to sample incident light conditions every second and logged the average incident light every 5 min over the 7-day period.

2.2.3 Spectral scan

A spectral scan of incident light (from 300 nm to 800 nm) in the Miers Valley was obtained using the EPP2000 Fibre Optic Spectrometer (StellarNet Inc.). A colonised quartz rock collected from the Miers Valley was thoroughly cleaned and cut into 1 cm x 1 cm cubes for spectral analysis of transmitted light using a UV-visible spectrophotometer (Cary 50, Varian, Inc.).

2.2.4 Carbon/Nitrogen analysis

Fractions (2–6 g) of hypolithic and control soil samples set aside for carbon/nitrogen analysis were ground in a Retsch MM 2000 ball mill to homogenise and reduce the particle size for efficient removal of soil carbonates. The equipment was thoroughly washed with water and 70% (v/v) alcohol and dried between samples. The acid digestion method described in Midwood & Boutton (1998) was used, with some modifications, to remove soil carbonates. The ground soil samples were placed in falcon tubes and sufficient 0.5 M HCl was added to saturate and immerse the ground soil. Saturated samples were vortexed then loosely capped and placed in a fume hood for 24 hrs, with intermittent agitation.

After 24 hrs, the samples were centrifuged at 2000 g for 1–2 min and the acid supernatant carefully discarded. The soil was washed by vigorous resuspension in dH_2O and centrifuged at 2000 g for 1–2 min and the supernatant discarded. The wash procedure was repeated until the supernatant pH reached 7.0.

The washed soils were dried at 60°C to constant weight, then re-ground in the bead mill (Retsch MM 2000). Samples were then weighed for analysis (0.25±0.002 g) into combustible foil sample packages. Percent organic carbon and nitrogen were

determined using a TruSpec Carbon/Nitrogen determinator (LECO Corp., St Joseph, MI, USA) at the Stable Isotope Laboratory at the University of Waikato, Hamilton, NZ.

2.3 Metagenomic DNA extraction

Metagenomic DNA refers to the total DNA from the collective microbial genomes extracted from a particular habitat/environmental sample (Handelsman *et al.*, 1998). In the field, metagenomic DNA extraction from all samples was conducted using the FastDNA® SPIN® Kit for soil (Bio101® Systems) or the UltraClean™ Soil DNA Kit (MO BIO Laboratories, Inc.). Both kits included a bead-beating physical lysis step, carried out using a FastPrep bead-beater (Bio101). Extractions were carried out as described in the manufacturer's instructions.

In the laboratory, metagenomic DNA was extracted from soils using a modification of the method described in Miller *et al.* (1999). Approximately 0.5 g quartz sand was placed in 2 ml screw-capped tubes and autoclaved then dried in an oven. Approximately 0.5 g of soil was added to the sterile sand, followed by 300 µl sodium phosphate (100 mM NaH_2PO_4) and 300 µl SDS-lysis buffer [100 mM NaCl, 500 mM Tris-HCl pH 8.0, 10% (w/v) SDS].

Samples were gently mixed by inversion before the addition of 300 µl chloroform-isoamyl alcohol (24:1, v/v). The vials were then mixed vigorously using a bead-beater (FastPrep Bio101) at 4.5 m s^{-1} for 30-40 s or vortexed at maximum speed for 1.5-2 min. The tubes were then centrifuged at 15000 g for 5 min to pellet the cell debris. The supernatants were removed to clean 1.5 ml tubes and 7 M ammonium acetate was added to a final concentration of 2.5 M.

Tubes were gently inverted until the appearance of white flocculates and centrifuged at 15000 g for 5 min to produce a clear supernatant with the SDS forming a thick gel-like interphase between the upper aqueous phase and the organic phase below. After transferring the clear supernatant to a clean 1.5 ml tube, 0.6 volumes of isopropanol was added.

The tubes were inverted a few times to mix then incubated at room temperature for at least 15 min. After incubation, samples were centrifuged at 15000 g for 5 min and the supernatant was carefully removed and discarded. The pellet was then washed with 1 ml 70% (v/v) ethanol to remove salts that might inhibit further processing. Complete resuspension of the pellet was achieved by pipetting slowly using a cut-off tip. The resuspended solution was centrifuged at 15000 g for 5 min, the wash supernatant removed and the pellet was air-dried.

The pellet was resuspended in 100 µl 10 mM Tris-HCl pH 8.0 for at least 2hrs before 5 µl of the DNA extract was analysed by agarose gel (1% w/v) electrophoresis and viewed using the AlphaImage (AlphaInnotech) imaging system. Extracts were then quantified using the Qubit™ fluorometer (Invitrogen™).

2.4 Amplification of phylogenetic marker genes

Table 2.1 lists the primer combinations used for amplification of phylogenetic marker genes for cloning and DGGE experiments. High molecular weight metagenomic DNA extracts from hypolithic and control samples were used as the template for amplification. The basic PCR reaction mix for 25 µl reactions consisted of: 10-20 ng template DNA, 1 x NEB ThermoPol reaction buffer [20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1% (w/v) Triton X-100], 0.2 mM each dNTP, 0.5 µM each primer and 0.5–1 µl *Taq* DNA polymerase (Fermentas). Negative controls containing all reagents and no template were always included in all rounds of PCR. Genomic DNA from the following sources were used as positive controls for the different primer combinations used:

- *E. coli* DH5α genomic DNA for all universal bacterial PCR reactions;
- Genomic DNA from an unknown filamentous fungus for all eukaryote PCR reactions;
- Recombinant plasmid DNA containing a cyanobacterial 16S rRNA gene insert for all cyanobacterial-specific primer combinations; and

- Genomic DNA from a *Sulfolobus* species for all archaeal-specific PCR reactions.

Table 2.1: Primer combinations and PCR parameters used in this study.

Primer Set	Sequence (5' to 3')	Target/Amplification region	Positions	Specificity	PCR cycling parameters	Reference
E9F	GAGTTTGATCCTGGCTCAG	16S rRNA gene	9 to 27[a]	Universal for bacteria	94℃ for 2 min **30 cycles**: 94℃ for 1 min, 50℃ for 1 min, 72℃ for 1 min 72℃ for 10min	Hansen *et al.*, 1998
U1510R	GGTTACCTTGTTACGACTT		1510 to 1492[a]			Reysenbach & Pace, 1995
341F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG	16S rRNA gene	341 to 357[a]	Universal for bacteria	94℃ for 5 min **20 cycles**: 94℃ for 45 s, 65℃ touchdown to 55℃ for 30 s, 72℃ for 1 min **20 cycles**: 94℃ for 30 s, 55℃ for 30 s, 72℃ for 1 min 72℃ for 20 min	Muyzer *et al.*, 1993
534R	ATTACCGCGGCTGCTGG		534 to 518[a]			Muyzer *et al.*, 1993

Table 2.1 continued

Primer Set	Sequence (5' to 3')	Target/Amplification region	Positions	Specificity	PCR cycling parameters	Reference
EukA	AACCTGGTTGATCCTGCCAGT	18S rRNA gene	1 to 21[b]	Universal for Eukaryotes	94℃ for 3 min **30 cycles:** 94℃ for 45 s, 50℃ for 1 min, 72℃ for 1 min 72℃ for 20 min	Diez *et al.*, 2001
EukB	TGATCCTTCTGCAGGTTCACCTAC		1795 to 1772[b]			Diez *et al.*, 2001
Euk1A	CTGGTTGATCCTGCCAG	18S rRNA gene	4 to 20[b]	Universal for Eukaryotes	94℃ for 3 min **8 cycles:** 94℃ for 45 s, 60℃ touchdown to 56°C for 45 s, 72℃ for 2 min **27 cycles:** of 94℃ for 45 s, 56°C for 45 s, 72℃ for 2 min 72℃ for 15 min	Diez *et al.*, 2001
Euk516R-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACCAGACTTGCCCTCC		563 to 548[b]			Diez *et al.*, 2001

Table 2.1 continued

Primer Set	Sequence (5' to 3')	Target/Amplification region	Positions	Specificity	PCR cycling parameters	Reference
ITS1	TCCGTAGGTGAACCTGCGG	5.8S, ITS1 and ITS2	N/A	Universal for Eukaryotes	94℃ for 3 min **30 cycles:** 94℃ for 30 s, 50℃ for 30 s, 72℃ for 1 min 72℃ for 20min	White *et al.*, 1990
ITS4	TCCTCCGCTTATTGATATGC		N/A			White *et al.*, 1990
ITS1F	CTTGGTCATTTAGAGGAAGTAA	5.8S, ITS1 and ITS2	N/A	Specific for fungi	94℃ for 5 min **35 cycles:** 94℃ for 45 s, 55℃ for 45 s, 72℃ for 1 min 72℃ for 20min	Gardes & Bruns, 1993
ITS4	TCCTCCGCTTATTGATATGC		N/A			White *et al.*, 1990

Table 2.1 continued

Primer Set	Sequence (5' to 3')	Target/Amplification region	Positions	Specificity	PCR cycling parameters	Reference
ITS1F-GC	CGCCCGCCGCGCCCC GCGCCCGGCCCGCCG CCCCCGCCCCTCCTCC GCTTATTGATATGC	ITS1	N/A	Specific for fungi	94℃ for 5 min **30 cycles:** 94℃ for 45 s, 55℃ for 45 s, 72℃ for 1 min 72℃ for 30min	Gardes & Bruns, 1993
ITS2	GCTGCGTTCTTCATCG ATGC		N/A			White *et al.*, 1990
A3Fa	TCCGGTTGATCCYGCC GG	16S rRNA gene	3 to 20[a]	Archaea except Nanoarchaea	94℃ for 4 min **25 cycles:** 94℃ for 45 s, 55℃ for 45 s, 72℃ for 1 min **10 cycles:** 94℃ for 30 s, 55℃ for 30 s, 72℃ for 1 min 72℃ for 20 min.	Baker *et al.*, 2003
Ab927r	CCCGCCAATTCCTTTA AGTTTC		906 to 927[a]			Baker *et al.*, 2003

Table 2.1 continued

Primer Set	Sequence (5' to 3')	Target/Amplification region	Positions	Specificity	PCR cycling parameters	Reference
A340F-GC	CGCCCGCCGCGCGCG GCGGGCGGGGCGGGG GCACGGGGGGCCCTA CGGGGYGCASCAG	16S rRNA gene	340 to 355[a]	Universal for Archaea	94℃ for 4 min **30 cycles:** 94℃ for 30 s, 67℃ for 30 s, 72℃ for 1.5 min 72℃ for 20 min	Ovreas *et al.*, 1997
A533R	TTACCGCGGCKGCTG		519 to 533[a]			Ovreas *et al.*, 1997
Cya359F-GC	CGCCCGCCGCGCCCC GCGCCCGTCCCGCCG CCCCCGCCCGGGGGG GAATTTTCCGCAATGG G	16S rRNA gene	359 to 378 [a]	Specific for Cyanobacteria	94℃ for 5 min **35 cycles:** 94℃ for 1 min, 64℃ for 1 min, 72℃ for 1 min 72℃ for 20min	Nubel *et al.*, 1997
Cya781R(a)	GACTACAGGGGTATCT AATCCCTTT		781 to 805 [a]			Nubel *et al.*, 1997
Cya781R(b)	GACTACTGGGGTATCT AATCCCATT		781 to 805 [a]			Nubel *et al.*, 1997

Table 2.1 continued

Primer Set	Sequence (5' to 3')	Target/Amplification region	Positions	Specificity	PCR cycling parameters	Reference
M13fw M13rev	AGCGGATAACAATTTC ACACAGG CCCAGTCACGACGTTG TAAAACG	Insert	N/A	Vector primers	94℃ for 5 min **10 cycles:** 94℃ for 30 s, 65℃ touchdown to 55℃ for 30 s, 72℃ for 1.5 min **25 cycles:** 94℃ for 30 s, 55℃ for 30 s, 72℃ for 1.5 min 72℃ for 5min.	InsT/Aclone™ PCR Product Cloning Kit (Fermentas)

[a] *E. coli* numbering of 16S rRNA gene.

[b] *Saccharomyces cerevisiae* numbering of 18S rRNA gene

2.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out essentially as described in Muyzer *et al.* (1993) with some modifications. PCR products amplified with primers containing a GC clamp were separated on 9% (w/v) polyacrylamide gels containing chemical denaturing gradient. Denaturing gradient gels were poured using a gradient mixer (Bio-Rad) containing 'high' (maximum amount denaturants required) and 'low' (minimum amount denaturants required) gel solutions. 'High' and 'low' solutions were prepared by mixing '0%' and '100%' denaturant gel stock solutions to give the required denaturant concentrations. The '0%' solution contained 40% acrylamide : *N,N'* bis-acrylamide (37.5:1) and 1 x TAE (40 mM Tris-HCl, 10 mM glacial acetic acid, 1 mM EDTA, pH 8.0) only, while the '100%' solution included the addition of 7 M urea and 40% (v/v) deionised formamide as the denaturants. The denaturing gradient for each set of primers used was determined by first using broad denaturing gradients such as 10% to 90%, then narrowing the range depending on the level of separation achieved.

Electrophoresis was performed using the Bio-Rad DCode™ DGGE system. The PCR products were separated out at a constant 100 V for 16 hrs in 1 x TAE at a constant temperature of 60°C. After electrophoresis, gels were stained in 1 x TAE containing ethidium bromide (0.5 mg l^{-1}) for 10–15 min, then destained in 1 x TAE for 15 min. Gels were viewed and the images captured under UV using the AlphaImage (AlphaInnotech) imaging system.

Analysis of DGGE profiles and banding patterns was performed using GelCompar® II, version 5.0 (Applied Maths). The same DGGE marker was electrophoresed on either end of all DGGE gels. This allowed for normalisation of the gel using GelCompar® II, which standardised the band migration in all gels in order for comparison of community profiles between different gels. Bands on the DGGE gels were considered as present or absent and recorded as a binary matrix. This matrix was used to generate distance matrices which were interpreted into dendrograms and multi-dimensional scaling plots to assess similarity of community profiles.

2.5.1 Diversity indices

Each biotype (DGGE band type) detected in this study was assigned as an operational taxonomic unit (OTU) as the definition of 'species' in terms of microbial diversity is debatable and obscure (Torsvik *et al.*, 1998). OTUs were therefore used to characterise and compare microbial populations. Diversity indices to compare biodiversity based on community fingerprints were calculated as follows:

- Species richness (S) – refers to the total number of different OTUs present in an environment. S does not take into account abundance or distribution of each OTU.
- Shannon index (H') – is a measure of the amount of information (entropy) in a system. The index is positively correlated with species richness and evenness. It also gives more weight per OTU to rare rather than common species. It is calculated by $H' = -\sum_{i=1}^{S} Pi \ln Pi$ where: n_i is the number of individuals in that OTU, S is the species richness (total number of OTUs) and N is the total number of individuals. P_i refers to the relative abundance of each OTU and is calculated as the proportion of individuals of a given OTU to the total number of individuals in the community i.e. $\frac{n_i}{N}$.
- The maximum Shannon index (H_{max}) was calculated using the equation $H_{\max} = \ln S$, where S is species richness.
- Evenness (E) – is a measure of the similarity of the abundance of individuals in different OTUs. When all OTUs contain similar numbers of individuals, evenness approaches 1. If distribution is uneven, the evenness value increases. Evenness is calculated by the equation $E = \frac{H'}{\ln S}$ where H' is the value for the Shannon index and S is species richness.

2.6 Preparation of competent cells

Competent *Escherichia coli* DH5α cells were prepared according to the Inoue method for the preparation of "ultra-competent" cells (Sambrook & Russell, 2001) with some modifications. *E. coli* DH5α was streaked onto Luria Bertoni (LB) agar (10 g l^{-1}

tryptone, 10 g l^{-1} NaCl, 5 g l^{-1} yeast extract, 15% w/v agar) and incubated at 37°C overnight. One colony (of 2–3 mm diameter) from the overnight plate culture was used to inoculate 25 ml SOB medium (20 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 0.5 g l^{-1} NaCl, 2.5 mM KCl, 100 mM MgCl) in a 250 ml flask. The flask was incubated at 37°C with vigorous shaking (200 rpm) for 6–8 hrs. Different volumes of this starter culture were used to inoculate 250 ml SOB medium in 1 l flasks. Flask 1 was inoculated with 10 ml starter culture, while Flasks 2 and 3 received 4 ml and 2 ml starter culture, respectively. The flasks were incubated overnight at 18-20°C with moderate shaking (90–120 rpm). The following morning, the OD_{600} of each flask was monitored every 30–45 min using a HELIOS spectrophotometer (ThermoSpectronic). Once the OD_{600} of one of the cultures reached 0.55 (indicating mid-logarithmic growth phase), the culture was immediately placed on ice for 10 min and the remaining two cultures were discarded.

The culture was centrifuged in a sterile centrifuge tube at 2500 g for 10 min at 4°C to harvest the cells. The supernatant was discarded and the tube inverted on a paper towel for 2 min to ensure that all the liquid medium was removed. The cell pellet was gently resuspended by swirling in 80 ml filter sterilised ice-cold Inoue transformation buffer (55 mM $MnCl_2.4H_2O$, 15 mM $CaCl_2.2H_2O$, 250 mM KCl and 10 mM PIPES pH 6.7). This suspension was centrifuged at 2500 g for 10 min at 4°C and the supernatant discarded. The cell pellet was resuspended in 20 ml ice-cold Inoue transformation buffer, 1.5 ml DMSO added, gently mixed and placed on ice for 10 min. Aliquots of the suspension were dispensed into sterile, chilled 1.5 ml reaction tubes and placed at -80°C. When required for use, competent cells were thawed on ice.

2.7 Cloning of phylogenetic marker genes

For cloning purposes, all PCR reactions were done in duplicate and the reactions pooled in order to minimise any effects due to sampling error. Once amplicons of the correct size were confirmed by agarose gel electrophoresis, pooled PCR products were electrophoresed in 1.5% w/v agarose. Bands were excised from the gel and the

products purified using the EZ-10 Spin Column DNA extraction kit (Bio Basic Inc.) or the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

5 µl volumes of purified PCR products were separated out on a 1.5% w/v agarose gel to confirm whether the amplicons purified were the correct size, and were quantified using the Nanodrop ND-1000 spectrophotometer.

Amplicons were directly cloned into the plasmid pTZ57R/T using the InsT/Aclone™ PCR Product Cloning Kit (Fermentas) as per the manufacturer's instructions. 10 µl ligation reactions were prepared and incubated at room temperature overnight. Ligations were chemically transformed in Inoue ultra-competent *E. coli* DH5α cells. Suitable positive and negative controls were included for all ligation reactions.

2.8 Chemical transformation

Chemical transformation of Inoue competent cells were carried out as per Sambrook & Russell (2001) with some modification. Up to 25 ng vector DNA was added per 50 µl Inoue chemically competent *E. coli* DH5α cells, gently mixed and incubated on ice for 30 min. Tubes were placed in a 42°C water bath for exactly 90 s and quickly transferred to an ice bath for 1–2 min. 800 µl SOC (SOB medium with 20 mM glucose) medium was added to each tube which was subsequently warmed for 1 min in a 37°C water bath. The reactions were incubated at 37°C for 45 min with gentle agitation.

200 µl transformation reactions were spread onto 15 cm LB agar plates containing 0.1 mg ml^{-1} ampicillin (LB-amp). The plates also included X-gal [100 µl 2% (w/v) X-gal] and IPTG [20 µl 20% (w/v) IPTG] spread-plated over the agar surface and dried before use. Suitable positive and negative controls, such as control vector or no vector added, were included for each round of transformation to ensure that transformation efficiency for library construction was sufficiently high (i.e. 1 x 10^7 cfu μg^{-1} DNA). Positive recombinant clones were picked, placed into a 96-well pattern on LB-amp agar and incubated at 37°C overnight. After incubation, a fresh replica of the

clones in the 96-well pattern was made on LB-amp plates and in 150 µl LB-amp broth in microtitre plates. After overnight incubation at 37°C, 150 µl sterile 50% v/v glycerol was added to the broth cultures and the glycerol stocks stored at -80°C.

2.9 Screening of phylogenetic marker genes

2.9.1 M13 colony PCR

M13 colony PCR (Table 2.1) was used to verify the presence of correct sized inserts. Single clone colonies were used as templates for PCR, with an initial boiling step incorporated in the PCR parameters to ensure cell lysis. Once the correct sized insert was verified via agarose gel electrophoresis, plasmid DNA was isolated using the silica plasmid minipreparation method (Brown, 1997).

2.9.2 Plasmid DNA extraction – Silica method

Clones were inoculated into 4 ml LB broth and incubated at 37°C overnight with shaking at 200 rpm. The 2 ml overnight cultures were centrifuged at 15000 g for 1 min in a 2 ml microfuge tube. The supernatant was removed and the remaining 2 ml culture was added and centrifuged at 15000 g for 1 min. Aspiration was used to ensure that all the supernatant was removed.

The cell pellet was thoroughly resuspended in 300 µl resuspension solution (50 mM Tris-HCl pH 8.0; 10 mM EDTA pH 8.0; 1 µg ml^{-1} RNaseA) before the addition of 300 µl freshly prepared lysis solution [200 mM NaOH; 1% (w/v) SDS]. The suspension was inverted to mix gently and incubated at room temperature for 2–5 min. 300 µl neutralisation solution (3 M potassium acetate, pH 5.5) was added and the suspension inverted to mix before incubation on ice for 2–5 min. The sample was centrifuged at 15000 g for 5 min, the supernatant removed and added to tubes containing 300 µl of silica suspension [2 g silica (Sigma, S5631) in 50 ml sterile water]. The samples were incubated at room temperature for at least 5 min before centrifugation at 15000 g for 30 s.

The supernatant was discarded and the pellet gently washed by inversion with 500 µl wash solution [50 mM NaCl, 10 mM Tris-HCl pH 7.5-8.0, 2.5 mM EDTA, 50% (v/v) ethanol]. The samples were centrifuged at 15000 g for 30 s and the wash solution discarded. A second wash step with 500 µl wash solution was done, with resuspension of the pellet by pipetting. The suspension was centrifuged at 15000 g for 30 s and the supernatant discarded. This was followed by a second centrifugation at 15000 g for 30 s. Any residual wash solution was removed by aspiration and the pellets were allowed to dry completely before proceeding.

The pellets were resuspended in 10 mM Tris-HCl (pH 8.0) using a vortex or pipetting and incubated at room temperature for at least 2 min. Suspensions were centrifuged at maximum speed for 1 min and the supernatant (plasmid minipreparation) transferred to a clean tube, avoiding the fine silica particles. Successful extraction of plasmid DNA (and complete elimination of *E. coli* genomic DNA) was confirmed by loading 5 µl volumes of the minipreparations onto 1% (w/v) agarose. Plasmid minpreparations were quantified using the Nanodrop ND-1000.

2.9.3 Amplified ribosomal DNA restriction analysis (ARDRA)

A nested PCR approach was used to amplify the phylogenetic marker inserts from ITS and 18S clone libraries for ARDRA analysis. The first round of PCR employed the vector primers M13fw and M13rev (Table 2.1). PCR products were verified on 1% (w/v) agarose gels before the second round of PCR. 1 in 10 dilutions of first round PCR products were prepared using sterile PCR-quality H_2O and 1-2 µl of the diluted product was used as the template in a nested reaction using the original insert primers (EukA with EukB for 18S rRNA gene inserts and ITS1 with ITS4 for ITS inserts) (Table 2.1).

Two different tetranucleotide restriction enzymes, *Hae*III and *MbO*I (Fermentas), were used separately for ARDRA. 25 µl restriction digest reactions consisted of 0.2 µl restriction enzyme, 2.5 µl of the appropriate 10 x buffer (according to the manufacturer), and 10 µl PCR product. The volume was made up to 25 µl using

sterile triple-distilled H_2O. *Hae*III and *MbO*I digest reactions were incubated at 37°C overnight.

The digests were electrophoresed through 4% (w/v) agarose gels containing ethidium bromide (0.5 μg ml^{-1}) at 100 V for 2–3 hrs, and the images viewed and captured using the AlphaImage system (AlphaInnotech). ARDRA patterns were analysed manually and clones containing inserts that generated distinctly different ARDRA banding patterns were sequenced.

2.10 Sequence analysis of phylogenetic marker genes

Cloned insert DNA was sequenced with the MegaBACE 500 Automated Capillary DNA Sequencing System (Amersham Biosciences) at the University of Cape Town Sequencing Facility, Cape Town. All sequencing reactions entailed the use of vector primers M13fw and/or M13rev. Sequences were manually edited using Chromas (Technelysium) and assembled in DNAMAN Version 4.15 (Lynann Biosoft).

Chapter 3: Physico-Chemical Analysis

3.1 Introduction

The coastal Miers Valley (78°06'S, 163°44'-164°12'E) in Eastern Antarctica is one of the number of ice-free valleys that together constitute the McMurdo Dry Valley region. The Miers Valley, located at the southern end of the region, is a typical glacially carved valley, approximately 25 km long and 1.2-2.5 km wide. The 20 m deep, permanently ice-covered Lake Miers situated on the valley floor is fed during the summer months by the melt-streams from two glaciers situated at the western end of the valley: the Miers Glacier on the northern side and the Adams Glacier on the southern side of the valley.

While no long-term temperature data exists for the Miers Valley, the mean annual air temperature of the coastal Dry Valleys is approximately -18°C (Dale *et al.*, 1999). During summer, maximum temperatures are around 0°C, with soil temperatures exceeding 14°C for short periods of time. Surface soil temperatures during winter fall to as low as -40°C. Snow is the sole form of precipitation, most of which sublimes quickly in the desiccating atmosphere. The region receives four months of continuous sunlight during summer and four months of complete darkness during winter, with each phase followed by transition twilight periods.

The extreme environmental conditions experienced in the Miers Valley are thought to be a challenge to the establishment and development of widespread biological communities. However, discrete hypolithic communities are present in the Miers Valley, confined to the translucent quartz rocks that occur in the desert pavement found in this Dry Valley (personal observation, based on the results from this thesis). The determination of temperature and humidity regimes in hypolithic and control environments may indicate whether these physical parameters are major drivers in

the establishment of hypolithic communities. Light conditions in the valley and in hypolithic habitats were also measured.

3.2 Temperature

Temperature was recorded at 4 hour intervals from January 2006 to December 2006 (Figure 3.1) in hypolithic and open soil environments in the Miers Valley (see Section 2.2.1). Figure 3.1 shows the average temperature in a hypolithic habitat and on the soil surface over the 12-month period.

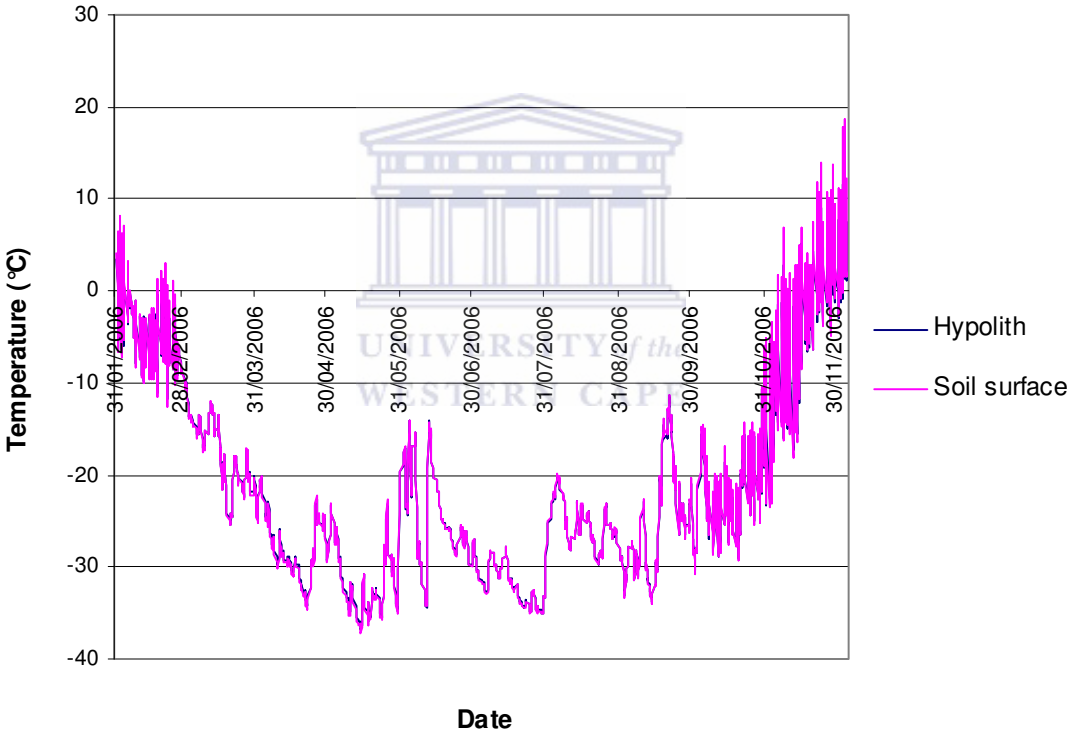

Figure 3.1: Average soil temperature over 12 months (Miers Valley, Antarctica, January to December 2006).

The figure (Figure 3.1) shows a decline in the average soil temperature to between -20 to -36°C during the winter months. During the summer months, soil temperatures increased to between -10 and 10°C, with highs of up to 18°C experienced in the open soil. It is clear that the average temperature, both on the soil surface and in the

hypolithic environment, decrease with reduced sunlight hours as the winter season approaches. Average temperatures increase with the approach to the summer season, when the number of sunlight hours increase. Although there was no significant difference in mean soil temperature in the hypolithic environment and on the soil surface over the 12 month period ($P>0.05$), there was a significant difference in temperature variance over the same period ($P<0.05$).

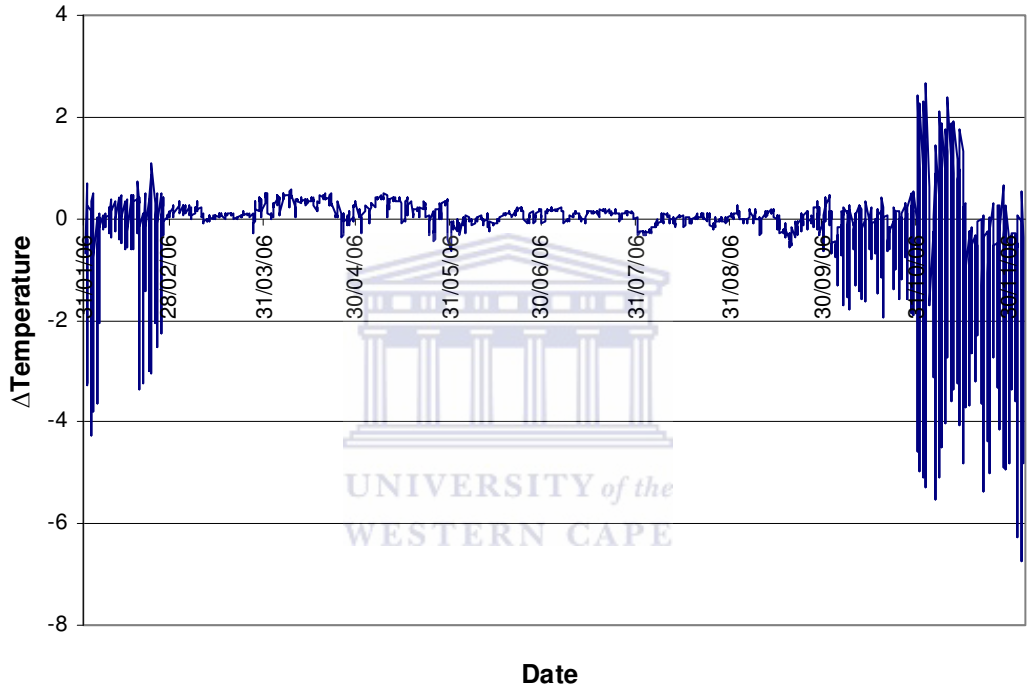

Figure 3.2: Difference in temperature between hypolithic and soil surface habitats over a 12 month period (Miers Valley, Eastern Antarctica, January to December 2006).

A plot of the temperature difference between hypolithic and soil surface habitats more clearly shows the differences between the two environments over the 12 month period (Figure 3.2). While the mean annual temperature in the hypolithic environment and at the soil surface were similar ($P>0.05$) and the difference between the two (~0) relatively stable over the winter months ($P>0.05$), the difference is more pronounced during the austral summer months (Figure 3.2). During the dark winter season, extremely cold but similar temperature conditions exist in hypolithic environments and on the soil surface. As the number of hours of sunlight increase with the approach of

the summer season, the difference between hypolithic and soil surface temperatures increase with up to 7°C differences occurring (Figure 3.2). Moreover, the negative values for during the summer months indicate that the hypolithic habitat is cooler than the soil surface during this period. This was expected as solar gain is the major determinant of soil surface temperature (de la Torre *et al*., 2003). Soil surfaces, which are directly impacted by solar irradiation will warm-up to a greater degree compared to the hypolithic habitat.

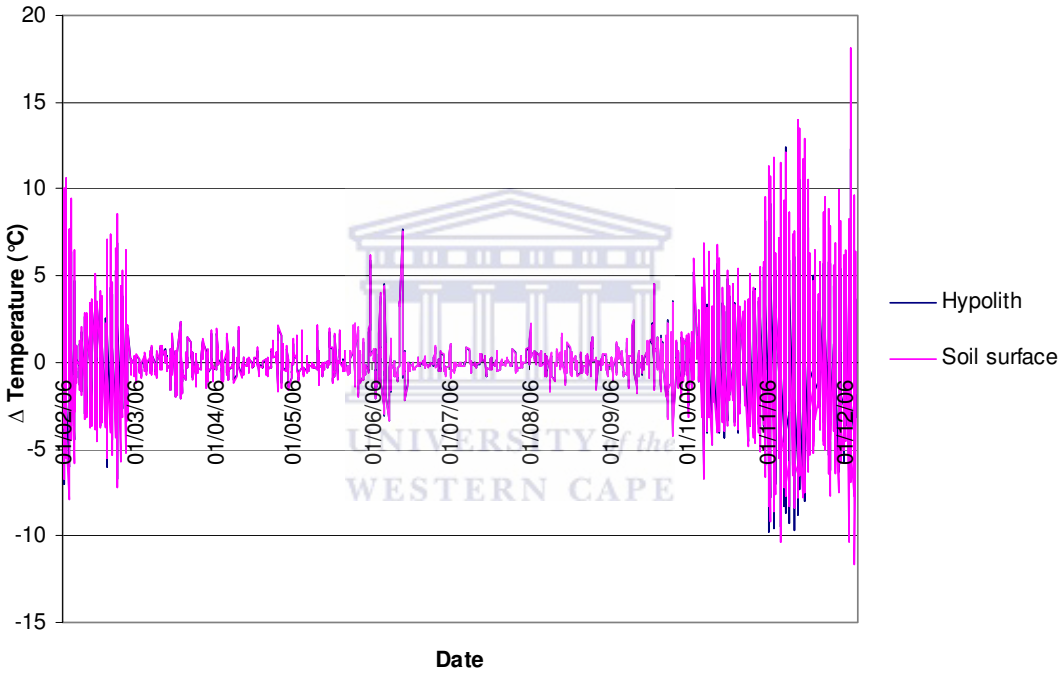

Figure 3.3: Change in temperature in hypolithic and soil surface environments at 4 hour intervals over 12 months (Miers Valley, January to December 2006).

Figure 3.3 illustrates the change in temperature in hypolithic and soil surface environments at the 4 hourly logging intervals over the 12 month period. The greatest changes in temperature over short time periods in both environments were experienced from October to February, when the maximum number of sunlight hours was experienced. As expected, the soil surface experienced much wider thermal fluctuations (with temperature oscillations of up to 18°C within 4 hrs occurring on the

soil surface) than the hypolithic environment (Figure 3.3), which was buffered from the direct effect of solar irradiation by the presence of the overlying rock.

In order to assess soil temperature fluctuations at higher resolution, and to establish the time-scales of soil temperature changes, temperature was also recorded in hypolithic and control environments at 2 min intervals over a 2 day period during January 2007 (Figure 3.4) and at 5 min intervals over 7 days in January 2008 (Figure 3.5) (see Section 2.2.1).

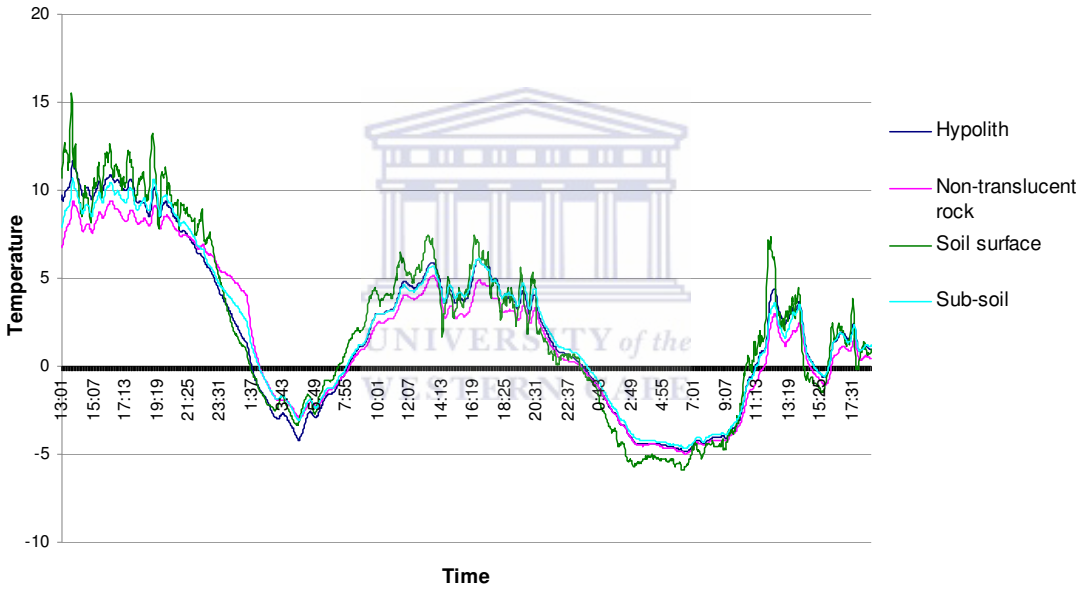

Figure 3.4: Temperature logged at 2 min intervals over 2 days in hypolithic and control environments (Miers Valley, Eastern Antarctica, January 2007).

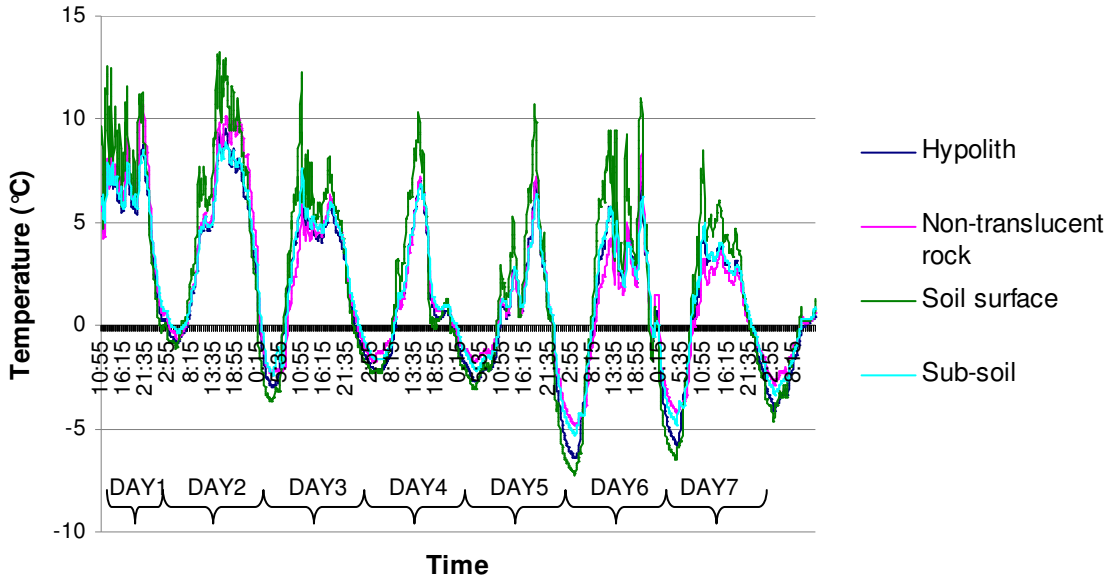

Figure 3.5: Temperature logged at 5 min intervals over 7 days in hypolithic and control environments (Miers Valley, Eastern Antarctica, January 2008).

Although the Antarctic continent, including the Dry Valleys, receives 24 hours of sunlight during the peak of the austral summer, diurnal fluctuations in temperature are evident in hypolithic and all control environments in the Miers Valley in January (Figures 3.4 and 3.5).

Temperatures increase to reach a maximum in the mid-afternoon and late evening (Figure 3.6), when the Miers Valley is subjected to more direct sunlight as the desert pavement lies on a 7° south-facing slope. The temperature then decreases to a minimum in the early hours of the morning when the sun is lower on the horizon and the lower portion of the south-facing slopes of the Miers Valley, where the study site was located, were shadowed. Sunlight and the angle of the incident irradiation is therefore important in determining the temperature regime of Dry Valley environments. The greatest diurnal temperature difference is experienced on the soil surface. For example, temperatures under the hypolithic rock vary from -2.7°C at the coolest point to 7°C at the warmest point during Day 4 (a difference of 9.7°C), while

temperatures on the soil surface goes from -3.1°C to 10.2°C (a difference of 13.3°C) within the same period (Figure 3.6).

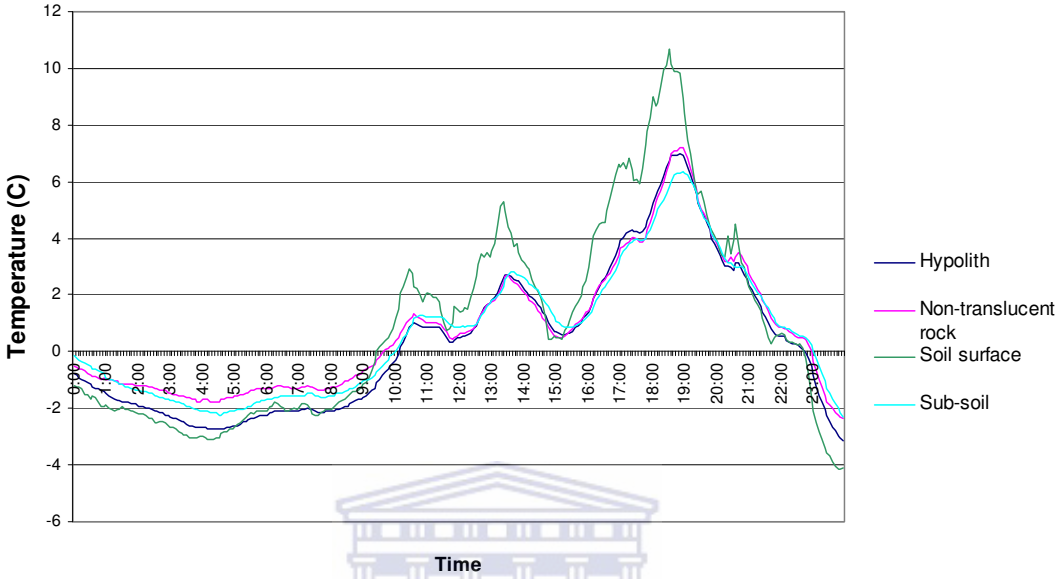

Figure 3.6: Expansion of a portion of Figure 3.5 (Day 4) showing the temperature regime in hypolithic and control habitats over 24 hours (Miers Valley, Eastern Antarctica, January 2008)

Based on the high resolution analysis (Figures 3.4 and 3.5) it is evident that temperature fluctuations are greater at the soil surface than beneath desert pavement rocks or 3cm below the soil surface (sub-soil). The temperature regimes in hypolithic, non-translucent rock and sub-soil environments are comparable with no significant difference ($P<0.05$).

The greatest temperature fluctuation occurs at the soil surface, with variance almost twice that of the other environments tested. However, there is no significant difference in temperature variance for the experimental hypolithic, non-translucent rock and sub-soil environments ($P<0.05$). This indicates that the buffering of temperature fluctuations experienced under hypolithic rocks is similar to the buffering effect under non-translucent rocks and at 3 cm below the soil surface.

Table 3.1: Analysis of temperature over 7 days in hypolithic and in control environments (Miers Valley, January 2008)

	Hypolithic environment	Non-translucent rock environment	Sub-soil environment	Open Soil environment
Average	1.45	1.73	1.76	2.19
StdDev[a]	3.64	3.54	3.40	4.66
Var(P)[b]	13.24	12.53	11.53	21.74

[a]StdDev = Standard deviation
[b]Var(P) = Variance

While a greenhouse-type effect was evident for hypolithic habitats in the hot desert environment (Schlesinger *et al.*, 2003), this was not the case in the Antarctic polar desert. The comparable temperature regime and variance beneath both types of rock and within the sub-soil (Figures 3.4 and 3.5) indicate that there is no greenhouse effect afforded by the presence of the overlying rock and the hypolithic habitat is, on average, cooler than the soil surface (Table 3.1). Together, the data suggests that temperature is not the main physical factor limiting terrestrial growth to the communities beneath quartz rocks as opposed to non-translucent rocks.

3.3 Relative Humidity (%RH)

Relative humidity (%RH) is the ratio of water vapour present in the atmosphere at a given temperature relative to the amount the air can hold at saturation at that temperature. %RH can be viewed as an indicator of potential water availability to biological communities. Temperature and %RH were simultaneously recorded using the iButton dataloggers in hypolithic and control environments over a 12 month period (January to December 2006) (see Section 2.2.1).

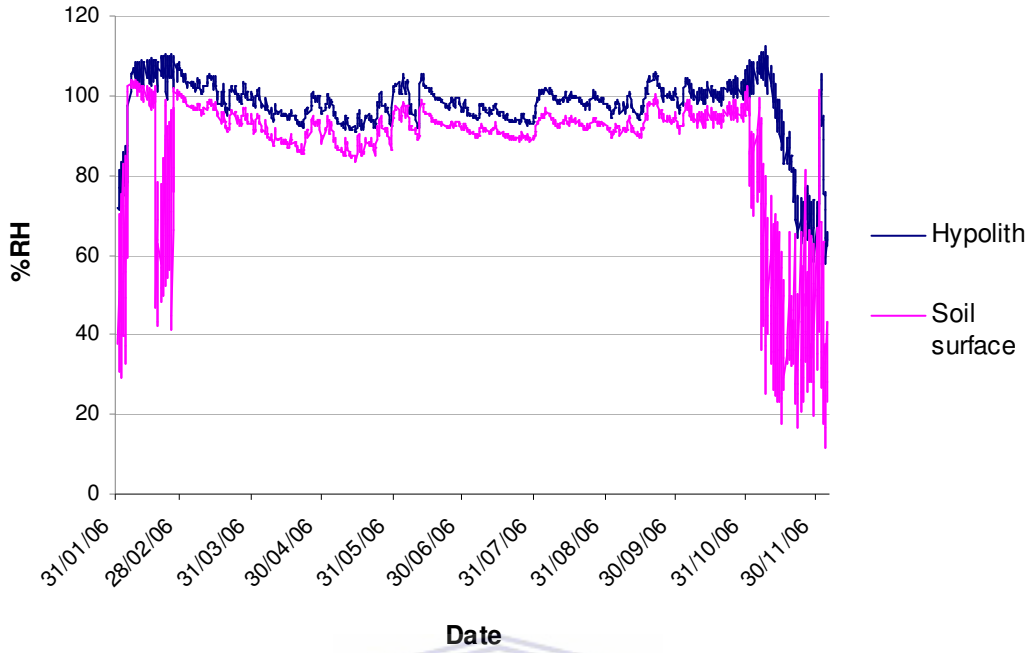

Figure 3.7: Average %RH on the soil surface and in a hypolithic environment over 12 months (Miers Valley, Eastern Antarctica, January to December 2006).

As predicted, the %RH pattern throughout the year (Figure 3.7) for both habitats appear to be the inverse of the patterns observed for temperature change in these environments (Figure 3.1). The %RH is highest over the winter season and decreases rapidly in summer. The unexpectedly high humidity during the winter months is believed to be due to unusually high snowfall persisting in the Miers Valley during the 2006 winter season. Nevertheless, %RH was consistently higher in hypolithic environments than the soil surface throughout the year (Figure 3.7) with a significant difference in the mean annual %RH ($P<0.05$). Furthermore, the soil surface experiences wider %RH fluctuations during the austral summer than the hypolithic habitat (Figure 3.7) with a significant difference in variance between the two environments ($P<0.05$).

Figure 3.8 shows the change in %RH at the 4 hourly logging intervals for both habitats over the 12 month period. %RH fluctuations in both environments were greatest during the summer season. It is also clear that %RH oscillations of up to 80% are possible within a 4 hr period on the soil surface (Figure 3.8).

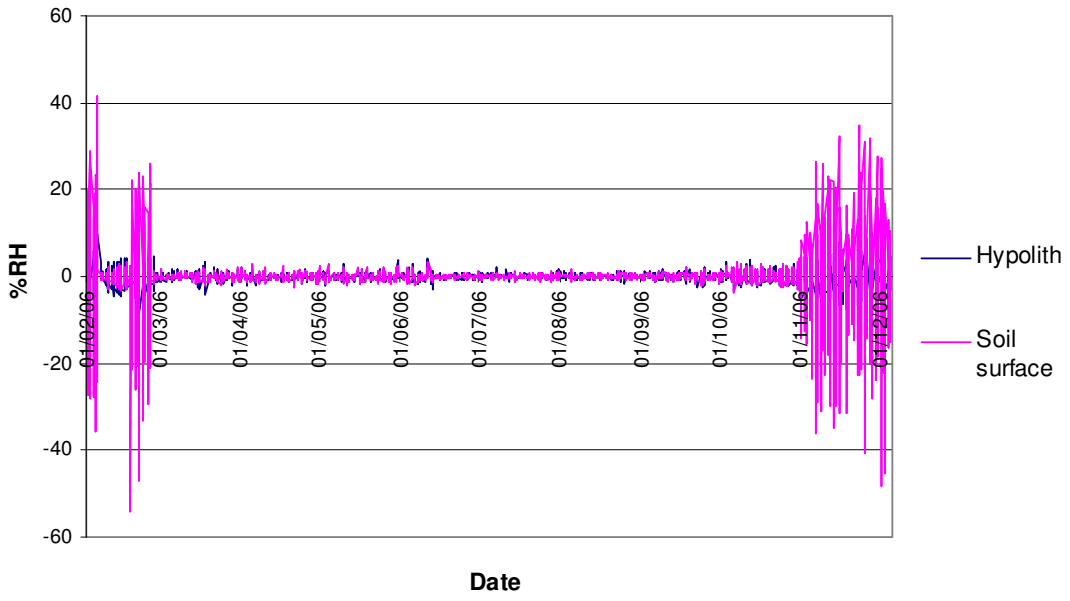

Figure 3.8: Change in %RH in hypolithic and soil surface environments over time (Miers Valley, January to December 2006).

The difference plot (Figure 3.9) shows a similar trend to that of the temperature regime experienced in the region with similar %RH in both environments over winter and greater differences during the austral summer. The difference in %RH remained stable over the winter months, with <10%RH difference between hypolithic environments and the soil surface (Figure 3.9). The start of the summer season in early November results in significant differences in %RH between the environments tested ($P<0.05$). Hypolithic communities are exposed to much higher atmospheric humidity during the austral summer season as shown by the largely positive value for the difference between hypolithic and open soil environments, [the %RH values were up to 70% above those at the soil surface (Figure 3.9)]. This implies that biological communities probably have greater water availability in hypolithic environments than on the soil surface.

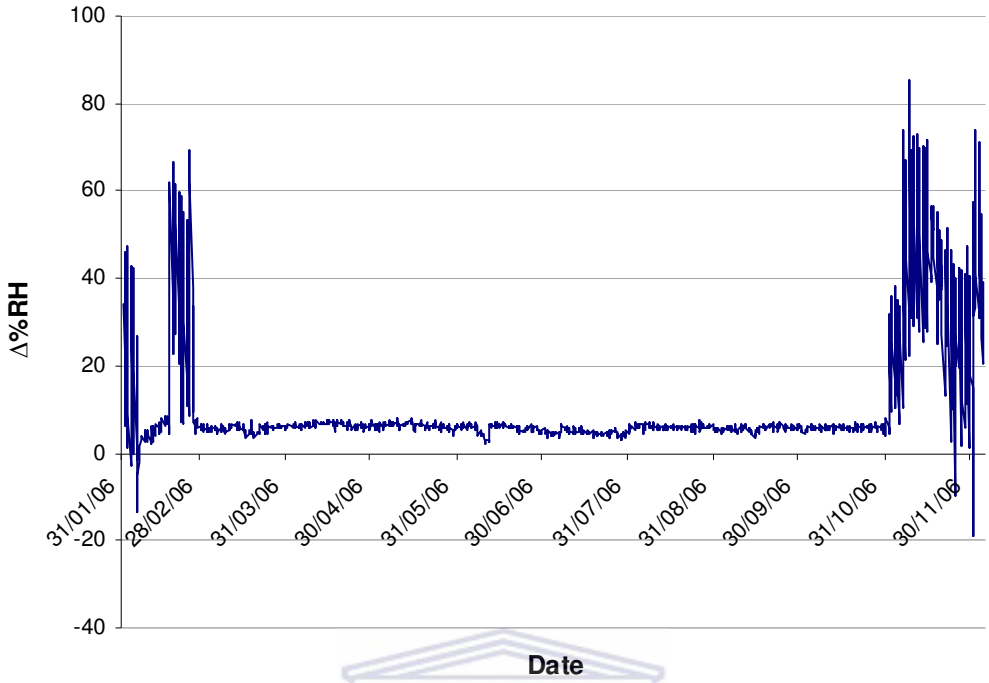

Figure 3.9: Difference in %RH values between the hypolithic and soil surface habitats over 12 months (Miers Valley, Eastern Antarctica, January to December 2006).

To observe high resolution humidity changes, %RH was logged at 2 min intervals over 2 days in January 2007 (Figure 3.10) and at 5 min intervals over 7 days in January 2008 (Figure 3.11) (see Section 2.2.1).

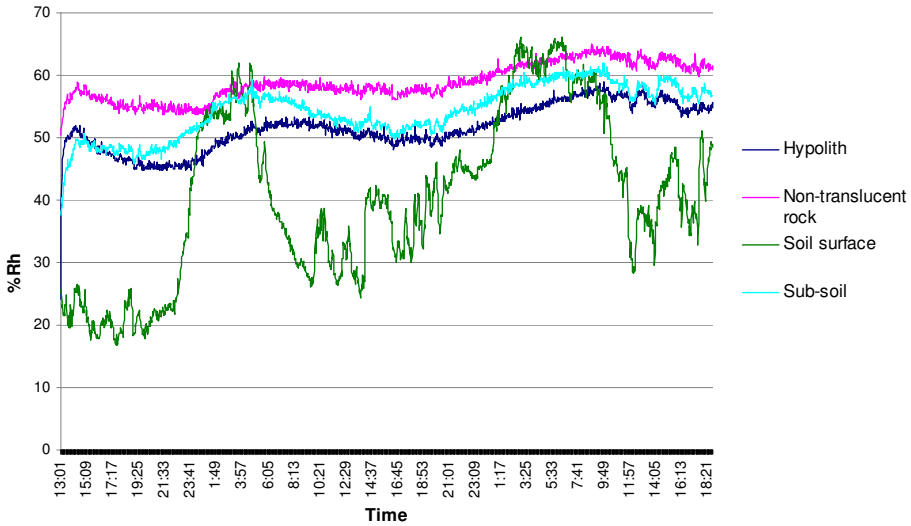

Figure 3.10: %RH logged at 2 min intervals over 2 days in hypolithic and control locations (Miers Valley, Eastern Antarctica, January 2007).

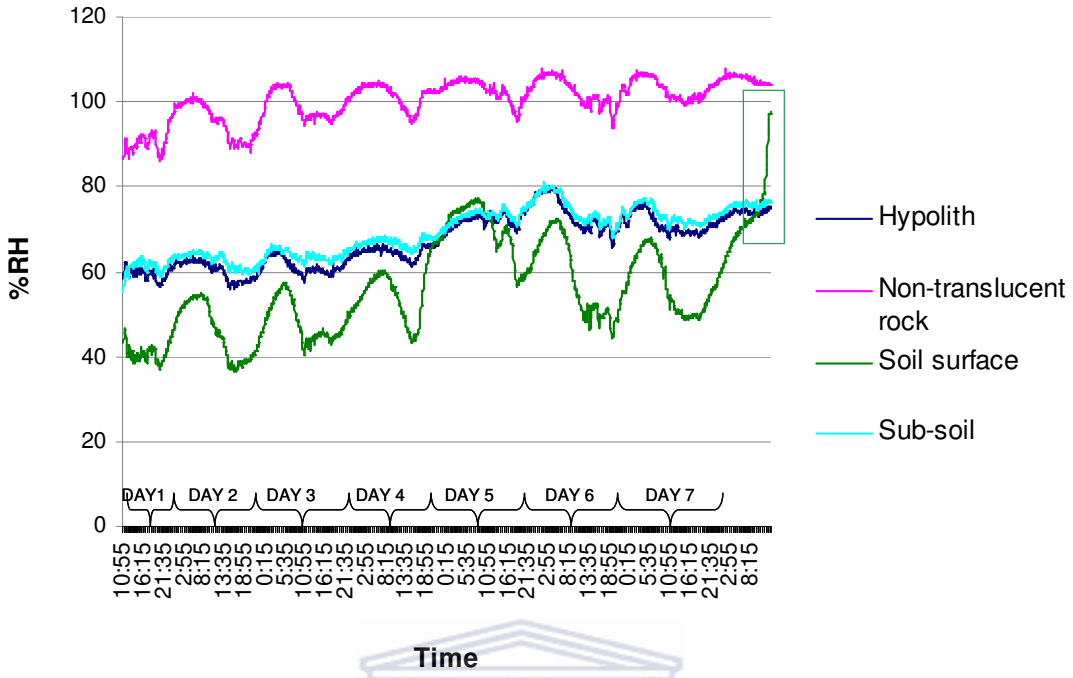

Figure 3.11: % RH logged at 5 min intervals over 7 days in hypolithic and in control locations (Miers Valley, Eastern Antarctica, January 2008).

Table 3.2: Analysis of %RH over 7 days in hypolithic and control environments (Miers Valley, January 2008)

	Hypolithic environment	Non-translucent rock environment	Sub-soil environment	Soil surface environment
Average	67.15	100.00	68.98	55.97
StdDev[a]	6.02	4.87	5.61	13.28
Var(P)[b]	36.28	23.71	31.41	176.38

[a]StdDev = Standard deviation
[b]Var(P) = Variance

Analysis of the average %RH in the tested environments shows that the highest mean %RH occurs beneath non-translucent rocks, while the lowest mean %RH occurs on the soil surface (Table 3.2). There was a significant difference in mean %RH under hypolithic and non-translucent rock. There was also a significant difference in mean %RH between the hypolithic environment and the soil surface ($P<0.05$). Furthermore, there was a significant difference in variation between all sites ($P<0.05$). This was particularly evident for the soil surface, where %RH fluctuated almost five times as much as the hypolithic environment (Table 3.2).

Although diurnal fluctuations in %RH are evident for all the environments tested, these changes are most prominent at the soil surface (Figures 3.10 and 3.11). While the hypolithic environment offers a more suitable environment for biological growth (based on field observations of biomass) than the soil surface in terms of greater %RH with a lower fluctuation; the hypolithic environment is similar to the sub-soil environment. This implies that %RH, as with temperature is not the limiting factor in the development of terrestrial biological communities in the Miers Valley.

3.4 Light

Light is believed to play a major role in the development of photosynthetic hypolithic communities (Schlesinger *et al.*, 2003; Thomas, 2005).The hypolithic communities in the Miers Valley are exclusively found beneath translucent quartz rocks. Non-translucent rocks in the region are not colonised by macrobiotic communities.

Colonised quartz stones collected in the Miers Valley region varied in dimensions and ranged from 0.9–3.4 cm in depth profile. Figure 3.12 shows the transmission of incident light through a range of hypolithic quartz rocks (see Section 2.2.2). It is evident that while the region receives high levels of incident light, transmission of incident light through hypolithic quartz rocks is low, averaging at 2.3% of the incident light (Figure 3.13). As all transmission values were taken from beneath colonised quartz rocks, it is evident that even though light transmission is very low, it is still sufficient to support the development of photosynthetic communities in hypolithic habitats.

Figure 3.13 shows a high resolution analysis of the diurnal incident light cycle in the region during January, with the highest incident light recorded during the mid-afternoon to late evening and the lowest recorded incident light in the early morning hours. Temperature regimes of all the habitats tested follow this pattern (Figure 3.5), with a strong positive correlation between temperature and incident light (0.73 to 0.86).

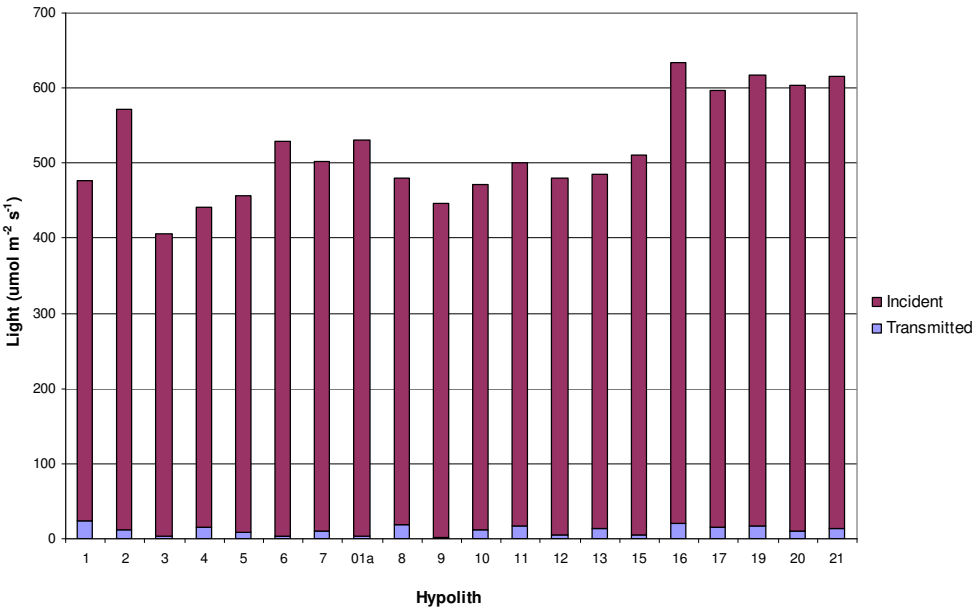

Figure 3.12: Transmission of light through hypolithic quartz rocks (Miers Valley, Eastern Antarctica, January 2008).

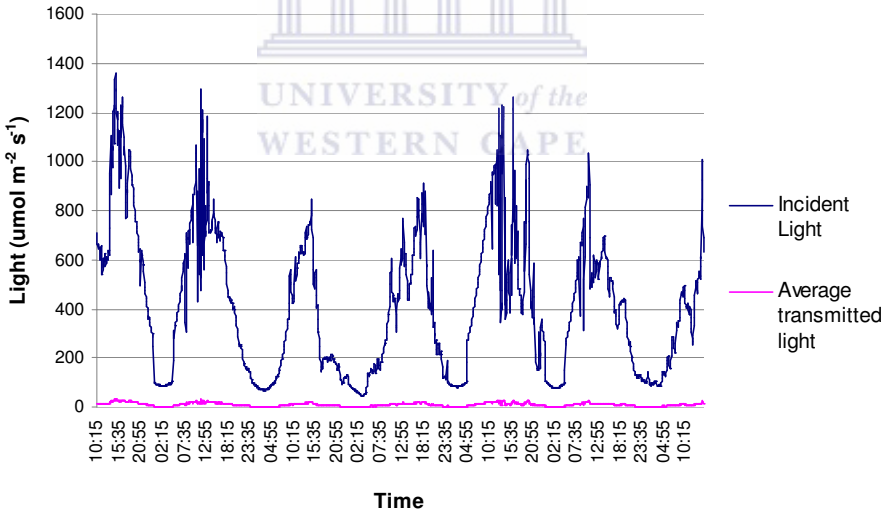

Figure 3.13: Average transmission of incident light through hypolithic quartz rocks over 7 days (Miers Valley, Eastern Antarctica, January 2008).

Furthermore, prolonged high light intensities such as that experienced on the soil surface during summer, may lead to a decrease in the number of photosystems present in cyanobacteria and therefore a decrease in photosynthetic activity (Yokoyama *et al.*, 1991). Proteins involved in photosynthesis can also be sensitive to

high irradiation levels. For example, the D1 protein found in photosystem II (PSII) reaction centres is particularly sensitive to high levels of irradiation. At low light intensities, the rate of photosynthetic electron transport is proportional to the photon flux. Therefore, damaged or denatured D1 polypeptide is removed from the PSII reaction centre and replaced with functioning D1 protein (Guenther & Melis, 1990). At high light intensities, there is a decrease in the quantum yield of photosynthesis as the rate of damage to the photosystem reaction centres exceeds the rate of repair (Reuter & Mueller, 1993; Bhaya *et al.*, 2000). This is particularly important in the Antarctic region, which receives constant, high levels of irradiation for extended periods of time (i.e. the austral summer period). Therefore, hypolithic environments, with lower light intensities, would provide a protected and more suitable environment for photosynthesis during the austral summer.

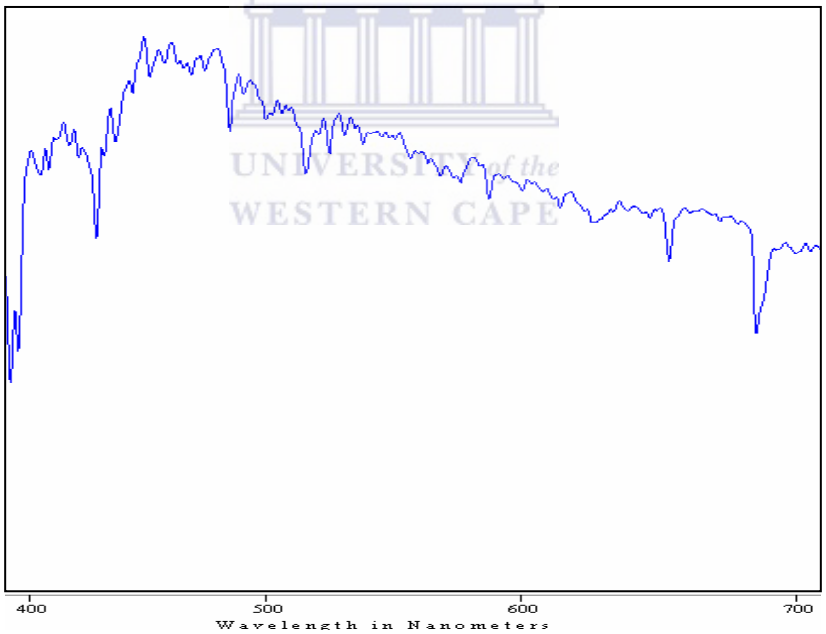

Figure 3.14: Spectral scan of incident light in the Miers Valley, Eastern Antarctica. Maximum light intensity at the time of measurement was 115 µmol m^{-2} s^{-1}.

Figure 3.14 shows the typical spectral analysis of incident light experienced on the soil surface in the Miers Valley. The figure shows the increased light intensity with decreasing wavelength (i.e. greater irradiation of shorter wavelength light approaching the UV range). Spectral analysis of light transmitted through quartz rock

indicates that the transmitted light spectrum is truncated by the presence of the rock (Figure 3.15).

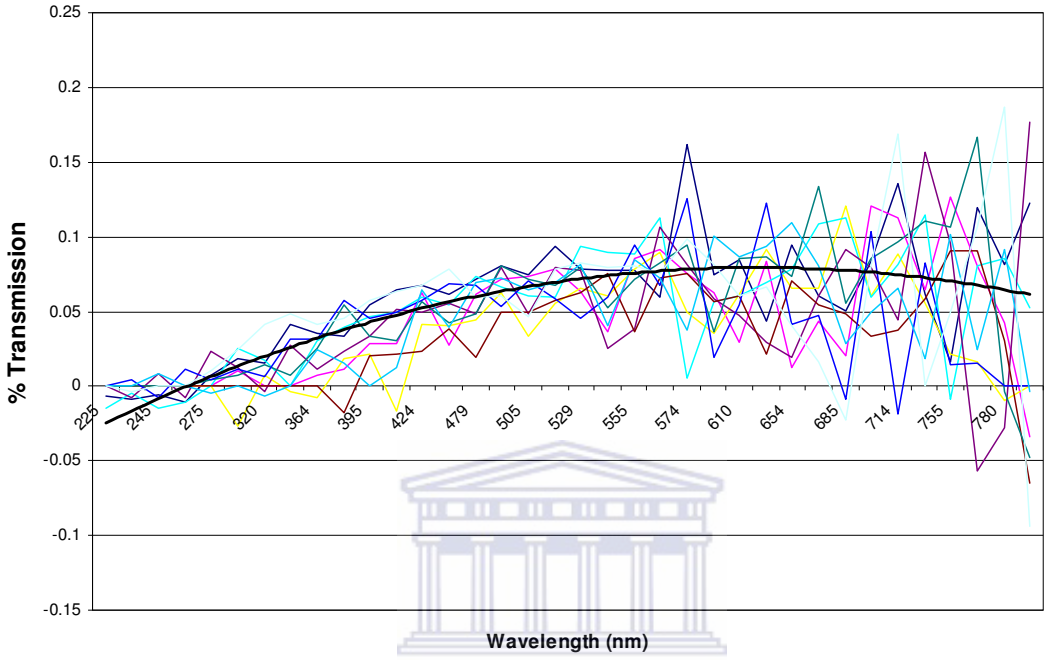

Figure 3.15: Spectral scan of %transmitted light through 1 cm x 1 cm quartz cut from a colonised quartz rock from the Miers Valley.

The figure (Figure 3.15) shows that, while transmitted light intensity over the entire spectrum is low, transmission of high-frequency light in the UV-range in particular is minimised. The effect of radiation on biological systems is dependent on the wavelength, the type of radiation and the organisms' ability to adapt. UV irradiation (280 nm to 400 nm) is particularly harmful to cellular processes. Growth, survival, pigmentation, morphology and adaptation of cyanobacteria, as well as enzymes for nitrogen metabolism and carbon fixation, are negatively affected by UV-irradiation (Donker & Hader, 1996; Sinha *et al.*, 1996; Wu *et al.*, 2005). The study by Donker & Hader showed that phycobilins (accessory cyanobacterial pigments) were least resistant to damage by UV-irradiation, followed by carotenoids and chlorophyll. Aside from photoprotection, these pigments transfer light energy to the photosystem reaction centres for photosynthesis (Donker & Hader, 1996). Reduction in

transmittance of short wavelength UV-irradiation in hypolithic environments is therefore advantageous to the photosynthetic communities in this micro-environment.

3.5 Carbon/Nitrogen Analysis

The presence of inorganic carbon in soils can strongly influence the measurement of organic carbon derived from biomass (Midwood & Boutton, 1998). Acid digestion was used to remove soil carbonates that may affect the measurement of organic carbon content as an indicator of biomass (see Section 2.2.4). Figure 3.16 shows the organic carbon content of hypolithic and control community samples. It is clear that, although low (<1g C kg^{-1} soil) when compared to temperate climates [e.g. 10.5 kg C m^{-2} in savannah grasslands (Zhong & Qiguo, 2001)], hypolithic community samples had a higher organic carbon content than other control desert soil environments in the Dry Valley.

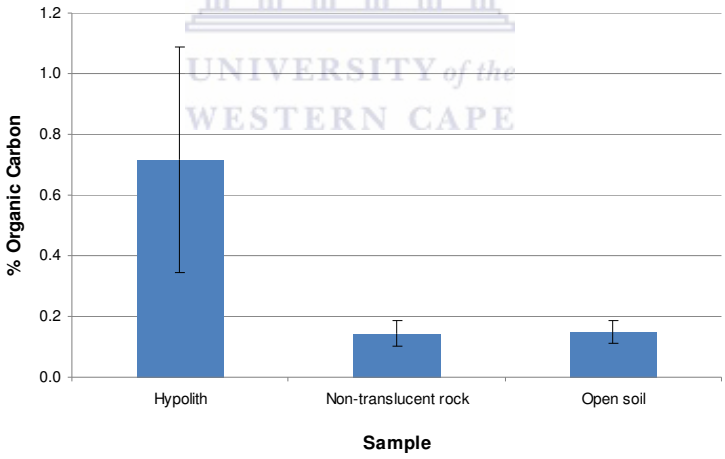

Figure 3.16: % Organic carbon content from hypolithic communities and control samples from the Miers Valley.

Analysis of the % organic carbon content (Table 3.3) shows that the average organic carbon content of hypolithic community samples were almost five times greater than control community samples. There was a significant difference in mean % organic carbon and variance between hypolithic and control samples ($P<0.05$), with no significant difference in mean organic carbon content or variance of control samples

(P<0.05). Hypolithic community samples, have a higher standard deviation (>50% of the average) and variance, indicating samples differ widely in their organic carbon content (Table 3.3). This could be a function of both the size and type of hypolithic community. For example, based on field observations, moss-dominated hypolithic communities contain greater biomass than cyanobacterial-dominated communities, therefore moss-dominated communities would reflect a higher organic carbon content. The results show that while non-translucent rock and open soil communities contain similar levels of biomass (based on organic carbon content), hypolithic communities differ from controls and each other in their biomass content.

Table 3.3: Analysis of % organic carbon content of hypolithic and control samples from the Miers Valley

	Hypolithic community	Non-translucent rock community	Open soil community
Average	0.72	0.14	0.15
StdDev[a]	0.37	0.04	0.04
Var(P)[b]	0.13	0.00	0.00

[a]StdDev = Standard deviation
[b]Var(P) = Variance

Total nitrogen content was also measured to determine if there was a difference in biomass or community size in hypolithic and control environments (Figure 3.17).

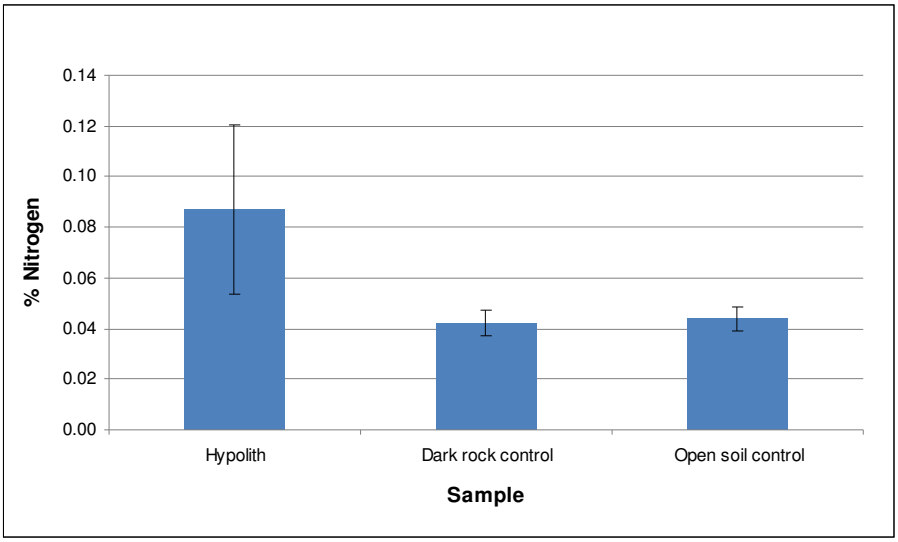

Figure 3.17: % Nitrogen content of hypolithic communities and control samples from the Miers Valley.

Table 3.4: Analysis of % nitrogen content of hypolithic and control samples from the Miers Valley

	Hypolithic community	Non-translucent rock community	Open soil community
Average	0.083	0.029	0.030
StdDev[a]	0.046	0.021	0.022
Var(P)[b]	0.002	0.001	0.000

[a]StdDev = Standard deviation
[b]Var(P) = Variance

There was a significant difference in mean total nitrogen content and variance between hypolithic and control samples ($P<0.05$). The average total nitrogen content from hypolithic community samples (Table 3.4) was low compared to those found in temperate regions [for example 0.75% total nitrogen content of temperate forest soils (Huygens *et al.*, 2008)]. The % total nitrogen data confirms the conclusions drawn from the % organic carbon data obtained: while non-translucent rock and open soil communities share similar biomass levels, hypolithic communities differ from these and from each other.

3.6 Conclusions

Analysis of temperature and %RH regimes beneath hypolithic and non-translucent rocks, in the sub-soil and on the soil surface, show that all environments experience diurnal patterns of change due to the position of the sun in relation to the valley. Furthermore, a strong positive correlation existed between incident light and temperature in all environments, where an increase in the incident light is related to an increase in temperature due to solar gain.

Thermal and %RH fluctuations are greatest on the soil surface in the Miers Valley. These fluctuations are buffered in the hypolithic microenvironment as well as beneath non-translucent rocks and in the sub-soil. This could indicate that, while minimised thermal and %RH fluctuations may be beneficial to the growth of biological communities, these factors are probably not the primary drivers for the development of hypolithic communities.

It is suggested that the availability of light is the determining factor that results in the development of communities beneath quartz stones as opposed to non-translucent rocks or the sub-soil While the % light transmission was shown to be low (<3% of the incident light), it is still sufficient for the development of photosynthetic hypolithic communities.

It is hypothesised that the combination of the following factors results in a microenvironment suitable for the growth and development of photosynthetic hypolithic communities in the Dry Valley polar desert ecosystem:

- Low but sufficient light availability for photosynthetic organisms. Quartz rocks allow for transmission of light at lower intensity levels than on the soil surface during summer, therefore reducing damage to the photosystems of the photosynthetic components of the community. Furthermore, hypolithic quartz rocks reduce transmission of UV-irradiation in particular, thereby preventing UV-induced damage to cells.
- Physical stability of the microenvironment and the biological community. The overlying rock prevents movement due to the eroding winds in the region.
- Minimised %RH fluctuations as the environment is not exposed to the desiccating atmosphere.
- Minimised temperature fluctuations as the hypolithic environment is not subject to fluctuations dependent on direct sunlight and cloud cover.

Chapter 4: Hypolithic Community Profiling

4.1 Introduction

The term biodiversity refers to the range of different types of organisms in an environment (Torsvik *et al.*, 1998). In order to determine the true biodiversity of an environment, all types of organisms present should be detected. Culture-independent methods are therefore important as they allow access to a greater variety of organisms, many of which cannot be detected via traditional culture-dependent methods (Section 1.5).

Metagenomic techniques are culture-independent methods that are increasingly being used to assess microbial diversity in various environments (Muyzer *et al.*, 1993; Duarte *et al.*, 1998; Yeates & Gillings, 1998; Krsek & Wellington, 1999; Niemi *et al.*, 2001; Aguilera *et al.*, 2006). The modified Miller bead-beating method uses a combination of physical (bead-beating) and chemical (detergent) lysis with organic solvents (phenol and chloroform) to liberate DNA from even highly recalcitrant cells (Borneman *et al.*, 1996; Yeates & Gillings, 1998). The combination of bead-beating with a lysis mixture that includes detergents and organic solvents has been shown to recover high yields of high quality DNA for downstream analysis (Miller *et al.*, 1999; Niemi *et al.*, 2001; Aguilera *et al.*, 2006). The ability of the PCR to amplify even small amounts of DNA has also greatly facilitated metagenomic DNA analyses since it allows access to rare organisms in a community and enables the analysis of small samples for the study of micro-habitats (van Wintzgerode *et al.*, 1997).

Denaturing gradient analysis (DGGE) is a simple and efficient method for the analysis of metagenomic DNA (Section 1.5). Phylogenetic marker genes are amplified and separated by electrophoresis on a polyacrylamide gel containing a gradient of denaturants. Amplicons are separated on the gel based on the differential melting of different sequences forming banding patterns or fingerprints where each band theoretically represents a different organism (Muyzer *et al.*, 1993). It was originally

developed for the detection of point mutations in DNA but was soon adapted to the study of microbial genetic diversity (Muyzer *et al.*, 1993; Kirk *et al.*, 2004). DGGE is a method that produces a community profile (or 'fingerprint') of community members. These community fingerprints can be used to assess community diversity and to help determine the similarity or differences between communities. Although the DGGE method has some drawbacks (see Section 1.5), it can be used as an effective method for the overall assessment of community diversity.

The use of modern culture-independent techniques can reveal extensive microbial diversity that previously remained undetected, but exhaustive inventories of microbial communities are currently impractical and often financially unfeasible. Microbial ecologists therefore, initially need to establish how well a particular sample represents a particular community, the sample size required in order to reliably analyse a particular biotope and the most appropriate method to reveal the diversity of the community. There are a finite number of species or biotypes in a community, and the number of biotypes detected increases with increased sampling effort until all the possible biotypes have been observed (Hughes *et al.*, 2001). However, while determining the actual, complete diversity may be useful, most ecological questions of diversity require knowledge of relative diversity (between habitats, over time, with environmental alteration, etc.) (Hughes *et al.*, 2001), which is altogether more realistic and achievable.

For culture-independent analysis of diversity, it is vital to consider the source of the metagenomic DNA used. It is likely that at least some proportion of the DNA isolated could originate from non-viable or dead cells and extracellular DNA often referred to as 'naked' DNA. While DNA extraction methods include isolation of total DNA, rigorous methods such as bead-beating decreases both the quantity and quality of 'naked' DNA.

Even though DNA that is released into the environment from dead or lysed cells is vulnerable to degradation by nucleases, naked DNA can persist in the soil

environment. For example, DNA remained retrievable from forest soils after three months (England *et al.*, 2004). Naked DNA can persist in cold soil environments for even longer time periods, as demonstrated by the detection of seeded *Staphylococcus epidermidis* DNA from Dry Valley soils for 23.5 weeks (Ah Tow & Cowan, 2005) and the recovery of plant and animal DNA from 400 000 and 30 000 year old sediments respectively in Siberia (Stokstad, 2003). Stability of exposed or exogenous DNA is facilitated by the formation of DNA-soil particle complexes (Lorenz *et al.*, 1994). The Dry Valley region incorporates all the optimal conditions for DNA preservation, which includes low temperatures, desiccating conditions and high salt concentrations (Shapiro, 2008). Furthermore, the action of nucleases may be reduced due to the extreme temperature conditions present in the polar desert environment. The extraction of RNA for the construction of rRNA gene libraries would aid in distinguishing the active population from the inactive one. Furthermore, isotope or radio-isotope labelling experiments could also be used to help differentiate between those phylotypes that represent the live, active population and to trace the metabolism through several trophic levels.

Although biodiversity indicators often use 'species' as a basic unit, this is impractical in the study of microbial diversity where the definition of species is debatable and obscure (Torsvik *et al.*, 1998). It is more practical to assign each biotype detected as an operational taxonomic unit (OTU), which can then be used to characterise and compare microbial populations (Torsvik *et al.*, 1998). Species richness, the Simpson's index of diversity (or its reciprocal) and the Shannon index are the most commonly used measures of biodiversity. Although these methods have been developed for the assessment of macro-organisms, they have been successfully used to compare microbial diversity (Torsvik *et al.*, 1998, Nubel *et al.*, 1999; Dilly *et al.*, 2004; Zhao *et al.*, 2008). Species richness (S) is the simplest measure as it refers to the total number of all the OTUs found in a community. While species richness gives an idea of diversity, it does not demonstrate how the diversity is distributed in a population. The Shannon index of diversity (H') (often called the Shannon-Weaver or Shannon-Wiener index) and the Simpson's index are general diversity indices that

quantitatively describe the biodiversity and how evenly the community members are distributed.

4.2 Field classification of hypolithic communities

All hypolithic communities observed in this study were found beneath quartz rocks that were > 3 cm across and partially embedded in the soil. It is believed that smaller translucent rocks (< 3 cm) do not present a physically stable microenvironment (i.e. still subject to wind movement) and are therefore not colonised. Based on extensive field observations, it is suggested that hypolithic communities in the Miers Valley can be divided into 3 types or classes:

- Type I: Cyanobacterial-dominated communities
- Type II: Moss-dominated communities
- Type III: Lichenised communities

Cyanobacterial-dominated communities (Type 1) adhere tightly to the underside of the quartz rock and appear as a thin, pigmented biofilm (Figure 4.1). Collection of the community involves scraping this biofilm from the rock.

Figure 4.1: An example of Type I cyanobacterial-dominated hypolithic communities. Pigmented cyanobacterial communities adhere tightly to the portion of the rock embedded in the soil. Inset shows light micrograph of *Oscillatorian* cyanobacteria found in Type I communities (Picture courtesy of D. A. Cowan, microscopy by S. A. Wood).

Type II communities are dominated by mosses (Figure 4.2). These communities do not adhere to the bottom of the rock, but reside directly underneath the rock with the perimeter of the community mirroring that of the overlying rock. Removal of the overlying rock exposes the upper surface of the moss community, where the tips of the moss leaves are visible as a greenish layer.

Figure 4.2: An example of Type II moss-dominated hypolithic communities. Removal of the overlying rock reveals the green moss community present directly underneath. Inset shows light micrograph of the moss *Hennediella heimii* found in the Type II hypolithic communities (Picture courtesy of D. A. Cowan, microscopy by S. A. Wood).

Type III communities adhere firmly to the underside of the quartz rock (Figure 4.3). They appear to be quite dry and fragile and crumble away from the rock quite easily. These are termed lichenised communities as the bulk of the biomass is formed from filaments of the fungal constituent/s (Figure 4.3 inset).

Figure 4.3: An example of Type III lichenised hypolithic communities. The community appears attached to the underside of the quartz rock. Inset shows a magnified view of the attached community, showing the filamentous nature of the fungal biomass associated with these communities (Picture by Cowan & Khan, 2008).

4.3 DNA extractions from soil

Metagenomic DNA was extracted from hypolithic and control soil communities using the modified Miller bead-beater method described in Section 2.3. Figure 4.4 shows typical metagenomic DNA extracts from hypolithic and control soil communities collected in the Miers Valley. This method produced high quality metagenomic DNA (A260/A280 ~1.9-2) that required no further purification for downstream processes.

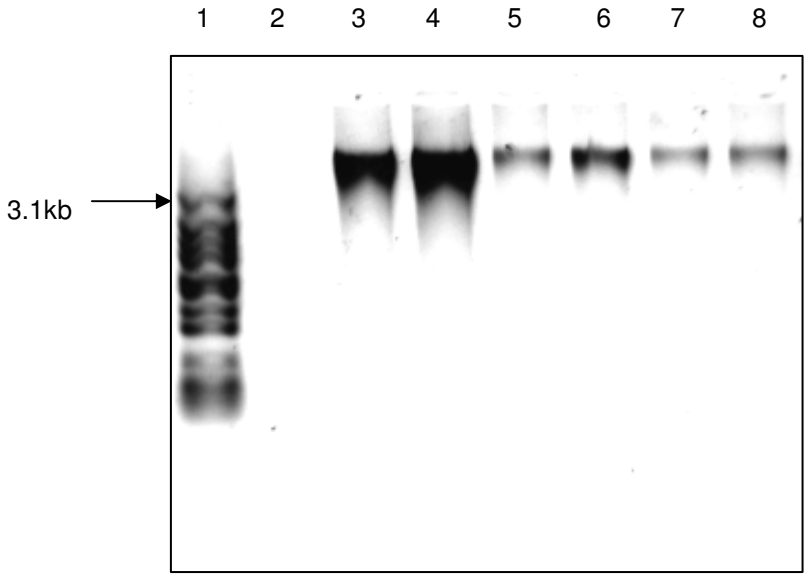

Figure 4.4: An agarose gel showing an example of typical metagenomic DNA extracts from Antarctic soils using the modified Miller bead-beating method described in Section 2.3. Lane 1: Molecular weight marker. Lane 2: Negative control (no soil added for extraction process). Lanes 3 and 4: Duplicate extractions from MVH 3-1A (hypolithic community). Lanes 5 and 6: Duplicate extractions from MVH 3-1B (non-translucent rock community). Lanes 7 and 8: Duplicate extractions from MVH 3-1C (open soil community).

The typical metagenomic DNA yields from hypolithic communities ranged from 8-16 µg DNA g^{-1} soil (Table 4.1). Extractions from hypolithic community samples produced higher DNA yield than the associated control communities (Table 4.1). There was a significant difference in DNA yields from hypolithic communities when compared to non-translucent rock and open soil communities ($P<0.05$). There was no significant difference in DNA yields from non-translucent rock and open soil communities. This indicates that biomass levels were greater in hypolithic communities, while biomass levels were similar in non-translucent rock and open soil communities.

Table 4.1: DNA extraction yields from hypolithic (H), non-translucent rock (NTR) and open soil (OS) community samples collected from the Miers Valley

Sample	µg DNA g^{-1} soil
H1	9.9
NTR1	2.4
OS1	1.8
H2	15.9
NTR2	3.8
OS2	4.0
H3	9.9
NTR3	1.7
OS3	0.6
H4	8.2
NTR4	1.9
OS4	1.8
H5	9.1
NTR5	1.6
OS5	3.9
H6	15.0
NTR6	2.6
OS6	1.3
H7	9.1
NTR7	2.0
OS7	0.7

4.4 Bacterial community profiles

A 193 bp fragment of the variable (V3) region of the 16S rRNA gene was amplified from metagenomic DNA using universal bacterial PCR primers (Figure 4.5) (see Section 2.4). Figure 4.5 shows a faint band in the negative control, which had no template DNA added to the amplification reaction. This false positive result was due to the presence of small amounts of *E. coli* genomic DNA in the recombinantly produced *Taq* polymerase. This was confirmed by DGGE, where the product from the negative control migrated to the same position as the positive control which contained genomic *E. coli* DNA as the template (Figure 4.6 – Lane 3). The reaction was repeated using various commercial *Taq* polymerases with the same result. The *E. coli* band was not prominent in the samples as the *E. coli* DNA contribution from the *Taq* polymerase was extremely low (Figure 4.6) and therefore did not interfere with the analysis.

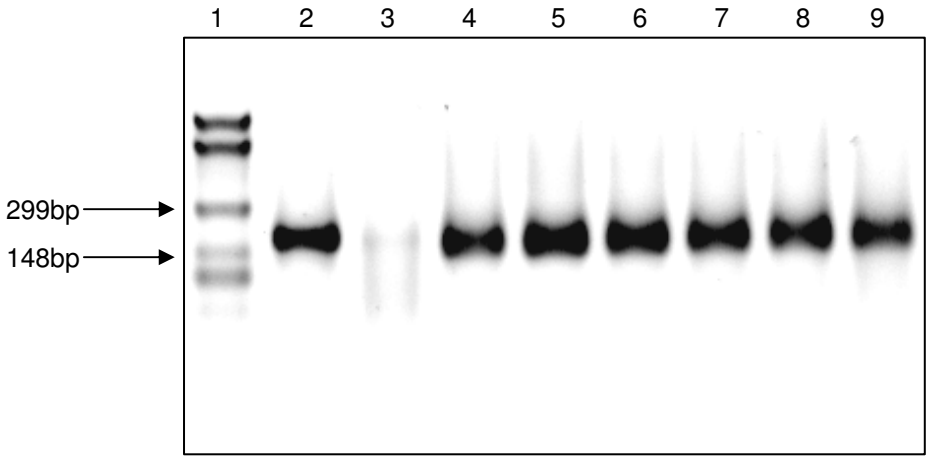

Figure 4.5: An agarose gel showing example of PCR amplification of a 193bp fragment of 16S rRNA genes from metagenomic DNA extracts using universal bacterial 16S rRNA gene primers for DGGE. Lane 1: Molecular weight marker. Lane 2: Positive control. Lane 3: Negative control. Lanes 4 and 5: PCR product from duplicate hypolithic community extracts. Lanes 6 and 7: PCR product from duplicate non-translucent rock community extracts. Lanes 8 and 9: PCR product from duplicate open soil community extracts.

PCR products were separated using 9% (w/v) polyacrylamide gels with a denaturing gradient of 35-75% (Figure 4.6) (see Section 2.5). The reproducibility of the DGGE fingerprints was tested, along with the effect of metagenomic DNA template concentration. In Figure 4.6, Lanes 5 (MVH 3-1A) and 8 (MVH 3-1A*) show fingerprinting patterns from amplicons produced using duplicate undiluted metagenomic DNA extracts from a hypolithic community as template. While template concentration for MVH 3-1A (37.5ng) was lower than for MVH 3-1A* (61.3ng), PCR amplification failed for MVH 3-1A. However, PCR products were obtained from diluted MVH 3-1A which possibly suggests that contaminants or salts that co-purified during the extraction process led to PCR inhibition.

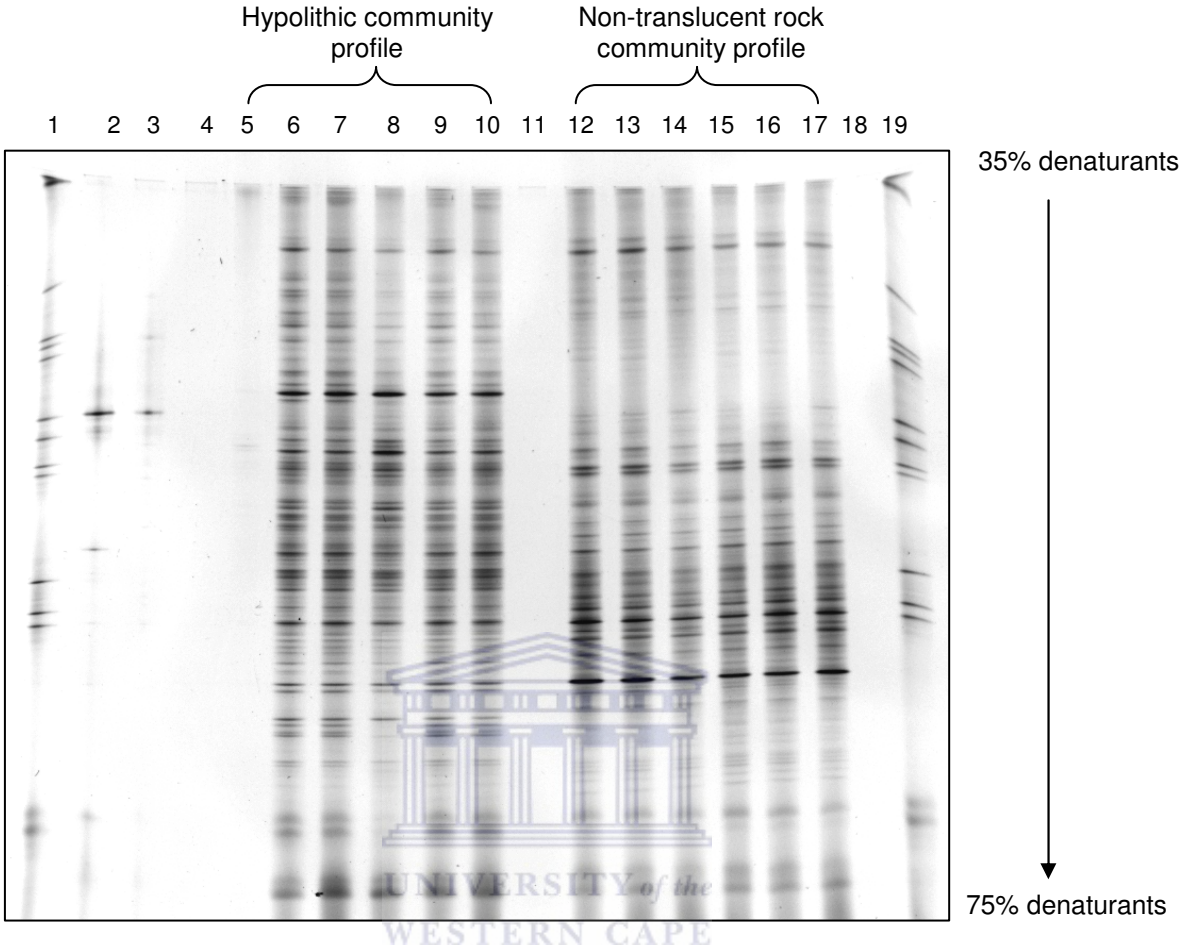

Figure 4.6: DGGE fingerprints on a polyacrylamide gel with a gradient of denaturants produced by amplicons using different template concentrations. Lane 1: DGGE marker. Lane 2: Positive control. Lane 3: Negative control. Lane 5: MVH 3-1A (undiluted template). Lane 6: MVH 3-1A (1:10). Lane 7: MVH 3-1A (1:100). Lane 8: MVH 3-1A* (undiluted template). Lane 9: MVH 3-1A* (1:10). Lane 10: MVH 3-1A* (1:100). Lane 12: MVH 3-1B (undiluted template). Lane 13: MVH 3-1B (1:10). Lane 14: MVH 3-1B (1:100). Lane 15: MVH 3-1B* (undiluted template). Lane 16: MVH 3-1B* (1:10). Lane 17: MVH 3-1B* (1:100). Lane 19: DGGE marker. *Indicates duplicate set of extractions.

Since template dilutions (up to 100 times dilution) did not appear to significantly affect banding patterns, while undiluted template could contain PCR inhibitors (Figure 4.6), an approximate 10-fold dilutions of template DNA was used for all further amplification reactions (5-20 ng template DNA).

Image analysis software was used to analyse all community fingerprints (see Section 2.5). Each band identified in a fingerprint was considered as an OTU. Bands were

indicated as present or absent and band matching was used as the comparison function. Band migration across gels were standardised using the same DGGE marker for all gels compared in order to adjust for gel to gel variation. All bands were assigned a band class, with bands having the same or similar migration grouped to a band class. Therefore every band belonged to a band class with every band class represented by a band in one or more community fingerprint. Figure 4.7 shows the 16S rRNA gene sequence community profiles and the resultant band matching from hypolithic and control community samples collected in the Miers Valley. The band matching analysis produced a similarity matrix (see supplementary material).

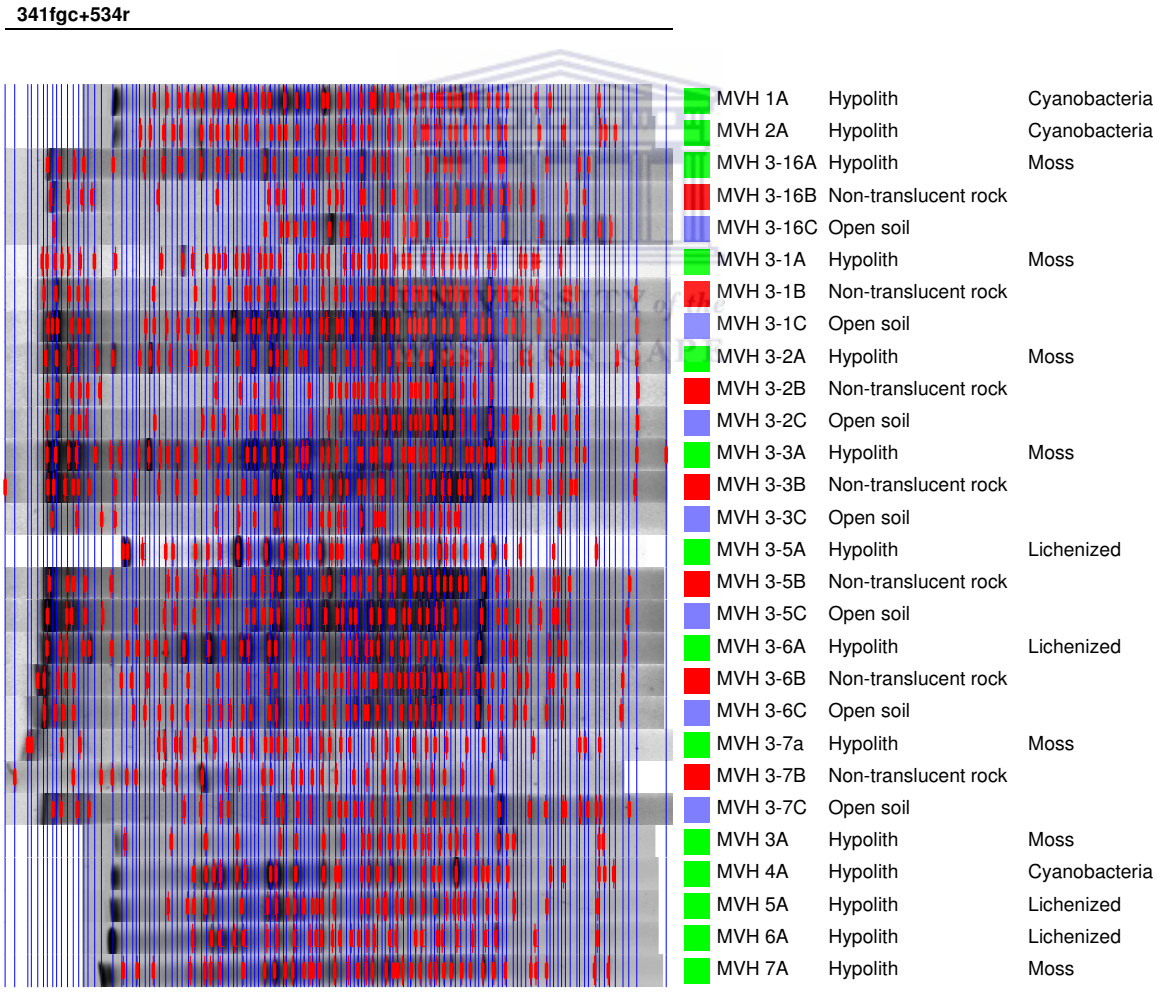

Figure 4.7: Band matching across hypolithic and control community fingerprints. Each band in a fingerprint appears as a red line. Band classes, matching bands across fingerprints, are represented by a blue line.

The Pearson correlation coefficient together with the Unweighted Pair Group Method using Arithmetic averages (UPGMA) algorithm was used for cluster analysis of the community fingerprint data. The dendrogram created and the cluster groups formed are shown in Figure 4.8.

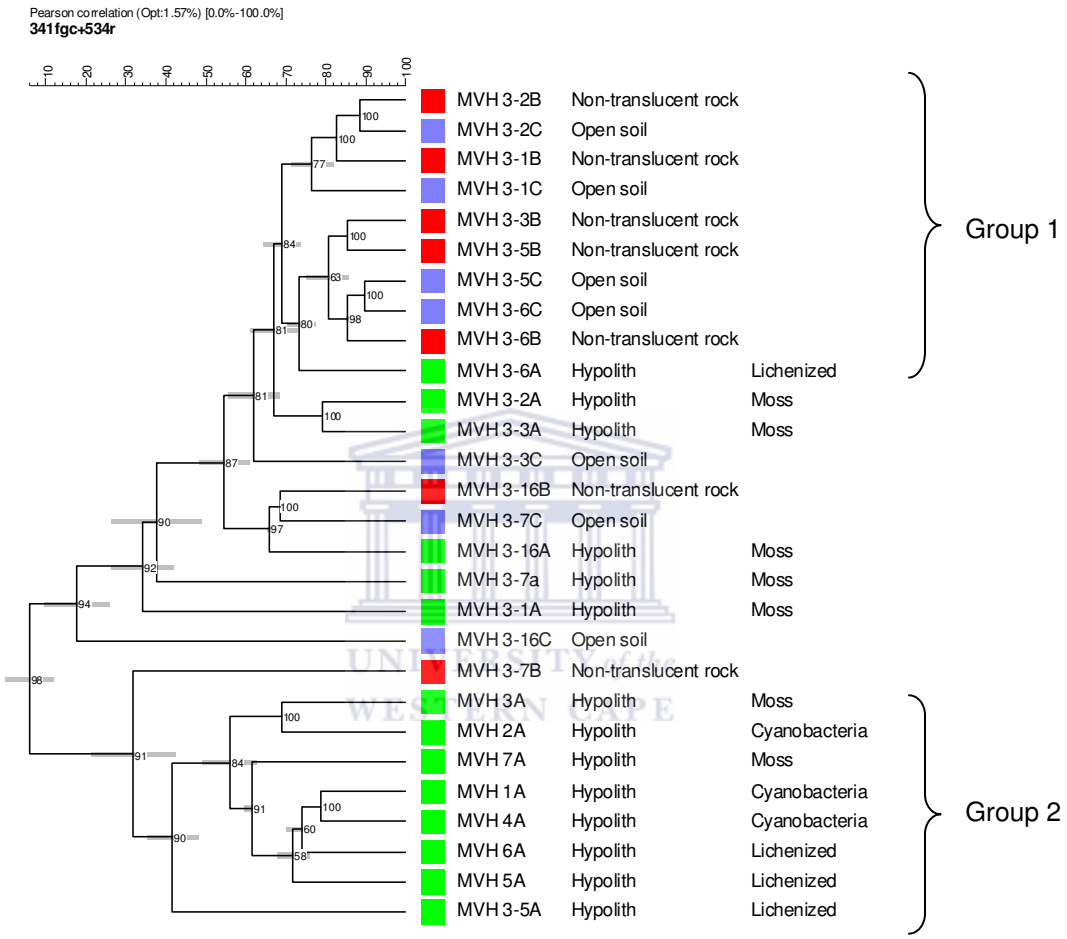

Figure 4.8: Cluster analysis of bacterial 16S rRNA gene hypolithic and control community fingerprints using the Pearson correlation and UPGMA algorithm.

While the dendrogram gives an indication of groups or clusters among the individual community fingerprints, the confidence or the reliability of the clusters in phylogenetic trees is traditionally expressed in terms of bootstrap values. Similarly, the numbers indicated on the nodes of the dendrogram above (Figure 4.8) indicate the cophenetic correlation of the branching. The cophenetic correlation is used to assess the consistency of a cluster by calculating the correlation between the dendrogram-derived similarities and the matrix similarities based on the band-matching (see

supplementary material). A value of 100% indicates complete correlation between the two, and the presence of a significant cluster. Branches formed with a cophenetic correlation >75% were considered significant clusters.

The branches that result in the clusters of Group 1 and 2 have cophenetic correlation values >80%, indicating confidence in these two groups. Group 1 consists largely of non-translucent rock and open soil communities, with one hypolithic community exception. Group 2 consists exclusively of hypolithic communities. This indicates that hypolithic communities share prokaryote communities similar to each other, while non-translucent rock and open soil prokaryote communities are most similar to each other.

Multi-dimensional scaling (MDS) was also used to visualise the similarity of the community fingerprints (Figure 4.9), without the hierarchical detail imposed by the use of dendrograms. The clustering was based on the proximity of samples in the 3-dimensional space.

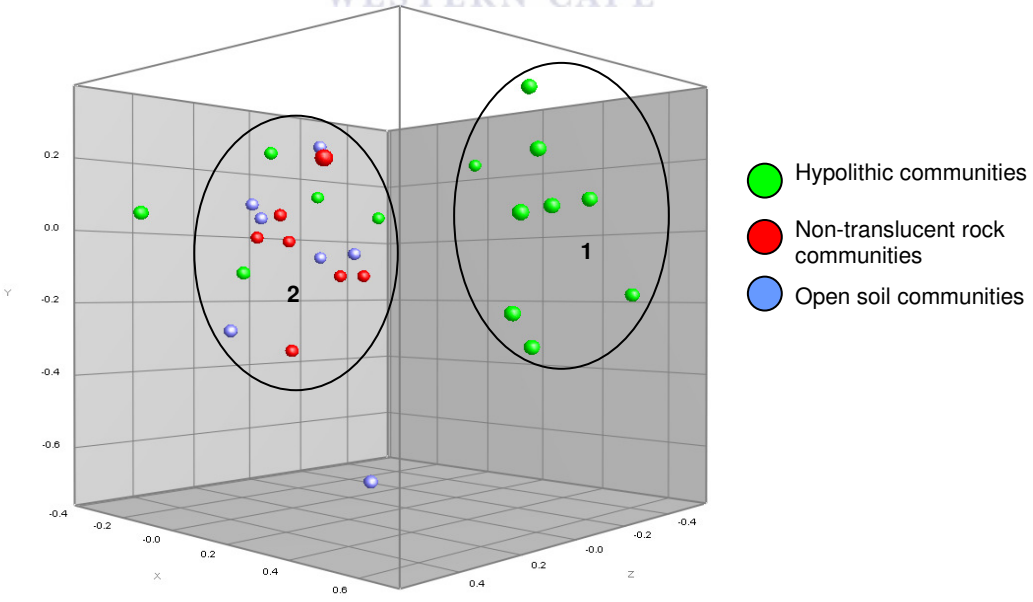

Figure 4.9: MDS analysis of bacterial 16S rRNA gene hypolithic and control community fingerprints.

Both the dendrogram (Figure 4.8) and MDS analysis (Figure 4.9) indicate that while some hypolithic communities are similar to each other, others cluster quite differently with regard to 16S rRNA gene community fingerprints. Two distinct groups are formed, with two outliers (Figure 4.9). 65% of the hypolithic community profiles cluster with Group 1, while 29% cluster with Group 2 and the remainder do not cluster with any group. 100% of the non-translucent rock communities and 86% of the open soil communities cluster exclusively in Group 2. This indicates that open soil communities and non-translucent rock communities are more similar to each other, while hypolithic communities are generally less similar to either open soil communities or non-translucent rock communities.

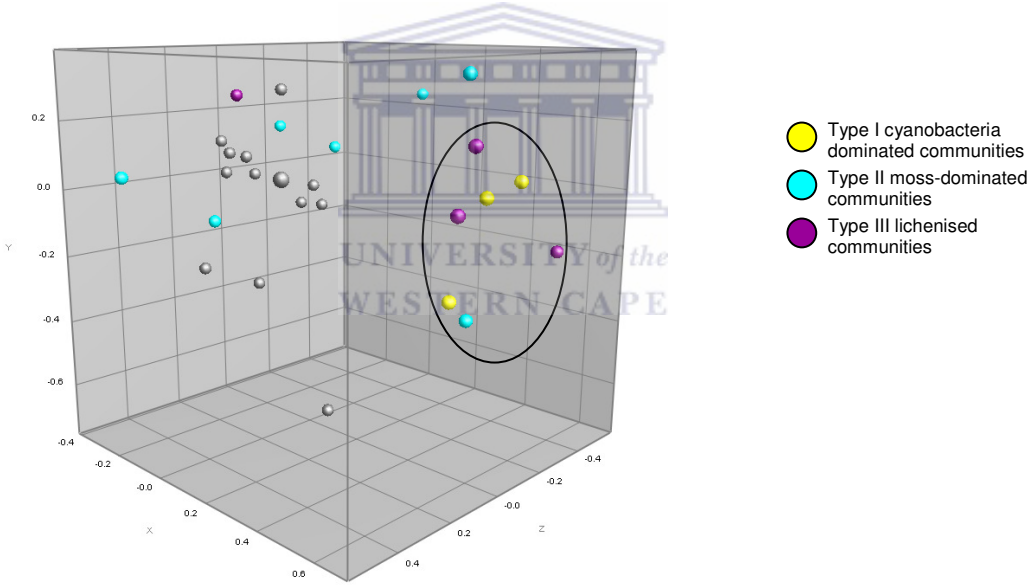

Figure 4.10: MDS analysis of proposed hypolithic community types based on similarity of 16S rRNA gene community fingerprints.

Figure 4.10 highlights the spatial distribution of the proposed hypolithic community types based on the similarity of DGGE fingerprints. While the Type I and Type III communities appear to cluster together (with one exception), Type II moss communities do not form a coherent cluster. This indicates that the cyanobacterial-dominated and lichenised hypolithic communities are more similar to each other, while Type II moss-dominated communities are more diverse.

The initial grouping of the community fingerprints into the three categories (hypolithic, non-translucent rock and open/bulk soil communities) was based on field observations of the hypolithic communities. Group separation statistics was used to assess the stability or confidence of the defined groups (i.e. whether the fingerprints fall into the separate groups as defined) (Table 4.2). The jackknife group statistic calculates the maximum similarity of each fingerprint with all other fingerprints and calculates the percentage of cases that fingerprints are identified to the group to which they were originally assigned. While the overall quality of the grouping was low (approximately 45%), this was mainly due to the insignificant grouping of the separate non-translucent rock and open soil community clusters.

The hypolithic community grouping was the most stable of the three groups, with almost 65% of the hypolithic community 16S rRNA gene fingerprints grouping with other hypolithic communities (Table 4.2). Almost 43% of non-translucent rock community profiles shared maximum similarity to the open soil community group and the same number (almost 43%) to each other. Less than 15% of the non-translucent rock community fingerprints clustered with the hypolithic community group (Table 4.2). The majority of open soil community profiles showed greater affinity to the non-translucent rock communities (almost 72%) than to its own group (almost 29%), while none shared maximum similarity with the hypolithic community group.

These results indicate that grouping non-translucent rock and open soil communities separately lacks confidence and is unreliable so these are better represented as a single group. In contrast, nearly 77% of hypolithic communities are likely to share more similarity to each other than other groups and therefore represents a reliable group. This data confirms the findings of the MDS analysis as it implies that there are two distinct groups formed, one encompassing the majority of the hypolithic communities only and the other composed of most of the non-translucent rock and open soil communities, with some hypolithic communities.

Table 4.2: Group separation statistics (jackknife method) indicating the stability of grouping the hypolithic, non-translucent rock and open soil bacterial communities separately

	Hypolithic community	Non-translucent rock community	Open soil community
Hypolithic community	64.29	14.29	0.00
Non-translucent rock community	21.43	42.86	71.43
Open soil community	14.29	42.86	28.57

It can therefore be concluded that, while all communities sampled in the Miers Valley (hypolithic, non-translucent rock and open soil) share some similarity based on 16S rRNA gene community profiles, non-translucent rock and open soil communities are most similar to each other. Furthermore, even though not all hypolithic communities are the same, they share more similarity with each other than other communities tested. Interestingly, the hypolithic communities were more similar to non-translucent rock than to open soil. The grouping of hypolithic communities into the three classes (Type I, Type II and Type III) was also tested using the jackknife (maximum similarities) method (Table 4.3).

Table 4.3: Group statistics (jackknife method) showing the stability of grouping Type I, Type II and Type III classes of hypolithic communities

	Type I	Type II	Type III
Type I	57.14	25.00	33.33
Type II	28.57	25.00	0.00
Type III	14.29	50.00	66.67

Overall, the grouping statistic based on fingerprint data analysis is low (49.06%). Type II appears the least stable grouping as 50% of samples identify more with Type III fingerprints and 25% identify more with Type I (Table 4.3). Type III is the most stable grouping as almost 70% of Type III communities identify with other members of the same group. Over 57% of Type I communities produce fingerprints that are most similar to other Type I communities.

The species richness (S), evenness (E), Shannon index (H') and maximum Shannon index (H_{max}) were calculated (see Section 2.6) for each community group based on

the total number of 16S rRNA gene OTUs detected for that community group (Table 4.4).

Table 4.4: Diversity indices calculated for the hypolithic, non-translucent rock and open soil communities

	Hypolithic communities	Non-translucent rock communities	Open soil communities
Species richness (S)	120	109	109
Evenness (E)	0.966	0.970	0.971
Shannon index (H')	4.625	4.548	4.556
Maximum Shannon index (H_{max})	4.787	4.691	4.691

The diversity indices indicate that hypolithic communities contain the greatest species richness while non-translucent rock and open soil communities have similar reduced species richness. This was not surprising considering that these communities share great similarity (Table 4.2 and Figure 4.9) and could be grouped together. The limitations of the use of PCR-DGGE for microbial community characterisation are discussed in Section 1.6.4. However, there was a significant difference in the Shannon biodiversity index for the three habitats indicating that the bacterial diversity in hypolithic habitats is significantly greater than non-translucent rock ($P<0.05$) and open soil communities ($P<0.05$). The evenness approaches 1 for all the communities indicating that the distribution within OTUs is very even, with each OTU represented by a similar number of individuals. H' approaches H_{max} for all the communities examined, which implies that while the species richness is high for all the communities the number of individuals represented by each OTU is low.

Based on 16S rRNA gene community fingerprint analysis, all soil communities in the Miers Valley are highly diverse. Non-translucent rock and open soil communities are more similar to each other compared to hypolithic communities. Group statistics data indicate that there is overlap in the community components for the three soil habitats. Furthermore, while distinction of hypolithic communities into three classes (Type I, Type II and Type III) based on field observations was possible, the clustering based

on 16S rRNA gene fingerprinting data did not distinguish the three classes into highly stable groups. This indicates some overlap in the community components between the three hypolithic community groups.

4.5 Cyanobacterial community profiles

Cyanobacteria have previously been found to constitute the major components of hypolithic communities in Antarctica (Smith *et al.*, 2000). Therefore, the cyanobacterial diversity in the metagenomic soil samples was explored by targeting the16S rRNA gene with cyanobacterial-specific primers for PCR-DGGE (see Section 2.4). Two primer sets were used, each with the same forward primer Cya359F-GC and either Cya781R(a) or Cya781R(b) (refer to Table 2.1) (Figure 4.11). PCR products were pooled if both sets of reactions produced amplicons for a particular sample before separation on 9% polyacrylamide gels with a 35-75% denaturing gradient.

Although the amount of cyanobacterial 16S rRNA gene PCR product generated from non-translucent and open soil communities was lower than that from hypolithic communities (Figure 4.11), fingerprint patterns were still clear and reproducible on DGGE gels. Furthermore, the differential amplification suggests that the concentration of cyanobacterial genomic DNA (and hence the cyanobacterial population) present in hypolithic communities is higher than that of the other soil environments.

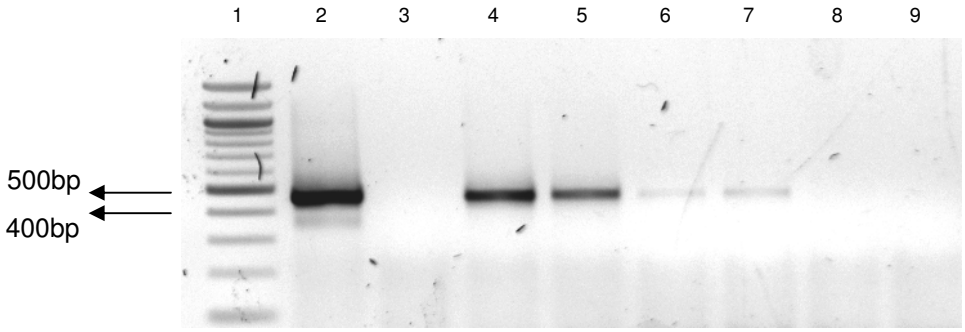

Figure 4.11: Pooled PCR product generated with cyanobacterial specific primers Cya359F-GC and Cya781R(AB) (a). Lane 1: Molecular weight marker. Lane 2: Positive control. Lane 3: Negative control. Lanes 4 and 5: PCR from duplicate hypolithic community metagenomic DNA extractions. Lanes 6 and 7: PCR from duplicate non-translucent rock community metagenomic DNA extractions. Lanes 8 and 9: PCR from duplicate open soil community metagenomic DNA extractions.

In the analysis of the cyanobacterial community fingerprints produced by DGGE each band was considered an OTU. Image analysis software (see Section 2.5) was used to match OTUs and assign all bands present to a band class (Figure 4.12). This produced a similarity matrix based on the band matching (see supplementary material).

The Pearson correlation and UPGMA algorithm were used for cluster analysis of the community cyanobacterial fingerprints (Figure 4.13) and MDS was used to visualise the clusters formed (Figure 4.14). A high cophenetic correlation value indicated the confidence of the clusters.

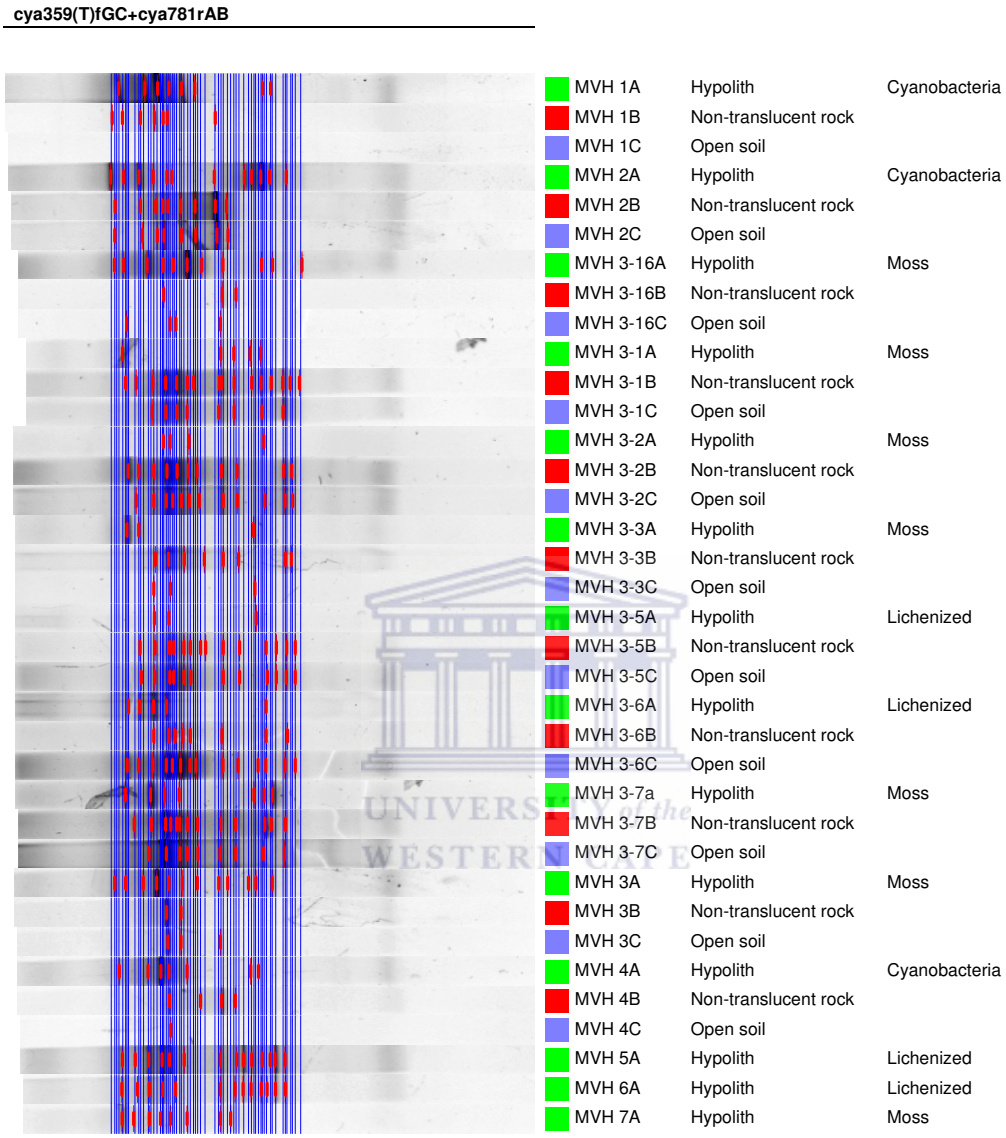

Figure 4.12: Band matching across cyanobacterial 16S rRNA gene fingerprints from hypolithic, non-translucent rock and open soil communities. Red lines represent bands in a fingerprint while blue lines indicate band classes, matching bands across fingerprints.

Figure 4.13 shows that there are five clusters formed (Groups 1-5). Group 1 is dominated by Type I (cyanobacterial) and Type III (lichenised) hypolithic communities with only one Type II (moss) community included. With few exceptions, non-translucent rock and open soil communities tend to cluster together into two groups (Groups 2 and 3). Groups 4 and 5 both contain hypolithic community profiles.

However while Group 4 consists exclusively of Type II (moss-dominated) communities, Group 5 consists of a single Type I and Type III community. Overall, it appears that non-translucent rock and open soil communities are most similar to each other, while hypolithic communities cluster separately. Furthermore, Type I and Type III communities tend to cluster together implying greater similarity between the cyanobacterial communities in these hypolithic community types. While some Type II communities cluster together, others show the least similarity to each other or other hypolithic, non-translucent rock or open soil communities.

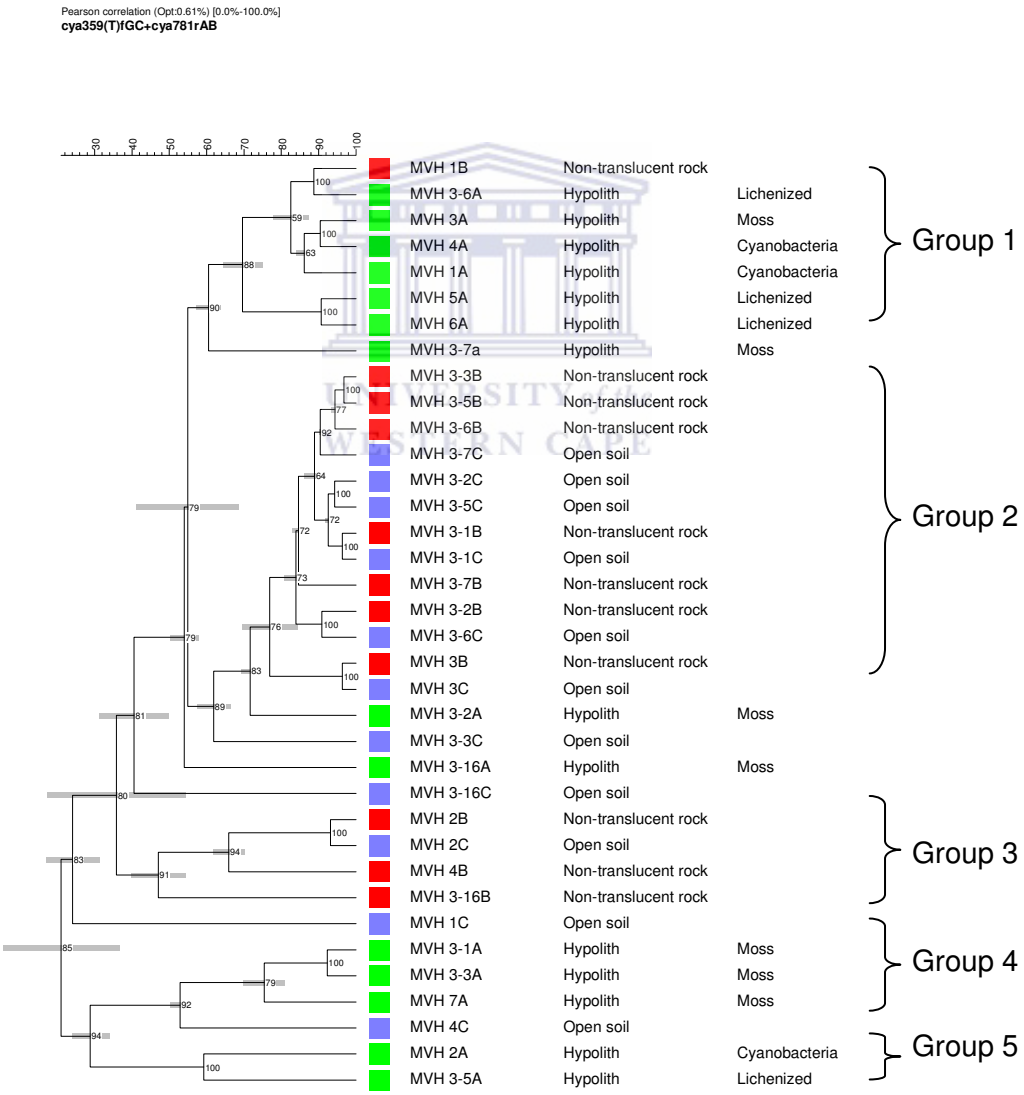

Figure 4.13: Cluster analysis of community cyanobacterial 16S rRNA gene fingerprints using the Pearson correlation and UPGMA algorithm.

MDS of the cluster analysis data (Figure 4.14) revealed a similar picture, but only two coherent clusters can be observed. Group 1 is dominated by hypolithic community cyanobacterial profiles while Group 2 is populated by both non-translucent rock and open soil community cyanobacterial profiles. This again shows the high similarity that exists in the cyanobacterial communities in the open soil and beneath non-translucent rock. Also, hypolithic cyanobacterial populations differ most from those detected in the open soil and beneath non-translucent rock. The hypolithic community cyanobacterial profiles that fall outside Group 1 are widely dispersed and indicate the differences in cyanobacterial populations within hypolithic communities.

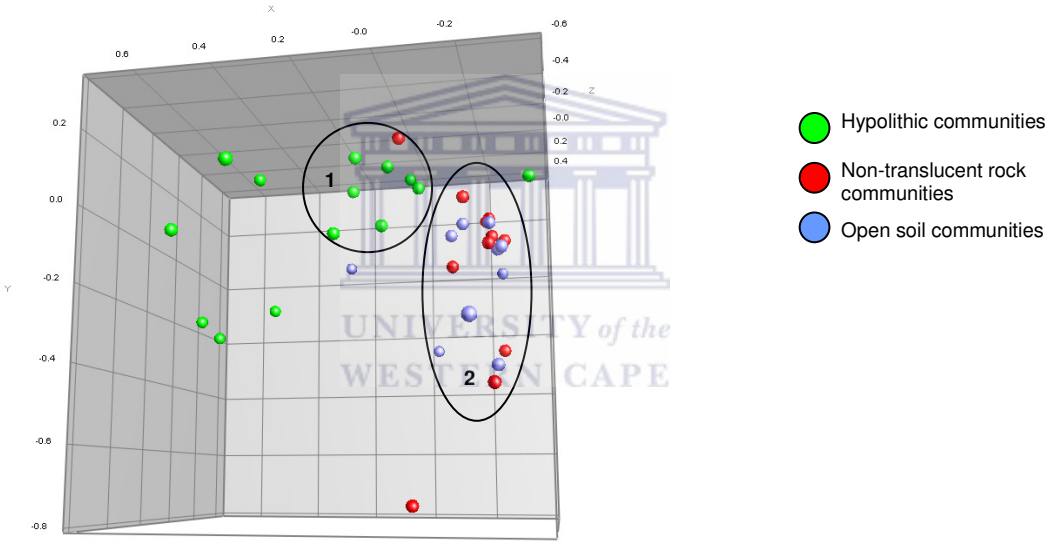

Figure 4.14: MDS analysis of hypolithic, non-translucent rock and open soil cyanobacterial 16S rRNA gene community fingerprints.

The jackknife method was used to establish whether the grouping of cyanobacterial profiles according to the sample community groups was stable (Table 4.5). As with the group statistic based on 16S rRNA gene community profiles, the overall quality of the group separation was low (53.46%) mainly due to the unstable grouping of non-translucent rock and open soil cyanobacterial community profiles as separate groups.

Again, the hypolithic community group is the most stable with almost 80% of hypolithic cyanobacterial community profiles having maximum similarity to other members in this group. In contrast, more than 50% of cyanobacterial profiles from non-translucent rock communities are more similar to open soil communities. The result indicates that grouping non-translucent rock and open soil cyanobacterial community profiles together may more reliably describe the community structure.

Table 4.5: Group separation statistics (jackknife method) showing the confidence of grouping the hypolithic, non-translucent rock and open soil cyanobacterial communities

	Hypolithic community	Non-translucent rock community	Open soil community
Hypolithic community	79%	9%	0%
Non-translucent rock community	14%	36%	55%
Open soil community	7%	55%	45%

Figure 4.15 highlights the three classes of hypolithic communities within the MDS cluster analysis of cyanobacterial communities from all habitats tested. The same clusters indicated in Figure 4.14 are again presented in Figure 4.15. Figure 4.15 indicates that, while some hypolithic cyanobacterial community profiles cluster, the distribution within the group is almost equally spread between the three hypolithic community classes. Therefore the cyanobacterial community profiles do not reflect the three classes of hypolithic communities assigned based on field observations.

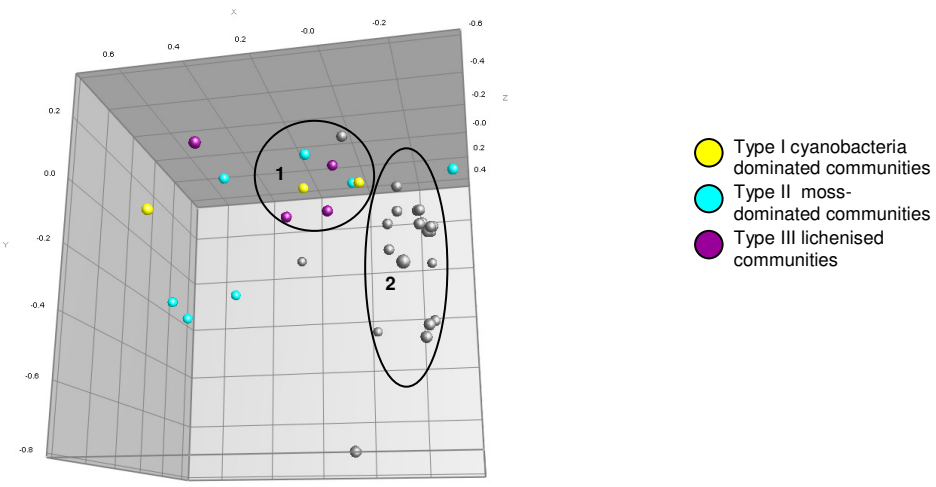

Figure 4.15: MDS analysis highlighting the positions occupied by the representatives of the three types of hypolithic communities proposed based on field observations (Type I, Type II and Type III).

In order to determine if the grouping of hypolithic communities into the three classes (Type I, Type II and Type II) is stable based on cyanobacterial community profiles, the jackknife method to test group stability was used (Table 4.6). Group statistics results indicated that none of the groupings are stable in terms of cyanobacterial community profiling. Type I cyanobacterial community profiles are equally similar to all the classes (Table 4.6). This indicates that these communities may be found in all classes of hypolithic communities. Type II cyanobacterial community profiles were found to have the greatest similarity within Type I and Type II communities, indicating that Type I and Type II communities share similar cyanobacterial communities. Type II cyanobacterial communities are largely different from those found in Type III as none of the Type II profiles showed the greatest similarity to Type III. Type III cyanobacterial populations are most similar to those found in both Type III communities and in Type I communities. Again, none of the Type III cyanobacterial profiles found maximum similarity with any Type II cyanobacterial community profile.

Table 4.6: Group statistics (jackknife method) showing the stability of the three classes of hypolithic communities with regard to cyanobacterial 16S rRNA gene community profiles

	Type I	Type II	Type III
Type I	33.3%	57%	50%
Type II	33.3%	43%	0%
Type III	33.3%	0%	50%

The diversity of cyanobacteria in the three habitats investigated was also assessed using the species richness, evenness and Shannon diversity indices (Table 4.7). As with total 16S rRNA gene-based analysis (Table 4.4), species richness in hypolithic communities was only slightly higher than the other two habitats. Furthermore, species richness for cyanobacteria was almost half that detected for the total bacteria 16S rRNA gene community profile. As the total 16S rRNA gene community profile includes cyanobacteria as well as other prokaryotes, this is not surprising. However, this does indicate that a high proportion of the bacterial population in the Miers Valley soil samples consists of cyanobacteria.

Table 4.7: Diversity indices calculated for hypolithic, non-translucent rock and open soil communities based on cyanobacterial 16S rRNA gene community fingerprints

	Hypolithic community	Non-translucent rock community	Open soil community
Species richness (S)	52	49	42
Evenness (E)	0.959	0.960	0.959
Shannon index (H')	3.791	3.736	3.586
Maximum Shannon index (H_{max})	3.951	3.892	3.738

The evenness approaches 1 for all habitats indicating very even distribution. As with diversity analysis of 16S rRNA gene fingerprinting data, the value of H_{max} was found to be the highest for hypolithic communities. This indicates that cyanobacterial diversity is slightly higher in hypolithic communities than other soil habitats tested in the Miers Valley. ANOVA shows no significant difference in H' between the habitats ($P>0.05$).

4.6 Archaeal community profiles

Archaeal signals have not previously been detected in the mineralised soils of the Miers Valley (Smith *et al.*, 2006). In this study, the amplification of *Archaeal* 16S rRNA genes for DGGE involved a nested-PCR approach in order to increase the sensitivity of detection. The first round of PCR employed *Archaeal*-specific 16S rRNA gene primers A3fa and Ab927r (see Section 2.4). These primers generated 924 bp fragments of the *Archaeal* 16S rRNA gene. However, amplicons were faint to invisible on agarose gel stained with ethidium bromide, probably due to a very small Archaeal population in the soils and therefore low Archaeal template DNA in the metagenomic extracts.

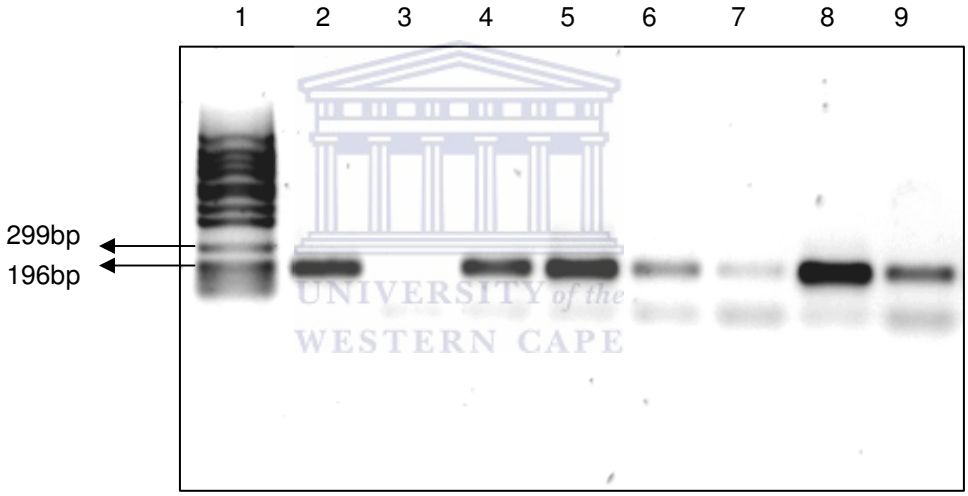

Figure 4.16: An agarose gel showing an example of PCR amplification of a 193bp fragment of *Archaeal* 16S rRNA genes from metagenomic DNA extracts using *Archaeal*-specific primers for DGGE. Lane 1: Molecular weight marker. Lane 2: Positive control. Lane 3: Negative control. Lanes 4 and 5: PCR product from duplicate hypolithic community extracts. Lanes 6 and 7: PCR product from duplicate non-translucent rock community extracts. Lanes 8 and 9: PCR product from duplicate open soil community extracts.

Although the PCR product concentration from the first round of PCR was low, the nested PCR (using the *Archaeal*-specific 16S rRNA gene DGGE primers A340F-GC and A533R in the second round PCR) produced sufficient product for detection on an agarose gel (Figure 4.16). In order to ensure that the amplicons produced were not an artefact due to the nested approach, the negative control in the nested-PCR

contained the negative control from the first round of PCR as the template (Figure 4.16, Lane 3). This control produced no PCR product.

The nested PCR products were then separated on 9% polyacrylamide gel with 30-65% denaturing gradient. Each band produced in the Archaeal fingerprints was defined as an OTU and band matching (Figure 4.17) produced a similarity matrix (see supplementary material).

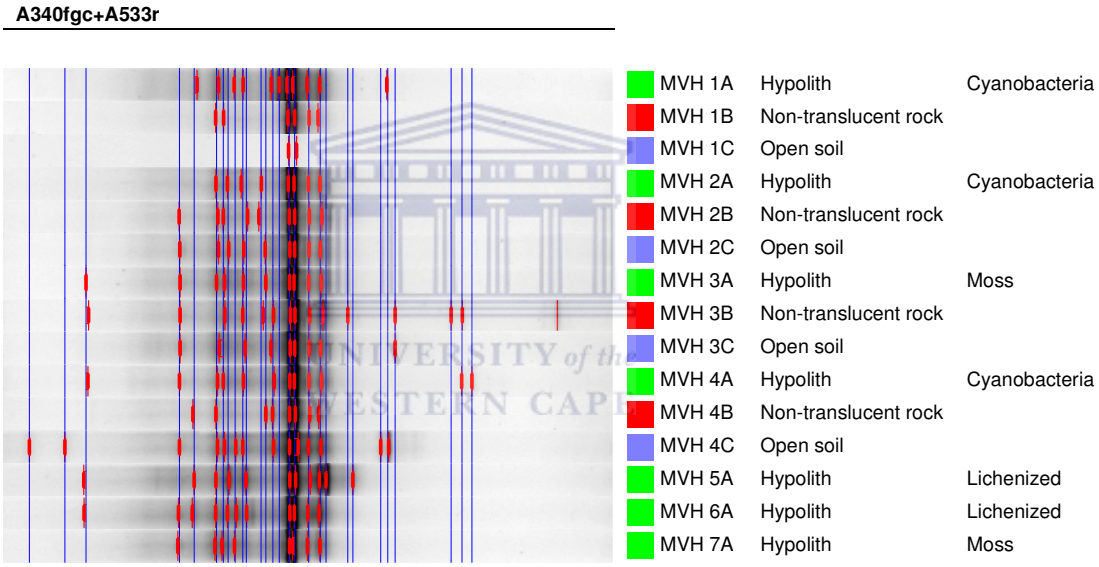

Figure 4.17: Band matching across hypolithic and control community *Archaeal* 16S rRNA gene fingerprints. Red lines indicate bands in each fingerprint while blue lines indicate band classes matching bands across profiles.

The Pearson correlation similarity coefficient with the UPGMA algorithm was used for cluster analysis of the *Archaeal* 16S rRNA gene community fingerprints (Figure 4.18).

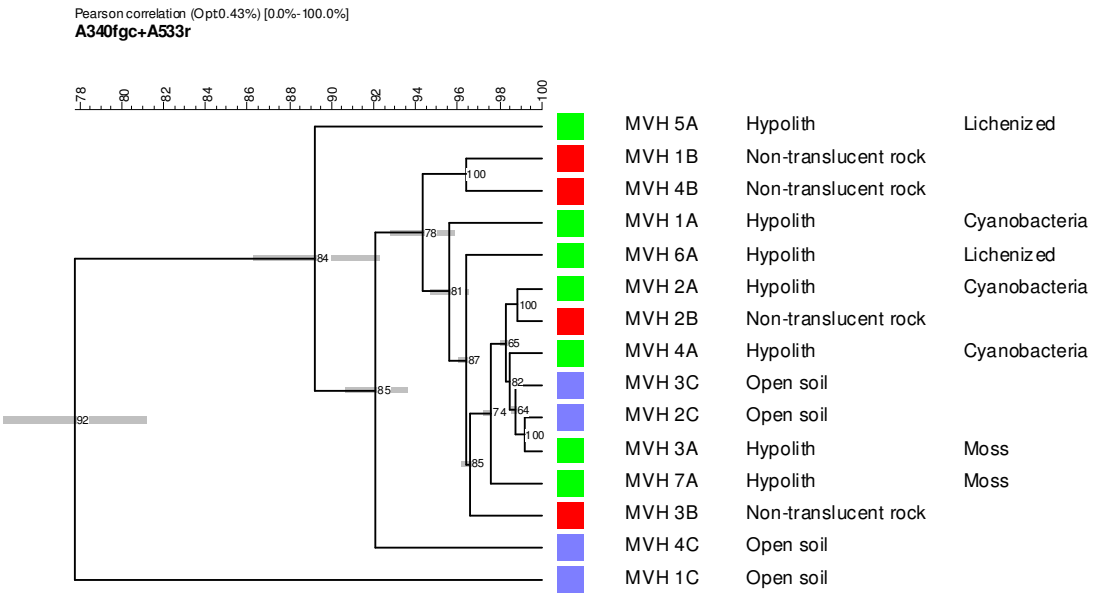

Figure 4.18: Cluster analysis of hypolithic, non-translucent rock and open soil *Archaeal* 16S rRNA gene community fingerprints (Pearson correlation, UPGMA).

Cophenetic correlation figures were all >75% indicating confidence in the designated clusters. However, similarity between all the Archaeal fingerprints was extremely high with an average similarity of 92.4 ± 6.6% between Archaeal fingerprints (see supplementary material). There are no separate clusters formed due to the high similarity. Aside from the exception of one open soil sample and one hypolithic community sample, all the other samples cluster with high confidence (cophenetic correlation of 85%).

MDS of Archaeal fingerprints (Figure 4.19) shows one apparent cluster that includes representatives from all three soil environments. However, many data points are widely dispersed indicating considerable differences in archaeal community profiles. Also, the scale for MDS is extremely small (<0.05) which indicates that all the Archaeal fingerprints are in fact very similar and would, on a larger scale, cluster together tightly. This means that if more diverse archaeal communities were examined, Miers Valley hypolithic, non-translucent rock and open soil archaeal communities would form a coherent, significant cluster. It can therefore be concluded that the Archaeal communities found in hypolithic, non-translucent rock and open soil

communities are highly similar (>90%). The jackknife (maximum similarity) method for group separation statistics was used to determine the stability of grouping the three environments tested separately (Table 4.8). As expected from the high similarity results, the overall group statistic is extremely low (27%) indicating that the grouping is very unstable.

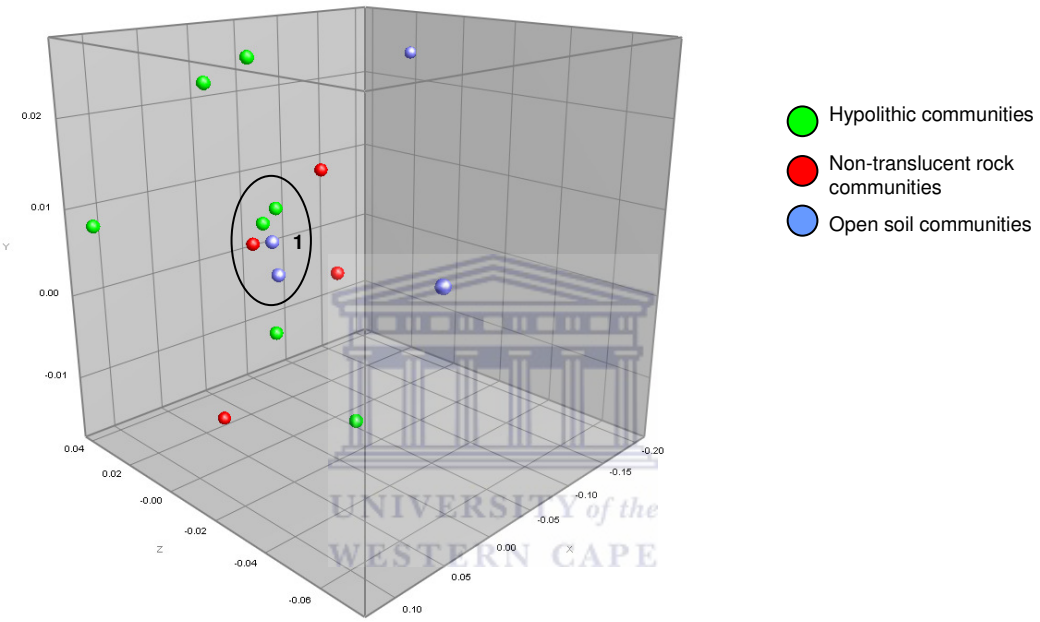

Figure 4.19: MDS of hypolithic, non-translucent rock and open soil *Archaeal* 16S rRNA gene community fingerprints.

While grouping hypolithic communities appears most stable, 100% non-translucent rock and 50% open soil archaeal fingerprints share greatest similarity with archaeal fingerprints in the hypolithic community group. This further confirms the overall high similarity in Archaeal communities present in Miers Valley soil habitats.

Table 4.8: Group separation statistics (jackknife method) indicating the stability of grouping hypolithic, non-translucent rock and open soil communities separately based on *Archaeal* 16S rRNA gene fingerprints

	Hypolithic community	Non-translucent rock community	Open soil community
Hypolithic community	57	100	50
Non-translucent rock community	14	0	25
Open soil community	29	0	25

MDS analysis of the proposed hypolithic community classes (Figure 4.20) showed no stable clustering. As mentioned, the scale of dimensioning is very small (<0.05). Inclusion of archaeal communities with greater diversity would more clearly show the clustering of archaeal community profiles from all Miers Valley soil habitats tested. Therefore all hypolithic archaeal communities are highly similar regardless of field observation-based separation of classes.

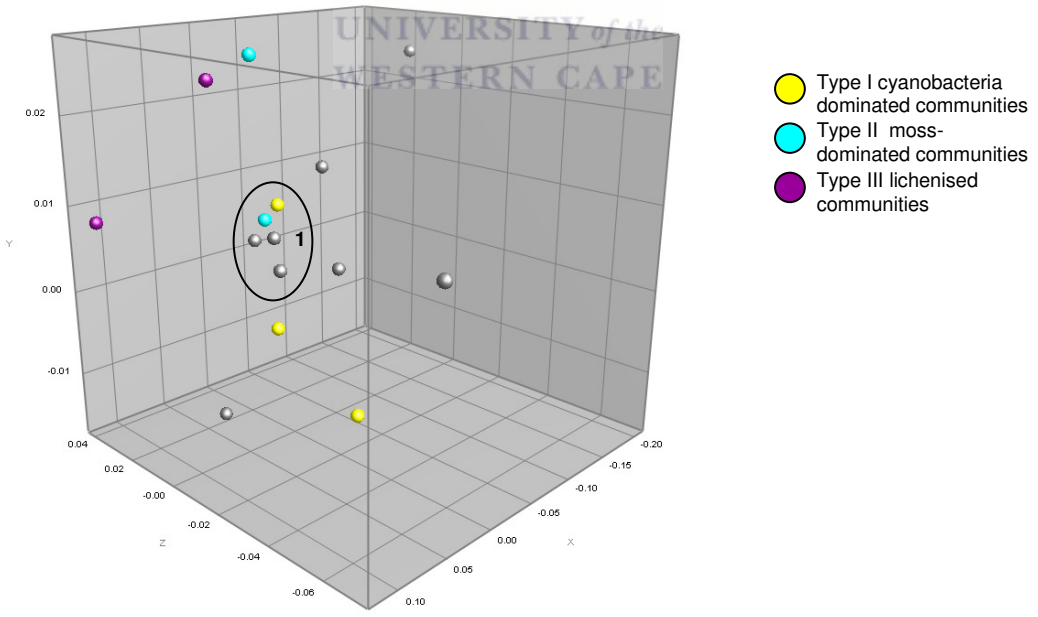

Figure 4.20: MDS analysis of *Archaeal* 16S rRNA gene community fingerprints highlighting the positions occupied by the representatives of the three types of hypolithic communities proposed based on field observations (Type I, Type II and Type III).

Group separation statistic analysis using the maximum similarity jackknife method (Table 4.9) showed that while the overall grouping statistic was low (45%), Type I communities appear to form the most stable grouping. Unlike grouping statistics from bacterial 16S rRNA gene and cyanobacterial-specific 16S RNA gene fingerprints, Type II and Type III communities show the lowest group stability and most similarity to each other. However, all hypolithic archaeal communities are highly similar to each other.

Table 4.9: Group separation statistics (jackknife method, maximum similarity) indicating the stability of grouping the three classes of hypolithic communities separately based on *Archaeal* 16S rRNA gene fingerprints

	Type I	Type II	Type III
Type I	64.29	14.29	0.00
Type II	21.43	42.86	71.43
Type III	14.29	42.86	28.57

Diversity indices indicated that archaeal diversity in all soil habitats examined is much lower than the prokaryotic diversity (Table 4.10). Hypolithic communities have slightly higher species richness than other soil environments, and a slightly lower evenness (i.e. a more uneven distribution of archaeal OTUs in hypolithic communities). The Shannon index (H') shows significantly lower archaeal diversity present in the soils compared to the cyanobacterial or overall bacterial diversity ($P<0.05$) (Tables 4.4 and 4.7). H' approaches H_{max} for non-translucent rock and open soil communities, but the difference between H' and H_{max} for hypolithic archaeal communities is greater. This is due to the lower evenness in hypolithic archaeal communities. Since the Shannon index is a function of evenness, the value of H' decreases with decreasing evenness. ANOVA of H' data shows no significant difference in archaeal diversity between the habitats.

Table 4.10: Diversity indices calculated for the archaeal populations in the different habitats based on community fingerprinting

	Hypolithic community	Non-translucent rock community	Open soil community
Species richness (S)	22	18	18
Evenness (E)	0.920	0.941	0.950
Shannon index (H')	2.844	2.721	2.746
Maximum Shannon index	3.091	2.890	2.890

4.7 Eukaryotic community profiles

The eukaryotic community diversity in the three soil habitats in the Miers Valley was assessed by targeting the 18S rRNA gene (the homologue of the prokaryotic 16S rRNA gene) for PCR-DGGE.

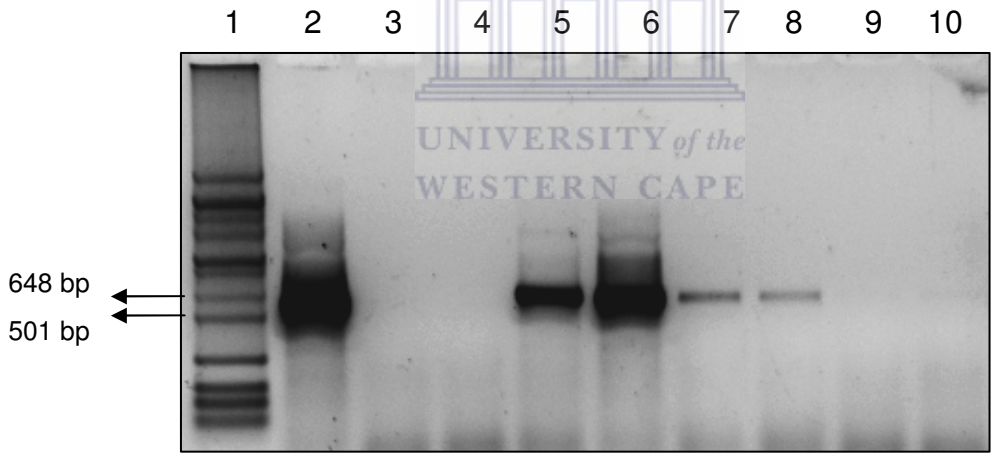

Figure 4.21: An agarose gel showing an example of PCR amplification of a fragment of the 18S rRNA genes from metagenomic DNA extracts using 18S rRNA gene-specific primers for DGGE. Lane 1: Molecular weight marker. Lane 2: Positive control. Lane 3: Negative control. Lane 4: Negative control using *E. coli* template DNA. Lanes 5 and 6: PCR product from duplicate hypolithic community extracts. Lanes 7 and 8: PCR product from duplicate non-translucent rock community extracts. Lanes 9 and 10: PCR product from duplicate open soil community extracts.

Figure 4.21 shows the 18S rRNA gene amplicons produced using the universal 18S rRNA gene-specific primers Euk1A and Euk516R-GC. To ensure that amplification

was specific to eukaryotes, a second negative control using *E. coli* genomic DNA as a template was added. This reaction did not produce any PCR product (Figure 4.21).

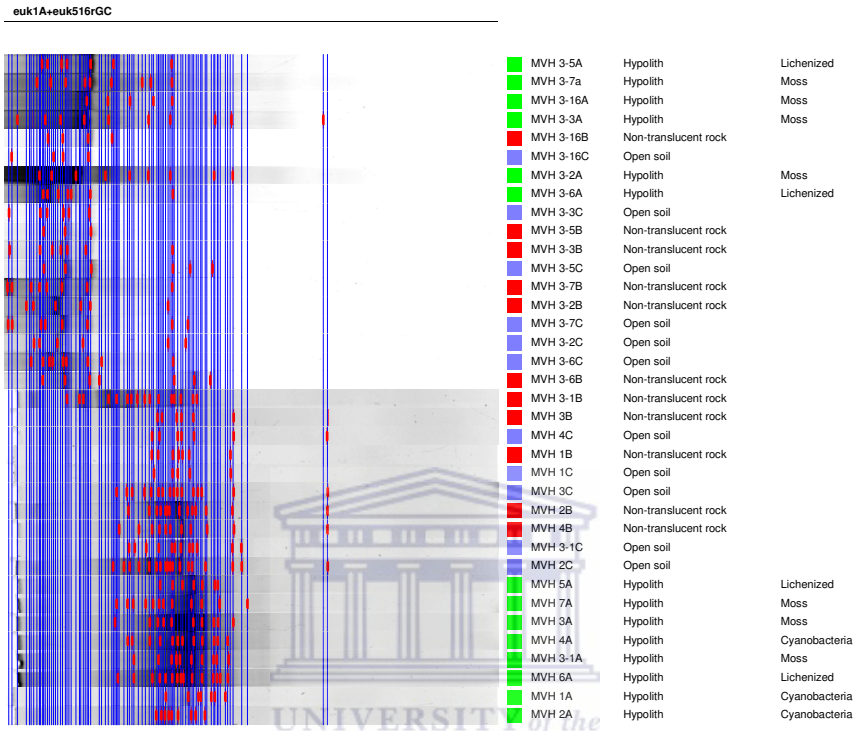

Figure 4.22: Band matching across hypolithic and control community 18S rRNA fingerprints. Red lines indicate bands in each profile while blue lines illustrate band classes matching bands across fingerprints.

Each band in the fingerprint profile was defined as an OTU and OTUs were matched based on their migration. This generated a similarity matrix (see supplementary material) which shows the similarity between each fingerprint profile. The densitometric curve of the profiles was then used to construct a dendrogram using the Pearson correlation similarity coefficient and UPGMA algorithm (Figure 4.23).

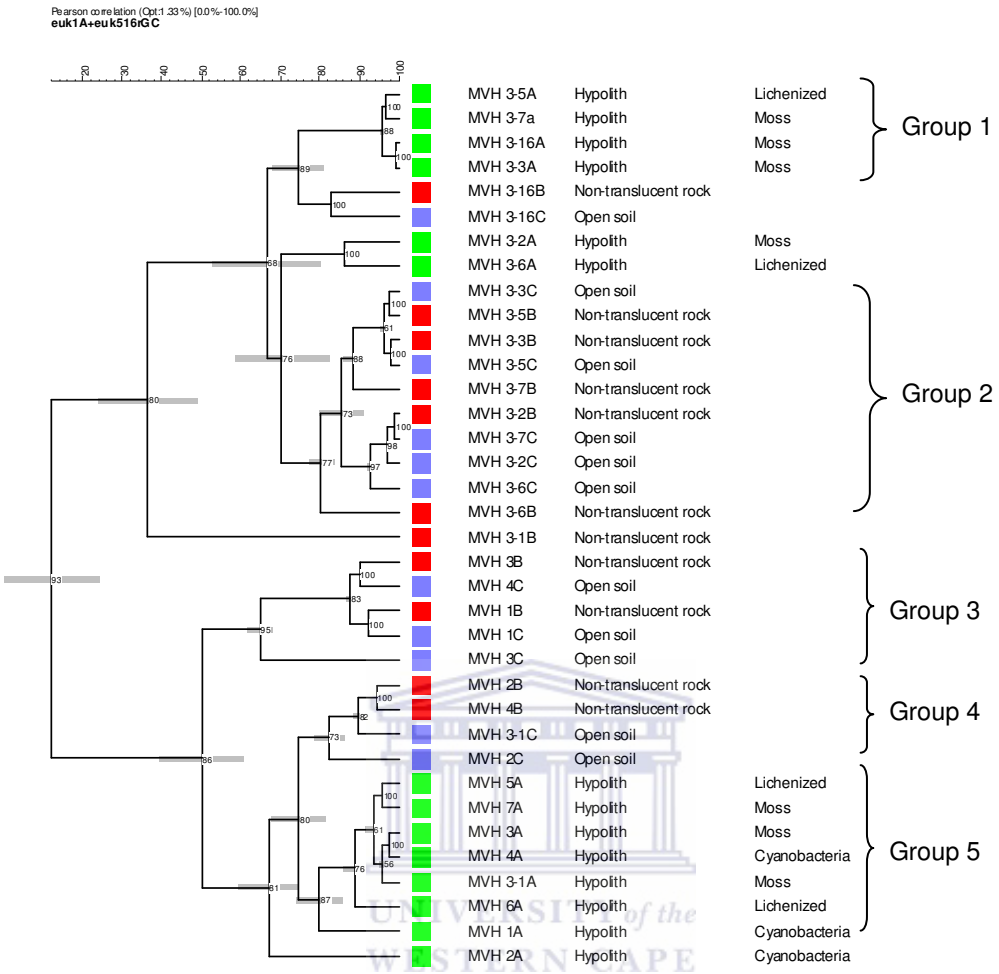

Figure 4.23: Cluster analysis of hypolithic, non-translucent rock and open soil eukaryote 18S rRNA community fingerprints (Pearson correlation, UPGMA).

Five major groups could be identified from the cluster analysis (Figure 4.23). Groups 1 and 5 consist only of hypolithic community 18S rRNA profiles, while the other groups consist of both non-translucent rock and open soil community 18S rRNA profiles. Three hypolithic community 18S rRNA profiles (one from each hypolithic community class) fall outside any group, although the Type II (moss) and Type III (lichenised) outliers are more similar to each other than the Type I (cyanobacteria) outlier. Group 1 consists of both Type II and Type III hypolithic communities, while Group 5 consists of members from all three hypolithic community classes.

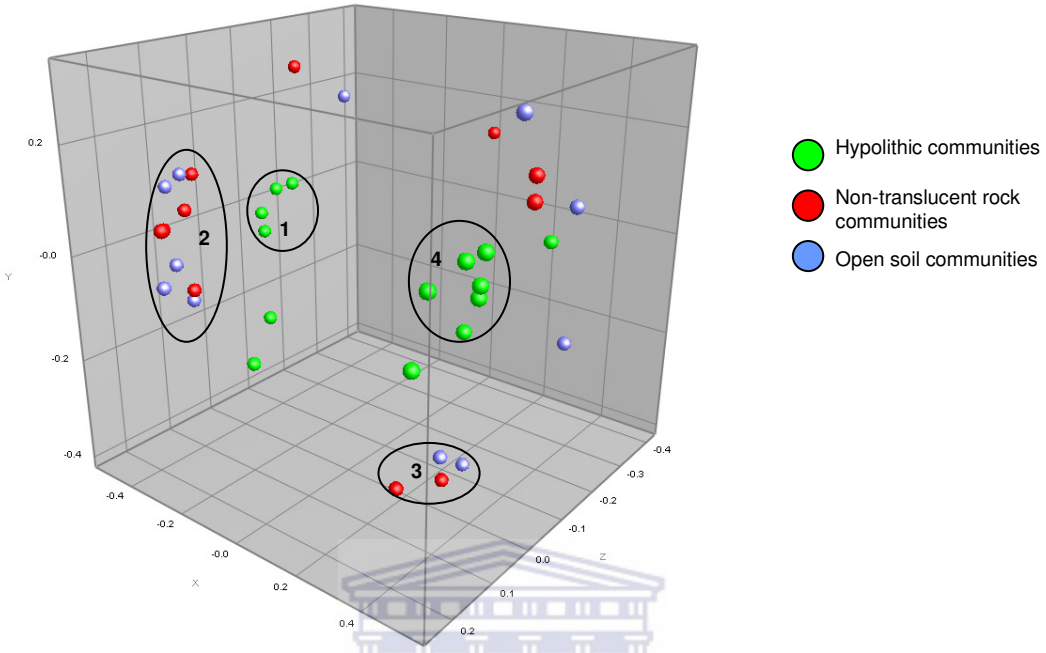

Figure 4.24: MDS analysis of hypolithic, non-translucent rock and open soil eukaryote 18S rRNA community fingerprints.

MDS analysis (Figure 4.24) showed only four clusters from the 18S rRNA community profiles. Groups 1 and 4 consist solely of hypolithic community 18S rRNA profiles, while Groups 2 and 3 consist of 18S rRNA fingerprints from both non-translucent rock and open soil communities. Therefore, while 18S rRNA eukaryotic communities found in hypolithic habitats form distinct clusters, those found in the open soil and beneath non-translucent rock are similar. Outliers consisted of 18S rRNA fingerprints from all three community types.

Table 4.11 shows the group separation statistics for the three communities based on 18S rRNA fingerprinting profiles. Although the overall grouping statistic suggests that the groupings were not very stable (40.7%), hypolithic communities formed the most stable group (86.7%). The overall statistic was low due to the high similarity between non-translucent rock and open soil eukaryote communities. The same trend was

found for group statistics based on bacterial- and cyanobacterial-16S rRNA gene fingerprints (Tables 4.2 and 4.5).

Table 4.11: Group separation statistics (jackknife method, maximum similarity) indicating the stability of grouping hypolithic, non-translucent rock and open soil communities separately based on 18S rRNA fingerprints

	Hypolithic community	Non-translucent rock community	Open soil community
Hypolithic community	85.7	18.2	0
Non-translucent rock community	14.3	27.3	90.9
Open soil community	0	54.5	9.1

MDS was also analysed with reference to the three proposed classes of hypolithic communities (Figure 4.25). It is evident from Figure 4.25 that Type II and Type III hypolithic eukaryote communities cluster together in two groups (Group 1 and 4; although Group 4 also contains one Type I hypolithic community). This implies that while there are some similarities in eukaryote populations between the hypolithic communities, Type II and Type III communities are most similar to each other.

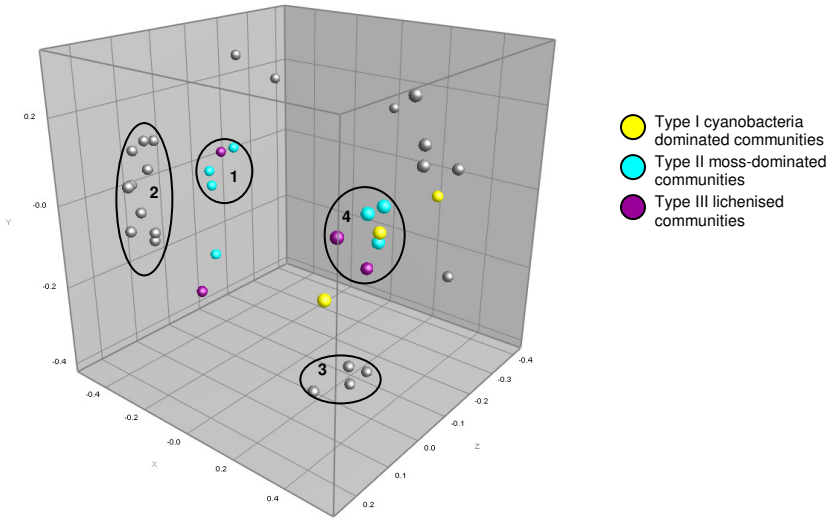

Figure 4.25: MDS analysis of 18S rRNA community fingerprints highlighting the positions occupied by the representatives of the three types of hypolithic communities proposed based on field observations.

Group separation statistics were calculated to determine the stability of grouping the three proposed classes of hypolithic communities separately based on 18S rRNA community profiles (Table 4.12). This revealed that the separation of the three proposed classes based on 18S rRNA fingerprint profiles is highly unstable (overall group statistic of 14.3%). Type I eukaryote communities do not form a separate group at all, while 100% of Type III eukaryote communities show maximum similarity to Type II communities. Group statistics indicate that separation of hypolithic communities into the three proposed classes based on eukaryote community profiles is unreliable in describing the community structure.

Table 4.12: Group separation statistics (jackknife method, maximum similarity) indicating the stability of grouping the three classes of hypolithic communities separately based on 18S fingerprints

	Type I	Type II	Type III
Type I	0.00	42.86	0
Type II	33.3	42.86	100
Type III	66.7	14.29	0

As with species richness calculated for the bacterial, cyanobacterial and archaeal populations, the eukaryote species richness is slightly higher for hypolithic communities than non-translucent rock and open soil communities (Table 4.13). Furthermore, the evenness approaches 1 for all communities implying a relatively even distribution of individuals in each OTU. The Shannon index for diversity (H') is higher for hypolithic communities, which reflects the higher species richness. However, ANOVA indicates no significant difference in H' between the three habitats ($P>0.05$). The difference between H_{max} and H' is similar for hypolithic and open soil eukaryote communities due to the similarity in evenness.

Table 4.13: Diversity indices calculated for the eukaryote populations detected in the different soil habitats in the Miers Valley

	Hypolithic communities	Non-translucent rock communities	Open soil communities
Species richness (S)	63	58	61
Evenness (E)	0.968	0.980	0.969
Shannon index (H')	4.011	3.978	3.981
Maximum Shannon index (H_{max})	4.143	4.060	4.111

4.8 Fungal community profiles

The fungal community diversity in the three soil habitats in the Miers Valley was assessed by targeting the fungal ITS1 region for PCR-DGGE using a nested approach (Anderson *et al.*, 2003). The first round of PCR used metagenomic DNA template with a forward primer specific for the ITS1 region (ITS1F) in fungi and a general eukaryote ITS4 reverse primer (see Section 2.4). The PCR products generated were then used as the template for the semi-nested second round PCR using the fungal-specific ITS1F primer with a 40 bp GC clamp (ITS1F-GC) and a reverse primer targeting the ITS2 region (Figure 4.26).

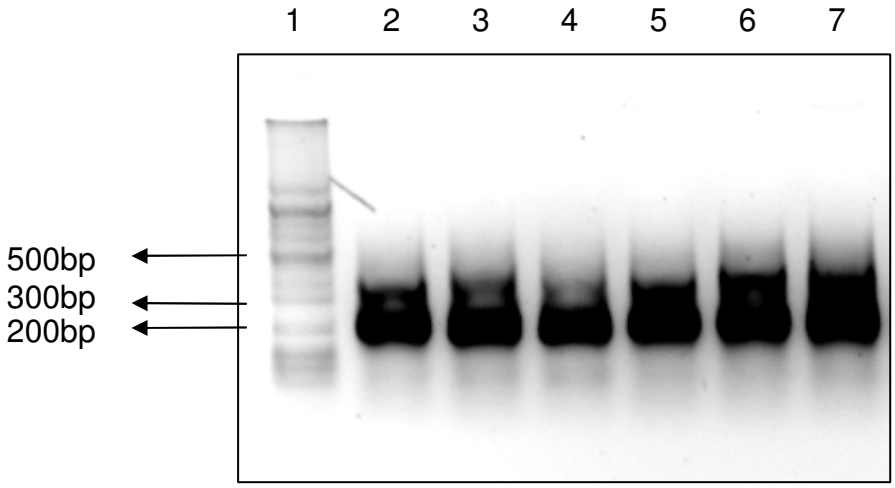

Figure 4.26: An example of nested-PCR amplification of a fragment of the ITS region from metagenomic DNA extracts using fungal-specific primers for DGGE. Lane 1: Molecular weight marker. Lanes 2 and 3: PCR product from duplicate hypolithic community extracts. Lanes 4 and 5: PCR product from duplicate non-translucent rock community extracts. Lanes 6 and 7: PCR product from duplicate open soil community extracts.

The amplicons were separated on 9% polyacrylamide gels with a 30-70% denaturing gradient. Band matching of the resultant fungal ITS community fingerprints was accomplished using imaging software (Figure 4.27). A similarity matrix was generated based on the similarity of the banding patterns (see supplementary material).

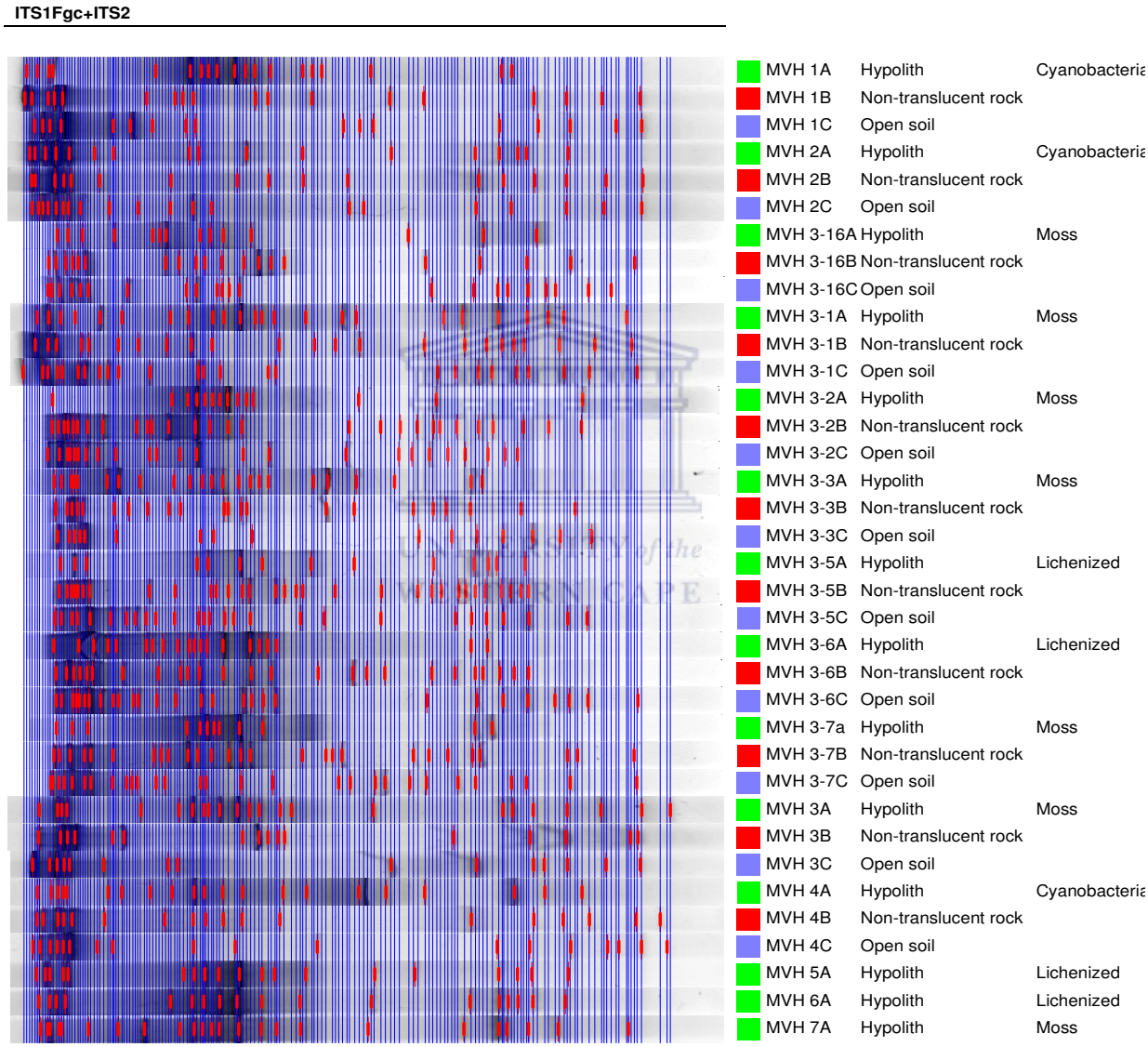

Figure 4.27: Band matching across hypolithic and control fungal ITS community fingerprints. Red lines indicate bands in each profile while blue lines indicate the band classes matching bands (or OTUs) across profiles.

The Pearson correlation coefficient and UPGMA algorithm were then used to generate a similarity dendrogram (Figure 4.28). Cophenetic correlation figures were

>75% indicating the stability of the cluster analysis. Cluster analysis showed four groups or clusters. Groups 1 and 2 consisted exclusively of hypolithic fungal ITS community profiles while Groups 3 and 4 consisted of both open soil and non-translucent rock fungal ITS profiles, with the exception of the inclusion of one hypolithic community profile in Group 4. The cluster analysis indicates that hypolithic fungal communities are different from the other soil communities assessed.

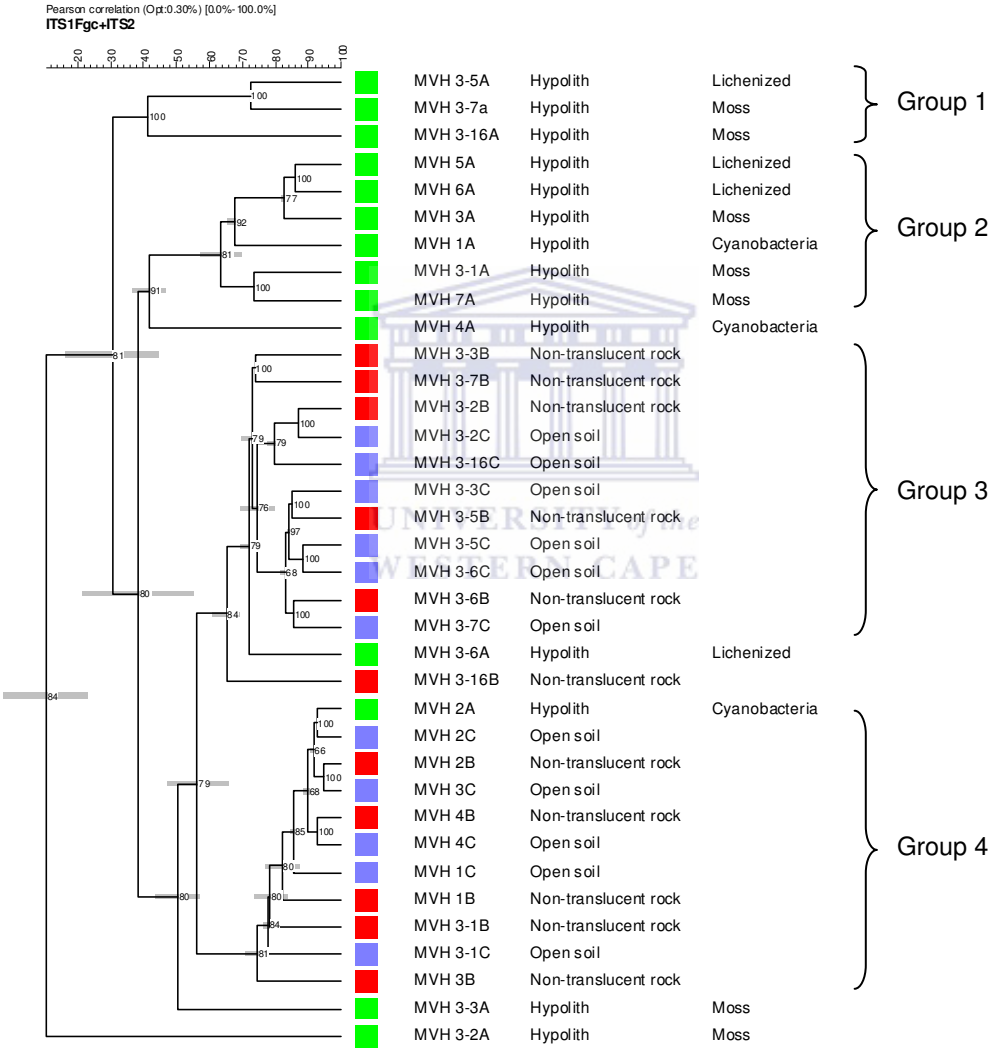

Figure 4.28: Cluster analysis of hypolithic, non-translucent rock and open soil fungal ITS community fingerprints (Pearson correlation, UPGMA).

MDS was also used for cluster analysis (Figure 4.29). MDS shows that there are in fact only two clusters formed. Both clusters (1 and 2) include almost equal

distributions of both non-translucent rock and open soil community profiles. Group 2 also contains a single fungal community profile from the hypolithic environment. Hypolithic fungal communities do not appear to form a coherent cluster.

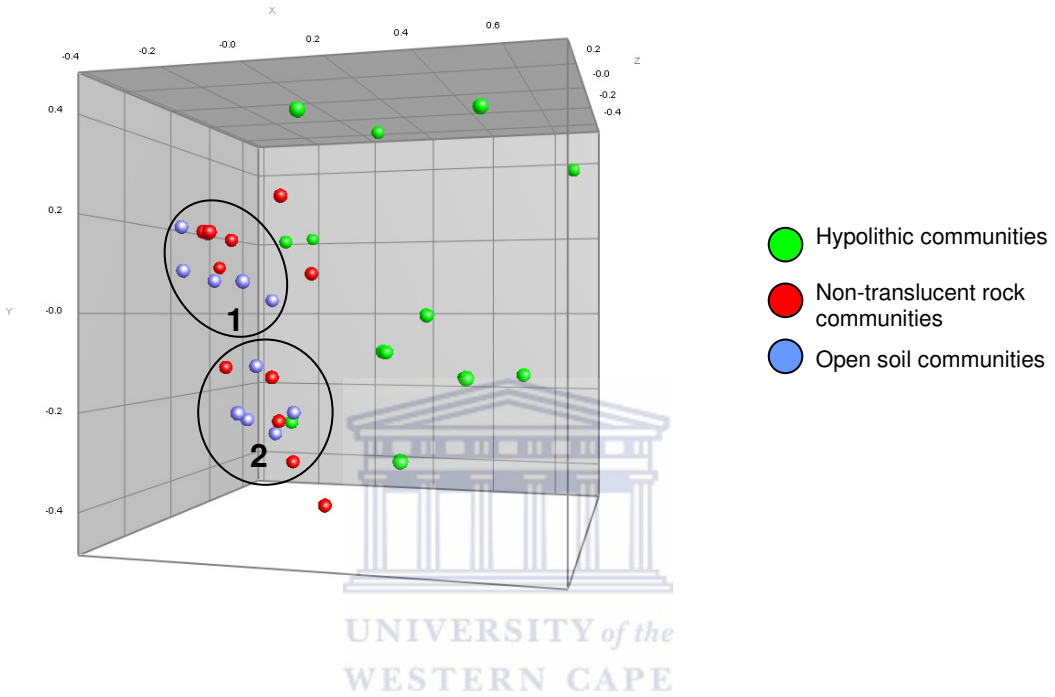

Figure 4.29: Multi-dimensional scaling of hypolithic, non-translucent rock and open soil fungal community fingerprints.

As with group statistics using other community profiles tested, the overall grouping statistic was low due to the high similarity between non-translucent rock and open soil communities. Hypolithic communities form the most stable grouping with almost 80% of hypolithic fungal community profiles sharing maximum identity with others in the hypolithic community group. As previously observed, grouping non-translucent rock and open soil communities separately is unstable as they share high similarity in the composition of their fungal community components.

Table 4.14: Group separation statistics (jackknife method, maximum similarity) indicating the stability of grouping hypolithic, non-translucent rock and open soil communities separately based on fungal 18S rRNA fingerprints

	Hypolithic community	Non-translucent rock community	Open soil community
Hypolithic community	78.6	18.2	9.1
Non-translucent rock community	21.4	18.2	36.4
Open soil community	0	63.6	54.5

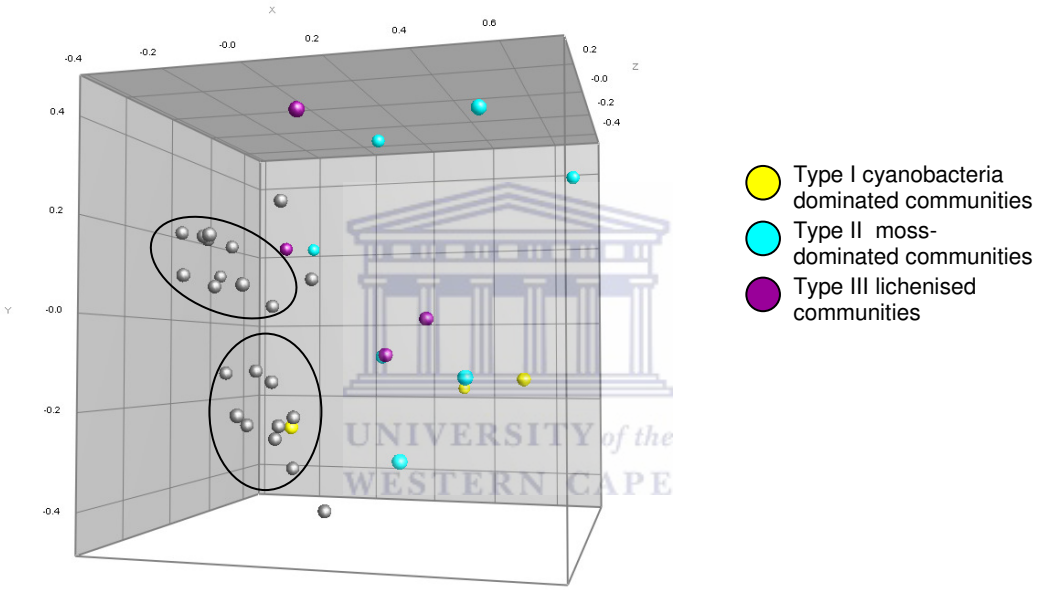

Figure 4.30: MDS analysis of ITS fungal community fingerprints highlighting the positions occupied by the representatives of the three types of hypolithic communities proposed based on field observations.

MDS analysis showed no significant clustering of hypolithic communities as a whole (Figure 4.29) and hypolithic communities did not cluster according to the proposed three hypolithic community classes (Figure 4.30 and Table 4.15).

Table 4.15: Group separation statistics (jackknife method, maximum similarity) indicating the stability of grouping the three classes of hypolithic communities separately based on fungal ITS fingerprints

	Type I	Type II	Type III
Type I	0	14.3	0
Type II	66.7	14.3	50
Type III	33.3	71.4	50

Hypolithic, non-translucent rock and open soil communities have a high fungal diversity with Shannon indices of >4.6 for all communities (Table 4.16). Species richness is high in all three communities, with the non-translucent rock communities containing species richness only slightly higher than the hypolithic habitats. The evenness index approaches 1 for all communities indicating that the all communities have an even distribution of OTUs. All three communities have high H_{max} values with H' approaching H_{max} which implies a high species diversity with an even distribution of individuals. ANOVA analysis of H' indicates that there is no significant difference in fungal species diversity within the three habitats ($P>0.05$).

Table 4.16: Diversity indices calculated for the fungal populations in the different soil habitats in the Miers Valley.

	Hypolithic communities	Non-translucent rock communities	Open soil communities
Species richness (S)	124	127	122
Evenness (E)	0.962	0.970	0.966
Shannon index (H')	4.639	4.698	4.638
Maximum Shannon index (H_{max})	4.820	4.844	4.804

4.9 Composite data analysis

The results of the different fingerprint types analysed in this study were combined to form a composite data set in order to determine the overall similarity of hypolithic, non-translucent rock and open soil communities. The composite data set was derived from bacterial 16S rRNA gene, cyanobacterial 16S rRNA gene, archaeal 16S rRNA gene, 18S rRNA gene and fungal ITS sequence fingerprint similarity matrices for

each community. The individual similarity matrices calculated for each fingerprint type were combined by averaging the similarity values to produce a combined matrix for all fingerprint types (see supplementary material).

Cluster analysis was then calculated from the composite data set using the average similarity coefficient and the UPGMA algorithm (Figure 4.32). The clustering was based on the average matrix of the combined similarity matrices of all the fingerprint types. While the average similarity was high between all community types, cophenetic correlation values were lower for branches clustering hypolithic communities with non-translucent rock and open soil communities than branches clustering solely hypolithic communities. For example, the cophenetic correlation for the branch that clusters Group 1 (which includes all three community types) is 40% while the cophenetic correlation for the branch clustering Group 4 (which consists only of hypolithic communities) is 74% (Figure 4.31). This indicates that the clustering of hypolithic communities together is a more reliable way of defining the community structure

MDS analysis was used to more accurately represent the cluster analysis without imposing a hierarchical structure of community similarities (Figure 4.32). MDS shows that all the communities assessed fall into one of two groups, with one outlier for each community type. Group 1 consists of all non-translucent rock and open soil communities (with one outlier from each) and 36% of the hypolithic communities assessed. Group 2 consists solely of hypolithic communities. It must be noted that the scale of dimensioning is small (<0.2) therefore the resolution for clustering is higher. This indicates that while all communities share a high degree of similarity, there is sufficient difference on a small scale to distinguish two distinct clusters.

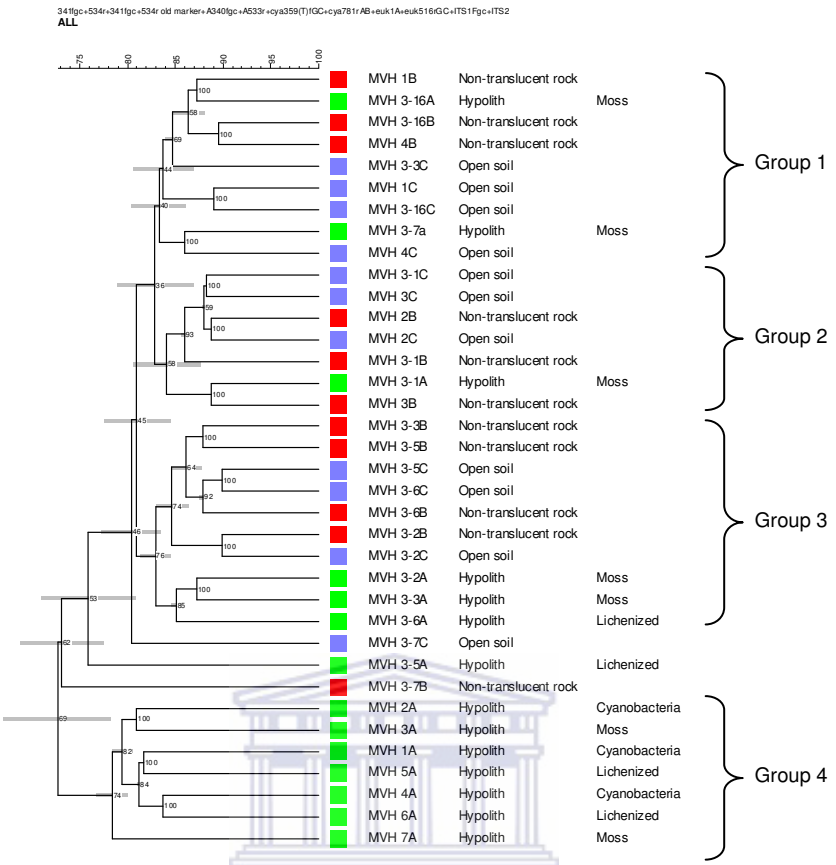

Figure 4.31: Cluster analysis based on the average similarity of hypolithic, non-translucent rock and open soil communities from the composite data set.

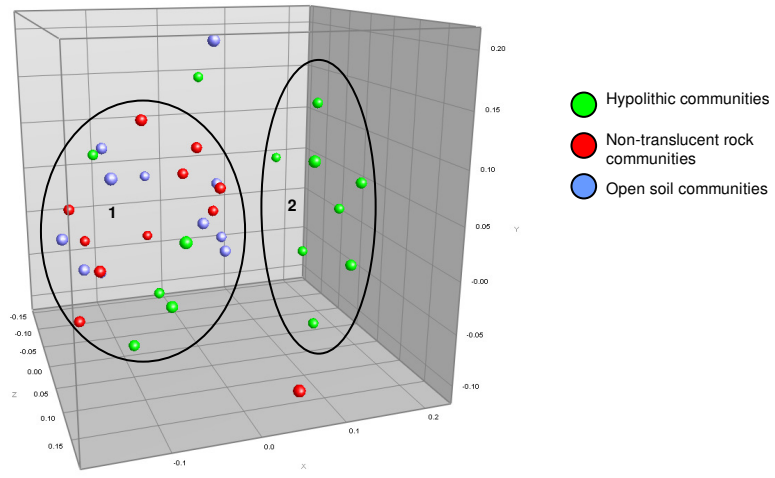

Figure 4.32: MDS of cluster analysis based on the average similarity of hypolithic, non-translucent rock and open soil communities from the composite data set.

The grouping statistics (Table 4.17) show that grouping the three communities separately is only 50% stable. Grouping stability increases if non-translucent rock and open soil communities are grouped together (Table 4.18). Non-translucent rock and open soil communities group together with 100% confidence (Table 4.18) while hypolithic communities form a separate stable group.

Table 4.17: Group separation statistics (jackknife method) based on the average similarity of hypolithic, non-translucent rock and open soil communities

	Hypolithic community	Non-translucent rock community	Open soil community
Hypolithic community	50	0	0
Non-translucent rock community	28.6	36.4	36.4
Open soil community	21.4	63.6	63.6

Table 4.18: Group separation statistics (jackknife method) showing stability of grouping when non-translucent rock and open soil communities are grouped together

	Hypolithic community	Non-translucent rock + open soil communities
Hypolithic community	71.4	0
Non-translucent rock + open soil communities	28.6	100

The MDS analysis of the three proposed classes of hypolithic communities shows that Group 1 contains one Type III and the majority of Type II hypolithic communities (Figure 4.33). These cluster with the non-translucent rock and open soil communities. Group 2 is composed of members from all three hypolithic community classes.

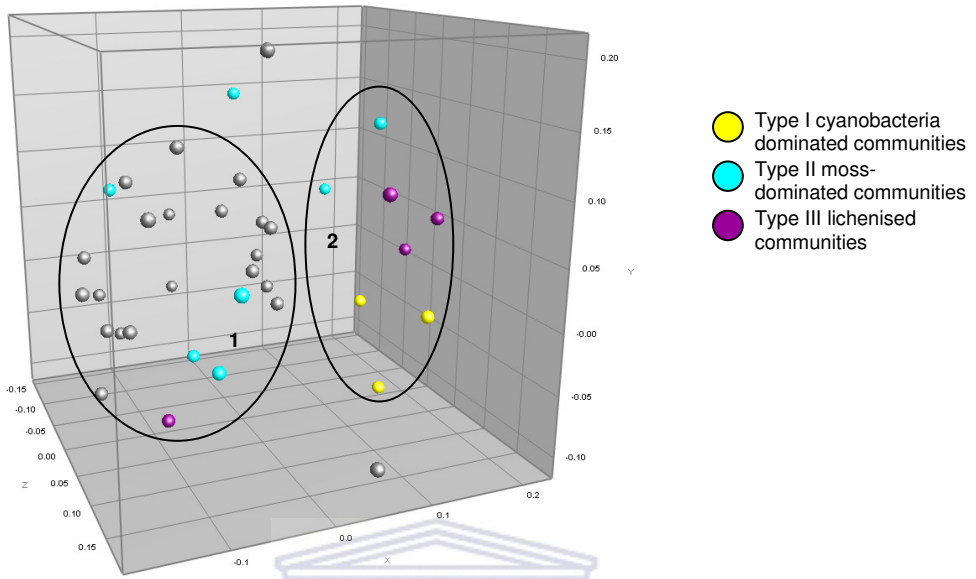

Figure 4.33: MDS analysis of composite data set highlighting Type I, Type II and Type III hypolithic community classes.

Group statistics indicate that Type I hypolithic community grouping is 100% stable (Table 4.19). Type II community grouping is also highly stable (71.4%) while Type III community grouping is totally unstable. This implies that, although field observations can differentiate Type III communities as a separate group, the overall community composition is most similar to Type I hypolithic communities.

Table 4.19: Group separation statistics (jackknife method) based on the average similarity of Type I, Type II and Type III hypolithic communities

	Type I	Type II	Type III
Type I	100	28.6	75
Type II	0	71.4	25
Type III	0	0	0

4.10 Conclusions

Extensive field observation led to the proposal that hypolithic communities can be divided into three classes:

- Type I cyanobacterial communities
- Type II moss-dominated communities
- Type III lichenised communities

However, the PCR-DGGE microbial profiling and cluster analyses indicate that, while hypolithic habitats may appear different due to the difference in dominant organisms, they contain similar species diversity. Qualitative phylogenetic analysis methods, such as PCR-DGGE used in this study, do not support the classification of hypolithic communities into the three proposed classes. As the field classification system is based on the observance of dominant macrobiotic biomass, a quantitative assessment of community structure may be required to better observe differentiation of the hypolithic classes into the three classes.

Analysis of DNA extraction yields showed that yields from hypolithic communities were significantly higher than those from non-translucent rock and open soil communities. This validates field observations of significantly higher biomass levels in hypolithic communities than other soil environments in the region.

PCR-DGGE microbial profiling was also used to assess the diversity of the hypolithic, non-translucent rock and open soil communities in the Miers Valley. Diversity indices showed that Bacteria, including cyanobacteria, contributed the greatest species richness to all the environments, followed by the Fungi and then the Archaea. The evenness values for all biotopes approached 1; indicating an even distribution of individuals among the OTUs. The species richness values for cyanobacteria were almost 50% of that of the total bacterial species richness indicating that a large proportion of the community in Miers Valley soils could be attributed to cyanobacteria. The Shannon diversity indices calculated for total bacterial, cyanobacterial and archaeal populations were always higher in hypolithic communities than non-translucent rock and open soil communities. The eukaryotic (particularly fungal)

diversity was high in all communities with Shannon index values >4.6. However fungal diversity may be overestimated as ITS sequences may yield more variants within species rather than greater species diversity (Huelsenbeck *et al.*, 1996). Overall, the Shannon diversity index was similar for each fingerprint type for the three soil communities assessed, with no significant difference except in the case of bacterial diversity. Therefore, while hypolithic communities certainly contain more biomass than other soil habitats in the Miers Valley, they do not contain a greater diversity of species.

Cluster analysis and multidimensional scaling revealed that the grouping of hypolithic communities together is more stable than grouping non-translucent rock and open soil communities separately. Grouping stability increases if non-translucent rock and open soil communities were grouped together since they share a high degree of similarity.

It can therefore be concluded that microbial diversity is similar in all the soil habitats tested in the Miers Valley. At high resolution, cluster analysis reveals that non-translucent rock and open soil communities are most similar to each other. Hypolithic communities on the other hand, cluster both separately from the other two communities and with them. This indicates that there are greater differences in fingerprint profiles within the hypolithic communities than between open soil and non-translucent rock communities. Therefore, compared to the non-translucent rock and open soil environments, hypolithic environments represent a distinct biotope. Quantitative methods of analysis are required to better define community structure. Furthermore, RNA-based studies (e.g. RT-PCR-DGGE) are required to differentiate between the active and dead or inactive members of the community.

Chapter 5: Phylogenetic characterisation of hypolithic communities

5.1 Introduction

Currently, a large proportion of the understanding of Antarctic mineral soil communities is based on culture-dependent studies. As culture-independent techniques can access organisms that are currently unculturable, these methods are important in order to gain a better perspective of microbial diversity within these environments.

5.1.1 Prokaryotic diversity

Cyanobacteria have been shown to be dominant members of Dry Valley ecosystems (Friedmann *et al.*, 1976, 1982, 1993; Broady *et al.*, 1981, 1991, 1996). Cyanobacteria are Gram-negative oxygenic phototrophs that have been found to prevail in the cold, extreme conditions of Antarctica (Broady & Kibblewhite, 1991; Nadeau *et al.*, 2001; Taton *et al.*, 2003, 2006a). The terrestrial lithic environment in particular, is dominated by cyanobacteria (Friedmann *et al.*, 1988; Smith *et al.*, 2000; de la Torre *et al.*, 2003). Classification of cyanobacteria based on morphology is often error-prone as they have simple morphologies with characteristics that exhibit plasticity with differences in environmental conditions (Zakhia *et al.*, 2008). Cyanobacteria were first described as algae and classified according to botanical nomenclature. With the advent of modern molecular techniques, the prokaryotic nature of cyanobacteria was revealed and subsequently, bacterial nomenclature has been applied (Zakhia *et al.*, 2008). The phylum *Cyanobacteria* currently consists of five principal groups (Rippka *et al.*, 1979; Zakhia *et al.*, 2008):

- *Chroococcales* – consisting of solitary and colonial unicellular cyanobacteria e.g. *Synechococcus* and *Gloeocapsa.*
- *Pleurocapsales* – unicellular to pseudo-filamentous, thallus-forming cyanobacteria capable of multiple and binary fission.
- *Oscillatoriales* – simple filamentous cyanobacteria lacking cell differentiation.

- *Nostocales* – filamentous cyanobacteria with cell differentiation to produce akinetes and heterocysts.
- *Stigonematales* – cell-differentiating cyanobacteria with complex multi-cellular organisation.

Studies have shown that the colonisation of Dry Valley soils by cyanobacteria results in increased soil stability and that cyanobacteria are the single largest contributor to biomass in Antarctica (Vincent & James, 1996). Cyanobacteria also increase nutrient concentrations in the ecosystem; they are photosynthetic primary producers and some cyanobacteria are able to fix atmospheric nitrogen (Zakhia *et al.*, 2008). The resilience of cyanobacteria to extreme polar desert conditions includes the ability to survive freezing and desiccation conditions (Davey, 1989).

Other prokaryote organisms detected in Antarctic soils include *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Chloroflexi* and *Proteobacteria* (Smith *et al.*, 2006).

Actinobacteria comprises one of the largest groups in bacterial phylogenetics in terms of the number and variety of species identified (Ventura *et al.*, 2007). These organisms present diverse morphologies, physiologies and metabolic properties that include the production of secondary metabolites (Schrempf, 2001), decomposition of refractory biomaterial and humus formation (Goodfellow & Williams, 1983; Stack & Bull, 2005).

Acidobacteria are an acidophilic group of bacteria that were recently recognised and described as a new phylum (Kuske *et al.*, 1997). While widely distributed in soil environments, this group contains few cultured representatives (e.g. *Acidobacterium capsulatum*, *Halophaga foetida* and *Geothrix fermentans*) and the majority of the 16S rRNA gene sequence representatives are obtained from environmental samples. Little is known about their physiology and metabolism. However, due to their abundance and global distribution, it is widely believed that these bacteria play a

major role in ecosystem processes and have versatile metabolic capacities (Quaiser *et al.*, 2003; Zhou *et al.*, 2003).

Verrucomicrobia is also a relatively recently recognised phylum of bacteria (since 1995) (Ward-Rainey *et al.*, 1995; Rappe & Giovannoni, 2003). Culture-independent methods based on the detection of 16S rRNA gene sequences, showed that these bacteria are widely distributed in soil communities, but there are currently only a few cultured representatives (Sangwan *et al.*, 2005). *Verrucomicrobia* are often major components of soil communities (Buckley & Schmidt, 2003; Sangwan *et al.*, 2005) and may play an important role in ecosystem functions and processes.

Chloroflexi, also known as the green non-sulphur bacteria, includes bacteria that display a diverse range of phenotypes (Rappe & Giovannoni, 2003). These organisms have been found to be ubiquitous in nature and have been detected in a vast range of habitats including soils (Dunbar *et al.*, 2002), sediments (Bowman *et al.*, 2000) and geothermal hotsprings (Hugenholtz *et al.*, 1998).

Proteobacteria form the second largest bacterial phylum and contain a diverse range of organisms in terms of physiology and metabolic activity. This group consists of Gram-negative bacteria and includes many of the bacteria responsible for nitrogen fixation e.g. *Rhizobium* spp.. Although *Proteobacteria* are widely distributed in soils from various sources, the prevalence or abundance of the alpha-, beta-, gamma- and delta- sub-divisions differ between sites (Borneman *et al.*, 1996; Borneman & Triplett, 1997; Zhou *et al.*, 2003).

Bacteroidetes are also commonly found in Antarctic soils (Saul *et al.*, 2005; Smith *et al.*, 2006; Aislabie *et al.*, 2006). This group of bacteria is well known for their ability to degrade organic polymers and play a role in decomposition (Buckley & Schmidt, 2001). In Antarctic soils, these organic polymers would be derived from wind-distributed microbial mats or lithic communities (Hopkins, *et al.*, 2005).

Archaea have not previously been detected in Antarctic hypolithic communities (Smith *et al*., 2000). While *Archaea* were once thought to exist exclusively in extreme environments such as hotsprings, the current influx of uncultured, unidentified archaeal sequences present in GenBank (>77% of all available archaeal sequences) indicate that these microorganisms are more widely distributed as they have been detected in almost all environments examined (Robertson *et al*., 2005). Furthermore, our knowledge of archaeal diversity and their ecological context is limited (Robertson *et al*., 2005). Currently, the archaeal phylogenetic tree consists of two phyla: *Crenarchaeota* and the larger group of *Euryarchaeota*. However, a large number of environmental archaeal sequences do not cluster with known groups, which indicates that archaeal diversity has not been fully sampled (Robertson *et al*., 2005). Little is known regarding the physiology of the *Archaea* and the ecosystem functions they provide since studies to address these issues are experimentally difficult to conduct (Robertson *et al*., 2005).

5.1.2 Eukaryotic diversity

Most molecular studies of Antarctic microbial diversity have focused on prokaryotic communities. The majority of studies of the terrestrial eukaryotic communities in the Antarctic have been based of traditional morphological or culture methods (Broady, 1996; Wynn-Williams, 1996). The coarse-textured, oligotrophic soils of the Dry Valleys contain relatively low biological activity compared to more temperate climates (Campbell *et al*., 1998). The majority of the terrestrial eukaryotic diversity studies that have been carried out mostly focused on nematodes. These studies have been limited to the more moist soil habitats near lakes and streams (Freckman & Virginia, 1997; Wall and Virginia, 1999). A limited number of studies on bryophytes (Dale *et al*., 1999; Skotnicki *et al*., 2002), algae (Broady *et al*., 1996) and fungi (Connell *et al*., 2006) in Antarctica have also been conducted. Mosses, lichens and algae are the predominant flora of the sparse terrestrial vegetation in the ice-free Dry Valleys of Antarctica.

Relatively few **moss** species are found in the Antarctic, most of which also occur in more temperate climates (Seppelt & Green, 1998). Mosses are widespread on the continent and inhabit a variety of ecological niches (Skotnicki *et al.*, 2000). Mosses in the Ross Sea region occur mostly in small clumps (and occasionally as turfs) in niche habitats where some moisture is available during summer such as in cracks and hollows in the ground surface, along drainage lines, and in habitats sheltered from the high winds in the area (Skotnicki *et al.*, 2000). Mosses rehydrate from their freeze-dried winter state and become metabolically active during summer, when moisture and light become available (Skotnicki *et al.*, 2000). Although it is widely accepted that mosses have a slow growth rate, little data is currently available regarding the growth rate of mosses in the Dry Valleys (Skotnicki *et al.*, 2000). Although mosses can reproduce both sexually and asexually, sexual reproduction (sporophyte production) is rare in Antarctica and vegetative dispersal, aided by high winds (over short and long distances) and water movement (local dispersion), are most likely (Selkirk *et al.*, 1998; Dale *et al.*, 1999, Skotnicki *et al.*, 2000). Phenotypic plasticity of mosses under extreme environmental conditions hinders their identification by morphology and molecular methods of identification are more suitable (Skotnicki *et al.*, 2005).

A significant proportion of terrestrial mosses in the McMurdo Dry Valleys are attributed to the moss *Hennediella heimii* (Dale *et al.*, 1999). *H. heimii* was formerly part of the genus *Pottia* until the taxonomy was reviewed and the species reclassified to the genus *Hennediella* (Zander, 1999). The study by Dale *et al.* (1999) showed that genetic variation within single *H. heimii* colonies which was attributed to somatic mutation rather than the establishment of propagules from other areas. Furthermore, while the Miers Valley *H. heimii* population was distinct from populations of the same species in neighbouring valleys, individuals in the Miers Valley were considered to constitute a single polymorphic population as no further genetic sub-divisions were detected. Wind was determined to be the most likely agent for dispersal of the moss in the Miers Valley due to the random genotypic distribution within the Miers Valley and nearby sites downwind of the Miers Valley. Similar patterns of genetic variation occur in the moss *Bryum argenteum* (Selkirk *et al.*, 1998) with high levels of genetic

variation, most of which occurs within rather than between colonies and populations (Skotnicki *et al.*, 1999). The extent of natural genetic variation in both species appears related to the amount of physical separation; adjacent shoots are more likely to be identical than shoots that are further apart (Selkirk *et al.*, 1998).

This high level of genetic variation found in Antarctic mosses was unexpected since these organisms are geographically isolated and lack sexual reproduction (Stevens *et al.*, 2007). A more recent study has offered an alternative solution and shows that genetic variations could be due to contamination of moss DNA extracts with DNA from fungi and protozoans that are naturally associated with mosses in Antarctica (Stevens *et al.*, 2007).

The first reports of *H. heimii* (*P. heimii* at the time) in the McMurdo Valleys (Seppelt *et al.*, 1992) showed that the two dominant mosses in a flush site near the Canada Glacier in the Lower Taylor Valley were *H. heimii* and *B. argenteum*. Overall, *B. argenteum* were found in the wetter sites, and absent from the drier margins of the flush where only salt-encrusted *H. heimii* occurred. Other mosses isolated from the ice-free regions of the coastal Dry Valleys include *Bryum pseudotriquetrum*, *Sarconeurum glaciale* and *Ceratodon purpureus* (Seppelt & Green, 1998).

Fungi are ubiquitous in Antarctica and have been detected and isolated from a range of Antarctic habitats (Sun *et al.*, 1978; Arenz *et al.*, 2006, Connell *et al.*, 2006; Yergeau *et al.*, 2007a). Yeast, yeast-like and filamentous fungi have also been found to be widespread, but occur in low abundance in Antarctica (Vishniac *et al.*, 1996). Contrary to Vishniac (1993), Arenz *et al.* (2006) detected equal numbers of both yeasts and filamentous fungi in Dry Valley soils. The distribution, importance and function of fungi in Dry Valley soils remains poorly characterised.

Most studies regarding **algal** diversity in the Antarctic are from marine or lake systems, but algae have been observed in Antarctic soils where no sign of macroscopic algal growth was evident (Cavacini, 2001). Direct microscopic evaluation

of soil samples from Northern Victoria Land and culture techniques detected the presence of twenty-three taxa belonging to *Chlorophyta*, nine to *Xanthophyta* and three to *Bacillariophyta* (rare).

5.2 Library construction and screening

Bacterial 16S rRNA gene, A*rchaeal*-specific 16S rRNA gene and eukaryotic ITS and 18S rRNA gene clone libraries of the following communities were constructed in order to determine the major phylotypes (composition) of each community (see Sections 2.7 and 2.8):

- Type I hypolithic community
- Type II hypolithic community
- Non-translucent rock community
- Open soil community

Ninety-six positive recombinant clones according to blue/white screening, were selected from each library for further analysis. Clones were first analysed for the possession of the correct sized insert. Figure 5.1 below shows an example of the amplification of the phylogenetic marker gene insert using the vector primers (M13fw and M13rev) (see Section 2.9.1). Plasmid DNA was isolated from clones containing the correct sized inserts (see Section 2.9.2).

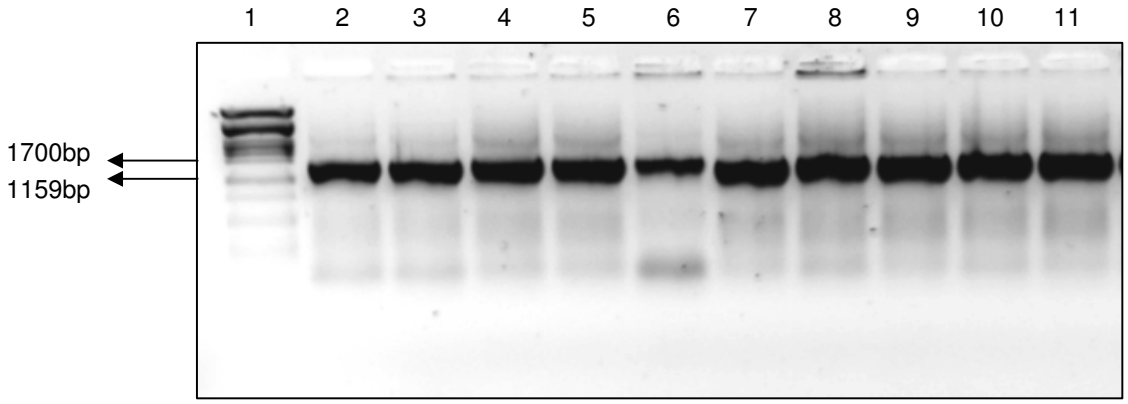

Figure 5.1: An example of colony PCR amplification from 16S rRNA gene clones using M13 primers to verify the presence of the correct sized insert. Lane 1: Molecular weight marker. Lanes 2-11: PCR products from Type I 16S rRNA gene clones (A1-A10).

Random plasmid minipreparations were separated out on a 1% (w/v) agarose gel to ensure plasmid preparations were free from genomic DNA contamination (Figure 5.2). Figure 5.2 shows that the minipreparation method used was successful in isolating plasmid DNA that was free from contaminating genomic DNA.

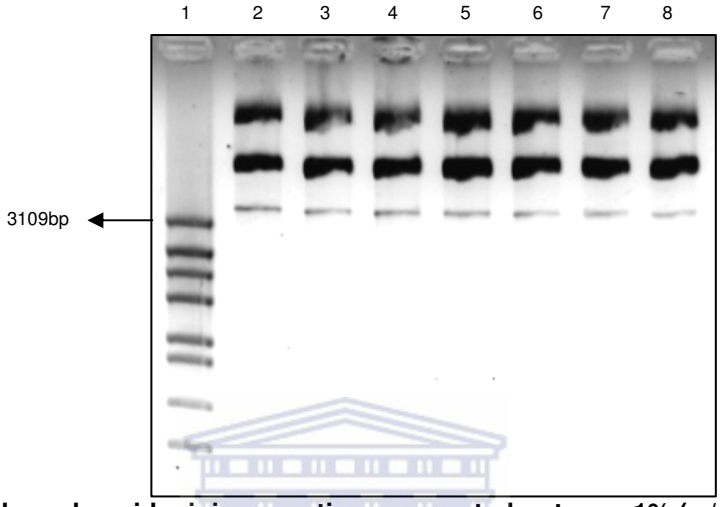

Figure 5.2: Random plasmid minipreparations separated out on a 1% (w/v) agarose gel to ensure the extracts were free from contaminating genomic DNA. Lane 1: Molecular weight marker. Lanes 2-8: Random minipreparations from 16S rRNA gene clones.

5.3 Bacterial 16S rRNA gene clone libraries

Ninety-six recombinant clones each from the Type I and Type II hypolithic community 16S rRNA gene clone libraries were sequenced using the primers targeting the M13 vector. The partial sequence data obtained included the variable regions V1, V2 and V3 of the 16S rRNA gene. Sequences that were less than 500bp in length were eliminated from further analysis. This resulted in a selection of seventy-three sequences for the Type I library (Table 5.1) and sixty sequences for the Type II library (Table 5.2). These sequences were analysed by searching the NCBI database for the most similar sequences using blastn (Altschul *et al.*, 1990). Table 5.1 presents the nearest match of the cloned sequence with the % identity to the top blastn hit and the phylogenetic grouping.

Table 5.1: Sequences obtained from a Type I hypolithic community 16S rRNA gene clone library.

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 1A-B7 MVH 1A-F5 MVH 1A-H11	*Calothrix* sp. KVSF5 (EU022730)	Free-living isolate - Norway	93 93 93	*Cyanobacteria*, *Nostocales*
MVH 1A-D9 MVH 1A-E10	*Dyadobacter* sp. VTT E-052912 (EF093135)	Biofilm on stone monument – Scotland, UK	98 97	*Bacteroidetes*
MVH 1A-E8	*Ehrlichia* sp. Belluno (AY098730)	Isolate	77	*Proteobacteria*, *α-proteobacteria*
MVH 1A-G4	*Iamibacter majanohamensis* strain NBRC 102561 (AB360448)	From sea cucumber host	97	*Actinobacteria*, *Acidimicrobidae*
MVH 1A-A5 MVH 1A-B3 MVH 1A-C10 MVH 1A-C12 MVH 1A-F10	*Leptolyngbya* sp. ANT.LH52.1 (AY493584)	Antarctica	97 97 97 96 96	*Cyanobacteria*, *Oscillatoriales*
MVH 1A-C8	*Mesorhizobium ciceri* strain C-2/2 (AY206686)	Effective nodules of *Cicer arietinum*	95	*Proteobacteria*, *α-proteobacteria*
MVH 1A-E9 MVH 1A-G1	*Nostoc* sp. '*Peltigera membranacea* 5 cyanobiont' (DQ185247)	Lichen specimen voucher	98 98	*Cyanobacteria*, *Nostocales*
MVH 1A-G2	Uncultured *Actinobacteria* bacterium clone AKYG403 (AY921844)	Farm soil – USA	97	*Actinobacteria*
MVH 1A-B1	Uncultured actinobacterium (DQ366004)	Soil – Victoria Land, Antarctica	98	*Actinobacteria*
MVH 1A-H10	Uncultured alpha proteobacterium clone D1B11 (EU753663)	Dry stromatolite – Ruidera Pools Natural Park, Spain	97	*Proteobacteria*, *α-proteobacteria*
MVH 1A-A7	Uncultured alpha proteobacterium clone E04_ELL01 (220106)	Non-vegetated soil – Ellsworth Mountains, Antarctica	99	*Proteobacteria*, *α-proteobacteria*
MVH 1A-B5	Uncultured bacterium clone: SRRT51 (AB240484)	Root tips – Japan	96	*Bacteria*, environmental samples
MVH 1A-F3	Uncultured bacterium clone d1-32 (AM409908)	Sediment – Lake Kinneret, Israel	96	*Bacteria*, environmental samples
MVH 1A-G10	Uncultured bacterium clone S011D (AM158337)	Rhizosphere in constructed wetlands – Spain	95	*Bacteria*, environmental samples
MVH 1A-H7	Uncultured bacterium clone 1-5A (EU289438)	Endophytes or symbionts enriched from stem bark – China	95	*Bacteria*, environmental samples
MVH 1A-H8	Uncultured bacterium clone 1-9B (EU289463)	Endophytes or symbionts enriched from stem bark – China	96	*Bacteria*, environmental samples
MVH 1A-D8	Uncultured bacterium clone 2uD_F04 (EU627940)	Water column near fish pens	82	*Bacteria*, environmental samples

Table 5.1 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 1A-G7	Uncultured bacterium clone 33H1 (EU676411)	Heavy metal contaminated soil	96	*Bacteria*, environmental samples
MVH 1A-D4	Uncultured bacterium clone BIhii45 (AJ318145)	Waste-gas biofilter, Germany	97	*Bacteria*, environmental samples
MVH 1A-E7	Uncultured bacterium clone BJ-169 (EU043224)	Uncultivated soil	96	*Bacteria*, environmental samples
MVH 1A-H6	Uncultured bacterium clone FFCH10699 (EU133698)	Soil from undisturbed prairie preserve – USA	87	*Bacteria*, environmental samples
MVH 1A-E5	Uncultured bacterium clone FFCH11085 (EU132685)	Soil from undisturbed prairie preserve – USA	97	*Bacteria*, environmental samples
MVH 1A-H4	Uncultured bacterium clone H6-B65 (FJ207094)	Microbial mat – Yellowstone National Park, Hillside Springs, USA	93	*Bacteria*, environmental samples
MVH 1A-H1	Uncultured bacterium clone JSC8-B11(DQ532226)	Johnson Space Centre – USA	98	*Bacteria*, environmental samples
MVH 1A-B10	Uncultured bacterium clone JulR-B69 (FJ206534)	Biofilm – hotspring run-off, Yellowstone National Park, USA	89	*Bacteria*, environmental samples
MVH 1A-G5	Uncultured bacterium clone LOXB-a02 (EU869573)	Sediment – Onyx River, Wright Valley, Victoria land, Antarctica	99	*Bacteria*, environmental samples
MVH 1A-F8	Uncultured bacterium clone LVH4-D9B (EF465023)	High productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	99	*Bacteria*, environmental samples
MVH 1A-G11	Uncultured bacterium clone LVH4-G2B (EF465030)	High productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	97	*Bacteria*, environmental samples
MVH 1A-D7	Uncultured bacterium clone LVL3-E1 (EF464867)	Low productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	96	*Bacteria*, environmental samples
MVH 1A-H2	Uncultured bacterium clone MeCl 62 (AY439192)	Soil	98	*Bacteria*, environmental samples
MVH 1A-F2	Uncultured bacterium clone N1512_68 (EU104236)	Activated sludge – New Zealand	96	*Bacteria*, environmental samples

Table 5.1 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 1A-E2 MVH 1A-E3	Uncultured bacterium clone ORSFC2_b08 (EF393416)	Sediments – Ohio River	96 96	*Bacteria*, environmental samples
MVH 1A-F11	Uncultured bacterium clone P3T_039 (EF552005)	Sulphate-reducing bioreactor treating mine drainage	94	*Bacteria*, environmental samples
MVH 1A-C4	Uncultured bacterium clone Pia-s-47 (EF632946)	Freshwater sediment – Chile	96	*Bacteria*, environmental samples
MVH 1A-A9	Uncultured bacterium clone sbrh_19 (FJ174988)	Soil rhizosphere - China	93	*Bacteria*, environmental samples
MVH 1A-D11	Uncultured bacterium GKS2-77 (AJ290040)	Lake Gossenkoellesee, Austria	99	*Proteobacteria*, *β-proteobacteria*
MVH 1A-C1	Uncultured bacterium partial isolate BF0002B067 (AM697117)	Indoor dust – Finland	96	*Bacteria*, environmental samples
MVH 1A-A6	Uncultured *Bacteroidetes* bacterium clone FB-1_E04 (EF220240)	Non-vegetated soil – Fossil Bluff, Antarctica	97	*Bacteroidetes*
MVH 1A-B6	Uncultured *Caldilineaceae* bacterium clone CL5.H57 (FM176315)	Rivulet – Hartz Mountain, Germany	91	*Chloroflexi*
MVH 1A-H5	Uncultured *Carnobacterium* sp. clone Hg5-30 (EU344940)	Larval gut	97	*Firmicutes*
MVH 1A-B4 MVH 1A-C7	Uncultured *Chloroflexus* sp. clone Dolo_07 (AB257633)	Endolith – Alps	97 96	*Chloroflexi*
MVH 1A-C6 MVH 1A-F9	Uncultured cyanobacterium clone FBP290 (AY250874)	Lichen-dominated cryptoendolith – McMurdo Dry Valleys, Southern Victoria land, Antarctica	85 96	*Cyanobacteria*
MVH 1A-H3	Uncultured cyanobacterium clone HAVOmat106 (EF032780)	Cyanobacterial mat – lava cave in Hawaii Volcanoes National Park	94	*Cyanobacteria*
MVH 1A-D1 MVH 1A-F6	Uncultured cyanobacterium clone H-B02 (DQ181686)	Microbial mat – Lake Heart, Larsemann Hills, East Antarctica	95 99	*Cyanobacteria*
MVH 1A-A10 MVH 1A-B11 MVH 1A-D5 MVH 1A-E4	Uncultured cyanobacterium clone R8-R13 (DQ181690)	Microbial mat – Lake Rauer, Rauer Islands, East Antarctica	96 96 96 97	*Cyanobacteria*
MVH 1A-A11 MVH 1A-B2 MVH 1A-C9	Uncultured cyanobacterium clone TAF-B22 (AY038732)	Epilithon – River Taff, UK	96	*Cyanobacteria*
MVH 1A-E1	Uncultured endolithic bacterium clone: 2B_WNS (AB374367)	Endolith – Alps	94	*Bacteria*, environmental samples

Table 5.1 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 1A-F4 MVH 1A-F7	Uncultured endolithic bacterium clone: 3B_WNS (AB374368)	Endolith – Alps	94 95	*Bacteria*, environmental samples
MVH 1A-A3	Uncultured gamma proteobacterium clone Dolo_15 (AB257640)	Endolith – Alps	97	*Proteobacteria*, *γ-proteobacteria*
MVH 1A-G8	Uncultured microorganism isolate SeaGull4 (EU181009)	Field seagull fecal sample – USA	88	Unclassified sequences, environmental samples
MVH 1A-D12	Uncultured proteobacterium clone 351F (AY571836)	Hydrocarbon-contaminated soil – Southern Victoria Land, Ross Island, Antarctica	98	*Proteobacteria*
MVH 1A-G6	Uncultured soil bacterium clone 2_H11 (EU589307)	Soil from rice paddy field	96	*Bacteria*, environmental samples
MVH 1A-E11	Uncultured soil bacterium clone 31A_MJK (EF540359)	Soil – Estonia	98	*Bacteria*, environmental samples
MVH 1A-D3	Uncultured soil bacterium clone C08_bac_con (EU861839)	Dry meadow soil, USA	98	*Bacteria*, environmental samples
MVH 1A-E12	Uncultured soil bacterium clone TA11 (DQ248298)	Carbon tetrachloride-contaminated soil – USA	94	*Bacteria*, environmental samples

Due to advancements in sequencing technology and the increased use of culture-independent methods in diversity studies, the GenBank database currently contains a large proportion of sequences that have not been assigned to a phylogenetic group. It is therefore not surprising that the majority of sequences from the 16S rRNA gene clone library (Table 5.1) can only be assigned as Bacteria, environmental samples. However, some conclusions and comparisons can be made using those sequences that have been assigned a phylogenetic group.

As predicted (based on field observations) the majority of the cloned sequences in the Type I hypolithic community 16S rRNA gene clone library belong to the phylum *Cyanobacteria*. Two orders of cyanobacteria were identified, *Nostocales* (*Nostoc* sp.) and *Oscillatoriales* (*Leptolyngbya* sp.), which together comprise approximately 45% of the cyanobacterial sequences detected. Wood *et al.* (2008) found that hypolithic communities in the Miers Valley contain multiple cyanobacterial species, with a clear

difference in cyanobacterial community structure amongst hypoliths. Furthermore, hypolithic cyanobacterial diversity was not significantly different from that of the lake and hydroterrestrial cyanobacterial mats in the region. This suggests that hypolithic cyanobacterial communities may be derivations of mat communities that find a suitable niche under quartz rocks.

Members of the *α*-, *β*- and *γ-Proteobacteria* were detected in the Type I 16S rRNA gene clone library. Members of *Bacteroidetes*, *Actinobacteria* and *Chloroflexi* were represented in equal abundance. A single representative of the *Firmicutes* was also detected.

Table 5.2 presents the top blastn matches for sequences obtained from the Type II bacterial 16S rRNA gene clone library.

Table 5.2: Sequences obtained from a Type II hypolithic community 16S rRNA gene clone library

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 3-1A-D3	*Bacteroidetes* bacterium P3 (DQ351728)	Soil – La Gorce Mountains, Antarctica	94	*Bacteroidetes*
MVH 3-1A-D8	*Ehrlichia* sp. Belluno (AY098730)	Italy	77	*Proteobacteria*, *α-proteobacteria*
MVH 3-1A-C7	*Flexibacteraceae* bacterium VUG-A141a (EU155016)	Glacial ice – Victoria Upper Glacier, Antarctica	97	*Bacteroidetes*
MVH 3-1A-A9	*Nocardioides* sp. (X94145)	Echo Lake, Vestfold Hills, Antarctica	97	*Actinobacteria*
MVH 3-1A-E7	Rhizosphere soil bacterium isolate RSI-21 (AJ252588)	Rhizosphere – Germany	97	*Bacteria*, environmental samples
MVH 3-1A-G6	Rhizosphere soil bacterium isolate RSI-21 (AJ252588)	Rhizosphere – Germany	99	*Bacteria*, environmental samples
MVH 3-1A-G12	Uncultured *Actinobacteria* bacterium clone AKYH1301 (AY921795)	Farm soil – USA	96	*Actinobacteria*
MVH 3-1A-D10	Uncultured actinobacterium (DQ366003)	Soil – Victoria Land, Antarctica	97	*Actinobacteria*
MVH 3-1A-E10	Uncultured actinobacterium (DQ366008)	Soil – Victoria Land, Antarctica	99	*Actinobacteria*

Table 5.2 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 3-1A-E12	Uncultured actinobacterium clone C10_MO03 (EF220795)	Non-vegetated soil – Mars Oasis, Antarctica	93	*Actinobacteria*
MVH 3-1A-H6	Uncultured actinobacterium clone D3A06 (EU753660)	Dry stromatolite – Ruidera Pools Natural Park, Spain	99	*Actinobacteria*
MVH 3-1A-B11	Uncultured actinobacterium clone FB-2_F04 (EF220342)	Non-vegetated soil – Fossil Bluff, Antarctica	97	*Actinobacteria*
MVH 3-1A-F9	Uncultured actinobacterium clone FBP234 (AY250866)	Lichen-dominated cryptoendolithic community – McMurdo Dry Valleys, Southern Victoria Land, Antarctica	93	*Actinobacteria*
MVH 3-1A-G5	Uncultured actinobacterium clone FI-2F_F04 (EF220433)	Non-vegetated soil – Falkland Islands	92	*Actinobacteria*
MVH 3-1A-B5	Uncultured actinobacterium clone: CH-18 (AB293383)	Anoxic rice field soil – Japan	98	*Actinobacteria*
MVH 3-1A-G4	Uncultured alpha proteobacterium clone g52 (EU979061)	Soil – China	99	*Proteobacteria*, *α-proteobacteria*
MVH 3-1A-C9	Uncultured alpha proteobacterium clone: Dolo_08 (AB257637)	Endolith – Swiss Alps	98	*Proteobacteria*, *α-proteobacteria*
MVH 3-1A-D5	Uncultured bacterium clone Rhag2-21 (AM940399)	Digestion system of Rhagium inquisitor larvae – Germany	94	*Bacteria*, environmental samples
MVH 3-1A-D12	Uncultured bacterium clone AYRV1-015 (DQ990929)	Rock varnish – Atacama Desert, Yungay	95	*Bacteria*, environmental samples
MVH 3-1A-F2	Uncultured bacterium clone FFCH2856 (EU135131)	Soil from undisturbed prairie preserve – USA	97	*Bacteria*, environmental samples
MVH 3-1A-E3	Uncultured bacterium clone FFCH7867 (EU135073)	Soil from undisturbed prairie preserve – USA	93	*Bacteria*, environmental samples
MVH 3-1A-H11	Uncultured bacterium clone JSC7-58 (DQ532203)	Johnson Space Center - USA	95	*Bacteria*, environmental samples
MVH 3-1A-G2	Uncultured bacterium clone JSC8-B11 (DQ532226)	Johnson Space Center – USA	98	*Bacteria*, environmental samples

Table 5.2 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 3-1A-D9	Uncultured bacterium clone LOXB-a06 (EU869575)	Sediment – Onyx River, Wright Valley, Victoria Land, Antarctica	97	*Bacteria*, environmental samples
MVH 3-1A-G10	Uncultured bacterium clone LVL3-H6B (EF464909)	Low productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land	99	*Bacteria*, environmental samples
MVH 3-1A-A6	Uncultured bacterium clone ORSFC2_d03 (EF393423)	Sediment – Ohio River, USA	98	*Bacteria*, environmental samples
MVH 3-1A-A10	Uncultured bacterium clone PH10-42 (DQ444079)	Sediment – Songhuajiang River	95	*Bacteria*, environmental samples
MVH 3-1A-C11	Uncultured bacterium clone TfC20L19 (EU362289)	Sediment – tidal flats, Germany	98	*Bacteria*, environmental samples
MVH 3-1A-H1	Uncultured bacterium clone UOXB-b04 (EU869693)	Sediment – Onyx River, Wright Valley, Victoria Land, Antarctica	99	*Bacteria*, environmental samples
MVH 3-1A-F6	Uncultured bacterium clone UOXB-h09 (EU869714)	Sediment – Onyx River, Wright Valley, Victoria Land, Antarctica	97	*Bacteria*, environmental samples
MVH 3-1A-H4	Uncultured bacterium clone UOXC-h04 (EU869725)	Sediment – Onyx River, Wright Valley, Victoria Land, Antarctica	98	*Bacteria*, environmental samples
MVH 3-1A-F8	Uncultured bacterium isolate N93-A05 (EU440515)	Calcrete – Haughton impact crater, Devon Island, Canada	91	*Bacteria*, environmental samples
MVH 3-1A-E1	Uncultured bacterium isolate N93-D08 (EU440517)	Calcrete – Haughton impact crater, Devon Island, Canada	96	*Bacteria*, environmental samples
MVH 3-1A-B4	Uncultured *Bacteroidetes* bacterium clone LF028 (EF417717)	Soil – China	95	*Bacteroidetes*
MVH 3-1A-F11	Uncultured *Chloroflexi* bacterium clone AKYG1672 (AY921843)	Farm soil – USA	98	*Chloroflexi*
MVH 3-1A-D4	Uncultured endolithic bacterium clone: NB16-WNS (AB374391)	Endolith – Swiss Alps	89	*Bacteria*, environmental samples
MVH 3-1A-H2	Uncultured *Flavobacteria* bacterium clone AUVE_11A10 (EF651408)	Crop land – Australia	98	*Bacteroidetes*

Table 5.2 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 3-1A-C4	Uncultured *Gemmatimonadetes* bacterium clone g45 (EU979054)	Soil – China	98	*Gemmatimonadetes*
MVH 3-1A-C3	Uncultured gold mine bacterium D12 (AF337866)	Gold mine – USA	97	*Bacteria*, environmental samples
MVH 3-1A-H8	Uncultured *Hymenobacter* group bacterium clone KL-27-1-11 (AF408277)	'Clean-room' facilities - USA	91	*Bacteroidetes*
MVH 3-1A-G8	Uncultured organism clone DLE054 (EF127617)	Glacial ice - Antarctica	99	Unclassified sequences, environmental samples
MVH 3-1A-A7	Uncultured planctomycete clone Amb_16S_1870 (EF019196)	Rhizosphere – USA	94	*Planctomycetes*
MVH 3-1A-F12	Uncultured planctomycete clone LF100 (EF417789)	Soil – China	88	*Planctomycetes*
MVH 3-1A-E6	Uncultured proteobacterium clone Amb_16S_460 (EF018126)	Rhizosphere – USA	94	*Proteobacteria*
MVH 3-1A-A3	Uncultured *Pseudonocardia* sp. clone 343G (AY571815)	Hydrocarbon-contaminated soil – Southern Victoria Land, Ross Island, Antarctica	99	*Actinobacteria*
MVH 3-1A-A4	Uncultured *Pseudonocardia* sp. Clone 343G (AY571815)	Hydrocarbon-contaminated soil – Southern Victoria Land, Ross Island, Antarctica	99	*Actinobacteria*
MVH 3-1A-F5	Uncultured *Pseudonocardia* sp. Clone 343G (AY571815)	Hydrocarbon-contaminated soil – Southern Victoria Land, Ross Island, Antarctica	99	*Actinobacteria*
MVH 3-1A-H7	Uncultured *Pseudonocardia* sp. Clone 343G (AY571815)	Hydrocarbon-contaminated soils – Southern Victoria Land, Ross Island, Antarctica	99	*Actinobacteria*
MVH 3-1A-E4	Uncultured soil bacterium clone 2_H11 (EU589307)	Rice paddy soil – China	96	*Bacteria*, environmental samples

Table 5.2 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 3-1A-A1	Uncultured soil bacterium clone bac2nit10 (EU861933)	Nitrogen amended meadow surface soil – Colorada, USA	99	*Bacteria*, environmental samples
MVH 3-1A-A8	Uncultured soil bacterium clone bac2nit10 (EU861933)	Nitrogen amended meadow surface soil – Colorada, USA	99	*Bacteria*, environmental samples
MVH 3-1A-C12	Uncultured soil bacterium clone bac2nit10 (EU861933)	Nitrogen amended meadow surface soil – Colorada, USA	99	*Bacteria*, environmental samples
MVH 3-1A-D1	Uncultured soil bacterium clone bac2nit10 (EU861933)	Nitrogen amended meadow surface soil – Colorada, USA	98	*Bacteria*, environmental samples
MVH 3-1A-D2	Uncultured soil bacterium clone bac2nit10 (EU861933)	Nitrogen amended meadow surface soil – Colorada, USA	99	*Bacteria*, environmental samples
MVH 3-1A-E8	Uncultured soil bacterium clone bac2nit10 (EU861933)	Nitrogen amended meadow surface soil – Colorada, USA	99	*Bacteria*, environmental samples
MVH 3-1A-F7	Uncultured soil bacterium clone bac2nit10 (EU861933)	Nitrogen amended meadow surface soil – Colorada, USA	99	*Bacteria*, environmental samples
MVH 3-1A-G3	Uncultured soil bacterium clone bac2nit10 (EU861933)	Nitrogen amended meadow surface soil – Colorada, USA	99	*Bacteria*, environmental samples
MVH 3-1A-A11	Uncultured soil bacterium clone H02_bac_con (EU861867)	Meadow surface soil – Colorada, USA	96	*Bacteria*, environmental samples
MVH 3-1A-B6	Uncultured soil bacterium clone M06_Pitesti (DQ378228)	Oil-polluted soil – Romania	96	*Bacteria*, environmental samples
MVH 3-1A-H10	Unidentified bacterium clone FB-2_B09 (EF220302)	Non-vegetated soil – Fossil Bluff, Antarctica	91	*Bacteria*, environmental samples

As with the Type I library, the majority of the sequences obtained could be assigned to the domain *Bacteria* with no further phylogenetic resolution. The top Blast results were often similar to sequences obtained from similar environmental phylogenetic studies of soil systems. The assigned sequences were dominated by the *Actinobacteria*, followed by almost equal abundance of representatives of *Bacteroidetes* and *Proteobacteria*. Low abundance of *Planctomycetes* and *Chloroflexi* were detected. Unlike the Type I library, where cyanobacteria were the major community components, cyanobacteria were not detected in the Type II moss-dominated community. This could imply that the moss species present out-compete cyanobacteria for the role of dominant primary producers in Type II hypolithic communities, therefore cyanobacterial abundance is decreased.

Culture-dependent analysis of hypolithic communities from the Vestfold Hills found three major bacterial groups: *Proteobacteria* (*α* and *γ* sub-divisions), *Cytophagales* and *Actinobacteria* (Smith *et al.*, 2000). While the order Cytophagales was not detected in the Miers Valley hypolithic communities, this group appears distantly related to the detected Bacteroidetes group and together these may constitute a phylum in the bacterial phylogenetic system (Reichenbach, 2006). *Proteobacteria* and *Actinobacteria* were also detected in 16S rRNA gene clone libraries of Miers Valley hypolithic communities constructed during this study.

16S rRNA gene clone libraries from two hypolithic communities in the Vestfold Hills, Antarctica predominantly consisted of cyanobacteria phylogenetically related to filamentous and *Chroococcidiopsis*-like cyanobacteria (Smith *et al.*, 2000). Other bacterial clones clustered with the *α*- and *γ*-*Proteobacteria*, the order *Cytophagales*, order *Planctomycetales* and *Actinobacteria*. While the hypolithic communities in the Vestfold Hills consist of mainly oscillatorian cyanobacteria with morphology typical of the *Lyngbya/Phormidium/Plectonema* group and of spherical *Chroococcidiopsis*-like cells, this study found that cyanobacteria were only dominant in Type I hypolithic communities with the majority belonging to the orders *Nostocales* and *Oscillatoriales*.

Sixty recombinant clones were selected from the non-translucent rock and open soil community 16S rRNA gene clone libraries and sequenced using the vector primers (M13fw and M13rev, Table 2.1). Sequences less than 500 bp were eliminated, resulting in fifty-four sequences for the non-translucent rock community and twenty-seven sequences for the open soil community. These sequences were analysed by the blastn function. Table 5.3 and Table 5.4 present the nearest match of the cloned sequence with the % identity to the top blastn result as well as the phylogenetic grouping.

Table 5.3: Sequences obtained from a non-translucent rock community 16S rRNA gene clone library

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH1B-C6B	Actinobacterium P2 (FM176595)	Soil – La Gorce Mountains, Antarctica	97	*Actinobacteria*
MVH1B-E3B MVH1B-A12B MVH1B-A6B	Bacterium G2DM-1(DQ416803)	Landfill site – India	93 93 93	*Bacteria*, environmental samples
MVH1B-D8 MVH1B-B5B	*Chamaesiphon subglobosus* PCC 7430 (AY170472)	Sediment – Onyx River, Wright Valley, Victoria Land, Antarctica	99 98	*Cyanobacteria*, *Chroococcales*
MVH1B-A5	*Flexibacteraceae* bacterium VUG-A124 (EU155015)	Glacial ice – Victoria Upper Glacier, Antarctica	98	*Bacteroidetes*
MVH1B-H10	*Flexibacteraceae* bacterium VUG-A141A (EU155016)	Glacial ice – Victoria Upper Glacier, Antarctica	93	*Bacteroidetes*
MVH1B-A3	*Flexibacteraceae* bacterium VUG-A142 (EU155017)	Glacial ice – Victoria Upper Glacier, Antarctica	100	*Bacteroidetes*
MVH1B-A5B MVH1B-D11B	*Janibacter* sp. strain IV-75 (FM179321)	Ultra pure water - Hungary	93 98	*Actinobacteria*
MVH1B-B12B	*Modestobacter* sp. CNJ794 PL04 (DQ448774)	Marine sediment - Palau	94	*Actinobacteria*
MVH1B-H8	*Nocardioides* sp. (X94145)	Isolate – Ekho Lake, Vestfold Hills, Antarctica	96	*Actinobacteria*
MVH1B-G8	*Planococcus* sp. JG07 (AF144750)	Isolate	86	*Firmicutes*
MVH1B-A2B	*Rubrobacter radiotolerans* (U65647)	Isolate	94	*Actinobacteria*

Table 5.3 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH1B-A11B	Uncultured *Acidobacteria* bacterium clone AKYH1176 (AY921881)	Farm soil - USA	98	*Acidobacteria*
MVH1B-B2	Uncultured *Acidobacteria* bacterium clone KL2-036 (EU122726)	TNT-contaminated soil – France	96	*Acidobacteria*
MVH1B-A10B	Uncultured actinobacterium clone F14_1G_FL (EF683029)	Atmosphere – Eastern Mediterranean	93	*Actinobacteria*
MVH1B-A7B MVH1B-B7B	Uncultured actinobacterium clone FI-2F_A07 (EF220380)	Non-vegetated soils – Falkland Islands	96 97	*Actinobacteria*
MVH1B-G3	Uncultured bacterium clone: BS144 (AB240274)	Soil, reed bed reactor	94	*Bacteria*, environmental samples
MVH1B-A3B MVH1B-A4B	Uncultured bacterium clone A15 (AM746693)	Mural - Italy	93 92	*Bacteria*, environmental samples
MVH1B-C12B	Uncultured bacterium clone 2005-WD-45-081407 (EU262373)	Sediment – Illinois River	97	*Bacteria*, environmental samples
MVH1B-B4B	Uncultured bacterium clone Atacama-colB1 (EF071505)	Atacama Desert	94	*Bacteria*, environmental samples
MVH1B-E1B	Uncultured bacterium clone JH-WHS131 (EF492942)	Soil - China	93	*Bacteria*, environmental samples
MVH1B-C5B	Uncultured bacterium clone JSC2-A6 (DQ532167)	Johnson Space Centre - USA	95	*Bacteria*, environmental samples
MVH1B-B8	Uncultured bacterium clone KIS.T91 (EU030494)	Sediment – West Antarctic ice sheet, Antarctica	97	*Bacteria*, environmental samples
MVH1B-F4	Uncultured bacterium clone LOXA-c10 (EU869550)	Sediment – Onyx River, Wright Valley, Victoria Land, Antarctica	98	*Bacteria*, environmental samples
MVH1B-B2B	Uncultured bacterium clone LVH3-A11 (EF464924)	High productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	87	*Bacteria*, environmental samples
MVH1B-E2B	Uncultured bacterium clone LVH3-H2 (EF464996)	High productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	99	*Bacteria*, environmental samples

Table 5.3 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH1B-B1B	Uncultured bacterium clone LVL3-F6 (EF464901)	Low productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	98	*Bacteria*, environmental samples
MVH1B-C4B MVH1B-E12B MVH1B-H4B	Uncultured bacterium clone LVL3-H11B (EF464913)	Low productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	99 99 99	*Bacteria*, environmental samples
MVH1B-B3B MVH1B-B9B	Uncultured bacterium clone LVL3-H6B (EF464909)	Low productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	99 99	*Bacteria*, environmental samples
MVH1B-C1B	Uncultured bacterium clone N74-I06 (EU883192)	Calcrete – Haughton impact crater, Devon Island, Canada	97	*Bacteria*, environmental samples
MVH1B-E12	Uncultured bacterium clone UOXB-c10 (EU869700)	Sediment – Onyx River, Wright Valley, Victoria Land, Antarctica	98	*Bacteria*, environmental samples
MVH1B-F8	Uncultured *Bacteroidetes* bacterium clone AKYG614 (AY922040)	Farm soil - USA	95	*Bacteroidetes*
MVH1B-E10B	Uncultured *Bacteroidetes* bacterium clone F15_8C_FL (EF683049)	Atmosphere – Eastern Mediterranean	93	*Bacteroidetes*
MVH1B-C2B	Uncultured *Bacteroidetes* bacterium clone J35H2 (DQ365987)	Soil – Victoria Land, Antarctica	95	*Bacteroidetes*
MVH1B-A2 MVH1B-C9	Uncultured cyanobacterium clone CL5.H402 (FM176595)	Rivulet – Germany	97 97	*Cyanobacteria*
MVH1B-D3	Uncultured cyanobacterium clone SSB-70 16S (FJ028692)	Sandstone microbial biofilm - Spain	98	*Cyanobacteria*
MVH1B-D2B	Uncultured *Gemmatimonadetes* bacterium clone g45 (EU979054)	Soil - China	98	*Gemmatimonadetes*
MVH1B-D5B	Uncultured planctomycete YNPRH1A (AF465649)	Environmental sample	94	*Planctomycetes*
MVH1B-B6B MVH1B-B11B	Uncultured *Pseudonocardia* sp. clone 343G (AY571815)	Hydrocarbon-contaminated soil, Ross Island, Southern Victoria Land, Antarctica	98 99	*Actinobacteria*

Table 5.3 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH1B-A8B MVH1B-B10B	Uncultured soil bacterium clone UE8 (DQ248230)	Carbon-tetrachloride-contaminated soil	97 96	*Bacteria*, environmental samples
MVH1B-F9B	Uncultured soil bacterium clone W4Ba36 (DQ643700)	Agricultural soil	93	*Bacteria*, environmental samples
MVH1B-F12B	Uncultured soil bacterium clone W4Ba40 (DQ643704)	Agricultural soil	99	*Bacteria*, environmental samples
MVH1B-F10B	Unidentified eubacterium clone GKS69 (AJ224989)	Isolate – Lake Gossenkoellesee	99	*Bacteria*, environmental samples

The assigned sequences in the non-translucent rock community 16S rRNA gene clone library are almost equally dominated by representatives of the *Actinobacteria* and *Bacteroidetes*. Other community members include the *Acidobacteria*, *Planctomycetes*, *Gemmatimonadetes* and *Cyanobacteria*. Interestingly, the one cyanobacterial sequence assigned to a phylogenetic group (*Chroococcales*) was not detected in the Type I 16S rRNA gene clone library.

Table 5.4 presents the sequence identities of clones from the open soil community 16S rRNA gene clone library.

Table 5.4: Sequences obtained from an open soil community 16S rRNA gene clone library

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 1C-H7B	*Kineococcus*-like bacterium AS3187 (AF060689)	Mojave Desert	94	*Actinobacteria*
MVH 1C-B4B	Uncultured *Acidobacteria* bacterium clone 32d1 (AY281353)	Soil	99	*Acidobacteria*
MVH 1C-B2B	Uncultured *Acidobacteria* bacterium clone 354C (AY571797)	Hydrocarbon-contaminated soil – Ross Island, Southern Victoria Land, Antarctica	73	*Acidobacteria*
MVH 1C-A9B	Uncultured *Actinobacteria* bacterium clone AKYH854 (AY922162)	Farm soil - USA	95	*Actinobacteria*

Table 5.4 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 1C-C5B	Uncultured actinobacterium clone C08_ELL01 (EF220068)	Non-vegetated soil – Ellsworth Mountains, Antarctica	99	*Actinobacteria*
MVH 1C-D10	Uncultured actinobacterium clone D02_MO03(EF220803)	Non-vegetated soil – Mars Oasis, Antarctica	95	*Actinobacteria*
MVH 1C-H11B	Uncultured actinobacterium clone FB-2_H09 (EF220368)	Non-vegetated soil – Fossil Bluff, Antarctica	99	*Actinobacteria*
MVH 1C-E7B	Uncultured bacterium clone 1969b-35 (AY917754)	Volcanic deposit – Hawaii, USA	87	*Bacteria*, environmental samples
MVH 1C-H11	Uncultured bacterium clone FFCH12132 (EU132747)	Soil – undisturbed prairie preserve, USA	92	*Bacteria*, environmental samples
MVH 1C-F11B	Uncultured bacterium clone LVH3-A7 (EF464920)	High productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	93	*Bacteria*, environmental samples
MVH 1C-G3	Uncultured bacterium clone LVL3-H11B (EF464913)	Low productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	98	*Bacteria*, environmental samples
MVH 1C-F3	Uncultured bacterium clone LVL3-H6B (EF464909)	Low productivity soil – Luther Vale, Admiralty Range, Northern Victoria Land, Antarctica	99	*Bacteria*, environmental samples
MVH 1C-F1	Uncultured bacterium clone N74-G03 (EU883182)	Calcrete – Haughton impact crater, Devon Island, Canada	97	*Bacteria*, environmental samples
MVH 1C-B9	Uncultured bacterium clone SAV05F04 (EU542270)	Pondweed leaves – Chesapeake Bay, USA	87	*Bacteria*, environmental samples
MVH 1C-D8B	Uncultured bacterium clone TfC20L19 (EU362289)	Sediment – tidal flats, Germany	98	*Bacteria*, environmental samples

Table 5.4 continued

Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
MVH 1C-E2B	Uncultured bacterium clone UOXB-b04 (EU869693)	Sediment – Onyx River, Wright Valley, Victoria Land, Antarctica	94	*Bacteria*, environmental samples
MVH 1C-C7B MVH 1C-D2B	Uncultured bacterium isolate N93-B08 (EU440476)	Calcrete – Haughton impact crater, Devon Island, Canada	92	*Bacteria*, environmental samples
MVH 1C-F9	Uncultured beta proteobacterium clone G05_SGPL01 (EF221214)	Vegetated soil – South Georgia, Antarctica	98	*Proteobacteria, β-Proteobacteria*
MVH 1C-E8	Uncultured *Brevundimonas* sp. clone: 14-91-ArvAB (AB425063)	Freshwater stones biofilm – Swiss Alps	98	*Proteobacteria*, *α-Proteobacteria*
MVH 1C-G10	Uncultured *Brevundimonas* sp. clone: 3-90-ArvAB (AB425055)	Freshwater stones biofilm – Swiss Alps	96	*Proteobacteria, α-Proteobacteria*
MVH 1C-C4B	Uncultured *Chloroflexi* bacterium clone g16 (EU979025)	Soil - China	94	*Chloroflexi*
MVH 1C-G9B	Uncultured cyanobacterium clone CL4.E303 (FM176126)	Rivulet – Hartz Mountain, Germany	99	*Cyanobacteria*
MVH 1C-E1	Uncultured *Gemmatimonadetes* bacterium clone AKYG781 (AY921899)	Farm soil - USA	96	*Gemmatimonadetes*
MVH 1C-G7B	Uncultured *Gemmatimonadetes* bacterium clone g45 (EU979054)	Soil - China	99	*Gemmatimonadetes*
MVH 1C-H3	Uncultured organism clone DLE100 (EF127619)	Glacial ice - Antarctica	98	Unclassified sequences, environmental samples
MVH 1C-D6B	Uncultured *Verrucomicrobia* bacterium clone D07-1 (FM253596)	Biofilm - Poland	97	*Verrucomicrobia*

The open soil 16S rRNA gene clone library (Table 5.4) was dominated by the *Actinobacteria*, followed by the *Proteobacteria*. There was a similar abundance of *Cyanobacteria*, *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes* and *Verrucomicrobia* detected. Interestingly, *Verrucomicrobia* were exclusively detected in the open soil 16S rRNA gene clone library. *Actinobacteria* were also found to be dominant in a previous study of Miers Valley soils (Smith *et al*., 2006). Furthermore, all the phylotypes detected in the previous study (*Actinobacteria*, *Verrucomicrobia*,

Acidobacteria, *Chloroflexi* and *Bacteroidetes*) (Smith *et al.*, 2006) were again detected in this study. In addition, this study detected the presence of *Gemmatimonadetes* which were not previously detected in Miers Valley soils.

To determine whether the sampling of clones has been exhausted or not, a collectors' curve can be constructed for each library, which plots the total number of clones observed (on the x-axis) against the number of unique clones observed (on the y-axis). The nature of the slope constructed is valuable as it indicates whether the sampling has been exhausted (slope is equal to or close to zero) or whether the sampling is insufficient (slope is rising). A slope close to or equal to zero indicates that further sampling is unlikely to yield additional phylotypes. The collectors' curve below (Figure 5.3) shows that the number of unique clones detected in all bacterial 16S rRNA gene clone libraries is still rising; therefore sampling of clones has not been exhausted. The coverage index (C) of each 16S rRNA gene clone library, calculated by $C = (1 - n/N)x100$ [where n is the number of different sequences represented by one clone and N is the total number of clones (Good, 1953; Taton *et al.*, 2003)] was low: 29.5% for the Type I library, 0% for the Type II library, 41% for the non-translucent rock library and 7.4% for the open soil library.

Clearly, the analysis of a greater number of clones is required in order for the collectors' curve to plateau for all four communities and to increase the library coverage. A clearer understanding of the bacterial community structure and dynamics can only be determined with more intense cloning and sequencing strategies.

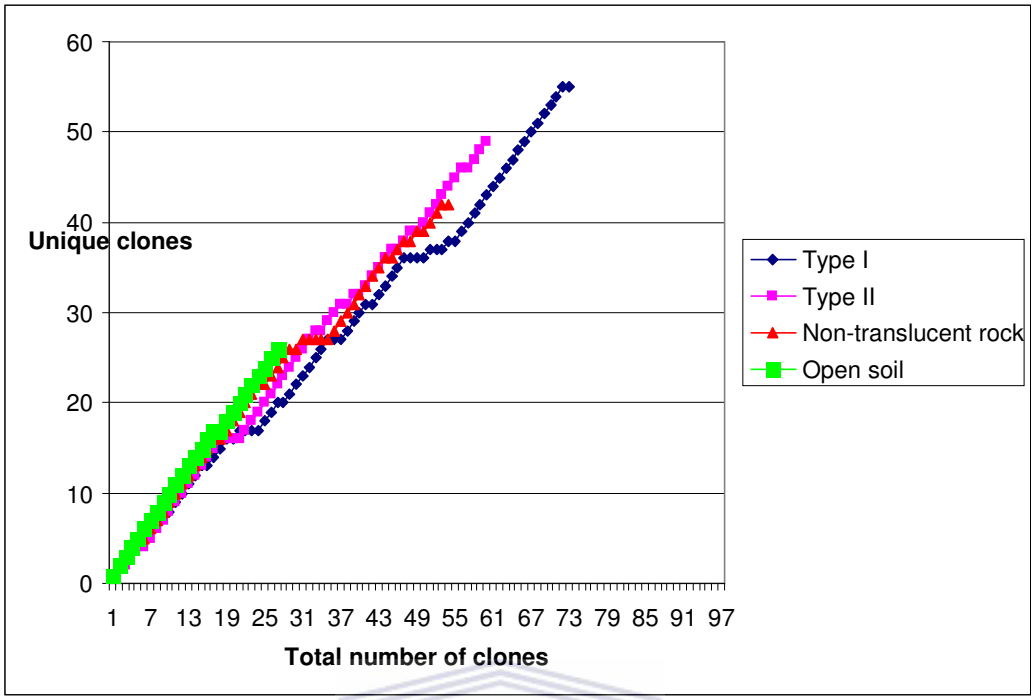

Figure 5.2: Collectors' curves for Type I, Type II, non-translucent rock and open soil community 16S rRNA gene clone libraries.

Although the coverage index was low and the collectors' curves for all the libraries are still increasing (Figure 5.3), differences in sequences recovered can still be detected and some preliminary observations of the community diversity can be made. Figure 5.4 compares the relative percentage distribution of phylotypes identified from the 16S rRNA gene clone libraries constructed and analysed. It is clear that while *Cyanobacteria* are the dominant phylotype in Type I hypolithic communities, they are less abundant in other Dry Valley soil environments, including the Type II hypolithic communities. *Bacteroidetes* are evident in the lithic community libraries and absent in the open soil clone library. While this could be a function of the smaller library from open soil, it may also be a feature of the fact that the *Bacteroidetes* require protection (UV irradiation and/or physical stability against wind mediated desiccation and erosion) and the organic matter from the primary producers as a food source, and therefore were more prominent in the lithic community libraries.

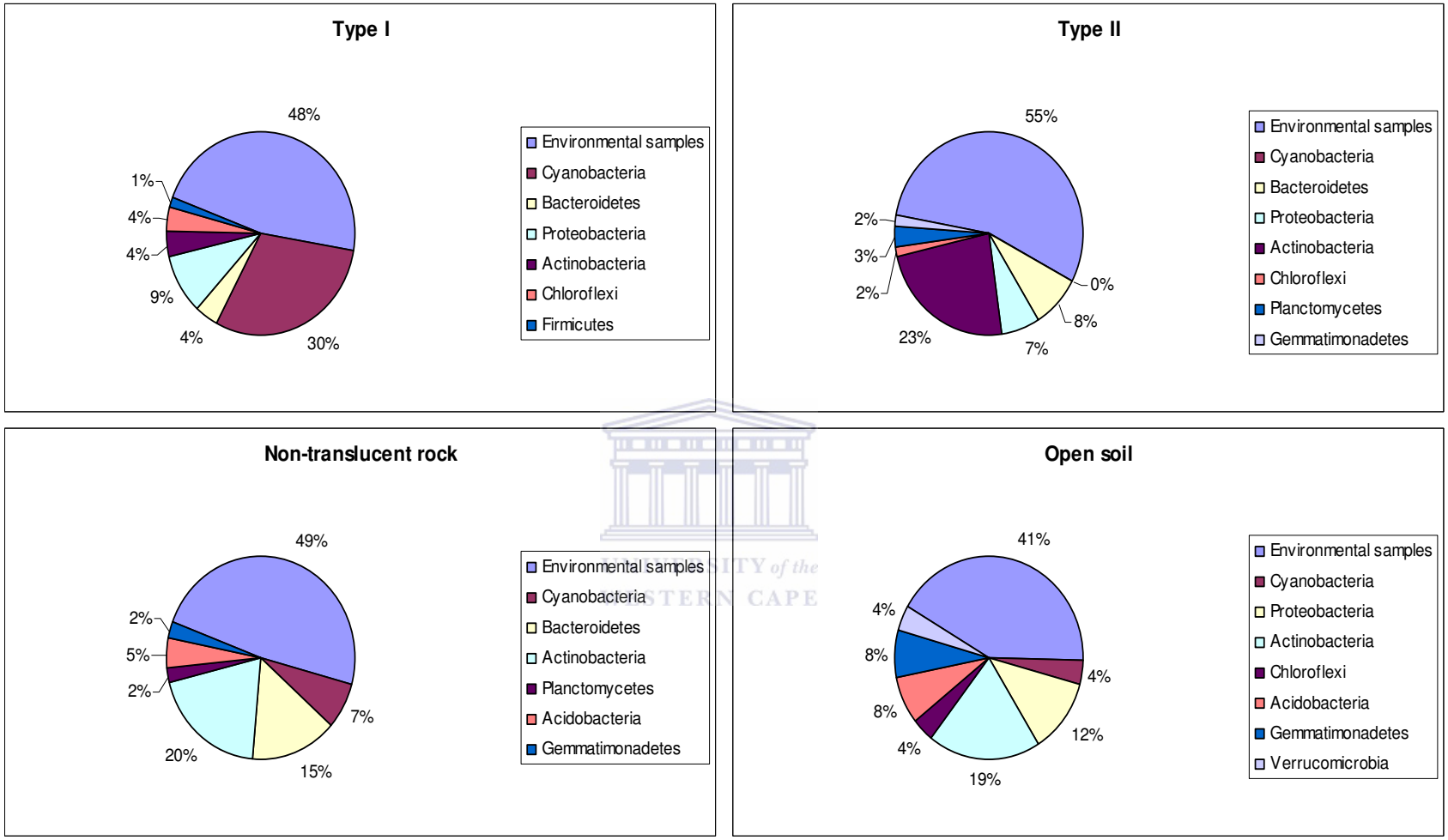

Figure 5.3: Relative percentage distribution of the phylotypes identified from the Type I (73 clones), Type II (60 clones), non-translucent rock (54 clones) and open soil community (27 clones) bacterial 16S rRNA gene clone libraries constructed.

Actinobacteria are as ubiquitous in Dry Valley soil environments (Figure 5.4) as in soil environments globally (Felske *et al.*, 1997; Rheims *et al.*, 1999; Piao *et al.*, 2008). This phylotype forms a major component of all communities aside from the Type I hypolithic community. Smaller, more divergent sub-divisions such as the *Acidimicrobidae* were detected in the Type I hypolithic community library (Table 5.1).

Gemmatimonadetes were detected in all libraries aside from Type I hypolithic communities, while *Verrucomicrobia* were exclusively detected in the open soil community. *Planctomycetes* were only detected in non-translucent rock and Type II hypolithic communities.

Chloroflexi, the major representatives of the filamentous anoxygenic phototrophs, were obtained from hypolithic and open soil communities only. This is perhaps not surprising as these green-pigmented bacteria require solar input to produce energy through photosynthesis which is unavailable beneath non-translucent rocks. *Acidobacteria* were detected in the open soil and non-translucent rock 16S rRNA gene clone libraries. This group of bacteria is found ubiquitously, in various soil environments and little is known about their function.

All four libraries lack representatives from readily culturable taxa such as *Bacillus, Achromobacter, Corynebacterium, Micrococcus, Planococcus* and *Pseudomonas* which were isolated in early culture-dependent studies of Antarctic Dry Valley mineral soils (Cameron *et al.*, 1972). Taxa found ubiquitously in hypolithic communities in the Vestfold Hills included *Pseudomonas, Psychrobacter, Stenotrophomonas, Arthrobacter, Achromobacter, Micrococcus, Rhodococcus, Janibacter* and *Gelidibacter* (Smith *et al.*, 2000). Aside from a single *Janibacter* sp. clone detected in the non-translucent rock community, these taxa were not detected in the clone libraries. However, phylogenetic groups such as *Verrucomicrobia* and *Acidobacteria* that have been detected by molecular methods in this study have not previously been detected by culture-based studies of Dry Valley soils.

5.4 Archaeal-specific 16S rRNA gene clone libraries

Twenty-four random clones from each archaeal-16S rRNA gene clone library were selected and the plasmids isolated by mini-preparations (see Section 2.10.2). PCR with the insert primers (A3fa and Ab927r) used extracted plasmid as template. A nested PCR approach using archaeal-specific 16S rRNA gene DGGE primers (A340F-GC and A533r) followed by DGGE was used for screening the selected clones. PCR products for three clones were pooled and separated out using DGGE (Figure 5.5).

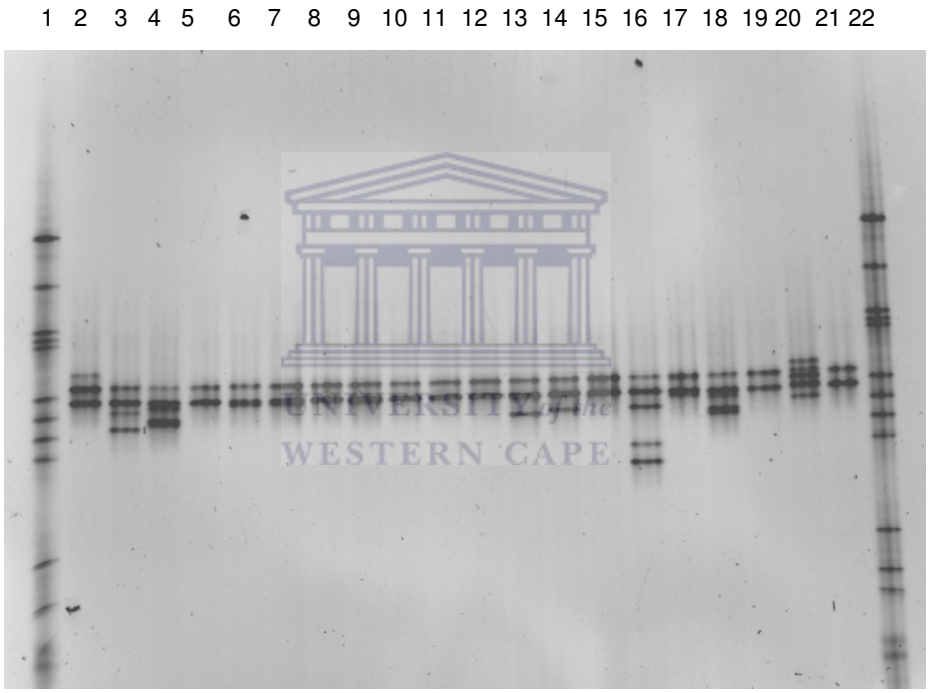

Figure 5.4: DGGE of pooled archaeal-specific PCR products from archaeal 16S rRNA gene clone libraries. Lanes 1 and 22: DGGE marker. Lanes 2-21: 3 pooled DGGE-PCR products from archaeal 16S rRNA gene clones.

In theory, if all three clones contain differences in the sequence, they should appear as three separate bands using DGGE (distinct Rf values). If fewer bands than the number of pooled products are detected using DGGE, this implies that one or more of the sequences are the same. As is evident from Figure 5.5, there are at least two clones that are common in all the samples. Furthermore, although only three clones were pooled, some lanes show more than three bands (Figure 5.5, Lanes 3, 4, 16, 18

and 21). This result can be attributed to the degeneracy of the archaeal-specific DGGE primers used (see Section 2.4.1). The forward primer (A340F-GC) contains two degenerate nucleotides (C/T and C/G) while the reverse primer contains one degenerate site (G/T). Mis-matching due to the degeneracy of the primers would alter the denaturation of the double-stranded DNA and results in two bands representing a single sequence. Table 5.5 lists the eight different archaeal signals detected in all four archaeal-specific 16S rRNA gene clone libraries.

All the archaeal signals detected were 98-99% identical to archaeal 16S rRNA gene sequences in the GenBank database. However, there were no cultured archaeal representatives found in the top 100 blastn results for any of the cloned sequences. This was not surprising as only a few *Archaea* have been cultured. While archaeal signals were not previously detected in Miers Valley soils (Smith, 2006), Antarctic endolithic communities (de la Torre *et al.*, 2003) or Antarctic hypolithic communities (Smith *et al.*, 2000), this study showed that archaeal DNA was present in all Miers Valley soil environments tested.

While the grouping of sequences according to the top blastn result may suggest that some archaeal sequences are only found in certain communities (for example, Group 4 consists of only hypolithic archaeal community clones) more extensive sequencing is required to draw these conclusions. The maximum coverage index for all libraries (since all sequences were represented by more than one clone) could be due to an insufficient number of clones initially picked for analysis. DGGE analysis from metagenomic DNA indicates a greater number of archaeal phylotypes present in all communities (Figure 4.17) than have been detected by the cloning-sequencing strategy. A comparison of DGGE analysis of archaeal 16S rRNA gene sequences detected in the clone libraries and from metagenomic DNA indicates that the archaeal populations in all habitats are dominated by only a few phylotypes.

Table 5.5: Sequences obtained from archaeal-specific Type I hypolithic, Type II hypolithic, non-translucent rock (NTR) and open soil (OS) community 16S rRNA gene clone libraries

	Clone	I.D. of nearest match (Accession number)	Isolation source	% I.D.	Phylogenetic group
Group 1	Type I – clone 1 Type I – clone 24 Type II – clone 2 Type II – clone 8 Type II – clone 13 Type II – clone 14 Type II – clone 20 NTR – clone 7 NTR – clone 8 OS – clone 17 OS – clone 18 OS – clone 19	Uncultured archaeon clone HTA-C5 (AF418930)	Freshwater reservoir – USA	99 99 99 99 99 99 98 99 99 99 99 99	*Archaea*, environmental samples
Group 2	Type I – clone 22 NTR – clone 1 NTR – clone 2 NTR – clone 3 NTR – clone 7 NTR – clone 9 NTR – clone 8 OS – clone 16	Uncultured archaeon clone Pia-s-39 (EF632926)	Freshwater sediment – Laguna de Piacota, Altiplano, Chile	99 99 97 99 99 98 99 99	*Archaea*, environmental samples
Group 3	Type I clone 23	Unidentified archaeon clone 218 (AJ831160)	Landfill sludge - France	99	*Archaea,* environmental samples
Group 4	Type I – clone 2 Type II – clone 1 Type II – clone 9	Uncultured archaeon clone SV2R_L2_A6 (EF503701)	Rhizosphere - USA	98 98 98	*Archaea*, environmental samples
Group 5	Type II – clone 19	Uncultured archaeon clone 1013-1-141 (AY534145)	Uranium-contaminated aquifer – USA	99	*Archaea*, environmental samples
Group 6	Type II – clone 21	Uncultured archaeon clone ARC_1SAF1-45 (DQ782354)	NASA spacecraft assembly facility – USA	99	*Archaea*, environmental samples
Group 7	Type II – clone 7 Type II – clone 15 NTR – clone 9 OS – clone 20 OS – clone 21	Uncultured haloarchaeon clone TX4CA_67 (EF690622)	Alkaline saline soil - Mexico	99 99 99 99 99	*Archaea*, *Euryarchaeota*, *Halobacteria*

Only one group of clones (Group 7) can be assigned to a phylotype based on the sequence from the blastn result (accession number EF690622). Group 7 is assigned to the *Halobacteria*, a group that belongs to the *Euryarchaeota*. Although *Haloarchaea* would be the more correct term, *Halobacteria* is still commonly used as these organisms were assigned before the realisation of the Kingdom *Archaea*.

Halobacteria are a diverse group within the family *Halobacteriaceae* (order *Halobacteriales*) that are known for their adaptation to extreme conditions (Oesterhelt, 1985; Grant *et al.*, 1998). This group of organisms consist of the most halophilic organisms known and are often the dominant population in microbial communities found in hypersaline environments such as salt ponds (Oesterhelt, 1985; Grant *et al.*, 1998). These flagellated archaea are often rod-shaped cells of variable length (2-10 µm) and approximately 0.5 µm in diameter (Oesterhelt, 1985). It is not surprising that *Halobacteria* would form part of the archaeal communities in Dry Valley soils as these oligotrophic soils are known to be both saline and alkaline.

5.5 ITS sequence clone libraries

There is often a debate surrounding the use of the 18S rRNA gene and ITS regions for the study of eukaryotic diversity due to differences in mutation rates within these DNA regions. Evolution of DNA sequences differs due to a number of reasons including whether the sequence is part of a coding or non-coding region (Huelsenbeck *et al.*, 1996). ITS regions mutate more rapidly than 18S rRNA genes as they are non-coding sequences. Sequences that evolve at different rates can be used to resolve different levels of phylogeny. Those sequences that evolved more slowly are useful to distinguish older evolutionary events or divergence (i.e. low level resolution of phylogeny) (Huelsenbeck *et al.*, 1996). More rapidly evolving sequences, such as ITS regions, may be used to resolve more recent speciation events (i.e. higher resolution of phylogeny) (Huelsenbeck *et al.*, 1996). In this study, both ITS sequence and 18S rRNA gene clone libraries were constructed for two hypolithic communities (Type I and Type II) to ensure maximum coverage of eukaryotic diversity in these niche habitats while enabling better phylogenetic resolution.

ITS libraries were constructed for a Type I and a Type II hypolithic community to assess the major components of the eukaryotic communities. ITS1 (forward primer annealing at the 3'-end of the 18S rRNA gene) and ITS4 (reverse primer annealing at the 5'-end of 26S rRNA gene) primers (White *et al.*, 1990) amplified the 5.8S rRNA gene and the non-coding regions ITS1 and ITS2. Amplicons were cloned into a

plasmid vector as described in Section 2.8 and ninety-six positive recombinant clones were selected and assessed for the presence of the correct insert via PCR using the vector primers (Table 2.1). Only clones with a positive PCR product were used for further analysis. Inserts were amplified via a nested PCR approach using the insert primers (ITS1 and ITS4) (Table 2.1) and the colony PCR product as template. Successful amplifications were subjected to ARDRA analysis to screen clones for sequencing. Amplicons were first digested with the *Hae*III restriction enzyme (Figure 5.6).

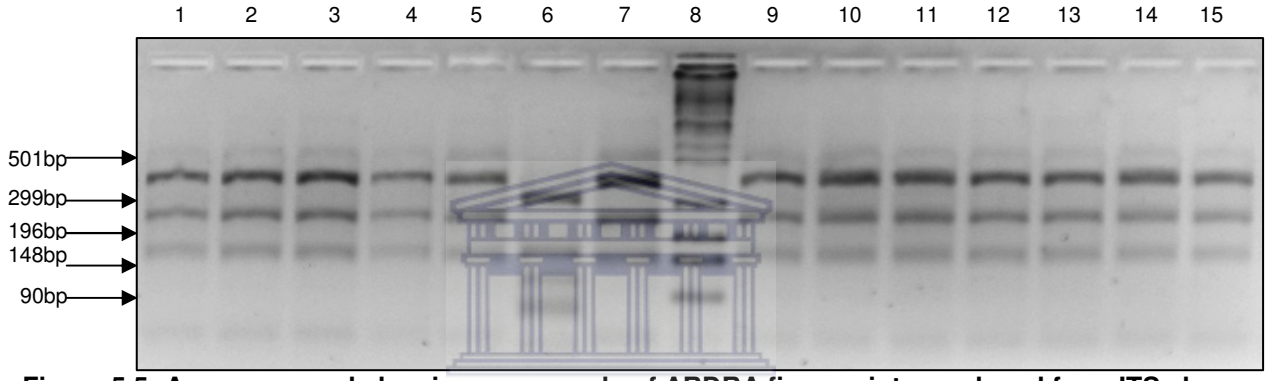

Figure 5.5: An agarose gel showing an example of ARDRA fingerprints produced from ITS clone inserts from a Type I hypolithic community ITS clone library digested with *Hae*III. Lanes 1-7 and 9-15: ITS ARDRA fingerprints. Lane 8: Molecular weight marker.

A second restriction enzyme digestion using *Mbo*I was carried out (Figure 5.7) to ensure that clones displaying the same ARDRA pattern with the *Hae*III digest were indeed the same and could be grouped together. For example, the *Hae*III ARDRA fingerprint from the clone in Lane 4 (Figure 5.6) is similar to the majority of ARDRA patterns. However, digestion with the second enzyme shows that this clone is different from the majority (Lane 4, Figure 5.7). Plasmids were extracted and the inserts sequenced from clones with different ARDRA patterns as well as representative clones with similar ARDRA patterns.

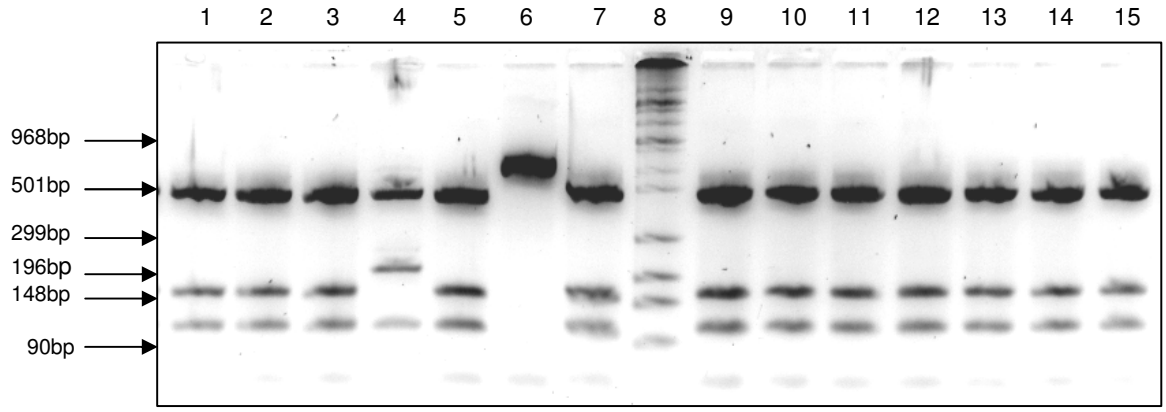

Figure 5.6: An agarose gel showing an example of ARDRA fingerprints produced from ITS clone inserts from a Type I hypolithic community ITS clone library digested with *Mbo*I. Lanes 1-7 and 9-15: ITS ARDRA fingerprints. Lane 8: Molecular weight marker.

A collectors' curve was constructed based on the number of clones picked and the number of different ARDRA patterns observed (Figure 5.8). It is evident that Type I hypolithic communities are composed of a greater number of eukaryote phylotypes than Type II hypolithic communities. Ten different eukaryote phylotypes were observed for the Type I community and six different eukaryote phylotypes for the Type II community. Field observations indicate that Type II hypolithic communities are dominated by moss, therefore it is probable that the dominant moss phylotype dominates the Type II community clone library. The Type I community on the other hand is dominated by cyanobacteria rather than a eukaryotic phylotype, therefore a greater number of eukaryotic phylotypes are evident in the ITS clone library.

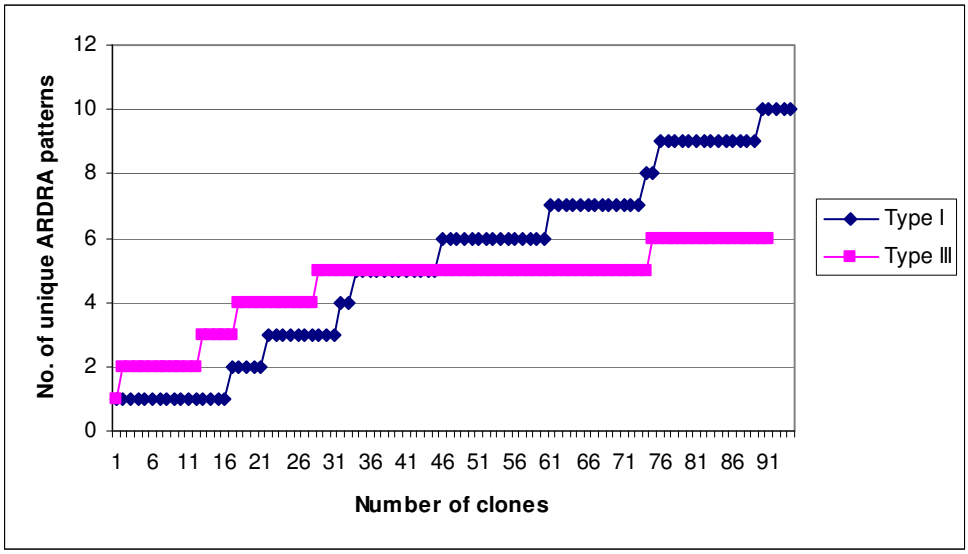

Figure 5.7: Collectors' curve for ITS libraries based on the number of different ARDRA patterns observed for two restriction enzyme digests. The coverage index (C), calculated by *C = (1 – n/N)x100* (where n is the number of different sequences represented by one clone and N is the total number of clones; Good, 1953) was 90% for the Type I library and 95% for the Type II library.

Furthermore, while the coverage index was high (90%), the rising collectors' curve for the Type I ITS library indicates that sampling of this library has not been exhausted. The Type II collectors' curve shows more of a plateau indicating that sampling may be approaching exhaustion. The coverage index (95%) also indicates that sampling approaches exhaustion. However, more extensive sampling may reveal less dominant or rare members of the communities as these communities appear to be dominated by few phylotypes.

While ARDRA analysis revealed ten and six different eukaryotic phylotypes for the Type I and Type II ITS libraries respectively, sequence analysis with blastn showed that all clones sequenced fell within fewer groups. The Type I library was dominated by the moss genus *Hennediella* (Table 5.6), while the Type II library was dominated by the moss genus *Bryum argenteum* (Table 5.7).

Table 5.6: Sequences obtained from a Type I hypolithic community ITS sequence clone library.

Clone	I.D. of nearest match (Accession number)	% I.D.	Phylogenetic group
ITS1A-C11 ITS1A-H8	*Stichococcus mirabilis* strain CCAP 379/3 (AJ431679)	77 78	*Eukaryota*; *Viridiplantae*; *Chlorophyta*
ITS1A-E2 ITS1A-F3 ITS1A-G6 ITS1A-A11 ITS1A-C9 ITS1A-D11 ITS1A-F4 ITS1A-G4 ITS1A-G8	*Hennediella heimii* voucher CC16 (AY613331)	99 98 99 96 96 98 99 96 98	*Eukaryota*; *Viridiplantae*; *Streptophyta*; *Embryophyta*; *Bryophyta*; Moss Superclass V; *Bryopsida*; *Dicranidae*; *Pottiales*; *Pottiaceae*; *Hennediella*.
ITS1A-F2	*Hennediella heimii* voucher Green & Seppelt #20336 1 (AY613330)	98	*Eukaryota*; *Viridiplantae*; *Streptophyta*; *Embryophyta*; *Bryophyta*; Moss Superclass V; *Bryopsida*; *Dicranidae*; *Pottiales*; *Pottiaceae*; *Hennediella*.

Clones that showed high similarity to the moss genus *Hennediella* (AY613331) included those representatives of the majority of ARDRA phylotypes as well as clones with different ARDRA patterns from the majority. The high similarity of sequences with different ARDRA patterns could be due to genetic variation within populations due to the higher mutation rates of non-coding ITS regions. While it can be concluded that the major eukaryotic phylotype in this hypolithic community belongs to the moss *Hennediella* (Table 5.6), further sampling is required to elucidate the less dominant and/or rare community members. Two clones in the Type I library (classified as different phylotypes according to ARDRA analysis) both showed a top blastn result to the algae *Stichococcus mirabilis*, but with very low identity (77-78%). The low identity and variation in ARDRA patterns indicates the potential for the discovery of novel eukaryotes or genotypes present in hypolithic communities.

Table 5.7: Sequences obtained from a Type II hypolithic community ITS sequence clone library

Clone	I.D. of nearest match (Accession number)	%I.D.	Phylogenetic group
ITS3-1A-A2 ITS3-1A-B1 ITS3-1A-C5 ITS3-1A-C9 ITS3-1A-C10 ITS3-1A-D5 ITS3-1A-D8 ITS3-1A-D9 ITS3-1A-F6 ITS3-1A-G7 ITS3-1A-G11 ITS3-1A-H1 ITS3-1A-H9 ITS3-1A-H12	*Bryum argenteum* voucher C474 (AY611433)	97 99 99 97 99 100 98 97 98 98 75 98 99 99	*Eukaryota*; *Viridiplantae*; *Streptophyta*; *Embryophyta*; *Bryophyta*; Moss Superclass V; *Bryopsida*; *Bryidae*; *Bryanae*; *Bryales*; *Bryaceae*; *Bryum*.
ITS3-1A-G12	*Bryum argenteum* voucher C363 (AY611434)	100	*Eukaryota*; *Viridiplantae*; *Streptophyta*; *Embryophyta*; *Bryophyta*; Moss Superclass V; *Bryopsida*; *Bryidae*; *Bryanae*; *Bryales*; *Bryaceae*; *Bryum.*

Despite differences in ARDRA patterns, all the clones sequenced for the Type II community ITS sequence clone library had up to 100% sequence identity to strains of *B. argenteum* (Table 5.7). The clones with high identity to *B. argenteum* (AY611433) include those phylotypes representing the majority of ARDRA types detected as well as several clones with different ARDRA patterns . Differences in ARDRA patterns could be due to mutations within the restriction sites within the ITS regions which have a higher mutation rate and therefore enable finer, sub-species resolution Clone ITS3-1A-G11 however, showed only 75% identity to *B. argenteum*. This indicates the need for further molecular characterisation of organisms in order to more fully define the phylogeny of eukaryotes in hypolithic communities. Furthermore, it highlights the potential for the discovery of novel eukaryotic species present in hypolithic communities.

Overall, the analysis of ITS sequence clone libraries indicates that hypolithic communities contain different eukaryotic communities, with different dominant moss populations. Furthermore, the lack of detection of fungi in these communities implies that fungi are minor components of Type I and Type II hypolithic eukaryotic communities. Further library construction employing fungal-specific PCR primers is required to reveal the fungal diversity of these communities. While it is thought that fungi and algae would dominate the Type III (lichenized) hypolithic eukaryotic community based on field observations of community components, the results suggest that mosses dominate the eukaryotic components of Type I and Type II hypolithic communities.

5.6 18S rRNA gene clone libraries

Libraries of 18S rRNA genes were constructed for four communities: Type I hypolithic community, Type II hypolithic community, non-translucent rock community and an open soil community. Ninety-six positive recombinant clones were selected and assessed for the presence of the insert using PCR with the vector primers as described in Section 5.2. Only clones with a positive PCR product were used for further analysis. The result of the initial screening process was:

- Ninety-four positive clones for the Type I hypolithic community 18S rRNA gene clone library;
- Eighty-seven positive clones for the Type II hypolithic community 18S rRNA gene clone library;
- Fifty-one positive clones for the non-translucent rock 18S rRNA gene clone library;
- Fourteen positive clones for the open soil 18S rRNA gene clone library.

The low numbers of clones that passed the initial screening for the open soil 18S rRNA gene clone library is thought to be due to the lower amount of DNA used for

cloning as PCR amplification of 18S rRNA genes from the metagenomic DNA extracts was low due to the fewer numbers of eukaryotic genes present.

A nested PCR approach was used to amplify the 18S rRNA gene inserts (primers EukA and EukB; Table 2.1) using the PCR product from the first round of amplification (primers M13fw and M13rev; Table 2.1) as the template. ARDRA analysis was used to de-replicate clones and a representative from each ARDRA type (using restriction enzymes, *Hae*III and *Mbo*I) were sequenced.

Figure 5.9 shows the collectors' curve for the 18S rRNA gene clone libraries constructed for each of the four communities assessed based on the number of different ARDRA patterns observed for the two restriction digests. Figure 5.9 shows that sampling has not been exhausted as all the curves have not reached a plateau. Unlike the ITS libraries (Figure 5.8), the analysis of 18S rRNA gene libraries of Type I and Type II hypolithic communities indicate that Type II communities consist of a greater number of eukaryotic phylotypes than Type I communities. Additionally, non-translucent rock communities harbour the greatest number of eukaryotic phylotypes. However, the eukaryotic diversity cannot be inferred as sampling was not exhausted for any of the 18S rRNA gene community libraries constructed (Figure 5.9). A coverage index of 100% for the open soil 18S rRNA gene clone library is probably an artefact due to the small library size.

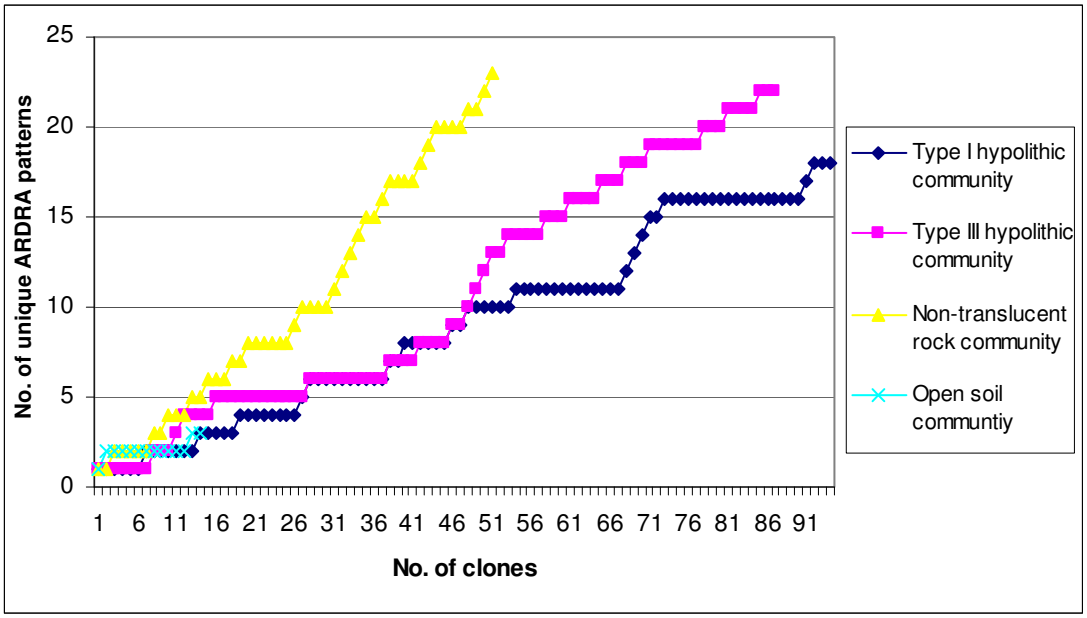

Figure 5.8: Collectors' curve for 18S rRNA gene libraries based on the number of different ARDRA patterns observed for two restriction enzyme digests. The coverage index (C), calculated by *C = (1 – n/N) x 100* [where n is the number of different sequences represented by one clone and N is the total number of clones (Good, 1953)] was 81% for the Type I library, 77% for the Type II library, 75% for the non-translucent rock library and 100% for the open soil library.

BLAST analysis of 18S rRNA gene sequences from a Type I hypolithic community library indicates that the community is dominated by the moss genus *Pottia* (Table 5.8). This corroborates the results of the ITS sequence clone library for this community since recent taxonomic revision has reclassified many species from the genus *Pottia* to the genus *Hennediella* (Zander, 1999). Furthermore, clone Euk-1A-A1 shows 97% identity to the algae from the genus *Stichococcus*, which confirms the low identity algal blastn result in the ITS sequence clone library (Table 5.6). Three clones showed the highest sequence identity to fungi (two *Ascomycetes* and one *Chytridiomycete*). Since fungi were not detected in the ITS library, this indicates that while present, fungi are not dominant components of Type I hypolithic communities.

Table 5.8: Sequences obtained from a Type I hypolithic community 18S rRNA gene clone library

Clone	I.D. of nearest match (Accession number)	% I.D.	Phylogenetic group
Euk-1A-A4 Euk-1A-A7 Euk-1A-B8 Euk-1A-C4 Euk-1A-C5 Euk-1A-C8 Euk-1A-C11 Euk-1A-D1 Euk-1A-D3 Euk-1A-D4 Euk-1A-D7 Euk-1A-E1 Euk-1A-E4 Euk-1A-F3 Euk-1A-F11 Euk-1A-G2 Euk-1A-H8 Euk-1A-H9 Euk-1A-H10	*Pottia truncata* 18S rRNA gene (X95935)	98 99 98 99 98 99 95 99 99 99 99 99 99 99 98 99 99 98 99	*Eukaryota*; *Viridiplantae*; *Streptophyta*; *Embryophyta*; *Bryophyta*; Moss Superclass V; *Bryopsida*; *Dicranidae*; *Pottiales*; *Pottiaceae*; *Pottia* (*Hennediella*).
Euk-1A-A1	*Stichococcus bacillaris* 18S rRNA gene (AB055865)	97	*Eukaryota*; *Viridiplantae*; *Chlorophyta*
Euk-1A-B3	*Tetracladium marchalianum* 18S rRNA gene (AF388580)	99	*Eukaryota*; Fungi; *Dikarya*; *Ascomycota*
Euk-1A-D5	*Tetracladium maxilliforme* strain F-13186 18S rRNA gene (EU883430)	97	*Eukaryota*; Fungi; *Dikarya*; *Ascomycota*
Euk-1A-F10	*Hyaloraphidium curvatum* 18S rRNA gene (Y17504)	98	*Eukaryota*; Fungi; *Chytridiomycota*

Despite the fact that the ITS sequence clone library (Table 5.7) indicated that the moss *B. argenteum* was the major eukaryote component of Type II communities, this taxon was not detected in the 18S rRNA gene clone library (Table 5.9). The majority of clones (including clones with similar ARDRA patterns and very different ARDRA patterns) were found to have up to 100% sequence identity to the moss *Dicranella staphylina*. Only one clone in this group (Euk-3-1A-E8) was less than 98% identical to *D. staphylina*. *D. staphylina* and the one other moss phylotype detected in the 18S library, *Mielichhoferia macrocarpa*, are included in the Moss Superclass V together with *B. argenteum* detected in the ITS sequence clone library. While fungi were not detected in the ITS sequence clone library for this community, three clones were most identical to fungal sequences in the database. As with the Type I ITS sequence clone library, two of these clones belong to the *Ascomycetes*. Ascomycete species have previously been found to be associated with the moss *B. argenteum* in Antarctica

(Bradner *et al.*, 2000). Two clones most closely identified to protists were identified (Euk3-1A-E3 and Euk3-1A-H6) in the Type II community 18S rRNA gene clone library while no protists were detected in the Type I community 18S rRNA gene clone library.

Table 5.9: Sequences obtained from a Type II hypolithic community 18S rRNA gene clone library

Clone	I.D. of nearest match (Accession number)	% I.D.	Phylogenetic group
Euk3-1A-E3	*Eimeriidae* environmental sample clone Amb_18S_1494 18S rRNA gene (EF024016)	98	*Eukaryota*; *Alveolata*
Euk3-1A-H2	*Alternaria alternate* (AF218791)	99	*Eukaryota*; Fungi; *Dikarya*; *Ascomycota* (*Pleosporales*)
Euk3-1A-G3	*Phaeosphaeriopsis nolinae* (AF250818)	97	*Eukaryota*; Fungi; *Dikarya*; *Ascomycota* (*Pleosporales*)
Euk3-1A-F8	*Pleosporales* sp. RMF2 (EF532931)	98	*Eukaryota*; Fungi; *Dikarya*; *Ascomycota* (*Pleosporales*)
Euk3-1A-D11	Uncultured fungus clone WIM38 (AM114817)	96	*Eukaryota*; Fungi; environmental samples
Euk3-1A-H6	*Sphaerothecum destruens* isolate SK (AY267346)	92	*Eukaryota*; *Ichthyosporea*
Euk3-1A-A9 Euk3-1A-H10	*Mielichhoferia macrocarpa* (AJ275006)	98 98	*Eukaryota*; *Viridiplantae*; *Streptophyta*; *Embryophyta*; *Bryophyta*; Moss Superclass V
Euk3-1A-A7 Euk3-1A-C4 Euk3-1A-D3 Euk3-1A-E6 Euk3-1A-E7 Euk3-1A-E8 Euk3-1A-F3 Euk3-1A-F5 Euk3-1A-F12 Euk3-1A-G6 Euk3-1A-B1 Euk3-1A-D7 Euk3-1A-E5	*Dicranella staphylina* (X89873)	99 99 99 96 98 93 97 97 96 99 100 100 99	*Eukaryota*; *Viridiplantae*; *Streptophyta*; *Embryophyta*; *Bryophyta*; Moss Superclass V

Table 5.10 shows the top blastn results for sequences obtained from the non-translucent rock 18S rRNA gene clone library. The diversity contained within the non-translucent rock community appears greater than that of the hypolithic communities analysed since a greater number of different sequences were obtained (Table 5.10). This could be due to the fact that, unlike hypolithic communities, this library was not

dominated by a single eukaryotic phylogenetic group. Instead, the distribution of the phylotypes was more even, with three major phyla: protists, green algae and fungi (Table 5.10). True comparisons of diversity cannot be achieved based on the cloning-sequencing data as the collectors' curve indicates insufficient sampling (Figure 5.9).

Table 5.10: Sequences obtained from a non-translucent rock community 18S library

Clone	I.D. of nearest match (Accession number)	% I.D.	Phylogenetic group
Euk3-1B-A2 Euk3-1B-H7	*Saccamoeba limax* (AF293902)	91 97	*Eukaryota*; *Amoebozoa*
Euk3-1B-H9	Uncultured eukaryotic picoplankton clone P1.18 (AY642694)	99	*Eukaryota*; environmental samples
Euk3-1B-D11	Uncultured eukaryote clone Elev_18S_4451 (EF024999)	93	*Eukaryota*; environmental samples
Euk3-1B-D7	Uncultured marine eukaryote clone NA1_1G7 (EF526863)	86	*Eukaryota*; environmental samples
Euk3-1B-E5 Euk3-1B-G8	*Powellomyces variabilis* strain BK91-11 (AF164241)	98 98	*Eukaryota*; Fungi; *Chytridiomycota*
Euk3-1B-F1	*Chlorellidium tetrabotrys* strain SAG 5.90 (AJ580949)	96	*Eukaryota*; Fungi; *Chytridiomycota*
Euk3-1B-A12	*Entophlyctis confervae-glomeratae* (EF014367)	98	*Eukaryota*; Fungi; *Chytridiomycota*
Euk3-1B-A4	Uncultured fungus clone WIM52 (AM114816)	99	*Eukaryota*; Fungi; environmental samples
Euk3-1B-E7	*Spongomonas minima* strain ATCC 50404 (AF411280)	91	*Eukaryota*; *Rhizaria*
Euk3-1B-A6 Euk3-1B-E1 Euk3-1B-H10	Uncultured stramenopile clone WIM103 (AM114810)	95 95 96	*Eukaryota*; stramenopiles; environmental samples
Euk3-1B-D6	*Pseudopleurochloris antarctica* (AF109729)	98	*Eukaryota*; stramenopiles; PX clade
Euk3-1B-B10	*Heterococcus caespitosus* (AF083399)	97	*Eukaryota*; stramenopiles; PX clade
Euk3-1B-E4	*Chlorella lobophora* strain Andreyeva 750-I (X63504)	95	*Eukaryota*; *Viridiplantae*; *Chlorophyta*
Euk3-1B-B6 Euk3-1B-C10 Euk3-1B-D8 Euk3-1B-H3 Euk3-1B-H4	*Pleurastrum insigne* (Z28972)	99 98 98 97 98	*Eukaryota*; *Viridiplantae*; *Chlorophyta*; *Chlorophyceae*; *Pleurastrum*
Euk3-1B-C8	Uncultured chlorophyte clone GHB103.20 (EU143976)	92	*Eukaryota*; *Viridiplantae*; *Chlorophyta*; environmental samples
Euk3-1B-E3	*Planophila laetevirens* strain SAG 2008 (AJ416102)	98	*Eukaryota*; *Viridiplantae*; *Chlorophyta*

The majority of non-translucent rock community clones based on ARDRA analysis were represented by the sequences obtained from clones Euk-3-1B-A2 and Euk-3-

1B-H7 (fifteen clones in total). This suggests that the eukaryote population of non-translucent rock communities is dominated by protists related to the phylum *Amoebozoa*. Other protists detected include members of the *Stramenophiles* and *Rhizaria* genera. The next largest component of these communities is the green algae, the majority of which belong to the genus *Pleurastrum*. Unlike the hypolithic community libraries, ascomycetes were not detected in the non-translucent rock 18S rRNA gene clone library. Here, the dominant fungal group was the *Chytridomycetes*.

Table 5.11 shows the top blastn results for sequences obtained from the open soil community 18S rRNA gene clone library. The majority of clones according to ARDRA analysis are represented by the sequences obtained from clones Euk3-1C-A3 and Euk3-1C-B6. This suggests that the major eukaryotic components are the green algae of the genus *Pleurastrum*. This is the same dominant green alga found in non-translucent rock communities. Furthermore, the only other clone assigned a phylogenetic group belongs to the protist Phylum *Alveolata*, which corresponds with the protist phylum detected in Type II hypolithic communities. While mosses were the predominant eukaryotic phylotypes detected in hypolithic communities, the green algae, protists and fungi were dominant in non-translucent rock and open soil communities.

Table 5.11: Sequences obtained from an open soil community 18S rRNA gene clone library

Clone	I.D. of nearest match (Accession number)	% I.D.	Phylogenetic group
Euk3-1C-A3 Euk3-1C-B6	*Pleurastrum insigne* (Z28972)	98 99	*Eukaryota*; *Viridiplantae*; *Chlorophyta* (*Pleurastrum*)
Euk3-1C-A7	*Enchelyodon* sp. (U80313)	98	*Eukaryota*; *Alveolata*
Euk3-1C-G4	Uncultured eukaryote clone Ha3_mtz6_A02 (EU740662)	92	*Eukaryota*; environmental samples

Although yeasts are known to be present in polar desert soils (Vishniac, 1996), they were not detected in the eukaryote clone libraries constructed for the different Miers Valley communities assessed. As with previous studies of terrestrial algae in Antarctica (Cavacini, 2001), the major algal component detected in this study belong to the division *Chlorophyta*. Algae were only detected in the non-translucent rock and

open soil community libraries, indicating that they are not major constituents of the eukaryotic component of hypolithic communities. Clearly, hypolithic communities differ in their eukaryote community structure in that they are dominated by mosses. Both Type I and Type II communities were dominated by mosses from the Moss Superclass V, but the moss genera identified in the clone libraries differed between the hypolithic communities analysed.

5.7 Conclusion

Analysis of the phylogenetic diversity using a cultured-independent approach revealed that there is considerable diversity in the soil communities, despite the harsh environment of the Miers Valley cold-desert soils. As expected on the basis of community fingerprinting methods (see Chapter 4), bacterial communities are highly complex, while archaeal and eukaryotic communities are considerably less diverse. The hypolithic habitat does not appear to offer a specific niche for archaea, as the archaeal signals detected were similar in all communities. *Actinobacteria* were dominant in Type II hypolithic communities, non-translucent rock communities and open soil communities, while cyanobacteria dominate the 16S rRNA gene clone library from a Type I hypolithic community. Both hypolithic communities are dominated by mosses of the Moss Superclass V, but contain different moss genera which could be due to the difference in phylogenetic resolution afforded by the different phylogenetic marker sequences used (ITS sequences and 18S rRNA genes).

The proposed division of hypolithic communities into classes based on field observations holds true based on phylogenetic sequence analysis of the bacterial and eukaryotic communities. Type I hypolithic communities are dominated by cyanobacteria, while cyanobacteria were not detected in Type II hypolithic communities. Type II communities therefore contain a different primary producer as the basis of the community structure, in the form of the moss species present. The dominance of mosses in the Type I hypolithic community could indicate that the components for succession to a moss-dominated hypolithic community are present

or, indeed, underway. In the future, phylogenetic marker libraries of Type II hypolithic communities will be helpful to determine the dominant organisms in these communities and paint a clearer picture of the succession of hypolithic communities.

It is also likely that some proportion of the DNA signals observed in the libraries originate from active and inactive cells, live cells and 'naked' DNA. Further investigation is required to determine the proportion of DNA acquired from these fractions. Furthermore, future research should include a larger clone sampling set and more intensive sequencing until the collectors' curve plateaus.

It must be noted that this study represents a 'snapshot' view of the four soil environments within the Miers Valley. Re-sampling the same communities at different time intervals will assist in determining if community structures change with changing environmental conditions and time. Rising collectors' curves for the bacterial and eukaryote libraries indicate that sampling of these communities has not been exhausted by the study. Furthermore, comparison of DGGE analysis of archaeal 16S rRNA gene sequence diversity from clone libraries and metagenomic DNA indicate that sampling of archaeal 16S rRNA gene clone libraries was not exhausted. This means that the comparisons made are not statistically valid since most libraries are under-sampled. However, this study does shed light on the community structure and possible dynamics of hypolithic communities in the Dry Valleys. Furthermore the detection of groups such as the *Actinobacteria*, which are major producers of useful secondary metabolites, should be further investigated in terms of their function in these environments.

Chapter 6: Conclusions, Future Work and a Hypothesis for Hypolithic Community Development

6.1 Physico-chemical analysis of hypolithic habitats

Quartz rocks larger than 3 cm wide and partially embedded in the soil in the Miers Valley create a niche habitat that is physically protected as the microenvironment is stabilised from movement or erosion by the strong katabatic winds in the region. Field observations have shown that rocks less than 3 cm wide or not firmly embedded in the soil are not colonised by macroscopic growth because the microenvironment is still susceptible to considerable physical disturbance and exposure.

6.1.1 Temperature and relative humidity (%RH)

Temperature and %RH fluctuations were greatest on the soil surface while the more buffered hypolithic, non-translucent rock and sub-soil environments showed greater stability in these parameters. A greenhouse effect was not observed in hypolithic environments as the temperatures were comparable to the sub-soil. Similarly, potentially available water (measured as %RH) was similar in hypolithic and sub-soil environments and, in fact, consistently higher beneath non-translucent rocks. This data indicates that the potentially available water may be preserved in the microhabitat due to the reduced exposure to the desiccating atmosphere. These results led to the conclusion that, while minimisation of temperature and %RH fluctuations may be important to the growth and development of communities in the Dry Valleys, they were not the primary factors resulting in macroscopic growth beneath translucent quartz rocks as opposed to beneath non-translucent rocks.

6.1.2 Light

Analysis of the physical parameters in hypolithic microenvironments indicates that light availability is the most important factor allowing for the macroscopic growth of

hypolithic communities beneath translucent quartz rocks as opposed to non-translucent rocks. While light transmission through the overlying quartz rock is low (average transmission was 2.3% of the incident light), it is sufficient for growth and development of photosynthetic communities. The study also showed that the quartz rock reduces the intensity of short wavelength light in the UV range of the spectrum. This is presumably advantageous to photosynthetic communities since, during the austral summer, the prolonged exposure to high light intensity, particularly in the UV range, could damage cellular contents and processes (Quesada *et al.*, 1998). This aspect of UV protection is also of increasing importance in context of the global ozone depletion that is concentrated in the polar regions and leads to an increasing amount of UV irradiation reaching the Earth's surface (Crutzen, 1992).

Light intensities transmitted to hypolithic communities are similar to those experienced at the base of Antarctic cyanobacterial lake mats (Vincent, 1993). Cyanobacteria found deep within these lake mats have high concentrations of light harvesting pigments that allow for substantial rates of photosynthesis under low light conditions: cyanobacteria within these mats all reached photosynthetic saturation at <15% incident light (i.e. 27 to 178 µmol m^{-2} s^{-1} light) (Vincent, 1993). The hypolithic habitats characterised in this study showed light intensities of up to 24 µmol m^{-2} s^{-1} which would suggest that hypolithic cyanobacteria also experience photosynthetic saturation at low levels of light intensity. Further investigation is required to test this hypothesis. Future studies on Antarctic hypolithic communities should also investigate the photosynthetic properties of cyanobacteria present, including rates of photosynthesis and carbon assimilation, the presence and concentrations of various light-harvesting pigments and the relationship between light, pigment concentrations and depth under the quartz rock. Together, this knowledge will increase our understanding of the primary autotrophs that drive the development of these communities.

In essence, it is proposed that the combination of sufficient light availability, physical stability and reduced temperature and %RH fluctuations leads to the development of

a suitable microenvironment in the harsh Antarctic desert environment for the development of macroscopic hypolithic communities.

6.2 Metagenomic DNA extraction

Metagenomic DNA extraction yields from hypolithic communities were significantly greater than yields from non-translucent and open soil communities. This results from the higher macroscopic biomass observed in hypolithic communities. While non-translucent rock and open soil environments harbour diverse microbial populations, these communities do not present the macroscopic growth observed in hypolithic communities.

It must be noted that the metagenomic DNA extracts used for fingerprinting and phylogenetic analysis in this study probably includes DNA from dead or inactive members of the community. Analysis of RNA extracts would give a clearer understanding of the active communities within these environments since RNA is less stable and has a greater turnover compared to DNA. This is an important aspect since many of the detected phylotypes(particularly in the open soil) may represent the remnants of microbes dispersed by the strong winds in the Miers Valley.

6.3 Community fingerprint analysis

Composite data sets, incorporating similarity matrices from PCR-DGGE community profiling using the 16S rRNA gene (bacteria, cyanobacteria and archaea), 18S rRNA gene and ITS sequence were constructed in order to compare the overall diversity of hypolithic communities with that of non-translucent rock and open soil communities. Analysis of the composite data set showed that all communities are highly diverse. Hypolithic communities are most different from non-translucent rock and open soil communities. Non-translucent rock and open soil communities are most similar to each other and can be grouped with confidence as a single group. Furthermore, while not all hypolithic communities were the same with regard to community composition, they shared more similarity to each other than the control communities. Interestingly,

the hypolithic communities also shared more similarity to non-translucent rock communities than open soil communities. Compared to the bacterial 16S rRNA gene DGGE profile, the archaeal diversity was much lower and all environments shared just a few phylotypes, suggesting a ubiquitous distribution in Dry Valley soils. The classification of hypolithic communities into three types based on field observations (Type I, Type II and Type III) was not reiterated by the microbial profiles. This indicates that while hypolithic communities may differ in the dominant population, there is some overlap in the community components. Future work should focus on more fully analysing the communities in a complete and quantitative manner so that the hypolithic biotypes can be more accurately described. A study of the development of these hypolithic communities over time would also be of interest since it may reveal differences in microbial composition during community succession or in response to changes in environmental factors. This may prove important as changes in community structure in response to environmental factors could present these communities as potential bio-indicators for climate change.

6.4 Phylogenetic analysis of communities

Bacterial 16S rRNA gene clone libraries constructed from metagenomic DNA extracted from non-translucent rock and open soil communities showed that *Actinobacteria* were the dominant phylotype in both environments. All the major phylogenetic groups detected in a previous study of Miers Valley soil communities (*Actinobacteria*, *Verrucomicrobia*, *Acidobacteria*, *Chloroflexi* and *Bacteroidetes*) (Smith *et al.*, 2006) were detected in this study, with the addition of *Gemmatimonadetes* which were previously not detected in these soils. *Actinobacteria* are the predominant bacterial in all soil communities, aside from Type I hypolithic communities that are dominated by the *Cyanobacteria* (especially *Nostocales* and *Oscillatoriales* spp.) which are the primary producers in these hypolithic communities.

While **cyanobacteria** have been thought to be the dominant primary producers in hypolithic environments (Smith *et al*, 2000), this study shows that is not the case in all hypolithic communities. The dominant primary producers in Type II hypolithic

communities are the moss phylotypes (*B. argenteum*; *D. staphylina*). The data suggests that the moss phylotypes could out-compete cyanobacteria as the dominant primary producer resulting in a decreased abundance of cyanobacteria in the Type II hypolithic community. This result could also be an effect of succession.

Archaeal sequences were detected in all the soil environments. Phylogenetic classification of *Archaea* was difficult due to the low numbers of cultured archaeal sequences in public databases (NCBI). There were no cultured *Archaeal* representatives within the top 100 most similar sequences from the NCBI database for the *Archaeal* 16S rRNA sequences retrieved from the clone libraries. Only one *Archaeal* phylotype was positively assigned to the *Halobacteria*. It is not surprising that halotolerant *Archaea* would occur in the Dry Valleys due the high salinity of the soils in this region.

The **eukaryotic** phylotypes in Type I and Type II communities were dominated by mosses, while non-translucent rock and open soil communities were dominated by protists, algae and fungi. The Type II hypolithic community ITS sequence clone library indicated that *Bryum argenteum* was the dominant moss while the 18S rRNA gene clone library showed *Dicranella staphylina* was dominant. Both bryophyte phylotypes belong to the Moss Superclass V and the difference in phylogenetic assignment could be due to the differences in resolution of the ITS and 18S rRNA gene sequences. The dominance of mosses in the Type I library could indicate that the components for succession of the community to a Type II community are present or, indeed, underway. Future work should include more intensive cloning and sequencing from multiple hypolithic communities. Moreover, PCR-DGGE with fungal-specific ITS primers revealed several phylotypes which were not revealed by cloning-sequencing analyses. This indicates the need for more intensive cloning-sequencing efforts using multiple primer sets and increased clone sampling. It is presumed, based on field observations, that Type III (lichenised) communities would be dominated by fungi and algae. Detailed analysis of clone libraries of Type III hypolithic communities are required to test this hypothesis.

The study revealed that hypolithic, non-translucent rock and open soil communities contain highly diverse populations of bacteria. This study was an extensive but not an exhaustive analysis of these environments; statistically valid comparisons are not yet fully viable. However, differences in community structure were noted and have led to the proposal for a hypothesis regarding the development of hypolithic communities.

6.5 Hypothesis regarding hypolithic community structure and development

Extensive field observations have led to the classification of hypolithic communities into three classes (Type I, Type II and Type III). Type I communities are dominated by cyanobacteria, while Type II communities are dominated by mosses. These observations have largely been confirmed by phylogenetic analyses. Type III (lichenised) communities appear to be dominated by lichen-associated fungi and algae but further phylogenetic analysis of these communities is required to test this hypothesis.

This study has led to the development of a hypothesis to describe the development of macroscopic hypolithic communities in the harsh desert environments found in the Antarctic Dry Valleys (Figure 6.1). It is proposed that the combination of available light, physical stability and buffered temperature and %RH regimes create a suitable microenvironment for the growth and development of photosynthetic cyanobacteria. Cyanobacteria are the first primary producers to colonise the microenvironment, resulting in the development of Type I hypolithic communities. Type I communities are then succeeded by Type II moss-dominated or Type III lichenised hypolithic communities. This evolution is probably slow and succession would be expected to result in an overlap in community composition of the three hypolithic community classes (as was observed). The rate and type of succession (to Type II or Type III communities) depends on numerous factors including the age of the community, the nature of the primary coloniser, microenvironmental factors or the presence of other determining microorganisms of the hypolithic community.

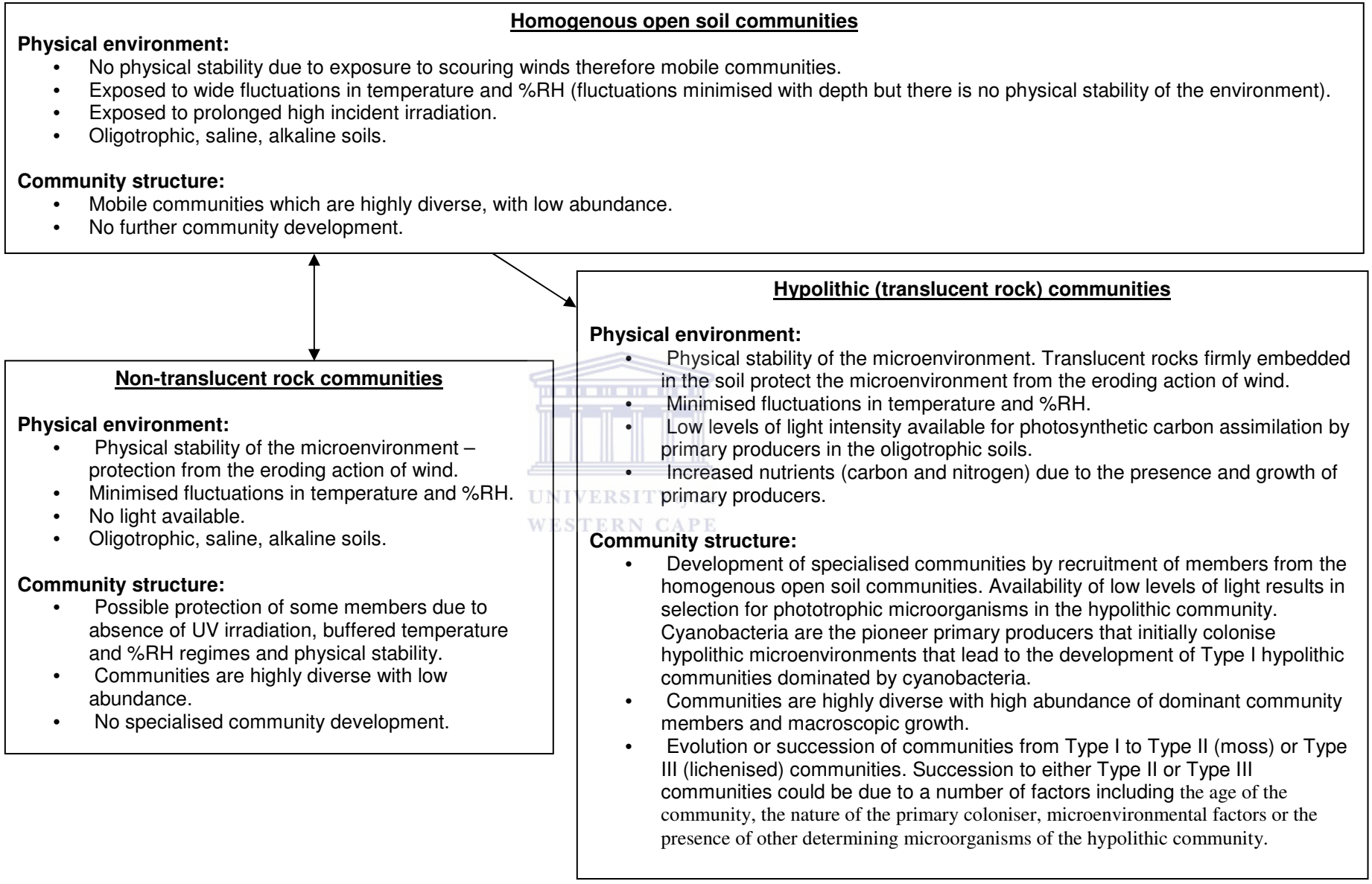

Figure 6.1: Hypothesis of microbial community development in the Dry Valleys, Antarctica

In summary, the main aims of this study have been successfully achieved. The main physical factor resulting in the development of hypolithic communities beneath translucent quartz rocks as opposed to non-translucent rock has been identified to be the availability of low, but sufficient levels of light for photosynthesis by phototrophic organisms. Based on PCR-DGGE community fingerprint analyses, not all hypolithic communities are the same although some phylotypes are shared. Non-translucent rock and open soil communities are highly similar and group together with confidence. Hypolithic communities differ from non-translucent rock and open soil communities, with some shared phylotypes. While sampling of clone libraries was not exhaustive, differences in community composition and structure between different hypolithic communities and between hypolithic and other soil environments were apparent. These results have led to the development of a hypothesis for the development of hypolithic communities in the Dry Valleys of Antarctica.

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