# COLD ADAPTATION STRATEGIES AND DIVERSITY OF ANTARCTIC BACTERIA

by

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B. Sc.


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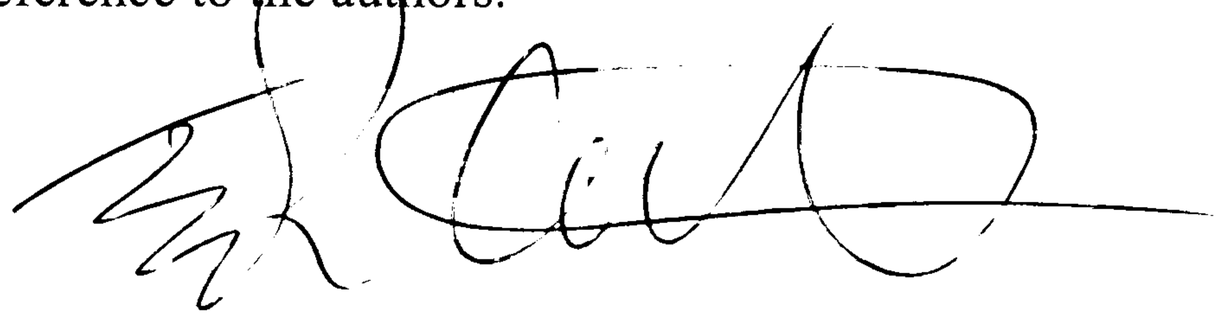
# DECLARATION

I hereby declare that the work presented in this thesis is my own and has not been submitted for any other degree. All sources of information have been acknowledged by reference to the authors.



Jack Anthony Gilbert

**"If you march your Winter Journeys you will have your reward, so long as all you want is a penguin's egg"**

*Apsley Cherry-Garrard (The Worst Journey in the World)*

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# List of Abbreviations

**AFP** - Antifreeze protein
**AFGP** - Antifreeze glycoprotein
**AFLP** - Amplified fragment length polymorphism
**AP-PCR** - Arbitrarily primed polymerase chain reaction
**ATCC** - American type culture collection
**ATP** - Adenine 5'-triphosphate
**BLAST** - Basic local alignment search tool for DNA DNA sequence comparisons
**CAP** - Cold acclimation protein
**CSP** - Cold shock protein
**CTAB** - Cetyltrimethylammonium bromide
**CTP** - Cytosine 5'-triphosphate
**DAPI** - 4', 6-diamidino-2-phenylindole
**DCM** - Deep chlorophyll maximum
**DDBJ** - DNA databank of Japan
**DFAA** - Dissolved free amino acids
**DFCHO** - Dissolved free monosaccharides
**DGGE** - Denaturing gradient gel electrophoresis
**DNA** - Deoxyribonucleic acid
**DOC** - Dissolved organic carbon
**EMBL** - European molecular biology laboratory
**FASTA** - DNA DNA sequence comparison program for computing similarity.
**FISH** - Fluorescent *in situ* hybridisation
**GES** - Guanidium isothiocyanate
**GM** - Genetically modified
**GTP** - Guanine 5'-triphosphate
**HNAN** - Heterotrophic nanoflagellates
**HTAP** - High-throughput antifreeze protein assay
**INA** - Ice nucleation agent
**INP** - Ice nucleation protein
**LRR** - Leucine rich repeat proteins
**NCBI** - National centre for biotechnology information
**OUT** - Operational taxanomic unit
**PCR** - Polymerase chain reaction
**PE** - Protein extract
**PGIPs** - Polygalacturonase inhibitor proteins
**PNAN** - Phototrophic nanoflagellates
**PUFAs** - Polyunsaturated fatty acids
**RAPD** - Random primed amplified polymorphic DNA
**RFLP** - Restriction fragment length polymorphism
**RI** - Recrystalisation inhibition, the act of inhibition of ice crystal growth
**RNA** - Ribonucleic acid
**SIMCO** - Sea ice microbial community
**SPLAT** - Ice recrystalisation inhibition assay determined using crossed polarised microscopy of a single crystal width sample

| | |
|---|---|
| **SRP** | - Soluble reactive phosphate |
| **SWA** | - Sea water agar |
| **½ SWA** | - ½ sea water salinity agar |
| **T-RFLP** | - Termination restriction fragment length polymorphism |
| **TGGE** | - Temperature gradient gel electrophoresis |
| **TOC** | - Total organic carbon |
| **TSA** | - Tryptic soya agar |
| **TTP** | - thymine 5'-triphosphate |
| **UPGMA** | - Unweighted pair group method with arithmetic averages |
| **YSA** | - Yeast selective agar |

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# ABSTRACT

Bacteria have been isolated from virtually every environment on Earth. The Antarctic continent is no exception. In this extremely cold and dry environment bacteria have colonised various refugia and have evolved a number of strategies for coping with the extreme physico-chemical fluctuations they are exposed to within the environment. These psychrophilic adaptations include cold adapted proteins and lipids which are interest for biotechnology in areas such as frozen foods, agriculture and cryogenic storage. One type of cold adapted protein of particular interest is the antifreeze protein (AFP) for its recrystalisation inhibition and thermal hysteresis activity. It was first isolated from Antarctic fish in the 1970, but has since been found in plants, fungi, insects and bacteria.

Over 800 bacterial isolates were cultured from lakes of the, Vestfold Hills, Larsemann Hills and MacRobertson Land, Antarctica. Approximately 87% were Gram negative rods. A novel AFP assay designed for high-throughput analysis in Antarctica, demonstrated putative activity in 187 isolates. Subsequent SPLAT analysis (qualification assessment of recrystalisation inhibition activity) of the putative positive isolates showed 19 isolates with significant recrystalisation inhibition activity. These 19 isolates were cultured from five separate lakes with substantial physico-chemical differences.

The 19 AFP active isolates were characterised, using amplified ribosomal DNA restriction analysis (ARDRA) and 16S rDNA sequencing, as predominantly belonging to genera from the α- and γ-Proteobacteria, although they were more prominent in the gamma subdivision. One of these isolates (213, *Halomonas* sp.) was shown as dominant within its community by DGGE analysis, indicating a possible selective advantage for AFP active bacteria.

This is the first report of the phylogenetic distribution of AFP activity within bacteria, thus providing information which could enable future bacterial AFP assessments to be aimed at specific taxonomic groups.

# Chapter 1: INTRODUCTION

## 1.1 - Temperature as a regulatory factor

Temperature is a fundamental factor in the regulation of metabolism in living organisms. Its influence can be seen in all cellular processes directly in terms of growth rates, enzyme activity and cell composition (Herbert, 1986), and in its effect on aqueous systems (solute concentrations, diffusion rates and osmotic effects) (Oppenheimer, 1970; Herbert, 1986; Russell, 1990). Organisms which survive, or even thrive, at extremes of temperature are known as extremophiles (Huber & Stetter, 1998; Russell and Hamamoto, 1998; Stetter, 1999). The major aim of the current investigation was to investigate the adaptations employed by organisms that enable them to proliferate and function in the extremely low temperatures of the Antarctic environment, with the goal of isolating novel anti-freeze proteins from a bacterial source and delineating the environmental parameters which have promoted such activity. Further to this, the study investigated the taxonomy, phylogeny and ecology of bacterial assemblages in Antarctic lacustrine ecosystems.

## 1.2 - Cold environments

The large global extent of cold environments (<5°C) provides a vast natural laboratory for the investigation of cold temperature adaptive mechanisms. Seventy percent of the Earth's surface is covered by water, more than 90% of which is at a temperature of less than 5°C (Herbert, 1986). The polar regions constitute 14% of the Earth's surface and have constantly low ambient temperatures (Herbert, 1986) with the polar seas at or near –1.9°C for the majority of the year. Thus more than 80% of the Earth's biosphere is permanently below 5°C (Russell, 1990). In extreme environments microorganisms (bacteria, protozoa, yeasts, algae, fungi and lichens) dominate because of their high turnover rates which provides them with a unique adaptive ability (Brock,1985).

The first microorganisms to be identified as showing growth at 0°C were cultures of luminous bacteria isolated by Forster (1887, *cited in* Morita, 1975) from marine fish. Subsequent studies (Fischer, 1888; Forster, 1892; Conradi and Vogt, 1901; Baur, 1902; Brandt, 1902 reviewed in Morita, 1975) showed such bacteria were ubiquitous in cold

environments. Cold adapted bacteria have since been isolated from many permanently cold environments such as Arctic and Antarctic fish, deep ocean waters. polar soils and Antarctic marine and fresh water systems (Herbert, 1986).

One of the most important conditions imposed by low temperature is the freezing point of water, which can fluctuate due to solute concentrations in the environment (pure water = 0°C, sea water (38 parts per thousand (ppt) salinity) = -1.9°C) (Feller and Gerday, 1997). The transition from fluid to crystalline state limits essential enzymatic reactions, and ice crystal growth can cause irreparable cellular damage (Russell, 1992). The following sections will describe the adaptive mechanisms used to survive in extreme low temperature environments, the evolution of such mechanisms and their biotechnological potential.

**1.3 - Psychrophiles and psychrotrophs**

Microorganisms constitute the most temperature-adapted organisms on the planet (Russell, 1990; Russell & Hamamoto, 1998). The majority of known bacterial phenotypes demonstrate optimal growth within the 30-40°C range, however, as a group, they show a continuum of thermal adaptation growing at temperatures from sub-zero to boiling point (Russell, 1990). In terms of temperature response, microorganisms can be classified into three groups, psychrophiles, mesophiles and thermophiles.

Psychrophilic microorganisms are further classified into two groups, psychrophiles and psychrotrophs. Individuals in both groups are capable of growing at or close to 0°C (Russell & Hamamoto, 1998). Morita (1975) defines psychrophiles as organisms having an optimal growth temperature <15°C with upper limits of <20°C or by definition 'cold adapted' (Herbert, 1986). Psychrotrophs, however, have an optimal temperature of >15°C with upper limits of 40°C suggesting a cold-tolerant strategy (Herbert, 1986). Psychrophiles tend to have a narrower growth temperature range (Russell, 1990).

Psychrophiles (this term will be used to describe both psychrotrophs and –philes throughout this report) have been isolated from the southern polar region on numerous occasions (Ellis-Evans, 1985; Hand & Burton, 1981; Franzmann *et al.*, 1990; Laybourn-Parry *et al.*, 1996; Russell & Hamamoto, 1998; Staley & Gosink, 1999). Many psychrophilic bacteria from Antarctic environments have been directly cultured (Mills.

1999 ; Hand & Burton, 1981) or identified using molecular techniques (e.g. DGGE. Murray *et al.*, 1998).

**1.4 - Antarctic oases – ice-free refugia.**

The Antarctic continent is one of the driest and coldest places on Earth. Less than 5% of the continent is free of ice. Seventy-five percent of the world's freshwater is locked up in its vast ice cap, which in places can reach a thickness of 4km. However. liquid water is available in its ice-free areas. Some areas have been ice-free for up to five million years such as the southern Victoria Land desert oasis (Parker *et al.*, 1982); others such as the Larsemann Hills, Syowa Oasis, Schirmacher Oasis, Bunger Hills and the Vestfold Hills are all considerably younger (Hand & Burton, 1981; Laybourn-Parry. 1997; Bell & Laybourn-Parry, 1999b) and mostly formed by iso-static uplift caused by the retreat of the Antarctic ice-cap approximately 9,000 years ago after the Winconsonian glaciation (Burton, 1981; Franzmann, 1991; Burgess *et al.,* 1994).

This study concentrates on the lakes of the Vestford Hills. Eastern Antarctica (68°S, 77-78°E, Fig. 2.1a), but also includes some lakes from the Larsemann Hills (69°23'S, 76°23'E, Fig 2.1b) and Beaver Lake, an epi-glacial lake in MacRobertson Land (70° 48'S, 68°15'E, Fig 2.1c). The Vestfold Hills contain around 300 lakes and ponds, most of which are of marine origin. During isostatic uplift pockets of seawater in hollows and fjords were cut off. Those lakes with inflows and outflows were progressively flushed by glacial and snow meltwaters and evolved into freshwater systems. However, lakes without outlets (i.e. endorehic basins) evolved into brackish, saline and hypersaline lakes some of which have undergone meromixis (Laybourn-Parry *et al.,* 2002). Therefore, the action of evaporation, freezing concentration and marine and melt water influx have altered the salinity and anion/cation ratio of each of the lakes (James *et al.,* 1994; Gore *et al.,* 1996a). The lakes vary from freshwater to 350 ‰ salinity (Pickard, 1986; Laybourn-Parry *et al*, 2002). Some lakes also show thermal and chemical stratification, which is either seasonal or meromictic (Burton, 1981; Pickard, 1986).

The microbial diversity of the lakes and soil in the Vestford Hills has been researched (Wright & Burton, 1981; Burke & Burton, 1988;Franzmann. 1991; Franzmann & Rohde. 1991; Franzmann & Rohde. 1992; Laybourn-Parry & Marchant, 1992a & 1992b; Laybourn-Parry *et al.,* 1992; Franzmann & Dobson, 1993; Perriss *et al.*.

1993; Bayliss & Laybourn-Parry, 1995; Laybourn-Parry *et al.*, 1995; Cavanagh *et al.*, 1996; Franzmann, 1996; Laybourn-Parry & Bayliss, 1996; Laybourn-Parry *et al.*. 1996; Perriss & Laybourn-Parry, 1997; Bowman *et al.*, 2000a & b; Laybourn-Parry *et al.*, 2000; Labrenz & Hirsch, 2001; Laybourn-Parry *et al.*, 2001a; Laybourn-Parry *et al.*, 2002; Pearce & Butler, 2002), and microorganisms (bacteria, yeasts, fungi, algae and lichens) have been isolated from most Antarctic environments, such as lake water (permanently and seasonally ice covered), sheet ice, sea ice, glacial ice, soil, rocks, benthic sediments and snow (Ellis-Evans, 1985a & b; Franzmann *et al*, 1990; James *et al.*, 1994; Laybourn-Parry *et al.*, 1996; Priscu *et al.*, 1998; Fritsen & Priscu, 1999; Murray *et al.* 1998; Mills. 1999; Staley & Gosink, 1999; Christner *et al.*, 2001). The planktonic and benthic biota of the lakes are dominated by bacteria and protozoa (Laybourn Parry *et al.*, 1991; Laybourn-Parry, 1997). Most of the lakes are oligotrophic to ultra-oligotrophic (Laybourn-Parry & Marchant, 1992a). The microbial population densities are several orders of magnitude lower than oligotrophic lakes from lower latitudes (Laybourn-Parry & Marchant, 1992a). Also, the biodiversity of the plankton in these lakes is extremely low compared to tropical or temperate lakes (Laybourn-Parry *et al.*, 1995; Parker *et al.*, 1982). Although bacterial species isolated from the lakes have been noted (Kerry *et al.*, 1977; Burton, 1980; Hand & Burton, 1981; Wright & Burton, 1981; Burke & Burton, 1988; McMeekin & Franzmann, 1988; Franzmann *et al.*, 1990; Mancuso *et al.*, 1990; Franzmann, 1991; Franzmann & Rohde, 1991; Franzmann & Rohde, 1992; Laybourn-Parry & Marchant, 1992b; Franzmann & Dobson, 1993; Franzmann, 1996; Bowman *et al.*, 2000a & b; Labrenz & Hirsch, 2001), few comprehensive biodiversity or biogeography assessments have been made of the bacterial populations to date.

**1.5 - Methods of cold adaptation**

There are many different methods used by living organisms to survive in cold environments. Organisms must contend with decreased growth rates, slowed enzyme activity, alteration of cellular composition and changes in nutritional requirements. They must also be able to cope with fluctuations in solubility of solute molecules, ion transport and diffusion, osmotic effects on membranes, surface tension, density and the colloidal properties of aqueous matter (Oppenheimer, 1970; Wilkins, 1973).

Cold adaptation is dependant on the adaptive changes in the cellular proteins and lipids in response to the conditions imposed on life by such extremely low temperatures (Russell, 1990). The following sections deal with the definition of lower growth-viable temperature and the range of different adaptations to allow psychrophiles to grow at these temperatures. Mindock *et al.* (2001) suggest two major physical or physio-chemical threats to bacterial survival when undergoing freezing:

1. Cell lysis due to increased water volume as ice.
2. Increased salinity outside of cell leading to an osmotic gradient across the membrane.

### 1.5.1 - Physio-chemical properties of water in confined spaces

Most theories on cold adaptation generally assume that water within a bacterial cell behaves in the same way as bulk water (Mindock *et al.*, 2001). This however is a fallacy. Water in confined spaces (e.g. a bacterial cell) has very different properties to those of bulk water systems (Mindock *et al.*, 2001). This is caused by the effect of an ordered surface upon water. The water molecules become highly ordered inducing a lower freezing point, which alters ionic solubilities, increases viscosity and reduces the dielectric constants compared to those of bulk water (Etzler, 1983). The ordering effect can extend up to 1 μm from the surface, therefore organising the majority of the water within a cell. The ordering of water on a cell membrane can dramatically reduce the freezing point of that water and as such acts as a form of cryo-protection. The highly ordered water near the cell membrane also has reduced solvent properties, thereby forcing solutes towards the centre of the cell causing an increase in solute concentration, thereby lowering the freezing point of the centre of the cell (Mindock *et al.*, 2001).

### 1.5.2 - Lower growth-viable temperature limit of psychrophiles

Reductions in temperature tend to reduce the conformation of cellular macromolecules and other constituents thereby determining the enzymatic reaction rate (Russell, 1990). This is described in the Arrhenius equation (Herbert, 1986):

$$K = A\,\mathrm{e}^{-\mathrm{Ea/RT}},$$

where, Ea is the activation energy, $A$ is a constant relating to steric factors and collision frequency, R is the universal gas constant, T is absolute temperature (K) and $K$ is the relation between reaction rate and temperature for a given enzyme.

The activation energy for most enzymes is 420 K J $mol^{-1}$, hence a fall in temperature from +20°C to 0°C would cause a four fold decrease in activity (enzyme rate constant). This means that temperature decrease does not completely inhibit activity; therefore, why do psychrophiles grow at 0°C and mesophiles not? In order to answer this question, it is important to look at the physical lower temperature limit for life.

There have been no reports of viable growth below -12°C (Russell, 1990). This is consistent with the known physical state of aqueous solutions at sub-zero temperatures (Mazur, 1980). Supercooling of intracellular fluid medium due to the exclusion of ice-nuclei (ice forming particles) via the plasma membrane, can result in an intracellular fluid state at -10 to -15°C (Russell, 1990). However, the intracellular supercooled water does have a higher vapour pressure than extracellular ice, resulting in water moving out of the cell, which causes an increase in solute concentration within the cellular medium (Mazur, 1980). Also extracellular ice formation causes an increase in salinity outside of the cell inducing an osmotic gradient across the cell membrane (Mindock *et al.*, 2001). The generally accepted adaptive response to this is to increase intracellular metabolites, such as glycine betaine, glycerol, mannitol and sorbitol both as cryo-protectants and osmolytes (Franks *et al.*, 1990).

At temperatures below -15°C the cell fluid begins to freeze causing super concentration (up to 3 Molar) of intracellular salts (Russell, 1990). This causes ionic imbalance, altered pH and a reduction in water activity, all of which has a toxic effect on the cell, which either inhibits biological function or kills it (Russell, 1990). Therefore, the lower growth-viable temperature limit is set by the physical properties of the aqueous solute system and not by the conformation properties of the cellular macromolecules (Russell, 1990).

It is the adaptive changes in proteins and lipid molecules, that is the maintenance of functional enzymes and structural molecules, within a cell which form the basis of all cold adaptations (Wilkins, 1973; Russell, 1990; Nichols *et al.*, 1993; Rotert *et al.*, 1993; Feller *et al.*, 1996; Feller & Gerday, 1997; Narinx *et al.*, 1997; Russell & Nichols, 1999). Even though the lower temperature limit to life is not a function of the chemical properties of the intracellular molecules, the ability of psychrophiles to grow at 0°C and

not at 25°C is a function of the adaptive changes of those molecules. Hochachka and Somero (1984) suggested three central biochemical aims for cold adaptation within a cell:

1. Preservation of the structural integrity of macromolecules and assemblies such as membranes.
2. Provision of the necessary energy, metabolites and intracellular environment for metabolism.
3. Regulation of the metabolism in response to the changing needs of the organism.

### 1.5.3 - Cold adaptation in proteins

The following sections outline the major adaptations in cellular proteins which permit biochemical function at cold temperatures. Anti-freeze proteins will be considered in detail because of their central role in this investigation.

#### *1.5.3.1 - Structural adaptations in psychrophilic enzymes*

Protein stability depends on the combined effect of many weak non-covalent interactions between the polypeptide backbone and between the amino-acid side chains (Russell, 1990). The interactions include hydrogen bonds, salt bridges, van der Waals interactions and hydrophobic bonding. Proteins inactivate and eventually destabilize due to loss of conformational entropy between these forces (Mathews, 1987; Gerday *et al.*, 1997). Metabolic enzymes associated with psychrophilic organisms are heat labile (Burton & Morita, 1963). However, proteins are shown to have thermal optima far in excess of the upper growth-viable temperature limit of the organisms themselves (Morita, 1975). The ability of these enzymes to function at temperatures close to or at 0°C appears to be inherent in their structure and not due to the action of other factors (Russell & Hamamoto, 1998). For example, studies of specific *Vibrio* strains have shown a series of amylase enzymes which show deactivation at temperatures in excess of 30°C, optimal activity at 25°C, whilst still providing 10-15% activity at 0°C (Hamamoto & Horikoshi, 1991).

Chessa *et al.* (2000) suggest that 'cold adapted enzymes' are usually characterised by a higher specific activity at low temperature when compared to their mesophilic counterparts, and also by a lower stability against temperature and denaturing agents. Russell & Hamamoto (1998) suggest that 'enzymes from psychrophilic microorganisms should be adapted to remain flexible at low temperatures in order to carry out the

conformational changes needed for catalytic action.' This was corroborated by Zuber (1988), who demonstrated that the psychrophilic lactate dehydrogenase (LDH) had more polar and charged residues and less hydrophobic and ion-paring residues than its mesophilic counterpart, which should give the active site more flexibility, but greater stability, at low temperatures. Recent investigations using protein modelling and X-ray crystallography, have shown that the most frequently encountered changes in protein structure consist of a decrease in the strength of hydrophobic clusters and in the number of salt bridges (Chessa *et al.*, 2000). Other investigations have shown further adaptations including additional glycine residues, a low arginine/lysine ratio, more hydrophilic surfaces and fewer aromatic interactions (Russell & Hamamoto, 1998). Some evidence suggests that the adaptation to low temperature is through alteration in the helix-capping residues (Rentier-Delrue *et al.*, 1993).

Ribosomal proteins in psychrophiles must be specially adapted to cope with protein production rates whilst under cold stress. It has been demonstrated that ribosomes from psychrophiles have special properties which allow them to function efficiently (Krajewska & Szer, 1967). Initialisation of protein synthesis rather than the ribosome itself is the more susceptible factor, as shown when *E.coli* is grown at 0°C causing a block in initiation (Russell, 1990). However, initialisation of protein synthesis is much more robust in response to a sudden drop in temperature in the psychrophile *Pseudomonas fluorescens* (Broeze *et al.*, 1978).

*1.5.3.2 - Cold induced psychrophilic protein production.*

A sudden, rapid decrease in temperature 'cold shock' will induce the expression of a series of cold-shock proteins (CSPs). These are initiated when the house-keeping genes are shut down and the cold-shock genes are transcribed and translated (Russell & Hamamoto, 1998). A similar situation occurs with heat stress, where a heat shock response causes the expression of heat-tolerant proteins. However, whereas the heat shock response, being a general stress response, can be induced by multiple stress events (ethanol, heavy metals, anaerobiosis, etc.), the cold-shock response is only triggered by cold (Russell, 1990). CSPs are expressed not only by psychrophiles, but also mesophiles and thermophiles though at correspondingly higher temperatures.

The most prolific literature on cold-shock proteins relates to the major CSP of *E.coli*, CspA, which is a transcriptional regulator of other cold-shock genes (Jones *et al.*, 1992; Jones & Inouye, 1994; Lee *et al.*, 1994). The synthesis of CspA is tightly regulated at the transcriptional level by low temperature (Tanabe *et al.*, 1992). Homologous proteins have been identified in other bacteria, e.g. CspB in *B. subtilis* (Willemsky *et al.*, 1992) and Antarctic psychrotolerant species (Ray *et al.*, 1994). The CspA protein achieves regulation by binding to the ATTGG sequences in the promoters of cold-shock genes (Russell & Hamamoto, 1998).

After long periods of cold exposure (isothermal growth at low temperature) the initial buffer zone created by CSPs is replaced by a semi-permanent adaptation, identified by cold-acclimation proteins (CAPs) (Russell & Hamamoto, 1998). For more information please refer to Whyte & Innis (1992) and Robert & Innis (1992).

*1.5.3.3 - Ice nucleation proteins*

Ice nucleation proteins or agents (INPs or INAs) act to promote freezing of super-cooled liquids by nucleating embryonic ice crystals before the homogenous ice nucleation event (spontaneous formation of ice nuclei at sub-zero temperatures during supercooling) (Warren & Wolber, 1987; Feeney, 1988; Warren & Wolber, 1991; Edwards *et al.*, 1994; INPs reviewed in Warren, 1994; Gehring *et al.*, 1998). Small volumes of water, such as those in cells, will not spontaneously freeze until they reach a temperature of –38°C (Cwilong, 1945); at this temperature the physiochemical processes in the cell will be irreparably damaged. To prevent this, the cell needs to initiate freezing at a higher temperature (Feeney and Yeh, 1993). The ability to nucleate ice at warmer temperatures gives the bacteria a selective advantage by allowing sufficient cellular dehydration (Edwards *et al.*, 1994) and warmer temperature will allow controlled propagation of freezing instead of a rapid freezing event which could cause cell rupture (Baertlein *et al.*, 1992). However, in order to initiate the phase transition from liquid to solid there needs to be the chance formation of a homogenous ice nucleation event in which water molecules align as they would in ice (Edwards *et al.*, 1994). This transition can occur at warmer temperatures (-12°C, Yankofsky *et al.*, 1997) when catalysed by a non-aqueous agent in a heterogeneous ice-nucleation event (Duman *et al.*, 1985; Edwards *et al.*, 1994; Wilson & Leader, 1995). The ice-forming agents within ice-nucleation active

(INA+ phenotype) bacteria are the most temperature-effective natural ice nuclei after ice itself (which nucleates at 0°C under standard conditions (Yankofsky *et al.*, 1997)). There are a large number of other naturally occurring ice nucleators including detritus (Upper & Vali, 1995), spider silk (Murase *et al.*, 2001) and soot particles (Gorbunov *et al.*, 2001). The INA+ phenotype has been identified in three Gram negative genera: *Erwina*, *Pseudomonas* and *Xanthomonas*. The protein itself is homologous between the genera, forming a beta-helical structure which could interact with water through the repetitive TXT motif (Graether & Jia, 2001). Water molecules are arranged into an ice-like array as the membrane bound heteronucleator (INP) acts as a template that mimics the structure of ice. INA+ bacteria are generally epiphytic and are often ubiquitous in agriculturally important crops. They play a significant role in causing frost damage by promoting ice formation at a higher temperature than would occur if normal super cooling temperatures (-4°C to-12°C) were allowed to occur, accounting for an excess of $1 billion in crop injury in the United States per year (Edwards *et al.*, 1994). As a result of this, much research has been performed to elucidate the mechanism behind ice nucleation in an effort to control or prevent crop damage by these bacteria. Research into crop protection led to the marketing of a number of commercial products including those used in the prevention of frost (Frostban™), manufacture of snow (Snomax®), reduction of the incidence of fire blight (Blightban®) and as an aid for food concentration and texturing (Skirvin *et al.*, 2000). For a comprehensive review of the history of ice nucleation and nucleation function please refer to Skirvin *et al.* (2000).

*1.5.3.4 - Anti-freeze proteins*

*1.5.3.4.1 – AFP structure and function*

Ice-nucleating proteins induce ice formation but anti-freeze proteins inhibit ice formation, and in some cases have been shown to inhibit the action of INPs (Parody-Morreale *et al.*, 1988). Anti-freeze proteins (also known as thermal hysteresis proteins) are a structurally diverse class of proteins, which all have the ability to bind to the surface of ice and inhibit its recrystalisation (Yeh & Feeney, 1996; Davies & Sykes, 1997; Smaglik, 1998). They act at the surface of ice by lowering the freezing point below the melting point (Davies & Sykes, 1997; Knight & DeVries, 1994; Haymet *et al.*, 1998), a

process known as thermal hysteresis. This mechanism is achieved non-colligatively, that is, due to the mechanism of the protein not by the concentration of particles. However, AFPs generally show a concentration-dependant effect on ice-crystal morphology, where low concentrations tend to produce hexagonal crystal morphologies and high concentrations tend to produce hexagonal bi-pyramidal and spicule morphologies (Meyer *et al.*, 1999). AFPs were first identified in Antarctic notothenoid fish in 1968 by DeVries, who noted that the fish were able to depress their freezing point below that of the surrounding seawater using non-colligative mechanisms (DeVries, 1971). This led to the isolation of the antifreeze glycoproteins (AFGPs) found in Southern ocean fish off the coast of Antarctica (Feeney & Yeh, 1993). Further work isolated four different AFPs, Type I, II, III and IV (which lack the glycotripeptide unit found in AFGPs) from polar fish (DeVries, 1971; Evans & Fletcher, 2001) and two types of thermal hysteresis proteins in some insects (Duman, 1977; Duman *et al.*, 1991; Graham *et al.*, 1997; Barrett, 2001), plants (Griffith *et al.*, 1992; Hon *et al.*, 1994; Meyer *et al.*, 1999), fungi (Duman and Olsen, 1993; Griffith & Ewart, 1995) and bacteria (Duman and Olsen, 1993; Sun *et al.*, 1995) (Table 1.1 outlines the five major AFPs and their structural diversity). The most researched AFP is the 37-residue, 3-5 kDa alanine rich (>60 mol%, Evans & Fletcher, 2001), amphipathic α-helical type I polypeptide from the winter flounder (*Pseudopleuronectes americanus*) (DeVries and Lin, 1977), shorthorn sculpin (*Myoxocephalus scorpius*) (Scott *et al.*, 1987) and grubby sculpin (*Myoxocephalus aeneus*) (DeVries & Cheng, 1992), and more recently the Atlantic snailfish (*Liparis atlanticus*) and dusky snailfish (*L.gibbus*) (Evans & Fletcher, 2001). In the winter, flounder AFP I is synthesized in the liver and then excreted into the blood to provide extracellular cryo-protection. This fish also produces the type I protein in its skin (Gong *et al.*, 1996).

The ice-crystal growth inhibition mechanism of Type I AFPs can be used to help describe the inhibitory mechanism for all AFPs. Firstly, it may be necessary to redefine the characteristics of type I AFPs. These AFPs have been described as small helical proteins/polypeptides that have a specific 11 amino acid repeating motif (Thr-$X_2$-Asn/Asp-$X_7$), where X is usually alanine but can be replaced by any amino acid that favours a helical formation (Fletcher *et al.*, 2001). However, based on current evidence

which shows type I AFPs considerably larger than previously thought and without the 11 amino acid repeating motif (Evans & Fletcher, 2001), it is best to simplify the characteristics that allow differentiation of type I proteins to those with a high alanine content and an α-helical secondary structure. Despite the structurally diverse nature of AFPs, they all tend to present a large proportion of their surface area exclusively for ice binding (Jia & Davies, 2002). It has been suggested that ice binding in AFPs is achieved by the matching of the ice-crystal lattice structure with the repeating amino acid sequence via polar residue interactions through hydrogen bonding, while the hydrophobic side is exposed to the water molecules inhibiting crystal growth (Fletcher *et al.*, 2001).

| **Type of Protein** | **Structure** |
|---|---|
| Type I | Low molecular weight proteins consisting of alanine rich amphipathic α-helices. |
| Type II | Similar to the carbohydrate-recognition domain of C-type lectins (see Drickamer, 1999), they are extensively disulphide bonded and contain two α-helices and two β-sheets. |
| Type III | Small, non-helical, globular proteins with a large hydrophobic core, several short β-sheets and one helical turn. |
| Type IV | 108 amino acids with a pyroglutamyl group blocking the end terminus. It is a four-helix bundle structure. |
| AFGPs | Polymers of an Ala-Ala-Thr repeat motif that is glycosylated at every threonyl hydroxy group with a disaccharide residue essential for AFP activity. |

**Table 1.1 - Types of AFPs and AFGP and their structures. Demonstrating the structural diversity in AFPs. Types I, II, III and AFGP descriptions were taken from Meyer *et al.* (1999), Type IV description taken from Zhao *et al.* (1998).**

However, more recent investigations have shown that non-polar residues may also be involved in the ice binding mechanism (Chao *et al.*, 1997). The most recent studies have postulated that the motif, previously suggested as directly associated with ice binding may actually play a functional role in protein solubility, and the hydrophobic site of the protein may bind to the ice via hydrophobic interactions of alanine residues (Evans & Fletcher, 2001). Zhang and Laursen (1999) suggested that the hydrogen bonding and hydrophobic interactions of the threonine sidechains and the interactions of the lysine residues may play specific roles in antifreeze activity. Haymet *et al.* (2001) also suggest that detailed simulations of the interactions of the diffuse ice/water interface do not support the original hydrogen bonding theory. In fact, through the creation of hydrophobic mutants they show that hydrophobic interactions play a significant, perhaps

dominant, role in the ice-growth inhibition mechanism (Warren *et al.*, 1993), whereas Baardsines *et al.* (1999) and Jia and Davies (2002) both suggest that methyl groups on the threonine residues might interact with ice via Van der Waals forces and play a greater role in binding that hydrogen bonding. There is still no conclusive theory to encapsulate the mechanism of AFP activity, however, there are many suggested theories (Burcham *et al.*, 1986; Knight *et al.*, 1988; Parody-Morreale *et al.*, 1988; Feeney & Yeh, 1993; Wilson *et al.*, 1993; Chao *et al.*, 1994; Laursen *et al.*, 1994; Dunker *et al.*, 1995; Sicheri & Yang, 1995; Wilson & Leader, 1995; Jia *et al.*, 1996; Madura *et al.*, 1996; Davies & Sykes, 1997; Haymet *et al.*, 1998; Meyer *et al.*, 1999; Grandum *et al.*, 1999, Evans and Fletcher, 2000; Jia & Davies, 2002).

*1.5.3.4.2 – Bacterial AFPs*

To date, only four bacterial strains have been characterised with AFP activity, *Pseudomonas putida* (Sun *et al.*, 1995), *Micrococcus cryophilus* and *Rhodococcus erythropolis* (Duman & Olsen, 1993) and *Marinomonas protea* (Mills, 1999). Sun *et al* (1995) showed that *P. putida* GR12-2 isolated from the rhizobia of plants in the high arctic, Canada, synthesised a protein with antifreeze activity. It was also suggested that the *P. putida* AFP may be similar to type II AFPs found in insects and plants, because it is a glycine- and alanine-rich glycoprotein (164kDa) with intermolecular disulfide bridges (Sun *et al.*, 1995; Ewart & Fletcher, 1990). The *P. putida* AFP was not fully characterised by Sun *et al.* (1995) and as such no definitive statements can be made. Duman and Olsen (1993) have also shown AFP activity in the well known psychrophiles *M. cryophilus* and *R. erythropolis*. Mills (1999) shows activity in the Antarctic psychrophile *M. protea*. To date there has been no analysis of AFP type from either *M.cryophilus, R.erythropolis* or *M.protea* (Duman & Olsen, 1993; Mills, 1999; Barrett, 2001). It is interesting to state that *P. putida*, *M. cryophilus* and *M. protea* all belong to the gamma sub-division of the superclass Proteobacterium (purple bacteria and relatives).

*1.5.3.4.3 – AFPs in biotechnology*

Biotechnology companies value AFPs for use in food such as ice cream (Sutton *et al.*, 1994; Feeney & Yeh, 1998), frozen vegetables and meats (to maintain texture and prevent nutrient leakage) (Payne *et al.*, 1994; Griffith and Ewart, 1995; Barrett, 2001).

cryopreservation and hypothermic storage of cold sensitive cells (Arav *et al.*, 1993; Wu & Fletcher, 2000; Barrett, 2001), farming (to improve cold tolerance in plants and animals) (Hew *et al.*, 1992; Griffith and Ewart, 1995), cryo-surgery (Koushafar *et al.*, 1997) and industry, for example the maintenance of acceptable ice-slurry characteristics for low-temperature energy storage and transport systems (Inada *et al.*, 2000). AFPs are very expensive to farm from fish, for example AFPs from Newfoundland fish are around $50 per gram (Feeney and Yeh, 1998). Carrot AFPs have been expressed in certain transgenic crops endowing the plants with thermal hysteresis activity and hence resistance to frost damage (Griffith and Ewart, 1995). Both these methods are problematic in terms of financial cost, sustainability or the controversial issue of genetically modified foods and fish stocks (based on ecological impact of GM fish farms (Feeney & Yeh, 1998)). Artificial type I AFPs have been synthesised (Barrett, 2001) and types I, II and III have been manufactured using recombinant techniques in both prokaryotic and eukaryotic systems (Duncker *et al.,* 1995). Transgenic expression of AFPs have been used to limited success because firstly there is a lack of suitable processing enzymes to convert the primary protein to its active form, and secondly there are generally low levels of protein production (winter flounder AFP levels – 8-15mg/ml of blood; transgenic fish – 2-30μg/ml) (Barrett, 2001). Bacterial AFPs would be an overall more attractive alternative. Not only are they cheap to produce and environmentally friendly (thousands of gallons of culture can be produced in hermetically sealed units at low production cost), but they are also an easy to cultivate natural source of AFP. However, only four AFP active bacterial strains have been isolated and characterised to date (Duman and Olsen, 1993; Sun *et al.*, 1995; Mills, 1999). The discovery of further AFP active bacteria would be extremely beneficial to the biotechnology industry. For a detailed review of the biotechnological applications of AFPs please refer to Griffith & Ewart (1995) or Barrett (2001).

*1.5.3.4.4 - Evolution of AFPs*

The five categorised AFP types are remarkably diverse in their 3D structures, having completely dissimilar folds and no sequence homology (Barrett, 2001). Even more distinct AFPs exist in plants and insects (Baardsines & Davies. 2001). One

explanation for this diversity is the number of different faces ice can present for binding, allowing for a great number of different protein structures to evolve for the same purpose (Baardsines & Davies, 2001).

The evolution of fish AFPs has been extensively researched (Scott *et al.*, 1985; Scott *et al.*, 1987; Davies *et al.*, 1993; Chen *et al.*, 1997; Davies and Sykes, 1997; Logsdon & Doolittle, 1997; Cheng, 1998; Meyer *et al.*, 1999; Baardsines & Davies, 2001). There are only a small number of structurally distinct proteins that act as AFPs. Their random distribution among bony Teleosts has been suggested as evidence for their recent, parallel and convergent evolution (Scott *et al.*, 1986; Davies *et al.*, 1993; Barrett, 2001). This was probably in response to the relatively recent cooling of the planet, leading to extensive glaciation around $10^6 - 10^7$ years ago (Davies and Sykes, 1997). Davies and Sykes (1997) suggest that certain fish proteins probably had an affinity for ice, and then by gene mutation and/or gene amplification in combination with natural selection, AFPs evolved within certain taxa. The appearance of two different types of AFP within two closely related taxa (as seen with AFP IV in *Myoxocephalus* spp.) suggests difficulties in predicting the presence of specific AFPs based on phylogenetic relationships (Davies and Sykes, 1997). There are also numerous examples of convergent evolution, for example AFGPs in Antarctic and Arctic species (Chen *et al.*, 1997; Cheng, 1998). The implication is that there are only a certain number of protein structures that are capable of altering the structure of ice to produce thermal hysteresis. The number of possible progenitors is therefore limited, since the majority of proteins have no affinity for ice. It is suggested that AFGPs in Antarctic notothenoid fish were derived from the pancreatic trypsinogen gene (Chen *et al.*, 1997). A recent discovery of a chimaeric antifreeze glyco-protein (AFGP)-protease gene in the giant Antarctic toothfish (*Dissostichus mawsoni*) could represent an evolutionary intermediate between the trypsinogen and AFGP (Cheng & Chen, 1999). High sequence homology between AFGP and notothenoid trypsinogen suggests a recent evolution (5-14 mya), this time frame being consistent with the divergence of notothenoids between 7-15 mya, which coincided with the cooling of the Antarctic ocean (10-14 mya) (Barrett, 2001). The AFGP in the northern cod shows homology with the AFGP in the Antarctic notothenoid fish, save the occasional Thr to Arg substitution in the northern cod (Chen *et al.*, 1997). However, in

the northern cod the AFGP shows no sequence homology with the trypsinogen gene (the precursor of the northern cod AFGP is therefore unknown), suggesting remarkable convergent evolution of AFGP from the northern cod and Antarctic notothenoid from two totally different precursor genes (Barrett, 2001). Type III fish AFPs show remarkable similarity to the C-terminal region of the mammalian sialic acid synthetase protein (Baardsines & Davies, 2001), type II show similarity to the carbohydrate recognition domain of the $Ca^{2+}$ dependant lectins (Meyer *et al.*, 1999), and type IV proteins are closely related to a helix-bundle serum apolipoprotein (Baardsines & Davies, 2001). These protein progenitors of the AFPs all have interactions with sugars and polysaccharides (Baardsines & Davies, 2001).

An example of plant AFP evolution was shown by Meyer *et al.* (1999) who demonstrated that carrot AFP shared significant sequence identity (50-65% of the sequence was shared between the proteins) with the polygalacturonase inhibitor proteins (PGIPs) family of plant leucine-rich repeat (LRR) proteins. Pathogenic resistance in plants is mainly the result of selection for hypervariability in the LRR domain of R-genes (those concerned with pathogenic resistance) (Meyers *et al.*, 1998). Therefore, if there is selection for variability in the LRR domain of resistance genes, there could also be an evolutionary selection for variability in the LRR region of PGIPs. Meyer *et al.* (1999) suggested that this evolutionary mechanism for divergent selection has at some point produced a PGIP-derived protein, which binds to the non-proteinaceous ligand ice, and therefore would have acted as an ideal template for a progenitor of AFPs (Meyer *et al*, 1999). Meyer *et al.* (1999) have also isolated a cold-induced PGIP-like protein from carrot that has 75% homology with the carrot AFP gene. This suggests that this protein could be a progenitor of carrot AFPs.

The apparent lack of taxa-specific AFP activity in fish does not preclude the possibility that bacteria may show taxa-specific AFP activity. Understanding phylogenetic relationships between AFP producers will enable further isolation of novel AFP active bacteria. If AFP activity is shown to be within distinct taxa then it may be possible to identify bacteria from environmental samples using genus or species specific DNA probes with techniques such as fluorescent *in situ* hybridisation (FISH) (Amann *et al.*, 1995; Ekong & Wolfe, 1998)

*1.5.3.5 - Membrane bound solute transport proteins*

Most microorganisms alter their membrane lipid composition as an adaptation to ambient temperature fluctuations. Such adaptations allow regulation of the solute transport system and the continued functioning of essential membrane bound proteins. It has been suggested that the minimum temperature for cell growth in mesophilic organisms is controlled by the low temperature inhibition of the solute transport mechanism (Morita, 1975). Baxter and Gibbons (1962) have demonstrated this in a comparison of respiratory activity between a psychrotrophic *Candida* sp. and a mesophilic strain of *C.lipolytica*. The psychrotroph was able to oxidize exogenous glucose at 0°C, where the mesophile showed no metabolic activity below 5°C, but was able to metabolise the glucose endogenously. This indicates that the low temperature had an inhibitory effect on transportation of glucose into the cell (Herbert, 1986).

Most studies since have shown that temperature has little or no effect on sugar transportation in the psychrophilic *Candida* spp. These studies have been confirmed to a wide range of Gram negative and positive bacteria with the uptake of 2-deoxy glucose and L-leucine (Wilkins, 1973). However, Herbert and Bell (1977) reported that *Vibrio* AF-1, a psychrophilic bacterium isolated from an Antarctic lake, demonstrated maximal [$^{14}$C]glucose and [$^{14}$C]lactose uptake rates at 0°C which decreased with an increase in temperature (Herbert, 1986).

Farrel and Rose (1967) postulated a series of mechanisms which might help to describe the differences of transport processes between mesophiles and psychrophiles. Only one of these postulates can be substantiated; it states that 'solute carrier proteins in mesophilic microorganisms are not abnormally cold sensitive but, owing to changes in the spatial organisation of the lipid bilayer, solute molecules are unable to combine with their respective carrier proteins'. As a result, most studies to date have concentrated not on the cold adaptability of the membrane bound proteins themselves but instead on the adaptations concerning membrane lipid composition and the liquid-crystalline state of the lipid bilayer of the cell membrane (Herbert, 1986).

**1.5.4 - Cold adaptation in membrane lipids**

Maintaining the optimal degree of fluidity of the lipid component of the cell membrane is important for normal functioning (Rotert *et al.*, 1993; Chattopadhyay &

Jagannadham, 2001). A decrease in temperature can cause formation of deleteriously strong lipid-lipid interactions (Russell & Hamamoto, 1998) causing rigidity through loss of liquid-crystalline phase which leads to loss of membrane transport mechanisms (impairing ion and solute regulation) and can cause membrane rupture.

The most important response of membrane lipids to temperature change are alterations in the fatty acyl composition (Russell, 1984, 1990). A decrease in growth temperature can cause one or a combination of the following responses:

- Increase in unsaturation
- Decrease in average chain length
- Increase in methyl branching
- Increase in the ratio of anteiso branching relative to iso branching. (Russell & Hamamoto, 1998).

Unsaturation of the fatty acids is the most common adaptation to cold stress (Rotert *et al.*, 1993; Russell & Hamamoto, 1998; Russell & Nichols, 1999, Mindock *et al.*, 2001), but changes in chain length frequently occur. Changes in methyl branching are found only in bacteria and mostly in Gram positive genera. As a rule Gram negative genera employ unsaturation and acyl chain length alteration in cold adaptation (there are relatively few Gram negative genera containing branched fatty acids), whereas Gram positive genera tend to alter the amount and type (anteiso/iso) of methyl branching whilst also utilising changes in chain length and unsaturation.

The majority of bacteria do not contain polyunsaturated fatty acids (PUFAs; Rotert *et al.*, 1993; Russell & Hamamoto, 1998; Russell & Nichols, 1999) therefore the above changes relate to monounsaturated fatty acids. Until 1977 it was considered that non-photosynthetic bacteria did not produce PUFAs (for a review of the history of discovery of polyunsaturated fatty acids refer to Russell & Nichols, 1999). However, since 1977 there have been numerous reports of PUFA in a range of eubacterial species (Johns & Perry, 1977; Delong & Yayanos, 1985; Wirsen *et al.,* 1987; Temara *et al.,* 1991; Intrigo, 1992). PUFAs are mainly found in psychrophilic bacteria, examples of which show an increase in PUFA with a decrease in temperature (Russell, 1990). Some examples of the complexity of adaptive mechanisms shown by Antarctic psychrophilic bacteria is provided by Nichols *et al* (1997). The presence of PUFAs in Antarctic psychrophiles has

been well established (Russell & Nichols, 1999), however, not all marine psychrophiles have PUFAs. It is therefore doubtful that this is a response to exposure to constant low temperature (-1.9°C). It seems more likely that, because the proportion of PUFAs in membrane lipids change with variations in temperature and may be maximal at the lowest temperature (Hamamoto, *et al.*, 1994; Russell & Nichols, 1999), they are part of a homoviscous adaptive response to maintain the correct membrane fluidity phase (Russell & Nichols, 1999). It appears that the increase in double bond formation with the PUFAs causes a 'hairpin' effect which reduces the effective acyl chain length (Keough & Kariel, 1987) providing a high degree of packing order whilst preventing the formation of an hexagonal-II phase packing order (non-bilayer phase, which would inhibit membrane permeability and kill the cell) and allowing sufficient molecular motion to lower the liquid-crystalline phase transition temperature (Russell & Nichols, 1999).

Another thermal adaptive mechanism for microbial lipids is the isomeric distribution of acyl chains between the *sn*-1 and *sn*-2 positions of the phospholipids which can alter their gel-to-liquid-crystalline phase transition temperature by up to 7°C (Russell, 1990). This combined with a high level of unsaturation can reduce the phase transition temperature to well below 0°C. An example of this adaptive ability can be seen in the psychrophilic bacterium, *Psychrobacter urativorans* (isolated from the Vestfold Hills, Antarctica), which shows the *sn*-1/*sn*-2 distribution of fatty acyl chains (permanently not just in response to temperature change) which provides it with a maintained lower-melting-point phospholipid state even after a sudden reduction of temperature (McGibbon & Russell, 1983).

*1.5.4.1 - Biochemical mechanisms of fatty acid induction*

Adaptations of the membrane lipid composition are important for enabling them to compete successfully within their ecological niche (Russell & Hamamoto, 1998). The ability to alter fatty acyl composition and the speed with which it can be achieved, are related to the biomechanism. Short term changes (aerobic desaturation) and long term changes (anaerobic desaturation, chain length and branching) can all be understood by looking at the biomechanism (Russell, 1984).

The anaerobic desaturation pathway involves the insertion of a double bond using fatty acid synthetase producing both saturated and unsaturated acids (Harwood and

Russell, 1984). The most important low temperature active thermo-regulation enzyme in this pathway is β-ketoacyl-ACP synthetase II, which elongates palmitoleate to *cis*-vaccenate. As the temperature falls, more carbon flows down the unsaturated arm relative to the saturated arm of the split pathway (Russell & Hamamoto, 1998). This increases unsaturated fatty acids and consequently diunsaturated phospholipids, causing the transition temperature of the membrane to fall (de Mendoza & Cronan, 1983). The pathway divergence occurs within seconds of any temperature reduction. However, once fatty acid synthetase products have been produced, they must be incorporated into the membrane lipids and this takes much longer (due to cellular growth and membrane formation) (Russell & Hamamoto, 1998).

Desaturases (aerobic pathway) can introduce double bonds into fatty acids much faster than the anaerobic pathway. They have been shown to use intact acyl lipids as their substrate for double bond insertion in bacteria and algae (Foot *et al.*, 1983). Therefore, because the fatty acids do not have to be made *de novo* the system is more rapid. The desaturase is activated by a decrease in membrane fluidity which is rapidly restored as exisiting fatty acids are desaturated (Russell & Hamamoto, 1998).

Other mechanisms for transition temperature reduction (i.e. changes in fatty acyl chain length and the amount or type (anteiso/iso) of methyl branching) must be introduced by the production of new fatty acids, because methyl branching is reliant upon the primer molecule and the chain length is governed by the termination mechanism (Harwood & Russell, 1984). The mechanism of temperature regulation of activity/specificity of fatty acid synthetase is not well understood (Kaneda, 1991), however for a review of current theories refer to Russell & Hamamoto (1998) and Russell & Nichols (1999).

**1.6 - Biodiversity and biogeography of bacteria**

There have been numerous extensive studies on microbial diversity and ecology within Antarctic ecosystems (Hand, 1980; Hand & Burton, 1981; Wright & Burton, 1981; Burke & Burton, 1988; Franzmann *et al.*, 1990; Mancuso *et al.*, 1990; Franzmann & Rohde, 1991, 1992; Laybourn-Parry & Marchant, 1992b; James *et al.*, 1994; Ellis-Evans, 1985a; Laybourn-Parry *et al.*,1996; Laybourn-Parry, 1997; Ellis-Evans *et al.*, 1998; Murray *et al.*, 1998; Nichols *et al.*, 1999; Bowman *et al.*, 2000a and b; Waters *et al.*, 2000; Brown

& Bowman, 2001; Labrenz & Hirsch, 2001). The current study attempts to describe the phenetic relationships between numerous bacteria from a suite of lakes in relation to cold adaptation. The following sections describe the molecular methods used to assess bacterial biodiversity and biogeography.

### 1.6.1 - Bacterial biodiversity

Biodiversity is the irreducible complexity of life. Staley and Gosink (1999) refer to it as the variety and abundance of life forms that live on Earth. Biodiversity is the diversity of units of life, the basic unit being the species. However, it is also measured as intraspecific genetic variability and by the richness of evolutionary lineages at high taxonomic levels (Staley & Gosink, 1999). Bacterial biodiversity is one of the most challenging aspects of microbiology. Traditionally, microbial diversity has been assessed using phenetic characterisation of a pure culture by determination of physiology and morphology (Priest & Austin, 1995) and by the use of biochemical tests, serotyping and enzymatic products (Jayarao *et al.*, 1992a). This approach is limited because of its inability to determine inter-species evolutionary relatedness (Staley & Gosink, 1999) and since traditional culturing techniques are not capable of isolating all bacteria from an environmental sample, there results a massive under-estimation of diversity in natural habitats (Staley & Gosink, 1999). Within the past twenty years there has been a gradual shift towards using molecular techniques to identify bacterial strains, which has revolutionised our concept of microbial ecology (Olsen *et al.,* 1986; Pace *et al.*, 1986). Woese (1987) pioneered the use of the RNA from the small ribosomal subunit, 16s or 18s rRNA. This allowed not only a phylogenetic tree to be produced to show bacterial evolutionary relatedness, but also provided a basis for revolutionising phenetic identification (Priest & Austin, 1995). Environmental monitoring has been dramatically improved with the use of direct nucleic acid assessment from environmental samples without prior culturing (van Elsas *et al.*, 2000). Woese (1987) described 12 divisions within the Eubacteria based on the analysis of cultivated organisms, but with the use of culture-independent phylogenetic surveys of environmental microbial communities the number of identifiable bacterial divisions has increased three fold to 36 (Pace, 1997; Hugenholtz *et al.*, 1998). There are a number of areas which have been significantly stimulated by the introduction and development of nucleic acid approaches:

1. Monitoring indigenous microorganisms of interest from an ecological or biotechnological perspective.
2. The study of natural microbial diversity and of factors that disturb that diversity,
3. The need to monitor genetically modified microorganisms based on their unique nucleic acid sequences.
4. The assessment of the expression of specific microbial genes in the environment (van Elsas *et al.*, 2000).

These molecular techniques are used to supplement the standard physiological information, which can then be used to improve accuracy of bacterial taxonomy. A more accurate understanding of bacterial taxonomy will enable a more effective understanding of microbial ecology, total global biodiversity and species richness, and will help to target bacterial taxa for exploitation by the biotechnological industries.

**1.6.2 - Bacterial biogeography**

Biogeography is the study of the global distribution of species living or extinct (Staley & Gosink, 1999). The basic premise is to map out the location of individual species within the geographic area. Species which are shown to exist in more than one geographic location are known as 'cosmopolitan' and those which are found in only one area are known as 'endemic' (Staley & Gosink, 1999). The smaller the area the better the bio-geographical resolution. Biogeography enables the study of how the environmental conditions influences species distribution. In reference to this current study, it can demonstrate how bacteria are distributed among the different Antarctic lakes based on the different environmental parameters associated with each body of water.

**1.6.3 - Molecular typing techniques**

The following section describes some of the major molecular techniques available for the phenetic/phylogenetic analysis of bacteria.

*1.6.3.1 – 16S rDNA sequencing*

Using sequencing techniques it is possible to elucidate the precise order of nucleotides in a block of DNA (Brown, 1996). The most common DNA sequencing method is the chain termination method developed by Sanger *et al.* in the late seventies (Sanger *et al.,* 1977, QIAGEN, 1995). The DNA of interest acts as a template for the enzymatic synthesis of new DNA starting at a defined primer binding site (Brown, 1996).

The reaction mix contains both deoxynucleotides (A, T, C & G) and dideoxynucleotides at concentrations which produce a finite probability that a dideoxynucleotide will be incorporated into each nucleotide position in a growing chain. The incorporation of a dideoxynucleotide inhibits further elongation, hence chain termination. This produces a number of truncated chains of varying length. This can then be read in four different reactions (ddATP, ddCTP, ddTTP, and ddGTP) to produce the order of nucleotides in the original template. Alternatively, a nucleotide-specific label can be attached to each dideoxynucleotides and the reaction read off on a polyacrylamide gel (QIAGEN, 1995).

The template used is generally the 16s rRNA gene. This gene is involved in the structure of ribosomes and the reading of mRNA for the production of proteins. There are three rRNAs used for taxonomic identification of bacteria; the 5S, 16S and 23S molecules. They are used to indicate relatedness in bacteria because they are present in all bacterial species. They have extensive regions of conserved sequence allowing relatedness to be assessed between species which otherwise share no sequence similarity, showing enough variation to allow differentiation of even closely related species. This allows phylogenetic relationships (suggested evolutionary relationships) to be inferred from the variations of these genes (Priest and Austin, 1995). The 16S rRNA molecule is generally accepted as the molecule upon which most of modern bacterial taxonomy is based (Priest & Austin, 1995; Palys *et al.,* 1997; Dalevi *et al.,* 2001). This is because the 5S molecule is too small (116-120bp) as it undergoes marked mutational changes which would be obscured by long sections of conserved sequence in the 16S rRNA, and the 23S molecule is too large to be easily sequenced (Priest and Austin, 1995). Over the last seven years, since the publication of the first bacterial genome sequence, over 60 bacterial genome sequences have been published and there are more than 200 current research projects concerning bacterial genome sequencing (Suerbaum *et al.*, *in press*). However, the 16S rRNA gene is still used as the primary identification tool for culture-dependant and –independent bacterial characterisation, this is exemplified by the presence of more than 30,000 16S rDNA sequences available in the main ribosomal databases, GenBank, EMBL and DDBJ (Dalevi *et al.,* 2001). When using the 16s rDNA gene, the amplified product can either be directly sequenced (Bevan *et al.*, 1992; Kocher,

1992), or cloned into another bacterium (e.g. *E.coli*) via a plasmid, and then sequenced directly from the plasmid (Brown, 1996).

*1.6.3.2 - Amplified ribosomal DNA restriction analysis (ARDRA)*

ARDRA uses the sub-species specific variations in the highly conserved 16S ribosomal RNA gene to delineate phenetic relationships between bacteria (Vaneechoutte *et al.*, 1995; Vila *et al.*, 1996; Ingianni *et al.*, 1997; Jawad *et al.*, 1998; Vinuesa *et al.*, 1998; Versalovic *et al.*, 1998). The 16S rDNA fragment is amplified from genomic DNA using PCR. The fragment is then digested using endonucleases to produce a sub-species specific DNA profile (Rademaker & de Bruijn, 1997). ARDRA was chosen based on its simplicity and flexibility because a large number of unknown specimens needed to be typed. ARDRA is an ideal tool for typing novel bacterial species (Ingianni *et al.*, 1997) and can be of help in elucidating the ecological significance of specific bacteria (Vaneechoutte *et al.*, 1993).

*1.6.3.3 - Southern hybridisation (ribotyping)*

Genomic DNA is digested using endonucleases and then run on an electrophoresis gel which is transferred (blotted) onto a nylon membrane. The DNA on the 'blot' is then hybridised with labelled 16S / 23S rDNA ($^{32}P$ or digoxigenin). The labelled DNA is then detected providing a unique fingerprint pattern for individual species or even strains. The technique was pioneered by Grimont and Grimont (1986). Whereas other randomly primed or restriction enzyme methods of total bacterial DNA (RFLP, rep-PCR, AP-PCR, etc.) produce numerous bands which all have to be resolved to enable sufficient species differentiation to be assessed, ribotyping produces only a small number of distinct bands allowing discrimination of individual species and sub-species (Grimont & Grimont, 1986).

*1.6.3.4 - Restriction fragment length polymorphism (RFLP/T-RFLP)*

Comparative analysis of 16S rDNA has shown that highly conserved sequences are interspersed with regions of variable sequences (Jayarao *et al.*, 1992b). Analysis of the variable regions can be used to determine the phylogenetic and evolutionary relationships between bacteria. This technique can be used to determine strain specific sequences between bacteria (Woese, 1987). Termination restriction fragment length polymorphism analysis (T-RFLP) is used to analyse the variable sequence regions of

gene mixtures and hence can be used to assess diversity within a simple bacterial community (Dunbar *et al.*, 2000).

*1.6.3.5 - Amplified fragment length polymorphism (AFLP)*

AFLP involves the digestion of total purified genomic DNA using a restriction endonucleases (Vos *et al.*, 1995; Janssen *et al.*, 1996; McLauchlin *et al.*, 2000: Rademaker *et al.*, 2000). The digest products are then ligated to a double stranded oligonucleotide adapter which is complementary to the base sequence of the restriction site (Zabeau & Vos, 1993). Selective PCR of the fragments is achieved using primers which correspond to the adapter/restriction site sequence. These sequences are then analysed by gel electrophoresis (McLaughlin *et al.*, 2000; Zhao *et al.*, 2000). This allows characterisation of the bacterial isolates under investigation to the species, sub-species and strain level (Rademaker & de Bruijn, 1997).

*1.6.3.6 - AP-PCR and RAPD*

Arbitrarily primed PCR (AP-PCR) (Welsh & McClelland, 1990) and random primed amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) are based on the amplification (using the polymerase chain reaction) of multiple DNA fragments from genomic DNA with random or arbitrary primers. This results in a characteristic pattern of amplicons for each strain of a given species. Thus these techniques permit the differentiation of isolates to the species, subspecies and strain level.

*1.6.3.7 - rep-PCR, ERIC-PCR and BOX-PCR*

These techniques rely on the amplification of conserved repetitive sequences found within the intergenic regions at sites dispersed throughout the genome of bacteria (Versalovic *et al.*, 1994). Such regions of noncoding, repetitive sequences can be used for multiple genetic targets for oligonucleotide primers, enabling the generation of unique DNA profiles or fingerprints for individual bacterial strains (Versalovic *et al.*, 1994; Rademaker & de Bruijn, 1997; Louws *et al.*, 1998; Rademaker *et al.*, 1998; Rademaker *et al.*, 2000). ERIC-PCR utilises amplification of the enterobacterial repetitive intergenic consensus, rep-PCR utilises the repetitive extragenic palindromes and BOX-PCR amplifies a BOX element found mainly in Gram positive bacteria (Tas & Lindström, 2000). The genomic fingerprints produced using these techniques allow the determination

of bacterial isolates to the species, subspecies and strain level (Rademaker & de Bruijn, 1997).

*1.6.3.8 - DGGE and TGGE*

The differentiation of multiple species from an environmental sample can be performed by analysing base pair variations within a specific gene common to all species. Microbial community diversity can be analysed by PCR amplification of the variable V3 region of the 16S rRNA gene (Neefs *et al.,* 1990) from the total community genomic DNA. This provides numerous copies of the V3 regions from every bacterial species in the community (unless selective amplification occurs), which are then separated on a gel by denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) (Muyzer *et al.*, 1993; Muyzer & Smalla, 1998). The resulting fingerprint indicates the major species within a given ecosystem and can be used to show temporal changes in microbial community structure and dominance (Øvreås *et al.*, 1997; Murray *et al.*, 1998; Muyzer & Smalla, 1998; Muyzer, 1999; Ampe & Miambe, 2000; Bosshard *et al.*, 2000; Ampe *et al.*, 2001; Cocolin *et al.*, 2001; Coppola *et al.*, 2001; Ercolini *et al.,* 2001a; Watanabe *et al.*, 2001).

**1.6.4 - Numerical taxonomy**

In order to classify living organisms, it is necessary to compare them. Molecular techniques can provide information on each organism which can then be compared with all other organisms, thus providing a dataset which can demonstrate the relatedness of a group of organisms. This is the basic principle behind numerical taxonomy. However, there is no single correct way of characterising organisms (Priest & Austin, 1995). In fact there are three distinct ways in which bacteriologists classify bacteria:

1. Special-purpose classifications, in which bacteria are classified based on characteristics which are ideal for their classification within a particular discipline. These are of no value to general microbiological classification (Priest & Austin, 1995).
2. Phenetic classifications, which refers to relationships between organisms (Operational Taxonomic Units , OTUs) based on comparison of the individual characteristics of the complete organism, both its phenotype and genotype. Placement of an organism within a group or 'phenon' is based on the organisms

displaying a high number of common characteristics with other members of the group. This is known as a polythetic grouping (Priest and Austin, 1995).

3. Phylogenetic classifications, which are based on genealogical relationships. These attempt to identify the true evolutionary tree that relates to the evolution of the organism or group of organisms in question. Phylogenetic classification is congruent with phenetic classification only if there is no parallel or convergent evolution and the rate of change proceeds constantly in all lineages (Priest & Austin, 1995).

Classifications are achieved through numerical taxonomy, which is defined as 'the grouping by numerical methods of taxonomic units into taxa on the basis of their characteristics' (Sneath & Sokal, 1973). The preference for numerical taxonomy as opposed to classical taxonomy comes from its equal weighting of all characteristics when constructing classifications (Priest & Austin, 1995). Ideally, classifications should be built on as many characteristics as it is possible to compile and compare. Therefore, using techniques which analyse a large number of characters such as DNA fingerprinting using molecular typing methods or by using total genomic or 16S rRNA sequence data, it is possible to assess the phenetic similarity between different organisms.

Assessment of similarity can now be done by computer analysis of characteristics (Rademaker *et al.*, 1999). A computer will assess the phenetic 'distance' between two pairs of OTUs using coefficients of resemblance (Priest and Austin, 1995). Three of the most common coefficients are shown in table 1.2. The coefficients are based on the relationship between positive (a), negative (d) and dissimilar matches (b, c) between OTUs (Fig. 1.1). When assessing taxonomy using DNA fingerprints, it is often advised to use the Dice coefficient as it weights positive matches and ignores negative matches (Sackin, 1987). This is ideal, as, when dealing with DNA fingerprints, the individual bands are being matched to other bands or identified as not matching to a band (symbols *a, b* and *c*, Fig. 1.1), so organisms are grouped on their matching bands and lack of matching bands, but not on the matching of spaces (symbol *b*, Fig. 1.1).

When the relationship distances have been calculated for all OTUs, the taxonomic structure can be demonstrated using techniques such as hierarchical groupings, where strains are grouped into species, species into genera, genera into families, etc. (Priest and

Austin, 1995), or ordination methods, which group organisms into taxa but with no hierarchical structure. There are three types of hierarchical structuring, these are:

| | | Results for OTU1 | |
|---|---|---|---|
| | | + | - |
| **Results for OTU2** | + | a | b |
| | - | c | d |

**Figure 1.1 – Diagrammatic representation demonstrating the relationships between the different symbols used in the formulae in Table 1.2. Reproduced from Priest and Austin (1995).**

| Coefficient | Abbreviation | Formula |
|---|---|---|
| **Simple Matching** | $S_{SM}$ | a + d / a + b + c + d |
| **Jaccard** | $S_J$ | a / a + b + c |
| ***Dice*** | $S_D$ | 2a / 2a + b + c |

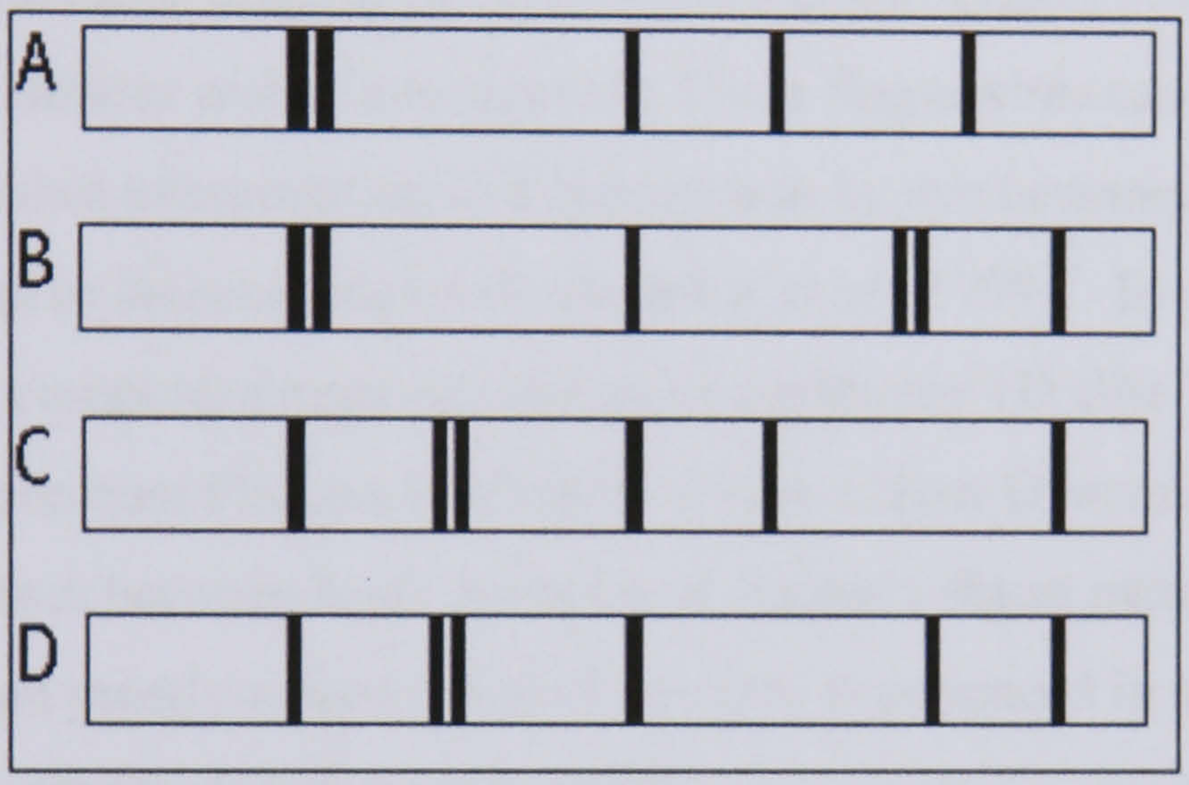
**Table 1.2 – Three of the most commonly used similarity coefficients in bacterial taxonomy (after Priest and Austin, 1995).**

1. Single Linkage (Nearest Neighbour) Clustering, in which OTUs form groups at the highest similarity between the OTU and any one member of the group, with no account taken of the other members of the group (Priest & Austin, 1995).
2. Complete Linkage (Furthest Neighbour) clustering, in which OTUs will only form groups when all members of the group are linked, that is clusters are formed at the lowest similarity level between the OTU and any member of the cluster (Priest & Austin, 1995).
3. Average linkage (UPGMA) clustering, or unweighted pair group method with arithmetic averages, is a compromise between single and complete linkage clustering. OTUs form groups at the average similarity between the OTU and all members of the group (Priest & Austin, 1995).

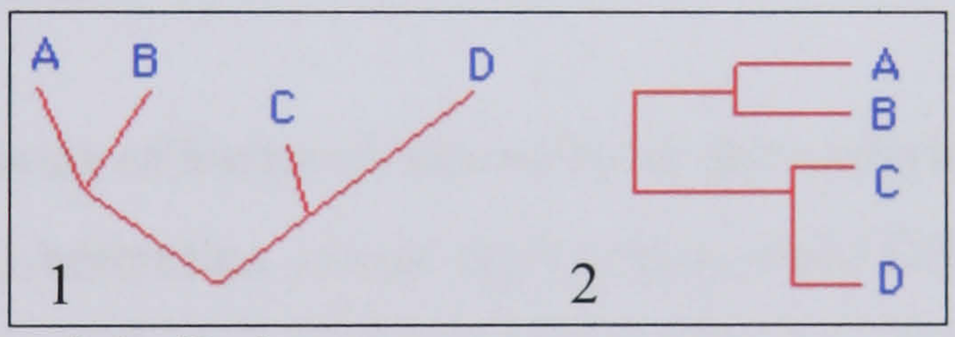
It has been demonstrated that UPGMA clustering provides the most accurate representation of the taxonomic structure and introduces the least distortion into the low-dimensional taxonomic space (Priest and Austin, 1995; for a description of taxonomic space please refer to Priest and Austin, 1995). Classification data can be represented by one of several methods. Firstly, the Similarity matrix, which is constructed by listing the

**AACGTCGAAA (Organism A)**
**AACCTCGAAA (Organism B)**
**AGGCTAGAAA (Organism C)**
**AGGCTAGTAA (Organism D)**

(A) – Sequence data




(B) – DNA fingerprint




(C) – Dendrograms of similarity.

**Figure 1.2 – Representation of molecular taxonomy. The sequence data (A) from organisms A, B, C and D, differs by only a few nucleotides. This arbitrary gene is digested by endonucleases to produce a DNA fingerprint (B). The similarity between these fingerprints can be calculated using coefficients of similarity to produce a similarity matrix, from which a dendrogram (C) can be calculated through hierarchical clustering (UPGMA). Two ways of representing the dendrogram are shown, (1) slanted, and (2) rectangular.**

OTUs according to the output from the cluster analysis (Priest and Austin. 1995). This can provide a shaded diagrammatic representation of the analysis; however, it is difficult to quickly assess the taxonomic structure from this. More common are the dendrograms which are produced from the cluster data via graph plotters or manual compilation. (Priest and Austin, 1995). They show relationships more clearly by indicating the joining of OTU clusters by a line (Fig. 1.2).

This can be performed for any of the molecular typing techniques described in section 1.5.3. For example, using ARDRA analysis to produce a sub-species specific fingerprint, which is compared with other fingerprints to produce a dendrogram of the relatedness between the bacterial isolates under investigation. These fingerprints can become so complex or numerous that interpretation and comparison by eye becomes too highly subjective and can lead to inconsistencies (Rademaker *et al.*, 1999). In order to prevent these inconsistencies, computer programs such as ImageMaster 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics) can be used to analyse the differences between large numbers of complex digest patterns. This numerical approach can greatly reduce the level of error experienced in such phylogenetic assessments (Rademaker *et al.*, 1999).

The functional analysis of bacterial taxonomy in the bacterial populations of the lakes of the Vestfold Hills, Antarctica, is best analysed by rapid DNA fingerprinting methods such as ARDRA and direct sequencing. These can be assessed using conventional numerical taxonomy tools such as clustering of OTUs using the Dice coefficient and UPGMA hierarchical groupings, which can be used to produce dendrograms to display interspecies relationships on the taxonomic scale. Analysis of bacterial community structures and their temporal and spatial variability can be assessed using DGGE analysis and culturing techniques. Similar studies have already been completed on Antarctic lakes (Bowman *et al.*, 2000a). This study, though, involves the estimation of the bacterial diversity of antifreeze protein (AFP) active bacteria in the lakes of the Vestfold Hills, Eastern Antarctica, as well as the demonstration of possible environmental impact on AFP activity, i.e. whether AFP activity is associated with a

particular set of environmental parameters. It also demonstrates the first attempt to outline the taxanomic groupings of AFP active bacteria and to determine if AFP activity confers a selective advantage within a community by suggesting abundance of AFP active bacteria within the lacustrine communities.

# Chapter 2: MATERIALS AND METHODS

## 2.1 – Study sites

Forty-one Lakes from the Vestfold Hills (68°S 78°E) and Larsemann Hills (69°24'S, 76° 20'E), and a single sample from Beaver Lake (latitudes and longitudes, and depths are shown in Table 2.1) were sampled during 2000 (Figure 2.1a, b and c). The salinity of these lakes ranged from freshwater to hypersaline (individual lake salinity is dealt with in Chapter 3). The lakes showed a range of stratification types from monomictic (Deep Lake), holomictic (Triple Lake) and meromictic (Ace Lake). Beaver Lake is a large epishelf lake, an unusual system found between ice shelves and continental land masses. They are freshwater lakes in hydrological contact with the sea, in that the lake sits on the colder denser seawater.

## 2.2 - Sampling

Water samples were obtained with a 2L Kemmener bottle through a hole drilled in the ice with a jiffy drill (AAA Products International, 7114 Harry Hines, Dallas, Texas 75235, United States). When it was possible to take depth samples (i.e. lake had ice cover capable of supporting personnel and equipment) water was collected from 0m, 2m, 4m, 8m and 10m (depth of sampling was dictated by the depth of oxygenated waters or depth of lake). 0m depth was taken as the base of the ice cover, and all other depths were taken from that point. In the absence of ice cover capable of supporting personnel and equipment, only surface water samples were taken from approximately 1-4m from the shore (depending on depth gradient of lake). For each sample, 4L of water was taken and stored in acid washed (10% HCl) polypropylene containers (for inorganic chemical analysis) and a further 100mL of water was taken in a sterilised glass Duran bottle (for microbiological analysis). Ice cores were taken where possible. The cores were obtained using a Sipre corer, which had been adapted for use with a Stihl drill (Stihl Inc, Virginia Beach, VA, USA) to provide faster and easier removal of ice cores. Holes were drilled into the ice using a Jiffy drill to approximately 1m above the ice-water interface. Then the ice core was taken from this depth to the ice-water interface.

| Lake | Maximum Depth (m) | Approximate Area ($km^2$) | Latitude (°S) : Longitude (°E) | Average salinity | Stratification type |
|---|---|---|---|---|---|
| Ace | 25 | 0.180 | 68.47: 78.19 | 20 ‰ [a] | Meromictic |
| Pendant | 12 | 0.16 | 68.46: 78.24 | 18 ‰ | Holomictic |
| Triple | 8 | 0.15 | 68.51: 78.23 | 170 ‰ | Holomictic |
| Deep | 36 | 0.64 | 68.56: 78.18 | 240 ‰ | Monomictic |
| Club | *na* | 0.91 | 68.54: 78.23 | 235 ‰ | Monomictic |
| Williams | 7 | 0.171 | 68.48: 78.16 | 20 ‰ [a] | Meromictic |
| Rookery | 2 | 0.177 | 68.50: 78.05 | 50 ‰ | Holomictic |
| Watts | 35 | 0.380 | 68.60: 78.19 | 4 ‰ | Monomictic |
| Crooked | 160 | 9 | 68.35: 78.25 | 0 ‰ | Monomictic |
| Organic | 7 | 0.047 | 68.46: 78.19 | 94 ‰ [a] | Meromictic |
| Stinear | 18 | 0.83 | 68.55: 78.15 | 154 ‰ | *na* |
| Dingle | 15 | 0.68 | 68.56: 78.05 | 136 ‰ | *na* |
| Beaver | ~435 | 150 | 70.80: 68.25 | 0 ‰ | Epi-shelf |
| Druzhby | 40 | 4 | 68.58: 78.33 | 0 ‰ | *unknown* |
| Nottingham | 18 | 0.22 | 68.45: 78.48 | 0 ‰ | *unknown* |
| LH73 | ~4 | 0.035 | 69.40: 76.38 | 0 ‰ | Holomictic |
| Pauk | *na* | *na* | 68.56: 78.45 | 0 ‰ | *na* |
| Zvezda | *na* | *na* | 68.53: 78.42 | 0 ‰ | *na* |
| Abraxas | 24 | 0.077 | 68.49: 78.29 | 10 ‰ [a] | Meromictic |
| Anderson | 21 | 0.119 | 68.61: 78.17 | 4 ‰ [a] | Meromictic |
| Ekho | 43 | 0.444 | 68.51: 78.27 | 24 ‰ [a] | Meromictic |
| Oval | 16 | 0.182 | 68.53: 78.28 | 34 ‰ [a] | Meromictic |
| Shield | 39 | 0.203 | 68.53: 78.27 | 4 ‰ [a] | Meromictic |
| Reid | *4* | 0.055 | 69.30: 76.30 [b] | 0 ‰ | *unknown* |
| Verenteno | *na* | *na* | 68.51: 78.18 | 4 ‰ | *na* |
| Collerson | *na* | *na* | 68.58: 78.18 | 4 ‰ | *na* |
| Jabs | *na* | *na* | 68.55: 78.25 | 96 ‰ | *na* |
| Lebed | 35 | 0.470 | 68.61: 78.21 | 195 ‰ | Holomictic |
| Oblong | 15 | 0.185 | 68.63: 78.24 | 70 ‰ [a] | Meromictic |
| Scale | 10 | 0.038 | 68.59: 78.18 | 4 ‰ [a] | Meromictic |
| Laternulla | 9 | 0.363 | 68.65: 77.97 | 146 ‰ [a] | Meromictic |
| Tassie | *na* | *na* | 68.53: 78.20 | 78 ‰ | *na* |
| Braunsteffer | *na* | *na* | 68.52: 78.35 | 4 ‰ | *na* |
| Tridant | *na* | *na* | 68.53: 78.21 | 60 ‰ | *na* |
| Pointed | *na* | *na* | 68.51: 78.28 | 4 ‰ | *na* |
| Calendar | *na* | *na* | 68.50: 78.43 | 4 ‰ | *na* |
| Hand | *na* | *na* | 68.56: 78.28 | 2 ‰ | *na* |
| Nicholson | 13 | 0.100 | 68.60: 78.23 | 0 ‰ | Holomictic |
| Medusa | *na* | *na* | 68.57: 78.23 | 2 ‰ | *na* |
| Cemetery | *na* | *na* | 68.62: 77.96 | 158 ‰ | *na* |
| Angel '2' | *na* | *na* | 68.64:77.91 | 186 ‰ | *na* |

**Table 2.1 – Location, selected physico-chemical properties of the study lakes All salinities based on current study. [a]average salinity in mixolimnion from current study records. [b] estimate. *na* = not available.**

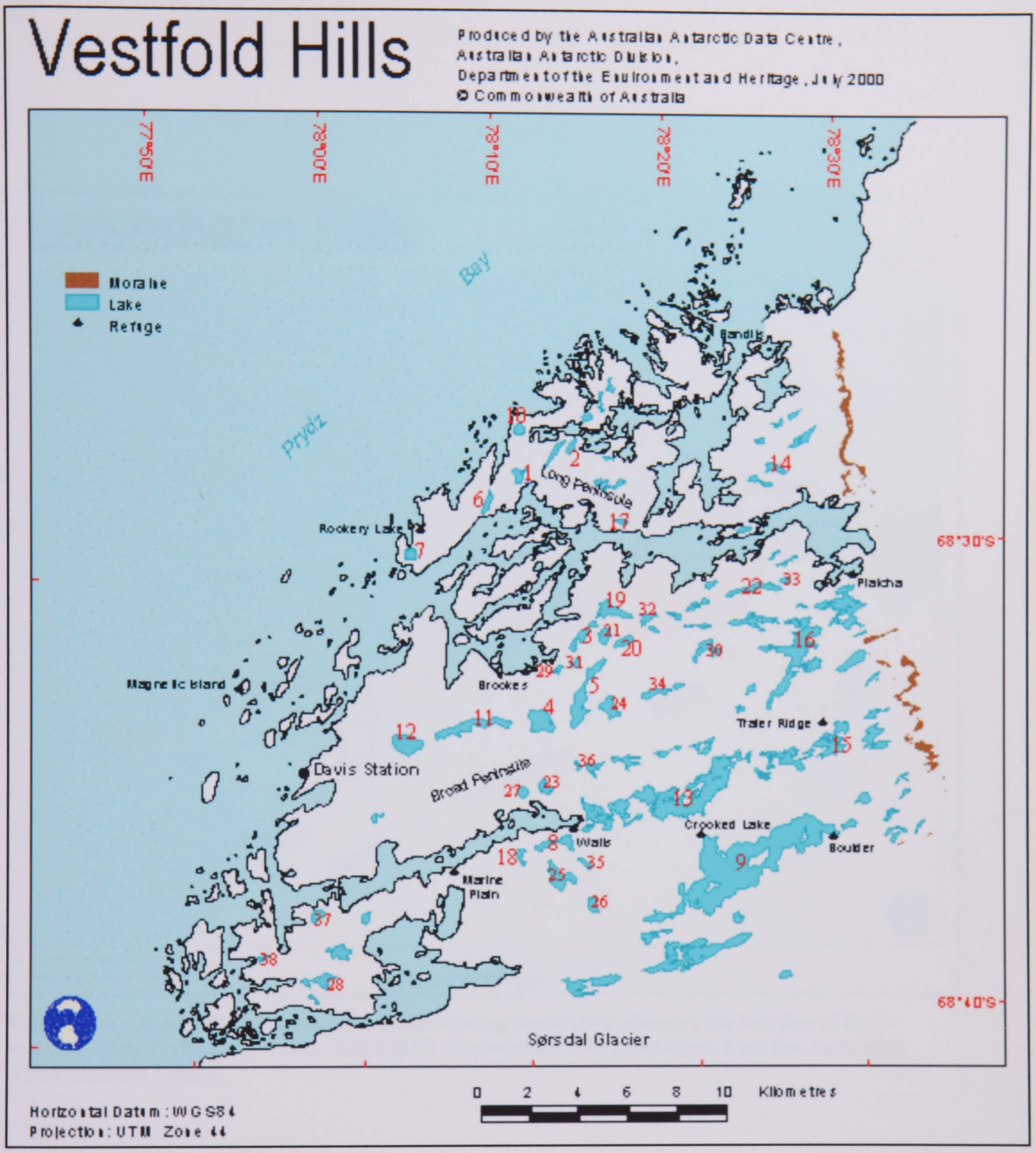



**Figure 2.1a – A map of the Vestfold Hills, Eastern Antarctica, showing the location of the 38 sampled lakes. (1) Ace Lake, (2) Pendant Lake, (3) Triple Lake, (4) Deep Lake, (5) Club Lake, (6) Williams Lake, (7) Rookery Lake, (8) Watts Lake, (9) Crooked Lake, (10) Organic Lake, (11) Lake Stinear, (12) Dingle Lake, (13) Druzhby Lake, (14) Lake Nottingham, (15) Pauk Lake, (16) Zvezda Lake, (17) Abraxas Lake, (18) Anderson Lake, (19) Ekho Lake, (20) Oval Lake, (21) Shield Lake, (22) Lake Verenteno, (23) Collerson Lake, (24) Lake Jabs, (25) Lake Lebed, (26) Oblong Lake, (27) Scale Lake, (28) Lake Laternulla, (29) Tassie Lake, (30) Braunsteffer Lake, (31) Tridant Lake, (32) Pointed Lake, (33) Calendar Lake, (34) Hand Lake, (35) Nicholson Lake, (36) Medusa Lake, (37) Cemetery Lake, (38) Angel '2' Lake. Reproduced with permission from the Australian Antarctic Data Centre.**

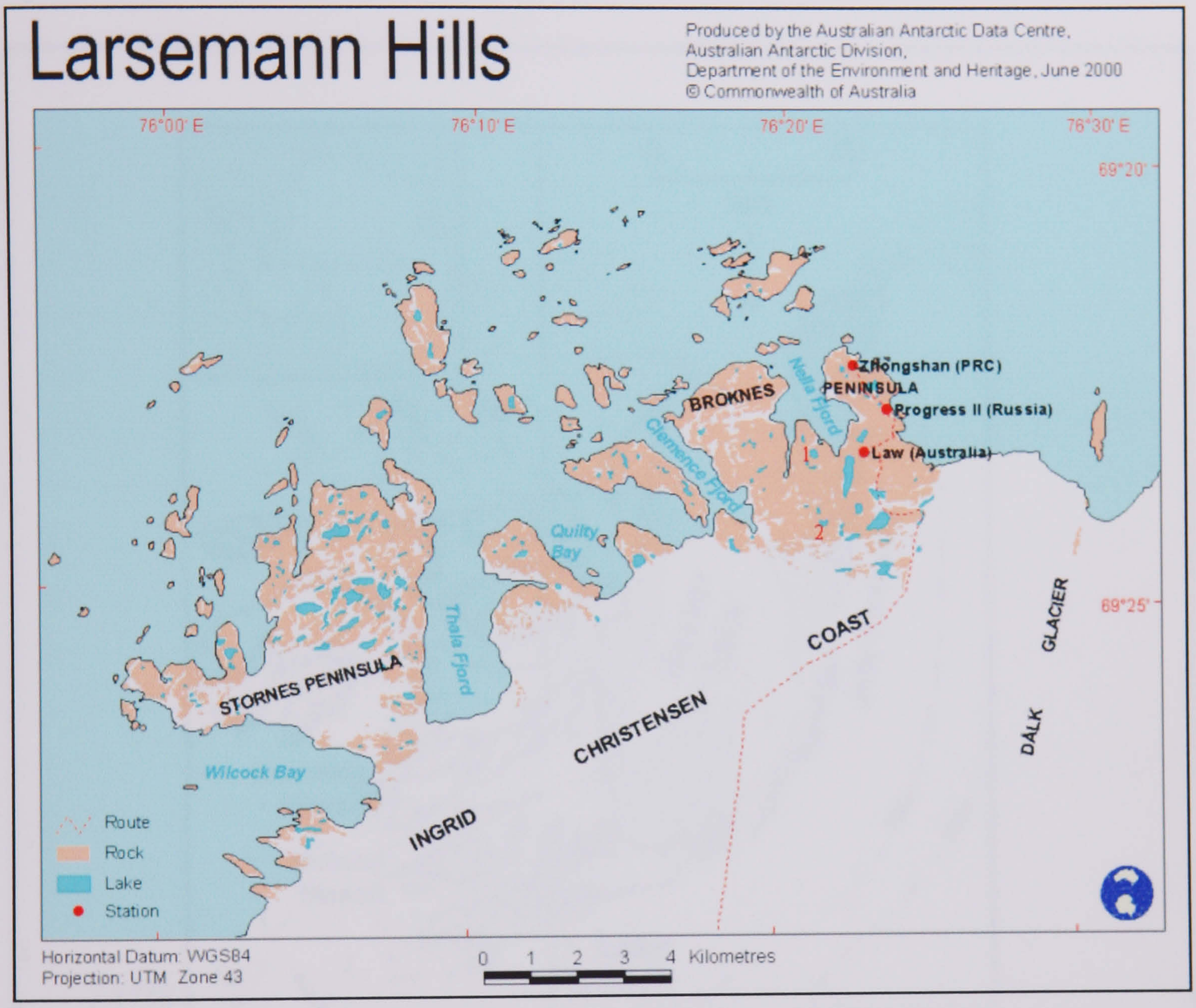



**Figure 2.1b – A map of the Larsemann Hills, Eastern Antarctica, showing the location of the 2 sampled lakes. (1) Lake Reid, (2) Lake LH 73. Reproduced with permission from the Australian Antarctic Data Centre.**

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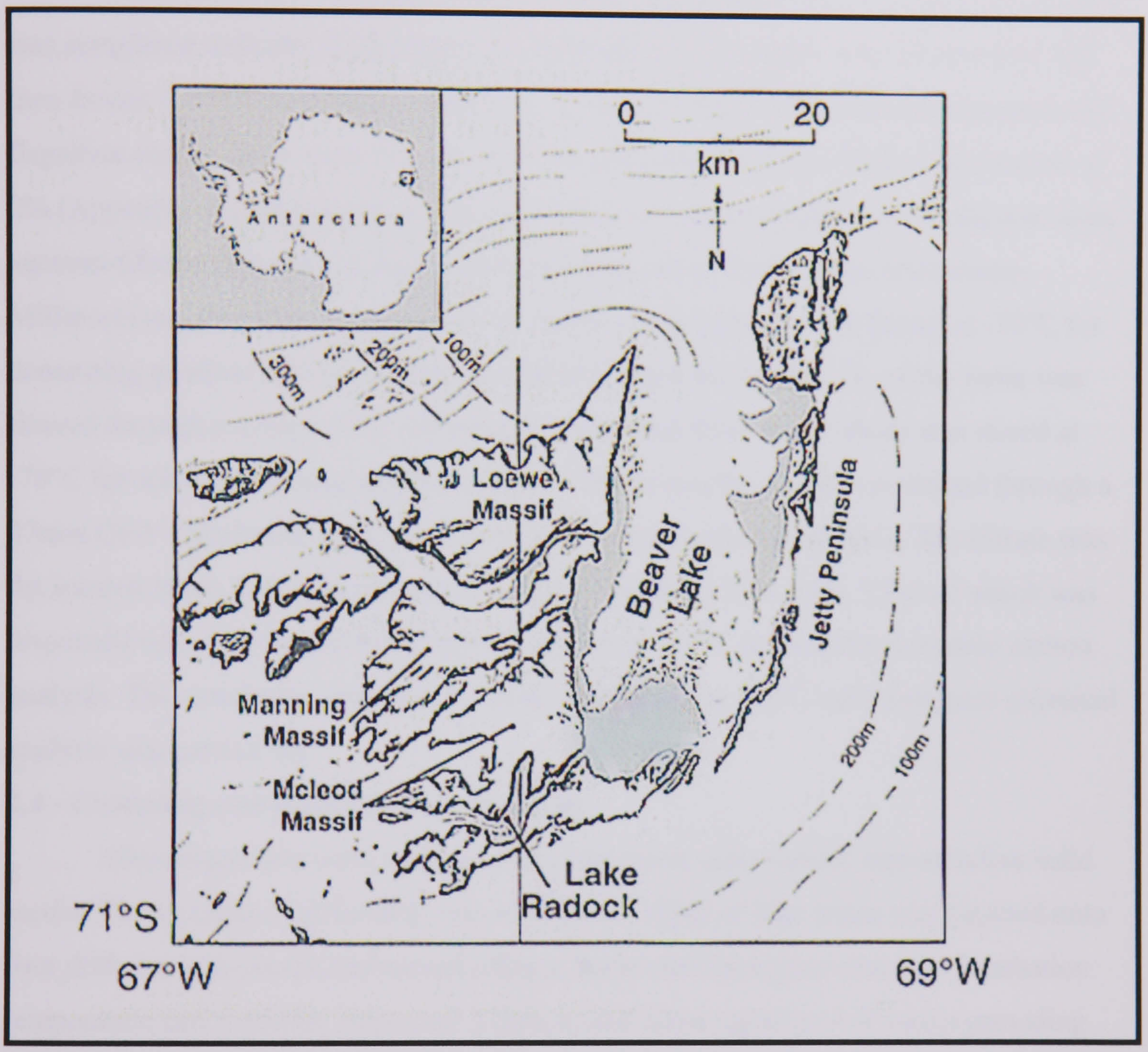


**Figure 2.1c – A map of the epishelf lake, Beaver Lake, MacRobertson Land (modified from Laybourn-Parry *et al.*, 2001b)**

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Sampling trips took two days during July, due to the lack of daylight (approx 3 hours) in the winter season, whilst in early summer (September and November) trips only took one day due to an increase in the number of hours of daylight per day.

**2.3 - Water sample processing**

On return to the laboratory, samples were stored at 1°C until all initial processing was completed. Initially 500mL aliquots were taken for inorganic nutrient analysis and then frozen (-20°C) for ammonia analysis. Aliquots (10mL) were taken for bacterial and flagellate counts; these were fixed in buffered glutaraldehyde to a final concentration of 2% (Appendix A2) and stored at 1°C in the dark. For each sample, 120mL aliquots were vacuum-filtered through a 0.2μm mixed cellulose esters filter (nitrate and acetate, Millipore) and the filter was stored in TE buffer (Appendix A2) and frozen at -70°C for denaturing gradient gel electrophoresis (DGGE) analysis. Exactly 1L of the water was filtered through a 47mm GF/C (Whatman) glass microfiber filter, which was stored at -70°C for subsequent chlorophyll *a* analysis. The remaining water was filtered through a 47mm GF/F (Whatman, heated at 450°C for ~5 hours) microfiber filter. The filtrate was decantered into a 2l polypropylene bottle (Acid washed, 10% HCl), 25ml of which was dispensed into a sterile universal tube and frozen at -20°C for dissolved organic carbon analysis. The remainder of the GF/F filtrate was frozen at -20°C until inorganic chemical analysis was carried out.

**2.4 - Culturing and maintenance of isolates**

Microorganisms were cultured from lake water onto various non-selective solid media. The inoculation procedure was as follows: 100μl of lake water was pipetted onto four different agar media and spread using a flame sterilised glass spreader. Incubation temperature and nutrients were used which would allow rapid growth whilst providing similar conditions to the environment from which they were isolated. This prevented the chances of adaptive radiation that may cause the loss of AFP activity in a previously active bacterium. The four types of agar used were tryptic soya agar, yeast agar, seawater agar (3.8% salinity) and ½ seawater agar (1.9% salinity) (TSA, YSA, SWA and ½ SWA respectively, Appendix A1). For the ice cores, a 4cm thick cross section of ice was taken from the ice-water interface and from 50-54cm from the ice-water interface. Each section was cut with a sterilized ice saw blade and then sterilized by flaming the exterior surface

with a Bunsen burner. The sections were then melted out at 1°C in filtered (GF/F, Whatman) and sterilized (120°C for 20 minutes in autoclave) lake water from the lake of origin, to prevent osmotic shock when the microbes were defrosted. The inoculation process was the same as for lake water. The plates were incubated at +17°C for 1 week, after which the plates were sub-cultured using a sterile streak plate technique, so that individual isolates (determined by colony morphology) were plated on to the media upon which they were originally grown. Each isolate was numbered, recorded in a database and then incubated at +17°C for 1 week. Original sample plates were stored at 1°C until verification of isolate growth was made from sub-cultures. Once sub-cultures were grown and verified, they were Gram stained (refer to Gram stain - section 2.6). All Gram stain results were recorded into a database along with basic observations of cellular and colony morphology (Appendix B1).

**2.5 - Preservation and transportation of the microbial collection.**

In order to maintain pure isolates and to transport them between Antarctica and England, it was necessary to place the cultures in cryogenic stasis. The isolates were cultured in their respective liquid media containing 10% glycerol (Appendix A1). Each isolate was inoculated into 500µl of medium, which had been aliquoted individually into sterile 1.5ml flip top Eppendorf tubes (autoclaved 121°C, 20 minutes). Once inoculated, the tubes were incubated at +15°C for 1 week. Following incubation, the tubes were frozen at -20°C for 1 week. After one week of storage, plating them out onto solid media tested the viability of each of the cryo-cultures. Once viability and correct isolate identification (via colony morphology) had been assessed, the original plated cultures were destroyed. All isolates were duplicated and putative AFP active isolates were quadruplicated.

**2.6 - Gram stain technique**

All isolates were Gram stained for basic phenetic classification using a basic Gram method (Rodina, 1972). Mid log phase growth cultures provide the best results with the Gram stain as variability can occur in very young or very old cells (Singleton & Sainsbury, 1997), therefore cultures incubated for up to 1 week were used. The isolates were tested using the following procedure. A heat-fixed smear of bacterial cells was stained for approximately 1 minute, using Crystal Violet dye (triphenylmethane dye 88%,

Sigma, Sigma-Aldrich Company Ltd., Dorset, England). This was rinsed with Milli Q (Millipore) water, after which, the smear was fixed for 1 min using Gram's Iodine (Sigma). The stained smear was rinsed and then run briefly under flowing acetone until no dye was freely flowing. The smear was then immediately rinsed under Milli Q (Millipore) water, as too much acetone can cause total decolourisation. A counter stain (safranin O, Sigma) was used for 30 seconds to stain any decolourised smears. The slide was then viewed under oil immersion at 100x magnification. The colouration of the cells and their cellular morphology was recorded.

Those smears that appeared purple/violet (crystal violet stain) in colour were termed Gram positive and those that underwent decolourisation and subsequent red safranin staining were termed Gram negative. Grams iodine is initially used as a mordant to bind the crystal violet to the cell wall. Acetone allows the increased permeability of the lipid rich Gram-negative cell wall causing decolourisation. The increased number of cross-linked teichoic acid residues found in the Gram-positive peptidoglycan boundary is believed to aid in initial dye retention during Gram staining (Singleton & Sainsbury, 1997).

## 2.7 –Water chemistry – analytical procedures

### 2.7.1 - Chlorophyll *a* analysis

All water samples were assessed for their chlorophyll *a* concentration using the following procedure adapted from *Standard Methods for the Examination of Water and Wastewater* (1998, section 10200H, Clesceri & Eaton (Eds.)). A litre of sample water was filtered through GF/C filter paper (45mm). The chlorophyll was then extracted using 97.8% methanol (giving a final volume of 15ml of 90% methanol allowing for water in paper) as a solvent at -20°C for 24h. After centrifugation (2000rpm for 10 minutes, Beckman J2-MC, JA-14 rotor) the absorbance (OD) of the supernatant was read at 665nm and 750nm (as background) (4cm cuvette path length, GBC UV/VIS 916 spectrometer). The concentration of chlorophyll was calculated using the following equation:

$$\text{Chlorophyll } a\ (\mu g l^{-1}) = 13.9 \times OD_{665\text{-}750} \times v/(V \times l)$$

Where v equals volume of methanol (ml), V equals volume of water (L) and 'l' represents cuvette path length (cm).

### 2.7.2 - Inorganic chemical analysis

Analyses of ammonia, nitrate, nitrite and soluble reactive phosphorus ($NH_4$-N, $NO_3$-N, $NO_2$-N, $PO_4$-P) concentrations were performed on the lake water samples.

*2.7.2.1 - Ammonia ($NH_4$-N)*

This method was taken from Mackereth *et al.* (1988, after Chaney & Marbach. 1962). The principle is that the ammonia reacts with phenol and hypochlorite to form indophenol blue (catalysed by nitroprusside). A calibration curve was established using an ammonium chloride standard. Absorbance was measured at 635nm in a 4cm cell.

*2.7.2.2– Nitrite ($NO_2$-N)*

This method is taken from Mackereth *et al.* (1988, after Bendschneider and Robinson, 1952). In principle, nitrite yields nitrous acid in acid solution which diazotises sulphanilamide; the diazonium salt that is formed binds to N-1-napthylethylenediamine dihydrochloride and forms a red azo dye. A calibration curve was established using a sodium nitrite standard solution. Absorbance was measured at 543nm in a 4cm cell.

*2.7.2.3 – Nitrate ($NO_3$-N)*

This method was taken from Mackereth *et al.* (1988). The principle is that nitrate is reduced to nitrite using spongy cadmium and the nitrite is then determined as shown above in the nitrite methodology. A calibration curve was established using a potassium nitrate standard solution. Absorbance was measured at 543nm in a 4cm cell.

*2.7.2.4 - Soluble reactive phosphorus ($PO_4$-P)*

This method was taken from Mackereth *et al.* (1988), (taken from Murphy & Riley, 1962). The principle is that an acidified phosphate solution reacts with molybdate to form molybdo-phosphoric acid. This is then reduced to form a coloured molybdenum blue complex, the absorption of which is determined spectrophotometrically. The concentration of ortho-phosphate was established by running the unknown samples against a calibration curve formed from standards of known concentration. The absorbance was measured at 880nm in a 4cm cell.

### 2.7.3 - Dissolved organic carbon analysis

DOC was analysed using a Total Carbon Analyser (Shimadzu). The analysis is based on the combustion/non-dispersive infrared gas analysis method (Najm & Marcinko, 1995). Non-purgeable organic carbon was measured. A liquid sample was

oxidized and the sample then converted to $CO_2$ and $H_2O$. A dehumidifier removed the water and a carrier gas swept the CO2 to a Non-Dispersive Infrared (NDIR) Detector, which measured the carbon concentration based on a Potassium Hydrogen Phthalate (KHP) standard.

**2.8 - Bacterial and flagellate counts**

Samples were stained with DAPI (4',6-diamidino-2-phenylindole) prior to filtration through a 0.2μm polycarbonate filter (Poretics) using a pressure no greater than 5 In Hg vacuum. Bacteria were enumerated under epifluoresence microscopy using UV excitation at x1200 magnification. Both UV and blue filter sets were used to discriminate the autoflouresence of chlorophyll in the autotrophic bacteria and flagellates.

For each preparation, ten replicate Whipple grids were counted and mean values calculated. Flagellates were counted as heterotrophic (no auto-fluorescence) or autotrophic (via auto-fluorescent) in twenty fields of view from which the mean of each was calculated.

**2.9 - Molecular typing techniques**

To enable assessment of the distribution of AFP activity within the taxa of isolated bacteria, molecular typing techniques were employed to produce a phenetic tree of relatedness and a phylogenetic tree to suggest evolutionary relationships between the bacterial isolates, which in turn provided insights not only into the relationships of AFP active bacterial strains, but also information to enable future expeditions more readily identify AFP active species in environmental samples.

**2.9.1 - Chromosomal DNA extraction**

The CTAB (Cetyltrimethylammonium bromide) method (Ausubel, 1999; from Wilson, 1987) was employed for chromosomal DNA extraction. 50ml of bacterial culture was grown in a conical flask to produce a turbid culture. The media upon which the bacteria were originally isolated (TSA, YSA, SWA, ½ SWA – section 2.4) were used for liquid media. The cultures were grown in a static incubator at 15°C. The cells were then centrifuged for 10 min at 4000 *g* (6000 rpm in JA-20 rotor). The supernatant was discarded and the pellet was resuspended in 9.5ml of TE buffer (Appendix A2). To this were added 0.5ml of 10% SDS (Sigma) and 50μl of 20mg/ml proteinase K (Sigma), which was then mixed. This was incubated for 1 h at 37°C. Following incubation, 1.8ml

of 5M NaCl (Ausubel *et al.*, 1999) was added and the solution was mixed thoroughly. Then 3ml of CTAB/NaCl solution (Ausubel *et al.*, 1999) was added and mixed. This was incubated for 1 h in a 65°C waterbath. Following incubation an equal volume (14.85ml) of 24:1 chloroform/isoamyl alcohol was added. The solution was allowed to extract for a few minutes and then centrifuged for 10 min at 6000 *g* (7000 rpm) at room temperature. The supernatant was transferred to a fresh tube with a wide bore pipette (5ml Gilson pipette). Isopropanol (0.6 vol) was added to the supernatant and then mixed gently until the DNA precipitated. This was centrifuged at 10,000 *g* (9000 rpm) for 5 min and the supernatant discarded. The pellet was resuspended in 1 ml of 70% ethanol and washed and then centrifuged again at 10,000 *g* (9,000 rpm) for 10 min and the supernatant was discarded again. The pellet was resuspended in 3ml of TE buffer and stored at 5°C overnight to allow the pellet to resuspend in the buffer. The following day the solution was transferred to a glass bijou bottle and stored at 5°C to prevent degradation of DNA. To determine if chromosomal DNA was extracted, 20µl of the solution was mixed with a 5x loading dye and then electrophoresed on a 1% agarose gel (TAE buffer) at 80V for 30 minutes (section 2.9.2).

**2.9.2 - Preparation, electrophoresis and recording of agarose gels**

This basic method (Sambrook *et al.*, 1989) was used for agarose gel electrophoresis throughout the project. The running voltage and length of running period were often altered for the varying types of analysis this protocol was employed in. For example, when assessing chromosomal DNA, the voltage was high (e.g.100V) and as a result the running time was short, but when analysing small DNA fragments from a restriction digest, it was necessary to run the gel at a low voltage (20V) often overnight to help maintain distinct bands for better analysis.

For electrophoresis, 1x TAE electrophoresis buffer was prepared from 50x stock solution (Sambrook *et al.*, 1989), which was used as running buffer and gel buffer. A 0.8-1% TAE agarose gel (Hi-pure, low EEO, Bio-Gene) was prepared and pre-stained using 0.5 µg $ml^{-1}$ ethidium bromide (Sigma) for DNA visualisation. Two sizes of gel casting tray (Anachem Origo), 50ml (6 x 7 cm) and 150ml (13 x 15 cm) and 12 well and 16 well casting combs (Anachem Origo) were used. The samples and standards were prepared in sterile 1.5ml Eppendorf tubes by mixing a 1:5 ratio of 5x loading buffer (Roche) to

sample. An appropriate volume was loaded depending on the expected DNA concentration, although it was usually between 5 and 50 µl. The gel was usually run at 80V/cm (power supply 500/400 = Amersham Pharmacia Biotech, Davy Avenue, Knowlhill, Milton Keynes, MK5 8PH, United Kingdom) for 30 min or until the dye had migrated approximately three quarters of the length of the gel. However, this varied (as stated above) depending on the samples were being observed.

Following electrophoresis, the gel was placed in the ImageMaster® VDS unit (Pharmacia Biotech) to be examined under a ultra-violet transilluminator and photographed using a video camera attached to the system and a computer, so that digital image could be taken.

### 2.9.3 - Amplified ribosomal DNA restriction analysis

#### *2.9.3.1 – PCR amplification of 16S rRNA gene*

The following procedure was adapted from Vaneechoutte *et al.* (1995) (ARDRA methodology outlined in figure 2.2). Bacterial specimens were grown in 30ml glass universal bottles in 10ml of Tryptic Soya Broth (Sigma). Of this culture, 1µl was added to a 49µl PCR reaction mixture consisting of 21µl sterile RO $H_2O$, 16µl dNTP mixture (Abgene, 98 College Avenue, Rochester, New York 14607, United States) (6.26µl of each dNTP made up to 1ml in sterile $H_2O$), 5µl Buffer IV (ABgene), 4µl 25mM MgCl (ABgene), 1µl universal primer 1.5 F (5'-TGG CTC AGA TTG AAC GCT GGC G-3') (Sigma-Genosys, 1442, Lake Front Circle, The Woodlands, Tx, 77380), 1µl universal primer 1.5 R (5'-TAC CTT GTT ACG ACT TCA CCC CA-3') (Sigma-Genosys) (Vaneechoutte *et al.*, 1995), 1µl *Taq* DNA Polymerase (ABgene).

Both primers were diluted 1:5 from their original supplied concentrations with sterile reverse osmosis water. *Taq* DNA polymerase was only added after 'hotstart' on the thermal cycler or PCR block (Techne Progene, Techne (Cambridge) Ltd, Duxford, Cambridge, CB2 4PZ, England). Total volume of PCR reaction mixture with DNA template was 50µl. Mixture was run on the PCR block using the following program. Initial denaturing step of 10 min at 95°C followed by the addition of ice cold *Taq* (1µl per reaction). This is followed by 30 cycles of 0.5 min at 95°C (denature), 1 min at 55°C (anneal), 1.5 min at 72°C (extension), after which there was a single cycle of 72°C for 8

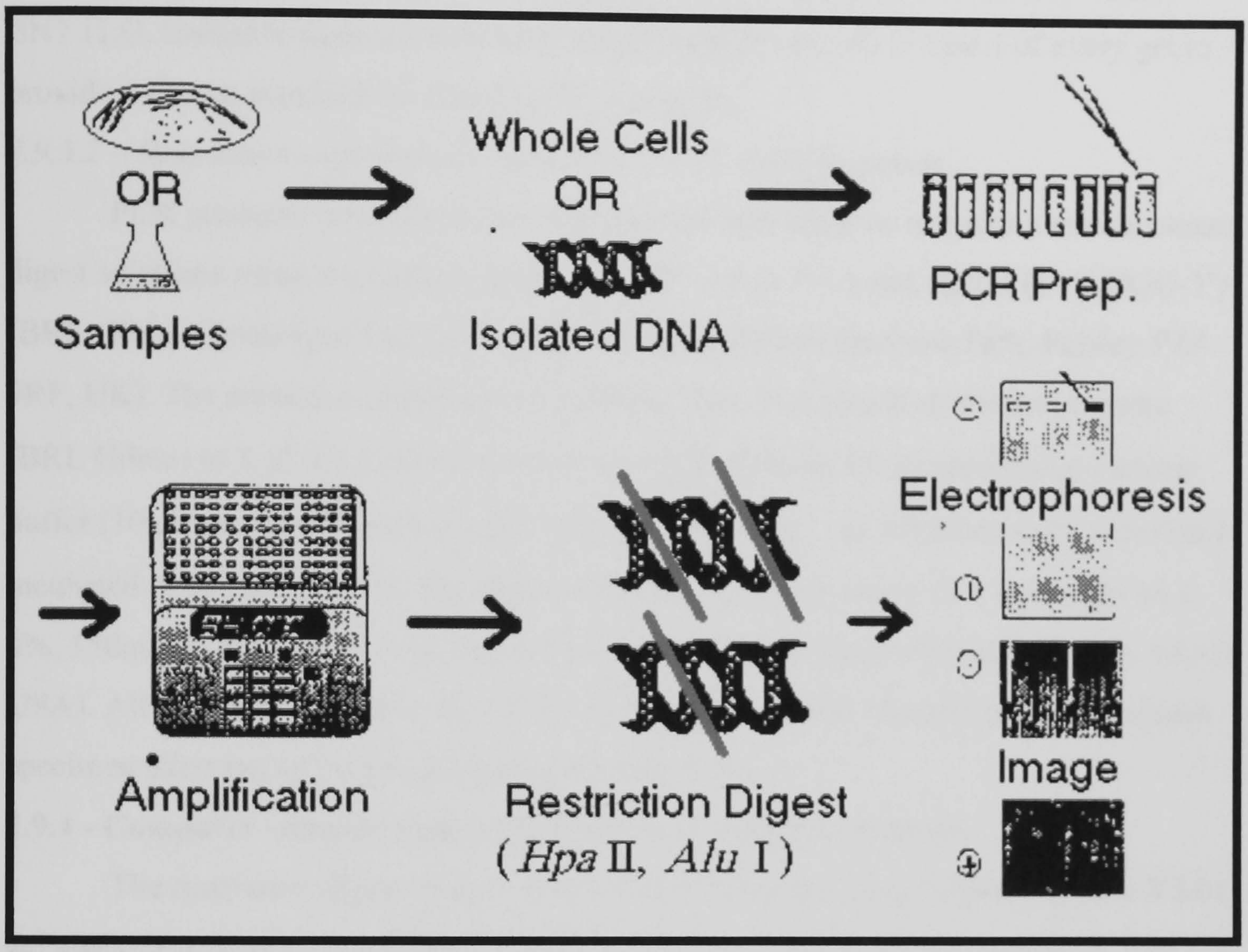


**Figure 2.2 - Diagrammatic representation of the ARDRA procedure. Chromosomal DNA is extracted from liquid media cultures, or whole cells are added from agar plate cultures to a PCR preparation. The target sequence, 16S rDNA, is then amplified by thermal cycling. The 16S rDNA sequence is then digested overnight using restriction enzymes (*Hpa* II & *Alu* I). The resultant fragment patterns are visualised by electrophoresis.**

min to ensure complete extension of all strands. The PCR reaction amplified a 1500 bp 16s rDNA fragment. To check for correct amplification, 5µl of PCR product was run on an electrophoresis gel (section 2.9.2). A 100bp DNA Ladder (molecular weight marker VIII - Boehringer Mannheim Roche Diagnostics Ltd., Bell Lane, Lewes, East Sussex BN7 1LG, United Kingdom) molecular weight marker was run in lane 1 of every gel to provide a known standard for sizing of PCR product.

*2.9.3.2 – Restriction endonuclease digestion of 16S rDNA fragment.*

PCR products were digested overnight with two separate restriction endonuclease digest solutions using the endonucleases *Alu* I (5'-AG/CT-3') and *Hpa* II (5'-C/CGG-3') (BRL Gibco - Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK). The solutions comprised 12µl sterile $H_2O$, 1 µl *Hpa* II restriction enzyme (BRL Gibco) or 1 µl *Alu* I restriction enzyme (BRL Gibco), 2.0 µl appropriate enzyme buffer (10x React 1, BRL Gibco) and 5.0 µl PCR product. The solutions were mixed and incubated overnight at 37°C. The digest solutions were then run at 20V overnight on a 2%, 150ml agarose gel (3:1 Nu-Sieve, FMC 1735 Market Street, Philadelphia, PA 19103, USA). All ARDRA gels were run with 3 100bp MW markers (Roche) and one standard specimen allowing cross-gel and inter-gel comparison.

**2.9.4 - Computer enhanced phenetic analysis of ARDRA patterns**

The restriction digest images were imported into the ImageMaster 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics). This program uses pixel intensity mapping to delineate DNA bands on 1D gels for use in composing dendrograms of phenetic similarity from comparisons on different patterns.

The analysis was divided into five stages: Firstly, *Lane delineation* (Fig. 2.3) where the lanes on the gel were outlined as specific areas for pixel intensity evaluation. Secondly, *Background reduction* (Fig. 2.4a & b) where a rolling disc algorithm, with a disc radius of 20 was used to digitally enhance the peaks of pixel intensity that relate to DNA bands. Thirdly, *DNA band detection* (Fig.2.5a & b), where bands were detected automatically by computer assessment of their pixel intensity. It was possible to remove artefacts such as anomalous high intensity points, afterwards. Fourthly, *Gel standardisation* (Fig. 2.6a & b) was applied to the gels using retardation factor (Fig 2.6a) (Rf – a measurement of a bands position along a lane relative to it's length) and

Molecular Weight (Fig 2.6b), three 100bp ladders were run with the restriction digests in the gel and were used to standardise MW across the gel. This standardisation allowed comparison between gels. Fifthly, *Band matching* (Fig. 2.7), where bands were matched to themselves and common bands in a computer generated reference lane that held all the standardised values for the bands in the gel being analysed. This provided data on similarities between lanes in relation to the position of bands, which allowed phenetic similarity calculations between the patterns based on band position.

This procedure was employed to produce a dendrogram showing the phenetic relatedness of the AFP active isolates ARDRA patterns. The data were then transferred to ImageMaster 1D database V2.12 (Amersham Pharmacia Biotech) to calculate a phenetic dendrogram created from the comparison of all the bands from all the lanes from more than one gel. The ARDRA patterns were matched by Rf value using the Dice similarity coefficient (Equation 2.1) and were displayed in a UPGMA (Unweighted pair group method with arithmetic averages) clustered dendrogram.

$$SD = \frac{2a}{2a + b + c}$$

**Equation 2.1 - Dice Coefficient equation. SD = coefficient of similarity based on the Dice equation; *a* = the number of positive matches. *b* and *c* = the number of non-matching characters between pairs of Operational Taxonomic Units (Priest and Austin, 1995).**

### 2.9.5 - Denaturing gradient gel electrophoresis

*2.9.5.1 – Collection of bacterial community*

To obtain a community fingerprint it was necessary to obtain a concentration of the bacterial community from each lake. This was performed by filtering 120mL of lake water through a 47mm diameter, 0.2 μm mixed cellulose esters (nitrate and acetate) filter (Millipore). The biologically inert mix of nitrate and acetate make the filters ideal for cellular harvesting. The filter was stored in 1ml of TE buffer in a 1.5ml Eppendorf tube at -70°C.

*2.9.5.2 - Genomic extraction for DGGE*

To provide a clean DNA template for the initial PCR step in DGGE analysis the DNA was extracted using a modified CTAB technique (Ausubel *et al.*, 1999), which is outlined here.

The stored filters in TE buffer were defrosted on ice. Once totally defrosted, the

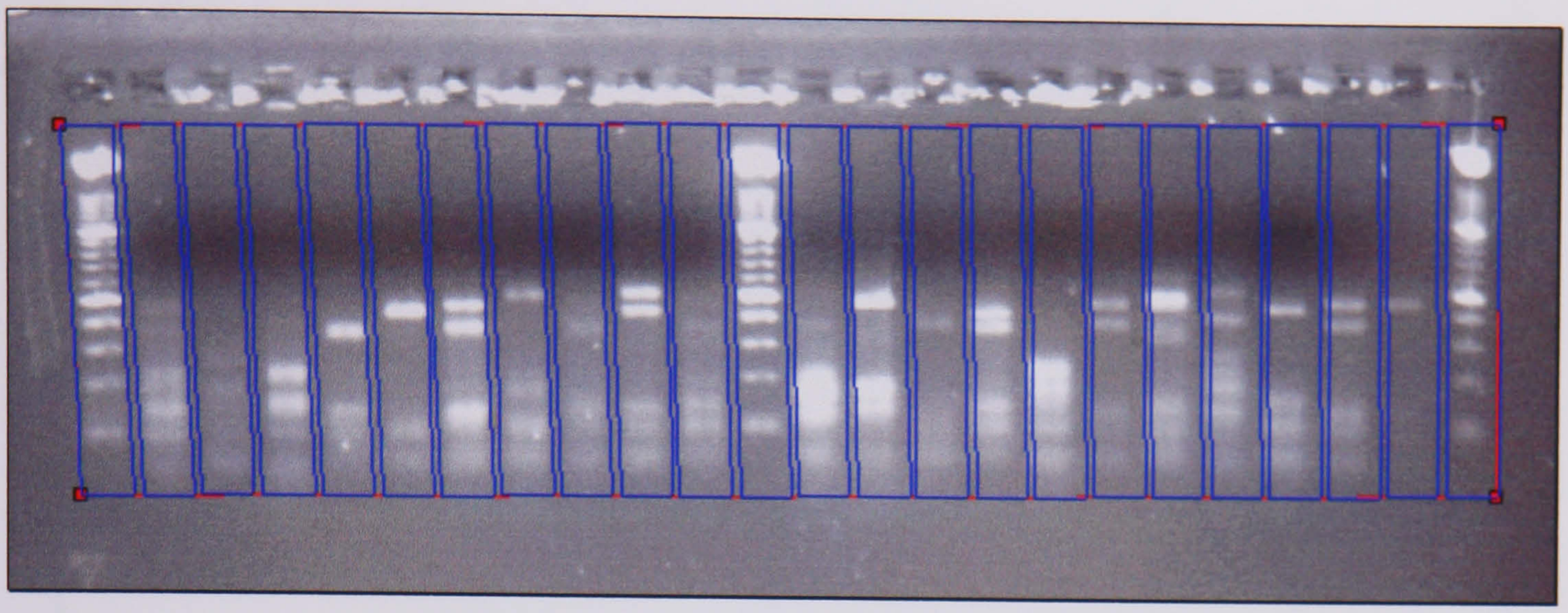

**Figure 2.3 - Lane delineation image from 1D elite gel analysis (Amersham Pharmacia Biotech). Each of the lanes is boxed to detail the specific areas for pixel intensity analysis.**

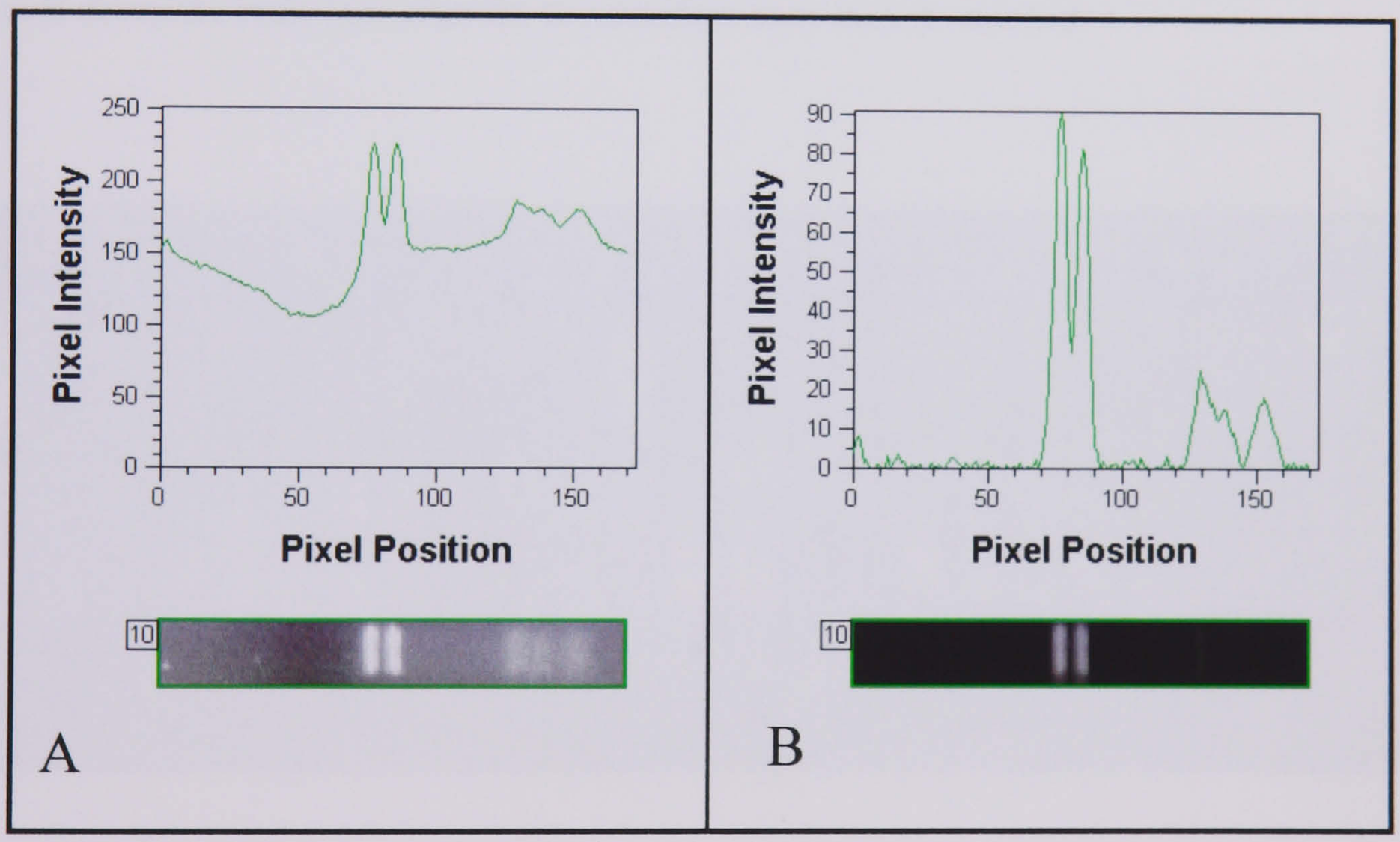


**Figure 2.4a & b. Figure A shows how the DNA bands in one lane are established by measuring pixel intensity. Figure B shows how the bands can be more clearly seen by removing the background pixel intensity using a rolling disc algorithm with a disc radius of 20.**



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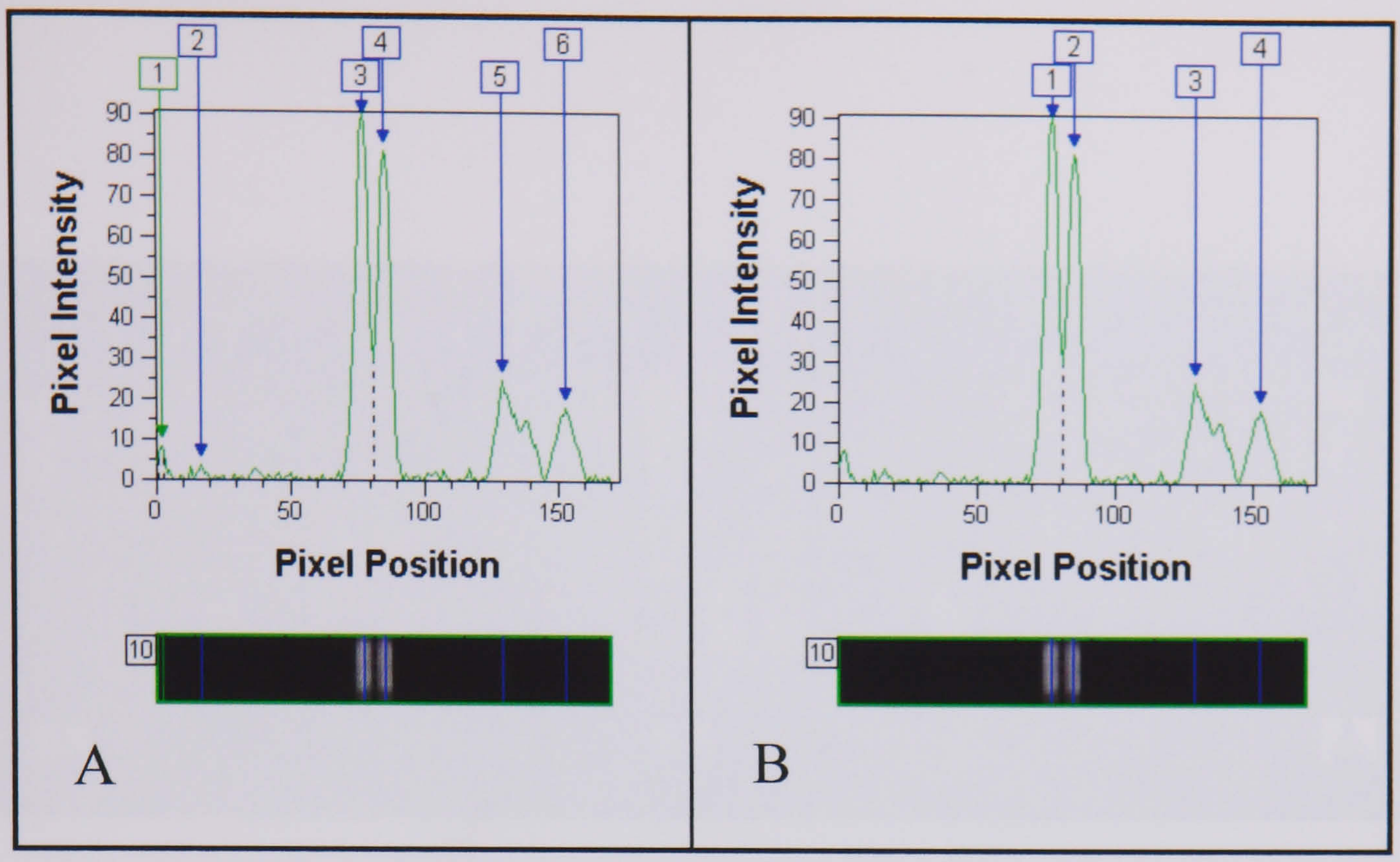


**Figure 2.5a & b - Figure A shows automatic band detection, the computer has automatically detected the bands in the lane, however, it has also detected two artefacts and represented them as bands 1 & 2; Figure B shows the same lane after the two artefacts have been de-marked.**

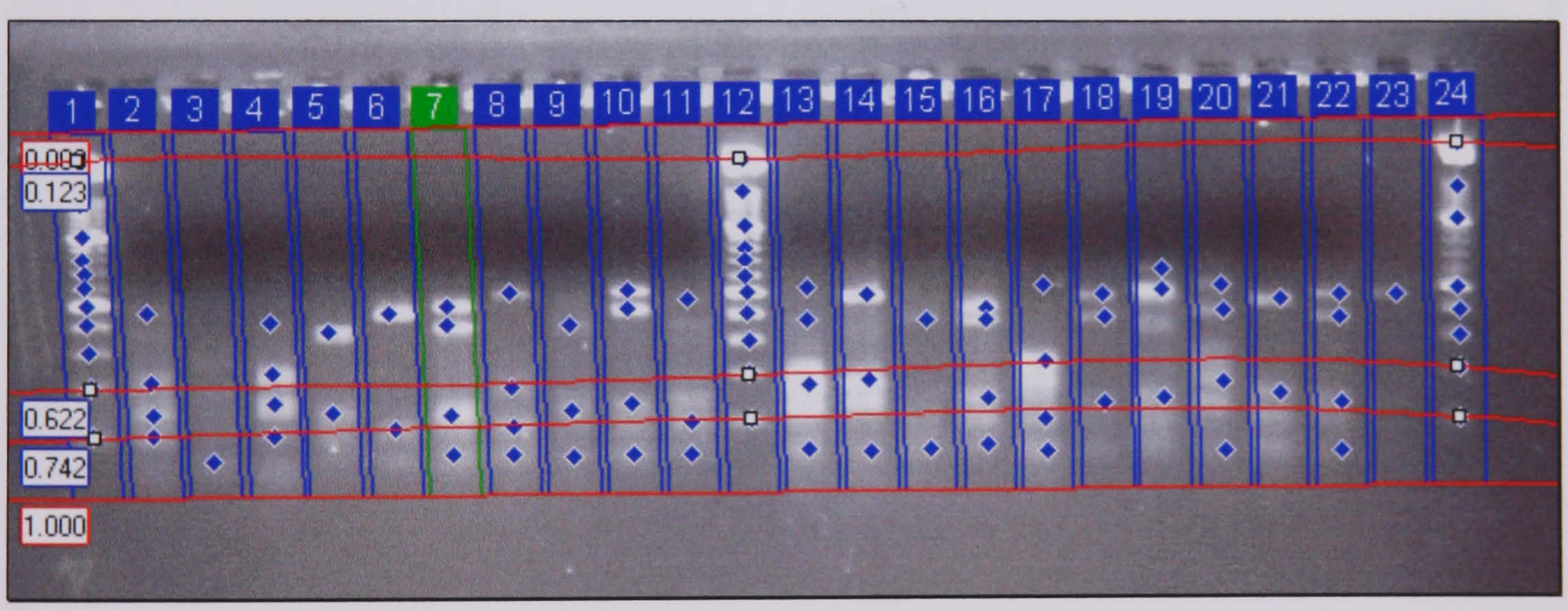


**Figure 2.6a - Standardisation across the gel using Retardation Factor, this alleviates any distortion due to uneven running or warping of the gel. The Rf points were anchored to molecular weight bands within the gel to provide cross-gel comparison.**

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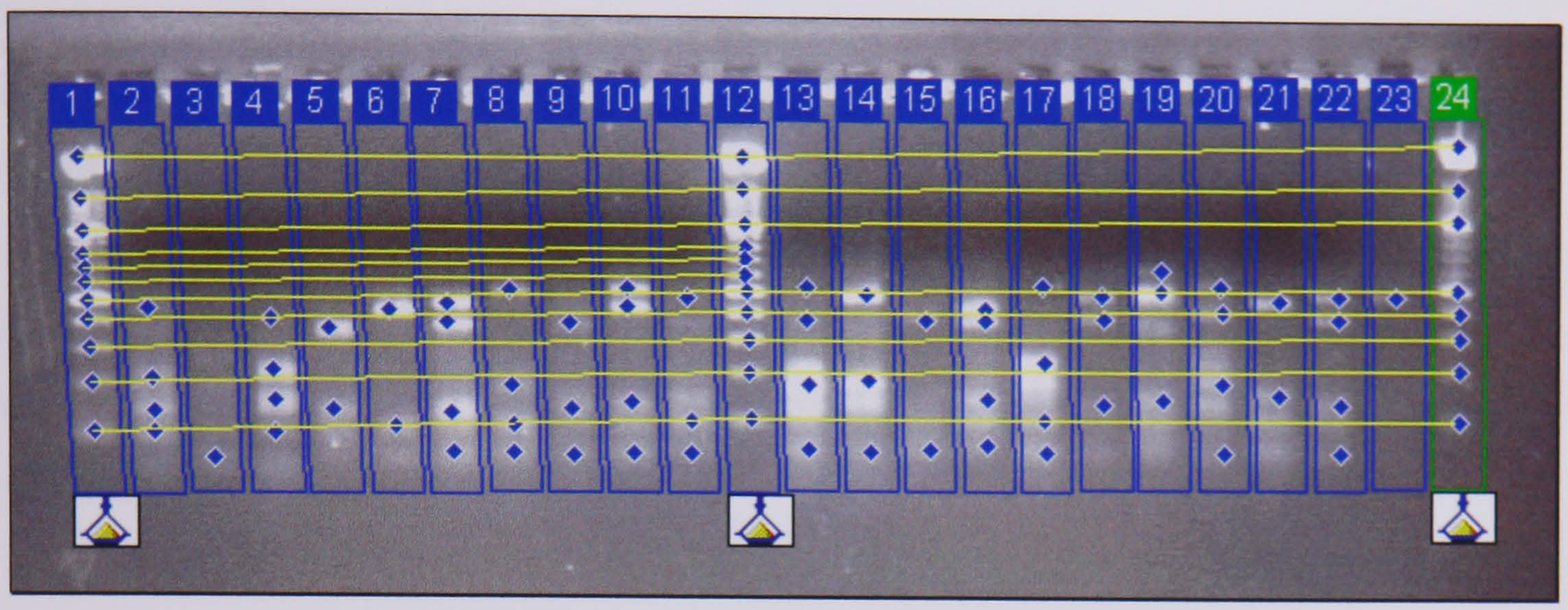


**Figure 2.6b – Molecular weights are assigned to each of the detectable bands within the three 100 base pair ladders, a curve is then computed which runs across the whole gel allowing MW values to be applied to each of the bands in all of the gels.**

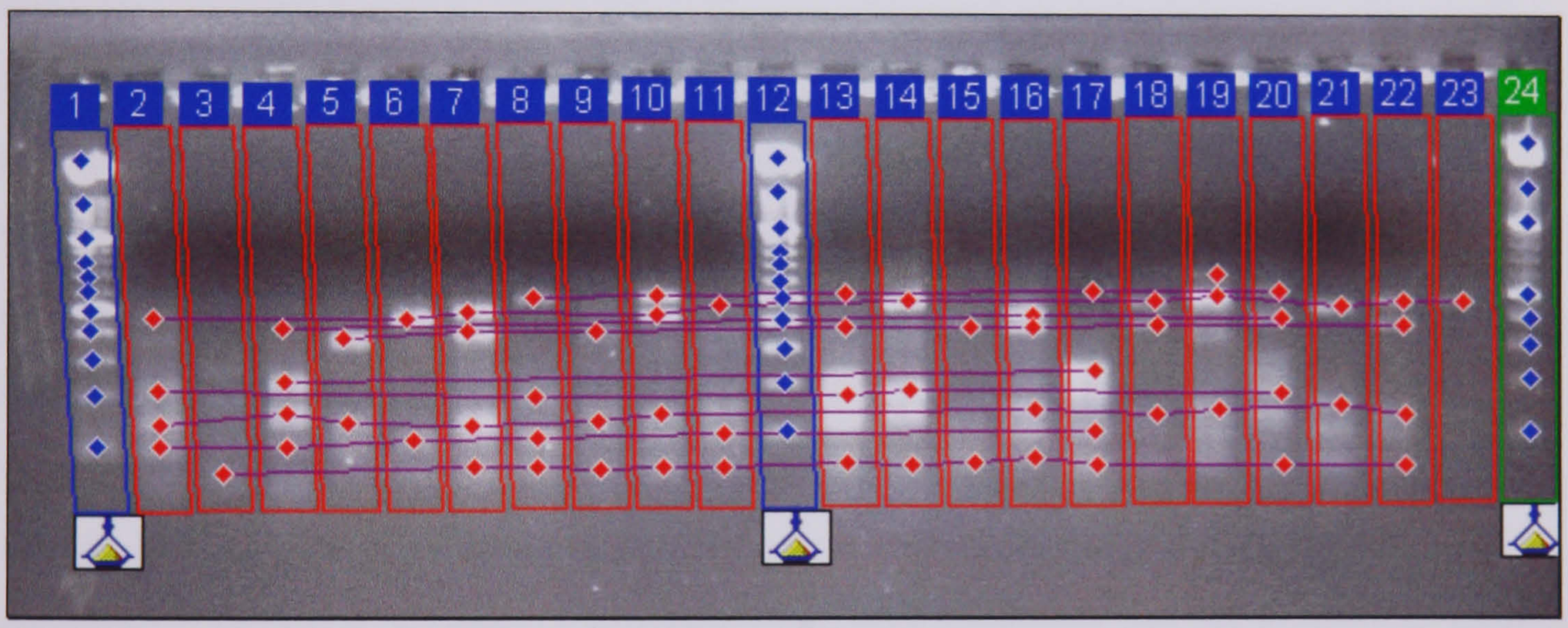


**Figure 2.7 - Each of the bands in each of the lanes is matched up to a band in a synthetic lane that is created by the program for the purpose of matching bands for comparison between lanes. Note only the specimens are matched and not the 100 base pair markers.**

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tubes were vortexed for 1 minute three times, in order to dislodge as many bacterial cells as possible from the filter membrane into solution. The filter was then squeezed to retain as much liquid as possible in the tube and removed to a new sterile Eppendorf tube and stored on ice. The initial tube was centrifuged at 13,000 *g* for 15 minutes (Biofuge pico, Heraeus) to form a pellet of available bacterial cells. If this pellet was not large enough for productive chromosomal extraction, then the filter was placed in a solution of 0.1% SDS and 0.2M Tris/HCl. This helped to lyse all remaining cells on the filter and free all DNA from the membrane surface. This was incubated at 55°C for 1 hour and then centrifuged at 13,000 *g* for 15 minutes to form a pellet or added to the initial tube and centrifuged to increase the pellet size.

Once the pellet was formed and the supernatant discarded, the pellet was resuspended in 567 µl TE buffer (Appendix A2) by repeated pipetting. Once in solution, 30 µl of 10% SDS and 3 µl of 20mg/ml proteinase K (Sigma) was added and incubated for 1 hour at 37°C. After incubation 100µl of 5M NaCl was added and then the solution mixed thoroughly by quick vortexing. This was followed by the addition of 160 µl of CTAB/NaCl solution (Ausubel *et al.,* 1999), the solution was mixed and then incubated at 65°C for 1 hour. This step was used to remove the polysaccharides and other contaminating macromolecules. After incubation, an equal volume (860µl) of chloroform/isoamyl alcohol was added and the solution was mixed using vortexing. This was then centrifuged (13,000 *g*, 10 min) and the top aqueous layer was collected and transferred to a new clean tube. To this was added 0.6 vol isopropanol and the solution was mixed very gently by inversion until the DNA had precipitated. This was then centrifuged (13,000 *g*, 10 min). The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol. This was centrifuged (13,000 *g*, 10 min) and the supernatant was discarded and the pellet was air-dried. After drying the pellet was resuspended in 50 µl of TE buffer and then stored at 4°C.

*2.9.5.3 – Nested V3 region PCR amplification for DGGE*

The variable V3 region of the bacterial 16S rRNA gene (Neefs *et al.*, 1990) was amplified from a 1kb fragment of the 16S rRNA gene which was previously amplified from the community genomic DNA. The 1kb fragment was amplified using the method outlined in section 2.9.3.1, except that two different universal primers were used, 16S

rRNA 41F (5'-GCT CAG ATT GAA CGC TGG CG-3') and 16S rRNA 1066R (5'-ACA TTT CAC AAC ACG AGC TG-3'), which amplified the 1025 bp fragment of the 16S rRNA gene which contained the V3 region. Amplification of a smaller fragment (i.e. not the whole gene) limited the possible amplification of non-specific products during PCR.

The PCR reaction for the amplification of the V3 region was as follows: To 3 µl of genomic DNA the following reagents were added, 2.5 µl of PCR buffer (Gibco BRL), 1.25 µl MgCl (Gibco BRL), 0.5 µl of dNTP mix (Gibco, BRL), 1 µl of primer V3F (5'-CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3' with GC clamp; Invitrogen), 1 µl of primer V3R (5'-ATT ACC CGC GCT GCT GG-3'; Invitrogen) (primers taken from Muyzer *et al.*, 1993) and 2.5 µl of *Taq* polymerase (Invitrogen). This was made up to 25 µl. The reaction mix was placed in 0.25 µl thin walled micro-Eppendorf tubes and positioned in a PCR block (Techne Progene). The following 'step-down' thermal cyclic program was taken from Ercolini *et al.* (2001a): 1 cycle of 5 min at 94°C; 10 cycles of 94°C (1 min), 66.0 – 56.0 (1 min) and 72°C (3 min); 20 cycles of 94°C (1 min), 56°C (1 min), 72°C (3 min); and finally one cycle of 72°C (10 minutes) to ensure complete extension of all strands. The V3 fragment was 233 bp long. The reaction was checked by gel electrophoresis of 2µl of PCR product (Section 2.9.2).

*2.9.5.4 - Denaturing gradient gel electrophoresis*

DGGE was performed using the D-code system apparatus (Bio-Rad, Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD) using a method from Muyzer *et al.* (1993). The PCR products were run in 8% (wt/vol) polyacrylamide gels in 1X TAE (Sambrook *et al.*, 1989) with denaturing gradients formed with 8% (wt/vol) acrylamide stock solutions (acrylamide-*N*,*N'* – methylenebisacrylamide 37:1) containg 0% and 100% denaturants (7 M urea (Sigma) and 40% (vol/vol) deionised formamide (Sigma). Gels were formed by the setting of a 1.5 mL 8% polyacrylamide solution with 0% denaturant as a base layer, upon which the 15 mL denaturing gradient gel was poured to produce a 30%-50% gel. Electrophoresis consisted of 50 V voltage for 10 min which allowed the DNA to migrate slowly out of the wells. Then electrophoresis was run at 140 V for 6 h. After electrophoresis, the gels were stained for 3 min in ethidium bromide (0.5 mg $L^{-1}$) and then rinsed for 15 minutes in

reverse osmosis water and photographed using the ImageMaster® VDS unit (Pharmacia Biotech) with UV transillumination (302nm). Position of bands was analysed using the ImageMaster 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics) as shown for ARDRA analysis (section 2.9.4).

**2.9.6 - Sequencing of bacterial 16S rDNA**

For each isolate of interest the 16S rRNA gene was amplified using PCR (section 2.9.3.1) and the nucleotide sequence of the gene was determined using chain-termination sequencing (Sanger *et al.*, 1977) performed at the biopolymer synthesis and analysis unit (BSAU) Nottingham University. For most of the isolates, sequencing was performed directly on the purified PCR product; however, in some cases, this did not produce satisfactory results and the product was cloned using the TOPO TA Cloning reaction (Invitrogen).

*2.9.6.1 – Purification of 16S rDNA PCR products*

It was necessary to purify the 16s rDNA PCR product prior to sequencing to remove any primers, excess salts and other contaminants (enzymes, unincorporated nucleotides, agarose dyes, etc.) which could interfere with the sequencing reaction. The QIAquick™ PCR purification kit (Qiagen) was used to purify the PCR amplicons. The following information is modified from the QIAquick™ handbook (Qiagen). The kit used a system in which nucleic acids were absorbed to a silica-gel membrane while the contaminants were passed through during microcentrifugation. The QIAquick PCR purification protocol was as follows: 5 volumes of buffer PB were added to one volume of PCR product, the solution was then mixed. The solution was added to a QIAquick spin column that was placed inside a 2ml collection tube. This was centrifuged at 10,000 *g* (13,000 rpm, Biofuge *pico*, Heraeus) for 1 min. The flow-through was discarded and 0.75ml of PE buffer (wash buffer) was added to the spin column, which was placed back in the empty 2ml collection tube. This was then centrifuged (13,000 rpm, 1 min). The flow-through was discarded and the spin column was centrifuged for one minute to remove the entire wash buffer. The column was then placed in a 1.5ml Eppendorf tube and 30µl of elution buffer (sterile reverse osmosis water) was added to the spin column (directly onto the membrane) and allowed to stand for 1 min. It was then centrifuged for

1 min. The spin column was removed and discarded and the purified product was frozen at -20°C to prevent autocatalytic degradation.

*2.9.6.2 – Purification of PCR products by gel extraction*

When spurious non-target DNA was found to occur within a PCR reaction, the target PCR product was purified by gel extraction, which was performed using the QIAquick gel extraction protocol (QIAGEN; QIAGEN, GmbH, Germany): The PCR reaction was electrophoresed on a 0.8% low-melting point agarose gel (Sigma) which was run at 50V for 1 h. The gel was then viewed using a UV transilluminator and the desired product was identified from the spurious product by molecular weight, using a 100bp molecular weight marker (Promega) as a guide. The desired product was excised from the gel with a clean, sharp scalpel. Care was taken to remove as much agarose as possible without losing DNA. The gel slice was weighed (Sartorius Balance) and 3 volumes of buffer QX1 were added to 1 weight of gel. This was then incubated at 70°C for 10 min to dissolve the gel fragment. Dissolution was aided by flicking the tube to mix the solution. When the gel was completely dissolved 1 vol of isopropanol was added and then mixed (the pH was checked at this time, if the pH was > 7.5 then 10μl of 3M sodium acetate, pH 5 was added). The solution was loaded into a QIAquick spin column and centrifuged (13,000 rpm, 1 min; Biofuge *pico*, Heraeus). The flow-through was discarded and 0.5 mL of buffer QX1 was added to the column, which was then re-centrifuged (13,000 rpm, 1 min). The flow-through was discarded and 0.75ml of buffer PE (wash buffer) was added to the column and then centrifuged (13,000 rpm, 1 min). The flow-through was discarded and the column was re-centrifuged (13,000 rpm, 1 min) to remove all trace of the wash buffer. The spin column was then placed in a sterile 1.5 mL Eppendorf tube, and eluted using 30 μl of sterile reverse osmosis water. The flow-through was frozen at –20°C until sequencing, to prevent autocatalytic degradation.

*2.9.6.3 – PCR product cloning*

In the event that direct sequencing of the 1500 bp 16S rDNA PCR product did not produce a satisfactory result, the fragment was cloned into a pCR®2.1 - TOPO® plasmid vector using the TOPO TA Cloning® kit (Invitrogen). TA cloning works by the ligation of a PCR product with a single deoxyadenosine (A) on the 3' ends (due to the terminal

transferase activity of *Taq* polymerase) to a vector with single overhanging 3' deoxythymidine (T) residues.

The following protocol is outlined in the TOPO TA Cloning Version M instruction manual (Invitrogen): 2 µl of PCR product was added to a solution of 1 µl salt solution, 1 µl TOPO vector and 2 µl sterile reverse osmosis water. This solution was incubated on ice for 5 min. Following incubation, 2 µl of the TOPO cloning mix was added to a vial of One Shot® chemically competent *Escherichia coli* (TOPO 10F) and mixed gently. This was then incubated on ice for 5 min, heat shocked at 42°C for 30 sec and then transferred back to ice and 250 µl of room temperature SOC medium (Invitrogen) was added and the tubes were shaken (200rpm) at 37°C for 1 h. Following incubation 50 µl was spread on pre-warmed selective agar plates (LB media (Oxoid) with 10 g $L^{-1}$ agar (Oxoid) and 50µg $ml^{-1}$ ampicillin (Sigma) and 40 µl of 40 mg $ml^{-1}$ X-gal (Sigma)) and incubated at 37°C overnight. Following incubation, white colonies were picked and cultured overnight in selective LB medium broth (Oxoid; 50µg $ml^{-1}$ ampicillin). Following incubation, the plasmid was isolated using the QIAprep® plasmid extraction kit (QIAGEN), which uses a protocol involving the alkaline lysis of bacterial cells (modified from Birnboim & Doly, 1979) followed by the adsorption of the DNA on to a silica membrane in the presence of a high salt concentration. DNA is then washed by buffers PB and PE which remove endonuclease and other contaminants. The plasmid was digested with *Eco*R I to excise the PCR product, this was then analysed on an electrophoresis gel (section 2.9.2) to determine if the cloning was successful. If cloning was successful then the plasmid extracts were sent for sequencing (see section 2.9.6.4).

*2.9.6.4 – Sequencing reaction*

The chain-termination method (Sanger *et al.*, 1977) was used to sequence the purified 16S rDNA PCR products, this was performed by the Nottingham University sequencing department. The chain-termination method requires single stranded DNA. The primers used for the amplification of the PCR product (1.5F and 1.5R – section 2.9.3.1) were used in the sequencing reaction. For sequencing of the cloned PCR product, the M13 forward and reverse primers were used.

The sequencing data was edited using the computer program Seqman (DNAstar Inc.) The data for the individual sequences and for the primed sequence areas were

compiled using Seqman and any erroneous nucleotides, ambiguous sections or nucleotides relating to the plasmid in sequences derived from cloned inserts, were either corrected or deleted based on manual comparison with the electropherograms. Sequences were then compared against the Genbank database of 16S rDNA sequences using the BLAST search engine (http://www.ncbi.nlm.nih.gov) to identify the nearest known phylogenetic relatives.

### 2.10 – Protein extraction and anti-freeze protein assessment technique

#### 2.10.1 – Total cellular protein extraction

In order to assess the thermal hysteresis activity of the isolated bacterium, it was necessary to obtain a solution containing a substantial concentration of the total cellular protein. The protein extraction protocol developed for the current study was as follows.

Isolates were inoculated into 250ml of appropriate broth (based on original agar media) in 500ml conical flasks. The cultures were incubated at 17°C for 1 week. The flasks were manually shaken once a day to allow adequate oxygen transfer to the broth solution. After 1 week of incubation, the cultures were transferred to 5°C for 1 week. This process is designed to cold shock the cells to induce cold adapted protein synthesis. Prior to protein extraction the cold shocked cultures were transferred to sterilized 250ml polypropylene centrifuge tubes. The glass bead protein extraction method was as follows:

The cultures (200 mL vol) were centrifuged at 15,300 *g* (10,000rpm JA-14) at 4°C, for 10 min (Beckman cooling centrifuge and JA-14 fixed angle rotor). The supernatant was then carefully removed so as not to disturb the pellet, and discarded. The pellet was then resuspended in 1ml of ice-cold Tris/HCl buffer (pH 7.0) and then centrifuged using the same centrifuge conditions stated above. The supernatant was carefully removed and discarded. The pellet was then resuspended in 1ml of ice-cold Protein Extraction Buffer (Appendix A2) and kept on ice for 5 minutes to allow cellular lysis due to osmotic shock. This suspension was then transferred to a sterile, ice-cold 1.5ml Eppendorf tube, to which was added 500mg of 106 microns or finer glass beads (Sigma). This was then shaken using a Mini Beadbeater™ (Biospec Products) for 1 min at 5000rpm and then chilled on ice for 1 minute to prevent heat denaturing of the protein. This was repeated 5 times for each suspension. To this solution was added 500µl of ice-cold protein extraction buffer, which was then vortexed to bring the cellular lysis

components into suspension. The Eppendorf tube was then centrifuged (13,000rpm, 4°C, 10 min, Biofuge *pico*, Heraeus) and the supernatant was transferred to a clean sterile ice-cold Eppendorf tube and frozen at -20°C until ready for AFP analysis.

All protein extracts were tested for protein using the BioRad Protein Assay Kit (BioRad). The assay was based on the Bradford method. It utilizes Coomassie Blue (G-250) as a dye reagent. The absorbance maximum shifts from 465nm to 565nm in an acidic solution as the dye binds to protein. Lyophilised Bovine Serum Albumin and Fish AFP III (10mg/ml) was used as a positive control, while Protein Extraction Buffer was used as a negative control. The sample (100µl) was transferred into a clean test tube to which 5ml of dilute BioRad dye reagent was added. The sample was vortexed and then measured at $OD_{595}$ verses a reagent blank using a spectrophotometer (Cecil CE 2021, 2000 series). The positive controls both showed a deep blue colouration, while both negative controls showed a brown/red colouration consistent with the cationic, double protonated state of the dye at the assay pH.

**2.10.2 – 'SPLAT' analysis**

The protein extractions (PE) were taken on ice to Unilever's Colworth laboratory where they were tested for recrystalisation inhibition activity using the 'SPLAT' assay equipment. The protocol is modified from Knight *et al.* (1988). PE solutions were standardised by adding 50 µl of 60% sucrose into solution with 50 µl of PE. These were made up in 1.5ml fixed lid Eppendorf tubes. The 30% sucrose/PE solutions were spun in an MSE Micro Centaur desktop micro-centrifuge for 10 sec (to make sure all liquid was at bottom of the tube and mixed well). Of the resulting solution, 5µl was placed between two circular 16mm diameter, numbered coverslips, which were blotted dry to remove any excess fluid. The coverslips were placed in 2,2,4 – trimethyl pentane pre-cooled to -70°C (using dry ice) for 2 minutes, to super-cool the samples. A circulating solvent bath was filled ¾ full of 2,2,4 – trimethyl pentane and pre-chilled to -6°C using a Haake C water bath circulator and two Haake Temperature control units (PG40 and F4). The coverslips were taken directly from the -70° C solvent baths to the -6°C solvent bath (as quickly as possible to prevent melting) and held there for one hour to allow for recrystalisation. The coverslips were then viewed whilst in the bath using an EF L 20/0.30 160/0-2 objective on a Leitz Dialux 20 EB stage. The observed crystal shape, size and density were

recorded and related to the level of AFP activity using the splat scoring system (Table 2.2).

| Observed crystal structure | Splat Score |
|---|---|
| Very small, very dense (e.g. Fish AFP III) | +++++ |
| Small not dense or small quite dense with mediums (*Marinomonas protea* AFP) | ++++ |
| Small not dense with medium-large or small-medium not dense | +++ |
| Mediums or Large with some smalls | ++ |
| Large round discrete crystals (e.g. 30% sucrose) | + |

**Table 2.2 – 'SPLAT' scoring key. The size and density of ice crystals relates to observations made after 1 hour of recrystalisation at -6°C.**

# Chapter 3: BIOLOGICAL AND PHYSICO/CHEMICAL CHARACTERISTICS OF THE STUDY LAKES

## 3.1 - Introduction

The lakes sampled during this study were Beaver Lake (MacRobertson Land, 70°48'S, 68°15'E, Fig. 2.1c), and 2 lakes from the Larsemann Hills (69°23'S, 76°23'E, Fig. 2.1b) and 39 lakes from the Vestfold Hills (68°30'S, 78°E, Fig. 2.1a). The vast majority of study was focused on the Vestfold Hills, an ice free 'oasis' created by isostatic uplift, which formed approximately 300 lakes and ponds from the sea as the land rose in the wake of the retreating polar ice cap. The marine origin of the lake water is still obvious in the ion ratios of a number of the saline and hypersaline lakes (Pickard, 1986). Evaporation, sublimation, melt-water input and various other factors have produced a wide variety of different limnological environments, including various stages of stratification (for example monomixis and meromixis (of which the Vestfold Hills may have the largest concentration in the world), various salinities (from freshwater to saline and hypersaline) and various levels of trophy (for example the eutrophic Rookery Lake and ultra-oligotrophic Crooked Lake).

Over the last thirty years the lakes and fjords of the Vestfold Hills have been extensively researched (Kerry *et al.,* 1977; Burton & Barker, 1979; Hand, 1980; Burton, 1981; Hand & Burton, 1981; Burke & Burton, 1988; McMeekin & Franzmann, 1988; Tucker & Burton, 1988; Volkman *et al.*, 1988; Franzmann *et al.*, 1990; Gibson *et al.*, 1990; Mancuso *et al.*, 1990; Franzmann & Rohde, 1991; Franzmann & Rohde, 1992; Laybourn-Parry & Marchant, 1992a; Laybourn-Parry & Marchant, 1992b; Laybourn-Parry *et al.*, 1992; Perriss *et al.*, 1993; Bayliss & Laybourn-Parry, 1995; Laybourn-Parry *et al.*, 1995; Perriss *et al.*, 1993; Perriss *et al.*, 1995; Laybourn-Parry & Bayliss, 1996; Bayliss *et al.*, 1997; Grey *et al.*, 1997; Laybourn-Parry, 1997; Tong *et al.*, 1997; Laybourn-Parry *et al.*, 1998; Bell & Laybourn-Parry, 1999b; McMinn *et al.*, 2000; Waters *et al.*, 2000; Labrenz & Hirsch, 2001). Much of the literature is focused on biological production rates, organism diversity and microbial ecology. There is paucity of

information regarding the cold adaptation mechanisms of bacteria from many of the 300+ lakes from this region. The current study was conducted on 41 lakes during January to November, 2000, to provide information on the antifreeze protein (AFP) activity status of bacteria from the lakes of the Vestfold Hills. AFPs act to inhibit the recrystalisation of ice, prevent cellular disruption, and induce thermal hysteresis, i.e. lowering the freezing point of the cell below the melting point (Knight & DeVries, 1994). AFPs are of interest to biochemical industries for uses such as frozen food preservation and transplant organ storage (Barrett, 2001).

As an integral part of the study, bacterial and flagellate abundance and the physico-chemical and biological characteristics of each lake were assessed. This enabled a relatively detailed analysis of the biogeography of the AFP active bacteria and provided information as to the physical, chemical and biological attributes of a lake system which might have forced the evolution of AFP activity in bacteria. All bacteria isolated from the 1999/2000 austral summer sampling trips were analysed for AFP activity (refer to Chapter 4). Those lakes from which high numbers of AFP active bacteria were isolated were chosen for a detailed study during the winter and spring of 2000 (June- November). The aim of this investigation was to provide information on the spatial and temporal variability of physical, chemical and biological characteristics for these lakes, and to try and isolate more AFP active bacteria. The five lakes chosen were, Ace Lake, Pendant Lake, Triple Lake, Deep Lake, and Club Lake. Oval Lake was not selected for the focus study but one bacteria isolated from the lake has subsequently been shown to be AFP active (Chapter 4). The focus study lakes as well as Oval Lake are dealt with in detail within this chapter; summer study lakes are analysed in less detail.

## 3.2 – Results

The results are divided into two sections, section 3.2.1 deals with those lakes sampled during the 1999/2000 austral summer, while section 3.2.2 deals with the focus study lakes as outlined in the introduction (3.1).

### 3.2.1 - Summer sampled lakes

The following lakes were only sampled during the summer of 1999/2000, they are divided into hypersaline lakes (*Rookery Lake, Lake Trident, Lake Tassie, Oblong Lake, Organic Lake, Lake Jabs, Dingle Lake, Lake Stinear, Lake Lebed, Lake Laternula, Angel*

*'2' Lake and Cemetery Lake*), saline lakes (*Pointed Lake, Lake Verenteno, Calendar Lake, Lake Williams, Ekho Lake, Shield Lake, Lake Anderson, Watts Lake, Scale Lake, Collerson Lake, Lake Braunsteffer, Lake Abraxas and Oval Lake*) and freshwater lakes (*Nottingham Lake, Lake Druzhby, Crooked Lake, Nicholson Lake, Pauk Lake, Lake Zvezda, Hand Lake, Lake Medusa* (all in the Vestfold Hills) *Larsemann Lake 73, Lake Reid* (in the Larsemann Hills) and *Beaver lake* (MacRobertson Land)). For locations of these lakes refer to Chapter 2. The physical, chemical and biological characteristics as recorded during the current study are dealt with here.

*3.2.1.1 - Physico-chemical characteristics*

*3.2.1.1.1 - Ice cover*

The percentage ice cover or ice thickness of the 36 hypersaline, saline and freshwater lakes sampled during the summer period, varied considerably between the lakes (Tables 3.1a, b and c). Several lakes (e.g. Lake Nottingham and Beaver Lake) had permanent ice cover. Other lakes had no ice cover during the summer only forming a thin ice sheet during the winter (e.g. Dingle Lake, Lake Stinear, Lake Lebed). All the other lakes showed some extent of melt-out from moating (e.g. Shield Lake and Oval Lake) to complete loss of ice-cover during the summer (e.g. Rookery Lake). Oval Lake showed an 20% loss of ice cover due to moating (Table 3.1b) but it is known that there was approximately 80% melt before refreezing.

*3.2.1.1.2 - Temperature*

Water temperature varied significantly between the lakes (Table 3.1a, b and c). All lake systems whether hypersaline, saline or freshwater were observed to be colder in January than February and then significantly colder in March. The saline and hypersaline lakes showed significant variation and those without ice cover the greatest variation. The freshwater systems were all slightly above freezing. Oval Lake had the coldest water temperature recorded during the summer, -1.9°C (Table 3.1b).

*3.2.1.1.3 - Salinity*

The salinity for the lakes ranged from extremely hypersaline to freshwater (Tables 3.1a, b and c). Twelve are considered hypersaline (>35ppt) with salinities ranging from

50ppt to 186ppt. Thirteen were saline (4-35ppt) and the remaining 11 lakes were freshwater (<4ppt). The hypersaline lakes were geographically grouped with Dingle, Stinear, Deep, Club, Triple, Tassie, Trident and Jabs all being in close proximity on Broad Peninsula; Oblong and Lebed were in the Watts Lake region; Cemetery, Laternula and Angel '2' were on the Mule Peninsula, and Organic and Rookery on the Long Peninsula (for locations of lakes refer to Fig. 2.1a). Oval Lake had a salinity of 34ppt (Table 3.1b).

*3.2.1.2 - Inorganic nutrients*

Inorganic nutrient levels for the summer sampled lakes are shown in Table 3.1a,b and c.

*3.2.1.2.1 – Nitrate ($NO_3$-N) and nitrite ($NO_2$-N)*

Nitrate was below the level of detection in most lakes, except Lake Jabs, Lake Stinear, Lake Laternula, Lake Verenteno, Lake Collerson and Lake Zvezda. There were no obvious similarities between these lakes. Nitrite concentrations remained relatively constant between the lakes. However, concentration was normally higher in the hypersaline lakes.

*3.2.1.2.2 – Ammonium ($NH_4$-N) and SRP ($PO_4$-P)*

Ammonium levels were relatively constant between the lakes. However, certain hypersaline lakes (Lake Jabs and Lake Laternula) had much higher concentrations than the rest. SRP concentrations were on average higher in the hypersaline lakes than saline or freshwater lakes. An extremely high concentration was noted in Rookery Lake, which is enriched by penguin faeces as it lies adjacent to a rookery.

*3.2.1.2.3 – Dissolved organic carbon and chlorophyll* a

DOC levels showed an overall increase with salinity; the hypersaline lakes had higher concentrations than the freshwater lakes. Chlorophyll *a* concentrations showed a similar increase with salinity although it was not so marked.

There was no apparent depletion of nutrients in lakes sampled towards the end of the summer (March) compared with those sampled in the middle of the summer (January)

| Lake | Air Temperature (°C) | Relative Humidity (%) | Wind Speed (km/h) | Ice Thickness (m) | Salinity (ppt) | Temperature (°C) | $NO_3$ (µg $L^{-1}$) | $NO_2$ (µg $L^{-1}$) | $NH_4$ (µg $L^{-1}$) | $PO_4$ (µg $L^{-1}$) | DOC (mg $L^{-1}$) | Standard Deviation for DOC (mg $L^{-1}$) | Chl *a* (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rookery | 1.9 | 68 | 21 | *nr*, 33% | 50 | -0.9 | 0 | 4.2 | 1.83 | 218.1 | 23.9 | 1.113 | 29.05 |
| Trident | -1.4 | 60 | 18 | *nr*, 90% | 60 | -1.8 | 0 | 4.64 | 1.44 | 10.54 | 28.068 | 0.5397 | 1.34 |
| Tassie | -1.4 | 60 | 18 | *nr*, 0% | 78 | 0.3 | 0 | 6.52 | 2.37 | 7.38 | 23.303 | 0.968 | 0.27 |
| | | | | | | | | | | | | | |
| Oblong | -1.5 | 53 | 30 | *nr*, 100% | 70 | -1.2 | 0 | 5.18 | 0.69 | 33.03 | 31.203 | 1.005 | 1.17 |
| Organic | -3.3 | 63 | 26 | *nr*, 0% | 94 | 0.4 | 0 | 5.05 | 0.87 | 10.05 | 52.712 | 1.558 | 0.678 |
| Jabs | 1.5 | 63 | 22 | *nr*, 0% | 96 | 1.5 | 104.69 | 7.09 | 22.59 | 63.22 | 23.546 | 0.3773 | 1.33 |
| Dingle | 1.5 | 63 | 22 | *nr*, 0% | 136 | 1.8 | 0 | 9.95 | 32.3 | 38.74 | 29.714 | 1.254 | 1.32 |
| Stinear | 1.5 | 63 | 22 | *nr*, 0% | 154 | 1.8 | 36.87 | 10.78 | 6.48 | 56.9 | 33.961 | 1.126 | 1.45 |
| Lebed | -1.5 | 53 | 30 | *nr*, 0% | 195 | 1 | 0 | 7.6 | 1.76 | 37.24 | 65.433 | 2.391 | 1.34 |
| | | | | | | | | | | | | | |
| Laternula | -5.5 | 67 | 13 | *nr*, 0% | 146 | -0.9 | 43.36 | 6.4 | 28.76 | 10.81 | 53.629 | 0.675 | 0.876 |
| Cemetery | -5.5 | 67 | 13 | *nr*, 0% | 158 | -1 | 0 | 3.84 | 2.08 | 42.62 | 9999 | 4.882 | 7.85 |
| Angel 2 | -5.5 | 67 | 13 | *nr*, 0% | 186 | -1.3 | 0 | 5.04 | 2.01 | 4.1 | *nr* | *nr* | 0.067 |

**Table 3.1a- Physical and inorganic data for the hypersaline lakes sampled during January (top), February (middle) and March (bottom) 2000. Each set is organised into increasing salinity. Lakes from each set were sampled within 2 weeks of each other. *nr – not recorded.* Ice thickness measured in meters unless too thin to record (*nr*) in which case the percentage of the lake which was ice covered is shown.**

| Lake | Air Temperature (°C) | Relative Humidity (%) | Wind Speed (km/h) | Ice Thickness (m) | Salinity (ppt) | Temperature (°C) | $NO_3$ (µg $L^{-1}$) | $NO_2$ (µg $L^{-1}$) | $NH_4$ (µg $L^{-1}$) | $PO_4$ (µg $L^{-1}$) | DOC (mg $L^{-1}$) | Standard deviation for DOC (mg $L^{-1}$) | Chl *a* (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pointed | -1.4 | 60 | 18 | *Nr*, 90% | 4 | -0.5 | 0 | 3.77 | 3.73 | 15.73 | 10.508 | 0.1152 | 2.83 |
| Verenteno | -1.4 | 60 | 18 | Nr, 0% | 4 | 2.3 | 26.39 | 2.32 | 9.59 | 12.1 | 3.062 | 0.2004 | 1.07 |
| Calendar | -1.4 | 60 | 18 | *Nr*, 95% | 4 | -0.2 | 0 | 2.68 | 3.26 | 8.07 | 5.094 | 0.3675 | 0.92 |
| Williams | 1.9 | 68 | 21 | *Nr*, 66% | 20 | 0.1 | 0 | 2.62 | 0.63 | 11.1 | 5.142 | 0.1193 | 11.41 |
| Ekho | 1.9 | 63 | 31 | *Nr*, 90% | 24 | -0.5 | 0 | 5.58 | 1.41 | 17 | 2.462 | 0.3692 | 1.13 |
| | | | | | | | | | | | | | |
| Shield | 3.3 | 45 | 37 | *Nr*, 95% | 4 | -0.2 | 0 | 4.77 | 4.24 | 7.422 | 3.817 | 0.5111 | 0.9340 |
| Anderson | -4.5 | 50 | 17 | *Nr*, 95% | 4 | 0.9 | 0 | 4.54 | 6.32 | 14.4 | 1.923 | 0.1644 | 0.74 |
| Watts | -4.5 | 50 | 17 | *Nr*, 90% | 4 | 4.9 | 0 | 3.9 | 4.09 | 0.85 | 28.339 | 0.2293 | 0.36 |
| Scale | -4.6 | 66 | 17 | *nr*, 100% | 4 | 2.5 | 0 | 4.04 | 7.93 | 22.06 | 2.637 | 0.4278 | 2.06 |
| Collerson | -4.6 | 66 | 17 | *nr*, 100% | 4 | 0.8 | 20.45 | 2.3 | 4.8 | 29.67 | 2.932 | 0.5791 | 0.88 |
| Braunsteffer | -4.6 | 66 | 17 | *nr*, 100% | 4 | 3.5 | 0 | 3.88 | 8.9 | 26.3 | 4.693 | 0.5422 | 2.45 |
| Abraxas | -3.3 | 63 | 26 | *nr*, 100% | 10 | -0.2 | 0 | 2.32 | 0.78 | 0 | 3.044 | 0.6913 | 0.33 |
| Oval | 3.3 | 45 | 37 | *Nr*, 85% | 34 | -1.9 | 0 | 5.41 | 0.89 | 52.45 | 10.607 | 0.4749 | 0.95 |

**Table 3.1b - Physical and inorganic data for the saline lakes of the Vestfold Hills sampled during January (top) and February (middle) 2000. Each set is organised into increasing salinity. Lakes from each set were sampled within two weeks of each other. Ice thickness measured in meters unless too thin to record (*nr*) in which case the percentage of the lake which was ice covered is shown.**

| Lake | Air Temperature (°C) | Relative Humidity (%) | Wind Speed (km/h) | Ice Thickness (m) | Salinity (ppt) | Temperature (°C) | $NO_3$ (µg $L^{-1}$) | $NO_2$ (µg $L^{-1}$) | $NH_4$ (µg $L^{-1}$) | $PO_4$ (µg $L^{-1}$) | DOC (mg $L^{-1}$) | Standard Deviation for DOC (mg $L^{-1}$) | Chl a (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nottingham | 1.9 | 63 | 31 | 1m | 0 | 0.1 | 0 | 3.29 | 1.83 | 6.05 | 3.749 | 0.6146 | 0.53 |
| Druzhby | 1.3 | 68 | 39 | *nr*,60% | 0 | 0.9 | 0 | 6.54 | 8.18 | 7.46 | 5.389 | 0.4968 | 0.7 |
| | | | | | | | | | | | | | |
| Crooked | -6.2 | 84 | 26 | 1.5m Moating seen round islands | 0 | 0.2 | 0 | 5.15 | 3.05 | 19.34 | 0 | 0 | 0.68 |
| Beaver | *nr* | *nr* | *nr* | 4m | 0 | 0.1 | 0 | 2.412 | 3.714 | 6.571 | 0.159 | *nr* | 0.55 |
| Nicholson | -1.5 | 53 | 30 | *nr*,100% | 0 | 0.6 | 0 | 2.76 | 3.9 | 10.56 | 2.799 | 0.1801 | 0.37 |
| Pauk | -4.6 | 66 | 17 | *nr*,100% | 0 | 1.9 | 0 | 2.12 | 1.29 | 0 | *nr* | *nr* | 0.28 |
| Zvezda | -4.6 | 66 | 17 | *nr*,100% | 0 | 2.2 | 12.65 | 3.8 | 1.57 | 1.4 | *nr* | *nr* | 0.44 |
| LH 73 | *nr* | *nr* | *nr* | 0.4m | 0 | 2.4 | 0 | 3.66 | 6.35 | 0 | 2.934 | 0.1427 | 0.314 |
| Reid | *nr* | *nr* | *nr* | 1m | 0 | 4.2 | 0 | 2.81 | 8.44 | 0 | 6.257 | 0.4233 | 0.324 |
| Hand | 1.5 | 63 | 22 | *nr*,95% | 2 | 0.9 | 0 | 5 | 6.6 | 49.08 | 2.063 | 0.2051 | 0.76 |
| Medusa | -4.6 | 66 | 17 | 100% | 2 | 0.8 | 0 | 3.13 | 6.84 | 25.19 | 2.943 | 0.1428 | 0.46 |

**Table 3.1c - Physical, inorganic nutrient and chlorophyll *a* data for freshwater lakes from the Vestfold Hills and Larsemann Hills sampled in January (top) and February (bottom) 2000. Each set is organised in to increasing salinity (where appropriate). Lakes from each set were sampled within two weeks of each other. Freshwater lakes are regarded as those whose salinity is <4ppt. *nr-not recorded.* Ice thickness measured in meters unless too thin to record (*nr*) in which case the percentage of the lake which was ice covered is shown.**

as would normally be expected, as biologically available nutrients are locked up in the increased biomass.

### 3.2.2 - Focus study lakes

Only five lakes were chosen for the detailed study from January to November 2000, these were Ace Lake, Pendant Lake, Club Lake, Triple Lake and Deep Lake. They were chosen because they revealed the highest number of AFP positive isolates (refer to chapter 4).

#### *3.2.2.1 - Physico-chemical characteristics*

##### *3.2.2.1.1 - Ice cover*

Ice cover on Ace Lake and Pendant Lake thickened during winter, with some fluctuation. This continued into November, with no observed melt (Fig 3.1a & b). Triple showed total melt-out during the summer months and the early winter months but ice thickness increased to 0.4m during the winter, followed by a rapid thinning in November (Fig 3.1c). Deep and Club had no ice cover throughout the year, apart from occasional greasy ice on extremely cold and still days (<-30°C/0kph).

##### *3.2.2.1.2 - Temperature*

Water temperature in Ace Lake (Fig. 3.2a) and Pendant Lake (Fig. 3.2b) varied only slightly throughout the year, with surface water temperature fluctuating between -2 and +1°C. Temperature was shown to steadily increase with depth throughout the year. A temperature anomaly was noted in both lakes during September with a extremely high temperature at ~10m. In Triple Lake (Fig. 3.2c), the depth profile for temperature was virtually uniform, but, as with the surface water recording of Deep Lake (Fig 3.3a) and Club Lake (Fig 3.3b), the temperatures were relatively warm in Summer (~3°C) and extremely cold in winter (-14°C).

##### *3.2.2.1.3 - Salinity*

Ace Lake, a meromictic lake, showed a distinct salinity profile in the mixolimnion (the monimolimnion was not sampled during this study) throughout the year with an increase in salinity with increase in depth, with the exception of the September and November when the water column was virtually isohaline (Fig. 3.4a). Pendant Lake (Fig.

3.4b) also showed an increase in salinity with depth on all sampling dates except November where a slight decrease in salinity with increasing depth was recorded. The average salinity was shown to increase from January to November in both lakes. The salinity profile for Triple Lake (Fig. 3.4c) is virtually isohaline; however, there was generally a small increase in salinity with depth. Deep Lake (Fig. 3.3a) and Club Lake (Fig. 3.3b) showed no more than a 7% fluctuation in surface water salinity throughout the year. Salinity was generally lower during the summer, indicating melt water input causing a temporary dilution.

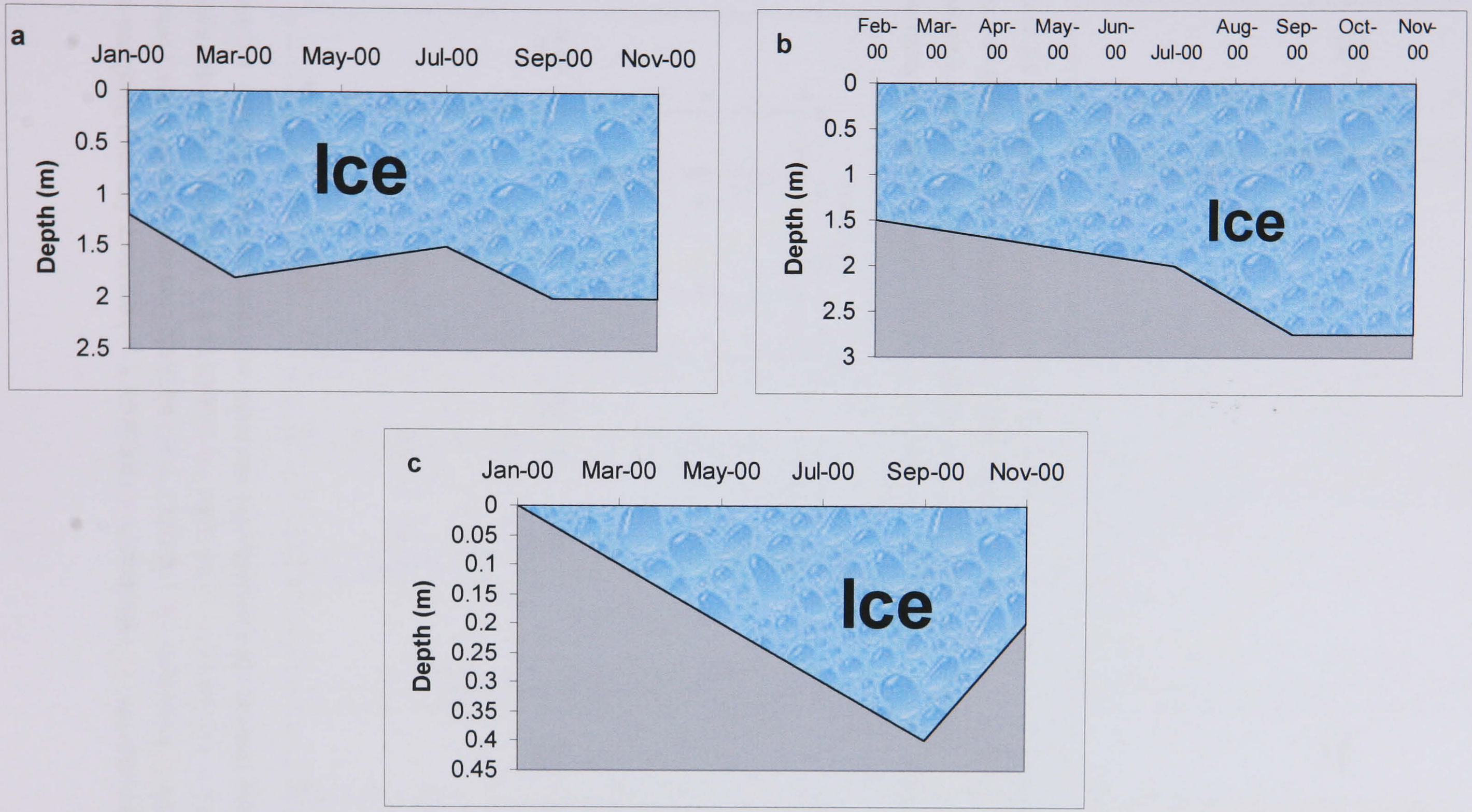


**Figure 3.1 – Ice thickness and date of recording for three of the five detailed study lakes throughout the 2000 sampling period. (a) Ace Lake, (b) Pendant Lake and (c) Triple Lake. Deep Lake and Club Lake are not shown as they had no ice cover throughout the year.**

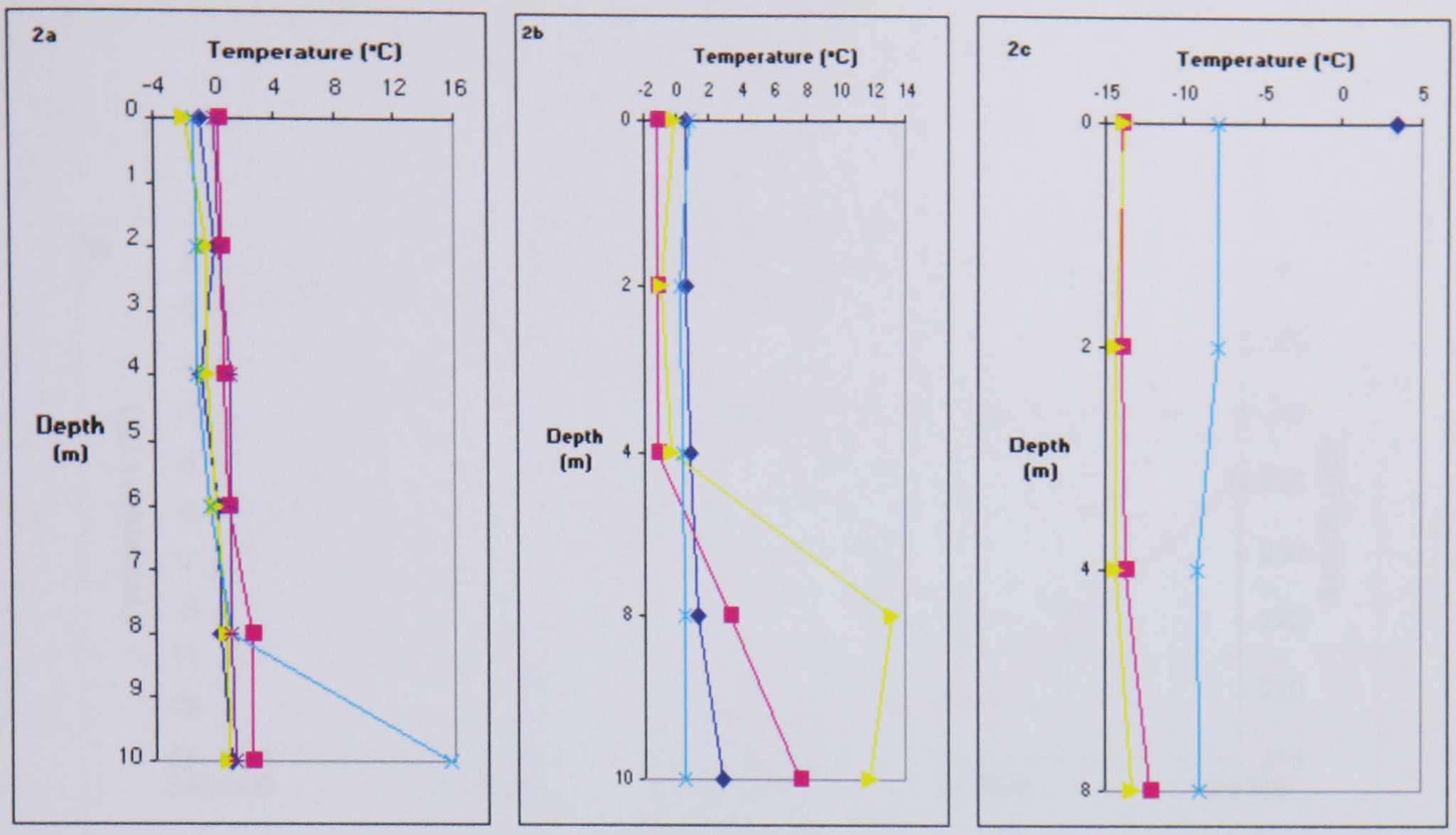


**Figure 3.2 - Temperature measurements for Ace Lake (a), Pendant Lake (b) and Triple Lake (c). Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

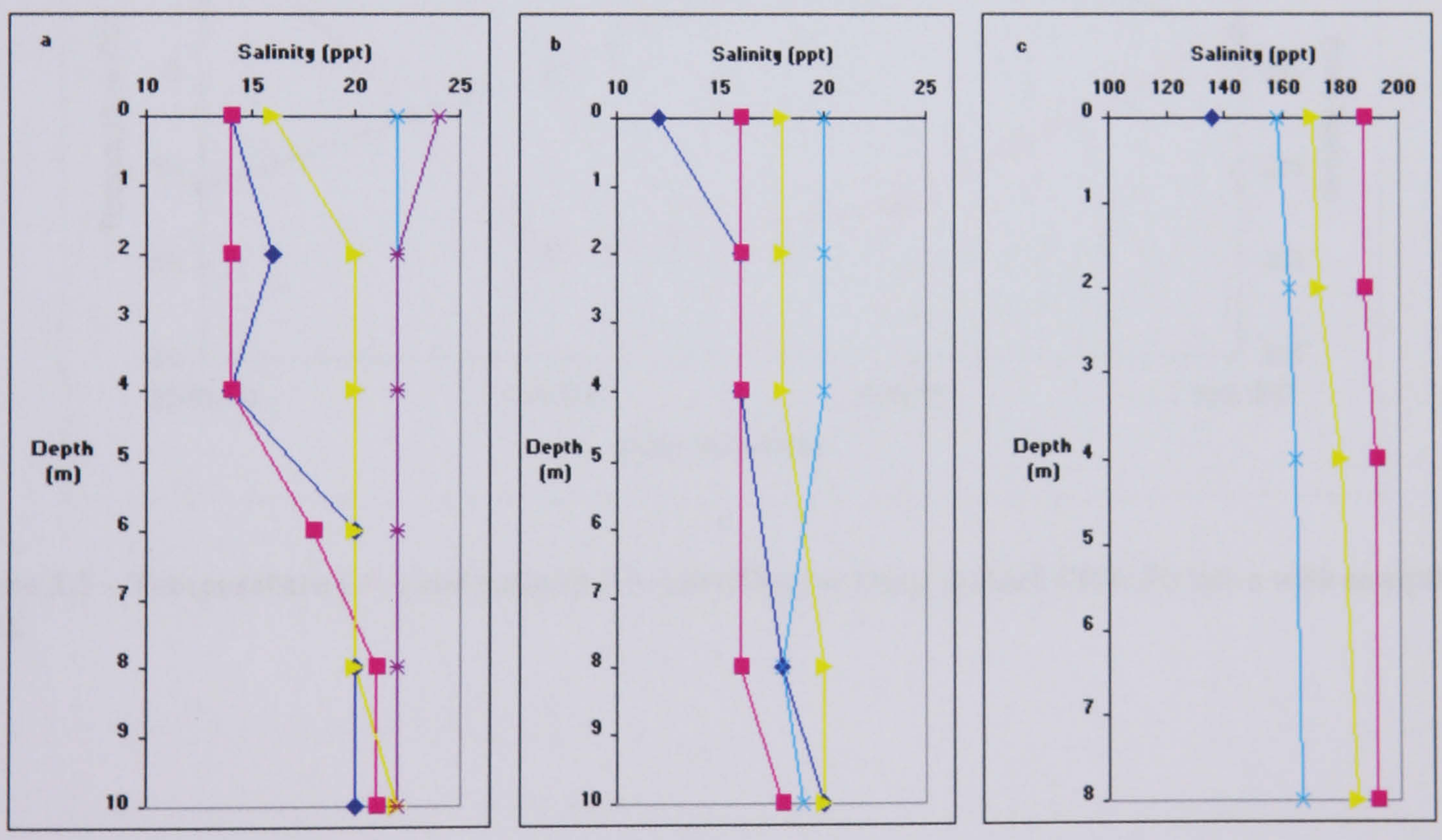


**Figure 3.4 – Salinity measurements for Ace Lake (a), Pendant Lake (b) and Triple Lake (c). Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

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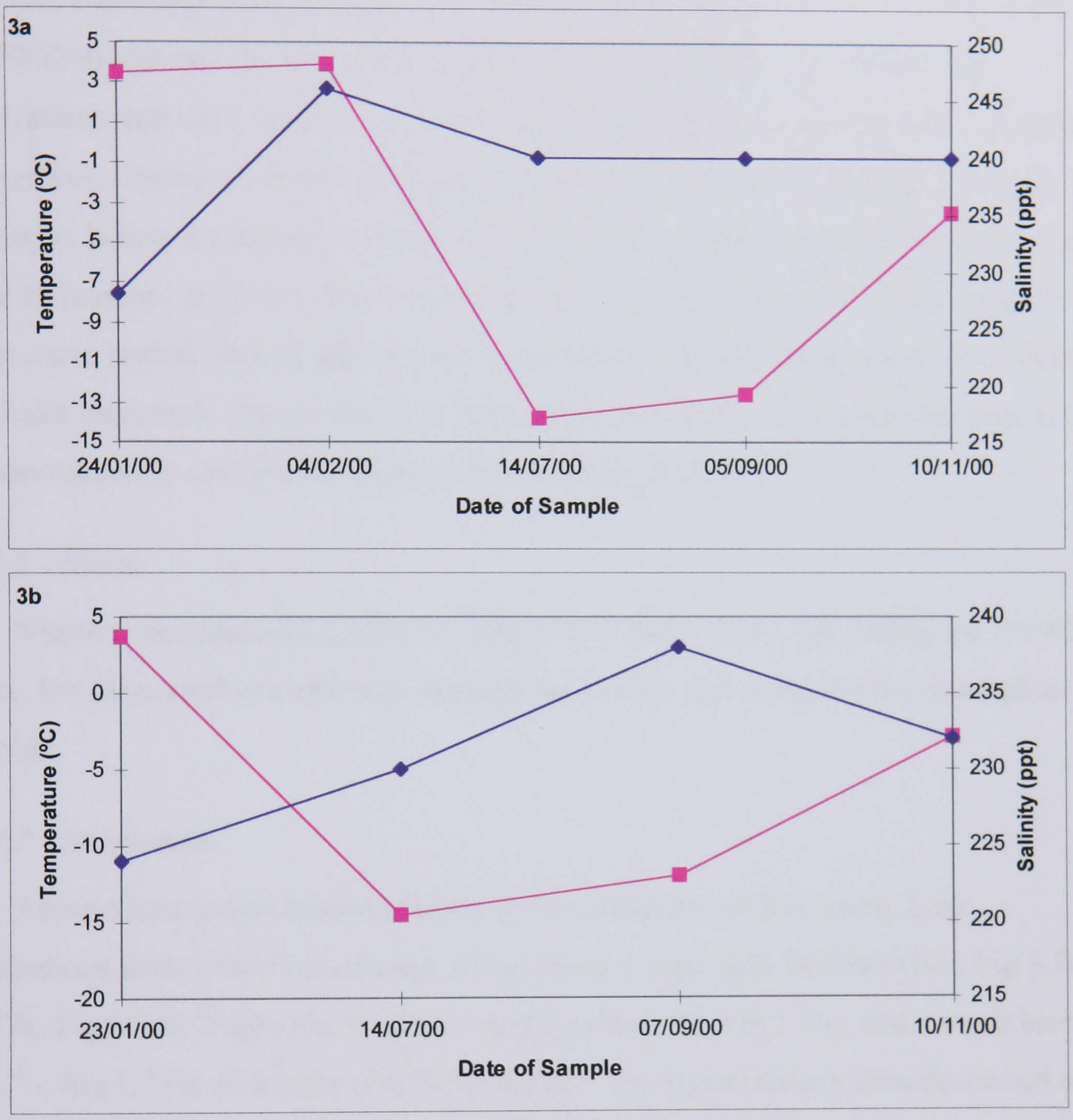


**Figure 3.3 – Temperature (-■-) and salinity (-◆-) profiles for Deep (a) and Club (b) lakes with sampling dates.**

*3.2.2.2 - Inorganic nutrients*

*3.2.2.2.1 - Nitrate*

Ace Lake (Fig 3.5a) and Pendant Lake (Fig 3.5b) both had low concentrations during the 1999/2000 summer, but those for Pendant Lake were higher. Both lakes had concentrations that were very low or below the level of detection during July, but increased by September. During November, Pendant Lake levels increased again but Ace Lake levels decreased to below the detection threshold. Triple Lake (Fig 3.5c) and Deep Lake (Fig 3.5d) showed an increase in levels from January to July. Club Lake (Fig 3.5e) showed a decrease over the same period. Deep Lake showed a reduction in levels during September whilst Triple Lake increased. The reverse was seen during November. The concentration in Club Lake was relatively uniform throughout the sampling period.

*3.2.2.2.2 - Nitrite*

Nitrite concentrations in all five lakes (Fig 3.6a-e) were high during the 1999/2000 summer, but decreased considerably through the winter, and remained low through to November.

*3.2.2.2.3 - Ammonium*

Ammonium concentration profiles were similar for all five lakes. Low concentrations during the first summer followed by a huge peak in Club (10x, Fig 3.7e), Deep (7x, Fig 3.7d), Triple (5x, Fig 3.7c) and Pendant (7x, Fig 3.7b), and a small increase ($4\mu g\ L^{-1}$ - $7\mu g\ L^{-1}$) in Ace Lake (Fig 3.7a) in July. The concentration then decreased to near the level of detection in September and remained low in November, except Ace Lake which had its highest concentrations during September (Fig. 3.7a).

*3.2.2.2.4 – Soluble reactive phosphate*

Soluble reactive phosphate (SRP) showed a similar profile to ammonium with concentrations higher in January to March than September to November, and peaking in July, this was indicative of all five lakes. Generally concentrations in November were the lowest recorded for the whole sampling season (Ace (Fig 3.8a); Pendant (Fig 3.8b); Triple (Fig 3.8c); Deep (Fig 3.8d); Club (Fig 3.8e)).

*3.2.2.2.5 - Dissolved organic carbon*

Concentration profiles for DOC were similar for both Ace Lake (Fig 3.9a) and Pendant Lake (Fig 3.9b), with a relatively constant level throughout the sampling season. However, concentrations were lower in July than September, and November had the lowest concentration for the year. Triple (Fig 3.9c), Deep (Fig 3.9d) and Club (Fig 3.9e) lakes all showed similar profiles with concentrations highest in January, lowest in July and all showed a steady increase in concentration from July to November. Out of these three hypersaline lakes, Triple (Fig 3.9c) had the lowest concentration, then Club (Fig 3.9e), and Deep (Fig 3.9d) had the highest.

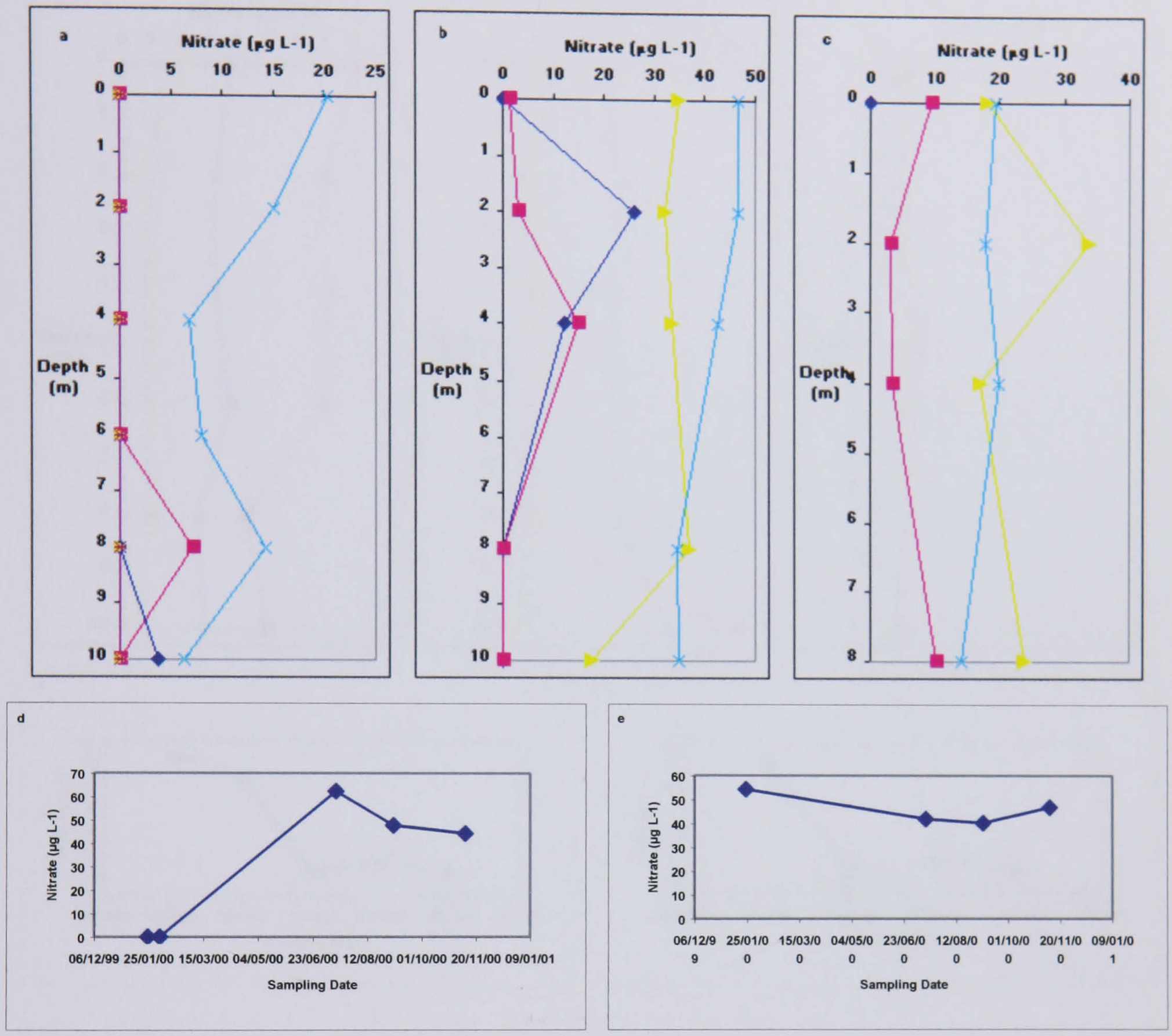


**Figure 3.5 – Nitrate concentration (µg $L^{-1}$) for the five detailed study lakes. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake (d) and Club Lake (e) were only surface water sampled (0m) over the sampling dates Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

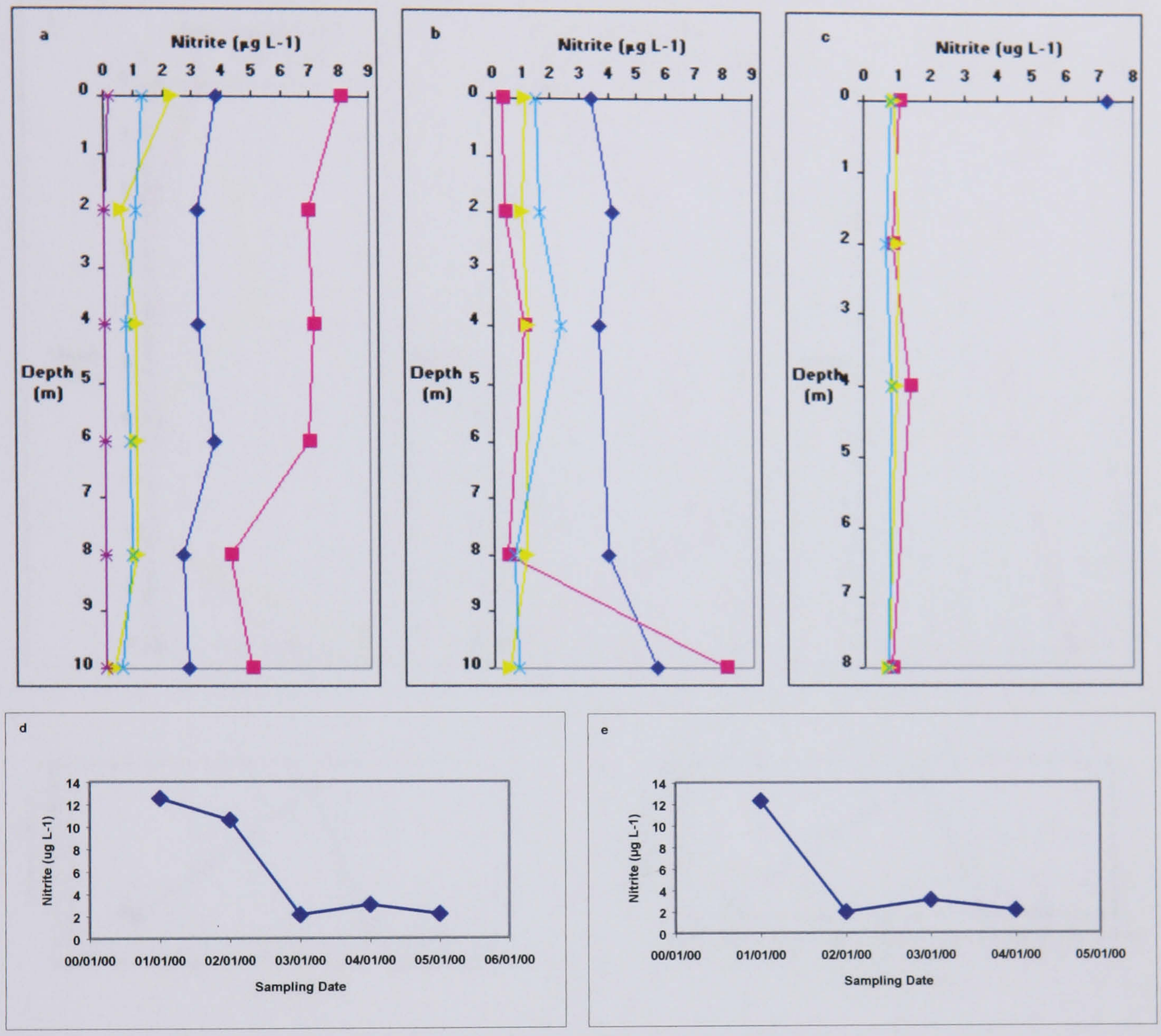


**Figure 3.6 – Nitrite concentration (µg $L^{-1}$) for the five detailed study lakes. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake (d) and Club Lake (e) were only surface water sampled (0m) over the sampling dates. Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

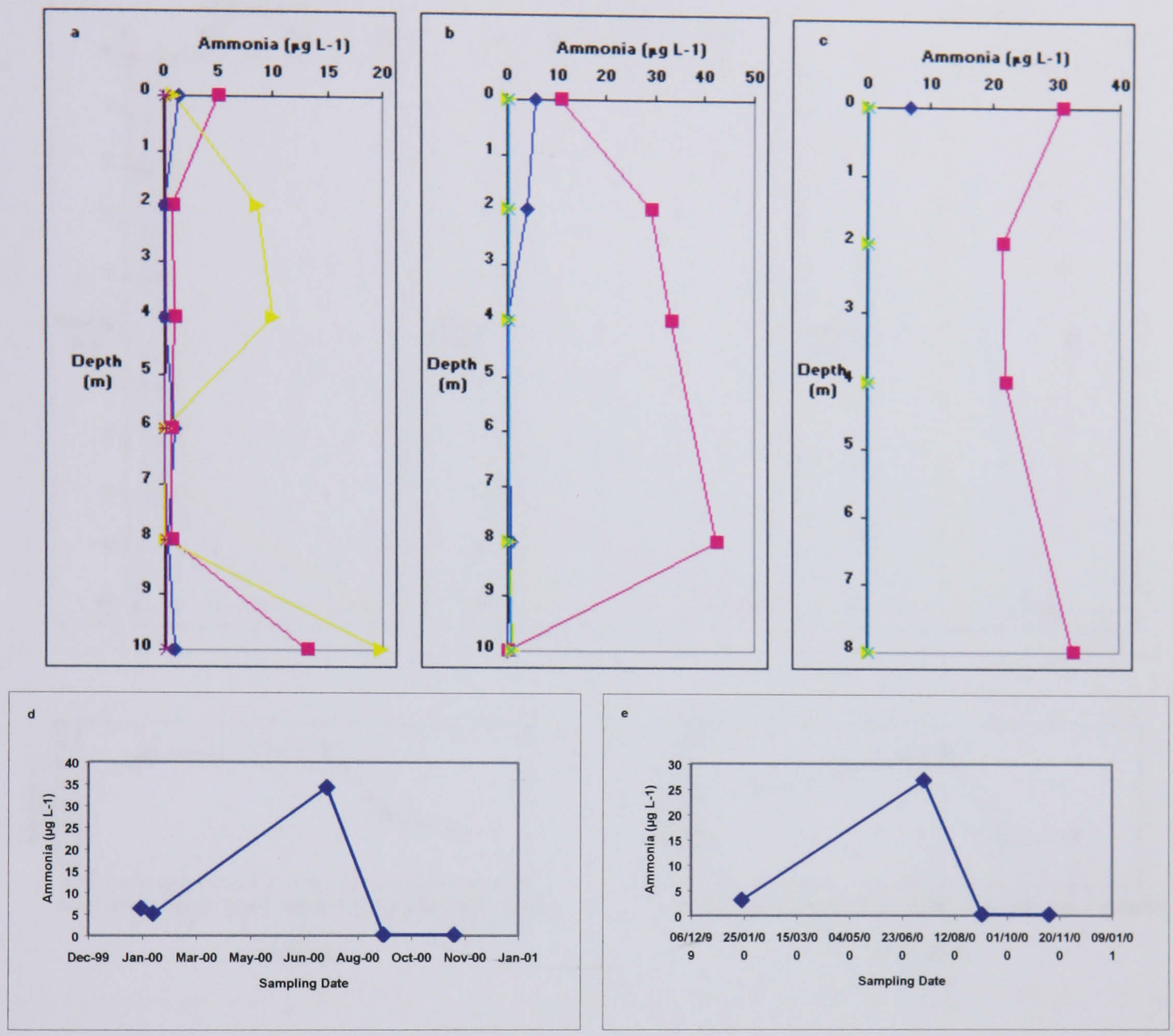


**Figure 3.7 – Ammonium concentration (µg $L^{-1}$) for the five detailed study lakes. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake (d) and Club Lake (e) were only surface water sampled (0m) over the sampling dates. Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

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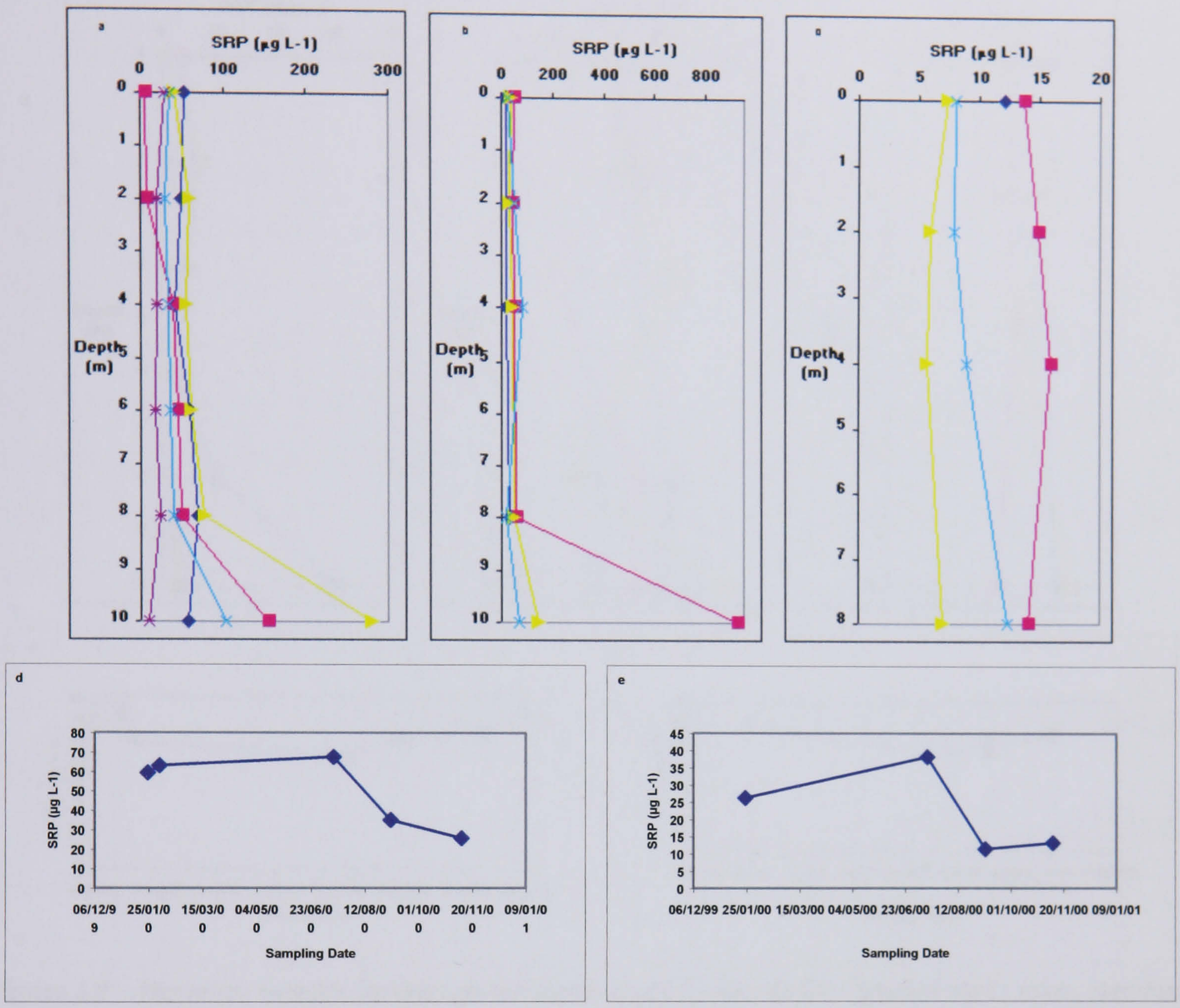


**Figure 3.8 – Soluble reactive phosphate (SRP) concentration (µg $L^{-1}$) for the five detailed study lakes. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake (d) and Club Lake (e) were only surface water sampled (0m) over the sampling dates. Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

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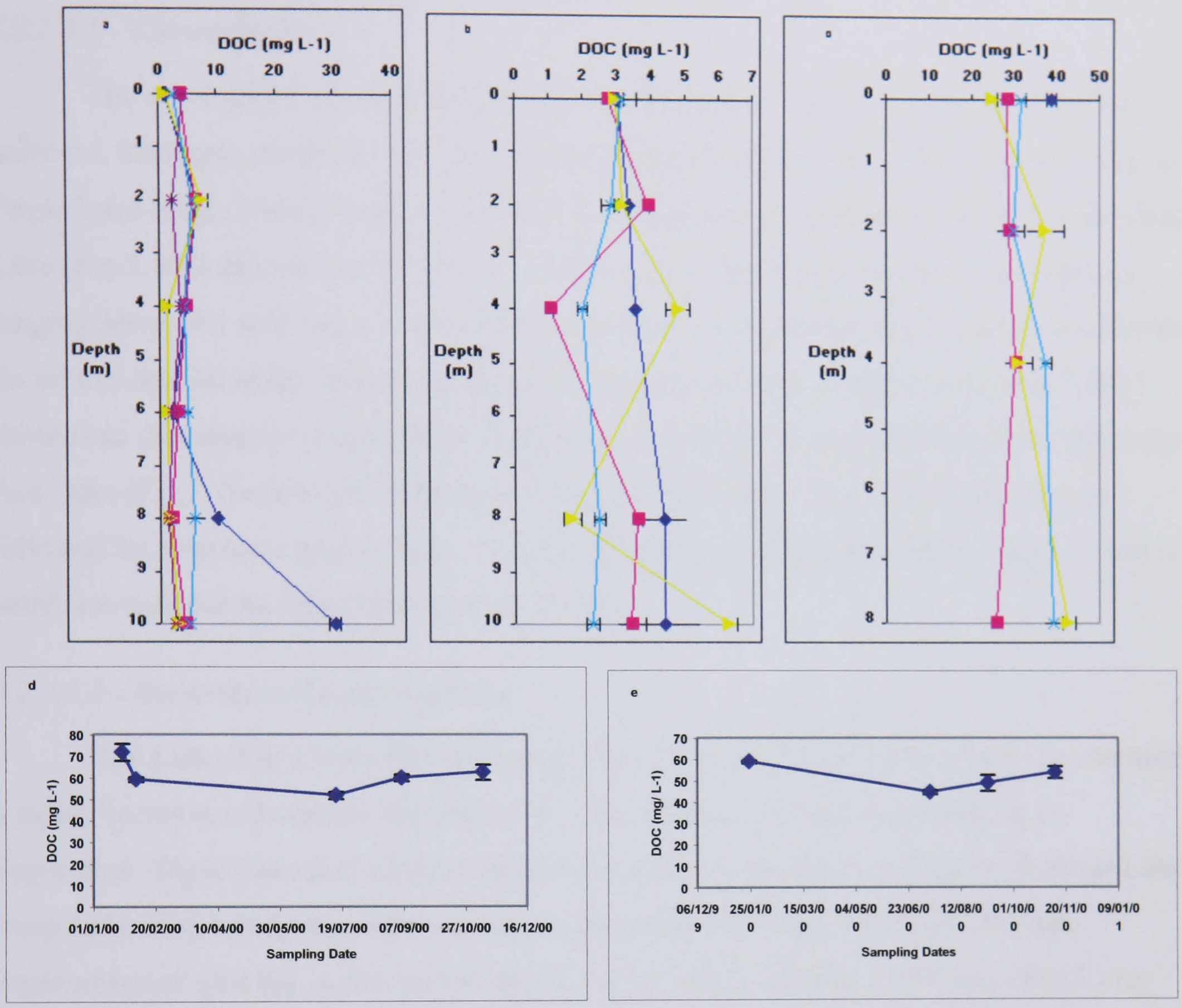


**Figure 3.9 – Dissolved organic carbon concentration (mg $L^{-1}$) for the five detailed study lakes, standard deviation (mg/ml) error bars are also given for each data point. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake (d) and Club Lake (e) were only surface water sampled (0m) over the sampling dates. Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**



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*3.2.2.3 - Biological characteristics*

*3.2.2.3.1 - Chlorophyll a*

The chlorophyll *a* concentrations showed significant variation across the five lakes; however, the depth profile of concentration change is similar in Ace Lake, Pendant Lake and Triple Lake (Figs. 3.10a, b and c). Levels in the hypersaline Club Lake (Fig 3.10e) and Deep Lake (Fig 3.10d) did not exceed 1μg $L^{-1}$. Triple Lake (Fig 3.10c) showed concentrations ranging between 1 and 5μg $L^{-1}$. Levels recorded for Triple Lake during July were the highest for all the detailed study lakes throughout the sampling period. Pendant Lake (Fig 3.10b) showed an increase in concentration of chlorophyll *a* from February to November. Whereas Ace Lake (Fig 3.10a) showed extremely low concentrations (<1μg $L^{-1}$) during January followed by a massive peak (~35μg $L^{-1}$) during March, a reduction in July (~3μg $L^{-1}$) and a small increase during September and November.

*3.2.2.3.2 - Bacterial cell concentrations*

Ace Lake (Fig 3.11a), Pendant Lake (Fig 3.11b) and Triple Lake (Fig 3.11c) showed a steady increase in bacterial abundance from the summer through July, peaking in September. Triple Lake had a higher bacterial concentration than Ace Lake or Pendant Lake. Deep Lake (Fig 3.11d) showed a increase in bacterial abundance from July through September and peaking in November with 2.1 x $10^6$ $mL^{-1}$, whereas Club Lake (Fig 3.11e) showed very little fluctuation in abundance.

*3.2.2.3.3 - Heterotrophic nanoflagellate (HNAN) abundance*

The HNAN abundance in Ace Lake (Fig 3.12a) and Pendant Lake (Fig 3.12b) showed a decrease from March to September, when no HNAN were observed in the Ace Lake mixolimnion. During November HNAN levels had recovered to ~50 x $10^5$ $L^{-1}$. Triple Lake (Fig 3.12c) showed an increase in abundance from July (50 x $10^5$ $L^{-1}$) to September (120 x $10^5$ $L^{-1}$) followed by a small decrease by November. Apart from a community abundance of 9.2 x $10^5$ $L^{-1}$ found in Club Lake in July, no HNAN were observed in the Deep and Club Lake water samples.

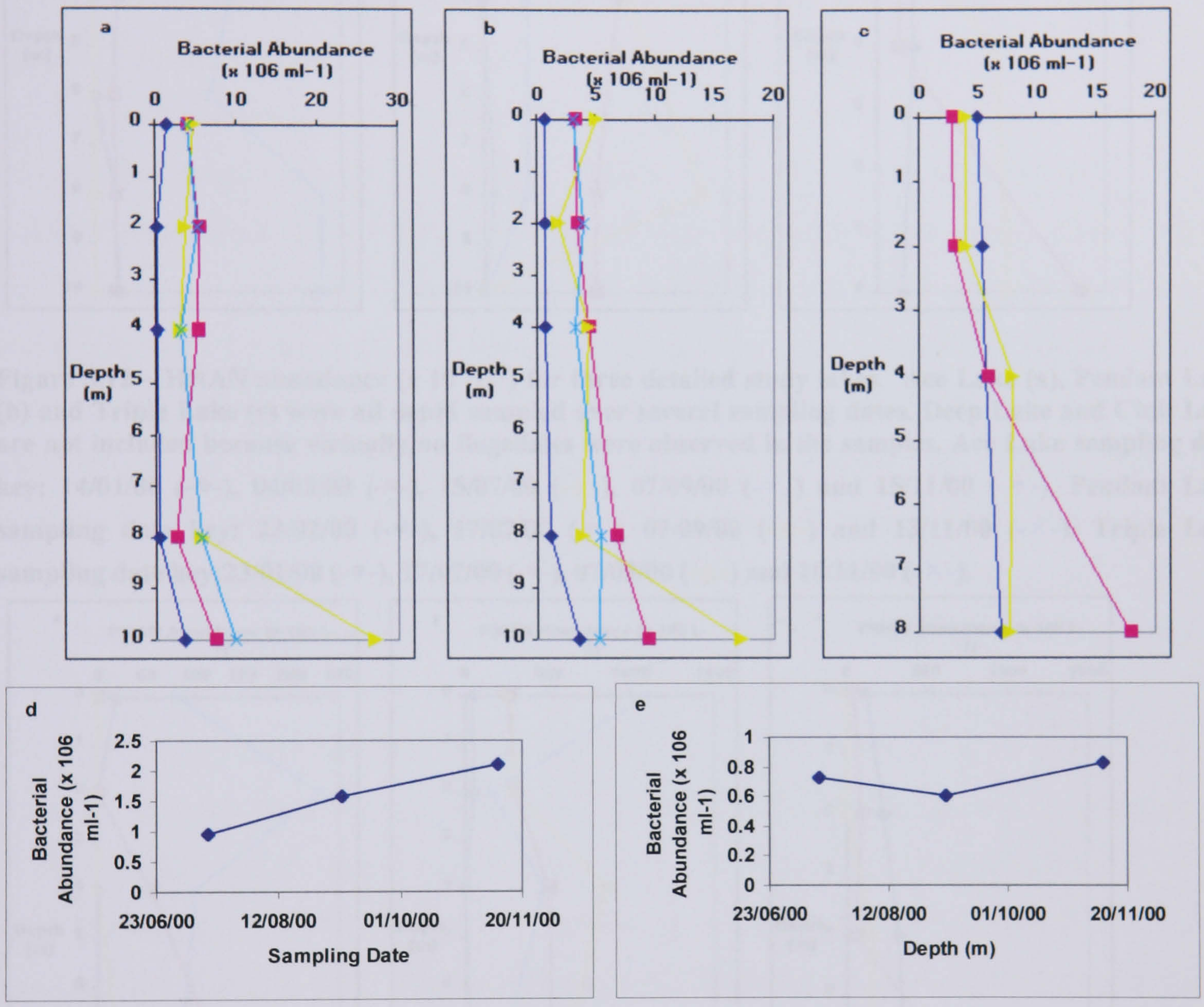


**Figure 3.11 – Bacterial abundance (x $10^6$ $ml^{-1}$) for the five detailed study lakes. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake (d) and Club Lake (e) were only surface water sampled (0m) over the sampling dates. Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

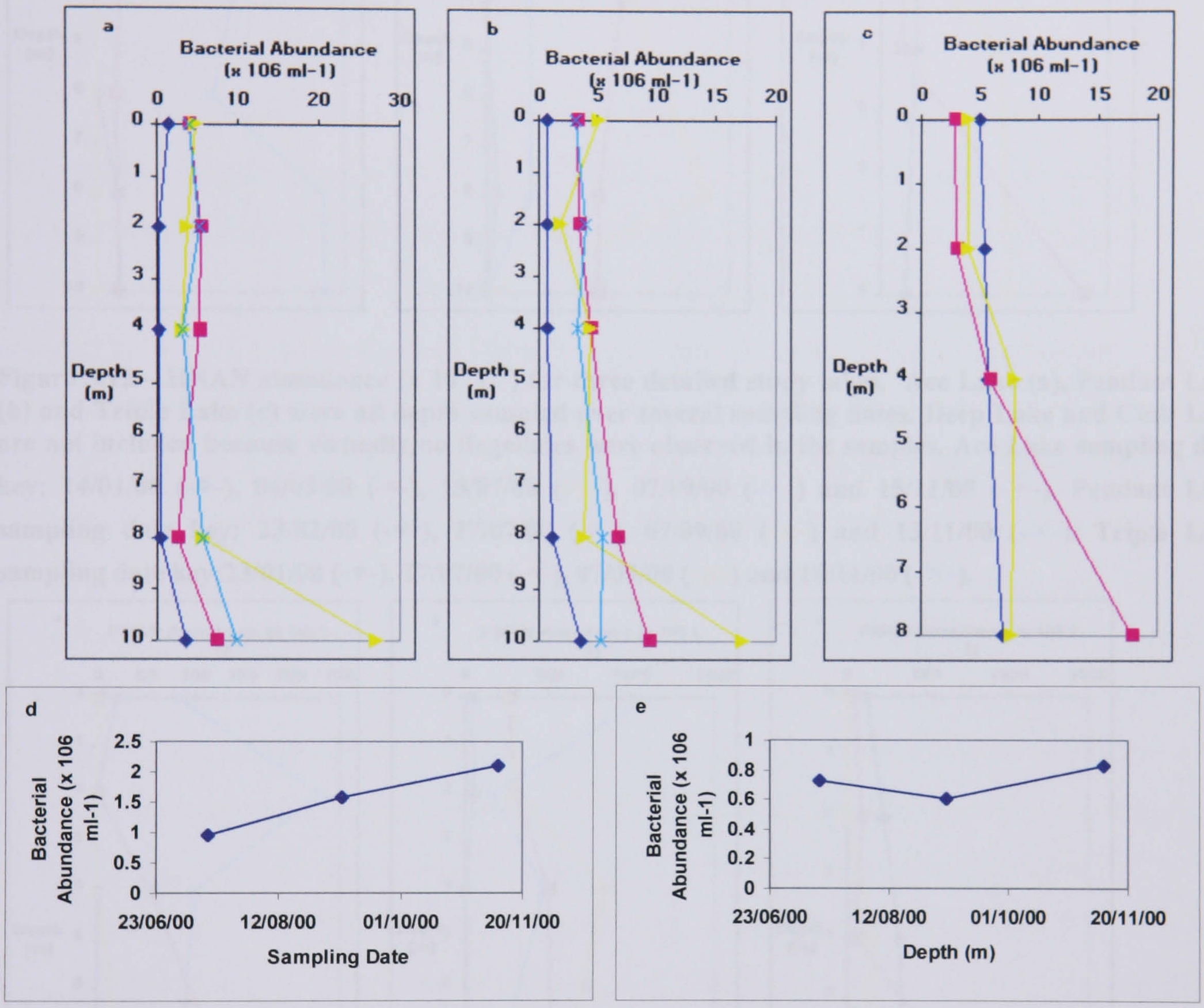


**Figure 3.11 – Bacterial abundance (x $10^6$ $ml^{-1}$) for the five detailed study lakes. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake (d) and Club Lake (e) were only surface water sampled (0m) over the sampling dates. Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

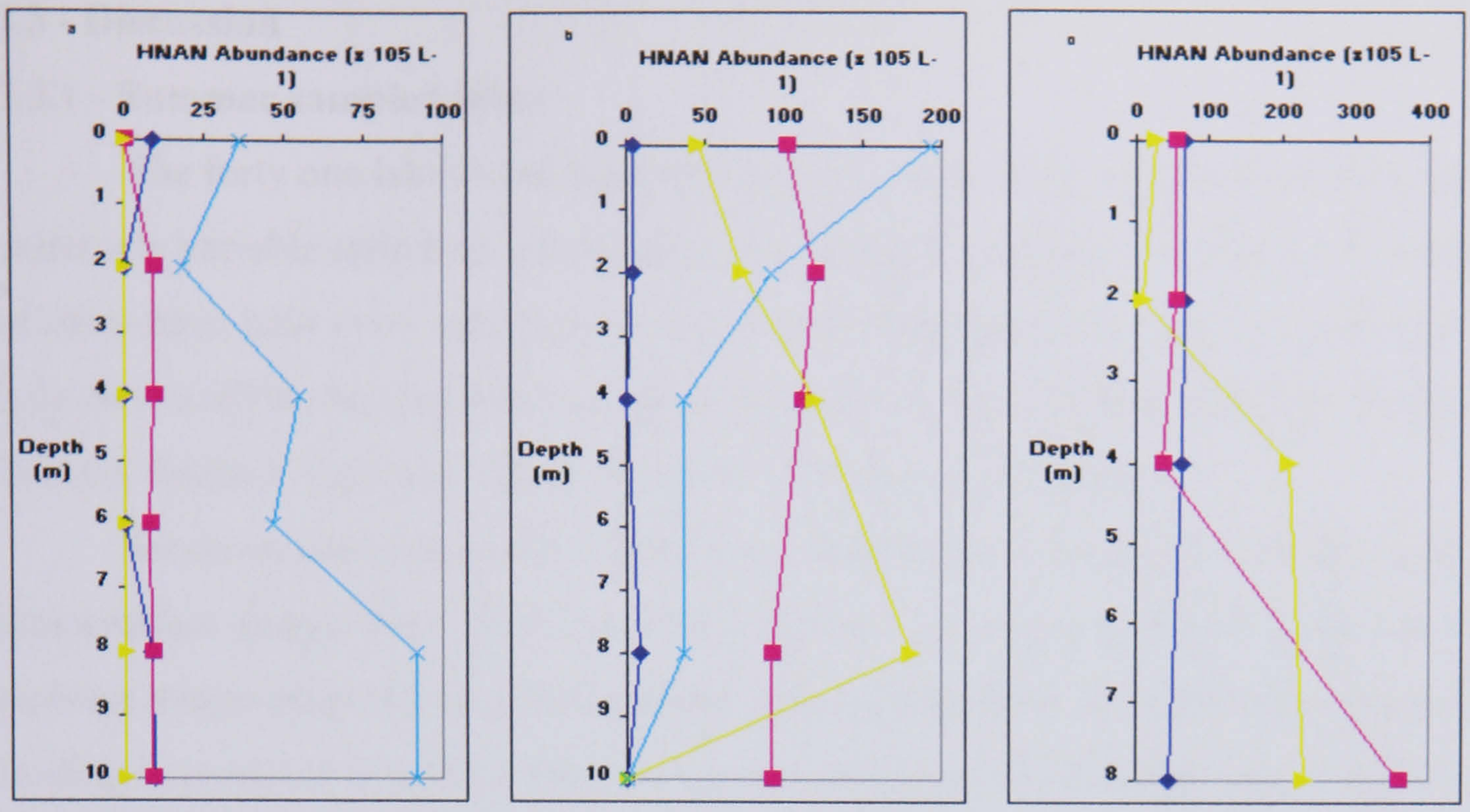


**Figure 3.12 – HNAN abundance (x $10^5$ $L^{-1}$) for three detailed study lakes. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake and Club Lake are not included because virtually no flagellates were observed in the samples. Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

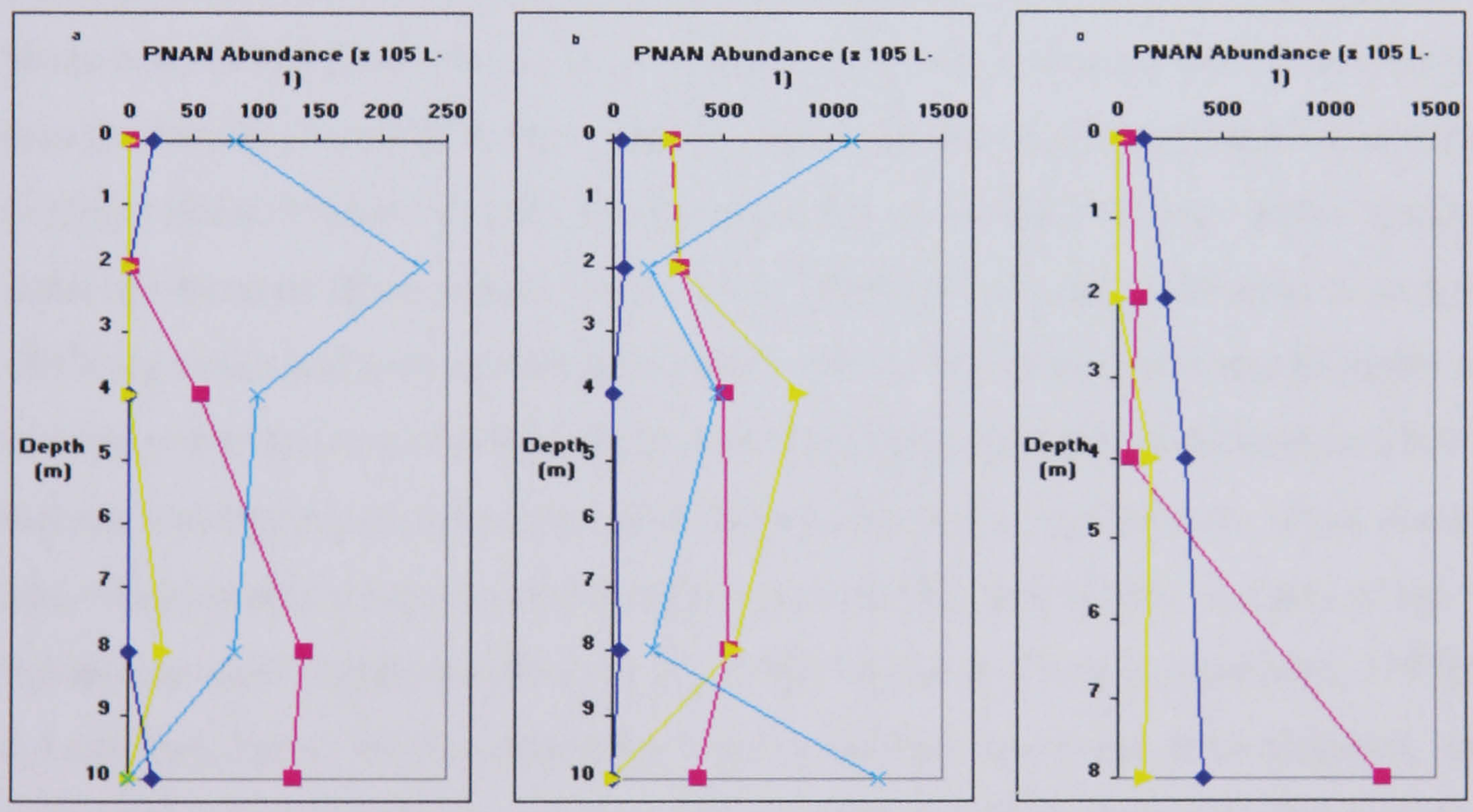


**Figure 3.13 – PNAN abundance (x $10^5$ $L^{-1}$) for three detailed study lakes. Ace Lake (a), Pendant Lake (b) and Triple Lake (c) were all depth sampled over several sampling dates. Deep Lake and Club Lake are not included because no flagellates were observed in the samples. Ace Lake sampling date key: 14/01/00 (-◆-), 04/03/00 (-■-), 15/07/00 (-▲-), 07/09/00 (-×-) and 15/11/00 (-✳-). Pendant Lake sampling date key: 23/02/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 13/11/00 (-×-). Triple Lake sampling date key:23/01/00 (-◆-), 17/07/00 (-■-), 07/09/00 (-▲-) and 10/11/00 (-×-).**

## 3.3 - Discussion

### 3.3.1 - Summer sampled lakes

The forty one lakes investigated displayed a wide range of characteristics. including extremely variable salinities, temperatures and inorganic nutrient conditions. The majority of these lakes have been sampled to some extent in the past as shown in the literature (Table 3.2). Seven of the sampled lakes are not mentioned in any available literature, these are Trident, Pointed, Calendar, Hand, Nicholson, Medusa and Cemetery.

Ice cover was assessed in all the lakes. During the summer (1999/2000) ambient temperatures ranged from –6.2°C to 3.3°C (Tables 3.1a, b and c). Sustained periods of ambient temperature above zero combined with high incident solar radiation caused ice melt leading to partial or complete loss of ice cover. Saline and hypersaline lakes (Tables 3.1a and b) showed a large reduction in the extent of ice cover or complete loss of cover (e.g. Oval, Lebed, and Cemetery, see table 1a). Salinity alters the freezing point of water (Williams and Sherwood, 1994), for example sea water has a salinity of 3.8% or 38ppt and does not freeze until the temperature falls to –1.9°C. This is because salinity increases the solute potential and allows better thermal conductivity within water (Williams and Sherwood, 1994), therefore extremely saline water will heat up or cool down much faster than freshwater (Ferris & Burton, 1988). Hence, the saline (4-35ppt) and hypersaline (>35ppt) lakes (Tables 3.1a and b) are more prone to melt out during 'warm' spells in the Antarctic summer (Franzmann, 1991; Gore, 1996a). Oval Lake at the time of sampling had ~85% ice cover and a water temperature of –1.9°C: this temperature was probably an isolated event due to a combination of high thermal conductivity (salinity) and loss of thermal insulation (ice cover). Ice cover has an extensive impact on the water chemistry of a lake. Maintenance of ice cover throughout most of the year as seen in many of the freshwater lakes (Laybourn-Parry *et al.*, 1992; Laybourn-Parry & Marchant, 1992b; Bayliss & Laybourn-Parry, 1995) isolates the water body from most external influences, such as wind induced mixing (Laybourn-Parry *et al.,* 2001a) and allochthonous input of, for example animal faeces and melt water (Bell & Laybourn-Parry, 1999a).

Surface water temperatures (Table 3.1a, b & c) are directly dependant on air temperature where ice cover is lacking. Periods of maintained high air temperature lead

| Lake | Published Information |
|---|---|
| Williams | Gibson (1999); Laybourn-Parry *et al.* (2001a); Laybourn-Parry *et al.* (2002) |
| Rookery | Bell & Laybourn-Parry (1999a); Laybourn-Parry *et al.* (2002). |
| Watts | Pickard *et al.*,(1986); Gore *et al.* (1996b); Gibson (1999). |
| Crooked | Laybourn-Parry *et al.* (1992,1995, 2001a); Laybourn-Parry & Marchant (1992a & b); Bayliss & Laybourn-Parry (1995); Gore *et al.* (1996b); Tong *et al.* (1997). |
| Organic | Burke & Burton (1988); McMeekin & Franzmann (1988); Bird *et al.* (1991); Roberts *et al.* (1993); Roberts & Burton (1993); Gibson & Burton (1996); Rogerson & Johns, 1996; Gibson (1999). |
| Stinear | McLeod (1964); Kerry *et al.* (1997); Gibson (1999) |
| Beaver Lake | Bayly & Burton (1993); Laybourn-Parry *et al.* (2001b) |
| Druzhby | Laybourn-Parry & Marchant (1992b); Bayliss & Laybourn-Parry (1995); Laybourn-Parry *et al.* (1996); Tong *et al.* (1997); Gibson (1999); Laybourn-Parry *et al.* (2002). |
| Nottingham | Laybourn-Parry *et al.* (2001a). |
| LH73 | Ellis-Evans (1998). |
| Pauk | Laybourn-Parry & Marchant (1992a). |
| Zvezda | Laybourn-Parry & Marchant (1992b). |
| Abraxas | Burke & Burton (1988); Perriss *et al.* (1995); Gibson (!999). |
| Anderson | Burke & Burton (1988); Gibson (1999). |
| Ekho | Burke & Burton (1988); Gibson (1999); Labrenz & Hirsch (2001). |
| Oval | Burke & Burton (1988); Gibson (1999). |
| Shield | Burke & Burton (1988); Gibson (1999). |
| Reid | Ellis-Evans (1998). |
| Verenteno | Perriss *et al.* (1995). |
| Collerson | Perriss *et al.* (1995). |
| Jabs | Perriss *et al.* (1995); Gibson (1999). |
| Lebed | Perriss *et al.* (1995); Gibson (1999). |
| Oblong | Gibson (1999). |
| Scale | Gibson (1999). |
| Laternula | Gibson (1999). |
| Tassie | Gibson (1999). |
| Braunsteffer | Laybourn-Parry & Marchant (1992b). |

**Table 3.2 – Shows literature in which lakes for the current study only sampled during the austral summer of 1999/2000 are mentioned.**

to high surface water temperatures (Fig 3.14a, b & c), but salinity acts as a buffer to the water temperature. Figure 3.14a, b and c shows how water temperature correlated to the air temperature fluctuations. However, it also shows how salinity modifies the water temperature, e.g. Lake Tassie, on the 31st January 2001, had a water temperature peak which correlates not to air temperature but to a peak in salinity on the graph. This is also shown by the more saline lakes (Lebed, Organic, Laternula, Cemetery and Angel 2) where a significant decrease in air temperature through February and March does not cause a significant decrease in water temperature. The high salinity has a higher latent heat potential than the less saline lakes, which helps to maintain water temperature significantly higher than the air temperature (Fig 3.14a). Watts Lake had a surface temperature of +5°C, this is significantly higher than the ambient temperature (-4.5°C) for the date of sampling (14/02/00), indicating that ambient air temperature on day of sampling had little affect on the water temperature. Therefore, most lakes require prolonged exposure to a constantly high or low ambient temperature to significantly modify water temperature, although other factors are important, e.g. wind speed and topography. The water temperatures seen in the saline lakes, Anderson, Scale, Collerson and Braunsteffer vary between 0°C and 5°C, whereas air temperature and salinity are constant. All four of these lakes had ice cover which isolates the surface waters from external meteorological influence. Therefore, water temperature could be affected by solar heating of the surrounding hills or thermal stratification (see below). This variation due to ice cover can also be seen in the freshwater lakes which apart from Druzhby, Crooked and Hand, were all covered in a relatively thick ice sheet for the whole year.

Water temperature is also affected by thermal stratification of the water column which is induced by salinity and temperature gradients. A number of the lakes of the Vestfold Hills are permanently stratified, or 'meromictic' (Gibson, 1999; Labrenz & Hirsch, 2001; Laybourn-Parry *et al.,* 2002). Meromixis is a condition in which distinct layers within the water remain permanently unmixed, separated by strong physical and chemical gradients. Meromictic lakes have an upper mixolimnion and a lower monimolimnion, which remain permanently unmixed. The two layers are separated by a steep density gradient or chemocline, which prevents mixing of the two layers. The monimolimnion of these lakes is usually warmer than the surface waters, but the temperature usually peaks at close to the

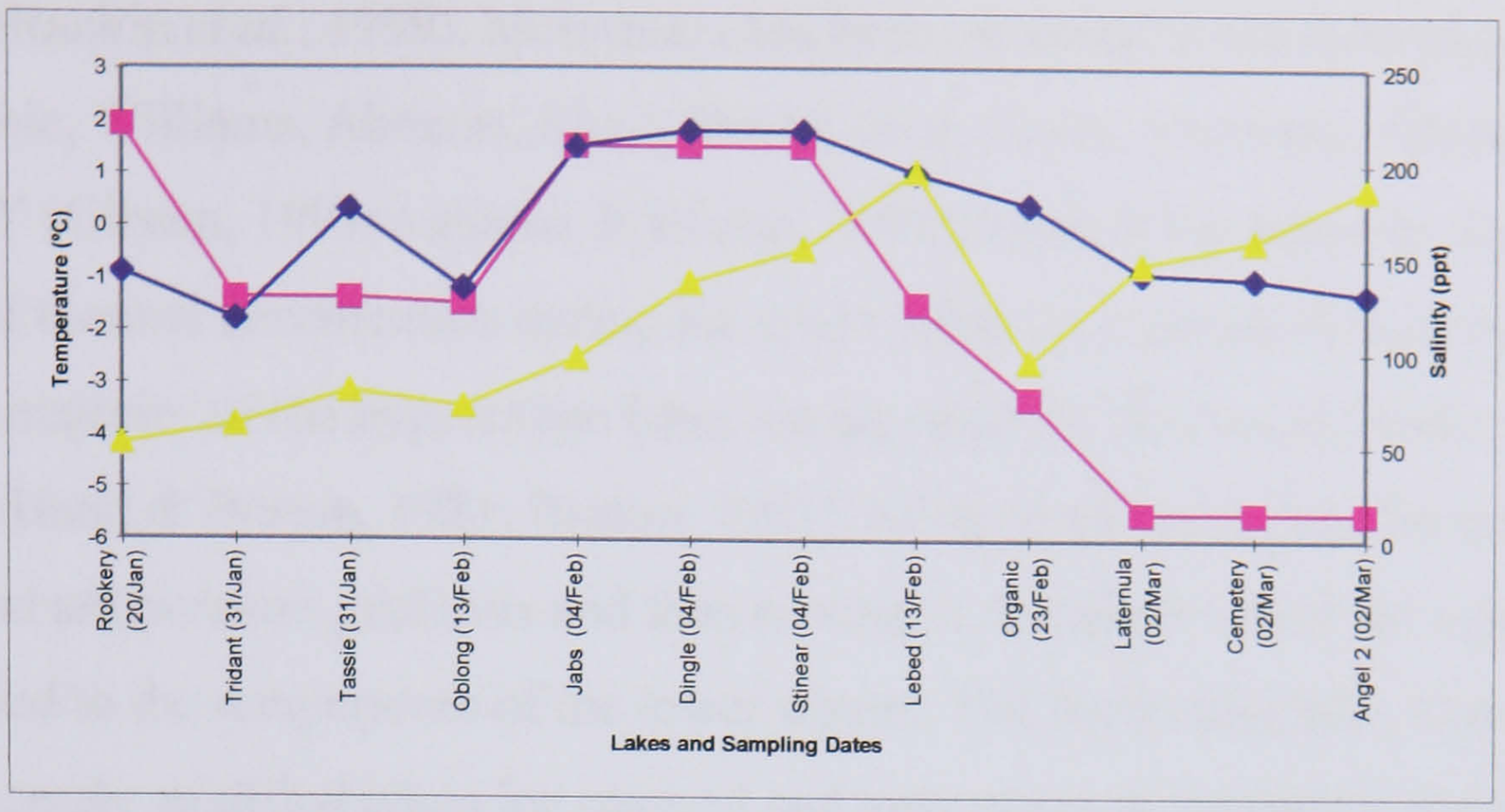


**Figure 3.14a– Air temperature (°C -■-), surface water temperature (°C -◆-) and salinity (ppt -▲-) for the 12 summer sampled hypersaline lakes.**

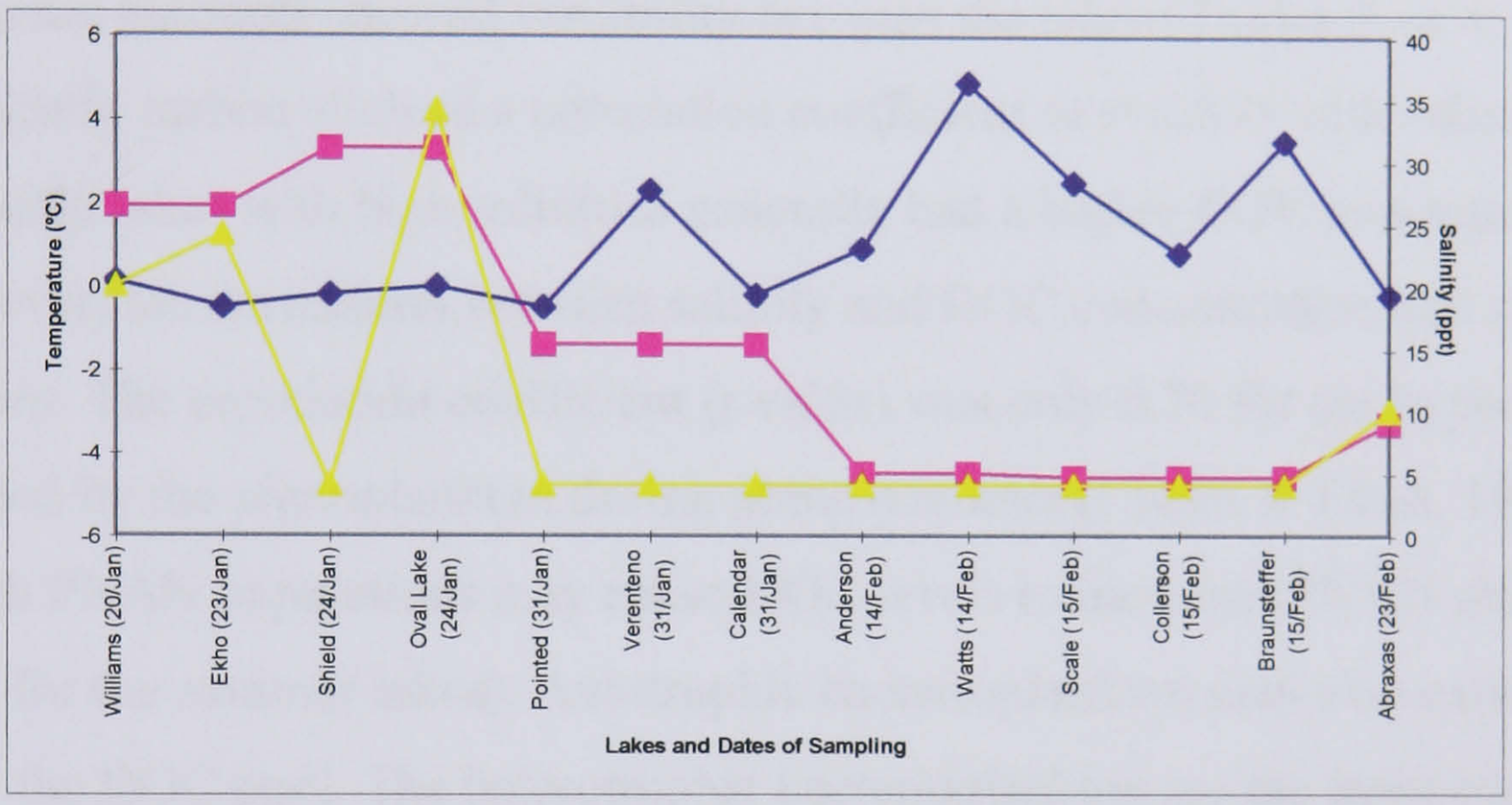


**Figure 3.14b– Air temperature (°C -■-), surface water temperature (°C -◆-) and salinity (ppt -▲-) for the 13 summer sampled saline lakes.**

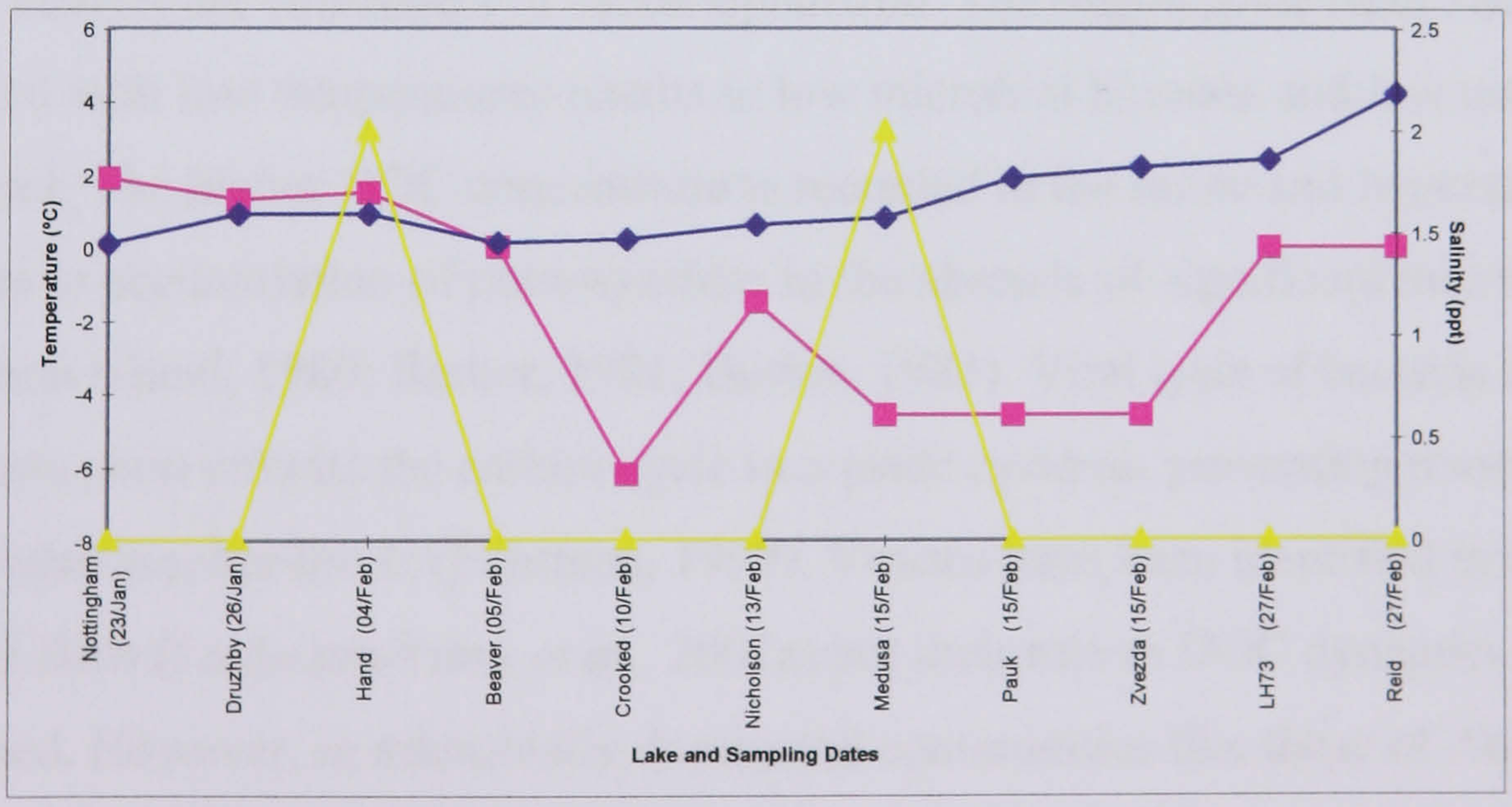


**Figure 3.14c - – Air temperature (°C -■-), surface water temperature (°C -◆-) and salinity (ppt -▲-) for the 11 summer sampled freshwater lakes.**

chemocline (Rankin *et al.*, 1999). Meromixis has been observed in the following sampled Lakes: Organic, Williams, Abraxas, Ekho, Shield, Oval, Scale, Anderson, Oblong, Laternula and Angel '2' (Gibson, 1999; Labrenz & Hirsch, 2001). Most of the lakes do demonstrate some form of thermal stratification during the year (Table 2.1), generally monomixis. For example the majority of the hypersaline lakes are monomictic (Burton & Barker, 1979; Hand, 1980; Hand & Burton, 1981; Burton, 1981), being stratified during the summer, due to salinity and temperature gradients and then mixing in the winter when the upper warm water is cooled to the temperature of the lower waters. The freshwater lake, Crooked Lake, becomes inversely stratified when ice covered and only mixes in summer if the ice breaks out (Laybourn-Parry *et al.*, 1992); it is, therefore, variably monomictic or amictic.

Inorganic nutrients showed variability between the lakes (Tables 3.1a, b and c). Dissolved organic carbon showed a correlation coefficient of r=0.895 with salinity (Fig 3.15a) indicating lakes with high salinities generally had a higher DOC concentration (Fig 3.15b). However, the correlation between salinity and DOC concentration was stronger at lower salinities. The correlation coefficient (r value) was only 0.70 for the hypersaline lakes. DOC is exuded by the phytoplankton during photosynthesis (Chróst & Faust, 1983), therefore high PNAN populations may cause DOC levels to increase (PNAN abundance was not recorded for the summer lakes). Autotrophic bacterioplankton may also exude DOC and contribute to the DOC pool. The heterotrophic bacterioplankton are the main consumers of DOC, but HNAN have also been known to exploit it (Laybourn-Parry, 1997), although the majority of HNAN are consumers of bacterioplankton. The oligotrophic nature of these lakes coupled with low temperatures results in low microbial biomass and low turnover of the DOC pool. The higher DOC concentrations recorded in the saline and hypersaline lakes could be due to accumulation of photosynthate in the absence of significant microbial decomposition (Hand, 1980; Barker, 1981; Burton, 1981). Viral lysis of bacteria and phytoplankton short circuits the carbon cycle in aquatic systems, preventing transfer of carbon to higher trophic levels (Fuhrman, 1999). Viruses have been identified in the lakes of the Vestfold Hills (Laybourn-Parry *et al.*, 2001a) but their role in DOC dynamics has yet to be determined. However, in microbially dominated communities like those of Antarctic lakes, viruses are likely to have a significant impact. This same trend can be seen for the major inorganic nutrients (Tables 3.1a, b and c), nitrate, nitrite, ammonium, and SRP, all of

which were, on average, highest in the hypersaline lakes, and lowest in the freshwater lakes. Therefore, the major limiting factor for metabolic processes in the hypersaline lakes was not nutrient availability but extreme high salinities. Oval Lake, although having a high salinity (34ppt), shows very similar inorganic and organic nutrient concentrations to the other saline lakes, except for a low concentration in ammonium, which is also seen in Lake Abraxas and Lake Williams as well as two hypersaline lakes (Table 3.1a and b).

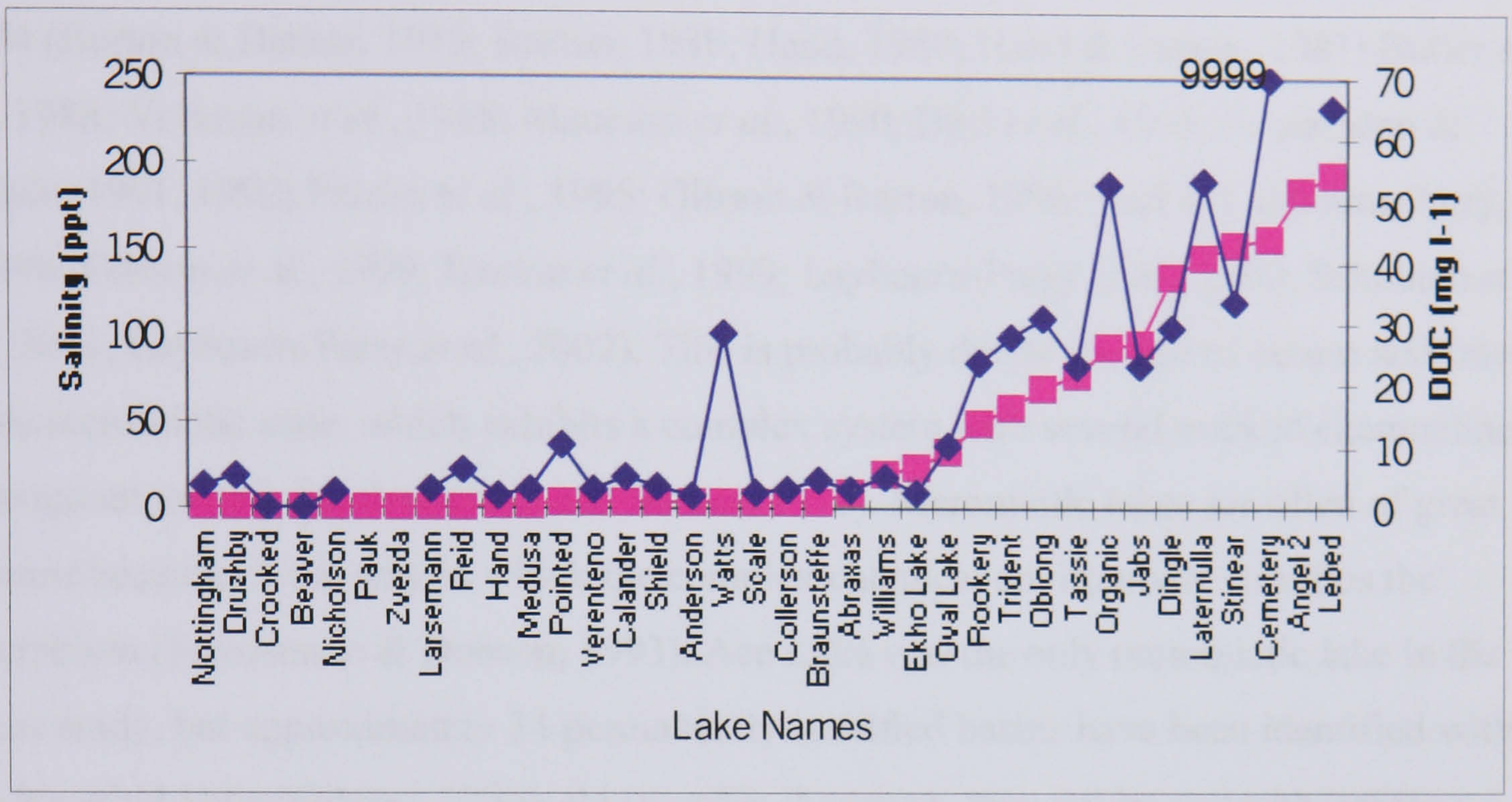


**Figure 3.15a – The correlation between salinity (-◆-) and DOC (-■-) concentrations in the summer sampled lakes. Note DOC concentration for Cemetery Lake was too high to record using the TOC analyser and is noted here as 9999 mg $l^{-1}$. The correlation coefficient for the two variables is r = 0.895.**

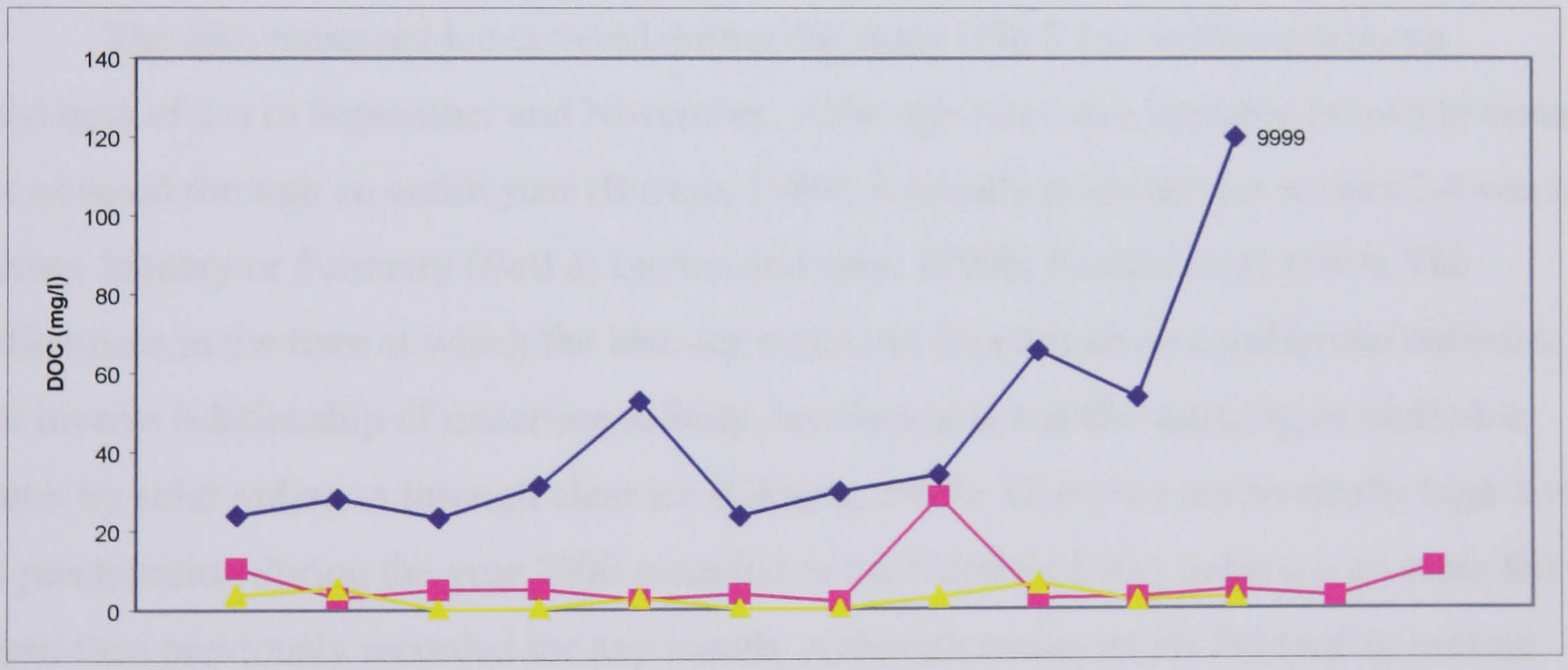


**Figure 3.15b – DOC levels for the summer-sampled hypersaline (-◆-), saline (-■-) and freshwater (-▲-) lakes. The lakes are shown in the order (left to right) in which they were shown in tables 1a, b and c. The last hypersaline lake (Cemetery Lake) recorded off the readable scale and is noted here as 9999µg $l^{-1}$.**

### 3.3.2 - Detailed study lakes

*3.3.2.1 - Ace Lake*

Ace Lake is the most studied lake in the Vestfold Hills, being first investigated in 1974 (Burton & Barker, 1979; Burton, 1980; Hand, 1980; Hand & Burton, 1981; Butler *et al.*, 1988; Volkman *et al.*, 1988; Mancuso *et al.*, 1990; Bird *et al.*, 1991; Franzmann & Rohde, 1991, 1992; Perriss *et al.*, 1995; Gibson & Burton, 1996; Bell & Laybourn-Parry, 1999b; Gibson *et al.*, 1999; Rankin *et al.*, 1999; Laybourn-Parry *et al.*, 2000; Schouten *et al.*, 2001; Laybourn-Parry *et al.*, 2002). This is probably due to its ease of access and interest in its meromictic state, which exhibits a complex system with several marked chemoclines throughout its 25m depth (Burton and Barker, 1979). Meromictic lakes are often of great interest because of the way in which the complex chemical environment stratifies the microbiota (Franzmann & Dobson, 1993). Ace Lake was the only meromictic lake in the focus study, but approximately 34 permanently stratified basins have been identified within the Vestfold Hills (Gibson, 1999); this number, however, may not be accurate (refer to section 3.3.2.2). There are approximately 40 described meromictic lakes in Antarctica which represents a large proportion of the global number (Rankin *et al.*, 1999).

The lake remained ice-covered during the study (Fig 3.1a), with a maximum thickness of 2m in September and November. Although Ace Lake has been known to remain ice covered through an entire year (Burton, 1980), it usually melts out for at least 2-4 weeks during January or February (Bell & Laybourn-Parry, 1999b; Rankin *et al*, 1999). The differences in the time at which the lake-ice melts out depends on an equilibrium between the inverse relationship of under-ice salinity, ice thickness and the warming of under-ice water by solar radiation through clear ice (Gibson, 1999). There was an unusually high level of precipitation during the year 2000 recorded in the Vestfold Hills; in fact more snow fell in April than previously recorded for any month. Although strong winds did tend to prevent massive build-up of snow on the lake ice, there was often at least 60% snow cover on the ice, which caused severe light attenuation. This was demonstrated by Burch (1988) who showed that approximately 21% of the incident radiation passed through 1.6m of snow-free ice, but only 7% when there was 30cm of snow cover. Hand and Burton (1981) show that only 20% of incident light penetrated the ice cover, but in times of melt out 10% of the light reaches 9m.

The temperature (Fig 3.2a) through the water column varied very little over depth and time. The temperature was always between -3 and 3°C, which is substantiated by previous studies (Laybourn-Parry *et al.*, 2002). Ace Lake's meromictic status greatly affects the water temperature. The monimolimnion is reported to start around 10-12m (Gibson, 1999; Laybourn-Parry, *unpublished A*), but has been reported as high as 7-9m (Hand, 1980; Bell & Laybourn-Parry, 1999b). An anomalous water temperature peak of 15.7°C was noted at 10m during the September sampling trip; this temperature is very unlikely and was probably due to equipment malfunction. The previous maximum temperature recorded in the lake was 11.42°C (24th Feb, 1992, Rankin *et al.,* 1999). Fluctuations in temperature correlate well with fluctuations in salinity throughout the mixolimnion during January, March and July (Fig. 3.16a, b and c). There tends to be an increase in temperature with depth, which should act to destabilise the stratified layers but proves insufficient to overcome the salinity gradient (Gibson, 1999; Rankin *et al.*, 1999). The salinity gradient (by virtue of its density) is the dominant force behind the maintenance of stratification (Gibson, 1999). The permanent stratification of the lake means that the mixolimnion does not undergo thermal inversion of its water column during the year, although mixing does occur as demonstrated by the iso-haline condition of the mixolimnion during September and November (Fig 3.4a). Annual temperature fluctuations in Ace Lake are discussed by Hand and Burton (1981) and Rankin (1999) who suggest that the mixolimnion reaches an isohaline state in winter when it is cooled to about -1°C. It heats during the spring and summer and heat is transferred to the thermocline at around 10m, where temperature increases sharply. Temperature at this depth is balanced between radiative input from heat at the surface and conductive loss to the surrounding cooler waters (Rankin *et al.*, 1999). This seasonal heat transfer to 10m results in the highest temperatures at this depth which are maintained throughout the year (Rankin *et al.*, 1999). This corresponds with the data from the current work (Fig. 3.2a and 3.16a, b and c).

Salinity within Ace Lake (Fig 3.4a) tends to remain relatively constant throughout the year (Bell & Laybourn-Parry, 1999b), due to a balance between an increase in salinity caused by evaporation and sublimation, and a decrease in salinity caused by melt water input, both from ice cover melt out and melt stream input from the catchment area (Roberts

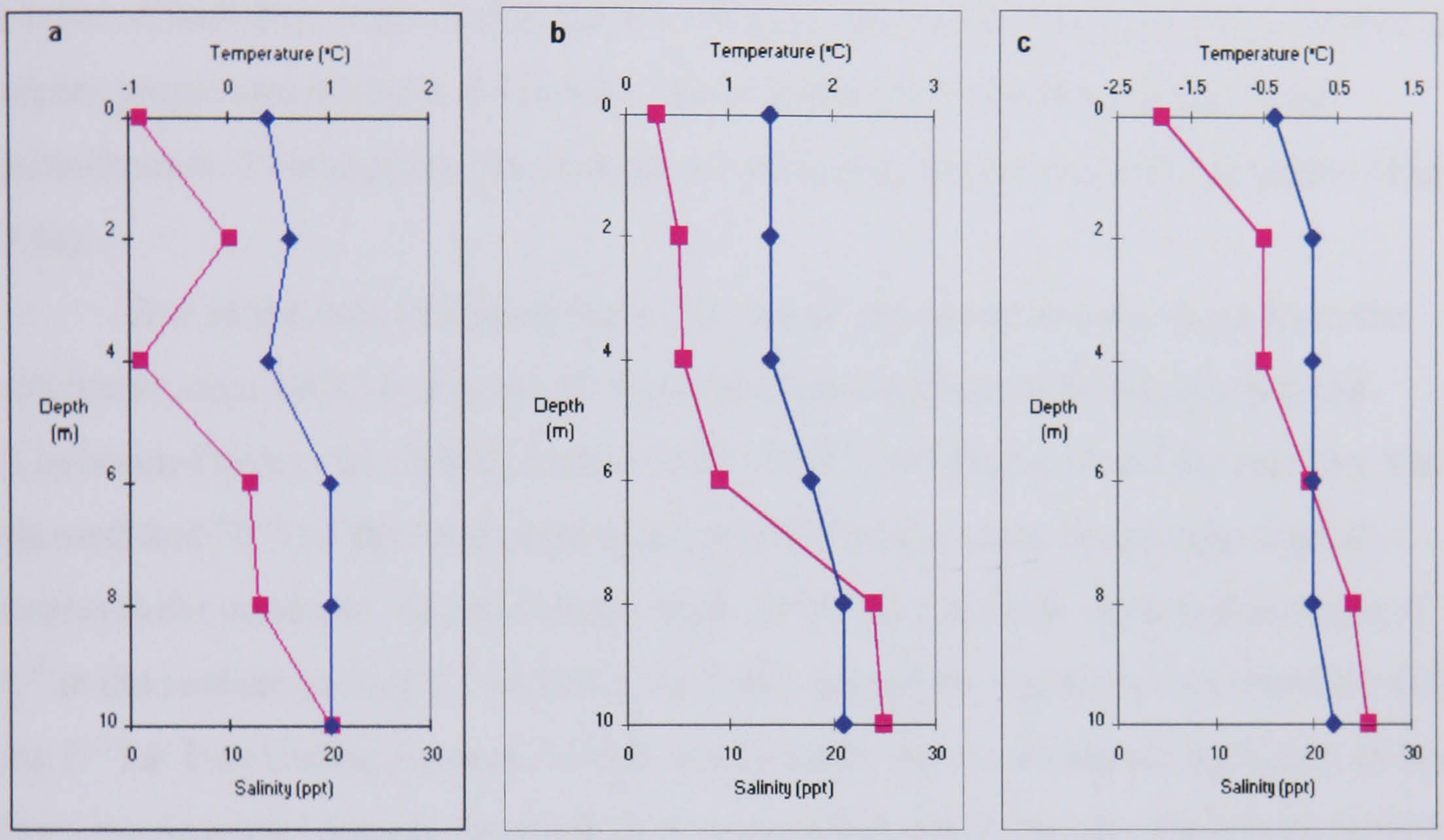


**Figure 3.16 – Shows how temperature fluctuations (-■-) in Ace Lake correlate with salinity fluctuations (-◆-) during January (a), March (b) and July (c). Correlation coefficients are (a) r = 0.927, (b) r = 0.955, (c) r = 0.899.**

*et al.*, 1999). No melt out was observed in Ace Lake and as such no known wind mixing occurred. However, mixing of the mixolimnion does occur with ice formation. A thermohaline convection cell is set up under new ice formation, due to the exclusion of brine increasing sub-ice salinity and thus density, producing a cell which is heated by transfer from deeper waters resulting in a convection cell (Gibson, 1999). Such a situation was seen with the increase in ice cover from July through November (Fig 3.1a); a layer of isohaline water forms, which becomes more saline and penetrates deeper as the winter progresses (Gibson & Burton, 1996), leading to complete mixing of the mixolimnion. This explains the isohaline state during September and November (Fig 3.4a).

Due to the lack of atmospheric $CO_2$ input and allochthonous input from the catchment area, DOC is entirely derived from carbon fixation by photosynthesis (Laybourn-Parry *et al.*, 2002). Parker *et al.* (1977), working on Lake Hoare (Dry Valleys) showed that 75% of the total photosynthetically fixed organic matter appeared as extracellular products. In the current study, DOC ranged from an annual average of 2mg $L^{-1}$ at the surface to 9mg $L^{-1}$ at 10m (Fig 3.9a), except for a peak in concentration (30.079 mg $L^{-1}$) at 10m during January, which was possibly due to accidental inclusion of water from the monimolimnion, but high DOC concentrations in January have been reported by Laybourn-Parry *et al.* (2002). DOC concentration reaches peaks at 10m, probably due to an increase in photosynthetic activity, producing DOC as by-products from photosynthesis. This peak may be brought on by deep light penetration to 10m in January when the snow coverage of the lake ice was minimal (Laybourn-Parry *et al.*, 2002). Photosynthetically active radiation (PAR) levels are much higher in summer (11.5m) compared with winter (2m), due to changes in incident light levels. High PAR during the summer may inhibit photosynthetic production in the surface waters. Therefore, motile organisms e.g. flagellates and ciliates (e.g. *Mesodinium rubidium*), move to a depth where they are not light inhibited (Wright & Burton, 1981; Bell & Laybourn-Parry, 1999b). The majority of phytoplankton are shade adapted due to the low light levels experienced for long periods during the Antarctic year. This is substantiated by the fact that the phytoplankton at 10m can still photosynthesise at <1% of the surface light intensity (Rankin *et al.*, 1999). The autotrophic bacteria, *Chlorobium vibrioforme*, *C.*

*limicola*, Rhodospirillaceae, *Chromatium* and *Desulfovibrio* (Hand, 1980; Hand & Burton, 1981; Volkman *et al.*, 1988) produce a deep chlorophyll maximum (DCM) at around 10-12m. This dense band of bacteria prevent light penetration deeper than 11.5m (Rankin *et al.*, 1999). The high DOC concentrations at 8-10m depth are greatly reduced with progression of the summer into autumn (Jan-Mar), possibly due to bacterial exploitation of the abundant carbon pool (Laybourn-Parry, *unpublished A*). DOC within the mixolimnion is primarily cycled by heterotrophic bacteria which return the carbon back to the dissolved inorganic carbon pool, by the 'sloppy' feeding of HNAN, degradation by decomposers and excretion. These bacteria are then consumed by HNAN, ciliates and possibly PNAN, due to mixotrophy during light limiting conditions (Rankin *et al.*, 1999; Laybourn-Parry *et al.*, 2000).

Inorganic nitrogen ($NO_2$, $NO_3$, $NH_3$) fluctuated with depth and time. Nitrate and ammonium increased with depth (Fig 3.5a and 3.7a), whilst nitrite showed a decrease in depth (Fig 3.6a). Total nitrogen has been shown to increase with depth (Hand and Burton, 1981). Nitrate was generally below the level of detection in the upper waters peaking at 8-10m (Fig 3.5a). Total oxidised nitrogen is generally low in the oxygenated waters (<0.4mM, Hand & Burton, 1981; Bell & Laybourn-Parry, 1999b). Hand and Burton (1981) show a sharp increase in inorganic nitrogen compounds below 7m; this corroborates the data from the current study. The seasonal trend showed an increase in levels during the winter due to accumulation, followed by a rapid decrease in summer due to increased primary productivity (Rankin *et al.*, 1999). However, nitrate and nitrite concentrations recorded in the current study (Fig 3.5a and 3.6a) tend to be higher in the summer months than winter months. This could be due to an abnormally low light level during the 1999/2000 summer (Laybourn-Parry *et al.*, 2002) which could have caused a depression in primary productivity and thus a reduction in the utilisation of total nitrogen.

Burton (1980) indicates there are adequate levels of ammonium in the water column, and therefore it is not limiting. However, Rankin *et al.* (1999) showed that ammonium had low concentrations in the mixolimnion, increasing dramatically in the monimolimnion (also shown by Bell & Laybourn-Parry, 1999b). This is corroborated by the current study (Fig 3.7a), which shows regeneration of ammonium during the winter with high levels in July and a tendency towards an increase in concentration at 10m. The

increase in ammonium is probably due to the deamination of proteins which accumulate due to a slow organic matter mineralisation rate (Rankin *et al.*, 1999). Figure 3.8a shows levels of ammonium to be close to the level of detection, except in July and at 10m in September, when massive increases in ammonium concentration are seen (Fig 3.7a). Increases in bacterial abundance from March to July could have been influenced by increasing ammonium concentrations (Fig 3.17a); however, the peak in bacterial abundance during September seems to coincide with a reduction in ammonium and other nitrogen compounds to levels below the limit of detection, which might indicate that bacteria were using their preferred nitrogen source, free amino acids.

Inorganic phosphate (SRP, Fig. 3.8a) is relatively high in the upper mixolimnion and peaks at 10m. SRP has been shown to not limit biological activity in the upper mixolimnion and to increase in concentration after 7m (Hand & Burton, 1981; Bell & Laybourn-Parry, 1999b; Rankin *et al.*, 1999).

DOC generally shows an increase with depth, but remains relatively stable throughout the year (Fig 3.9a). Hand and Burton (1981) also show an increase in TOC (total organic carbon) with depth. In February, the DOC was comprised of only 28% of dissolved free amino acids (DFAA) and dissolved free monosaccharides (DFCHO), much lower than Pendant Lake (Laybourn-Parry *et al.*, 2002), which may explain why bacterial abundance was considerably lower in Ace Lake compared to Pendant Lake despite the high concentrations of available DOC. This probably relates to the state of meromixis in the lake, which imposes severe nutrient restrictions on the euphotic zone (Laybourn-Parry *et al.*, 2002). High bacterial abundance appeared to coincide with high DOC concentration (Fig. 3.17b), possibly because bacterial production relies on concentration and composition of the DOC pool as well as nitrogen and phosphorus (Rankin *et al.*, 1999). Also, high bacterial abundance at 10m (Fig 3.11a) coincides with high DOC, which is released from the metabolism of $H_2S$ by the anaerobic sulphate reducers (e.g. *Desulfovibrio*). This DOC pool produced by the sulphate reducers near 10m is also fed on by other heterotrophs. It is also exploited by heterotrophic bacteria as a direct or indirect food source and fixed by phototrophic bacteria (Hand & Burton, 1981).

Bacterial abundance was low in the upper mixolimnion (>6m) (Fig. 3.11a) compared with abundance in the lower mixolimnion (6-10m), rarely peaking above 5.5

$x10^6$ cells $mL^{-1}$. Previous abundance measurements have confirmed this with <2 $x10^6$ cells $mL^{-1}$ (Hand, 1980; Hand & Burton, 1981). High bacterial abundance over the chemocline (10-12m) may be partly due to populations of *Chlorobium vibrioforme* and *C. limicola*, and the anaerobic Rhodospirillaceae, *Chromatium* and *Desulfovibrio* (Hand, 1980; Hand & Burton, 1981; Volkman *et al.*, 1988; Bell & Laybourn-Parry, 1999b). Heterotrophic sulphate reducers in the monimolimnion, of the genus *Desulfovibrio*, combine with phototrophs (e.g. *Chromatium*) in the mixolimnion, to produce a sulphur cycle, where the latter use $H_2S$ as an electron donor oxidising it to sulphate, which is used as an electron acceptor by the former (Hand and Burton, 1981). This cycle produces DOC as a by-product, which is utilised by the heterotrophic bacteria in the mixolimnion, increasing the abundance around 10m. A massive peak in bacterial abundance at 10m during September (Fig 3.11a) is difficult to explain, it does not correlate with an increase in chlorophyll *a* or PNAN abundance (and thus DOC), so it possibly corresponds to an increase in the sulphur cycle described above. However, there is no data to support this. Overall high abundance of bacteria during September to November is also noted by Hand (1980).

Bacteria are a food source for HNAN (Bell & Laybourn-Parry, 1999b), therefore bacterial abundances are directly influenced by HNAN grazing. However, HNAN growth and production is not limited by the abundance of bacteria; temperature appears to be a more limiting factor (Laybourn-Parry *et al.*, 2000). HNAN tend to reach maximum abundance after a prolonged period of high bacterial abundance (Fig 3.18). However, there is a peak in bacterial abundance during July (mid-winter) which is not exploited by the HNAN population which remained low. This is probably due to the low winter temperatures, which reduce grazing pressure (Bell & Laybourn-Parry, 1999b). HNAN also tended to peak at 10m due to abundant food resources (Fig. 3.12a; Bell & Laybourn-Parry, 1999b). Laybourn-Parry *et al.* (2002) suggest that HNAN tended to peak in abundance in late summer after a peak in abundance of bacteria in early summer. The current study however notes a peak in bacterial abundance in late winter/early spring (September) followed by a peak in HNAN and PNAN abundance in spring/early summer (November) (Fig 3.18, Fig. 3.12a & 3.13a). High PNAN abundance recorded during November (Fig 3.13a) correlates well with previous data (Bell & Laybourn-Parry,

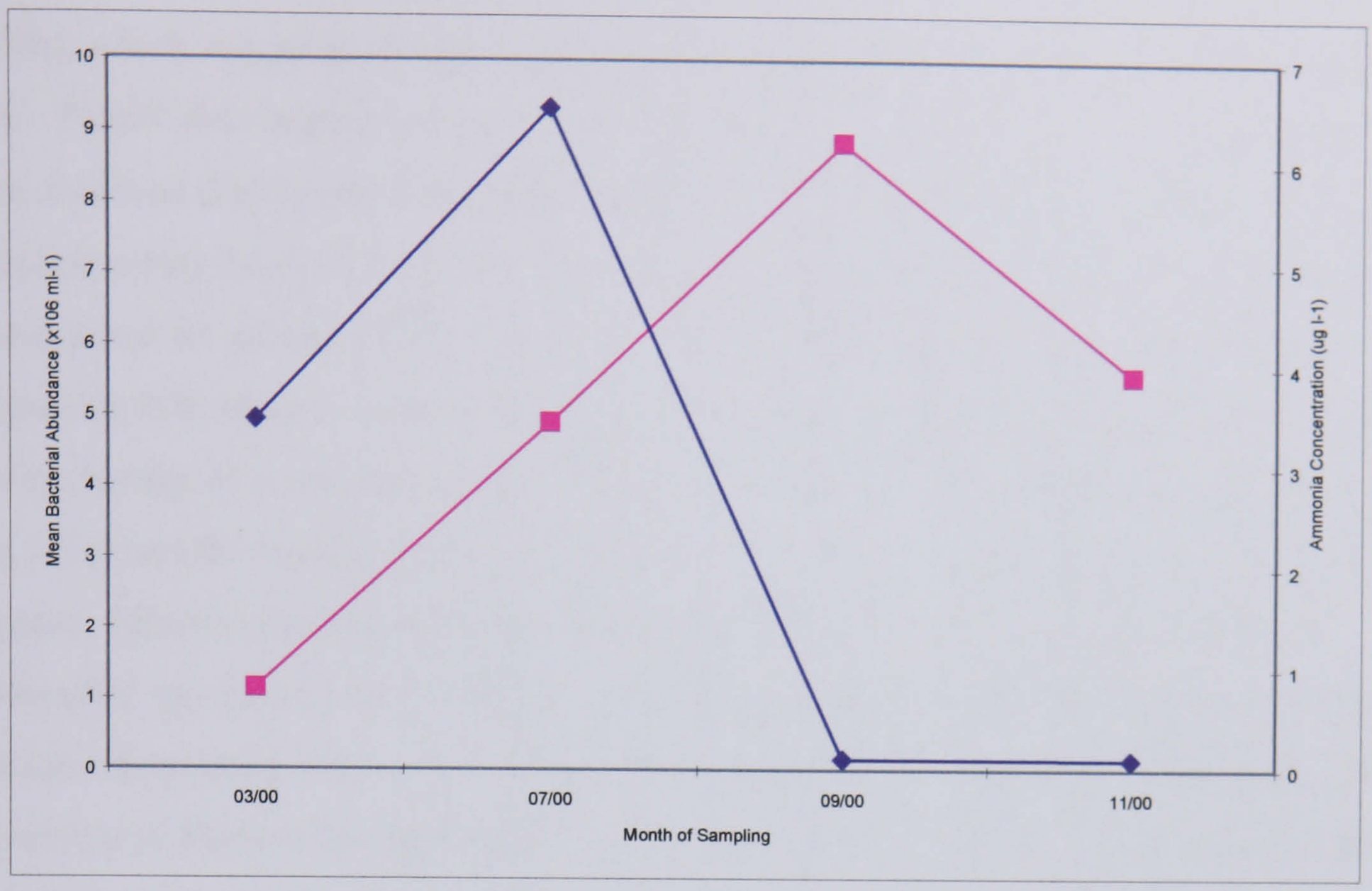


**Figure 3.17a – Mean bacterial abundance (-■-) against mean ammonium concentrations (-◆-) for Ace Lake.**

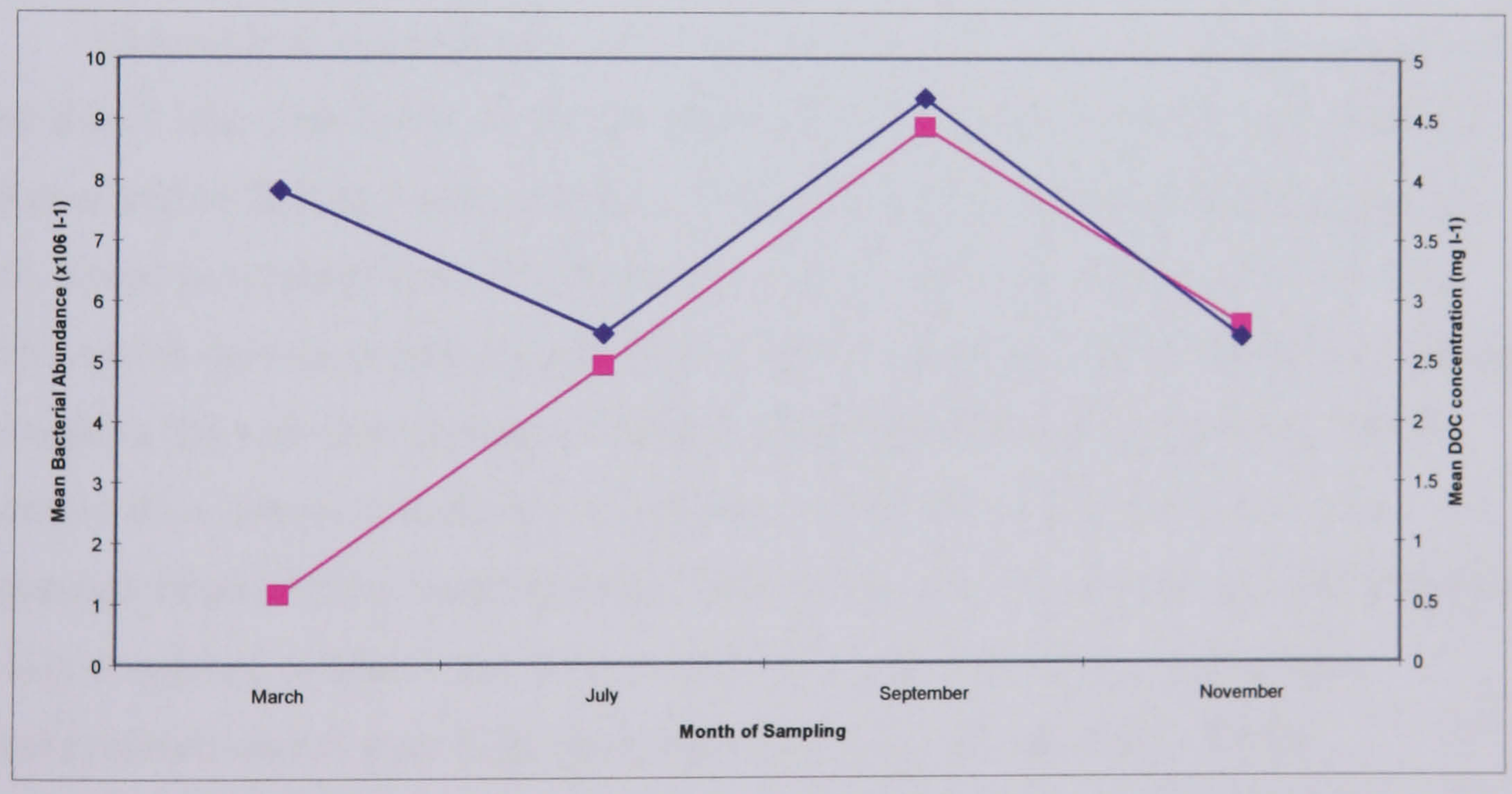


**Figure 3.17b – Relationship between average bacterial abundance with depth (-■-) and average DOC concentration with depth (-◆-) over four sampling dates in Ace Lake.**



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1999b), which suggests a PNAN peak in early summer and maximum abundance at 8-10m. PNAN abundances in Ace Lake tended to be low (<200 x$10^5$ $L^{-1}$). This has also been observed during the 1999/2000 summer by Laybourn-Parry *et al.* (2002). The low abundance may be due to reduced light penetration caused by the increase in snow cover as suggested for chlorophyll *a*. However, July showed a dramatic increase in PNAN abundance following 6 weeks of darkness. It is unknown why this occurred, but it may be due to clearing of snow cover from the lake ice combined with a decrease in ice thickness (Fig 3.1a) and the small increase in light levels, which could have triggered a population increase. Mixotrophy may also have allowed an increase in abundance, as normally autotrophic species turn to mixotrophy to supplement their metabolism during the winter darkness (Laybourn-Parry *et al.*, 2000). However, the sudden decrease in population in September is likely to be experimental error, as abundance shows a further increase in November presumably due to increased light levels. The PNAN community in Ace was dominated by *Pyramimonas gelidicola* (which has been shown to be mixotrophic, Laybourn-Parry & Marshall, in prep) and cryptophytes (Bell & Laybourn-Parry, 1999b; Laybourn-Parry *et al.*, 2002).

Chlorophyll *a* concentrations were generally <2μg $L^{-1}$ in the surface waters all year (Fig 3.10a) (this is also noted by Laybourn-Parry *et al.* (2002) for the 1999/2000 summer and by Bell & Laybourn-Parry (1999) for the '95/'96 and '96/'97 summers). This results in nutrient limitation in the mixolimnion. The DCM around 8-10m (Fig 3.10a, which correlates with data shown by Bell & Laybourn-Parry, 1999b) is maintained throughout the year due to photosynthetic bacteria (Bell & Laybourn-Parry, 1999b). Summer snow cover on the lake ice (Laybourn-Parry *et al.*, 2002) will have also depressed chlorophyll *a* concentrations. Chlorophyll *a* concentrations showed a decrease in July compared to March and November (Fig 3.10a), inferring a reduction in photosynthesis due to poor light conditions (Bell & Laybourn-Parry, 1999b).

Distinctive chlorophyll pigments, as well as carotenoid and lipids can be used to assess abundances of specific bacterial taxa in an ecosystem. Volkman *et al.* (1988) used these organic geochemical techniques to assess Ace Lake and showed bacterial and phytoplankton communities to be stratified throughout the water column. At 10m the

main pigments were chlorophylls *a* and *c*, the latter of which is bacteriochlorophyll (Burke & Burton, 1988). This corroborates the peak in bacterial abundance at 10m noted in the current study.

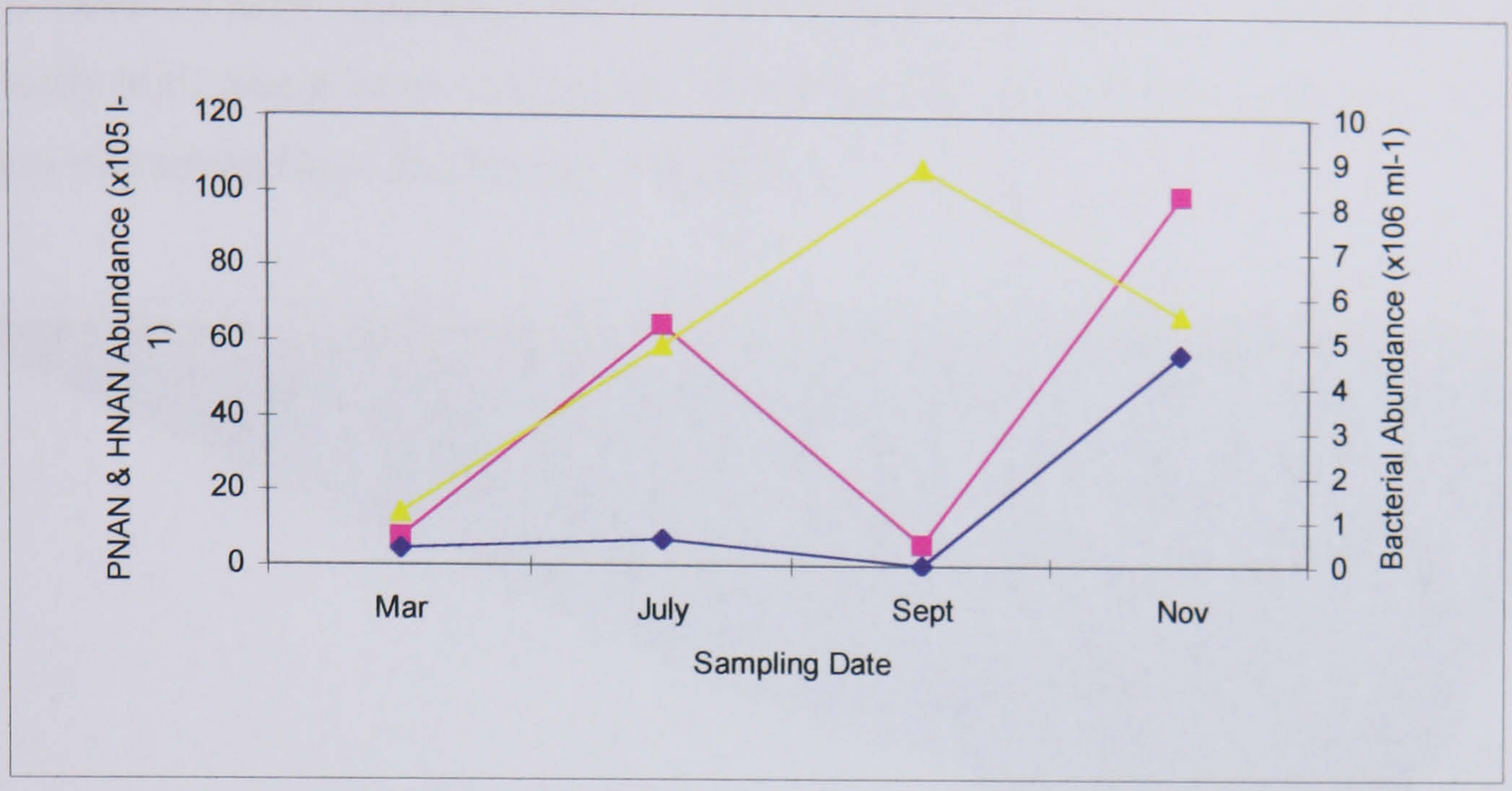


**Figure 3.18 – Relationship between bacterial abundance (-▲-), PNAN abundance (-■-) and HNAN abundance (-◆-) in Ace Lake**

### *3.3.2.2 - Pendant Lake*

Compared with Ace Lake, Pendant Lake is relatively unstudied (Burke & Burton, 1988; Perriss *et al.*, 1995; Gibson, 1999; Laybourn-Parry *et al.*, 2002). It is, therefore, not a surprise that the physico-chemical properties of Pendant Lake are not well defined; for example, Gibson (1999) suggests that Pendant Lake is a 23m deep, meromictic lake with a salinity of 120 ppt. This does not correspond with the current study or those of Laybourn-Parry *et al.* (2002), in which Pendant Lake has a maximum depth of 12m and a maximum recorded salinity of 20 ppt (Fig 3.4b; Laybourn-Parry *et al.*, 2002). The lake is also not considered meromictic, although there is a small anoxic sump at the bottom of the main southern depression in it (Fig 3.19)(Laybourn-Parry, *per.com.*) Based on the current study, Pendant Lake is considered unstratified (amictic) as no stratification was observed in the water column throughout the year.

In common with Ace Lake, Pendant Lake had thick ice cover for the entire study period. A maximum thickness of 2.75m was recorded in September and November, 2000 (Fig. 3.1b). Ice thickness records for the previous summer (Nov, '99 – Feb, 2000) show



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that the ice did not melt out and had a thickness of 2m in November (Laybourn-Parry *et al.*, 2002), indicating inter-annual variation in ice thickness. The increase could be due to lower temperatures for the 2000 winter compared to the 1999 winter. The 1999 winter was considered to be unusually mild. As previously mentioned, the Vestfold Hills saw unusually high precipitation throughout 1999 and 2000 (information courtesy of the Bureau of Meteorology, Melbourne, Australia).

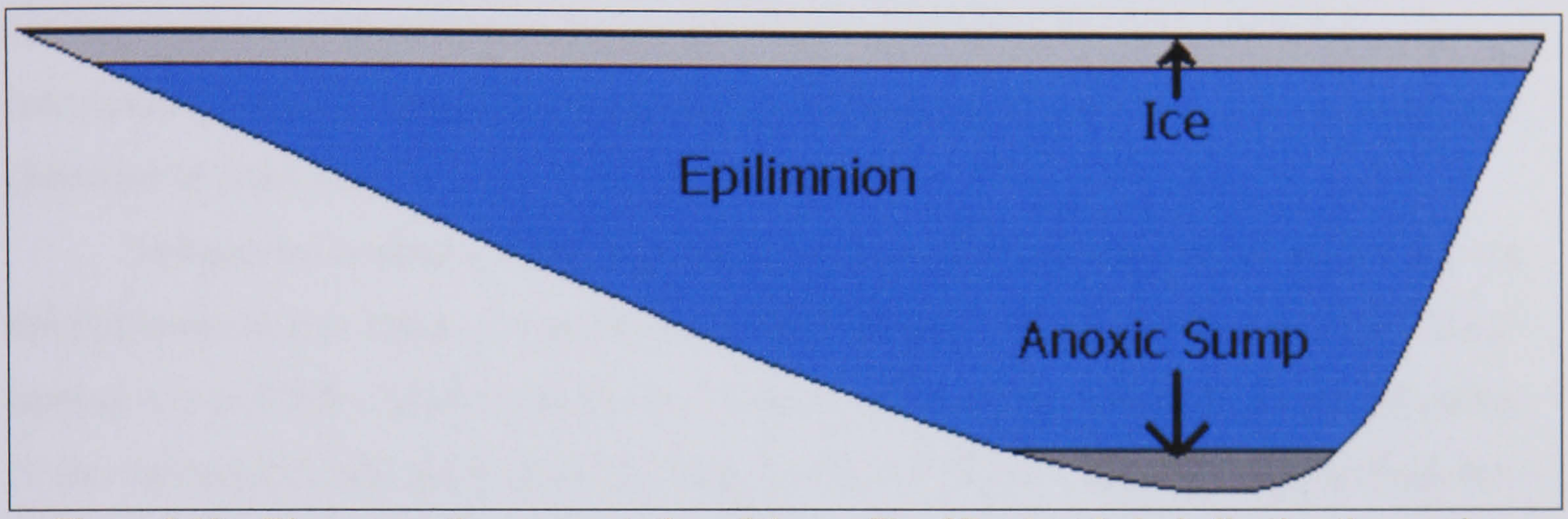


**Figure 3.19 – Diagrammatic representation of the profile of Pendant Lake indicating the anoxic sump.**

The annual water temperatures in Pendant Lake (Fig. 3.2b) were, on average, higher than those in Ace Lake. This may be due to the near complete mixing of the water column during the winter compared to the permanently stratified Ace Lake. Ace Lake had a higher summer temperature (Laybourn-Parry *et al.*, 2002), possibly due to solar heating through the thinner ice cover and surrounding rocks, compared with the relatively thick ice cover on Pendant Lake which, due to whitening of the ice (produced by repeated freeze/thaw during summer), would act as a reflective barrier to solar heating.

Salinity (Fig 3.4b) was on average lower in Pendant Lake (17.75ppt) than the mixolimnion of Ace Lake (19.66ppt). Also, salinity throughout the water column was generally more uniform, with only a small increase at 10m where the sampling bottle collected some water from the more saline anoxic sump. An exception was in November, 2000, when there was a small decrease in salinity with depth, which could be caused by an increase in salinity in the surface waters due to exclusion of brine through increased ice formation (Gibson, 1999; Rankin *et al.*, 1999). The salinity profile during February shows the opposite trend, with fresher water at the surface due to ice melt (Fig 3.1b; Laybourn-Parry *et al.*, 2002).



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Inorganic nitrogen ($NO_2$, $NO_3$ and $NH_4$) was generally higher in Pendant Lake than Ace Lake (Gibson, 1999; Laybourn-Parry *et al.*, 2002). Ammonium and nitrate concentrations were probably higher due to the complete mixing of the Pendant Lake water column; whereas in Ace Lake, inorganic nitrogen settles out of the mixolimnion into the monimolimnion through the breakdown and diffusion of organic matter down the water column (Hand & Burton, 1981). However, nutrients do diffuse upwards across the chemocline, providing a localised nutrient pool just above the chemocline (Bell & Laybourn-Parry, 1999b). Nitrite concentrations (Fig. 3.6b) were virtually the same in both lakes (~2μg $l^{-1}$). Generally, inorganic nitrogen species were always measurable, and therefore not limiting.

Inorganic phosphate (SRP; Fig 3.8b) was higher in Pendant Lake compared to the mixolimnion of Ace Lake. It was not nutrient limiting, reaching its highest concentration during July at 8-10m. SRP dynamics are heavily influenced by biogeochemical processes in the sediment (Laybourn-Parry *et al.,* 2002). Since Pendant Lake was not stratified, the levels of SRP in the water column would have been influenced by the sediment processes which would not have occurred in the permanently stratified Ace Lake.

Dissolved organic carbon (DOC; Fig 3.9b) showed little fluctuation with depth and time. Overall there was less DOC in Pendant Lake than Ace Lake (Fig 3.9a & b; Laybourn-Parry *et al.*, 2002). There was a slight decrease in concentration from 0m to 10m during November, which might coincide with an increase in phytoplankton activity in the upper waters due to increased light levels during the early summer. Laybourn-Parry *et al.* (2002) recorded the highest concentrations in November during the summer of 1999/2000. However, the ice thickness during this month was only 72% of November 2000 (Fig 3.1b), therefore the greater ice thickness would cause greater light attenuation, which could reduce photosynthetic activity and thus DOC output. DOC was higher during February compared with the winter, due to an increase in light conditions and a reduction in ice thickness allowing increased light penetration and intensity.

Chlorophyll *a* concentrations (Fig 3.10b) were generally higher in Pendant Lake than Ace Lake, showing that the meromictic status of Ace Lake probably limits primary productivity within the oxylimnion (Laybourn-Parry *et al.*, 2002). Chlorophyll *a* peaked at 10m during September (~35μg $l^{-1}$); levels then decreased during November,

presumably with increasing light levels or maintained ice thickness causing photosynthetic inhibition. During the 1999/2000 summer Pendant Lake showed significantly higher primary production than Ace Lake (Laybourn-Parry *et al.*, 2002). However, chlorophyll *a* concentrations were also shown to decrease throughout the 1999/2000 summer from November through February, which correlates with the data from the current study where February 2000 levels were lower than November 2000 (Fig 3.10b).

Bacterial abundance (Fig 3.11b) fluctuated considerably throughout the year. However, there was a general trend to an increase in abundance with depth, which might indicate the presence of a deep chlorophyll maxima at 10m, as was seen in Ace Lake. This correlates well with chlorophyll *a* data which shows a maximum concentration at 10m throughout the year (Fig 3.10b).

HNAN abundance showed a strong correlation with bacterial abundance (Fig 3.20). HNAN utilise bacteria as a food source, but there was a peak in bacterial abundance during September which is not utilised by the HNAN (Fig 3.20). However, the peak is mainly influenced by a huge abundance of bacteria at 10m where no HNAN were isolated during September (Fig 3.12b). This point at 10m during September also shows no recorded PNAN. The PNAN population was virtually entirely made up of *Stichococcus bacillaris* (Laybourn-Parry *et al.*, 2002). PNAN were recorded at moderately low abundance during February (<100 $x10^5$ $L^{-1}$) (Fig 3.13b); this is lower than other recorded figures for February (>500 $x10^5$ $L^{-1}$) (Laybourn-Parry *et al.*, 2002). Reasons for this low abundance are unknown.

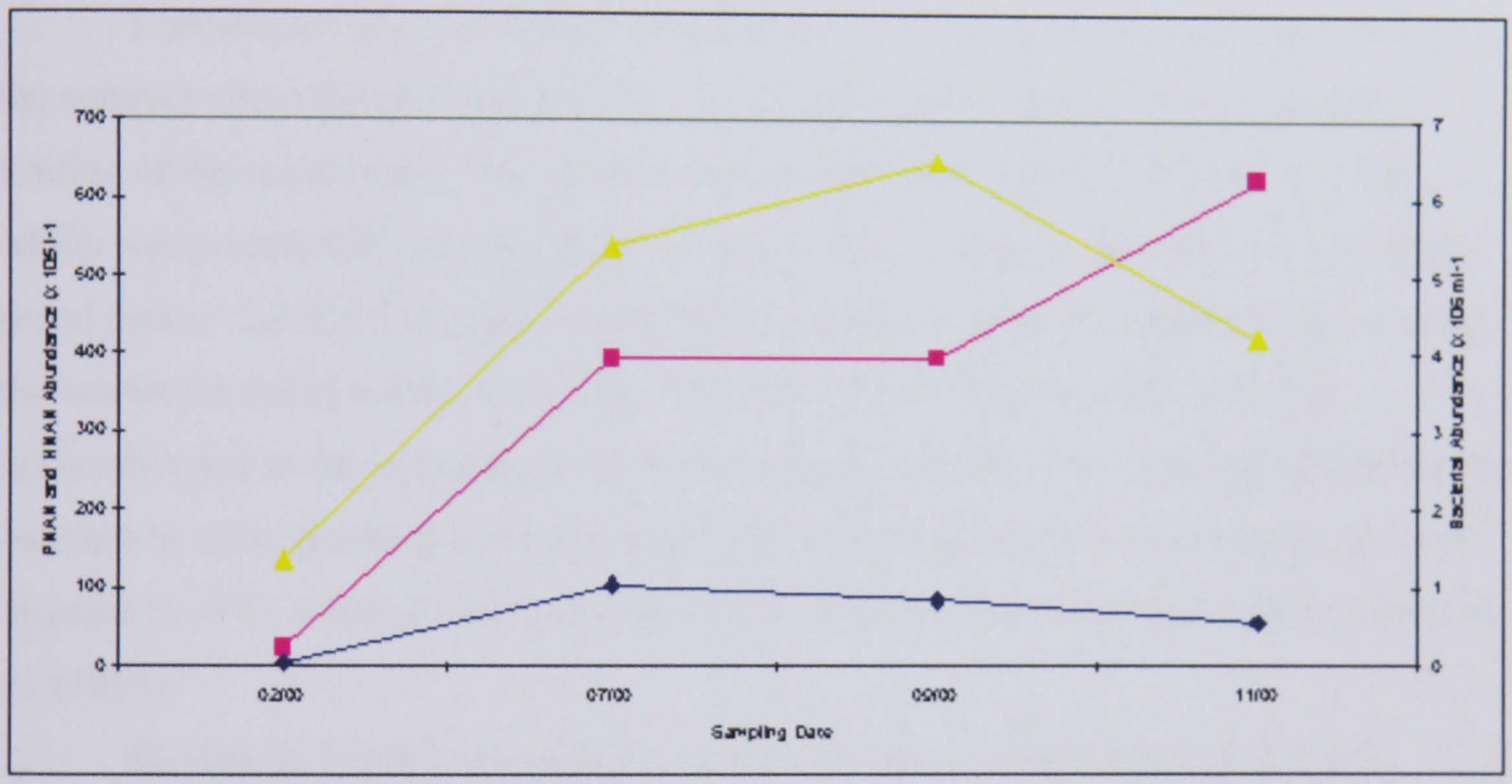


**Figure 3.20 – Relationship between bacterial (-▲-), PNAN (-■-) and HNAN (-◆-) abundances for Pendant Lake over time as averages of depth (0-10m).**



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*3.3.2.3 - Triple Lake*

There is little published work on Triple Lake. In the available literature, there is only one reference (Perriss *et al.*, 1995), in which certain physico-chemical parameters were outlined. Triple Lake is a tri-basin hypersaline lake, during the current study the most northerly basin was depth sampled to its maximum depth (8m) throughout the winter of 2000. During the summer there was no ice cover (Fig 3.1c) due to the extreme hypersalinity of the lake water (136ppt; Fig.3.4c). As previously described, salinity inhibits the formation of ice during warm periods due to the lowered freezing point (Gibson, 1999). During the winter approximately 30cm of ice had formed over the lake (Fig 3.1c) which was sufficient for depth sampling.

Temperature was influenced considerably by the hypersaline condition. During the summer when the lake was ice free, the temperature reached 3.4°C, due to solar heating of the water body. The summer sample was taken on the 23rd January, 2000; which was preceded by a particularly warm 2 weeks (average temperature at 14.00 hrs (local time) = 2.0°C). This caused the high temperature within the surface water. During the winter the water temperature (Fig. 3.2c) fell to -14°C in July and -14.3°C in September due to the reduction in ambient temperature (Fig. 3.21). During November the increase in solar radiation and ambient air temperature caused the water temperature to increase to -9°C, which is similar to the temperature for November reported by Perriss *et al.* (1995).

Salinity in Triple Lake varied considerably throughout the year (Fig 3.4c). Initially, during January, the salinity was 136ppt; however, this increased dramatically by July to a surface salinity of 188ppt. This increased with depth to 192ppt at 8m. This was probably due to an increase in ice thickness by 30cm from January to July, which excludes brine increasing the salinity of the water body. Due to the shallow depth of this basin the formation of ice would have caused an increase in salinity through the entire water column. The shallow link (<1m) between the basin of study and the middle basin in Triple Lake was completely frozen during the winter months, therefore the basin of study essentially became an isolated system. Salinity increased with depth due to the sinking of denser saline water, although, the water column remained relatively isohaline (Fig 3.4c – compared with Ace Lake and Pendant Lake) and no pycnocline was formed. The surface

salinity decreased from July to September, but the difference in salinity between 0m and 8m increased dramatically from 4ppt to 15ppt. This could be due to settling out of denser saline waters, although overall salinity decreased from July to September despite an increase in ice thickness (Fig 3.1c). Salinity showed a further decrease during November, as warmer ambient temperatures started to melt the ice cover and thickness halved from 40cm to 20cm. This input of freshwater in to the lake decreased the overall salinity of the whole water column (Fig. 3.4c).

Inorganic nitrogen ($NO_2$, $NO_3$ and $NH_4$), e.g. nitrate, was much lower (35μg $L^{-1}$) for Triple Lake than previously recorded nitrate concentrations of >100μg $L^{-1}$ (Perriss *et al.*, 1995) (Fig 3.5c). However, it is not known which of the three basins that comprise Triple Lake was sampled by Perriss *et al.* (1995). It is thought to of been one of the other basins, as the maximum depth sampled during their study was 10m, when maximum depth during this study was 8m. As none of the other basins were measured in the current study and each basin is a virtually isolated system during the winter, it is possible that inorganic nitrogen concentrations could be significantly higher in them. Nitrite levels were uniform with time and depth, indicating a well mixed system. These concentrations were similar to those seen in Ace and Pendant Lakes during September (Fig 3.6c). Ammonium concentrations showed significant fluctuation throughout the year (Fig 3.7c). Concentrations were higher in January than those seen in Ace Lake and Pendant Lake during the first summer (Fig 3.7a and b). During July the concentrations increased more than 4x to an average of 26μg $L^{-1}$. These fluctuated with depth, but similar concentrations were seen at 0m and 8m while there was a small decrease between these depths. It is presumed that low biological activity during the winter enabled the ammonium to re-accumulate (Hand, 1980).

Soluble reactive phosphate (SRP) concentration (Fig 3.8c) showed significant fluctuation during the year. Concentrations during January were initially high for Triple Lake (~12μg $L^{-1}$), but low compared with Ace (~52μg $L^{-1}$) and Pendant Lakes (~17μg $L^{-1}$), which may have been caused by high photosynthetic activity as seen by high chlorophyll *a* levels (~1.6μg $L^{-1}$) in the surface water during January (Fig 3.10c) compared with Ace Lake (~0.7μg $L^{-1}$) or Pendant Lake (~0.2μg $L^{-1}$). This may be a consequence of the warm temperatures seen in Triple Lake during January (Fig 3.2c).

During July SRP increased, along with chlorophyll *a* concentration (Fig 3.8c & Fig 3.10c). However, SRP was still lower than in Ace Lake or Pendant Lake while, overall, chlorophyll *a* was higher. By September SRP concentrations dropped considerably, but the mean chlorophyll *a* concentration in September was approximately 50% that of July and remained low through November (Fig 3.10c). It is possible that bacteriochlorophyll could be responsible for the decrease in SRP. As bacterial abundance increases, phosphate decreases, despite the decrease in chlorophyll *a*. Therefore it is suggested that bacterioplankton, not the PNAN, have the biggest impact on phosphate concentrations in Triple Lake.

DOC concentrations (Fig 3.9c) were significantly higher than in Ace or Pendant Lake (the correlation between high salinity and high DOC levels is shown in section 3.3.1, figure 3.15a). It is believed this is because of the lack of biological activity in hypersaline lakes such as Triple Lake, which causes a reduction in the utilization of DOC leading to accumulation (Hand, 1980).

Chlorophyll *a* shows an increase in concentration with depth throughout the year (Fig 3.22a). This indicates a shade adapted system. With similar inorganic nutrient concentrations throughout the lake, it is more likely that the phototrophic organisms are functioning with greater efficiency at depth due to reduced light intensities (Fig 3.10c). Bacterial abundance correlates well with chlorophyll *a* concentrations during July and November, 2000, with r values of 0.96 and 0.95 respectively (Fig 3.22a). However, during November there is no correlation between the two variables, although they still show a similar relationship, i.e. an increase with depth.

HNAN and PNAN (Fig 3.12c & 3.13c) abundances were similar to those found in Pendant Lake and Ace Lake. HNAN abundance correlated well with the bacterial abundance (September r = 0.97, November r = 0.99), which was expected, as HNAN use bacteria as a food source (Hand & Burton, 1981).

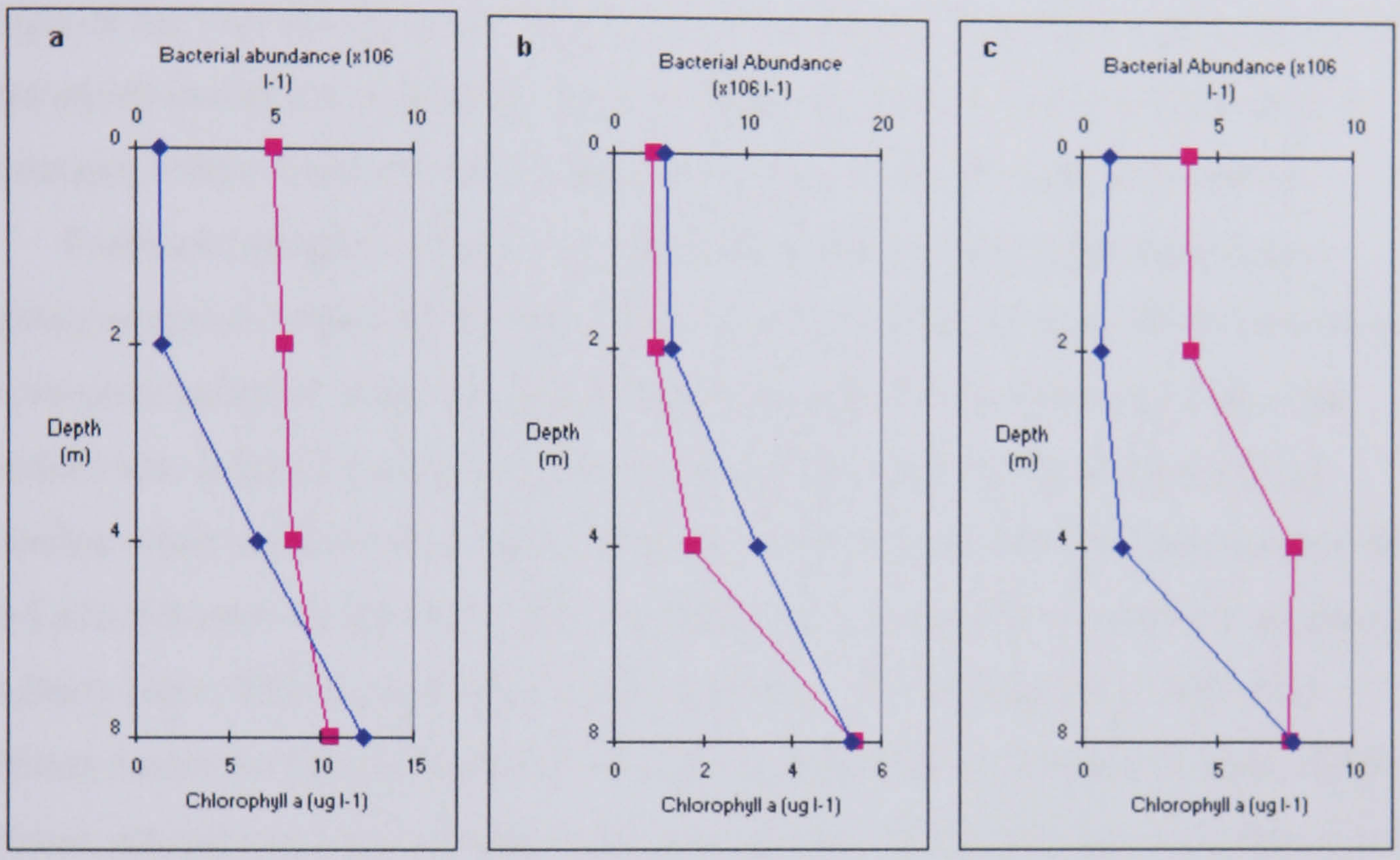


**Figure 3.22 – Correlation between bacterial abundance (-■-) and chlorophyll *a* concentration (-◆-) for three sampling dates in 2000, July (a), September (b) and November (c), in Triple Lake. Correlation coefficients are as follows, (a) r = 0.96, (b) r = 0.95 and (c) r = 0.61.**

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*3.3.2.4 - Deep Lake and Club Lake*

Both Deep Lake and Club Lake are hyper-saline (246ppt & 231ppt respectively (Fig 3.3a & b)). Only exceptionally halotolerant bacterial species can survive in these lakes (Hand, 1980; Ferris & Burton, 1988; McMeekin & Franzmann, 1988), therefore most propagules brought into the lakes by wind, melt streams or animal deposits will die immediately. Several desiccated penguin carcasses are to be found on the western most beach of Deep Lake, therefore penguins do travel to the lake, albeit infrequently. The majority of Deep Lake is too cold and too saline to support life (~ -15°C below 15m throughout the year (Kerry *et al.*, 1977)), however, the top 1m of the lake has suitable temperatures during the summer to support bacterial growth (~+3°C) (McMeekin & Franzmann, 1988). Therefore, the surface water was ideal for bacterial isolation.

Fourteen bacterial isolates were cultured from Deep Lake throughout the sampling season (Chapter 4). Eleven of the 14 isolates cultured from Deep Lake were from summer samples when the surface water was relatively warm (Fig 3.3a). One bacterium was isolated during July, none during September and then three during November when surface water began to warm again. Fifteen bacteria were isolated from Club Lake (Chapter 4). Eleven of these isolates were cultured from summer samples as with Deep Lake. This suggests that low temperature, not high salinity is the main inhibitory factor for bacterial growth in the hypersaline lakes (Ferris & Burton, 1988). However, attempts to culture bacteria from Deep and Club Lake water samples on solid media at the same salinity as the lake water itself were not effective. Subsequent attempts to culture bacteria in liquid enriched media (nutrient broth) using Deep Lake water at varying concentrations (100%, 50% and 25% of original 246ppt sterilized and GF/F filtered lake water) proved negative. Hand (1980) also showed that bacteria could not be cultured *in vitro* from Deep Lake if the salinity of the media was more than half of the lake itself. Of course the numbers of bacterial isolates found from direct culturing may not represent the entire bacterial assemblage. As has been suggested previously (Muyzer *et al.*, 1993; Amann *et al.*, 1995; Hugenholtz & Pace, 1996; Hugenholtz *et al.*, 1998), it is estimated that >99% of bacteria in the environment are not cultivatable using traditional techniques. Attempts were made to address this problem by looking at community profiles for 16s rDNA DGGE-PCR (Chapter 6).

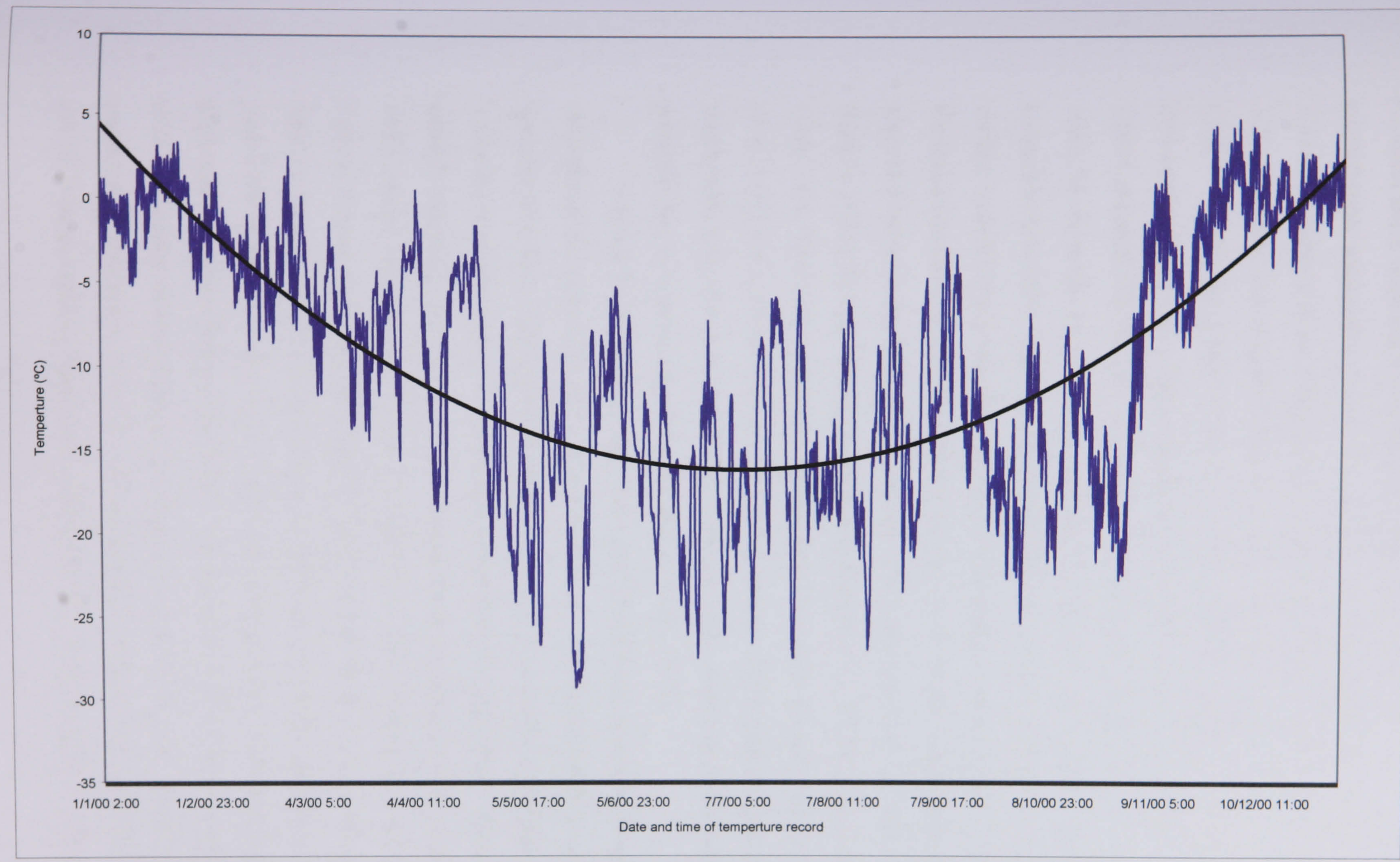


**Figure 3.21 – Ambient air temperature graph for Davis station, Vestfold Hills, Antarctica. Data is shown as 8 time points for each day from 1st January 2000 to 31st December 2000 (data reproduced with the permission of the Bureau of Meteorology, Melbourne, Australia). A polynomial trend line has been applied to the graph to show the general trend in temperature change.**

Temperature in the two lakes showed significant seasonal fluctuation (Fig 3.3a & b). Both lakes were ice free throughout the year, therefore surface waters were subject to considerable seasonal temperature changes. The temperature profiles in both lakes (Fig 3.3a & b) shows a close relationship with the seasonal ambient temperature chart (Fig 3.21). Extreme low temperatures recorded during the winter (Deep = -13.8°C, July 2000; Club = -14.5°C, July, 2000) probably had an inhibitory effect on biological activity. Previously, the lowest recorded surface temperatures were –19.6°C (9th August 1973) for Club Lake and –18.3°C (9th August 1973) for Deep Lake (Kerry *et al.*, 1977). Deep Lake water has been shown to freeze at about –28°C (Kerry *et al.*, 1977) and exhibits a monomictic stratification cycle (Ferris & Burton, 1988). Increase in temperature in the surface waters during summer produces a pycnocline with the colder denser water and the lakes becomes thermally stratified (Kerry *et al.*, 1977). Summer temperatures recorded between the surface and bottom of the lake can differ by up to 26°C (Ferris & Burton, 1988). Surface water reached temperatures of 3.9°C in Deep Lake and 3.7°C in Club Lake. However, temperatures have been shown to exceed 11°C in Deep Lake (Kerry *et al.*, 1977). As surface waters cool during autumn and winter the pycnocline increase in depth until the column is isothermal, allowing vertical mixing of the whole water column possibly due to wind induced mixing (Kerry *et al.*, 1977).

Salinity in Deep Lake and Club Lake (Fig 3.3a & b) was relatively stable throughout the year (McLeod, 1964; Kerry *et al.*, 1977), but it was generally lower during summer (Jan-Feb, 2000), probably due to snowdrift and melt water input – these produce a thin layer of less dense, lower salinity water over the main body of water, which is quickly assimilated with the surface waters due to wind induced mixing (Kerry *et al.*, 1977; Hand, 1980; Ferris & Burton, 1988). Burton & Campbell (1980) suggest that Deep Lake is a snow-drift-trap, which might explain why there is a small decrease in surface salinity from February to July, a period which comprised the highest snow fall for the year 2000 (courtesy of the Bureau of Meteorology, Melbourne, Australia). The extremely high salinity causes interesting density variations within the water column. Compared with freshwater, density change in water from Deep Lake is 11 times greater over a temperature increase of 4 to 9°C (Ferris & Burton, 1988). This means that density change per °C is more rapid in Deep Lake compared with lower salinities; the same is

undoubtedly true of Club Lake. The exceptional thermal gradients set up during the summer combined with the rapid change in density found in Deep Lake produces a very stable stratification. This explains why only the surface waters of the lake are ever heated above 0°C. The stability of the thermal stratification means that heating of the water column is exceptionally inefficient (Ferris & Burton, 1988). This is despite the retention of 50% of solar radiation between September-December, 1977 (Ferris & Burton, 1988).

Inorganic nitrogen ($NO_2$, $NO_3$, $NH_4$) was generally higher than in Ace Lake, Pendant Lake or Triple Lake (Fig. 3.5, 3.6 & 3.7) possibly due to low biological activity allowing accumulation (Hand, 1980). It has been suggested (McLeod, 1964; Kerry *et al.*, 1977) that inorganic nitrogen is added through melt water streams, which have been shown to have considerably higher concentrations of these nutrients (Kerry *et al.*, 1977). It is not thought that decomposition of mummified remains of seals and penguins found on the west shore of Deep Lake contributes any significant input (Kerry *et al.*, 1977). Ferris & Burton (1988) suggest that nitrogen is not limiting within the Deep Lake water column - this is supported by the current data. However, they also suggest that the inorganic nitrogen may not be available to photosynthetic organisms and absence of detectable ammonium, as shown during September and November in the current study, may inhibit photosynthesis, as ammonium is probably the preferred form of nitrogen for the photosynthetic organisms (Brezonik, 1972).

Soluble reactive phosphate (SRP) concentration was generally lower in Deep Lake and Club Lake than in Ace Lake or Pendant Lake, but higher than was found in Triple Lake (Fig. 3.8). SRP has an inverse relationship with bacterial abundance ($r = -0.97$); thus as bacterial abundance increased, SRP concentration decreased, presumably due to exploitation of SRP by photosynthetic bacteria. Kerry *et al.* (1977) suggest that in Deep Lake the maximum production occurs when water temperatures are between 3 and 6°C. Although this is not supported by the chlorophyll *a* or SRP data it is supported by the DOC data (Fig 3.9d) and by the number of bacteria cultured from water samples during this period. DOC is generally higher in the summer when surface water temperatures are within this range. DOC concentrations (Fig 3.9d & e) were considerably higher in Deep and Club Lake than the other focus study lakes. Although the majority of this large DOC pool is probably recalcitrant, a proportion of it is probably due to low

biological activity leading to accumulation (Hand, 1980). The seasonal fluctuation in both lakes was minimal, although generally concentrations were lower during the winter. possibly due to vertical mixing of the water column.

Chlorophyll *a* concentrations (Fig 3.10d & e) fluctuated dramatically for each lake. However, the overall concentrations were considerably lower than in other focus study lakes, suggesting inhibition of autotrophs, due to low temperatures and high salinity. In Deep Lake concentrations were very low during January but doubled during February, even though there was only an 11 day gap between these two sampling dates. Reasons for this apparent increase are unknown. Concentrations remained close to 1 $\mu g\ L^{-1}$ throughout the winter, falling again during November, possibly due to light inhibition with the increased solar radiation during the summer. Club Lake showed a similar plot with concentrations high from February through September, falling to almost half the concentration during November. Deep Lake and Club Lake are both very clear and in Deep Lake 45 to 50% of incident light has been shown to penetrate to 2m (Kerry *et al.*, 1977) which would cause severe inhibition to shade tolerant photosynthetic species (Kerry *et al.*, 1977).

Bacterial abundance was significantly lower in Deep and Club Lakes throughout the year, compared with Ace, Pendant and Triple Lakes (Fig 3.11). This is probably due to inhibition of bacterial growth by high salinities and extremely low temperatures. Only two species of the genus *Halobacterium* have been isolated in the lake previously (Ferris and Burton, 1988); however, as previously suggested, the culture bias must be taken into consideration. McMeekin & Franzmann (1988) indicate that *Halobacterium sp.* may be of significant abundance within Deep Lake, as ether-linked isoprenoid glycerol lipids, which are specific to this genus, are found in high concentrations within the sediment. Hand (1980) suggests that bacterial abundance is generally around $10^5$ cells per mL during the summer and reaches $10^6$ cell $mL^{-1}$ during winter, this data correlates with the current study where a greater bacterial abundance is shown during the winter (Fig 3.11 d & e). Hand (1980) also suggests that this increase in bacterial abundance might be due to resuspension of sedimented cells during holomixis. There were no PNAN or HNAN recorded in either lake. Although Kerry *et al.* (1977) did report 'relative abundance of phytoplankton' they found no HNAN which correlates with the current study. It is

suggested that input of bacteria and PNAN may occur from melt water streams in which PNAN are especially abundant (Kerry *et al.*, 1977; Hand, 1980). but the 1999/2000 summer was abnormally cold and overcast (Laybourn-Parry *et al.*, 2002) and most lakes did not melt out during this season. It is therefore possible that there was a severe reduction in melt water input into Deep Lake which would have meant a severe reduction in biotic input during favourable growth conditions. This would have left the lake impoverished and could account for the overall low bacterial population and absence of PNAN.

**3.3.3 - Oval Lake**

Oval Lake was not studied during the focus study but has subsequently been shown to support one AFP+ bacterial isolate within its culturable biota (Chapter 4). Therefore it was considered prudent to compare this lake to Ace Lake, Pendant Lake and Triple Lake (Table 3.2 – subsequent AFP analysis has shown that only these four lakes have AFP active bacteria in their populations, refer to chapter 4), to note any similarities between them.

Oval Lake is meromictic (permanently stratified) (Burke and Burton, 1988; Gibson, 1999), therefore its salinities and temperatures in the mixolimnion were expected to increase with depth as is seen in Ace Lake (Laybourn-Parry *et al.*, 2000).

Inorganic nitrogen ($NO_2$, $NO_3$, $NH_4$) (Table 3.2) showed a similar concentration at 0m during the summer as in Pendant Lake, Ace Lake and Triple Lake (Fig 3.5-3.7). There was no detectable nitrate, and ammonium was significantly lower than Pendant Lake or Triple Lake. This is indicative of Oval Lake's meromictic status. Nitrite concentrations were very similar to those of Ace Lake, Pendant Lake and Triple Lake. Oval Lake had an identical concentration of SRP to Ace Lake, strengthening the similarity between these two meromictic lakes. Higher levels of DOC in Oval Lake compared with Ace Lake could be due to inefficient degradation of DOC due to the high salinity inhibition of activity as seen in Triple Lake.

Chlorophyll *a* concentration was similar to Ace Lake, although slightly higher, possibly due to increased nutrient levels as seen in higher salinity lakes, e.g. Triple Lake (Table 3.2) (Laybourn-Parry *et al.*, 2002). There are no records for bacterial, PNAN or HNAN abundance as these were not recorded for the summer lake samples. Further study

will have to be performed on Oval Lake, as a single surface water measurement is not sufficient to understand the complex chemistry of its circulating mixolimnion.

| Lake | Ice Cover (thickness & percentage) | Salinity (ppt) | Temperature (°C) | Nitrate (µg $L^{-1}$) | Nitrite (µg $L^{-1}$) | Ammonium (µg $L^{-1}$) | SRP (µg $L^{-1}$) | DOC (mg $L^{-1}$) | Chl *a* (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| Pendant Lake | 1.5m, 100% | 12 | 0.5 | 0 | 3.44 | 5.9 | 17.96 | 3.045 | 0.243 |
| Ace Lake | 1.2m, 100% | 14 | -0.9 | 0 | 3.84 | 1.39 | 52.5 | 1.079 | 0.74 |
| Oval Lake | *Nr*, 85% | 34 | -1.9 | 0 | 5.41 | 0.89 | 52.45 | 10.607 | 0.95 |
| Triple Lake | 0% | 136 | 3.4 | 0 | 7.22 | 6.69 | 12.17 | 38.676 | 1.68 |

**Table 3.3 – Comparison of the major recorded physico-chemical factors for the lakes from which AFP active bacteria were isolated. All measurements are from 0m during the summer. This allows comparison between the lakes because Oval Lake was only sampled at 0m. *Nr – not recorded.***

## 3.4 - Conclusions

Only four of the lakes were shown to contain AFP active bacteria within their biota - Pendant Lake, Ace Lake, Oval Lake and Triple Lake (refer to Chapter 4). Table 3.2 outlines the differences between these four lakes. However, apart from the two meromictic lakes (Ace Lake and Oval Lake), there were very few similarities between them; they have different salinities, stratification types and inorganic / organic chemical concentrations.

Ace Lake is a meromictic, saline lake. Its meromixis induces a very interesting water chemistry profile. The bacterial populations within the lake were zoned due to the permanent stratification. The mixolimnion appears to have lower concentrations of ammonium and DOC and a higher SRP concentration than Pendant Lake or Triple Lake, possibly due to meromixis.

Oval Lake is also a meromictic, saline lake. Only a surface water sample was collected from the lake during the summer, so there was a lack of information regarding the physico-chemical characteristics of the water column. However, Oval Lake had a moderately high salinity and low ammonium concentrations, with high DOC and chlorophyll *a* concentrations. As observed in Ace Lake, SRP was also relatively abundant compared to Pendant Lake and Triple Lake.

Pendant Lake is an unstratified, saline lake. It had a similar salinity to Ace Lake but generally had higher primary production, and hence a more abundant biota. It had a small anoxic sump at the bottom of the lake.

Triple Lake is a shallow hypersaline holomictic lake. It had moderately high bacterial and flagellate abundance due to high nutrient concentrations which were higher than Ace Lake or Pendant Lake, probably due to low levels of biotic degradation and therefore accumulation.

Deep Lake and Club Lake were not shown to have AFP active bacteria in their bacterial populations, but were part of the detailed study and so are still summarised here. They had similar surface water environments; they were both extremely hypersaline and had high nutrient concentrations, which suggests that nutrient levels did not inhibit biological activity within the lakes. Biological activity during the summer period indicated that some organisms were capable of surviving the high salinity. However, for about 9 months of the year the surface water temperature reached approximately -14°C - this is thought to be the main inhibitory factor to life in the lakes.

# Chapter 4: ANTIFREEZE PROTEIN ANALYSIS

## 4.1 - Introduction

Bacteria were isolated from a series of lakes located in the Vestfold Hills (68°30'S, 78°E), Larsemann Hills (69°23'S, 76°23'E) and MacRobertson Land (70°48'S, 68°15'E), Eastern Antarctica . These bacterial isolates were analysed for antifreeze protein (AFP) activity. AFPs have the ability to inhibit the growth of ice (recrystallisation inhibition (RI)) by binding on to its surface and inhibiting further interaction with water molecules (Smaglik, 1998) and to depress the freezing point of an aqueous solution below that of the melting point (thermal hysteresis) (Davies and Sykes, 1997). Thermal hysteresis can be assessed by analysing the reduction in freezing temperature of a sample in comparison to pure water (Barrett, 2001). Recrystallisation inhibition activity can be assessed by observing the growth of ice crystals, following supercooling, as the sample temperature is increased towards 0°C (Barrett, 2001).

In the current study, cell lysates from Antarctic bacteria were assessed for AFP activity for potential use in the food industry. The most important property of AFPs for the food industry is their ability to inhibit recrystallisation, because ice crystal growth during defrosting of frozen foods causes loss of texture, nutrients and flavour (Feeney & Yeh, 1993). Therefore, the recrystallisation-inhibition activity screening method was used to assess the Antarctic bacterial isolates for AFP activity.

The basic premise behind the assay is that if a sample is supercooled to -70°C then embryonic ice crystals will form. These crystals will undergo recrystallisation when the solution is held at -6°C, because recrystallisation of ice is most potent at temperatures just below freezing due to the presence of slow moving free water molecules (Smaglik, 1998). In the presence of anti-freeze proteins the crystals show limited recrystallisation. The basic assay technique for conformation of crystal-growth inhibition activity is the 'SPLAT' assay. This is based on a method developed by Knight *et al.* (1988) and utilises a single layer of ice crystals whose growth is assessed using crossed polarized microscopy to distinguish the crystals. This method involves standardisation of all protein samples to 30% sucrose concentrations to, firstly, provide enough free liquid to prevent

non-AFP active peptides from inhibiting ice-crystal growth (Knight *et al.*, 1995) and, secondly, to prevent false positive results.

### 4.1.1 - Disadvantages of the SPLAT assay

The 'SPLAT' assay, although not as accurate as the 'Colworth RI assay' (which employs computer analysis of photomicrographs of ice crystals to ascertain quantitative measurements of recrystallisation), is still a relatively rapid means of qualifying the recrystallisation inhibition activity of a protein sample. However, the equipment required is both expensive and cumbersome to transport. Samples also need to be supercooled to -70°C in dry-ice cooled solvent, which for field conditions such as Antarctica where dry-ice has to be made *in situ*, means the transportation of $CO_2$ cylinders which is both dangerous and expensive. The protocol is also labour intensive and time consuming. All of which make the 'SPLAT' assay unfavourable for rapid field analysis in 'difficult to get to' environments such as Antarctica.

### 4.1.2 - Development of the high-throughput AFP analysis protocol (HTAP)

A protocol was required which would overcome the disadvantages of the 'SPLAT' assay. The new protocol had to be cheaper, more easily transportable and able to analyse large numbers of bacterial protein extracts in a short period of time in order to make the most of the valuable field sampling time.

#### *4.1.2.1 – Premise of methodology*

As it was still necessary to record a sample's RI activity, the new assay was based on the 'SPLAT' assay. Whereas the SPLAT assay uses crossed polarized microscopy to qualify samples for gradated AFP activity, the new assay utilised direct visual identification of activity by increasing the volume of the sample and observing the refraction of light through the frozen sample. A supercooled sample has very small crystals. When a non-AFP active solution is recrystallised, the lack of ice-growth inhibition means that the crystals increase in size (Fig.4.1a) as opposed to solutions containing AFPs which show a limited increase in crystal size (Fig. 4.1b). The highly dense, small crystals resulting from AFP activity, cause a high level of light refraction so that they appear opaque as opposed to the transparent non-AFP active solutions.

*4.1.2.2 - Development of assay*

Through a series of tests using 1mg $mL^{-1}$ solutions of Fish AFP III (acquired from Unilever) as a positive control and total cellular protein extraction from the AFP negative *E.coli* strain JM109 and sterile reverse osmosis (RO) water as a negative controls, it was found that 100 μL solutions (50 μL of protein extract + 50 μL of 60% sucrose solution) in 96 well microtitre plates provide the most effective, practical guide for rapid visual identification of AFP activity. This volume was a compromise between the visual acuity of activity (small volumes, 25-50 μL, show greater contrast between positive and negative) and the melting-time for the sample (large volumes, 150-200 μL, melt slower).

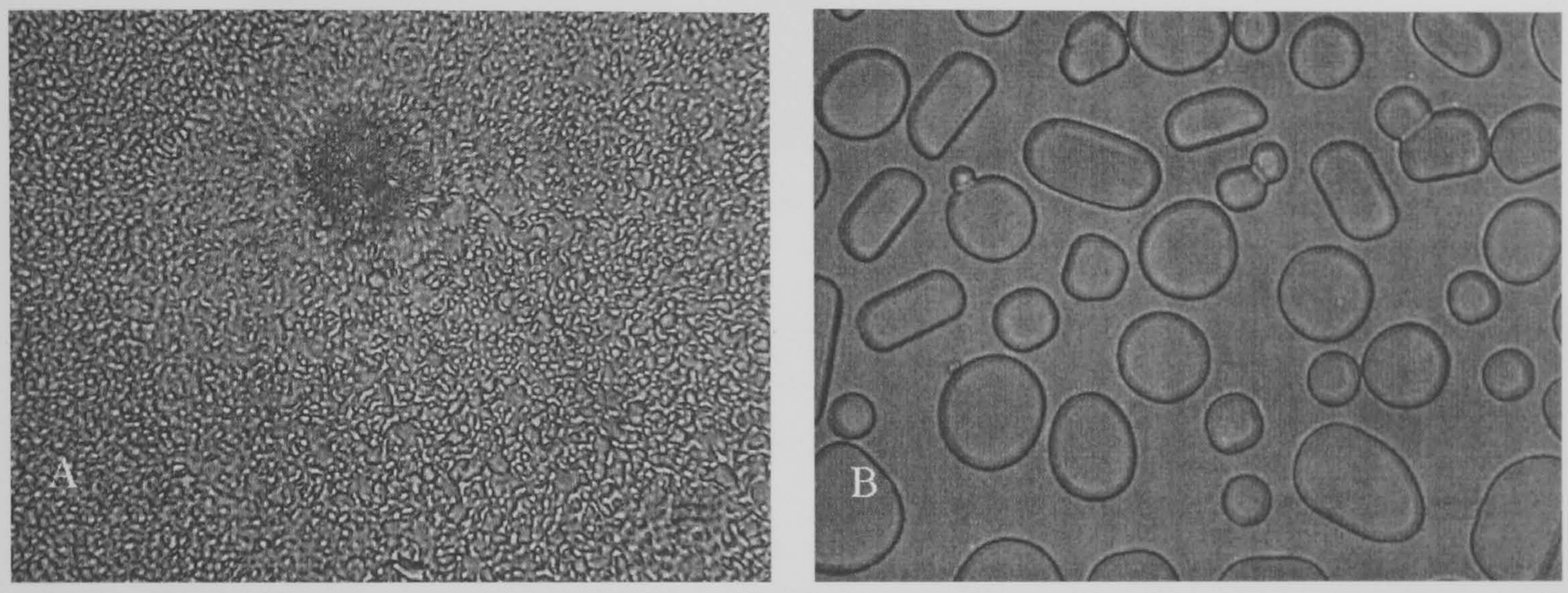


**Figure 4.1 – Ice recrystallised at -6°C for 30 minutes in a (A) AFP solution of 30% sucrose and 1mg/ml Fish AFP III, where the crystals are very small and dense; and (B) non-AFP solution of 30% sucrose and sterile $H_2O$ where the ice crystals are large and round (reproduced from Mills, 1999).**

Melting time was important in trials as the sample had to be viewed and recorded, during which time it was not possible to maintain it at -6°C. However, in field trials a dedicated cold room was available at -6°C, so observation could be performed without the sample melting (Fig. 4.2). Sample size was not decreased from 100 μL in this situation, so the samples could be directly compared with those assayed without a dedicated cold room.

When a light was shone from below the microtitre plate the wells with AFP active solutions appear turbid, whereas the AFP inactive solutions appear clear (Fig.4.3). To make sure that high levels of light refraction were due to AFP activity, two tests were performed. Firstly, serial dilutions were made of fish AFP III (1/10, 1/100 and 1/1000); these were compared against the standard 1mg $mL^{-1}$ fish AFP III solution. This confirmed that a decrease in AFP concentration produces a decrease in RI activity (indicated by a decrease in opacity of solution) and that, even at low concentrations of the

**Figure 4.2 –Fryka KP 281 cold block (CamLab) *in situ* with HTAP microtitre plates during recrystallisation in a -6°C cold room.**

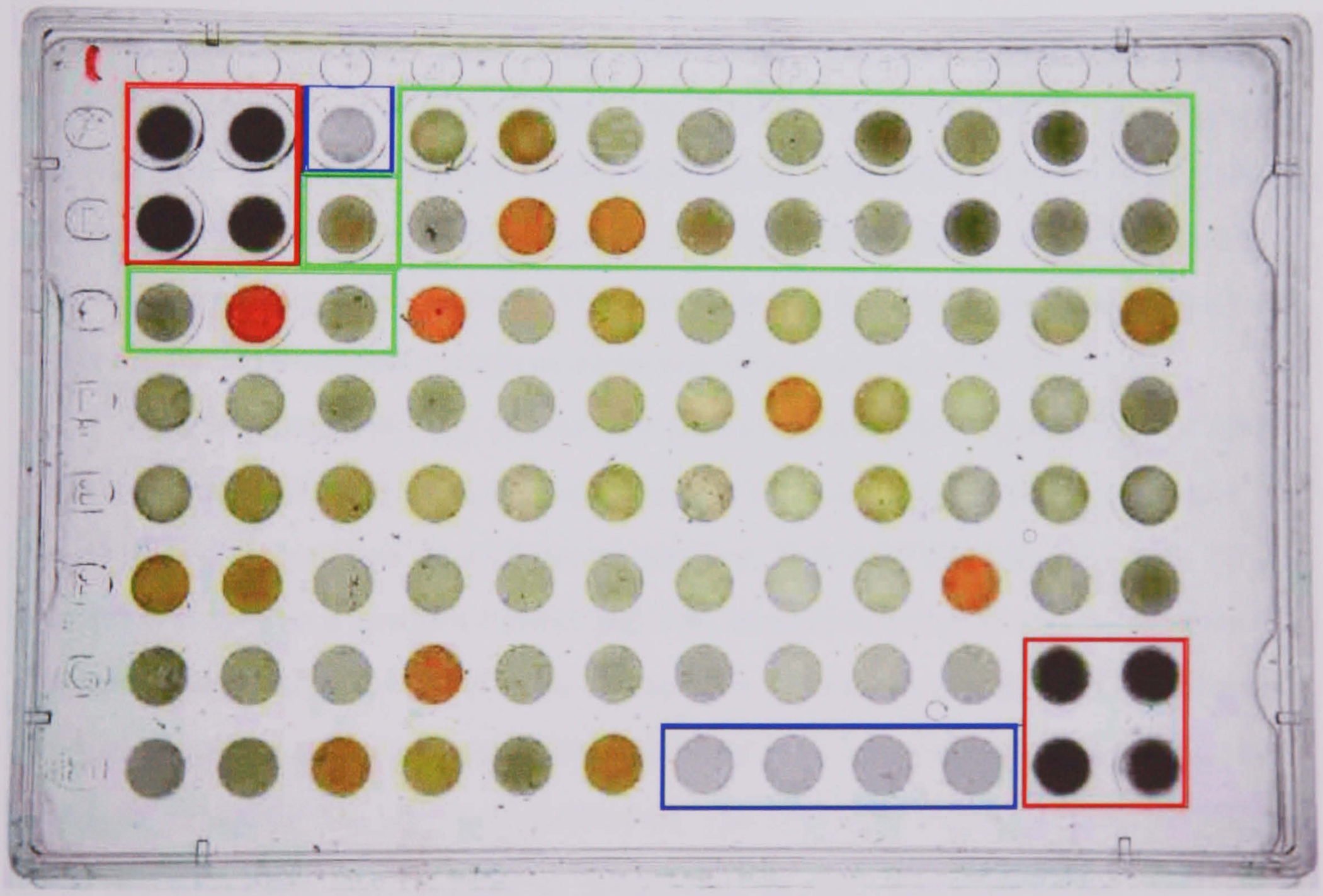

**Figure 4.3 – Photographic image of high-throughput AFP assay in 96 well microtitre plate. Wells surrounded in red contain fish AFP III; these wells are therefore nearly completely opaque due to high refraction of light through the sample. Wells surrounded in blue are 30% sucrose negative controls; these have low refraction and thus appear clear. Wells surrounded in green are isolates with SPLAT confirmed AFP activity; all appear active or coloured. Those samples not surrounded are APP negative protein extracts; some appear positive - this is an artefact of the protocol. All false positives must be confirmed using the SPLAT assay.**

protein (as might be found in a total cellular protein extract), the inhibitory activity was still present and could be visualised against a negative control. This suggests that while the mechanism of action is non-colligative, the level of activity and ice crystal morphology are altered by AFP concentration. Secondly, Proteinase K (20mg $mL^{-1}$, Sigma) was added to a fish AFP III sample (incubated at 37°C for 1 hour) to destroy the protein. After this treatment no diffraction was seen in the sample, indicating that ice-growth inhibition was a result of the fish AFP III.

False negatives were ruled out based on the fact that positive controls never appeared negative, unless there was some practical error when running the protocol. To make sure that there were no errors, the experiment was always duplicated. However, it is possible to concede that some bacteria that were AFP active may have appeared falsely negative because of some step of the protein extraction protocol or indeed in the HTAP analysis itself (this will be discussed more in section 4.3).

In field trials using Antarctic bacterial isolates, 1mg $mL^{-1}$ fish AFP III was used as a positive control and as an indicator of the opacity which AFP activity might produce in the test solutions. The negative control was a sterile solution of 30% sucrose. If the test solutions were darker/more opaque than the negative control then it was assumed that these solutions had possible activity. Distinguishing the solutions was often difficult as activity in the total cellular protein extracts was often extremely low (Fig. 4.4).

The actual methodology for the new protocol, named the high-throughput AFP analysis protocol (HTAP), is as follows. 50 μL of 60% sucrose and 50 μL of protein extract were aliquoted into 96 well microtitre plate. Fish AFP III (1mg $mL^{-1}$ with 30% sucrose) was used as a positive control and 30% sucrose solution as a negative control. The plates were placed at –70°C (cryo-freezer) for 10 minutes to supercool the samples (producing embryonic ice crystals). The microtitre plate was then placed on a cold plate (CamLab, Fryka KP 281) for recrystallisation. The cold plate was pre-cooled to -6°C and stored in a cold room with an ambient temperature of -6°C, this ensured that there was no thermal difference (caused by ambient air temperature) within the solution. The plate was viewed after 5 days using a light box in the cold room, so observation could occur at -6°C to prevent temperature change within the sample altering ice-crystal size. The microtitre plates were placed on a transparent plastic platform 15cm from the surface

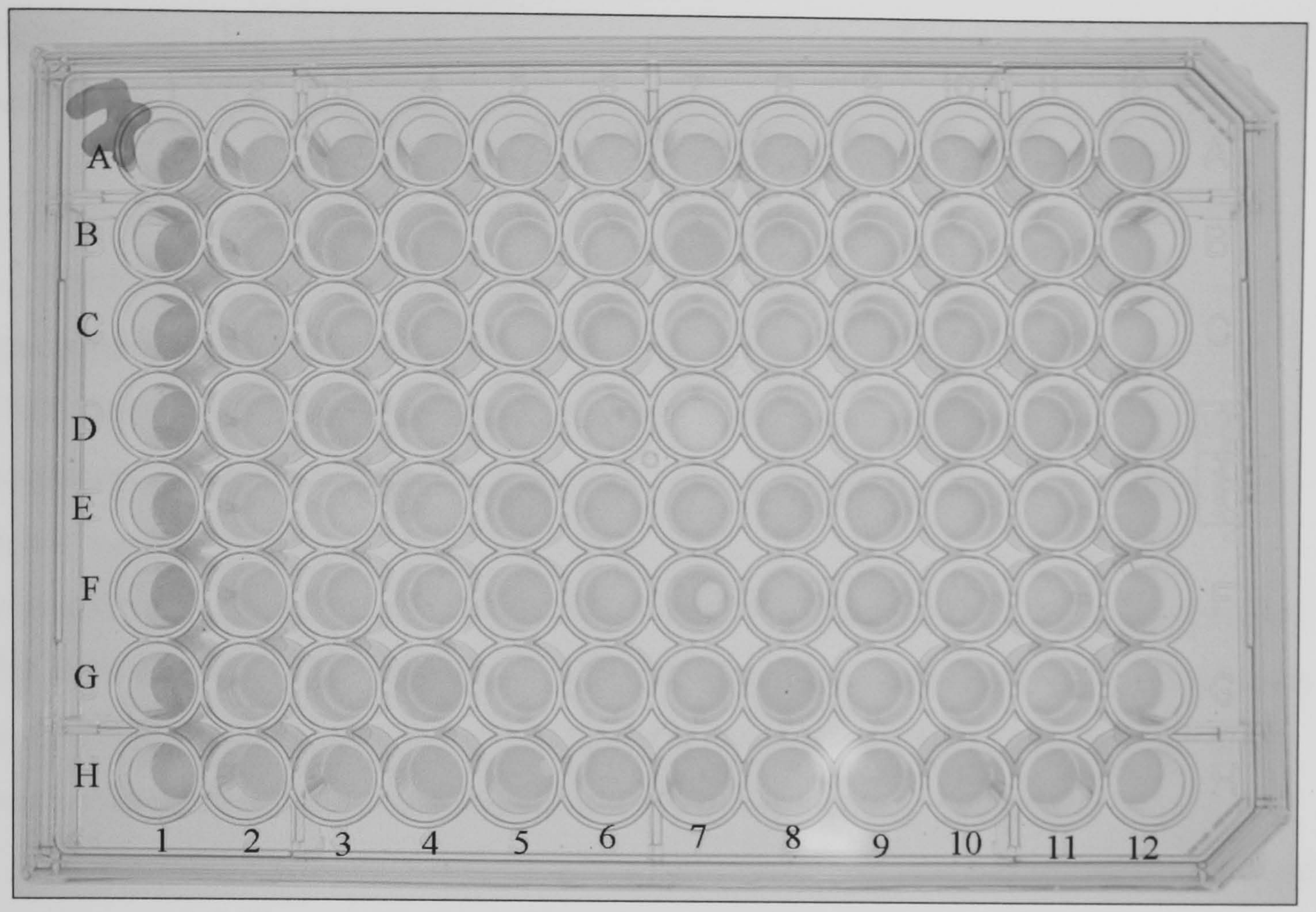


**Figure 4.4 – HTAP assay in a 96 well microtitre plate. The low contrast between negative and positive samples means that analysis for this plate is difficult. However, direct visual analysis can be more effective at discerning positive from negative, the following were considered positive, A12, B7, C4, D3, D6, D10, D12, E5, E8, F3, F5, F6, F10, G4, G8, H7, H10.**

of the light box to prevent radiated heat from the light source altering the temperature of the samples. Visual observations were recorded and a digital photograph was taken of each plate.

This protocol was used when in the field; however, as previously stated, a dedicated cold room was not always available and as such the freeze plate apparatus had to be stored in a 4°C cold room and the metal surface of the plate sealed from the external environment. A polystyrene ice-box was used to isolate the air space above the cold plate and the air temperature was recorded using a thermometer. When the internal temperature had reached -10°C, the microtitre plates were taken from the -70°C freezer and quickly placed on the cold-plate. On average, the internal temperature rose only 4°C. After the plates were placed on the block, the temperature control was set at -6°C and the apparatus was left for 5 days. Observation and recording of results was carried out in the cold room by quickly placing the microtitre plate onto a light source, making a rapid visual assessment of the refraction status of each sample, then taking a photograph using a

digital camera (Fig. 4.3 & 4.4). After several pictures were taken, the plate was placed back on the cold block in case the digital pictures were not satisfactory.

*4.1.2.3 – Advantages and disadvantages of the assay*

The HTAP assay is much cheaper than the 'SPLAT' assay as the only pieces of specialised equipment necessary were the freeze plate (CamLab, Fryka KP 281) and -70°C freezer (which is common place in most well equipped laboratories). Apart from consumables (sucrose, microtitre plates, etc.) and the equipment involved in the protein extraction methodology, there are no further costs. The 'SPLAT' assay required expensive $CO_2$ cylinders, solvents (2,2,4 – trimethyl pentane) and microscope objectives (EF L 20/0.30 160/0-2). Although the -70°C freezer was an advantage in the HTAP assay, tests using a -20°C freezer proved successful at establishing activity, although the test was not as discriminatory as when using -70°C to supercool the samples.

Identification of AFP activity within the protein samples was sometimes hindered by colouration, caused by carotenoid pigments (found extensively in Antarctic bacteria as protection against UV damage; Vincent & Quesada, 1994; George *et al.*, 2001), which were not removed by the protein extraction protocol. Cultured Antarctic bacterial samples within this study showed high levels of carotenoid pigmentation. Colouration does not affect a 'SPLAT' assay because the sample used is so thin and analysis is done at the microscopic level, but with a HTAP assay the large volume of sample required (50 μL) means that coloration can make it very difficult to differentiate between an active or non-active sample. Normally a darkened sample, i.e. one which has greater opacity, would be considered positive, but, if a sample has colouration, it is difficult to ascertain the level of refraction. This artefact meant that samples with refraction inhibitory colouration were recorded as positive for AFP activity and then assessed using the 'SPLAT' assay at a later date. Due to the large sample size and uncertainty of location of pigment within the cell, fractionation of the solution to remove pigmentation was not performed. Despite the fact that a large number of false positive are incurred using this protocol, it is still more advantageous as a first step field screen for reducing the number of isolates which require 'SPLAT' analysis. It therefore provides more time for field analysis and bacterial isolation and prevents the need for costly and cumbersome 'SPLAT' analysis in the field.

## 4.2 - Results

### 4.2.1 - Sampling and bacterial isolation

During a two month sampling period over the 1999/2000 austral summer, 41 lakes were sampled with the aid of helicopter support. Two of these lakes, Ace and Pendant, were depth sampled because they had sufficient ice cover thickness to support the necessary ice drilling/coring equipment, depth sampling equipment and personnel. The majority of other lakes did not have sufficient ice cover thickness to support depth sampling activity. These were, *Williams, Rookery, Club, Ekho, Triple, Deep, Shield, Oval, Druzhby, Tassie, Trident, Pointed, Verenteno, Calendar, Stinear, Jabs, Hand, Dingle, Crooked, Oblong, Lebed, Anderson, Scale, Collerson, Medusa, Pauk, Zvezda, Braunsteffer, Abraxas, Organic, Cemetery, Laternula*, and *Angel 2*. For a number of lakes, i.e. Watts, Nicholson, Nottingham, the lakes from the Larsemann Hills region (Larsemann 73 and Reid), and Beaver lake, samples were taken during other sampling programmes and as such these were not targeted for depth analysis. Surface water (within the top 1 meter from the surface) sampling provided a cursory analysis of bacterial AFP activity from each lake, a spot test from which a more thorough, focused study could be developed on those lakes which demonstrated surface bacterial populations with a high instance of AFP activity (Chapter 3).

Over the whole sampling period 866 bacterial cultures were isolated from surface water samples of the 41 individual lakes and depth samples with replicate samples on five detailed study lakes.

### 4.2.2 - Gram staining and cellular/colony morphology

Approximately 87% of bacterial isolates were Gram negative. Colony morphology was dominated by mucoid colonies. These polysaccharide rich colonies could indicate a UV protective strategy. Noted morphologies included irregular, undulate, dry colonies, and punctiform, entire, moist/dry colonies.

Gram negative cellular morphology was dominated by rods (~98%), however, these rod morphologies did vary in length (actual sizes were not recorded) and type, such as straight, club-shaped and long, thin curved rods. The remaining bacterial isolates were reported as coccoid (Appendix B1). It was possible that some of the Gram positive bacteria could have been contaminants.

### 4.2.3 - High-throughput AFP assay

The assay was applied to all 866 isolates from the Antarctic lake environments. Of these, 187 (21.6%) proved to be positive for AFP activity by this assay. Activity was demonstrated by a reduction in transparency of the protein extracts when compared with a 30% sucrose solution without protein extract. The samples could not be quantified or qualified for level of activity as with a 'SPLAT' assay, however, protein extracts were marked as either active or non-active. Those with reduced transparency (increased opacity) or colouration which were difficult to confirm were classed as positive, and those extracts with refraction equal or less than the negative control (30% sucrose) were classed as negative. The 187 bacteria strains, which were identified as positive using the HTAP assay, are shown in table 4.1. These isolates were returned to England and analysed using the 'SPLAT' assay to confirm AFP activity within the bacteria. Of the 187 bacteria identified as AFP active using the HTAP assay, only 12 were Gram positive.

### 4.2.4 - SPLAT assay

The 187 bacterial isolates from which positive protein extracts (using the HTAP assay) were extracted, were brought back to Colworth (Unilever) and assessed for activity using the 'SPLAT' assay. Nineteen of the bacterial isolates were confirmed to have some level of AFP activity within their total cellular extracts (Table 4.2), which equates to 10.2% of those isolates showing a positive protein extract using the HTAP assay. Ten of these 19 isolates showed a SPLAT score of 4 or 5 (Section 2.10.2), indicating high AFP activity, comparable with pure fish AFP III protein (1mg $mL^{-1}$) or total cellular protein extracts of *Marinomonas protea* (Mills, 1999). The rest of the samples had an activity of 3. Samples with an activity of less than 3 were considered to have insufficient activity for further analysis. AFP active bacteria with a score $\geq 3$ were isolated from Ace Lake, Triple Lake, Pendant Lake, Pendant Ice cores and Oval Lake (Table 4.2 and Fig. 4.5). All AFP active bacteria, as confirmed by the SPLAT assay of total cellular protein extracts, were Gram negative.

The concentrations of protein extracts, prepared from each putative AFP active isolate, were assessed using the 'SPLAT' analysis and were calculated using the BioRad Protein Kit® (BioRad, see Chapter 2 section 2.10.1). Figure 4.6 shows that there was no direct relationship between total cellular protein concentration used for analysis and

SPLAT score. It has been shown that activity level is affected by AFP concentration. However, the AFP active quotient of these samples does not appear to be affected by the total cellular protein concentration.

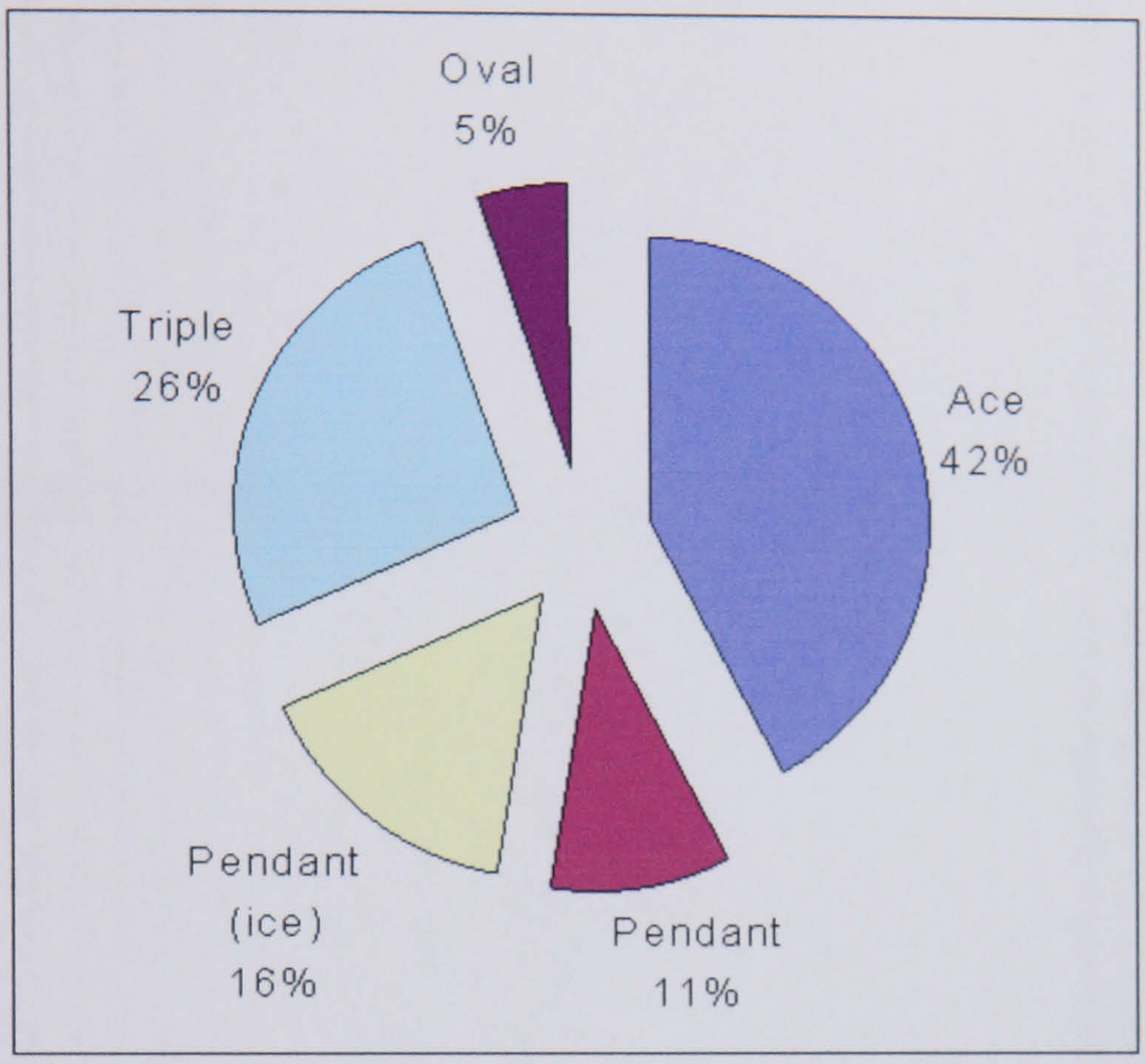


**Figure 4.5 – Percentages of 'SPLAT' assay confirmed AFP active bacteria isolated from each lake.**

### 4.2.5 - Crystal morphology

The majority of the AFP positive protein samples produced small round crystal morphologies, as can be seen in figures 4.1 and 4.7. This morphology is consistent with crystal morphologies seen in SPLAT analysis, as crystals are formed and recrystalise after crash-cooling in a liquid medium. The interface between ice and water is molecularly rough and the transport of water molecules to the growing crystal surface is limited by diffusion, therefore circular discs often form (Whitworth & Petrenko, 1999). Isolate number 494 had a SPLAT activity of between 4 and 5 and produced small round crystals as far as could be ascertained (Fig. 4.7).

| Lake of Origin | Number of bacteria originally isolated from the lake. | Number positive isolates from HTAP assay | Number positive isolates confirmed by SPLAT assay. |
|---|---|---|---|
| Ace | 194 | 50 | 8 |
| Ace (Ice) | 63 | 5 | 0 |
| Pendant | 106 | 28 | 2 |
| Pendant (Ice) | 10 | 6 | 3 |
| Triple | 95 | 35 | 5 |
| Club | 15 | 5 | 0 |
| Deep | 15 | 5 | 0 |
| Crooked | 22 | 3 | 0 |
| Medusa | 15 | 3 | 0 |
| Nottingham | 21 | 3 | 0 |
| Shield | 12 | 2 | 0 |
| Beaver | 12 | 2 | 0 |
| Abraxas | 10 | 2 | 0 |
| Tassie | 8 | 2 | 0 |
| Rookery | 20 | 2 | 0 |
| Stinear | 16 | 2 | 0 |
| Hand | 10 | 2 | 0 |
| Oval | 12 | 2 | 1 |
| Williams | 11 | 2 | 0 |
| Organic | 6 | 1 | 0 |
| Cemetery | 4 | 1 | 0 |
| Scale | 16 | 1 | 0 |
| Pointed | 8 | 1 | 0 |
| Collerson | 12 | 1 | 0 |
| Lebed | 8 | 1 | 0 |
| Laternula | 9 | 1 | 0 |
| Calendar | 11 | 1 | 0 |
| Jabs | 13 | 1 | 0 |
| LH73 | 9 | 1 | 0 |
| Pauk | 4 | 1 | 0 |

**Table 4.1 - The proportion of HTAP positive bacteria isolated from each lake. Most of the putative positive bacteria assessed as RI active using the HTAP assay were isolated from Ace Lake and Triple Lake, followed by Pendant Lake, then Deep and Club. The rest of the lakes produce negligible numbers of RI active isolates.**

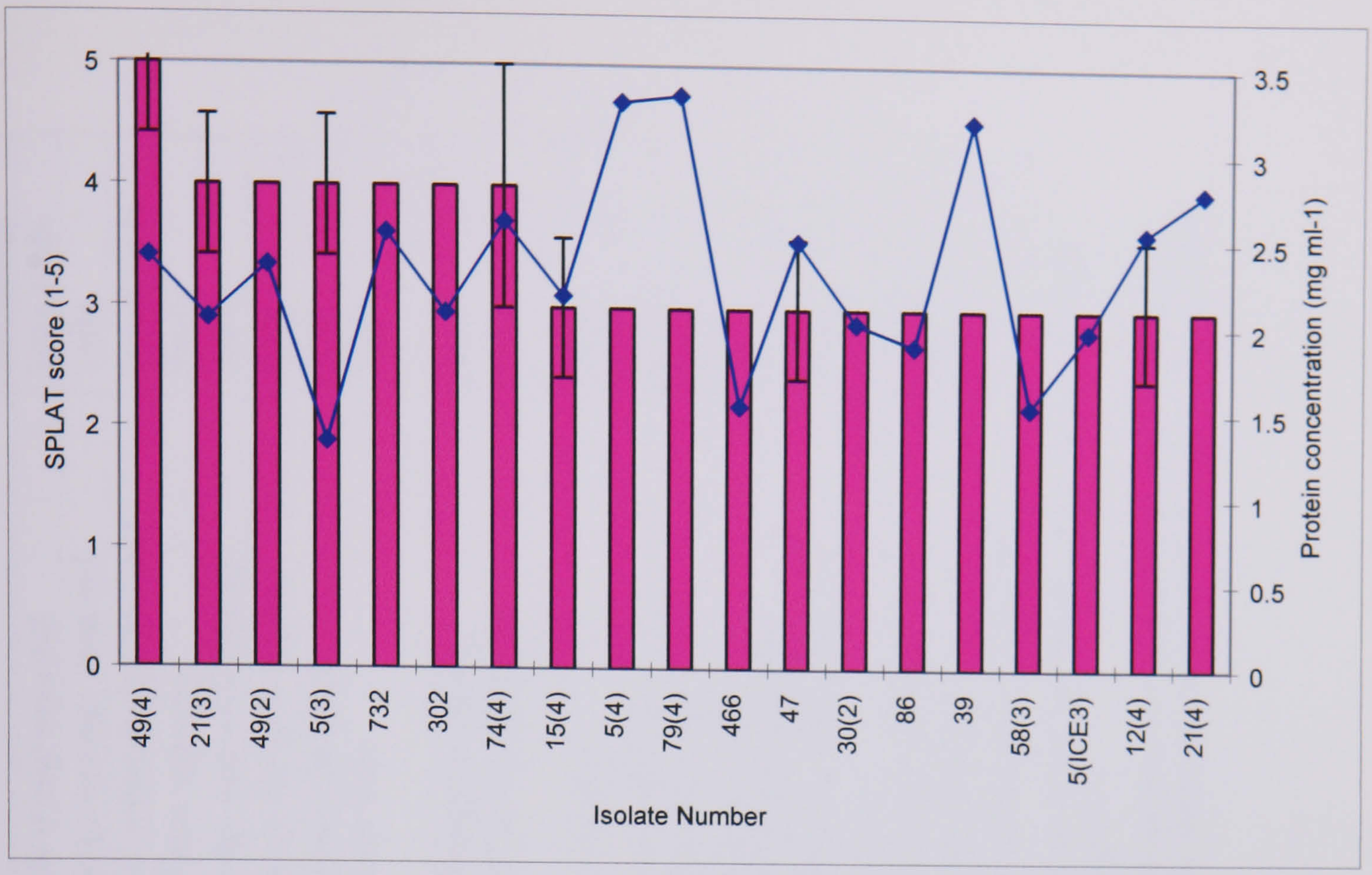


**Figure 4.6 – Total cellular protein extraction concentration (mg $ml^{-1}$) (blue line) with SPLAT score (red bars) with error bars (1 = no activity, 5 = activity of 1mg $ml^{-1}$ fish AFP III) for AFP active isolates.**

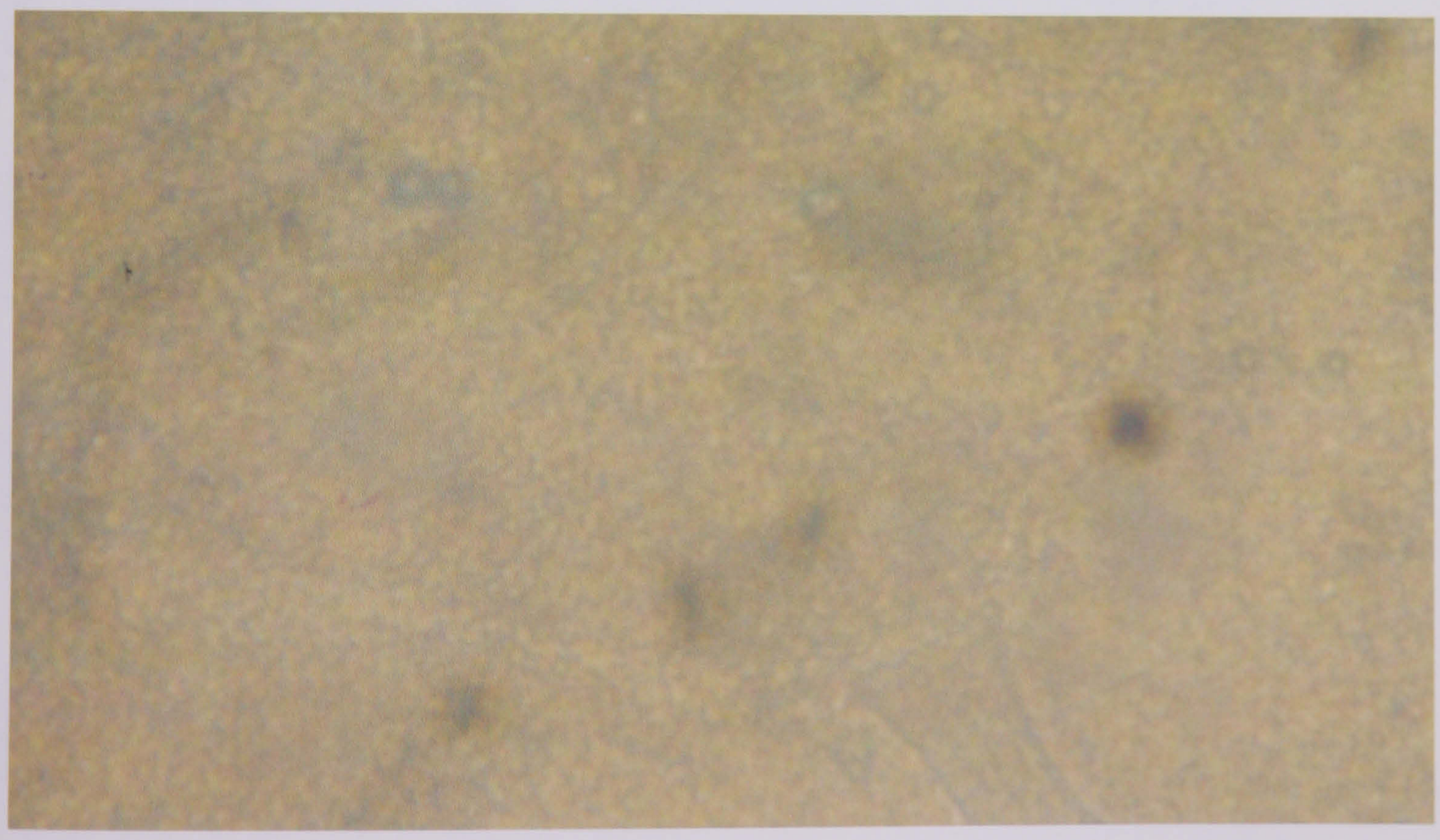

**Figure 4.7 – Photograph of SPLAT analysis of isolate 494. This isolate was designated with a SPLAT score of 4-5 based on the small size and extreme density of the ice crystals. There are several artefacts in the picture due to the preparation of the protein extract for SPLAT analysis. Ice crystal size was not recorded.**

| *Isolate Number* | *SPLAT Score* | *Lake of Isolation* | *Depth of isolation* | *Date of isolation* | *Media used for culturing* | *Colonial colour and morphology* | *Cellular morphology* |
|---|---|---|---|---|---|---|---|
| **21(3)** | **++++** | **Triple** | **4m** | **07/09/00** | **½** | **Off white mucoid** | **long rods** |
| **49(2)** | **++++** | **Ace** | **0m** | **15/07/00** | **TSA** | **Beige/orange mucoid** | **rods** |
| **5(3)** | **++++** | **Triple** | **8m** | **07/09/00** | **SWA** | **Off white/beige mucoid** | **small rods** |
| **732** | **++++** | **Ace** | **2m** | **04/03/00** | **YSA** | **White mucoid** | **Rods** |
| **302** | **++++** | **Triple** | **0m** | **23/01/00** | **TSA** | **Beige mucoid/ lumpy** | **Rods** |
| **15(4)** | **+++/++++** | **Ace** | **4m** | **13/11/00** | **½** | **Beige distinct white** | **rods** |
| **5(4)** | **+++/++++** | **Pendant** | **4m** | **13/11/00** | **½** | **Cream distinct** | **rods** |
| **74(4)** | **++++** | **Pendant Ice core** | **50cm** | **13/11/00** | **SWA** | **Beige fine** | **rods** |
| **49(4)** | **++++/+++++** | **Pendant** | **0m** | **13/11/00** | **YSA** | **Orange mucoid** | **rods** |
| **79(4)** | **+++/++++** | **Pendant Ice core** | **Ice/water interface** | **13/11/00** | **½** | **Off white distinct** | **rods** |
| 466 | +++ | Oval | 0m | 04/02/00 | SWA | White fine | Rods |
| 47 | +++ | Ace | 6m | 14/01/00 | YSA | Beige mucoid | Rods |
| 30(2) | +++ | Triple | 2m | 17/07/00 | TSA | Clear Mucoid | Rods |
| 86 | +++ | Ace | 4m | 14/01/00 | TSA | Beige mucoid | Rods |
| 39 | +++ | Ace | 4m | 14/01/00 | SWA | Off White distinct | Rods |
| 58(3) | +++ | Triple | 0m | 07/09/00 | SWA | Off white beige distinct Mucoid | Rods |
| 5(ice3) | +++ | Pendant | 50cm | 07/09/00 | ½ | Off white distinct | Thin rods |
| 12(4) | +++ | Ace | 8m | 13/11/00 | ½ | Beige Distinct | rods |
| 21(4) | +++ | Ace | 2m | 13/11/00 | ½ | Off white mucoid distinct | rods |

**Table 4.2 - Isolates which tested positive for some level of RI activity. Also provided are the location from which the bacteria were isolated, the agar which was used to culture them, the colony and cellular morphologies. All these isolates were gram negative.**

**RI activity key (for SPLAT assay) - +++++ - very small/dense crystals (e.g. fish AFP III); ++++ - small not dense or quite small dense with medium crystals; +++ - small not dense with medium large or small medium not dense crystals; ++ - medium or large with some small crystals; + - large round discrete crystals (e.g. 30% sucrose).**

## 4.3 – Discussion

Of the 866 bacteria isolated from the 41 lakes of the Vestfold Hills. Larsemann Hills and MacRobertson Land, 86.5% were Gram negative. This high instance of Gram negative bacteria in Antarctic bacterial collections has been noted in previous literature (Herbert, 1986; Russell, 1992). The fact that all the AFP active bacteria. as confirmed by the SPLAT assay, were also Gram negative supports the fact that Gram negative bacteria are generally well adapted to extreme conditions. However, the presence of Gram positive bacteria in Antarctica is also supported (Franzmann, 1996; Sheridan & Brenchley, 2000).

Despite the limitations of the HTAP assay, it is inexpensive, transportable and provides an effective first-stage high through-put analysis technique. However, the method still involves cellular protein extraction, which is the rate limiting step in any protein analysis methodology. During the current study, HTAP analysis was developed and employed as a first-stage AFP analysis protocol (recrystallisation inhibition analysis protocol) for analysing bacterial samples in Antarctica. Total cellular protein extractions were performed for 866 bacterial isolates cultured from the study lakes in the Vestfold Hills. 50ml liquid media cultures were grown for each isolate; each took approximately one week to produce a turbid culture. This was followed by one week of cold storage (1°C), in an attempt to induce cold shock gene transcription (Russell & Hamamoto, 1998). Due to limited numbers of glassware for culture growth, it took approximately 1 month to extract the protein from all 866 isolates. It took approximately three hours to set up all 866 isolates in the HTAP assay which was then allowed to recrystalise for 5 days at -6°C. Therefore, although the actual protocol is rapid and labour free, the protein extraction protocol limits the speed with which isolates can be analysed for activity. The same is true for the SPLAT assay, which also requires the same protein extraction protocol. However, the actual SPLAT protocol can take up to 7-8 hours to analyse 50 isolates. Thus, the minimum time needed for analysis of 866 isolates would be 138.5 man hours.

The disadvantages of the HTAP assay include problems with analysing protein extracts with colouration. Figure 4.3 shows some extreme forms of colouration in crude protein extracts and, as can be seen, it is virtually impossible to discern the level of

refraction. They are, therefore, considered positive and analysed using the SPLAT assay to ascertain AFP activity. Such colouration existed within the protein extracts because it was necessary to analyse all the proteins from the bacterial cells as it is unknown where within the cell the AFP resides. However, the inclusion of all cellular proteins meant that any pigment-protein complexes, such as cytochrome or carotenoid complexes (Poggese *et al.*, 1997; Boronowsky *et al.*, 2001) were also included. Hence, the colouration within the crude protein extracts could only be removed by degrading these various protein complexes, which may inactivate the AFPs. It is possible to suggest the location of the AFP by SPLAT analysing fractionations of the different cellular components, but this was not performed for the current study due to time constraints.

Only 10.2% of the HTAP positive isolates proved to be RI active using the SPLAT assay. That equates to 2.1% of the isolated bacteria from Antarctic lakes showing some degree of RI activity. This seems a very small percentage, unless it is considered that, until a phenetic assessment of all 866 isolates is produced, it will be impossible to suggest how many species comprise the 866 isolates. It is not expected that there are 866 individual species; indeed, phenetic analysis of the 19 AFP active isolates has identified only 11 individual taxa (Chapter 5). Therefore, it may be suggested that only a small percentage of the isolates are actually individual species. This is because bacterial species within a particular lake are not likely to vary considerably throughout the year, as only a limited number of taxa can actually survive and thrive in such a harsh environment, as suggested by a relatively low species diversity and biomass within Antarctic lakes (Laybourn-Parry, 1997). Of the bacteria isolated, there was still only a very small percentage which showed AFP activity. This may suggest that AFP is not the dominant method of cold adaptation in Antarctic bacteria. There are a number of other cold adaptation techniques, as reviewed in the introduction (Chapter 1). These mainly involve structural adaptation in enzymes and other proteins to maintain functionality at cold temperatures, as well as specific proteins which manipulate and control ice formation, e.g. ice nucleation proteins. They also involve cold adaptations in membrane lipids. It is possible, therefore, that the vast majority of bacteria in Antarctic lakes utilise one or more of these cold adaptations to allow for growth and multiplication in the extreme cold. This would mean that AFP activity is actually a relatively rare adaptation which has only

arisen in very few species due to chance mutation. During this study AFP activity has been shown to occur in strains of common bacterial species, such as *Pseudomonas fluorescens* (Chapter 5), which is known to inhabit household refrigeration systems (Dodd, *pers. com.*). It is assumed that the *P.flourescens* isolated in Antarctica is a separate strain from that isolated from refrigeration systems, but it suggests that AFP activity might not be a rare condition, but in fact a cold induced protein that might occur in all psychrophilic bacteria. It is indeed possible that all of the Antarctic isolated bacteria may be genotypically AFP positive, but the conditions under which they were cultured may not have been those necessary for AFP induction. If this is correct, then there could be a very high proportion of false negatives within the 866 bacteria cultivated. There could also be bacteria which do express the protein under the culture conditions but the protein is degraded or inactivated in someway during protein extraction or during the AFP analysis itself. However, there is no evidence to suggest this. Isolates known to have activity, e.g. *Marinomonas protea*, do show activity using these conditions and these assays. As AFP from other sources (e.g. fish, insects, plants) are known to be extremely variable in structure (Yeh & Feeney, 1996; Davies & Sykes, 1997; Smaglik, 1998), it is possible that the conditions used to assess Antarctic bacteria during the current study, may have been ineffective at identifying activity of certain types of AFP. More detailed analysis will have to be performed to isolate the proteins and, subsequently, the genes which code for them, to help assess whether AFP activity is present within these putative non-active isolates, using specific oligonucleotide probes which identify specific conserved regions of the AFP gene. To date there has been no known successful published work on a bacterial AFP gene and, therefore, it is unknown as to whether there are conserved regions.

AFPs have shown an ability to alter the structure of ice crystals. Barrett (2001) suggests that AFPs alter the ice growth morphology significantly, producing bypyrimidal crystallites and columnar spicules. The visualisation required to observe crystal shapes was not performed for the current study due to time constraints. It is suggested that AFPs bind to the bypyrimidal planes, so that when crystal growth occurs close to the hysteresis temperature (lowered freezing point), it occurs along the *c*-axis of the crystal (Grandum *et al.*, 1999). Therefore, AFPs bind to a particular face of the embryonic ice crystal and

inhibit growth along the axis upon which they act perpendicularly. Thus when the ice crystal is held at a temperature, close to its hysteresis temperature the corresponding increase in the rate of crystal growth produces extensive growth along the axis not 'protected' by the influence of AFPs (Grandum *et al*, 1999; Barrett, 2001; Jia & Davies, 2002).

**4.4 - Conclusions**

To increase the number of culturable isolates which could be screened for AFPs when in Antarctica, a new protocol was developed based on the SPLAT assay, but without the disadvantages which made SPLAT analysis in inaccessible field locations unfavourable. The new assay, the high-throughput AFP protocol (HTAP), was cheaper, faster, less labour intensive and was able to analyse large numbers of bacterial isolates at one time. 866 isolates were screened for AFP activity using the HTAP assay, only 187 showed RI activity. One of the drawbacks to using the HTAP assay is that it produces false positives due to sample colouration. Therefore, all putative positive isolates had to be re-analysed using the conventional SPLAT assay upon return to England. Only 19 of the HTAP positive isolates were proved to be positive using the SPLAT assay.

The HTAP assay is an excellent first stage assessment technique for reducing the number of isolates which have to be assessed using the more accurate, but slower and more labour intensive, SPLAT assay.

# Chapter 5: BACTERIAL MOLECULAR TYPING

## 5.1 – Introduction

Bacterial isolates that were shown to be antifreeze protein (AFP) active (Chapter 4) were characterised using a polyphasic taxonomic approach, combining amplified ribosomal DNA restriction analysis (ARDRA) with 16S rRNA molecular sequencing. These molecular phenetic and phylogenetic techniques were used to identify the isolates that show AFP activity, to enable an understanding of the taxonomic distribution of Antarctic bacterial AFP activity.

Characterisation of the isolates was performed using ARDRA, in which endonucleases restriction of the 16S rRNA gene was used to delineate phenetic relationships between bacterial isolates. The 16S rRNA gene is highly conserved, approximately 1500bp long and contains sub-species specific variations (Vaneechoutte *et al.*, 1995; Vila *et al.*, 1996; Rademaker & de Bruijn, 1997; Jawad *et al.*, 1998). ARDRA was used because it is a well established, rapid and repeatable technique which enables sub-species identification between isolates (Vaneechoutte *et al.*, 1995; Rademaker & de Bruijn, 1997) and therefore could determine the phenetic relatedness of the AFP active species, down to the sub-species level.

Sequencing of the 16S rRNA gene was performed using the chain-termination method (Chapter 2, section 2.9.6.4; Sanger *et al.*, 1977; Brown, 1996). The unidentified sequence can then be compared against 16S rRNA sequences from known species and a percentage similarity can be calculated to identify the isolate. By comparing the sequence of an unidentified isolate with those of a number of similar identified sequences, it is possible to infer phylogenetic relationships between the sequences. These relationships can be represented in a phylogenetic tree (Priest & Austin, 1995), which is used to graphically represent a possible evolutionary pathways which link the represented species to the unidentified isolate.

In the current study all 19 AFP active isolates were characterised using ARDRA and molecular sequencing of the 16S rRNA gene to ascertain if AFP activity is present

only in specific taxa or if it is found in many divergent taxa. By identifying taxa which have a propensity for AFP activity, it may be possible to aid future isolation of AFP active bacteria.

## 5.2 – Results

### 5.2.1 - Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA analysis was performed on the 19 Antarctic bacterial isolates with AFP activity (Chapter 4). Two separate restriction digest enzymes, *Hpa* II and *Alu* I (Gibco BRL) were used to demonstrate the repeatability of digestion, in terms of the relationships derived between isolates, regardless of the restriction site.

#### *5.2.1.1 – Analysis of Hpa II and Alu I restriction digestion patterns.*

The *Hpa* II and *Alu* I digestions of the 1500bp 16S rDNA fragments from the 19 AFP active isolates were analysed using the ImageMaster 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics) (Fig 5.1). The procedure for analysis is outlined in Chapter 2 (section 2.9.4). A matrix of relatedness was calculated from the comparison of the ARDRA patterns of each isolate to every other isolate using the Dice coefficient of relatedness. A dendrogram was calculated from the matrix to graphically represent the relationships between the isolates. However, for the *Hpa* II and *Alu* I digestions it was necessary to run each digestion on two gels (Fig 5.1), therefore each gel from the Image Master 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics) was exported to a database program, ImageMaster 1D database V2.12 (Amersham Pharmacia Biotech). This allowed the information from each gel to be combined to produce a similarity matrix (Appendix B2) and a dendrogram (Fig 5.2a & b, *Hpa* II & *Alu* I respectively) for all the isolates from a single digest.

Twenty restriction digests were run, 19 individual isolates (494, 213, 302, 22, 53, 86, 39, 732, 492, 47, 583, 124, 154, 214, 5ice3, 54, 744, 794 and 466) and *Marinomonas protea* (Mills, 1999) which was an AFP active γ-Proteobacteria which was isolated from Ace Lake during 1996/97. Ace Lake was one of the detailed study lakes from the current study and the lake from which 8 of the AFP active bacteria were isolated. Hence it was

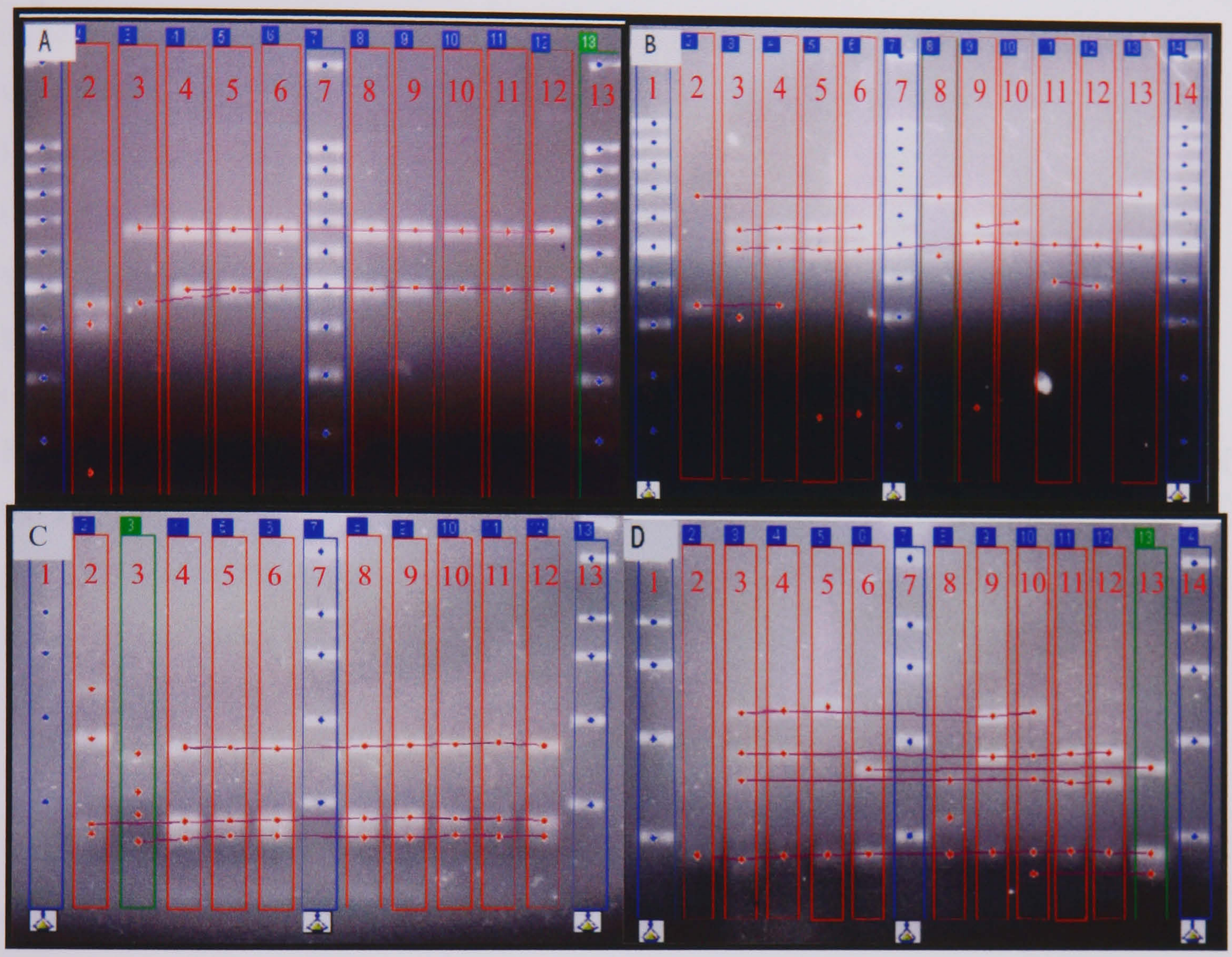


**Fig 5.1 – Photographs of ARDRA gel patterns used for phenetic analysis of band position along gel. (a) and (b) gels resulting from *Hpa* II restriction digestion of isolates. (c) and (d) gels resulting from *Alu* I restriction digestion of isolates. On both gels a control digestion of *Marinomonas protea* was used to enable inter-gel comparisons. Gels A and C: Lane1: molecular weight (MW) marker, Lane2: 466, Lane3: 47, Lane 4: *M. protea*, Lane5: 154, Lane6: 124, Lane7: MW marker, Lane8: 5ice3, Lane9: 744, Lane10: 794, Lane11: 214, Lane12: 54, Lane 13: MW marker. Gels B and D: Lane1: MW marker, Lane2: 494, Lane3: 583, Lane4: 213, Lane5: 302, Lane6: 33, Lane7: MW marker, Lane8: 492, Lane9: 53, Lane10: 732, Lane11: 39, Lane12: 86, Lane13: *M. protea*, Lane14: MW marker. MW marker in gels A and B comprises 100 bp increments from 100 bp to 1000 bp then a 1500 bp fragment at the top. The MW marker in gels C and D comprises 5 bands, 250, 500, 750, 1000 and 1500 bp.**

considered important to run this species with the un-characterised isolates. *M. protea* was also used as a standard for inter-gel analysis.

Some small molecular weight bands on the gels were very faint due to opposite migration of ethidium bromide during electrophoresis and low DNA concentration during initial restriction digestion. If DNA concentration was deemed to be too low then the volume of 1500bp 16S rDNA PCR product added to the digestion reaction was increased. However, very small molecular weight bands (<200bp) still had low intensity. This was overcome by taking multiple exposures of the gel at steadily increasing light intensities until the low molecular weight bands were visible (Fig. 5.3). These high light intensity gel images were analysed using the ImageMaster 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics) and the molecular weight and Rf value of these bands were determined and standardised with respect to other images of the same gel with less light intensity, so that bands represented on high intensity gels could be represented on low intensity gels. High light intensity images could not be used for complete analysis as bands with an initial high intensity became obscured (Fig 5.3).

*5.2.1.2 – Relatedness of AFP active isolates from ARDRA*

Dendrograms (Fig 5.2a & b, *Hpa* II & *Alu* I) were calculated to graphically represent the relatedness of the restriction patterns of each isolate to each other isolate as demonstrated in the similarity matrix (Appendix B2). The restriction pattern for the isolates using *Hpa* II (Fig 5.1a & b) indicated that there were 10 groups, 6 of which were single isolate groups (53, 732, 583, 213, 466 and 494) (Fig 5.2a). The rest were clustered into four groups, these were (1) 302 and 33; (2) 39 and 86; (3) 47 and 492; and (4) 154, 124, 5ice3, 744, 794, 214 and 54. Group 4 clustered with *Marinomonas protea* suggesting that it was a single species group.

Using the restriction enzyme *Alu* I a similar but slightly different pattern (Fig 5.2b) was produced (Fig 5.1c & d) with 12 individual taxa. These were isolates 47, 492, 732, 583, 213, 53, 494, 466, 302, 33 and several isolates grouped into single taxon, these were 39 and 86, and the *M. protea* taxon including isolates 154, 124, 5ice3, 744, 794, 214, and 54.

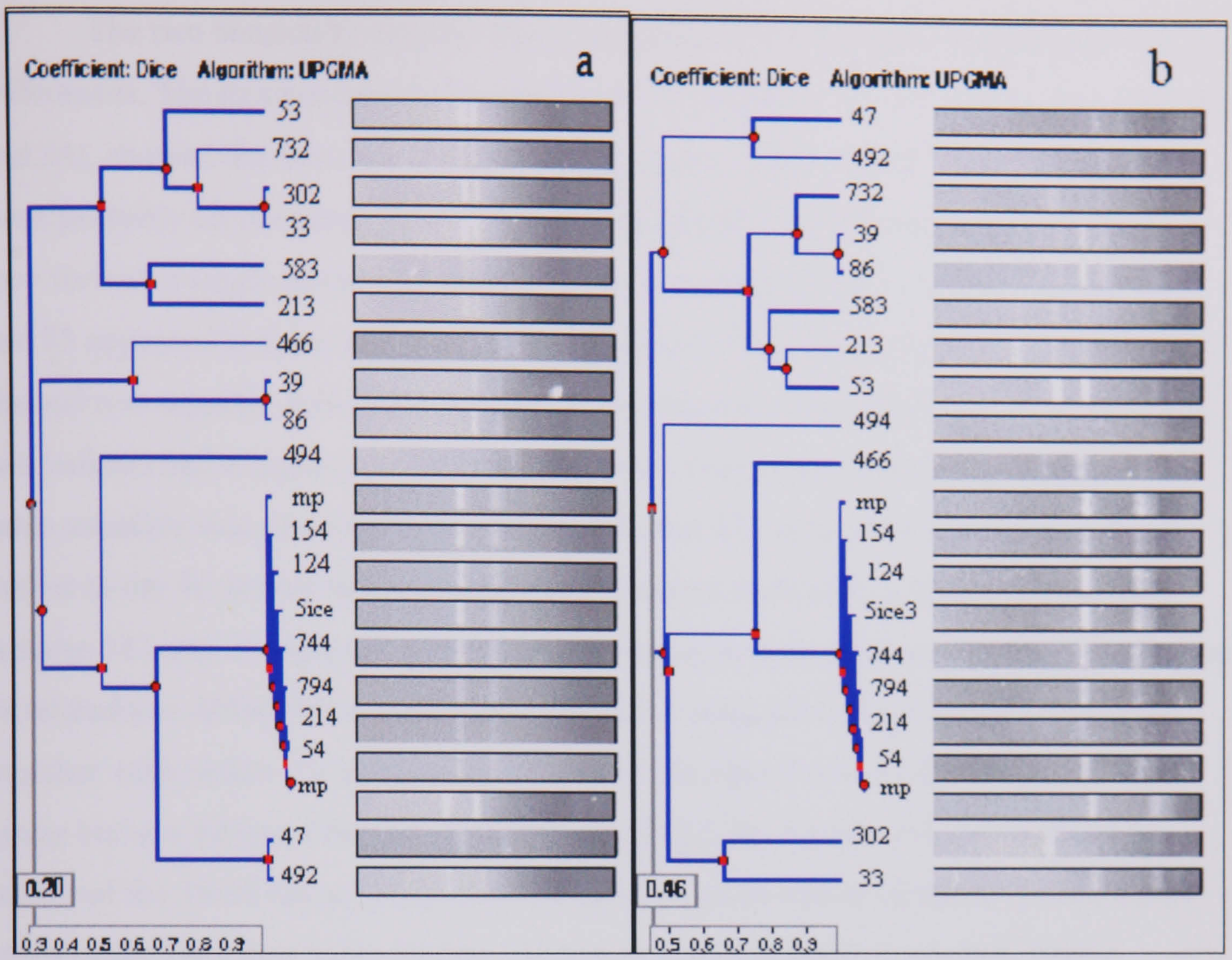


**Figure 5.2 – (a) Dendrogram calculated using the unweighted pair group method with arithmetic averages (UPGMA) from the similarity matrix produced by similarity assessment of *Hpa* II digestions of AFP active isolates; (b) Dendrogram calculated using UPGMA from the similarity matrix produced by similarity assessment of *Alu* I digestions of AFP active isolates. mp – *Marinomonas protea.***

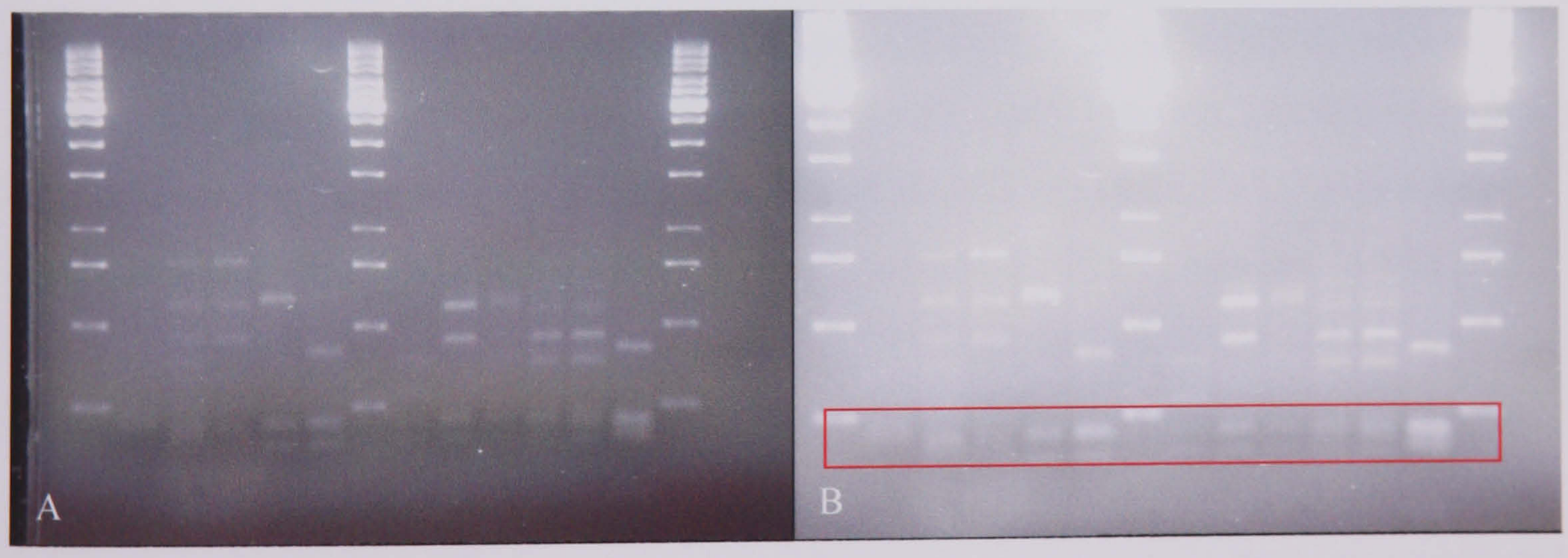


**Figure 5.3 – Photograph of an over exposed gel image (B) next to a normal exposure gel image (A), indicating the low molecular weight bands or bands of low DNA concentration (red box) which can be better identified when the image is overexposed.**

The two restriction enzyme digest patterns were very similar but did show some differences. The isolates which clustered with *M. protea* (154, 124, 5ice3, 744, 794, 214 and 54), showed the same clustering for both digests (Figs. 5.2a & b) indicating they were probably all the same species. Isolates 39 and 86 were probably also identical as they formed a single taxon with both restriction enzymes (Figs. 5.2a & b). Isolates 302 and 33 appeared to form a single taxon with *Hpa* II (Fig 5.2a), however, with *Alu* I they formed two separate taxa while still clustering together (Fig 5.2b), indicating that these two isolates had a higher similarity to each other than to any of the other isolates. The same situation was also shown by isolates 492 and 47. Isolate 494 appears as a direct outlier to the *M. protea* taxon in both digest patterns indicating a distant relationship. Isolates 583 and 213 appear within the same group in both patterns, however, the degree of relatedness differs for the different enzymes. Using *Alu* I (Fig 5.2b) they clustered together with isolate 53 which when digested with *Hpa* II was found within the same group but at a different degree of relatedness (Fig 5.2a). Isolate 466 moved from an outlier of the 39/86 taxon using *Hpa* II (Fig 5.2a), to an outlier of the *M. protea* taxon when using *Alu* I (Fig 5.2b). Isolate 732 shows a similar jump in position from a taxonomic grouping with the 302/33 taxon when using *Hpa* II (Fig 5.2a), to a grouping with the 39/86 taxon when using *Alu* I (Fig 5.2b).

**5.2.2 –16S rRNA nucleotide sequencing**

PCR products were purified using the QIAquick PCR purification centrifugation protocol (QIAGEN), eluted in water and then sequenced directly (Chapter 2, section 2.9.6.4). The majority of the isolates provided approximately 1kb of nucleotide sequence directly from the PCR product. However, some of the PCR products failed to produce adequate sequence using this method; these sequences were cloned into the TOPO 2.1 vector (Chapter 2, section 2.9.6.3) using the TOPO TA cloning kit (Invitrogen), and sequenced using the M13 forward and reverse primers. This provided better sequencing results for the remaining isolates. Isolates 494, 213, 492, 5(ice3) and 466 proved difficult to clone and so these sequences have not be adequately sequenced, as time constraints did not allow further attempts.

The available sequence data for the 16S rDNA gene was entered into the Genbank database (http://www.ncbi.nlm.nih.gov) and assigned an accession number (Table 5.1).

The closest published relative for each isolate was determined by comparison with the Genbank nucleotide database using the FASTA (http://www.ebi.ac.uk) and BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ools) search algorithms which compare multiple nucleotide sequences for sequence similarity and homology (Altschul *et al.*, 1990).

Isolates, 124, 154, 214, 5ice3, 54, 744 and 794 were all 100% identical to *Marinomonas protea* (AJ238597), confirming that these are all one species. Similarly isolates, 86 and 39 were 99.4% and 99.6% identical respectively to an unspeciated *Pseudoalteromonas* sp. (U85856); therefore it is probable that these two isolates were the same species. Isolates 33 and 302 were identified as an Antarctic seawater bacterium (AJ293825) and *Pseudomonas fluorescens* (AF228367) respectively. However, isolate 33 was also 97.8% related to an unspeciated *Pseudomonas* sp. (AB021318) and therefore shows a close relationship with isolate 302. Isolate 302 has been further identified as *Pseudomonas fluorescens* by culturing the bacterium on *Pseudomonas* agar F (fluorescens agar) and CFC agar (Table 5.2; Bridson, 1998). Isolate 33 was identified as a pseudomonad but did not produce fluorescein pigment. *Pseudomonas* agar F stimulates the production of fluorescein and reduces that of pyocyanin and/or pyorubin and therefore delineates between pseudomonads which produce fluorescein and those which produce pyocyanin and/or pyorubin. CFC agar selects for pseudomonads whilst inhibiting most other bacteria.

Isolates 492 and 47 showed more than 99% similarity to *Stenotrophomonas maltophilia* (Ac. No. AJ293464). However, the sequence data for isolate 492 only comprised 408 bp and so were not sufficient to make a definitive identification (genus identification is supported by ARDRA, discussed in section 5.3.2.4). However, identification of isolate 47 was based on 1182 bp and was therefore sufficient for putative speciation. Isolate 732 showed 99.5% similarity to *Enterobacter agglomerans* (AF348161) based on 1493 bp, which is nearly a complete gene sequence, it is therefore unlikely that this identification would be inaccurate. Isolate 53 was identified as 99.3% related to *Idiomarina loihiensis*, which is a hydrothermal vent bacterium. Isolate 494 was 100% related to an unidentified *Sphingomonas* sp (AF395031). However, this

| Bacterial Isolate Number | Genbank accession number | Size of sequenced 16S rDNA fragment (bp) | Closest relative (EMBL prokaryote nucleotide database, using FASTA search engine) |
|---|---|---|---|
| 494 | *na* | 153 | *Sphingomonas* sp. Accession number AF395031 (100%) |
| 213 | *na* | 179 | *Halomonas sp.* Acc. No. AB016049 (97.2%) |
| 302 | AY092072 | 1370 | *Pseudomonas fluorescens* Acc. No. AF228367 (99.92%) |
| 33 | AY092073 | 1370 | Antarctic Seawater Bacterium Acc. No. AJ293825 (99.927%) |
| 53 | AY092077 | 1381 | *Idiomarina loihiensis* Acc. No. AF288370 (99.348%) |
| 86 | AY092080 | 962 | *Pseudoalteromonas* sp. Acc. No. U85856 (99.476%) |
| 39 | AY092074 | 1376 | *Pseudoalteromonas* sp. Acc. No. U85856 (99.636%) |
| 732 | AY092079 | 1493 | *Enterobacter agglomerans* Acc. No. AF348161 (99.512%) |
| 492 | AY092076 | 408 | *Stenotrophomonas maltophilia*. Acc. No. AJ293464 (99.020%) |
| 47 | AY092075 | 1182 | *Stenotrophomonas maltophilia* Acc. No. AJ293466 (99.833%) |
| 583 | AY092078 | 814 | *Psychrobacter* sp. Acc. No. AJ272303 (94.711%) |
| 124 | AY092065 | 886 | *Marinomonas protea*. Acc. No. AJ238597 (100%) |
| 154 | AY092066 | 1372 | *Marinomonas protea*. Acc. No. AJ238597 (100%) |
| 214 | AY092067 | 972 | *Marinomonas protea*, Acc. No. AJ238597 (100%) |
| 5ice3 | AY092068 | 277 | *Marinomonas protea*, Acc. No. AJ238597 (100%) |
| 54 | AY092069 | 965 | *Marinomonas protea*, Acc. No. AJ238597 (100%) |
| 744 | AY092070 | 1019 | *Marinomonas protea*. Acc. No. AJ238597 (100%) |
| 794 | AY092071 | 970 | *Marinomonas protea*. Acc. No. AJ238597 (100%) |
| 466 | *na* | 567 | *Bacillus aquamarinus* Acc. No. AF202056 (87.223%) |

**Table 5.1 – Original isolate number, Genbank accession number, the size of the sequenced 16S rDNA fragment used for identification and the closest relative identified by FASTA search of the EMBL prokaryote nucleotide database. na = not available, these sequences were not entered into the Genbank database due to their shortness of length. Isolate 466 shows only 87% similarity to *Bacillus aquamarinus*, thus although included here it is not considered to be an accurate identification.**

identification was made based on 153 bp of sequence and therefore the identification is considered to be of low confidence. Isolate 583 showed a 94.7% similarity to an unspeciated *Psychrobacter* sp. (AJ272303). Isolates 213 had a sequence similarity with an unspeciated *Halomonas* sp. (AB016049) of 97.2% from a sequence length of 179bp. Isolate 466 showed no close similarity to any sequence in the database for the 567bp entered. Therefore, isolate 466 will require further investigation to provide an accurate identification.

| Growth media | Isolate 302 | Isolate 33 |
|---|---|---|
| Fluorescens Agar | + | - |
| CFC Agar | + | + |

**Table 5.2 – Growth results for suspected pseudomonad species isolates on pseudomonad selective agar and fluorescein selective agar.**

### 5.2.3 – Phylogenetic tree

16S rDNA sequences for each of the 19 AFP active bacterial isolates from this study were aligned and analysed against the electropherograms from the sequencing results (see chapter 2). The consensus sequences for each of the 19 isolates were then compared against the Genbank nucleotide database and the nearest known phylogenetic relatives for each isolate were determined (Table 5.3). Only 13 of the isolates had sufficient sequence length for analysis, which was determined as being over 800 bp of contiguous sequence. Therefore, isolates 494 (153bp), 213 (179bp), 492 (408bp), 466 (567bp) and 5ice3 (277bp) could not be included in the alignment. Also removed was isolate 47 as although it comprised 1182bp of sequence, that sequence was not contiguous, it included a 300bp 'gap' in the middle and so was not of sufficient contiguous length for alignment. An 887 bp consensus sequence for each of the remaining 13 isolates were re-aligned with the sequences of the nearest known relatives using PileUp (GCG package), which aligns the sequences using progressive pairwise alignment. This was the shortest contiguous sequence length in the analysis. The same 887 bp region was used for all isolates to enable an accurate phylogeny to be calculated. A total of 18 published species were aligned (Table 5.3) these included species that were putatively related to the 6 isolates which could not be included in the alignment. Isolate 494 was identified as *Sphingomonas* sp, which is the only identification belonging to a

group other than the γ-Proteobacteria. Therefore, this species was used as the outlier group in the phylogeny to root the tree. The multiple sequence alignment from PileUp was transferred into ClustalX 1.64b (Higgins *et al.*, 1996), which was used to create a Phylip file for the production of the phylogenetic tree using the default weight parameters to produce a sequence distance matrix, which estimated the evolutionary distance of a particular pair of sequences based on differences in base pair substitutions. GeneDoc (Nicholas and Nicholas, 1997) was then used to examine and demonstrate that the alignment was correct by directly comparing the aligned sequences. Tree Puzzle version 4 (Strimmer and von Haeseler, 1996) was then used to analyse the phylogeny and compute the maximum likelihood tree, i.e. the tree that best fits the sequence data. The maximum likelihood algorithm corrects for the superposition of multiple mutations at a single sequence position, so that the observed number of nucleotide differences do not underestimate the number of mutational events that may have occurred since the evolutionary separation of the genes (Olsen *et al.*, 1986). The tree was created using the assumption that all the genes used in the alignment are mutating at a similar rate. The maximum likelihood tree was then viewed using TreeView 1.6.6 (Page, 2001) (Fig 5.4). It must be stressed that due to time limitations this may not be the most optimal tree, i.e. it may not be the best representation of the data. However, it is a good approximation of the phylogenetic relatedness of the taxa.

The phylogenetic tree (Fig 5.4) shows a similar pattern of relatedness to the ARDRA analysis (Fig 5.2a & b). Both isolates 302 and 33 cluster together in the *Pseudomonas* group. Isolate 732 clusters with the Enterobacteriaceae indicating its near identical similarity with *Enterobacter agglomerans* (syn. *Pantoea agglomerans*), also being closely related to *Escherichia coli*. Both isolates 86 and 39 cluster with *Pseudoalteromonas* sp. (Alteromonadaceae) as the most closely related group to the Enterobacteriaceae. The next group to join this clustering is the *Idiomarina loihiensis*/ isolate 53 group (Alteromonadeceae). The Alteromonadeceae / Enterobacteriaceae group then joins on to the rest of the phylogeny, which comprises the Moraxellaceae family (*Psychrobacter*, *Moraxella* and isolate 583), Oceanospirillium group family (*Marinomonas protea* taxon) and the Halomonadaceae family (*Halomonas* sp. and Arctic seawater bacterium). The Halomonadaceae family represent isolate 213 which could not

| **Isolate number** | **Closest phylogenetic relatives used in phylogenetic tree analysis.** |
|---|---|
| 124, 154, 214, 5ice3, 54, 744, 794 | *Marinomonas protea* (AJ238597) |
| 86, 39 | *Pseudoalteromonas* sp (U85856) |
| 494 | *Sphingomonas* sp (AF395031) |
| 213 | *Halomonas* sp (AB016049) |
| | Arctic seawater bacterium (AJ293828) |
| 302 | *Pseudomonas fluorescens* (AF228367) |
| | *Pseudomonas azotoformans* (D84009) |
| | *Pseudomonas putida* (AB016428) |
| 33 | *Pseudomonas* sp MBIC3 (AB021318) |
| 53 | *Idiomarina loihiensis* (AF288370) |
| 492, 47 | *Stenotrophomonas maltophilia* (AJ293464) |
| | *Xanthomonas* sp 1018 (AB054969) |
| 583 | *Psychrobacter* sp (AJ272303) |
| | *Moraxella lacunata* (AF05169) |
| 732 | *Enterobacter agglomerans* (AF348161) |
| | *Pantoea agglomerans* (AJ233423) |
| | *Citrobacter freundii* (AF025365) |
| | *Escherichia coli* (J01859) |

**Table 5.3 – Table shows the close relatives used for creation of a maximum likelihood phylogenetic tree. Also shows which isolates (from the current study) that the relatives relate to. Isolate 466 was not used in the phylogenetic tree as no close relative could be ascertained from the available sequence.**

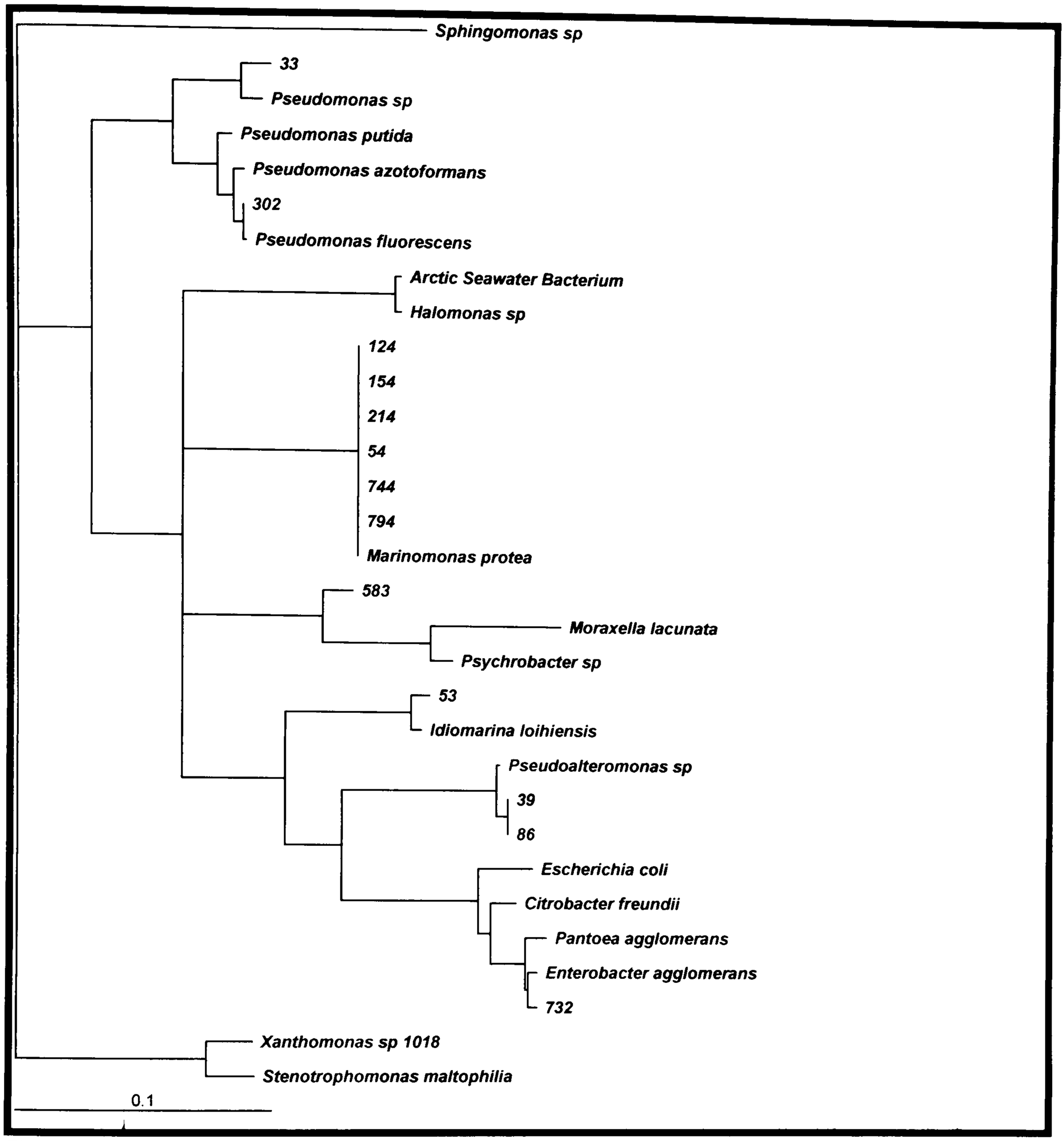


**Figure 5.4 – Maximum likelihood phylogenetic tree of relatedness for 13 AFP active isolates aligned against 18 published close relatives. Sequence comparisons comprise a common 887 bp region from the 16S rDNA of all strains in analysis. *Stenotrophomonas maltophilia* (AJ293464); *Xanthomonas* sp 1018 (AB054969); *Sphingomonas* sp. (AF395031); *Pseudomonas* sp MBIC3 (AB021318); *Pseudomonas putida* (AB016428); *Pseudomonas azotoformans* (D84009); *Pseudomonas fluorescens* (AF288367); Arctic Seawater Bacterium (*Halomonas* sp – AJ293828); *Halomonas* sp. (AB016049); *Marinomonas protea* (AJ238597); *Moraxella lacunata* (AF05169); *Psychrobacter* sp (AJ272303); *Idiomarina loihiensis* (AF288370); *Pseudoalteromonas* sp (U85856); *Escherichia coli* (J01859); *Citrobacter freundii* (AF025365); *Pantoea agglomerans* (syn. *Enterobacter agglomerans* – AJ233423); *Enterobacter agglomerans* (AF348161). All isolate numbers are referred to in the text. Scale shown at bottom of picture equates to a Jukes-Cantor coefficient of 0.1. Note isolates 5ice3, 494, 213, 492 & 47 were not included in this analysis due to insufficient size of their sequence data which would not of clustered accurately.**

be included due to its small sequence length. *Xanthomonas* sp 1018 and *Stenotrophomonas maltophilia* (Xanthomonas group family)are still members of the gamma sub-group of the Proteobacteria but are represented here as a closely related outlier to the main group. The Xanthomonas group family represent isolates 47 and 492 which could not be included due to their small sequence length. *Sphingomonas* (Sphingomonadaceae family) belongs to the alpha sub-group of the Proteobacteria and hence is shown here as relatively distant outlier (compared with the Xanthomonas group family) to the main group (Fig 5.4).

## 5.3 – Discussion

### 5.3.1 – ARDRA limitations

The phenetic similarity between the AFP active isolates was determined using multiple ARDRA analysis with two restriction enzymes to generate two distinct molecular 'fingerprints'. The ARDRA analysis proved successful at delineating 11 groups, 8 of which were single isolate groups. Subsequent 16S rDNA sequences proved that the proposed phenetic similarities (Fig 2a & b) represent the taxanomic structure with reasonable accuracy. This is especially shown by the delineation of the *Marinomonas protea* grouping (section 5.3.2.1) and the *Pseudoalteromonas* grouping (isolates 39 and 86, section 5.3.2.2), both of which are robust with both endonucleases. However, the phenetic relatedness of some other of the isolates showed variation between the two restriction digestions (*Hpa* II & *Alu* I). These fluctuations in the relatedness of species between the digests are based on small variations in the way the taxa clustered for the dendrogram, and some small differences in the restriction sites for the different endonucleases that induce variation in the relatedness of two isolates when using different enzymes. Variability in clustering patterns is noted by Jawad *et al.* (1998) who showed that variation in clustering did occur when five enzymes were used to analyse 32 strains of *Acinetobacter* spp. This is a basic limitation of the ARDRA protocol: it is recommended that additional phenotypic tests be used to reconcile these variations (Vaneechoutte *et al.*, 1995).

| Isolate | Closest relative (EMBL prokaryotic nucleotide database, using FASTA search engine) | Lake of isolation | Depth of isolation | Date of isolation |
|---|---|---|---|---|
| 124 | *Marinomonas protea* | Ace Lake | 8m | 13/11/00 |
| 154 | *Marinomonas protea* | Ace Lake | 4m | 13/11/00 |
| 214 | *Marinomonas protea* | Ace Lake | 2m | 13/11/00 |
| 5ice3 | *Marinomonas protea* | Pendant Ice Core | 50cm from ice/water interface | 07/09/00 |
| 54 | *Marinomonas protea* | Pendant Lake | 4m | 13/11/00 |
| 744 | *Marinomonas protea* | Pendant Ice Core | 50cm from ice/water interface | 13/11/00 |
| 794 | *Marinomonas protea* | Pendant Ice Core | Ice/Water interface | 13/11/00 |
| 39 | *Pseudoalteromonas* sp. | Ace Lake | 4m | 14/01/00 |
| 86 | *Pseudoalteromonas* sp. | Ace Lake | 4m | 14/01/00 |
| 302 | *Pseudomonas fluorescens* | Triple Lake | 0m | 23/01/00 |
| 33 | Antarctic seawater bacterium | Triple Lake | 2m | 17/07/00 |
| 53 | *Idiomarina loihiensis* | Triple Lake | 8m | 07/09/00 |
| 732 | *Enterobacter agglomerans* | Ace Lake | 2m | 04/03/00 |
| 47 | *Stenotrophomonas maltophilia* | Ace Lake | 6m | 14/01/00 |
| 492 | *Stenotrophomonas maltophilia* | Ace Lake | 0m | 15/07/00 |
| 583 | *Psychrobacter sp.* | Triple Lake | 0m | 07/09/00 |
| 494 | *Sphingomonas sp* | Pendant Lake | 0m | 13/11/00 |
| 213 | *Halomonas sp.* | Triple Lake | 4m | 07/09/00 |
| 466 | *Bacillus sp.* * | Oval Lake | 0m | 04/02/00 |

**Table 5.4 – Isolate number with closest relative from the EMBL prokaryotic nucleotide database using the FASTA search engine, lake of isolation and the depth in the water column or ice core of isolation. *This identification is extremely tenuous.**

### 5.3.2 – Identification of the AFP active isolates

*5.3.2.1 – The Marinomonas protea group, 124, 154, 214, 54, 744, 794 and 5ice3.*

Both the *M. protea* taxon (which comprises isolates 124,154,214,54,744,794 & 5ice3) and the *Pseudoalteromonas* sp. taxon (comprising isolates 86 and 39) have a robust phenetic relationship when compared using ARDRA, demonstrated by the fact that the grouping remains the same using the 2 different restriction enzymes. Based on subsequent 16S rRNA analysis it can be stated that these isolates are identical species. 124, 154, 214, 5ice3, 54, 744 and 794, they are all *Marinomonas protea* (Fig 5.4). This species was first isolated from Ace Lake (Vestfold Hills, Antarctica) in 1996 (Mills, 1999). However, these bacterial strains were not all isolated from Ace Lake (Table 5.4). Although three of these isolates, 124, 154 and 214 were isolated from various depths in Ace Lake on the 13th November 2000, the rest were isolated from Pendant Lake on the same date except 5ice3 which was isolated in September 2000. Three of these isolates came from the Pendant Lake ice cores (5ice3, 744 & 794). These lakes were sampled throughout the year 2000 and yet the majority of AFP active *M. protea* was isolated on the 13th November 2000. This suggests that *M. protea* is more abundant within the bacterial population around late winter/early summer and hence is more readily isolated (Dang & Lovell, 2002). However, it is also possible that experimental procedure could have caused such an anomaly, for example through unintentional preferential culturing of *M. protea*. Until the remainder of the bacterial isolates, which were isolated from the lakes of the Vestfold Hills for the current study, are characterised to species level it will be impossible to determine whether the dominance of *M. protea* during the November sampling period for AFP active bacteria is an experimental artefact or represents actual variation in bacterial dominance. Two bacterial isolates (AF277471 & AF277469) closely related (97%) to *M. protea*, have also been isolated from the sea ice microbial communities (SIMCO, Brown and Bowman, 2001), and the majority of *Marinomonas* species are found directly associated with the marine environment (Van Landschoot and De Ley, 1983) suggesting that the genus could have evolved from a marine based organism, which is supported by the fact that both Ace and Pendant Lakes are marine

derived. The phylogeny of the *M. protea* group can be seen in the phylogenetic tree (Fig 5.4). All the isolates (except 5ice3, see section 5.3.2) are shown to group as an identical taxon with *M. protea.*

*5.3.2.2 – The Pseudoalteromonas group, isolates 39 and 86.*

Isolates 86 and 39 show repeatable identical ARDRA patterns using both restriction enzymes and therefore appear to be the same species. Comparison of the 16S rDNA sequence with the EMBL prokaryotic nucleotide database identifies both isolates as *Pseudoalteromonas* sp. (U85856). Phylogenetic comparison of these two isolates against *Pseudoalteromonas* sp. showed that they were identical and shared a near identical relationship with *Pseudoalteromonas* sp (Fig 5.4). The statistical estimate of the likelihood of this branch being accurate was 99%. It is therefore suggested that these two isolates are an identical species. Isolate 86 and 39 were both isolated from the same lake, at the same depth, on the same date, which may explain their equal sequence similarity. The genus *Pseudoalteromonas* is mainly associated with marine ecosystems and is particularly prevalent in Antarctic coastal bacterial communities, most notably within the SIMCO (Bowman *et al.*, 1997; Bozal *et al.*, 1997; Bowman, 1998; Ivanova *et al.*, 1998; Nichols *et al.*, 1999; Brown & Bowman, 2001). As Ace Lake, from which 39 and 86 were isolated, is marine-derived (refer to chapter 1) the presence of isolates belonging to the genus *Pseudoalteromonas* is not unexpected. Species belonging to the genus *Pseudoalteromonas* are also generally psychrotrophic (Bozal *et al.*, 1997), therefore the presence of AFP activity within these isolates is also not remarkable.

*5.3.2.3 – The Pseudomonas group, isolates 302 and 33.*

Isolates 302 and 33 showed a varying level of relatedness between the ARDRA gels, this would appear to indicate that they were not identical species but were closely related. Characterisation of the isolates using 16S rDNA sequence data indicated that isolate 302 is a strain of *Pseudomonas fluorescens* this was further substantiated by growth of isolate 302 on pseudomonad selective agar and fluorescens agar. It has also been shown that isolate 33 is 97% related to a *Pseudomonas* sp., whilst being most closely related to an unidentified Antarctic marine bacterium (Mergaert *et al.*, 2001, AJ293825). It therefore can be considered to be related to the *Pseudomonas* genus. This

explains the fluctuation in relatedness between the two digest patterns using the ARDRA technique. In the phylogenetic tree (Fig 5.4) isolate 302 forms a virtually identical taxon with *Pseudomonas fluorescens*, and shows a close relationship with *P. azotoformans* and *P. putida*. This confirms that 302 is closely related to the pseudomonads. Isolate 33 groups in a phenon with its closest relative, *Pseudomonas* sp (AB021318), which in turn groups with the remaining *Pseudomonas* species. This confirms the relationship between isolate 33 and the pseudomonads. However, it also highlights the evolutionary distance between isolates 302 and 33, which pushed the identification of isolate 33 towards the unidentified Antarctic marine pseudomonad (AJ293825) (not included in the phylogram). Examples of the genus *Pseudomonas* are readily isolated from the Antarctic environment (Shivaji *et al.*, 1989; Meyer *et al.*, 1998; Voss *et al.*, 1999; Chessa *et al.*, 2000; Brown & Bowman, 2001; Mergaert *et al.*, 2001; Panicker *et al.*, 2002) and have been shown to be capable of growth at temperatures less than 5°C (Yabuuchi *et al.*, 1993) and hence are known to be psychrotrophic and psychrophilic. Cold adaptation is therefore well documented in the pseudomonads (Krajewska & Szer, 1967; Kozloff *et al.*, 1983; Deininger *et al.*, 1988; Turner *et al.*, 1991; Chessa *et al.*, 2000), especially *Pseudomonas fluorescens* which has been shown to demonstrate ice-nucleation activity and cold adapted gene expression (Deininger *et al.*, 1988; Obata *et al.*, 1998). It is therefore not remarkable that AFP activity should be present in this genus. Indeed, AFP activity has been shown to exist in *Pseudomonas putida* GR12-2, which was isolated from the rhizobium of plants growing in the Canadian high arctic (Sun *et al.*, 1995). The AFP protein from this isolate was shown to be similar to AFP II which is also found in plants and insects (section 1.4.3.4) it would therefore be prudent to characterise the AFP active protein from isolates 302 and 33 to see if it correlates with the protein characterised by Sun *et al.* (1995) from *P. putida*, because as shown by the phylogenetic tree isolate 302 and 33 have a close relationship with *Pseudomonas putida*.

*Pseudomonas fluorescens* has been previously isolated from soil samples around Antarctic Lakes of the Schirmacher oasis in Antarctica (Shivaji *et al.*, 1989). There are no known Antarctic *P. fluorescens* strains on any public access nucleotide databases and as such it was not possible to compare the sequence similarity of this strain against an Antarctic strain compared to strains isolated from more common environments such as

aquifers (AF336349) and river water (AF228367) to determine whether there is a specific Antarctic strain. It would be valuable to assess non-Antarctic isolated *P. fluorescens* strains for AFP activity to determine whether AFP activity is solely a characteristic of Antarctic strains of *P. fluorescens*. Suspected regions for the isolation of non-Antarctic AFP active strains would be the Arctic, as previously stated one AFP active species of *P. putida* was isolated from plant roots in the Canadian high Arctic (Sun *et al.*, 1995). However, to date no Arctic *P. fluorescens* strains have been submitted onto the public domain nucleotide databases.

*5.3.2.4 – The Stenotrophomonas maltophilia group, isolates 492 and 47*

Isolates 47 and 492 also showed fluctuation in relatedness with different restriction enzymes using the ARDRA technique (Fig 5.2a & b). Both isolates were identified as *Stenotrophomonas maltophilia* by 16S rDNA sequencing. However, the identification for isolate 492 was based solely on a 408bp fragment of the 16S rRNA gene, which was considered too small for absolute identification. Therefore based on the ARDRA analysis it is more likely that 492 is in fact a separate species of the *Stenotrophomonas* genus as it exhibits the same fluctuation of relatedness as 302 and 33 when digested with different enzymes (Fig 5.2 a & b). Both isolates were cultured from Ace Lake. This constitutes the first isolation of the *Stenotrophomonas* genus from Ace Lake water column. The only other recorded isolation of *S. maltophilia* from Antarctica is from the hypersaline lake sediments of Deep Lake (Bowman *et al.*, 2000a), also located in the Vestfold hills (during the current study water samples were taken from the surface of Deep Lake, but *S. maltophilia* was not isolated; Chapter 2). However, Bowman *et al.* (2000a) have suggested that the clone they identified as having close sequence identity with *S. maltophilia* is possibly a PCR contamination, because it was cloned from a 'very low yield' of DNA suggesting that it might be more vulnerable to contamination by common lab based genera, e.g. *Pseudomonas, Stenotrophomonas, Enterobacter* and *Delftia* (Tanner *et al.*, 1998; Bowman *et al.*, 2000a). However, they also state that their PCR negative controls were negative and therefore there is no proof of contamination (Bowman *et al.*, 2000a). The actual isolation of the strains as a pure culture of *S. maltophilia* in the current study meant that the susceptibility to PCR

contamination was lower as the PCR was prepared from high quality genomic DNA, and the negative controls on the 16S rRNA PCRs were also negative. Therefore, as this was the second isolation of *S. maltophilia* from a related environment in close proximity to the first site of isolation, it was possible to suggest that the isolate is present in that environment. The AFP activity of isolates 47 and 492 demonstrates a cold adapted strategy presumably in response to a cold environment. Therefore, it is unlikely that these two isolates were contaminants, but it would be necessary to determine if non-Antarctic *S. maltophilia* strains are AFP active before AFP activity can be used to confirm isolates 47 and 492 as Antarctic strains. Further identification of isolates 492 and 47 is required to confirm that PCR contamination did not occur. It would be prudent to perform standard biochemical characterisation of the original isolate to confirm it as *S. maltophilia*.

Phylogenetic analysis of isolates 47 and 492 was not possible as the 16S rRNA sequence length was not adequate for inclusion in the phylogenetic alignment. However, two examples of the Xanthomonas group family were included (Fig 5.4), *Stenotrophomonas maltophilia* (AJ293466) and *Xanthomonas* sp 1018 (AB054969). This group clusters outside of the main γ-Proteobacteria phylogeny, and constitute a partial outlier to the main grouping, This is also shown by the ARDRA analysis, where isolates 492 and 47 continually show a low level of relatedness with the rest of the patterns (Fig 5.2a & b). This suggests that this phylogeny is robust and that the identification of these isolates is accurate.

*S. maltophilia* is a pseudomonad like bacterium that is commonly found as a commensal organism of birds (as well as aquatic ecosystems), however, there are very few bird species in Antarctica; only three species were observed around the Ace Lake sampling site, Adelie penguins (*Pygoscelis adeliae.*), South Polar Skuas (*Catharacta maccormicki*) and the Snow Petral (*Pagodroma nivea*). Although it is not currently known whether *Stenotrophomonas maltophilia* could have been introduced into Ace Lake from avian fauna, it is still a possibility.

*5.3.2.5 – The Sphingomonas group, isolate 494.*

Isolate 494 was identified as *Sphingomonas sp.* (Ac No. AF395031) using 16S rRNA sequencing data. However this identification was based on 153 bp which as with

isolate 492, was considered too small a fragment for confident identification. Isolate 494 produced the most AFP active protein extract of the 19 isolates identified by SPLAT analysis as being AFP active (Chapter 4). Therefore it was considered important to identify at least the genus of the isolate. Isolate 494 showed an identical relationship with two other bacterial species using multiple restriction enzymes for ARDRA analysis (Fig 5.5). These two bacterial strains M3C203B-B (AF395031) and SIA181-1A1 (AF395032) (Christner *et al.*, 2001) were also sequenced and shown to be members of the genus *Sphingomonas.* This suggested that isolate 494 was probably also a member of the *Sphingomonas* genus. This genus is characterised as Gram negative, non-spore forming rods, with yellow pigmentation (Yabuuchi *et al.,* 1990) which correlates with the phenotypic characteristics of isolate 494. The genus *Sphingomonas*, which belongs to the α-Proteobacteria, has been readily identified in certain Antarctic environments, specifically oil contaminated soil (Panicker *et al.*, 2002) and the accretion ice above Lake Vostok (Christner *et al.*, 2001). Bowman *et al.* (2000b) have also shown that α-Proteobacteria are one of the most common groups of eubacteria isolated from the saline lake sediments of the Vestfold Hills, Antarctica, although they did not isolate any examples of the genus *Sphingomonas*. Strains of the genus *Sphingomonas* have been isolated from a wide variety of sources including soil, marine and fresh water, marine organisms, and from plants (http://www.jgi.doe.gov/JGI_microbial). Isolate 494, being the only AFP active isolate from the current study which did not belong to the γ-Proteobacteria, was isolated from Pendant Lake, which is brackish and marine derived and therefore the presence of this isolate in such a lacustrine environment is not remarkable. Isolate 494 showed 99.02% sequence similarity with a *Sphingomonas* sp (AF395031) which was isolated from samples of melt water from the accretion ice above Lake Vostok, this ice originated in the lake, indicating that the bacterial isolate may also have originated in the lake (Christner *et al.*, 2001), which further supports the isolation of 494 from a lacustrine system. In the phylogenetic tree analysis (Fig 5.4) the *Sphingomonas* species, which was entered to propose the positioning of isolate 494, was a distantly related outlier to the main group. This is as expected because *Sphingomonas* is a genus belonging to the α-Proteobacteria, whereas the rest of the sequences in the phylogenetic alignment belong to the γ-Proteobacteria as already mentioned. This was

seen to a certain extent in the ARDRA analysis (Fig. 5.2a & b) where isolate 494 forms an outlier to the main *Marinomonas* group but shows a distant relationship with all other groups in the assemblage.

*5.3.2.6. – The Psychrobacter group, isolate 583.*

Isolate 583 was identified as belonging to the genus *Psychrobacter* using 16S rRNA sequence analysis (Table 5.1). The closest relative was identified by comparison with the Genbank database as *Psychrobacter proteolyticus* (AJ272303), however this was identified at 94% similarity (Table 5.1). *P. proteolyticus* was isolated from Antarctic Krill, it is a γ-Proteobacterium belonging to the family Moraxellaceae (Denner *et al.*, 2001). Therefore, the closest relative to this isolate was found in association with the Antarctic marine environment and does exhibit the characteristics suitable for a cold-adapted AFP active bacterium from a saline lake; it is psychrotrophic and halotolerant. Isolate 583 was isolated from Triple Lake with a water temperature of -14°C and a salinity of 175ppt, therefore the bacterium would need to be psychrotrophic and halotolerant. However, it must be stated that this identification is based on the sequence similarity of only 814bp (Table 5.1), which limited the confidence of this identification. In the phylogenetic tree, isolate 583 was aligned against two close relatives from the family Moraxellaceae (*Psychrobacter sp.* (AJ272303) and *Moraxella lacunata* (AJ247228), Table 5.3) and clustered with the Moraxellaceae group. It was not closely related to either of the two relatives but was closer to the *Psychrobacter* sp. compared with *Moraxella lacunata* (Fig 5.4). This indicated that isolate 583 is probably an unidentified member of the *Psychrobacter* genus, but until the complete 16S rRNA gene has been sequenced this cannot be known. Duman & Olsen (1993) showed that *Micrococcus cryophilus*, which is synonymous with *Psychrobacter urativorans* (Cavanagh *et al.*, 1996), demonstrated thermal hysteresis activity. *M. cryophilus* was originally identified as a Gram positive large coccus (McLean *et al.*, 1951), however, the Gram stain was variable and was shown to be predominantly Gram negative and as such was re-grouped into the *Psychrobacter* genus, which is characterised by Gram negative, aerobic, oxidase positive coccobacilli (Juni & Heym, 1986; Cavanagh *et al.*, 1996). *Psychrobacter urativorans* was isolated from ornithogenic soils (derived from the

**Figure 5.5 – ARDRA analysis gel of *Hpa* II restriction digest of (1) isolate 494, (2) M3C203B-B (AF395031), (3) *Marinomonas protea*, (4) SIA181-1A1 (Ac. No. AF395032). M3C203B-B and SIA181-1A1 have both been putatively identified as *Sphingomonas* sp. based on sequence similarity assessments (Christner *et al.*, 2001).**

American Type Culture Collection (ATCC) (Duman & Olsen, 1993) and isolated from fresh pork sausages (McLean *et al.*, 1951) and therefore although the Antarctic strain of *P. urativorans* has not been identified as AFP active, there is a possibility it could demonstrate activity based on the apparent AFP activity of isolate 583.

The grouping of isolate 583 with the other taxon within the phylogenetic tree (Fig 5.4) also resembled the grouping shown between the associated isolates using the ARDRA analysis, especially the *Alu* I digestion (Fig 5.2b). This further substantiates the identification of isolate 583 as belonging to the genus *Psychrobacter*. Examples of the *Psychrobacter* genus have been isolated from such diverse environments as freshwater fish and vacuum-packed beef in cold storage (Sakala *et al.*, 2002; Gonzalez *et al.*, 2000) and deep sea trenches whose deep-ocean circulation currents link it to surface waters in Antarctica (Maruyama *et al.*, 2000). There have been several isolations of *Psychrobacter* from different Antarctic marine and terrestrial environments (Cavanagh, 1996; Duilio *et al.*, 2001; Camardella *et al.*, 2002) indicating that *Psychrobacter* is readily associated with the Antarctic marine environment, which links this genus to the marine derived lakes of the Vestfold Hills.

*5.3.2.7 – The Enterobacter agglomerans group, isolate 732.*

Isolate 732 shows a 99.5% similarity with *Enterobacter agglomerans* (AF348161), which is a member of the Enterobacteriaceae family. When aligned with *Escherichia coli* (J01859), *Pantoea agglomerans/Enterobacter agglomerans* (syn. AJ233423/AF348161) and *Citrobacter freundii* (AF025365), 732 clustered in a single taxon with *E. agglomerans* (AF348161) and *P. agglomerans* (AJ233423), and showed a close relationship with *E. coli* and *C. freundii* (Fig 5.4). In the cluster alignment the Enterobacteriaceae family phenon showed close relationship with the *Pseudoalteromonas* genus (Fig 5.4), the statistical estimate of the likelihood of branching for the *Pseudoalteromonas / Enterobacter* branch is 93% which suggests this is rather robust

phylogeny. This same relationship was demonstrated by the *Alu* I ARDRA analysis, where isolate 732 (Enterobacter) demonstrated a close relationship with isolates 39 and 86 (*Pseudoalteromonas*; Fig 5.2c). Bowman *et al.* (2000) identified the species *Enterobacter aerogenes* from a clone library of the hypersaline sediment community of Deep Lake (Vestfold Hills), but it was suggested that this clone might be a PCR contaminant (Bowman *et al.,* 2000; as with *Stenotrophomonas maltophilia*, section 5.3.2.4, isolates 492 & 47). However, the isolation of *E. agglomerans*, which has a 94% homology with *E. aerogenes,* from the nearby Ace Lake in the current study adds further precedent to the presence of this genus in the Antarctic environment. Further to this, the PCR preparations in the current study were made from high quality genomic DNA, and therefore were not subject to the same level of PCR contamination probability as with the Bowman *et al.* (2000) study where PCR preparations where made from 'very low yield' DNA (Bowman *et al.*, 2000). *Enterobacter agglomerans* or as it is now known *Pantoea agglomerans*, is more commonly found in associations with plant materials, animals and humans (Lindh *et al.*, 1991), however it has been shown to be ice-nucleation active and be cold adapted, showing a wide range of cold acclimation protein changes (Kozloff *et al.*, 1983; Deininger *et al.*, 1988; Koda *et al.*, 2000; Koda *et al., 2001*). It is also known to have been isolated from a saline pond (Francis *et al.*, 2000) and therefore has shown the ability to be halotolerant as well as psychrotrophic. Therefore, this identification is not remarkable, as *E. agglomerans* has shown the ability to adapt to such an environment as Ace Lake. However, there is also a possibility that this isolate could be a PCR contamination but there is nothing to support this.

*5.3.2.8 – The Idiomarina loihiensis group, isolate 53.*

Isolate 53 was identified as being 99.3% related to *Idiomarina loihiensis*, a bacterial isolate which was first isolated from the hydrothermal fluids of a submarine volcano in Hawaii (Donachie *et al.*, unpublished). Isolate 53 was isolated from Triple Lake on September 7$^{th}$ 2000 at 8m. The water temperature was –13.3°C and the salinity was 185ppt. Although the sequence relationship between isolate 53 and *I. loihiensis* suggests they are the same species it is almost certain that they are different strains as they exhibit an ability to survive in two very different extreme environments.

Phylogenetic cluster alignment of these two strains suggests they are very closely related but not identical (Fig 5.4). The alignment places isolate 53 and *I. Loihiensis* as an outgroup to the *Pseudoalteromonas* and Enterobacteriaceae family phena. This relationship was also demonstrated by the *Alu* I ARDRA analysis (Fig 5.2b), suggesting this is a stable relationship. The genus *Idiomarina* was first described by Ivanova *et al.* (2000) with two species, *I. abyssalis* and *I. zobellii*. Both were isolated from 4000-5000m in the north-western Pacific Ocean (Ivanova *et al.*, 2000). Phylogenetic characterisation of these two novel species placed them in a clade containing the genera *Alteromonas*, *Colwellia* and *Pseudoalteromonas* (Ivanova *et al.*, 2000; Ivanova & Mikhailov, 2001). This same clade formation is also shown in the current study with isolate 53 forming a close relationship with *Idiomarina loihiensis*, which groups in a clade with *Pseudoalteromonas* (Fig. 5.4). Therefore based on the sequence analysis and phenotypic characterisation (Gram negative rods, as demonstrated by Ivanova *et al.*, 2000) it could be suggested that isolate 53 is a species of *Idiomarina*. Further to this, to date all examples of the *Idiomarina* genus have been isolated from saline, marine environments, which correlates with the marine derivation of Triple Lake from which isolate 53 was cultured. It is possible that isolate 53 is a novel strain of *Idiomarina loihiensis* based on the high sequence similarity between them. The extreme differences in their respective environments suggests that possibility that they are separate species, as no single species can survive across the entire temperature range found in the biosphere (Fields, 2001). This is because certain adaptations which allow the species to survive at extreme high temperatures conflict with those required for extreme low temperatures, for example homeoviscous adaptation in membrane fluidity. At high temperatures adaptations to increase membrane viscosity include increasing the acyl-chain length, saturation, and the ratio of *iso* branching (Tolner *et al.*, 1997; Stetter, 1999). In contrast at extreme low temperatures adaptations to increase the fluidity of the membrane include decreasing the acyl chain length, decreasing saturation and increasing the ratio of *anteiso* branching (Russell & Hamamoto, 1998). This would all lead to suggest that it would be impossible for isolate 53 to be *I. loihiensis*, insinuating a misidentification. However, this is not supported by the sequence data. By comparison of the sequences of the 16S rRNA genes for the four *Idiomarina* species available on the Genbank database (*I. loihiensis*

(AF288370), *I. abyssalis* (AF052740), *I. zobellii* (AF052741), *I. baltica* (AJ440214)) it was possible to identify the regions of the 16S rRNA gene which showed the greatest level of differentiation between species,. These were identified as bp 424-472, bp 1114-1123 and bp 1242-1257 (*Escherichia coli* numbering). These regions of variation were all included within the 16S rDNA sequenced from isolate 53. Therefore, further characterisation of this species is necessary to determine its complete identification and discern whether this is a highly cold adapted strain of *Idiomarina loihiensis* or a different species of *Idiomarina*.

*5.3.2.9 – The Halomonas sp group, isolate 213.*

Isolate 213 could not be definitely identified due to the lack of 16S rRNA sequence; only 179bp were retrieved for this particular isolate. However, those 179 bp did identify the bacterial strain as belonging to the genus *Halomonas* (Table 5.1). The ARDRA analysis clustered isolate 213 into a group with isolates 53 (*Idiomarina loihiensis*), 583 (*Psychrobacter* sp.), 732 (*Enterobacter agglomerans*), 39 and 86 (*Pseudoalteromonas* sp.). The relationship between these isolates varied with the two different restriction enzymes. Although isolate 213 could not be utilized for direct assessment in the phylogenetic alignment (Fig 5.4) the species *Halomonas variablis* (AJ306893) was included and indeed it did show a relationship with the genera *Psychrobacter*, *Idiomarina*, *Pseudoalteromonas*, *Marinomonas* and *Enterobacter* (Fig 5.4). *Halomonas* showed a closer grouping with *Marinomonas* in the phylogenetic tree (Fig 5.4), however, the estimated statistical likelihood of the occurrence of this grouping was 53%. Therefore there is a possibility that the *Halomonas* sp and the Arctic seawater bacterium grouping could of clustered with the *Psychrobacter, Idiomarina, Pseudoalteromonas, Enterobacter* clade if a more robust branching pattern was calculated, however time constraints would not allow for this.

The genus *Halomonas* has been readily identified as ubiquitous in the marine habitat (Kaye & Baross, 2000; Maruyama *et al.*, 2000; Teske *et al.*, 2000; Sass *et al.*, 2001; Ivanova *et al.*, 2002), and has shown particular abundance in the deep-sea habitat and hydrothermal vents (Kaye & Baross, 2000; Teske *et al.*, 2000). The *Halomonas* genus has also been identified in lakes of the Vestfold Hills (Bowman *et al.*, 2000; James

*et al.*, 1994) and the Antarctic SIMCO (Brown & Bowman; 2001). However, it has never been isolated from Triple Lake, Vestfold Hills, which demonstrates such an extreme hypersaline and cold environment, and it may therefore be a novel species or strain. Bowman *et al.*. (2000) did demonstrate a 16S rRNA clone from the sediments of Deep Lake (the most hypersaline lake in the Vestfold Hills) which showed a close relationship with *Halomonas subglaciescola*. This indicates not only that *Halomonas* species can exist in extremely saline and cold environments, but also that *Halomonas* species have been isolated from the lakes of the Vestfold Hills, thus increasing the likelihood of the identification of isolate 213 as a member of the genus *Halomonas*. The identification of isolate 213 as *Halomonas* sp is therefore not unexpected, but complete sequencing of the 16S rRNA gene would be necessary for this identification to be definitive.

*5.3.2.10 – Isolate 466*

Isolate 466 is shown using ARDRA analysis to be a distant relative of the *M. protea* grouping this was demonstrated in both enzymatic digestions, but was more obvious in the *Alu* I digestion, where the phenetic similarity was ~75%. Due to this assessment it was considered that it is possible that this strain does belong to the gamma Proteobacteria, however, sequence assessment of the 153bp 16S rRNA gene fragment for isolate 466 demonstrated a very weak relationship (87.2%) with *Bacillus aquamarinus* (AF202056) which is part of the *Bacillus / Clostridium* group, which has been noted in Antarctic lakes (Brambilla *et al.*, 2001) and psychrophilic species have been noted in deep-sea sediments (Rüger *et al.,* 2000), and while it is a marine based species (Yoon *et al.*, 2001) the sequence similarity is to small to realistically consider it as a possibility. More conclusively isolate 466 was Gram negative whereas members of the genus *Bacillus* are Gram positive, and hence it is extremely unlikely that it could be related to this genus. It is thus possible that the sequence obtained is the result of PCR contamination. Hence no definitive identification of isolate 466 can be given at this point; further work was required to identify this isolate, but as it had a low level of AFP activity it was considered to be superfluous and so was not pursued further.

## 5.4 - Conclusions

The ARDRA patterns demonstrated putative relationships between the isolates but did not show a robust pattern; there was fluctuation between the relatedness of isolates and their position within the dendrogram. This is probably due to the way the DNA fingerprints produced from the different restriction sites for the different enzymes cluster in the analysis. DNA sequencing was used to identify the bacterial taxa. Phylogenetic assessment using the 16S rRNA gene sequences for the bacterial isolates showed a more similar pattern of relatedness between the isolates to the *Alu* I ARDRA pattern, suggesting that this pattern may be a more accurate representation of the relatedness of the isolates. The 19 bacterial isolates that demonstrated AFP activity from the 866 isolates cultured and screened for activity were found predominantly within the γ-Proteobacteria, with one isolate from the α-Proteobacteria. To date, all characterised AFP active bacteria have been shown to belong to the γ-Proteobacteria (Sun *et al.*, 1995; Duman & Olsen, 1993; Mills, 1999) except *Rhodococcus erythropolis* which belongs to the Actinobacteria (High G+C Gram-positive class of the phylum Firmicutes; Duman & Olsen, 1993) suggesting that either there is a phylogenetic relationship for AFP proteins within the γ-Proteobacteria or that there is a bias towards AFP activity assessment of γ-Proteobacteria through their psychrophilic marine and lacustrine environmental isolation. This shows a relatively close phylogenetic relatedness for AFP activity, suggesting that the gene for the antifreeze protein has evolved in only one limited eubacterial phylum. However, γ- and α-Proteobacteria are considered to be the dominant eubacterial groups within the Antarctic marine and aerobic lacustrine systems. Therefore, if environmental factors are selecting for these groups based on halotolerant and psychrotrophic function, then it is possible that AFP would evolve only in these groups. However, selective culturing techniques should not be ruled out. Other dominant groups within the Antarctic ecosystems e.g. the order Verrucomicrobiales, the class Actinobacteria, the *Clostridium/Bacillus* subphylum of the Gram positives and the *Cytophage-Flavobacterium-Bacteroides* phylum, are not represented within the characterised AFP strains. It is therefore possible that the culturing techniques used may have inadvertently selected for Proteobacteria. Further discussion of this will be presented in Chapter 7.

# Chapter 6: BACTERIAL COMMUNITY ANALYSIS

## 6.1 – Introduction

To assess whether antifreeze protein (AFP) activity provided a selective advantage to those bacterial isolates that were shown to have activity, and to identify the spatial and temporal fluctuations in the lacustrine bacterial communities, a rudimentary molecular study of the eubacterial community was done.

The application of molecular biological techniques to microbial ecology has revolutionized our view of microbial diversity. It is now generally accepted that only a small fraction of the actual bacterial diversity has been identified using traditional culturing techniques (Muyzer *et al.*, 1993; Øvreås *et al.*, 1997; Hugenholtz *et al.*, 1998; Muyzer & Smalla, 1998; Bosshard *et al.*, 2000). The apparent underestimation of bacterial diversity and abundance within a community has been called "the great plate count anomaly" whereby the use of viable plate counting and most-probable-number techniques has proved inadequate to assess bacterial communities because current culturing techniques fail to isolate the majority of environmental bacterial species (Amann *et al.*, 1995). Using molecular techniques the number of characterised bacterial divisions has tripled to about 40 due to the identification of novel taxa without the need for cultivation (Hugenholtz & Pace, 1996; Hugenholtz *et al.*, 1998). Studying the bacterial diversity of a community has therefore been dramatically advanced by the use of environmental clone libraries (Bano & Hollibaugh, 2002) and techniques such as fluorescent *in situ* hybridisation (FISH, Amann *et al.*, 1995; Ekong & Wolfe, 1998; Hugenholtz *et al.*, 1998; Bano & Hollibaugh, 2002), which enable the study of un-culturable bacterial species. Using techniques such as denaturing gradient gel electrophoresis (DGGE, Muyzer *et al.*, 1993) it is possible to rapidly analyse structural and species composition change within a community over time and along physicochemical gradients, such as those found within the lakes of the current study (Chapter 3).

The use of DGGE in studying complex microbial populations was developed by Muyzer *et al.* (1993) and has since been used to study microbial communities from many different environments by separation of the variable V3 region of the 16S rRNA gene

(Neefs *et al.*, 1990), amplified by PCR from community genomic preparations. The DNA is separated through the mobility of partially melted DNA during electrophoresis in a polyacrylamide gel, which will cease migration at specific locations along a denaturing gradient (Muyzer *et al.*, 1993; Muyzer & Smalla, 1998). DGGE can detect up to 95% of all possible single base substitutions in sequences up to 1000bp (Myers *et al.*, 1985). As stated this has been applied to a wide variety of microbial populations including meromictic lacustrine systems (Øvreås *et al.*, 1997; Bosshard *et al.*, 1999). hypersaline pools (Nübel *et al.*, 2000), Antarctic coastal waters (Murray *et al.*, 1998). hot springs (Ferris *et al.*, 1996), varied marine systems (Muyzer *et al.*, 1995; Schäfer *et al.*, 2001; Campbell & Cary, 2001), food products (Cocolin *et al.*, 2001; Coppola *et al.*, 2001; Ercolini *et al.* 2001a & 2001b) and agriculture (Ampe & Miambi. 2000; Ampe *et al.*, 2001; Duineveld *et al.*, 2001). DGGE analysis has also been used to study the diversity of specific species groups (e.g. ammonia-oxidizing bacteria, Nicolaisen & Ramsing, 2002). analyse sequence variation between type strains of the human immunodeficiency virus (Andersson *et al.*, 1993) and for the identification of specific genes of interest from natural ecosystems (Muyzer, 1999).

In the current study, community samples were taken at 2m intervals from three of the detailed study lakes (Ace Lake, Pendant Lake and Triple Lake, Chapter 3) and the surface water of Deep Lake and Club Lake, at two time intervals between the late austral winter and early austral summer. This was to determine the presence and nature of spatial and temporal fluctuation within the microbial population and to observe how the AFP active bacterial strains were represented in the community.

## 6.2 – Results

DGGE analysis was performed using the method outlined in Muyzer *et al.*, (1993; Section 2.9.5.4). Community and pure culture chromosomal DNA was extracted using the CTAB protocol (Ausubel *et al.*, 1999). Nested PCR of the V3 region for DGGE analysis was performed using a protocol outlined in Ercolini *et al.* (2001a). from a 1kb 16S rDNA fragment (PCR protocol outlined in section 2.9.5.3).

### 6.2.1 – Detection of AFP active isolates in DGGE community profiles

Fourteen AFP active isolates were compared against 7 community profiles taken from the lakes from which the cultivated strains were isolated. Two DGGE gels were run, firstly a gel which comprised the 10 isolates which demonstrated an AFP activity of 4-5 (SPLAT scoring, section 2.10.2) with 3 community profiles (Fig 6.1); secondly a gel comprising 9 isolates, which represented the taxonomic groups demonstrated by the amplified ribosomal DNA restriction analysis (ARDRA, refer to Chapter 5) with 4 community profiles (Fig 6.2). The seven community profiles were not taken from the depths and times from which the majority of the AFP active strains were isolated, because community samples were only isolated during September and November, 2000 and eight of the AFP active bacteria were isolated from dates other than these. Five of the remaining isolates were taken from depths at which due to the lack of community profile DNA, it was not possible to include the appropriate community profile in this analysis. Only one isolate, 213, was compared against a community profile from the lake, depth and time from which it was isolated. However, it was assumed that the other 13 isolates may have been represented in the communities throughout the depth of their respective lakes.

In DGGE gel 1 (Fig 6.3a), lanes 4, 5 and 8 each showed a single band match with bands in the community profiles. However, none of the lanes show complete matching which indicates that these isolates were either not present within the selected community profiles (Fig 6.3a, Lanes 1, 2 & 3) or were not represented by the DGGE community analysis due to biases inherent in the protocol (see section 6.3.2 for a discussion of bias within DGGE analysis). DGGE gel 2 (Fig 6.2/Fig 6.3b) indicated that 5 of the AFP active isolates were present within the community profiles. It was observed that the majority of the isolates showed more than one putative 16S rDNA copy (putative because they were not confirmed through 16S rDNA sequencing). Only isolates 54 (Fig 6.1, Lane 10), 794 (Fig 6.1, Lane 13) and 583 (Fig 6.2, Lane 9) showed a single copy of the 16S rRNA gene.

Isolate 302 (Fig. 6.3b, Lane 3) showed 2 bands that were also present in the community profile for Ace Lake, 4m from September (Fig. 6.3b, Lane 10), even though this isolate was isolated from Triple Lake, 0m during January (Table 5.2). Isolate 732 (Fig. 6.3b, Lane 6) was isolated from Ace Lake at 2m in March (Table 5.2), but matched

with two bands in the Triple Lake, 4m September community profile (Fig. 6.3b, Lane 13). Isolate 494 (Fig. 6.3b, Lane 7) showed three bands present within the community profile of Ace Lake, 4m September (Fig. 6.3b, Lane 10), but was isolated from Pedant Lake at 0m during November. Isolate 583 (Fig 6.3b, Lane 9) matched with a single band from the Pendant Lake, 0m September community profile (Fig 6.3b, Lane 11), however, it was isolated from Triple Lake at 0m during September (Table 5.2). Isolate 213 (Fig 6.3a, Lane 4) matched with two bands from the Triple Lake, 4m September profile (Fig 6.3b, Lane 13). As previously stated, this is the only isolate which was identified as being present within a community profile from the lake, depth and time from which it was actually isolated.

The pure culture AFP profiles which were present within the community profiles (Fig 6.3b) were not represented by high intensity bands, possibly indicating that the AFP active isolates were generally not the dominant bacteria within the DGGE community profiles, except isolate 213 which was represented by the most dominant bands in the Triple Lake, 4m September profile (Fig 6.3b, Lane 13).

In figure 6.3a, there is a single band present in multiple lanes (Lanes 4, 5, 6, 7, 8, 9, 10, 11, 13 & 14) which contain pure culture profiles. The presence of this band was considered to be artefactual as it was not present in any of the replicate profiles in figure 6.3b. It is possible that this band could represent a PCR contaminant that affected all these isolates during PCR (Section 6.3.2.4.4).

*6.2.1.1 – Assessment of 16S rRNA operon heterogeneity in M. protea*

Isolates 154, 54, 744, 794 and *Marinomonas protea* (Fig 6.1, Lanes, 9-11 & 13-14 respectively) all demonstrated 100% 16S rDNA sequence similarity to each other (Chapter 5), however, they show variation in the number of 16S rRNA genes. Isolates 154, 744 and *M. protea* all showed four putative copies of the gene whereas, as already stated, isolates 54 and 794 showed only a single copy.

A DGGE gel was run to compare isolates 154, 54, 744 and 794 against *Marinomonas protea* (Fig 6.3a – lanes 9-11, 13 & 14 respectively). The resultant bands for each isolate were excised from the DGGE gel, the DNA was extracted (by suspension of the excised band in water, at 4°C, overnight) and the V3 region was then re-amplified

using the same PCR protocol as originally used (Section 2.9.5.3). The top two bands from isolates 154, 744 and *M. protea* (Fig. 6.3a) both re-amplified all four of the bands from the original profile, except that the top two bands were less intense. The bottom two bands (Fig 6.3a) could both be isolated and re-amplified separately without inclusion of any other bands in the resultant profile. This indicated that the top two bands contained trace DNA from the bottom two bands, which was causing re-amplification of the bottom two bands. It is possible that the top two bands were single stranded artefacts because no single PCR product could be obtained when the bands are excised and re-amplified. However, it is unlikely that the same size single stranded DNA artefact would continue to appear in repeated experiments. Another hypothesis is that these bands relate to amplicons from a secondary binding site within the 1kb 16S rRNA gene fragment used for nested PCR. This unspecific product would therefore be repeatably amplified in each PCR. This could explain why the bands were not amplified to the same intensity as the bottom two bands, which would have been preferentially amplified as they contained the exact target sequence. However, without further analysis this cannot be proven, and it is possible that the top two bands while still containing DNA from the bottom two bands could be 16S rDNA, with a quite a different sequence (based on location in DGGE gel compared to bottom two bands), but it was not possible to isolate and identify them definitively.

The bottom two bands were both excised from the DGGE gel, extracted and purified. They were then sequenced using the chain-termination method (Sanger *et al.*, 1977). Both of the single bands from isolates 54 and 794 proved to be identical showing 100% similarity to each other over the 172 bp of the sequenced V3 region (230bp) of the 16S rRNA gene. They also showed 100% sequence similarity to *M. protea* when compared using a BLAST search on the Genbank ribonucleotide database (Altschul *et al.*, 1990). The lower band of the sequenced pair of bands from isolates 154, 744 and *M. protea* showed 100% sequence similarity to the single band from isolates 54 and 794. Therefore, this band which is in the same position in the DGGE gel is identical for all five isolates. The upper band of the pair of sequenced bands in isolates 154, 744 and *M.* protea, showed a 95% sequence similarity to the lower band and to the single band of isolate 54 and 794. It also showed only a 98% similarity to *M. protea*, using a Genbank

BLAST search. In fact it showed a 100% sequence similarity to *M. primoryensis*. However, it was not possible to make a definitive identification based on only 173 bp of the 16S rRNA gene (Ercolini *et al.*, 2001a).

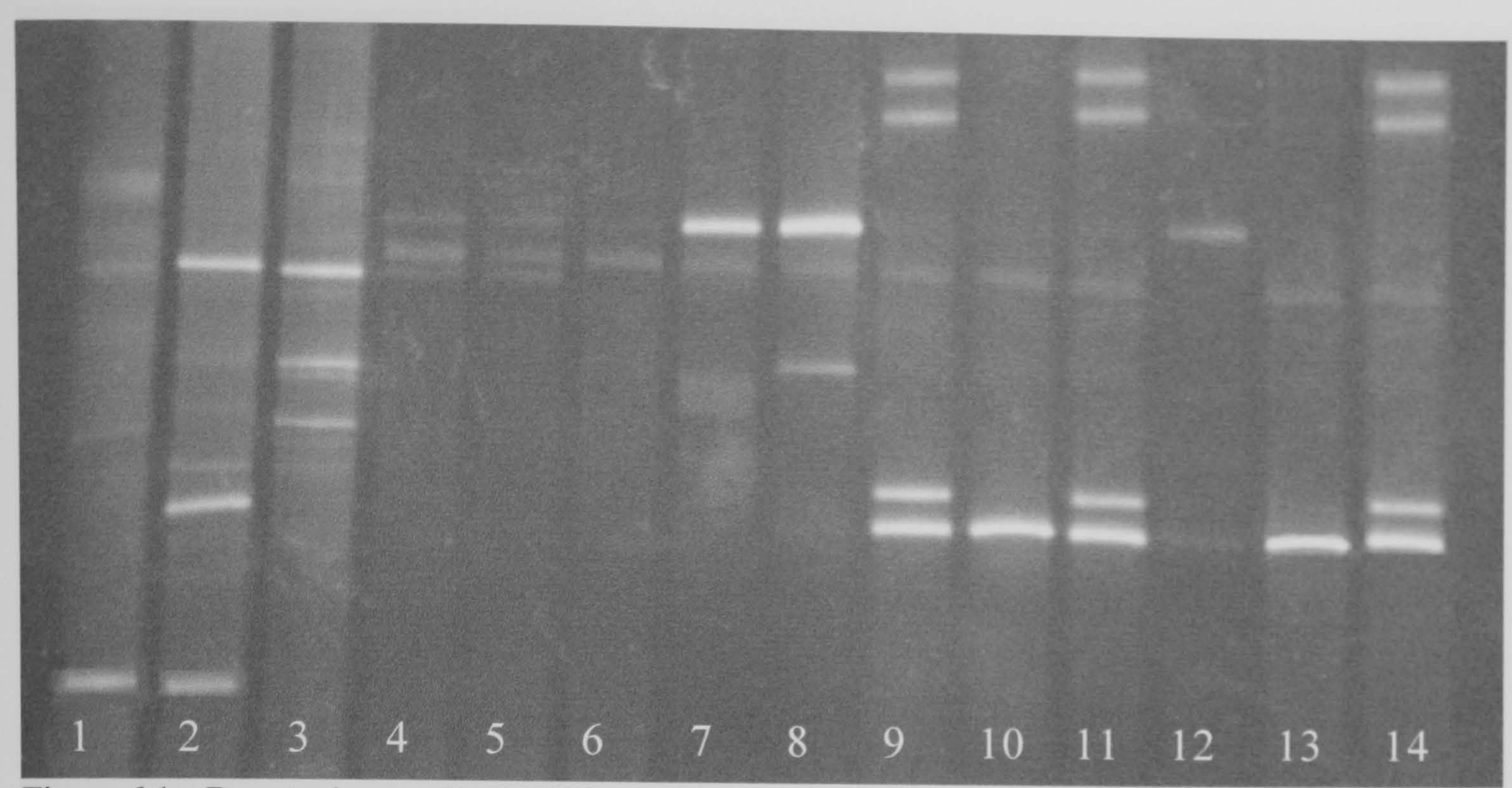


**Figure 6.1 – Denaturing gradient gel electrophoresis acrylamide gel showing 10 highly AFP active Antarctic lake bacterial isolates against 3 community profiles from the lakes from which they were isolated. Lanes 1-3 comprise community profiles: Lane 1 – Ace Lake 8m November; Lane 2 – Pendant Lake 10m November; Lane 3 – Triple Lake 8m November. Lanes 4-14 comprise 16S rDNA V3 region patterns for individual cultivated species: Lane 4 – isolate 213; Lane 5 – isolate 492; Lane 6 – isolate 53; Lane 7 – isolate 732; Lane 8 – isolate 302; Lane 9 – isolate 154; Lane 10 – isolate 54; Lane 11 – isolate 744; Lane 12 – isolate 494; Lane 13 – isolate 794; Lane 14 – *Marinomonas protea*. For details of gel composition and running conditions refer to section 2.9.5.4.**

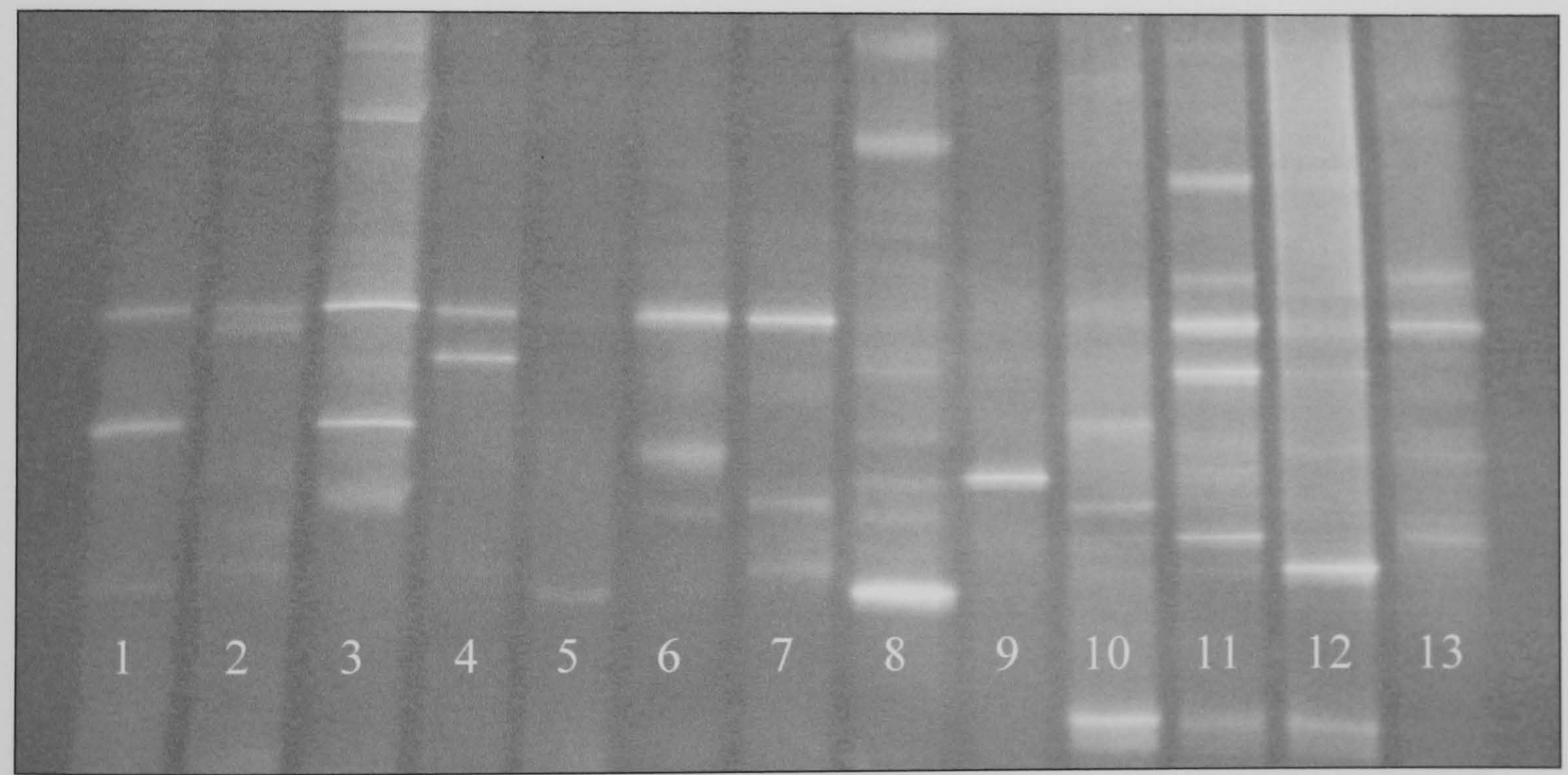


**Figure 6.2 – Denaturing gradient gel electrophoresis acrylamide gel showing 9 representatives of phena identified within the AFP active isolate grouping (except *Marinomonas protea* related species) against 4 community profiles from the lakes from which they were isolated. Lanes 1-9 comprise 16S rDNA V3 region patterns for individual cultivated species: Lane 1 – isolate 466; Lane 2 – isolate 47; Lane 3 – isolate 302; Lane 4 – isolate 492; Lane 5 – isolate 53; Lane 6 – isolate 732; Lane 7 – isolate 494; Lane 8 – isolate 86; Lane 9 – isolate 583. Lanes 10-13 comprise DGGE community profiles: Lane 10 – Ace Lake 4m September; Lane 11 – Pendant Lake 0m September; Lane 12 – Pedant Lake 10m September; Lane 13 – Triple Lake 4m September.**

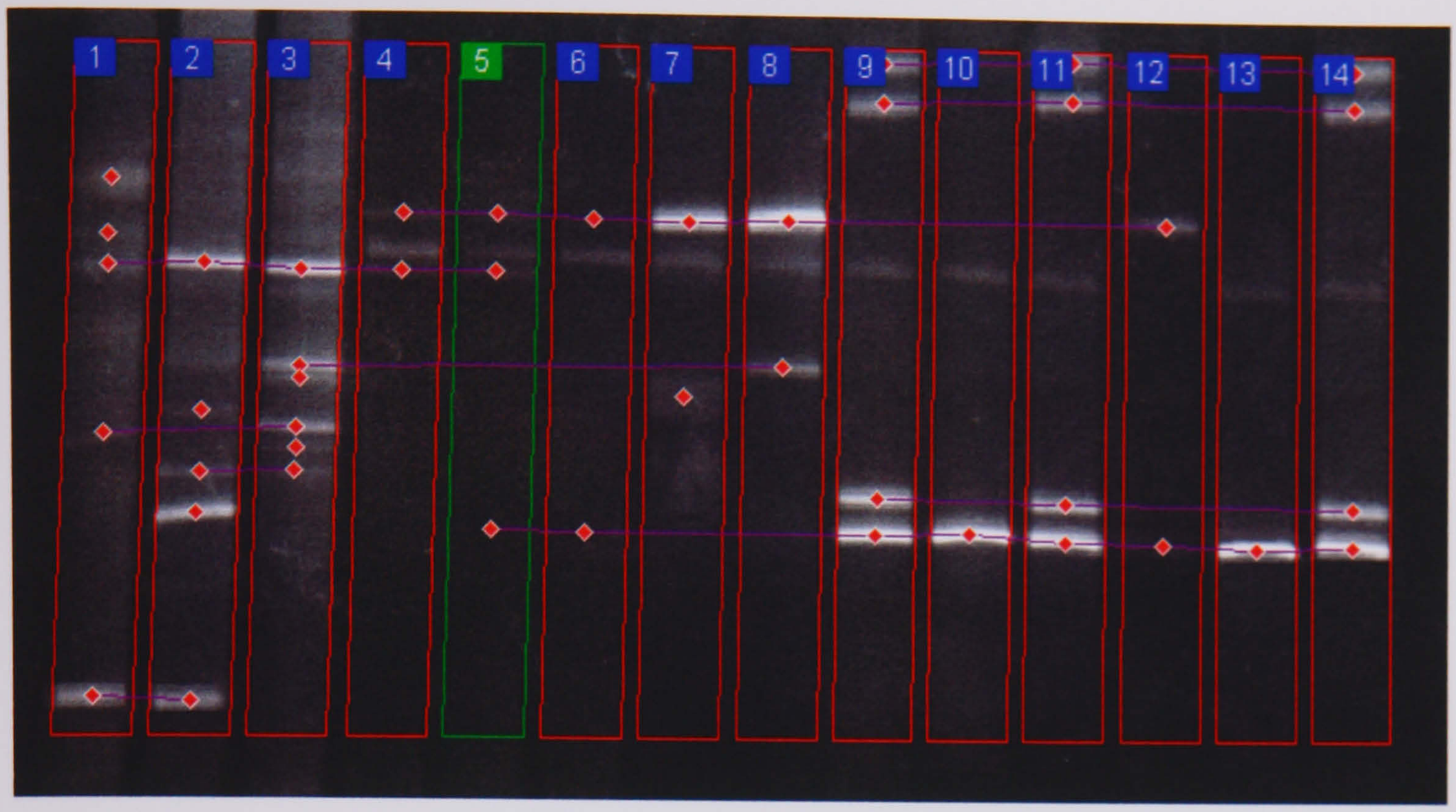


**Figure 6.3a – DGGE gel 1. Matching analysis by Rf values to assess for the presence of pure culture AFP active isolates within community fingerprints. Note single band in lanes 4-11 and 13-14 which was not included in the analysis as it was considered to be a contaminant. Lane 1 – Ace Lake 8m November; Lane 2 – Pendant Lake 10m November; Lane 3 – Triple Lake 8m November. Lanes 4-14 comprise 16S rDNA V3 region patterns for individual cultivated species: Lane 4 – isolate 213; Lane 5 – isolate 492; Lane 6 – isolate 53; Lane 7 – isolate 732; Lane 8 – isolate 302; Lane 9 – isolate 154; Lane 10 – isolate 54; Lane 11 – isolate 744; Lane 12 – isolate 494; Lane 13 – isolate 794; Lane 14 – *Marinomonas protea.***

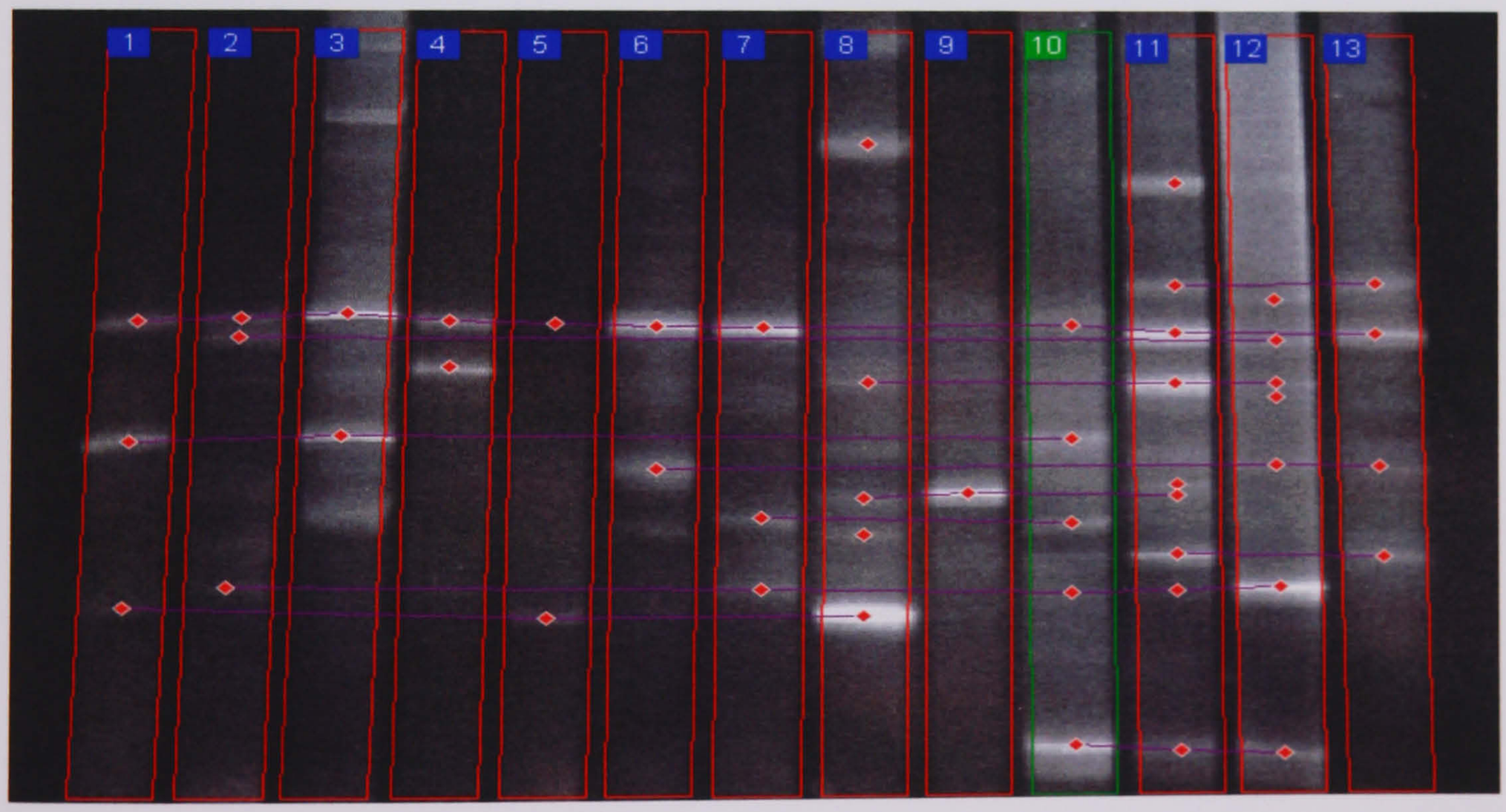


**Figure 6.3b – DGGE gel 2. Matching analysis by Rf value to assess for the presence of pure culture AFP active isolates within community fingerprints. Lane 1 – isolate 466; Lane 2 – isolate 47; Lane 3 – isolate 302; Lane 4 – isolate 492; Lane 5 – isolate 53; Lane 6 – isolate 732; Lane 7 – isolate 494; Lane 8 – isolate 86; Lane 9 – isolate 583. Lanes 10-13 comprise DGGE community profiles: Lane 10 – Ace Lake 4m September; Lane 11 – Pendant Lake 0m September; Lane 12 – Pedant Lake 10m September; Lane 13 – Triple Lake 4m September.**

### 6.2.2 – Temporal and spatial variation in microbial communities

Community samples were taken for each lake at 2m depth intervals on two different dates and then analysed by computer assessment of the DGGE profiles for the eubacterial communities of each sample (Fig 6.4a & b). Both images show complex microbial communities. However, there is some distinct change between the profiles in each lane. The original profile images were analysed using the Image Master 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics) to demark lanes within the profiles and then analyse the similarity between the profiles by matching the Rf values of each band (Fig 6.4c, Gel 1 and Fig 6.4d, Gel 2). Similarity matrices were based on the Dice coefficient which weights positives matches (refer to section 2.9.4).

Inter-lane comparison of the two gels was undertaken by standardisation of two replicates present in both gels, the V3 community fingerprint for Ace Lake 10m during September (Fig 6.4a Lane 8; Fig 6.4b Lane 4) and Pendant Lake 4m during September (Fig 6.4a, Lane 13; Fig 6.4b, Lane 5) were standardised using Rf (retardation factor) values so that the two gels could be compared. This inter-gel comparison was performed using ImageMaster 1D database V2.12 (Amersham Pharmacia Biotech). A similarity matrix was produced (Fig 6.5a) using the Dice coefficient and a dendrogram was derived (Fig. 6.5b).

For the following descriptions of temporal and spatial variation of similarity values between communities refer to figure 6.5a. Ace Lake showed a similarity between communities throughout its mixolimnion during September, 2000, ranging from 0.72 between 4m and 8m, to 0.9 between 0m and 2m. The only exception was 8m to 10m which was only 0.61 which correlated with the introduction of the bacterial community associated with the chemocline. The temporal variation between depths, however, showed a low similarity indicating a change in the community between September and November, 2000. During November, 2000 the similarity between communities at different depths throughout the mixolimnion in Ace Lake was very low compared with September, suggesting that the communities had undergone zonation due to an increase in the salinity gradient and temperature gradient caused by changing environmental conditions with the start of the austral summer.

**Figure 6.4a – DGGE community profiles for Ace Lake and Pendant Lake. Lanes 1-9 are Ace Lake profiles, Lane 1 = 0m, September, followed sequentially paired lanes of 2m, 4m, 8m and 10m. First of each pair relates to a September sample, second of each pair relates to a November sample. Lane 10-15 are Pendant Lake profiles, 10 & 11 are 0m, Sept/Nov; 12 is 2m, Sept; 13 & 14 are 4m, Sept/Nov, and Lane 15 is 8m, Sept.** ***Note loss of Ace, 0m, November and Pendant Lake, 2m, November. Lost in transit from Antarctica. Pendant Lake 10m, September and November and Pendant Lake, 8m, November are shown in figure 6.4b.***

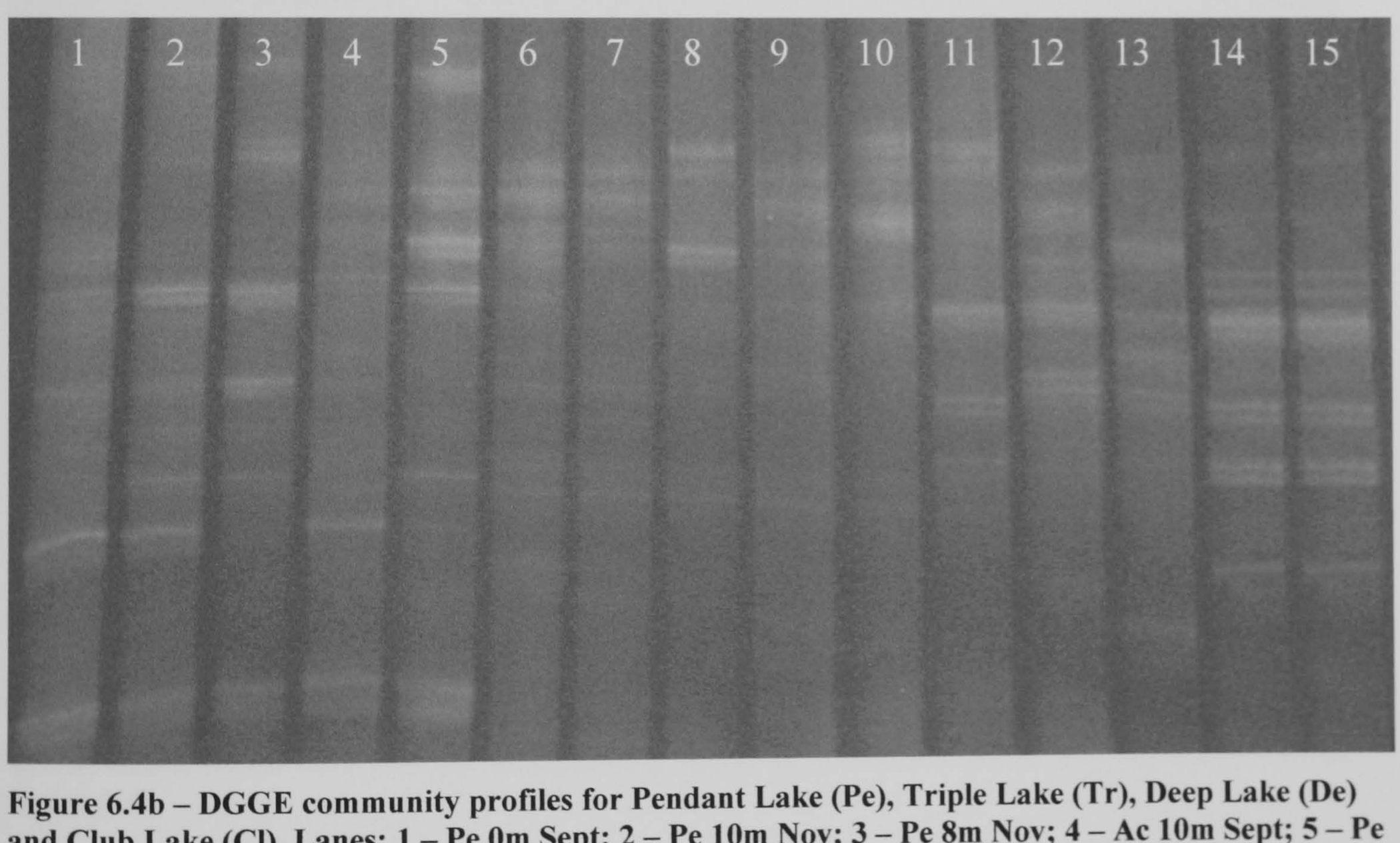


**Figure 6.4b – DGGE community profiles for Pendant Lake (Pe), Triple Lake (Tr), Deep Lake (De) and Club Lake (Cl). Lanes: 1 – Pe 0m Sept; 2 – Pe 10m Nov; 3 – Pe 8m Nov; 4 – Ac 10m Sept; 5 – Pe 4m Sept; 6 – Tr 0m Sept; 7 – Tr 2m Sept; 8 – Tr 4m Sept; 9 – Tr 4m Nov; 10 – Tr 8m Sept; 11 – Tr 8m Nov; 12 – De 0m Sept; 13 – De 0m Nov; 14 – Cl 0m Sept; 15 – Cl 0m Nov.** ***Triple Lake, 0m & 2m, November, had identical patterns as indicated by previous DGGE assessment (not shown) and as such only one of each were included.***

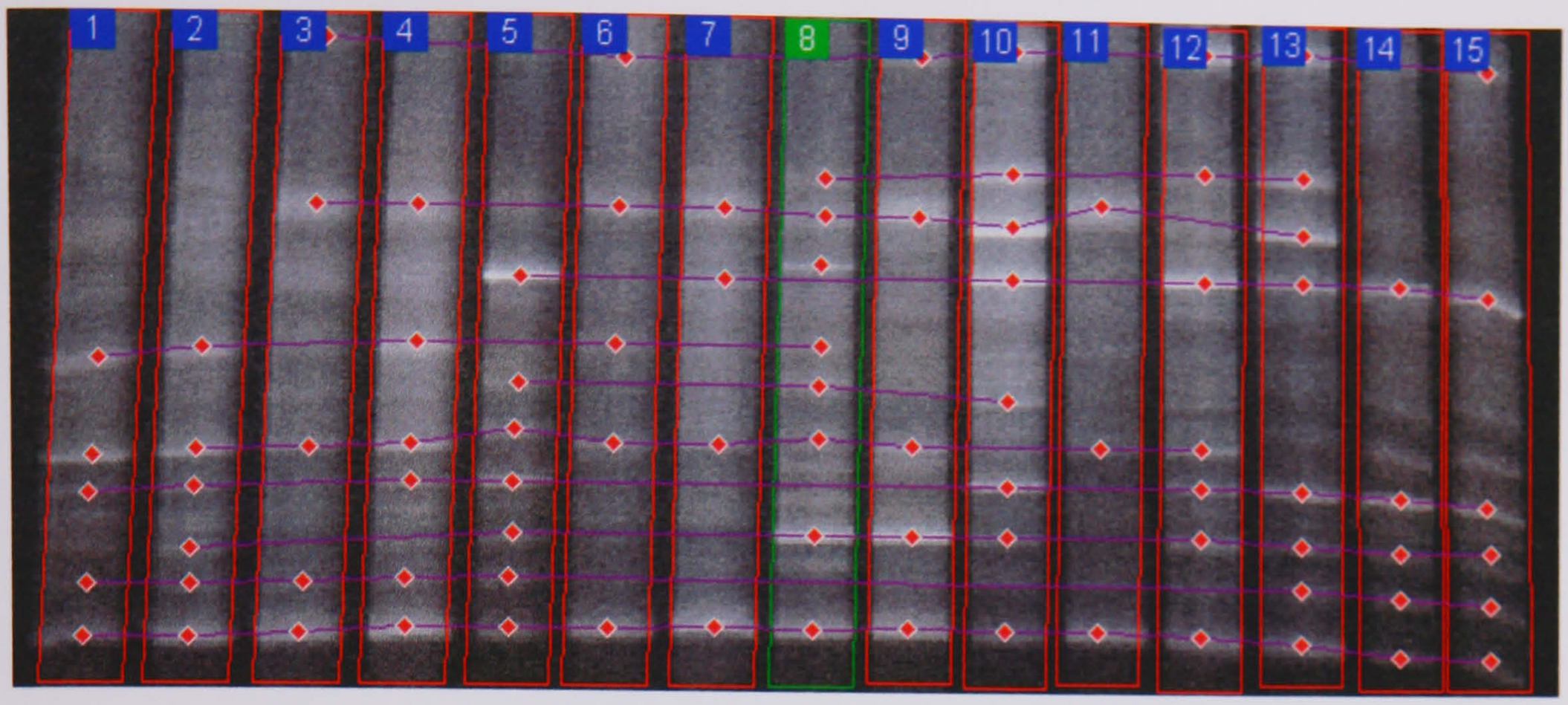


**Figure 6.4c – Gel 1 image showing band matching of Rf values using the Image Master 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics). Red dots denote bands which were selected automatically based on a threshold pixel intensity. Layout as of Figure 6.4a.**

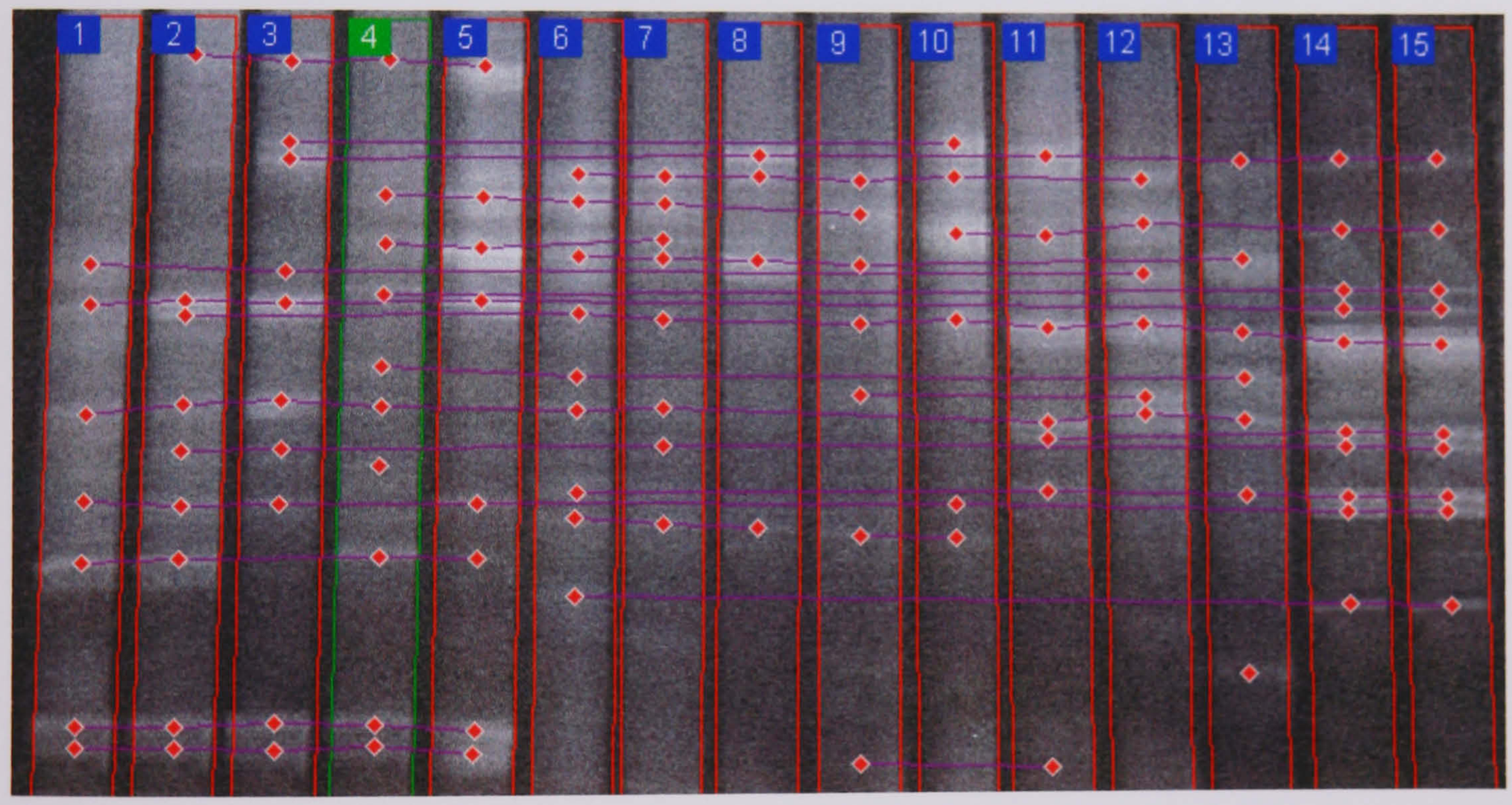


**Figure 6.4d – Gel 2 image showing band matching of Rf values using the Image Master 1D elite V3.01 gel analysis software (Amersham Pharmacia Biotech & Non-Linear Dynamics). Red dots denote bands which were selected automatically based on a threshold pixel intensity. Layout as of figure 6.4b.**

| C.P.s | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 / 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 Ac0m4 | 1 | 0.9 | 0.6 | 0.9 | 0.5 | 0.6 | 0.44 | 0.46 | 0.4 | 0.3 | 0.5 | 0.5 | 0.46 | 0.6 | 0.54 | 0.33 | 0.28 | 0.26 | 0.13 | 0.3 | 0.42 | 0.3 | 0.22 | 0.5 | 0.36 | 0.33 | 0.18 | 0.33 | 0.26 |
| 2 Ac2m4 | | 1 | 0.54 | 0.83 | 0.61 | 0.54 | 0.4 | 0.42 | 0.36 | 0.28 | 0.44 | 0.46 | 0.42 | 0.54 | 0.5 | 0.46 | 0.26 | 0.25 | 0.37 | 0.28 | 0.53 | 0.14 | 0.2 | 0.3 | 0.16 | 0.3 | 0.16 | 0.46 | 0.37 |
| 3 Ac2m5 | | | 1 | 0.72 | 0.5 | 0.8 | 0.66 | 0.46 | 0.8 | 0.3 | 0.75 | 0.5 | 0.61 | 0.4 | 0.54 | 0.16 | 0.28 | 0.26 | 0.53 | 0.46 | 0.28 | 0.15 | 0.22 | 0.16 | 0.18 | 0.5 | 0 | 0.5 | 0.26 |
| 4 Ac4m4 | | | | 1 | 0.61 | 0.72 | 0.6 | 0.57 | 0.54 | 0.28 | 0.66 | 0.46 | 0.57 | 0.54 | 0.5 | 0.3 | 0.26 | 0.25 | 0.5 | 0.42 | 0.53 | 0.28 | 0.4 | 0.3 | 0.33 | 0.46 | 0.16 | 0.61 | 0.37 |
| 5 Ac4m5 | | | | | 1 | 0.33 | 0.54 | 0.66 | 0.5 | 0.66 | 0.4 | 0.71 | 0.66 | 0.66 | 0.76 | 0.71 | 0.75 | 0.58 | 0.58 | 0.53 | 0.37 | 0.26 | 0.18 | 0.28 | 0.3 | 0.42 | 0.3 | 0.28 | 0.35 |
| 6 Ac8m4 | | | | | | 1 | 0.66 | 0.61 | 0.8 | 0.3 | 0.75 | 0.5 | 0.46 | 0.2 | 0.36 | 0.16 | 0.28 | 0.26 | 0.66 | 0.46 | 0.42 | 0.15 | 0.22 | 0.5 | 0.18 | 0.5 | 0.18 | 0.5 | 0.26 |
| 7 Ac8m5 | | | | | | | 1 | 0.66 | 0.66 | 0.33 | 0.85 | 0.54 | 0.5 | 0.22 | 0.4 | 0.36 | 0.3 | 0.28 | 0.57 | 0.5 | 0.46 | 0.33 | 0.25 | 0.54 | 0.4 | 0.72 | 0.2 | 0.54 | 0.42 |
| 8 Ac10m4 | | | | | | | | 1 | 0.61 | 0.75 | 0.36 | 0.66 | 0.62 | 0.46 | 0.42 | 0.53 | 0.58 | 0.44 | 0.88 | 0.62 | 0.58 | 0.37 | 0.16 | 0.66 | 0.14 | 0.53 | 0.42 | 0.4 | 0.22 |
| 9 Ac10m5 | | | | | | | | | 1 | 0.61 | 0.5 | 0.66 | 0.61 | 0.4 | 0.54 | 0.33 | 0.42 | 0.26 | 0.66 | 0.61 | 0.28 | 0.15 | 0.22 | 0.33 | 0 | 0.33 | 0 | 0.33 | 0.13 |
| 10 Pe0m3 | | | | | | | | | | 1 | 0.18 | 0.8 | 0.87 | 0.61 | 0.71 | 0.66 | 0.58 | 0.44 | 0.66 | 0.87 | 0.47 | 0.62 | 0.33 | 0.53 | 0.28 | 0.4 | 0.28 | 0.4 | 0.33 |
| 11 Pe0m4 | | | | | | | | | | | 1 | 0.4 | 0.18 | 0.25 | 0.22 | 0.2 | 0.33 | 0.15 | 0.3 | 0.18 | 0 | 0.18 | 0 | 0.2 | 0.22 | 0.6 | 0.22 | 0 | 0.15 |
| 12 Pe2m3 | | | | | | | | | | | | 1 | 0.8 | 0.66 | 0.76 | 0.57 | 0.75 | 0.58 | 0.7 | 0.8 | 0.37 | 0.4 | 0 | 0.42 | 0.3 | 0.28 | 0.15 | 0.28 | 0.23 |
| 13 Pe4m3 | | | | | | | | | | | | | 1 | 0.76 | 0.85 | 0.53 | 0.58 | 0.44 | 0.66 | 0.87 | 0.47 | 0.37 | 0.16 | 0.53 | 0.28 | 0.26 | 0.14 | 0.26 | 0.22 |
| 14 Pe4m4 | | | | | | | | | | | | | | 1 | 0.9 | 0.5 | 0.57 | 0.4 | 0.4 | 0.61 | 0.28 | 0.15 | 0 | 0.33 | 0.36 | 0.16 | 0.18 | 0.16 | 0.26 |
| 15 Pe8m3 | | | | | | | | | | | | | | | 1 | 0.46 | 0.66 | 0.5 | 0.5 | 0.71 | 0.26 | 0.28 | 0 | 0.3 | 0.33 | 0.3 | 0.16 | 0.3 | 0.25 |
| 16 Pe10m3 | | | | | | | | | | | | | | | | 1 | 0.75 | 0.7 | 0.58 | 0.66 | 0.25 | 0.26 | 0.18 | 0.28 | 0.3 | 0.42 | 0.3 | 0.28 | 0.47 |
| 17 Pe10m4 | | | | | | | | | | | | | | | | | 1 | 0.73 | 0.73 | 0.7 | 0.33 | 0.47 | 0.15 | 0.25 | 0.26 | 0.37 | 0.26 | 0.25 | 0.52 |
| 18 Pe8m4 | | | | | | | | | | | | | | | | | | 1 | 0.6 | 0.55 | 0.42 | 0.55 | 0.42 | 0.35 | 0.5 | 0.47 | 0.5 | 0.47 | 0.5 |
| 19 Ac10m4 r | | | | | | | | | | | | | | | | | | | 1 | 0.77 | 0.52 | 0.44 | 0.14 | 0.58 | 0.12 | 0.35 | 0.37 | 0.35 | 0.1 |
| 20 Pe4m3 r | | | | | | | | | | | | | | | | | | | | 1 | 0.47 | 0.37 | 0.16 | 0.53 | 0.28 | 0.4 | 0.28 | 0.26 | 0.22 |
| 21 Tr0m3 | | | | | | | | | | | | | | | | | | | | | 1 | 0.7 | 0.46 | 0.75 | 0.4 | 0.37 | 0.66 | 0.62 | 0.42 |
| 22 Tr2m3 | | | | | | | | | | | | | | | | | | | | | | 1 | 0.5 | 0.8 | 0.57 | 0.53 | 0.71 | 0.4 | 0.44 |
| 23 Tr4m3 | | | | | | | | | | | | | | | | | | | | | | | 1 | 0.54 | 0.6 | 0.18 | 0.4 | 0.36 | 0.28 |
| 24 Tr4m4 | | | | | | | | | | | | | | | | | | | | | | | | 1 | 0.61 | 0.28 | 0.76 | 0.42 | 0.23 |
| 25 Tr8m3 | | | | | | | | | | | | | | | | | | | | | | | | | 1 | 0.61 | 0.5 | 0.3 | 0.5 |
| 26 Tr8m4 | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | 0.46 | 0.57 | 0.58 |
| 27 D0m3 | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | 0.46 | 0.25 |
| 28 D0m4 | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | 0.47 |
| 29 Cl0m3 / 4 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |

**Figure 6.5a – Similarity matrix for the community fingerprint patterns of DGGE analysis gels from fig 6.4a and 6.4b. Values represented as a Dice coefficient value of similarity. Numbers along top row correspond to the community numbers down first column. Ac – Ace Lake, Pe – Pendant Lake, Tr – Triple Lake, D – Deep Lake, Cl – Club Lake. For Ace lake, if the end number is 4 the sample was taken in September, if it = 5, the sample was taken in November. For all other lakes, if the end number is 3, the sample was taken in September, if it is 4 the sample was taken in November. Similarity calculated from Dice coefficient. C.P.s = Community profiles.**

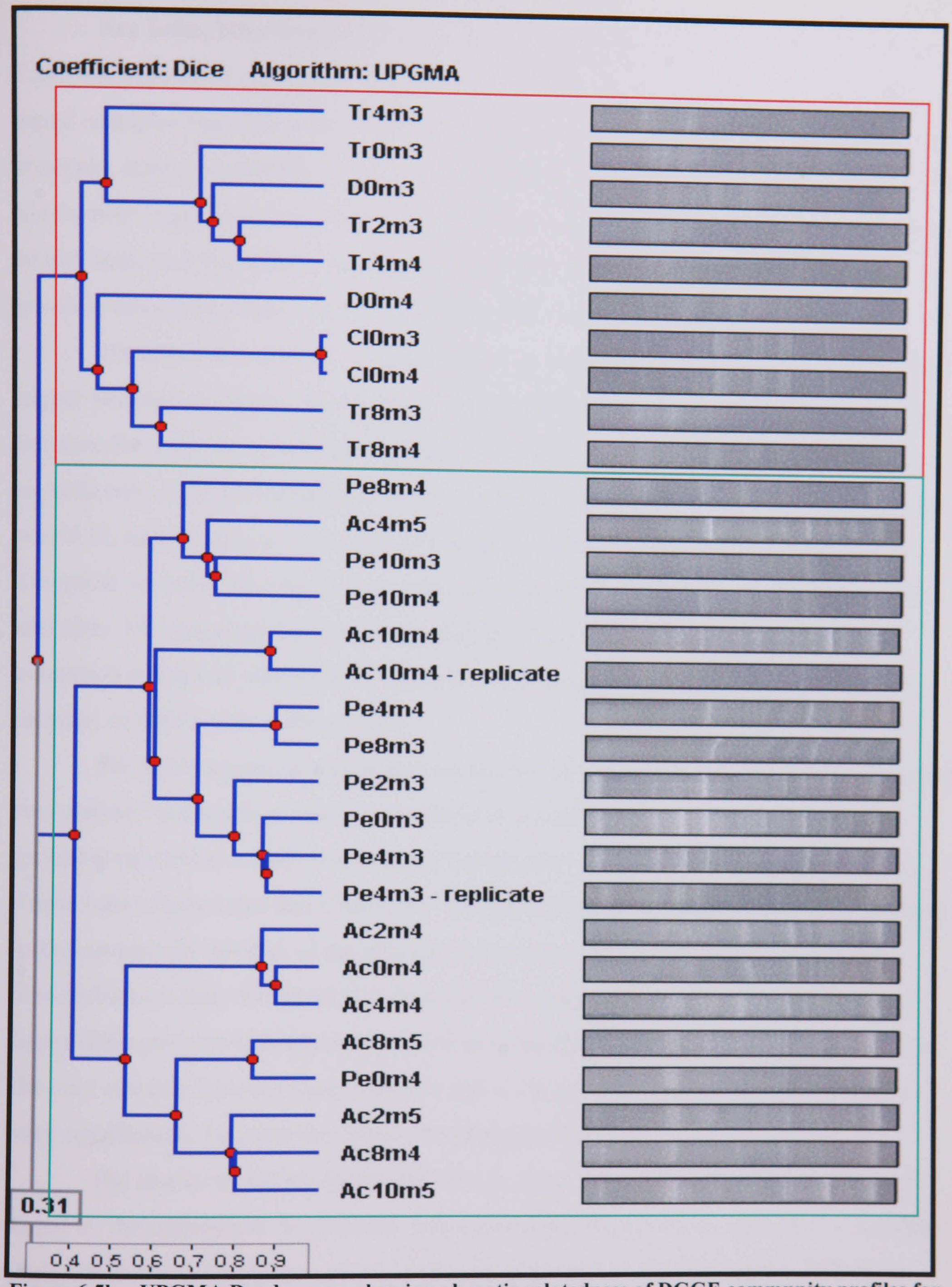


**Figure 6.5b – UPGMA Dendrogram showing phenetic relatedness of DGGE community profiles for Ac = Ace Lake, Pe = Pendant Lake, Tr = Triple Lake, D = Deep Lake and Cl = Club Lake (Vestfold Hills Antarctica), From September to November. For Ace Lake: Ac10m4 relates to Ace Lake, 10m depth sampled during September; Ac10m5 relates to Ace Lake, 10m during November. All the other lakes utilise the end code '3' for September sampling and '4' for November sampling. For descriptions of red box and green box refer to text.**

Ace Lake, 10m, September replicates showed 0.88 similarity, and the Pendant Lake 4m September replicates showed 0.87 similarity. Therefore any similarity value equal or higher than this would have been considered to be an identical community. For example, during September, Pendant Lake 0m, 2m and 4m showed between 0.8 and 0.87 similarities suggesting that these communities were extremely similar to identical, which in turn indicated that there was significant mixing in this part of the water column, possible caused by saline convection current induced by ice cover melt.

Pendant Lake showed a similar pattern to Ace Lake, in that spatial similarity was higher between the depths during September than November. The exception to this was between 8m and 10m which during September showed a similarity of 0.46 indicating a significantly different community. In November the similarity between these two depths was 0.73, indicating a possible converging of the different communities at this depth. Temporal variation was significant at 0m and 8m, but considerably less variable at 4m and 10m. This suggests that the community at 0m and 8m changed considerably compared to 4m and 10m possibly caused by changing salinity due to ice melt and an increase in light levels at the surface.

The dendrogram of relatedness for all the community profiles (Fig. 6.5b) indicates two distinct taxonomic groupings separated by a similarity level of 0.31. The first grouping (encircled in red) relates to the community profiles of the hypersaline lakes, Triple Lake, Deep Lake and Club Lake. The second grouping (encircled in green) relates to the community profiles of the brackish lakes, Ace Lake and Pendant Lake. This demonstrates a clear differentiation between the communities adapted to the extremely hypersaline environment and those which have moderate saline tolerance. Salinity is not the only variable between these two lake types, the general physicochemical properties vary significantly between the brackish and hypersaline lakes (refer to Chapter 3).

The similarity values for 0m and 2m in Triple Lake (Fig 6.5b) were calculated based on the community fingerprints being identical between September and November (data not shown). Therefore, only one of each was used for the community DGGE assessment because it was considered important to perform the DGGE analysis for all lake samples at the same time and there were only 15 viable wells on the DGGE gels. The spatial variation for each date showed a similar profile with depth. The similarity

between 0m and 2m was obviously the same during September and November at 0.7, which was probably caused by mixing due to saline convection cells caused by the melting of the ice cover. Between 2m and 4m the similarity varied from 0.5 in September to 0.8 in November, which suggested that the bacterial community in the top four meters was showing greater mixing during November, possible due to more ice-melt. The similarity between 4m and 8m was low in both September and November, indicating a substantially different bacterial population at the bottom of the lake compared with the water column, possibly caused by the interaction with the bacterial population in the sediment. There was high temporal variation for 4m and 8m suggesting that at these depths the population was undergoing significant change between September and November, possibly caused by increases in temperature and light levels.

The spatial fluctuation in the community composition of Deep Lake and Club Lake could not be recorded because there was no ice cover on these lakes throughout the year and therefore depth sampling could not be performed. However the temporal fluctuation at 0m in Deep Lake between September and November was 0.46 indicating that there was significant change in community between these two dates, possibly caused by influx of bacterial species via melt streams during November, 2000. This is substantiated by an increase in the number of bands detected in the Deep Lake community in November compared with September (Fig. 6.4d). There was no temporal fluctuation in the community in Club Lake.

## 6.3 – Discussion

### 6.3.1 – Detection of AFP active isolates in community profiles.

Only five out of fourteen AFP active isolates analysed using DGGE were shown to be present in the DGGE community profiles of the lakes of the Vestfold Hills (Figs 6.3a & b). Only one of the five (isolate 213) was represented by particularly intense bands within the profile, which is commonly associated with abundance of template. Template abundance is believed to be proportional to the abundance of a particular species within the original community (Muyzer *et al.*, 1993). Based on this assumption, that band intensity equates with abundance, the majority the AFP active strains were not abundant within the communities in which they were identified. Isolate 213 was the only

AFP active bacterium which was represented by bands of the greatest intensity within its community. This suggests that AFP activity within this bacterium did provide it with a selective advantage within its community. However, as for all the isolates detected within the community profiles, the link between band intensity and abundance relies on the absence of bias which, as described later (section 6.3.2), can significantly affect the authenticity of such an assumption (Ercolini *et al.*, 2001b).

As previously stated, the DGGE profiles against which the isolates (except isolate 213) were analysed were not the communities from which they were cultured. Thus it is possible that the 13 isolates were not present within those communities, as they were not represented in the community profiles. This would mean that the initial assumption that those 13 isolates may have been represented in the communities throughout the depth of their respective lakes is possibly false. The similarity scores for the DGGE community profile analysis (Fig 6.5a & b, section 6.2.2) show that there is considerable change within these communities over short periods of time. For example, Isolate 53 was isolated from Triple Lake at 8m during September. It was analysed against the community profile for Triple Lake at 8m from November (Fig 6.3a). The community profiles of Triple Lake at 8m between September and November had a similarity of 0.61%, indicating the community had changed considerably. This change in community is probably due to the changing environmental conditions during the period September – November; the water temperature in Triple Lake increased by nearly 7°C from -14°C to –7.5°C. The ice cover was halved due to melt and therefore the salinity decreased from 170 ppt to 160 ppt (Section 3.3.2.3). This probably had a substantial impact on the bacterial community composition. A similar situation was shown for isolates 154, 54, 494 and 583 (all isolated from different depths or times), which suggests why they were not present in the community profiles selected for analysis.

Isolates 732, 302, 494 and 583 were represented in the community profiles of lakes from which they were not isolated (section 6.2.1). It is possible that even though they were not isolated from those lakes they may have been present within the community and the culturing conditions used may not have been ideal to isolate them. This could have been because they may have been competitively excluded from the initial environmental culture by a species which could utilise the culture medium more

efficiently (Amann *et al.,* 1995). This is one reason for the culture bias seen in environmental sampling, it is also another reason why traditional culturing techniques should always be used in conjunction with molecular techniques (Hugenholtz *et al.*, 1998).

### 6.3.2 – DGGE bias

All PCR-based rRNA molecular techniques have substantial bias (Amann *et al.*, 1995). This is exemplified by the current DGGE study. As stated, 9 AFP active isolates were not present within the community profiles of the lakes from which they were isolated, which suggests that the DGGE analysis does not represent the full diversity of the community. As has already been suggested it is possible that, because the community profiles selected for analysis were not directly sampled from the communities from which the AFP active isolates were cultured, the AFP active isolates may in fact not be present within those communities. However, the ubiquitous presence of *Marinomonas protea* within Ace Lake during November (as indicated by its isolation in Ace Lake at 8m, 4m and 2m during November (Table 2 – Chapter 5)) suggests it is unusual that this species should not be identified in the Ace Lake community profiles from November.

DGGE as with all molecular rRNA analyses has bias at virtually every physical, chemical and biological step, all of which can cause artefacts and distortion of the observed community structure (van Wintzingerode *et al.*, 1997; Ercolini *et al.*, 2001b). This can be seen in the current study, where the cultivated record does not correlate with the molecular 'view' of the community. There are five major steps in all small subunit ribosomal molecular analysis and all involve some level of bias; the following sections outline the areas of bias in the context of the current study.

#### *6.3.2.1 – Species composition of community*

The selection of a molecular technique is largely dependant on knowing what it is that the technique will be selecting for, e.g. Archaea or Eubacteria. This is the first step where bias can occur. It is important to design a protocol which will select for the species present in that community. This can be determined by analysis of the environment, for example, observation of the different morphotypes present within the community by fluorescent microscopy and physical and chemical analysis of the environment, which

can help to predict the types of bacteria that will be found there (van Wintzingerode *et al.*, 1997). During the current study a detailed analysis of the environment from which the communities were isolated was performed (Chapter 3). The main assessment of these analyses is that the communities were dominated by Gram-negative rods which were psychrotrophic or psychrophilic and halotolerant. Characterisation of the AFP active isolates using ARDRA and molecular sequencing (Chapter 5) also provided information on what types of bacteria were present in the community. Therefore DGGE was performed using nucleic acid extraction techniques and PCR universal primers (Section 2.9.5.3) that would select for these bacterial communities, i.e. cold, saline, aquatic ecosystem communities (Øvreås *et al.*, 1997; Bosshard *et al.*, 1999; Schäfer *et al.*, 2001).

*6.3.2.2 – Sample isolation and maintenance*

The second step where bias can occur is during sample collection and maintenance of the samples until analysis (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998). Certain environments, especially extreme ecosystems contain bacterial communities that are specifically adapted to that environment, therefore, changing that environment, i.e. changing the salinity, pressure, temperature in which a community is maintained, can cause the enrichment of certain bacterial groups within the community, thus changing the abundance ratios of the groups within the community or more drastically changing the composition of the community so that it no longer reflects the original structure (Rochelle *et al.*, 1994). During the current study water samples were taken directly from the lake and stored in a thermally insulated 'cool box', to prevent them from freezing, for transportation back to the laboratory, where they were immediately processed and then frozen. It was necessary to prevent the samples from initially freezing so that the bacteria were kept viable. It is likely that the temperature of the water samples did change from the point of their isolation to when they were processed. However, because during transportation (<2 hours) the samples were maintained at refrigeration temperatures (<4°C) it is assumed that the bacterial communities would not undergo any significant change in abundance ratios or composition, but it is recognised that within this time frame, based on the cold adaptation of the bacteria within the communities, some compositional or abundance change could have occurred (Herbert *et al.*, 1986).

*6.3.2.3 – Cellular lysis and DNA purification*

The third step where bias can occur is in the act of lysing the cells and the subsequent extraction of DNA from the cells (Hugenholtz & Pace, 1996). It is recognized as important that all the cells within a community must be lysed if the true community structure is to be discerned. If total cellular lysis can be confirmed then it is just as important that the DNA thus extracted must be of high quality, i.e. free of contaminants which could inhibit or effect subsequent PCR analysis (Amann *et al.*, 1995). Many varied lysis techniques have been developed to deal with the different bacterial types (e.g. Gram negatives and positives) from different environments (e.g. inorganic particles and organic matter can affect lysis efficiency (van Wintzingerode *et al.*, 1997)). Aquatic systems are relatively devoid of inorganic and organic particles compared to soil ecosystems and so generally lysis is more efficient (Somerville *et al.*, 1989), this is especially true of Antarctic aquatic systems (Laybourn-Parry *et al.*, 2000). During the current study complete cellular lysis was determined by observation of cell suspensions under a microscope prior to DNA purification. Two main methods were considered for DNA isolation, the guanidium isothiocyanate (GES) method and the hexadecyltrimethylammonium bromide (CTAB) method (Ausubel, 1999; after Wilson, 1987). The CTAB method was chosen because it is applicable to most bacterial species, whereas the GES method can fail to eliminate DNAase produced by some bacterial strains (Owen & Hernandez, 1993). As the majority of the bacterial species were probably unidentified it was considered that CTAB would be a more appropriate method. The method was adapted from Ausubel *et al.* (1999) using SDS, in a high salt buffer in the presence of CTAB and Proteinase K and then purified by chloroform extraction and precipitation with isopropanol (Section 2.9.1). A similar protocol developed by Somerville *et al.* (1997) was used for the isolation of bacteria from Antarctic coastal waters (Murray *et al.*, 1998). CTAB is a quaternary ammonium compound, which purifies the DNA by removing the polysaccharide compounds. This was important in the current study due to the high polysaccharide content of psychrophilic bacteria (Mindock *et al.*, 2001). The DNA had to be of high quality (i.e. not fragmented) as fragmentation of DNA can cause artefacts in subsequent PCR, and not associated with contaminants.

which could have inhibited PCR by interfering with *Taq* polymerase and DNA hybridisation efficiencies (Steffan & Atlas, 1988; Porteous & Armstrong, 1991).

*6.3.2.4 – Polymerase chain reaction (PCR) amplification*

The fourth step and probably the most bias inducing step in any molecular protocol is the use of the polymerase chain reaction (PCR) to amplify small DNA fragments for analysis. The major elements which induce bias in PCR are dealt with the following sub-sections.

*6.3.2.4.1 – Amplification inhibition*

Firstly, PCR amplification can be inhibited by contaminants, but these should be removed by the DNA purification steps. However, in the current study PCR reactions were performed in a final volume of 50µl, which should of diluted any contaminating compounds to below their inhibitory concentration (Tsai & Olsen, 1991).

*6.3.2.4.2 – Preferential amplification*

Secondly, there is the possibility of preferential amplification within the community. Amplified DNA can only reflect the true diversity and abundance ratios of a community if amplification efficiencies are identical for all species (van Wintzingerode *et al.*, 1997). The possibility of preferential amplification during PCR was virtually impossible to ascertain for the current study due to the lack of information regarding the bacterial population under assessment. Van Wintzingerode *et al.* (1997) have suggested that the lack of information on genome size and *rrn* copy number (section 6.3.2.4.5) for uncultured bacteria means that currently it is impossible to determine the true abundance ratios for a microbial community. Suzuki and Giovannoni (1996) have suggested that amplified DNA, for example in a DGGE profile, can only reflect species abundance if the following are assumed: firstly that all DNA molecules are equally accessible to primer hybridisation; secondly that all primer-template hybrids form with equal efficiency; thirdly that the extension efficiency of the *Taq* polymerase is identical for all templates; and fourthly that limitations to amplification by substrate exhaustion are equal for all templates. No degenerices were used in the primers for amplification of the V3 region for DGGE (Section 2.9.5.3) in the current study and as such formation of primer-template

hybrids should be equal. However, Suzuki and Giovannoni (1996) have shown that bias can occur due to successive reannealing of a single template, which would progressively inhibit the formation of other primer-template hybrids. Van Wintzingerode *et al.* (1997) have suggested that this should not occur if the community genomic DNA template contains sufficient diversity. Hansen *et al.* (1998) suggested that DNA bias during PCR originated in the DNA which flanked the PCR amplicons, i.e. some species may have DNA inserts prior to the 16S rDNA which inhibits the amplification of the target DNA. For the current project it is unknown if this bias has affected the results. It is possible that preferential amplification of the gene products of specific species within the community acted to inhibit the amplification of other species, which could explain the absence AFP active cultured isolates from the community profiles. Bosshard *et al.* (1999) have suggested that only numerically dominant groups will be amplified in community PCR. Ercolini *et al.* (2001b) have suggested that DGGE using the variable V3 region is prone to selective amplification, they suggested using culture dependant analysis to support culture-independent analysis due to the bias present in both analyses. This has been previously substantiated by other research (Liesack *et al.*, 1997). During the current study nested PCR was used, which incorporates two PCR steps (Section 2.9.5.3). This was performed as V3 PCR amplification from genomic DNA was not providing repeatable results, possibly due to contamination in the genomic DNA preparation. The inclusion of multiple PCR steps in the current study would have increased the likelihood of bias. The probability of differential amplification could also suggest why, for example, isolate 302 was represented in the community profile for Ace Lake, 4m from September despite not being cultured from this community. It may not have been cultivatable from the environmental conditions in Ace Lake, but was preferentially amplified by the multiple DGGE-PCRs.

*6.3.2.4.3 – Artefactual PCR products*

Thirdly the production of artefactual PCR products such as chimeric molecules and erroneous base pair insertions during the extension phase can affect a DGGE profile by altering the composition of the target sequence. During the current study a 'proof reading' enzyme was associated with the *Taq* polymerase which should have

significantly reduced the likelihood of erroneous insertions during the extension phase of PCR. However, there were still bands within the profiles which could not be explained. These bands were usually very faint and so were not designated for analysis by the automated band selection software. The majority of artefactual bands on a DGGE gel especially high up in the gel (closer to the wells) are often single stranded DNA due to incomplete extension during PCR amplification (Muyzer & Smalla, 1998). The final extension time for the DGGE-PCR in the current study was 10 minutes which was considered long enough to ensure complete extension of all PCR products (Ercolini *et al.*, 2001a). Incomplete extension of the DNA strand during PCR can be caused by the GC-clamp, which leads to multiple bands from a single product (Nübel *et al.*, 1996). The confirmation of the absence of incomplete strand synthesis by attempted re-amplification of bands within the DGGE profiles (incomplete strands would not re-amplify) was not determined in the current study due to time constraints which prevented assessment of each band within the community profiles.

*6.3.2.4.4. - PCR contamination*

Fourthly, the possibility of contaminating DNA being amplified within the PCR which would cause a complete misrepresentation of the community diversity. The sensitivity of PCR required strict aseptic technique to prevent the amplification of DNA from bacterial contaminants (van Wintzingerode *et al.*, 1997). However, bacterial contaminants in the current study cannot be ruled out as time constraints prevented full analysis of the DNA bands present within the DGGE profiles. Characterisation of PCR contaminants in DGGE profiling can be achieved by sequence identification of the bands within a profile. Identification of a common PCR contaminant (e.g. *Bacillus* sp.) would mean re-amplification of the DGGE profiles. To prove a putative contaminant conclusively would require the use of techniques such as FISH, which utilise labelled species specific DNA probes applied *in situ* to confirm or deny the presence of an isolate in a community (Dang & Lovell, 2002). The diversity of the DGGE community profiles obtained during the current study and the distinct division of community profiles between brackish and hypersaline lakes (section 6.2.2) infers that the community profiles do not

contain contaminants. If the profiles were contaminated they may show a closer similarity to each other.

*6.3.2.4.5. – 16S rRNA operon heterogeneity*

Lastly, sequence heterogeneity within multiple 16S rRNA operons can lead to a biased reflection of microbial diversity (van Wintzingerode *et al.*, 1997). As demonstrated by figures 6.1 and 6.2, most of the AFP active species studied as pure culture DGGE profiles demonstrate more than one copy of the rRNA gene. Multiple *rrn* copies have been identified in many bacterial species (Condon *et al.*, 1995; Farrelly *et al.*, 1995; Nübel *et al.*, 1996; Afseth & Mallavia, 1997; Lin & Tseng, 1997; Fegatella *et al.*, 1998; Ueda *et al.*, 1999; Klappenbach *et al.*, 2000; Klappenbach *et al.*, 2001; Moreno *et al.*, 2002). Farrelly *et al.* (1995) showed that genomic properties such as genome size and *rrn* gene copy number can have an effect on PCR amplification efficiencies. Nicolaisen and Ramsing (2002) have suggested that it is important to evaluate any gene used in DGGE analysis for the presence of multiple copies of that gene before it can be used effectively in community fingerprinting. The number of rRNA operons varies significantly between taxa, for example the pathogenic bacteria *Rickettsia prowazkeii* only has one copy, whereas the *Escherichia coli* has seven copies and *Clostridium paradoxum* possess 15 copies (Klappenbach *et al.*, 2001). It is generally assumed that multiple copies of rRNA operons in prokaryotes are indicative of high growth rates (Farrelly *et al.*, 1995), however, inactivation of operons in multiple copy number species shows marginal impact on growth rates, and species with a single copy still have short doubling rates (Condon *et al.*, 1995; Klappenbach *et al.*, 2001). It has been suggested that the number of rRNA operons may indicate the ability of a bacterium to adapt to favourable changes in growth conditions by the rapid synthesis of ribosomes (Condon *et al.*, 1995). Klappenbach *et al.* (2000) demonstrated this, showing direct correlation between the rates at which a variety of phylogenetically diverse bacterial species respond to changes in resource availability and the number of rRNA genes in the genome, suggesting that the number of rRNA operons in a bacterial genome represents an adaptive strategy to changing resource availability. It was therefore considered that Antarctic bacterial species should contain multiple copies of the 16S rRNA gene as they have to

adapt to the extreme environmental conditions prevalent in Antarctica, which are accompanied by, for example, rapid changes in temperature and light availability throughout a year (Russell & Hamamoto, 1998). This was substantiated by the current study in which all but three of the assessed AFP active isolates showed multiple copies of the 16S rRNA gene.

One genus isolated in the current study, *Sphingomonas* (isolate 494), an ultramicrobacterium (α-Proteobacteria) usually found in oligotrophic marine waters (Fegatella *et al.*, 1998), has been twice isolated in Antarctica, once in the accreted ice above the sub-glacial Lake Vostok (Christner *et al.*, 2001) and then in the current study from the sub-ice, surface water of Pendant Lake. This isolate was shown to have 3 copies of 16S rRNA operon (Fig 6.1 & 6.2, lanes 7 and 12 respectively). The *Sphingomonas* sp. strain RB2256, which is undoubtedly a different species from that of the current study, was isolated from cold marine waters in Resurrection Bay, Alaska, and has been shown to have only 1 copy of the *rrn* operon (Fegatella *et al.*, 1998). Despite this it has also been shown to maintain a higher concentration of ribosomes per cell volume than *Escherichia coli*, and maintained the ability to show rapid response to nutrient fluctuation within its substrate (Fegatella *et al.*, 1998). Fegatella *et al.* (1998) has suggested that this may be a characteristic of the oligotrophic ultramicrobacteria, but the current study suggests that this may be inaccurate, unless the multiple-banding pattern shown in figures 6.1 and 6.2 was not accurate. It is possible that two of the bands in the tri-banded profile may be artefactual (refer to section 6.3.2.4.3).

This possibility was demonstrated in the heterogeneity in the 16S rRNA gene from the *Marinomonas protea* strain patterns in the current study (section 6.2.1.1). Where three of the strains produced a four banded profile, which by further investigation through sequencing was considered to be only a two banded profile. The fact that three of these five strains produced a possible two banded profile and the other two strains produced a single band profile suggests that there was operon copy number heterogeneity within a single species from the same and similar environments. The presence of two bands within three of the strains could be due to a mixed culture, however, this was not substantiated by analysis of cellular and colony morphology which indicated pure cultures. The production of a second band by erroneous nucleotide insertions introduced during PCR

(Section 6.3.2.4.3 ) could not be ruled out, but the use of a 'proof reading' *Taq* enzyme and maintenance of heterogeneity through repetition does suggest that PCR error was unlikely. It was not ascertained why isolates which proved by near complete 16S rRNA sequencing and ARDRA (Chapter 5) to be identical species, should have one strain with one 16S rDNA copy and one strain with two copies. This suggested that there were two separate strains. It also suggested however, that during PCR, the gene represented by the lower band (in isolates 154, 744 & *M. protea*) and the single band (in isolates 54 & 794) is preferentially amplified. This was demonstrated by comparison of the sequence for this band with the complete 16S rDNA sequence from each isolate, which found that only this band showed 100% similarity. The upper band, from isolates 154, 744 and *M. protea* could not be found within the complete sequence for the 16S rRNA gene from these isolates, which suggested that this copy was not amplified during PCR of the 16S rRNA gene.

Heterogeneity between copies of the 16S rRNA gene within a single species of bacteria could interfere with the interpretation of a DGGE profile, because individual bands within a DGGE pattern would not necessarily equate to individual species. This is demonstrated with the pure culture DGGE analysis of *M. protea* (Section 6.2.1.1). If the two bands present in 3 of *M. protea* isolates were sequenced for identification from a DGGE profile they would suggest the presence of two different strains, which would cause inaccuracies in the assessment of diversity (Priest & Austin, 1995; Nicolaisen & Ramsing, 2002). This is dramatic flaw in the analysis. The presence of rRNA operon heterogeneity within a single species also has consequences for other methods in microbial ecology as well as placing doubt on the use of 16S rRNA as an identification tool in unculturable microorganisms (Nübel *et al.*, 1996).

Multiple copies of the 16S rRNA gene have been shown to have a sequence variability of <1% in most species (Carbon *et al.*, 1979; Ogasawara *et al.*, 1994). Only a few studies have shown a sequence divergence greater than 1%. Mylvaganam & Dennis (1992) demonstrated an overall sequence divergence of 5% between the two *rrn* operons of *Haloarcula marismortui*. Moreno *et al.* (2002) demonstrated approximately 2% sequence variation in the 16S rRNA sequences of single strains of the genus *Vibrio*. Nübel *et al.* (1996) showed a sequence variation of 2.3% between *rrn* operons of

*Paenibacillus polymyxa*. In the current study the two 16S rRNA operons of *M. protea* showed a sequence dissimilarity of 4.7% over 179bp. It is generally accepted that 95% sequence similarity and above is sufficient to indicate a single species with 16S rDNA comparisons, therefore, all the percentage dissimilarities mentioned above are still within the single species similarity threshold. The presence of heterogeneity in multiple copies of a single gene has been suggested to be caused by selective pressure on RNA function which cause differential evolution on selective operons causing the fixation and spread of sequence variants (Nübel *et al.*, 1996). However, horizontal gene transfer between similar species has not been ruled out as a possible cause of heterogeneity (Mylvaganam & Dennis, 1992; Moreno *et al.*, 2002), although this may cause greater sequence divergence than has been observed.

The presence of a number of sequence variants of the 16S rRNA molecule with some degree of independent evolution in a single genome has a significant impact on prokaryotic systematics, which is based on the assumption that sequences of such genes reflect the evolution of the species of origin (Nübel *et al.*, 1996). The use of DGGE for the analysis of pure cultures to detect sequence heterogeneity in single species is suggested as a support technique for phylogenetic determination, as it is important to know which copy of the gene is being used for determination (Nübel *et al.*, 1996). This is obviously not possible for unculturable prokaryotes, and it is therefore suggested that community assessments using 16S rRNA sequences of unculturable microorganisms be augmented by traditional culture studies (Amann *et al.*, 1995; Ercolini *et al.*, 2001b).

**6.4 - Conclusion**

Without further analysis of the DGGE community profiles it is not possible to make definitive statements about the results, due to the possibility of bias within this molecular technique. However, it is important to note that the majority of AFP active bacterial isolates were not present within the analysed DGGE community profiles. Those that were present were not represented by significantly bright bands and as such it could be assumed that they are not dominant within those communities. Isolate 213 was present within the DGGE community profile from the community of its isolation, and was represented by the most intense bands in the profile, possibly indicating that it was the

most dominant species in the community. This may suggest that AFP activity did impart some level of selective advantage to that species within that community at the time of isolation. Further analysis of all the bacterial strains which demonstrated AFP activity within their communities would need to be performed, using such techniques as DGGE and fluorescent *in situ* hybridisation (FISH), to determine if AFP activity did provide a selective advantage. The assessment of spatial and temporal variation of the different communities suggested that between September and November, 2000, the communities of Ace Lake, Pendant Lake, Triple Lake and Deep Lake generally underwent significant change in species composition, whereas Club Lake showed no change in its community profile. During September the communities showed limited variation through the measured water column. However, the lowest depth recorded for Ace Lake, Pendant Lake and Triple Lake demonstrated a significant change in community composition compared with the rest of the measured water column, most likely due to the presence of different bacterial communities in the sediments, in the case of Pendant and Triple Lakes, and the chemocline, in the case of Ace Lake. During November each lake showed significant variation in the communities recorded at each depth, indicating a zonation of communities, possibly due to the increase in light levels (hence zonation based on light sensitivity), an overall decrease in the salinity due to ice melt (hence zonation along a steeper salinity gradient) and increase in temperature (hence zonation along a steeper temperature gradient).

Denaturing gradient gel electrophoresis (DGGE) is a useful technique for identifying spatial and temporal dynamics in community structure. However, its limitations due to bias mean that it requires support from traditional culturing methods and the determination of 16S rRNA operon heterogeneity (polyphasic taxonomic approach).

# Chapter 7: GENERAL DISCUSSION

## 7.1 – Discussion

The strategies adopted by micro-organisms in adapting to survive and, in some cases thrive, in cold environments is of great interest to biologists; not only from an academic perspective, but also for the novel compounds and biological pathways of biotechnological interest, which can be gained from the study of such adaptations (Herbert, 1986; McMeekin & Franzmann, 1988; Nichols *et al.*, 1993; Feller & Gerday, 1997; Russell & Hamamoto, 1998; Naganuma, 2000; Ásgeirsson & Andrésson, 2001; Demirjian *et al.*, 2001; Duman & Serianni, 2002). Prokaryotes have colonised Antarctica through birds and air-borne transportation (Abyzov, 1993; Marshall, 1996) as well as transportation through the marine ecosystem. It is likely that the majority of bacterial isolates from the current study are marine derived, as the lacustrine systems under study were formed from isolated pockets of seawater (Laybourn-Parry & Marchant, 1992b). In Antarctica, the continuous exposure to extreme cold moulds not only the environment but also the microbial communities therein. The ubiquitous nature of bacteria within the Antarctic microbial community is well demonstrated by the characterisation of bacterial species from this extreme environment (Wright & Burton, 1981; Franzmann, 1991; Franzmann *et al.*, 1990; Franzmann & Dobson, 1992; Dobson *et al.*, 1993; Franzmann & Dobson, 1993; Franzmann, 1996; Bowman *et al.*, 1997; Franzmann *et al.*, 1997; Labrenz *et al.*, 1998; McCammon *et al.*, 1998; Labrenz *et al.*, 1999; Bowman *et al.*, 2000b; Labrenz *et al.*, 2000; Lawson *et* al., 2000; McCammon & Bowman, 2000; Pearce & Butler, 2002; ). The methods which these bacteria employ for cold adaptation are largely unidentified. The current study demonstrated the occurrence of antifreeze proteins (AFPs) as a cold adaptive mechanism in the bacterial populations from the lakes of the Vestfold Hills, Eastern Antarctica (68°S 78°E).

Nineteen bacterial isolates, comprising eleven characterised taxa, were identified as being positive for AFP activity during the current study. Previously, only four other bacterial species have been identified as AFP active, *Psychrobacter urativorans*, *Rhodococcus erythropolis* (Duman & Olsen, 1993), *Pseudomonas putida* (Sun *et al.*, 1995) and *Marinomonas protea* (Mills, 1999). One of the eleven species from the current

study was characterised as *M. protea*, and comprised 7 of the 19 AFP active isolates. Although it was suggested, based on DGGE analysis of the variable V3 region of the 16S rRNA gene (Chapter 6), that the 7 isolates comprised two separate strains of this species. With the four previously identified AFP active species, the current study brings the total number of identified AFP active species up to 14 (Table 7.1).

One isolate from the current study was identified as belonging to the *Psychrobacter* genus. Duman and Olsen (1993) identified *M. cryophilus* as being AFP active, but this species was re-characterised as *Psychrobacter urativorans* (McLean *et al.*, 1951; Juni & Heym, 1986), which was isolated from ornithogenic soils in the Vestfold Hills (Cavanagh *et al.*, 1996). It is possible that *Psychrobacter urativorans* (isolated by Cavanagh *et al.*, 1996) and the *Psychrobacter sp.* isolated from Triple Lake during the current study may be a similar strain, but species identification of the isolate from the current study would be necessary to prove this.

All the bacterial isolates in the current study were identified as belonging to the Proteobacteria (α and γ subgroups) and both *Pseudomonas putida* (Sun *et al.*, 1995) and *Psychrobacter urativorans* (Duman & Olsen, 1993) belong to the γ-Proteobacteria. This may suggest that AFP active phenotype is present only in the Proteobacteria and prevalent in the γ-subgroup. However, *Rhodococcus erythropolis* (Duman & Olsen, 1993) belongs to the class Actinobacteria (high G+C gram positive bacteria),which suggests that the AFP active phenotype although abundant in the Proteobacteria, is not exclusively contained therein. The four studies performed on AFP activity bacteria to date (Duman & Olsen, 1993; Sun *et al.*,1995; Mills, 1999; current study) suggest that AFP activity in bacteria is solely contained within the α- and γ- Proteobacteria and the Actinobacteria. However, the presence of many other bacterial genera from Antarctic ecosystems (e.g. *Flavobacterium* (Dobson *et al.*, 1993; McCammon *et al.*, 1998; McCammon & Bowman, 2000), *Carnobacterium* (Franzmann *et al.*, 1991) and many other novel and existing genera (Franzmann & Dobson, 1992; Bowman *et al.*, 2000; Labrenz *et al.*, 2000; Lawson *et al.*, 2000), provides the possibility that AFP activity is more widespread within bacterial taxonomy than is suggested from the characterised AFP active species. During the current study, when an assessment of AFP activity was made on 866 individual environmental isolates, only 19 isolates (which were characterised as

| AFP Active Species | Location of Isolation | Reference |
|---|---|---|
| *Pseudomonas putida* | Rhizosphere in high Arctic | Sun *et al.*, 1995 |
| *Rhodococcus erythropolis* | Midgut of beetle larvae | Duman & Olsen, 1993 |
| *Micrococcus cryophilus* | Fresh pork sausages (ATCC) | Duman & Olsen, 1993 |
| *Marinomonas protea* | Ice/water interface, Ace Lake, Vestfold Hills, Antarctica | Mills, 1999. |
| *Pseudoalteromonas* sp. | 4m, Ace Lake, Vestfold Hills, Antarctica | *Current Study* |
| *Pseudomonas fluorescens* | 0m, Triple Lake, Vestfold Hills, Antarctica | *Current Study* |
| Antarctic sweater bacterium (*Pseudomonas)* | 2m, Triple Lake, Vestfold Hills, Antarctica | *Current Study* |
| *Idiomarina loihiensis* | 8m, Triple Lake, Vestfold Hills, Antarctica | *Current Study* |
| *Enterobacter agglomerans* | 2m, Ace Lake, Vestfold Hills, Antarctica | *Current Study* |
| *Stenotrophomonas maltophilia* | 6m, 0m, Ace Lake, Vestfold Hills, Antarctica | *Current Study* |
| *Psychrobacter sp.* | 0m, Triple Lake, Vestfold Hills, Antarctica | *Current Study* |
| *Sphingomonas sp* | 0m, Pendant Lake, Vestfold Hills, Antarctica | *Current Study* |
| *Halomonas sp.* | 4m, Triple Lake, Vestfold Hills, Antarctica | *Current Study* |
| Isolate 466 | 0m, Oval Lake, Vestfold Hills, Antarctica | *Current Study* |

**Table 7.1 – AFP active bacterial species, their isolated location and study from which they are cited. *Micrococcus cryophilus* is synonymous with *Psychrobacter urativorans* and is referred to as this in the text; *P. urativorans* was isolated from ornithogenic soils in the Vestfold Hills, Eastern Antarctica (Cavanagh *et al.*, 1996). Isolate 466 was not definitively identified and so is not characterised here, for further information refer to section chapter 5, section 5.3.3.10.**

11 species) showed AFP activity. This suggests one of two possibilities. Firstly, that the culture conditions used to isolate the bacteria from these environments were unintentionally selecting for Proteobacteria, which are considered dominant in cold, aquatic ecosystems (López-Garcia *et al.*, 2001). Secondly, that all or the majority of bacteria within the community had an AFP active phenotype, but the cold-shock conditions used to induce expression of the AFP gene were not sufficient. This second point is supported by the studies of Duman & Olsen (1993) and Sun *et al.* (1995), who both suggested that variation in the length of cold-shock exposure did affect the AFP activity of the respective bacterial species.

The nineteen bacterial isolates which showed AFP activity were isolated from four different lakes. The majority of the isolates came from Ace Lake, the site of previous isolation of the AFP active bacterium *Marinomonas protea* (Mills, 1999). This is a meromictic (permanently stratified) saline lake with low inorganic nitrogen concentrations ($N\text{-}NO_2$, $N\text{-}NO_2$ and $N\text{-}NH_4$) and high soluble reactive phosphate (SRP) concentrations, compared with the other lakes from which AFP active bacteria were isolated (Pendant, Triple and Oval). Pendant Lake, from which five AFP active bacteria were isolated, had a similar salinity to Ace Lake, but it generally had higher concentrations of inorganic nitrogen and lower concentrations of SRP. The variation between the two lakes is attributed to the meromictic status of Ace Lake, which accumulates nutrients within the anoxic monimolimnion due to downward flux of particulate and dissolved matter across the chemocline (Laybourn-Parry *et al.*, 2002).

One of the AFP active isolates was isolated from Oval Lake. This isolate has not been fully characterised; however, in ARDRA analysis it does appear to have a Dice coefficient of similarity of approximately 75% with the *Marinomonas* group which could indicate that it belongs in the Proteobacteria. Oval Lake is also a meromictic lake (Burke and Burton, 1988; Gibson, 1999) and although at 8m it is considerably more shallow than Ace Lake (25m), it was expected to show a similar chemical profile as Ace Lake. The current study demonstrated that inorganic nutrient concentrations in the surface waters during the summer were very similar between Oval Lake and Ace Lake. However, Oval Lake has a considerably higher salinity than Ace Lake, which at 34 ppt is close to marine salinity.

The remaining five AFP active isolates were cultured from the extremely hypersaline Triple Lake. All five isolates were characterised as belonging to genera which have shown an association with extreme environments, i.e. *Halomonas, Psychrobacter, Idiomarina* and *Pseudomonas* (James *et al.*, 1994; Meyer *et al.*, 1998; Obata *et al.*, 1998; Ivanova *et al.*, 2000; Kaye & Baross, 2000; Maruyama *et al.*, 2000; Teske *et al.*, 2000; Brown & Bowman, 2001; Duilio *et al.*, 2001; Mergaert *et al.*, 2001; Panicker *et al.*, 2002; Camardella *et al.*, 2002) . The hypersalinity of Triple Lake causes severe temperature fluctuation throughout the year; during the summer the water temperature can reach 3 to 4°C and during the winter it can fall to -14°C. It has been suggested that the AFP activity of the characterised isolates should be considered one adaptation to survival of the bacterial cell in the extreme cold (Sun *et al.*, 1995). However, due to the extreme hypersalinity of the environment the chances of freezing are low, unless the isolate exists in the surface water, where a 30cm thick ice cover forms during the winter. The possibility of the bacteria freezing is low, because adaptations to extreme salinity generally involve an increase in intracellular solute potential to allow biochemical functioning within the high osmotic stress environment (Kushner, 1978; Wright and Burton, 1981). Thus, AFP activity is possibly redundant in their current environment, but it is suggested that the bacteria evolved AFP activity during a time when they were exposed to freezing stresses. The salinities within Ace Lake, Pendant Lake and Oval Lake are low enough to allow substantial freezing of the surface waters during the winter and so AFP activity could have evolved in such an environment. To date there have been no recorded isolations of *Pseudomonas, Halomonas, Psychrobacter* or *Idiomarina* from either Ace Lake, Pendant Lake or Oval Lake. This could imply that, while the species from the hypersaline lakes have evolved AFP activity, they did not originate within the saline lakes. This in turn could suggest that the saline lakes - Ace Lake, Pendant Lake and Oval Lake - are not the only locations in which AFP activity has evolved. This is substantiated by *Psychrobacter urativorans,* which was isolated from ornithogenic soils in an Adelie penguin colony in the Vestfold Hills (Cavanagh *et al.*, 1996), and as such would have been exposed to freezing stresses. The close association of the predatory South Polar Skua (*Catharacta maccormicki*) with the penguin colony, means that it could have acted as a vector for the bacteria to Triple Lake. This suggests

that AFP activity could have evolved in any location in which freezing stress acted as a selective pressure for its evolution. However, it is also recognised that the lack of isolation of *Pseudomonas, Halomonas, Psychrobacter* or *Idiomarina* from Ace Lake, Pendant Lake and Oval Lake does not necessarily mean that they do not exist within those communities, as culturing bias could have negated their isolation (Amann *et al.*, 1995). Indeed, characterisation of the remainder of the bacterial isolates cultured from these lakes during the current study, may indicate the existence of these genera in these communities. However, the lack of AFP activity within the remainder of the isolates under the conditions used, suggests the absence of the AFP active species of these genera from the hypersaline lakes.

DGGE community profiling of the Ace Lake, Pendant Lake and Triple Lake bacterial communities, demonstrated the distinct difference between saline (Ace Lake & Pendant Lake) and hypersaline (Triple Lake) Antarctic lake communities. Triple Lake showed a distinct clustering with two other hypersaline lake communities (Deep Lake and Club Lake) when the similarity coefficients for the DGGE community profiles were compared in an UPGMA dendrogram (Fig. 6.5b). *Pseudomonas* and *Halomonas* isolates have been cultured from Deep Lake, Organic Lake and Ekho Lake, which are all hypersaline lakes from the Vestfold Hills (Bowman *et al.*, 2000b). However, if these species were cultured from these lakes during the current study (which cannot be known as the majority of the 866 environmental isolates were not characterised), then they did not demonstrate AFP activity under the culturing and AFP assessment conditions.

The AFP activity within these species should provide freeze tolerance which would allow the bacteria to survive in a niche in which other species are unable to survive and thus provide a selective advantage. Due to its enhanced freeze-resistance, the AFP active species would be able to out-compete freeze-intolerant species. In the current study, the identification, using DGGE, of the *Halomonas* sp. within the community from which it was isolated (Triple Lake, 8m), confirmed the presence of that AFP active species within its community. The DGGE analysis also suggested that the *Halomonas* sp. was the most dominant species in the Triple Lake, 8m community, suggesting AFP activity may have enabled it to become dominant. The inability to isolate AFP active species from some lake communities supports the hypothesis that these species have

evolved AFP activity as a selective advantage over other non-AFP active bacteria, suggesting that they were competitively excluded from environments which were warmer and had less freezing stress. However, the presence of AFP active species in various depths of each lake from which they were isolated suggests that some AFP active species may also be able to successfully compete for nutrients in parts of the lake where AFP activity is redundant.

Including the 19 AFP active environmental isolates thus far described, a further 847 cultured isolates were maintained during the current study. The potential biotechnological resource is therefore considerable, as these psychrotrophic and psychrophilic bacterial species are possible sources of novel biochemical compounds. Not only have a series of bacterial species producing possibly novel AFPs been isolated and characterised, but also a huge potential reservoir of cold tolerant enzymes (e.g. proteinases, lipases and cellulases), polyunsaturated fatty acids (for food additives) and various pharmaceuticals (Feller *et al.,* 1996; Feller & Gerday, 1997; Russell & Hamamoto, 1998; Gerday *et al.,* 2000) is now available. The potential use of AFPs in industry is also well documented, e.g. in preservation of frozen foods, such as meat and ice cream (Griffith & Ewart, 1995; Feeney & Yeh, 1998), cryopreservation of living tissue (Wu & Fletcher, 2000), maintenance of ice slurries as cooling agents in industrial plants (Inada *et al.,* 2000) and protection of freeze-intolerant food animals and agricultural crops (Feeney & Yeh, 1993). The development of the AFPs from the current study into working biotechnological products will require considerable future research to identify and purify the proteins with antifreeze activity, understanding of post-translational modifications and then research the possible applications of these purified proteins. Bacterial AFPs from the current study could provide a readily exploitable source of AFP. The bacterial species involved require no specific growth requirements for the production of AFP activity (save those reported in the current study) and as such could be easily produced on an industrial scale, especially if further research was conducted into the optimisation of AFP yield from bacterial cultures.

## 7.2 – Future Work

The 19 AFP active bacterial isolates were characterised using ARDRA and partial 16S rDNA sequencing; however, it would be prudent to complete the characterisation of these isolates through biochemical and molecular techniques to allow a complete taxonomic understanding of these species. This would involve phenotypic characterisation through biochemical tests including carbohydrate utilisation (differentiated into assimilation, oxidation and fermentation), oxidase, catalase and antibiotic susceptibility. Sequencing of the complete 1500bp 16S rRNA gene would allow complete identification of the bacterial species by comparison with the GenBank nucleotide database (NCBI, EMBI). Sequencing should be completed by cloning the 1500bp product for subsequent sequencing. Further to this, utilisation of the 16S rDNA sequence for phylogenetic analysis to delineate evolutionary pathways for AFP isolates should also be conducted. Although this work has been carried out to a certain level in the current study, it would be prudent to complete the species identification to provide a clearer understanding of the taxonomy of the AFP active species.

To increase understanding of Antarctic bacterial diversity, it would also be useful to perform molecular characterisation of the remaining 847 isolates from the current study. This would provide information on the spatial and temporal changes in diversity and distribution of culturable bacterial species within the lakes of the Vestfold Hills. If this was combined with the detailed physico-chemical analysis of each of the lakes from the current study, it would be possible to analyse the biogeography of the bacterial isolates. This would provide a more comprehensive understanding of the culturable bacterial communities of the diverse lacustrine ecosystems of the Vestfold Hills than is currently known. It might also be possible to discern how the different bacterial communities developed within the different ecosystems, and therefore track the evolution of the bacterial communities of this recently (~9000 years) formed environment (refer to Chapter 1 for a description of the formation of the Vestfold Hills).

Identification of the protein responsible for AFP activity in each of the AFP active bacterial isolates is also considered important. Determining the nature of the protein and subsequent identification (through screening of gene libraries) of the gene which codes for the protein, would provide valuable information on bacterial AFPs. To date, 5 AFP

types have been identified from fish, insects and plants (Chapter 1, section 14.3.4.1), and they show significant structural variation. Therefore, identification and characterisation of the bacterial AFPs from the current study would enable a greater understanding of the structure and function of all AFPs, providing a clearer overall picture of AFP diversity.

Identification of the genes encoding the AFPs in the bacterial isolates of the current study would also enable the identification of bacterial species with the same AFP genes *in situ,* using fluorescently labelled DNA probes. This would provide a rapid method for the identification of potential AFP activity in different environments. It does rely, however, on the same AFP gene being present in different AFP active bacterial species.

Further work on the DGGE profiles used for analysis of the temporal and spatial variability in the community composition within the lakes (Chapter 6) is also necessary. Sequence identification of the bacterial 16S rDNA fragments within the gel would provide more information on the location of confirmed AFP active species within the communities, as well as increasing the knowledge and understanding of Antarctic lacustrine bacterial diversity and ecological function.

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# APPENDICES

## A1. Media

Tryptic Soya Agar (TSA)

| | |
|---|---|
| Tryptic Soya Broth (Sigma) | 30 g |
| Agar (Difco) | 15 g |
| Sterile RO Water | 1 L |

Yeast Malt Agar (YMA)

| | |
|---|---|
| Yeast Extract (Sigma) | 3 g |
| Malt (Sigma) | 3 g |
| Peptone (Sigma) | 5 g |
| Dextrose (Sigma) | 10 g |
| Agar (Difco) | 20 g |
| Sterile RO Water | 1 L |

Seawater Agar (SWA)

| | |
|---|---|
| Yeast Extract (Sigma) | 1 g |
| Peptone (Sigma) | 1 g |
| Salt (Coral Life) | 38 g |
| Agar (Difco) | 15 g |
| Sterile RO Water | 1 L |

½ Seawater Agar (½ SWA)

| | |
|---|---|
| Yeast Extract (Sigma) | 1 g |
| Peptone (Sigma) | 1 g |
| Salt (Coral Life) | 19 g |
| Agar (Difco) | 15 g |
| Sterile RO Water | 1 L |

For each recipe the corresponding liquid media was made without the agar.

For each recipe the corresponding cryo-preservation liquid medium was made containing 10% glycerol (Sigma), which was added after incubation time immediately prior to freezing.

## A2 Buffers and Reagents

Protein Extraction Buffer (PEB)

25 mM Tris (Tris{hydroxymethyl}amino-methane)/HCl pH 7.0
1 mM EDTA (Ethylenediamine-tetraacetic acid)
1 mM PMSF* (Phenylmethylsulphonyl fluoride) from a 100mM stock in ethanol.
2 $\mu g\ ml^{-1}$ Pepstatin A* from a 2.5mg stock in methanol.

* added prior to use.
PEB recipe above was used in Antarctica, but PMSF and Pepstatin A were replaced with a Complete ® protease inhibitor tablet (Roche) when the protocol was performed in laboratories in Nottingham University.

20% Buffered Glutaraldehyde
Na2HPO4 – 14.145g in 500ml (A)
NaH2PO4 – 10.585g in 500ml (B)
Then: 152.5ml A + 97.5ml B + 250ml distilled H20 = 500ml buffer
Then: 800ml glut + 200ml Buffer = 20% Buffered gluteraldehyde
Adjust Ph to 7.0 with NaOH

TE Buffer (for protein and genomic DNA extractions)
10 mM Tris-HCl
1 mM EDTA
Adjust pH to 8

**B1 – Isolate numbers, lake and depth of isolation, colony and cellular morphology, and Gram stain result. This is raw data and as such may contain some irregularities. na = not applicable.**

| Lake name | Depth (m) | Culture medium agar | Colonial morphology and colour | Cellular morphology | Isolate number | Gram Stain Result |
|---|---|---|---|---|---|---|
| Ace | 0m | SWA | Off White Distinct | rod | 1 | - |
| Pendant | 4m | TSA | Beige Mucoid Lumpy | rods | 12 | - |
| Ace | 8m | SWA | off white beige mucoid distinct | short rods | 13 | - |
| Pendant | 2m | TSA | Beige Mucoid | Rod | 14 | - |
| Ace Ice | na | YSA | beige mucoid lumpy | rods | 1ice3 | - |
| Ace | 0m | TSA | Beige Mucoid Distinct | rod | 10 | - |
| Pendant | 8m | TSA | Beige Mucoid | thin rods | 102 | - |
| Triple | 8m | YSA | Beige ,mucoid lumpy | short rods | 103 | - |
| Pendant | 8m | TSA | Beige Mucoid Lumpy | Rods | 104 | - |
| Ace | 10m | SWA | Off White Distinct | rod | 100 | - |
| Ace | 10m | 1/2 | Off White Distinct | rod | 101 | - |
| Ace Ice 1 | na | TSA | Beige Mucoid Lumpy | rod | 104 | - |
| Ace Ice 1 | na | YSA | Orange mucoid | rod | 107 | - |
| Ace Ice 1 | na | YSA | Beige Mucoid | rod | 108 | - |
| Ace Ice 2 | na | 1/2 | Off White Distinct | cocci | 109 | + |
| Ace | 0m | TSA | Beige Mucoid Lumpy | rod | 11 | - |
| Pendant | 8m | SWA | Off white distinct mucoid | cocci | 112 | - |
| Triple | 8m | 1/2 | Off white distinct mucoid | short rods | 113 | - |
| Ace | 8m | TSA | Beige Mucoid Lumpy | rod | 114 | - |
| Ace Ice 2 | na | SWA | off white distinct | cocci | 114 | + |
| Ace Ice 2 | na | YSA | Beige Mucoid Lumpy | rod | 115 | - |
| Ace | 0m | TSA | Beige Mucoid Distinct | rod | 12 | - |
| Pendant | 8m | 1/2 | Off White Distinct | v. thin rods | 122 | - |
| Triple | 8m | 1/2 | Off white distinct mucoid | thin rods | 123 | - |
| Ace | 8m | 1/2 | Beige Distinct | Rods | 124 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace Ice 3 | na | TSA | White distinct mucoid | cocci | 120 | + |
| Ace Ice 3 | na | SWA | beige mucoid | rod | 121 | - |
| Ace Ice 3 | na | YSA | Beige Mucoid | rod | 122 | - |
| Ace Ice 3 | na | YSA | White mucoid distinct | rod | 123 | + |
| Ace Ice 3 | na | YSA | beige Distinct mucoid | rod | 124 | - |
| Ace Ice 4 | na | 1/2 | white fine distinct | cocci | 125 | + |
| Ace Ice 4 | na | TSA | Beige off white mucoid distinct | rod | 126 | - |
| Ace Ice 4 | na | TSA | White mucoid | rod | 127 | - |
| Ace Ice 4 | na | YSA | Beige Mucoid | rod | 129 | - |
| Ace | 2m | TSA | Beige mucoid lumpy white | rod | 13 | - |
| Pendant | 2m | 1/2 | Off white distinct mucoid | rods | 132 | - |
| Triple | 8m | 1/2 | Yellow mucoid distinct | cocci | 133 | - |
| Triple | 8m | 1/2 | White Distinct | rod | 134 | - |
| Ace Ice 4 | na | SA | Clear v fine distinct | rod | 130 | - |
| Ace Ice 5 | na | 1/2 | white fine distinct | cocci | 132 | + |
| Ace Ice 5 | na | TSA | white fungi | na | 133 | na |
| Ace Ice 5 | na | TSA | Beige cream distinct mucoid | rod | 134 | - |
| Ace Ice 5 | na | TSA | Beige Mucoid Distinct | rod | 135 | - |
| Ace Ice 5 | na | TSA | White distinct | cocci | 136 | + |
| Ace Ice 5 | na | SWA | Green Fungus White Patches | na | 138 | na |
| Ace Ice 5 | na | YSA | Beige Mucoid | rod | 139 | + |
| Ace | 2m | TSA | Beige Mucoid Lumpy | rod | 14 | - |
| Pendant | 2m | TSA | Beige Mucoid Distinct | long rods | 142 | - |
| Triple | 4m | SWA | Beige mucoid off white distinct | small rods | 143 | - |
| Ace | 4m | TSA | Beige Mucoid | Rods | 144 | - |
| Ace Ice 5 | na | SWA | Green Fungus White Patches | na | 140 | na |
| Ace Ice 1 | na | 1/2 | clear v fine distinct | rod | 142 | - |
| Ace Ice 1 | na | TSA | Beige Mucoid | rod | 143 | + |
| Ace Ice 2 | na | 1/2 | Off White Distinct | rod | 148 | - |
| Ace Ice 1 | na | YSA | Off White Distinct | rod | 148 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace Ice 2 | na | TSA | Beige Mucoid Distinct | rod | 149 | - |
| Ace | 2m | TSA | Beige Mucoid Lumpy | rod | 15 | - |
| Pendant | 2m | TSA | Beige mucoid | helical rods | 152 | - |
| Triple | 4m | TSA | Beige Mucoid | small rods | 153 | - |
| Ace | 4m | 1/2 | Beige Ditsinct White | Rods | 154 | - |
| Ace Ice 3 | na | 1/2 | Off White Distinct | rod | 152 | - |
| Ace | 2m | TSA | Beige distinct | rod | 16 | - |
| Pendant | 2m | SWA | Off White Distinct | cocci | 162 | - |
| Triple | 4m | TSA | Beige Mucoid Lumpy | small rods | 163 | - |
| Pendant | 0m | TSA | Beige Mucoid | Rods | 164 | - |
| Ace Ice 1 | na | 1/2 | Off White Distinct | rods | 162 | - |
| Ace Ice 1 | na | TSA | White mucoid distinct | cocci | 163 | + |
| Ace Ice 1 | na | TSA | Off White Distinct | cocci | 164 | + |
| Ace Ice 1 | na | SWA | Off White Distinct | cocci | 166 | + |
| Ace Ice 1 | na | YSA | Beige Mucoid Distinct | rods | 167 | - |
| Ace | 2m | YSA | Beige Mucoid | rod | 17 | - |
| Triple | 0m | 1/2 | Off White Distinct | cocci | 172 | - |
| Triple | 4m | TSA | Beige Mucoid Lumpy | rods | 173 | - |
| Pendant | 0m | TSA | Beige Mucoid | Rods | 174 | - |
| Ace Ice 2 | na | 1/2 | Off White Distinct | rods | 170 | - |
| Ace Ice 2 | na | TSA | White mucoid | cocci | 171 | + |
| Ace Ice 2 | na | TSA | Beige Distinct | rods | 172 | - |
| Ace Ice 2 | na | TSA | Off White Beige Distinct | cocci | 173 | + |
| Ace Ice 2 | na | SWA | Off white distinct mucoid | cocci | 174 | + |
| Ace Ice 2 | na | SWA | Off white distinct mucoid | cocci | 174 | - |
| Ace Ice 2 | na | SWA | Off White Distinct | cocci | 175 | - |
| Ace Ice 3 | na | 1/2 | Off White Distinct | cocci | 179 | - |
| Triple | 0m | 1/2 | Off white vv fine distinct | small rods | 182 | - |
| Triple | 4m | YSA | Beige Mucoid Lumpy | rods | 183 | - |
| Pendant | 0m | YSA | Yellow Mucoid | Cocci | 184 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace Ice 3 | na | TSA | Beige Off white distinct | Cocci | 180 | + |
| Ace Ice 3 | na | TSA | Beige Mucoid Lumpy | Cocci | 181 | + |
| Ace Ice 3 | na | TSA | Beige distinct | rods | 182 | - |
| Ace Ice 3 | na | YSA | Beige distinct | rods | 184 | - |
| Ace Ice 4 | na | 1/2 | pink distinct | rods | 187 | - |
| Ace Ice 4 | na | TSA | Beige White mucoid | Cocci | 188 | + |
| Ace Ice 4 | na | TSA | Beige White distinct | Cocci | 189 | + |
| Ace | 2m | YSA | Orange Yellow mucoid | rod | 19 | - |
| Triple | 0m | YSA | white Mucoid Distinct | rods | 192 | - |
| Triple | 4m | YSA | Off white beige mucoid lumpy | long rods | 193 | - |
| Pendant | 0m | 1/2 | Off white distinct | Small Rods | 194 | - |
| Ace Ice 4 | na | TSA | Beige Mucoid Distinct | rods | 190 | - |
| Ace Ice 4 | na | SWA | fine white distinct | Cocci | 191 | + |
| Ace Ice 5 | na | TSA | Beige Lumpy | rods | 193 | - |
| Ace Ice 5 | na | TSA | Beige Lumpy | Cocci | 194 | + |
| Ace Ice 5 | na | SWA | Beige Lumpy | rods | 195 | - |
| Pendant | 4m | YSA | Beige Mucoid | rods | 22 | - |
| Ace | 8m | YSA | Beige Mucoid Lumpy | rods | 23 | - |
| Ace | 10m | TSA | Beige Mucoid Thin | Rods | 24 | - |
| Ace Ice | na | TSA | beige mucoid lumpy | Cocco bacilli | 2ice3 | - |
| Ace | 2m | YSA | Cream beige mucoid | rod | 20 | - |
| Triple | 0m | SWA | Off white distinct mucoid | rods | 202 | - |
| Triple | 4m | 1/2 | Off white mucoid lumpy | short rods | 203 | - |
| Ace | 2m | TSA | Beige Mucoid and Lumpy | Rods | 204 | - |
| Rookery | 0m | SWA | Cream White distinct | rods | 200 | - |
| Rookery | 0m | 1/2 | Yellow White distinct | rods | 201 | - |
| Rookery | 0m | 1/2 | Off White Distinct | rods | 203 | - |
| Rookery | 0m | TSA | Beige Mucoid Lumpy | rods | 204 | - |
| Williams | 0m | SWA | Off White Distinct | rods | 207 | - |
| Williams | 0m | SWA | Off White Distinct | rods | 208 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Williams | 0m | 1/2 | Yellow Distinct | rods | 209 | - |
| Ace | 2m | YSA | Orange Mucoid distinct | rod | 21 | - |
| Triple | 0m | SWA | Off White Distinct | rods | 212 | - |
| Triple | 4m | 1/2 | Off white distinct mucoid | long rods | 213 | - |
| Ace | 2m | 1/2 | Off white mucoid distinct | Rods | 214 | - |
| Williams | 0m | TSA | Orange Distinct | rods | 210A | - |
| Williams | 0m | TSA | Mucoid Beige Lumpy | rods | 210B | - |
| Williams | 0m | TSA | Beige Mucoid Distinct | rods | 211 | - |
| Williams | 0m | YSA | Orange mucoid Lumpy | rods | 212 | - |
| Rookery | 0m | SWA | off white fine | rods | 214 | - |
| Rookery | 0m | SWA | Off White Distinct | rods | 215 | - |
| Rookery | 0m | 1/2 | Off white distinct mucoid | rods | 216 | - |
| Rookery | 0m | 1/2 | Off white orange distinct | rods | 217 | - |
| Rookery | 0m | TSA | Beige Mucoid Lumpy | rods | 218 | - |
| Rookery | 0m | TSA | Cream white mucoid distinct | cocci | 219 | + |
| Ace | 2m | YSA | Transparent mucoid beige | rod | 22 | - |
| Triple | 0m | TSA | Beige Mucoid | rods | 222 | - |
| Ace | 10m | SWA | Off white distinct mucoid | rods | 223 | - |
| Ace | 2m | SWA | Off white distinct | Rods | 224 | - |
| Rookery | 0m | YSA | Yellow Orange Mucoid Lumpy | rods | 222 | - |
| Rookery | 0m | YSA | Yellow beige Mucoid distinct | rods | 223 | - |
| Rookery | 0m | YSA | Orange distinct mucoid | rods | 224 | - |
| Williams | 0m | SWA | Off White Distinct | rods | 225 | - |
| Williams | 0m | SWA | Orange pink white distinct | rods | 226 | - |
| Williams | 0m | TSA | beige mucoid lumpy | rods | 229 | - |
| Triple | 0m | TSA | Beige Mucoid Distinct | coccoid | 232 | - |
| Ace | 10m | SWA | Yellow distinct | rods | 233 | - |
| Ace | 0m | TSA | Beige Mucoid Distinct | Coccoid | 234 | - |
| Rookery | 0m | TSA | Beige Mucoid Distinct | rods | 238 | - |
| Triple | 0m | TSA | Clear distinct mucoid | rods | 242 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace | 10m | TSA | beige mucoid lumpy | rods | 243 | - |
| Ace | 0m | TSA | Beige Mucoid Lumpy | Long Rods | 244 | - |
| Rookery | 0m | YSA | beige mucoid lumpy | rods | 241 | - |
| Triple | 0m | 1/2 | Off White Distinct | rods | 243 | - |
| Triple | 0m | SWA | Off White Distinct | rods | 244 | - |
| Triple | 0m | YSA | Beige Mucoid | rods | 247 | - |
| Triple | 0m | YSA | Beige Mucoid | rods | 248 | - |
| Triple | 0m | YSA | Beige Mucoid Lumpy | rods | 249 | - |
| Ace | 2m | 1/2 | Off White Distinct | rods | 25 | - |
| Triple | 0m | TSA | Beige Mucoid Distinct | long rods | 252 | - |
| Ace | 10m | YSA | Beige Mucoid Lumpy | rods | 253 | - |
| Ace | 0m | 1/2 | Off white mucoid distinct | Small Rods | 254 | - |
| Nottingham | 0m | YSA | Orange distinct | rods | 251 | - |
| Nottingham | 0m | YSA | Orange Distinct / pink | rods | 252 | - |
| Nottingham | 0m | YSA | Off White Distinct | rods | 253 | - |
| Nottingham | 0m | TSA | Beige Mucoid | rods | 255 | - |
| Nottingham | 0m | TSA | Orange Mucoid distinct | rods | 256 | - |
| Nottingham | 0m | TSA | Beige Mucoid | rods | 257 | - |
| Nottingham | 0m | TSA | Beige Mucoid Lumpy | rods | 258 | - |
| Triple | 2m | SWA | Off white distinct mucoid | rods | 262 | - |
| Ace | 10m | 1/2 | off white clear fine and distinct | rods | 263 | - |
| Ace | 0m | 1/2 | Yellow Off white Mucoid Distinct | rods | 264 | - |
| Nottingham | 0m | 1/2 | Off White Distinct | rods | 260 | - |
| Club | 0m | TSA | Off White Distinct | rods | 261 | - |
| Club | 0m | SWA | Off White Distinct | rods | 264 | - |
| Ekho | 0m | SWA | Off White Distinct | rods | 267 | - |
| Ekho | 0m | TSA | Beige Transparent mucoid | rods | 268 | - |
| Ekho | 0m | TSA | Beige Mucoid Distinct | rods | 269 | - |
| Triple | 2m | SWA | Off White Distinct | rods | 272 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace | 2m | TSA | Beige Mucoid Distinct | vv thin rods | 273 | - |
| Triple | 0m | TSA | White Mucoid Distinct | cocci | 274 | + |
| Ekho | 0m | YSA | Beige Mucoid Distinct | rods | 272 | - |
| Triple | 0m | 1/2 | Off White Distinct | rods | 274 | - |
| Triple | 0m | TSA | Transparent Beige Mucoid | rods | 276 | - |
| Triple | 0m | TSA | Beige Mucoid Lumpy | rods | 277 | - |
| Triple | 0m | YSA | Off white distinct mucoid | rods | 278 | - |
| Ace | 4m | TSA | Beige Mucoid | rods | 28 | - |
| Triple | 2m | TSA | Beige Mucoid | rods | 282 | - |
| Ace | 2m | YSA | Cream beige mucoid lumpy | rods | 283 | - |
| Ekho | 0m | 1/2 | off white distinct | rods | 280 | - |
| Ekho | 0m | YSA | Beige Distinct | rods | 285 | - |
| Ekho | 0m | YSA | Beige Distinct | rods | 286 | - |
| Club | 0m | TSA | Beige distinct | rods | 288 | - |
| Club | 0m | YSA | Beige Distinct | rods | 289 | - |
| Ace | 4m | TSA | Beige Mucoid Lumpy | rods | 29 | - |
| Triple | 2m | TSA | Off white distinct mucoid | cocci | 292 | - |
| Ace | 2m | 1/2 | Off white distinct mucoid | rods | 293 | - |
| Triple | 2m | YSA | White Mucoid Distinct | ovoid cocci | 294 | + |
| Nottingham | 0m | 1/2 | Off White Distinct | rods | 293 | - |
| Nottingham | 0m | TSA | Beige Mucoid | rods | 295 | - |
| Nottingham | 0m | YSA | Pink Mucoid | rods | 296 | - |
| Nottingham | 0m | YSA | Orange/Beige Distinct Mucoid | rods | 297 | - |
| Nottingham | 0m | YSA | Beige Mucoid Lumpy | rods | 298 | - |
| Nottingham | 0m | YSA | Beige Mucoid Lumpy | rods | 298a | - |
| Triple | 0m | 1/2 | Off White Distinct | rods | 299 | - |
| Ace | 0m | 1/2 | Off white distinct mucoid | rods | 3 | - |
| Pendant | 4m | SWA | Beige Off White mucoid Distinct | rods | 32 | - |
| Ace | 8m | 1/2 | Off white distinct mucoid | rods | 33 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace | 10m | 1/2 | Cream Distinct | rods | 34 | - |
| Pendant Ice | 50cm | TSA | beige mucoid distinct | rods | 3ice3 | - |
| Ace | 4m | TSA | Beige Mucoid Lumpy | rods | 30 | - |
| Triple | 2m | TSA | Clear mucoid distinct | rods | 302 | - |
| Ace | 0m | SWA | off white fine distinct | rods | 303 | - |
| Triple | 2m | SWA | Off white distinct Fine | rods | 304 | - |
| Triple | 0m | SWA | Off White Distinct | rods | 301 | - |
| Triple | 0m | TSA | Beige Mucoid Lumpy | rods | 302 | - |
| Triple | 0m | TSA | Beige Mucoid Lumpy | rods | 303 | - |
| Triple | 0m | TSA | Beige Mucoid | rods | 304 | - |
| Triple | 0m | YSA | Beige Mucoid Lumpy | rods | 305 | - |
| Ekho | 0m | 1/2 | Off White Distinct | rods | 306 | - |
| Ekho | 0m | SWA | Off White Distinct | rods | 307 | - |
| Ekho | 0m | TSA | Beige Mucoid Lumpy | rods | 308 | - |
| Ekho | 0m | TSA | Off white distinct mucoid | rods | 309 | - |
| Ace | 4m | TSA | Beige Mucoid Lumpy | rods | 31 | - |
| Triple | 2m | 1/2 | off white mucoid | rods | 312 | - |
| Ace | 0m | SWA | off white beige mucoid distinct | rods | 313 | - |
| Triple | 4m | TSA | White Fungi | na | 314 | na |
| Ekho | 0m | TSA | Beige Mucoid Lumpy | rods | 310 | - |
| Club | 0m | TSA | Beige Mucoid Lumpy | rods | 313 | - |
| Club | 0m | TSA | Beige Mucoid Lumpy | rods | 314 | - |
| Club | 0m | YSA | Beige Mucoid Distinct | rods | 316 | - |
| Club | 0m | YSA | Beige Mucoid Lumpy | rods | 317 | - |
| Club | 0m | SWA | Off White Distinct | rods | 318 | - |
| Club | 0m | 1/2 | Off White Distinct | rods | 319 | - |
| Ace | 4m | TSA | Beige Mucoid | rods | 32 | - |
| Triple | 2m | 1/2 | Off White Distinct | cocci | 322 | + |
| Ace | 0m | TSA | off white beige mucoid distinct | cocci | 323 | - |
| Triple | 4m | 1/2 | Beige Mucoid distinct | rods | 324 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Club | 0m | 1/2 | Off White Distinct | rods | 320 | - |
| Nottingham | 0m | 1/2 | Off White Distinct | rods | 321 | - |
| Nottingham | 0m | TSA | Yellow Mucoid distinct | rods | 322 | - |
| Nottingham | 0m | TSA | Beige Mucoid Lumpy | rods | 323 | - |
| Nottingham | 0m | TSA | Beige Mucoid Lumpy | rods | 324 | - |
| Nottingham | 0m | YSA | Beige Mucoid Lumpy | rods | 325 | - |
| Nottingham | 0m | YSA | Yellow Mucoid Lumpy | rods | 326 | - |
| Nottingham | 0m | YSA | Pale Pink Mucoid Distinct | rods | 328 | - |
| Ace | 4m | YSA | Beige Mucoid | rods | 33 | - |
| Triple | 2m | 1/2 | Off White Distinct | rods | 332 | - |
| Ace | 0m | TSA | Beige Mucoid Lumpy | rods | 333 | - |
| Pendant | 2m | TSA | Beige Mucoid | cocci | 334 | - |
| Ace | 4m | YSA | Beige Mucoid | rods | 34 | - |
| Triple | 2m | YSA | white Mucoid distinct | short rods | 342 | - |
| Ace | 0m | TSA | Beige Mucoid Distinct | rods | 343 | - |
| Pendant | 2m | YSA | White mucoid distinct | short rods | 344 | - |
| Rookery | 0m | YSA | Beige Mucoid Distinct | rods | 344 | - |
| Rookery | 0m | YSA | Red Mucoid Distinct | rods | 346 | - |
| Rookery | 0m | SWA | Off White Distinct | rods | 347 | - |
| Rookery | 0m | SWA | Beige off white distinct | rods | 348 | - |
| Ace | 4m | YSA | Beige Mucoid | rods | 35 | - |
| Triple | 2m | YSA | beige mucoid | rods | 352 | - |
| Ace | 0m | YSA | Beige cream mucoid distinct | rods | 353 | - |
| Pendant | 2m | 1/2 | dark orange mucoid distinct | rods | 354 | - |
| Williams | 0m | SWA | Beige Mucoid Lumpy | rods | 350 | - |
| Druzhby | 0m | YSA | Beige Mucoid Lumpy | rods | 351 | - |
| Druzhby | 0m | YSA | Beige Distinct | rods | 352 | - |
| Druzhby | 0m | YSA | White Mucoid distinct | rods | 353 | - |
| Druzhby | 0m | YSA | Yellow Orange distinct | rods | 354 | - |
| Druzhby | 0m | YSA | Beige Mucoid Lumpy | rods | 355 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Druzhby | 0m | TSA | Beige Mucoid Lumpy | rods | 356 | - |
| Druzhby | 0m | TSA | Beige Distinct | rods | 357 | - |
| Druzhby | 0m | TSA | Beige Mucoid Lumpy | rods | 358 | - |
| Druzhby | 0m | TSA | Beige Distinct large colonies | rods | 359 | - |
| Ace | 4m | YSA | Beige Mucoid Lumpy | rods | 36 | - |
| Triple | 4m | TSA | Beige Mucoid | V long rods | 362 | - |
| Ace | 0m | YSA | Beige mucoid lumpy distinct | rods | 363 | - |
| Pendant | 2m | 1/2 | orange distinct | rods | 364 | - |
| Druzhby | 0m | TSA | orange distinct | rods | 360 | - |
| Druzhby | 0m | TSA | Beige Mucoid Distinct | rods | 361 | - |
| Tassie | 0m | SWA | Off White Distinct | rods | 362 | - |
| Tassie | 0m | 1/2 | Off White Distinct | rods | 363 | - |
| Tassie | 0m | YSA | Beige Mucoid Lumpy | rods | 364 | - |
| Tassie | 0m | YSA | Beige Mucoid Lumpy | rods | 365 | - |
| Tassie | 0m | YSA | Pink mucoid | cocci | 366 | + |
| Tassie | 0m | TSA | Transparent Beige Mucoid | rods | 367 | - |
| Tassie | 0m | TSA | Beige Mucoid Lumpy | rods | 368 | - |
| Tassie | 0m | TSA | Beige Mucoid Lumpy | rods | 369 | - |
| Triple | 4m | SWA | White mucoid distinct | rods | 372 | - |
| Ace | 0m | YSA | Orange distinct | thin rods | 373 | - |
| Pendant | 4m | 1/2 | orange mucoid distinct | rods | 374 | - |
| Pointed | 0m | SWA | Off White Distinct | rods | 370 | - |
| Pointed | 0m | 1/2 | Off White Distinct | rods | 371 | - |
| Pointed | 0m | YSA | Beige Mucoid Lumpy | rods | 372 | - |
| Pointed | 0m | YSA | Beige Mucoid Cream | cocci | 373 | + |
| Pointed | 0m | YSA | Beige Mucoid | rods | 374 | - |
| Pointed | 0m | TSA | Beige Mucoid Lumpy | rods | 375 | - |
| Pointed | 0m | TSA | White Distinct | cocci | 376 | + |
| Pointed | 0m | TSA | Beige Mucoid | rods | 377 | - |
| Calendar | 0m | SWA | Off White Distinct | rods | 378 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Calendar | 0m | 1/2 | Yellow orange distinct | rods | 379 | - |
| Ace | 4m | SWA | Off White Distinct | rod | 38 | - |
| Triple | 4m | YSA | Beige Mucoid Lumpy | rods | 382 | - |
| Ace | 0m | 1/2 | off white mucoid distinct | rods | 383 | - |
| Pendant | 4m | 1/2 | off white mucoid distinct | rods | 384 | - |
| Calendar | 0m | 1/2 | Off white distinct mucoid | rods | 380 | - |
| Calendar | 0m | 1/2 | Off white mucoid distinct | rods | 381 | - |
| Calendar | 0m | YSA | Orange Yellow Mucoid | rods | 382 | - |
| Calendar | 0m | YSA | Beige Mucoid Lumpy | rods | 383 | - |
| Calendar | 0m | YSA | Transparent Beige mucoid | rods | 384 | - |
| Calendar | 0m | YSA | Rusty Orange lumpy mucoid | cocci | 385 | + |
| Calendar | 0m | TSA | Beige Mucoid | rods | 386 | - |
| Calendar | 0m | TSA | Beige Mucoid | rods | 387 | - |
| Calendar | 0m | TSA | Rusty Orange lumpy mucoid | cocci | 388 | + |
| Tridant | 0m | SWA | Off White Distinct | rods | 389 | - |
| Ace | 4m | SWA | Off White Distinct | rod | 39 | - |
| Triple | 4m | 1/2 | Off White Distinct | rods | 392 | - |
| Ace | 0m | 1/2 | Off white distinct mucoid | short rods | 393 | - |
| Pendant | 4m | SWA | off white mucoid distinct | rods | 394 | - |
| Tridant | 0m | 1/2 | Off White Distinct | rods | 390 | - |
| Tridant | 0m | YSA | Beige Mucoid Lumpy | rods | 391 | - |
| Tridant | 0m | YSA | Beige Mucoid | rods | 392 | - |
| Tridant | 0m | YSA | Beige Mucoid Lumpy | rods | 393 | - |
| Tridant | 0m | TSA | Beige Mucoid | rods | 394 | - |
| Tridant | 0m | TSA | Beige Mucoid | rods | 395 | - |
| Verenteno | 0m | SWA | Off White Distinct | rods | 396 | - |
| Verenteno | 0m | SWA | Off White Distinct | rods | 397 | + |
| Verenteno | 0m | 1/2 | Off White Distinct | rods | 398 | - |
| Verenteno | 0m | 1/2 | Off White Distinct | rods | 399 | - |
| Pendant | 4m | 1/2 | Off white mucoid distinct | rods | 42 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Triple | 8m | SWA | off white beige mucoid distinct | short rods | 43 | - |
| Pendant | 4m | TSA | Beige Mucoid | Long thin Rods | 44 | - |
| Pendant Ice | 50cm | YSA | Beige off white mucoid distinct | cigar shaped rods | 4ice3 | - |
| Triple | 8m | 1/2 | Off White Distinct | rods | 402 | - |
| Ace | 4m | SWA | Beige Mucoid Lumpy | rods | 403 | - |
| Pendant | 10m | YSA | dark yellow mucoid | rods | 404 | - |
| Verenteno | 0m | 1/2 | dark yellow mucoid | rods | 400 | - |
| Verenteno | 0m | 1/2 | White Distinct | cocci | 401 | + |
| Verenteno | 0m | 1/2 | Creamy white distinct | cocci | 402 | + |
| Verenteno | 0m | TSA | Beige Lumpy | rods | 403 | - |
| Verenteno | 0m | TSA | Beige Mucoid | rods | 404 | - |
| Verenteno | 0m | TSA | white distinct Mucoid | cocci | 405 | + |
| Verenteno | 0m | TSA | Beige mucoid | rods | 407 | - |
| Verenteno | 0m | TSA | Pink orange distinct mucoid | cocci | 408 | + |
| Verenteno | 0m | TSA | Pink orange distinct mucoid | cocci | 409 | + |
| Ace | 0m | SWA | Beige Mucoid | rods | 412 | - |
| Ace | 4m | TSA | Beige Mucoid Lumpy | rods | 413 | - |
| Pendant | 10m | TSA | transparent beige mucoid | rods | 414 | - |
| Verenteno | 0m | TSA | Transparent Beige | rods | 410 | - |
| Verenteno | 0m | YSA | Transparent Beige | rods | 411 | - |
| Verenteno | 0m | YSA | Yellow mucoid | rods | 412 | - |
| Verenteno | 0m | YSA | Transparent Beige | rods | 413 | - |
| Verenteno | 0m | YSA | Beige Transparent lumpy | rods | 414 | - |
| Verenteno | 0m | YSA | white Beige distinct mucoid | rods | 415 | - |
| Stinear | 0m | SWA | Off White Distinct | rods | 416 | - |
| Stinear | 0m | 1/2 | Off White Distinct | rods | 417 | - |
| Stinear | 0m | 1/2 | Off White Distinct | rods | 418 | - |
| Stinear | 0m | YSA | Beige Mucoid Lumpy | rods | 419 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace | 6m | TSA | Beige Mucoid Lumpy | rods | 42 | - |
| Ace | 0m | SWA | Beige Mucoid Distinct | rods | 422 | - |
| Ace | 4m | TSA | Beige Mucoid Lumpy | rods | 423 | - |
| Pendant | 10m | 1/2 | orange mucoid distinct | rods | 424 | - |
| Stinear | 0m | YSA | pink grey green fungus | na | 420 | na |
| Stinear | 0m | YSA | Beige Mucoid | rods | 421 | - |
| Stinear | 0m | YSA | Beige Mucoid | rods | 422 | - |
| Stinear | 0m | YSA | Beige Mucoid | rods | 423 | - |
| Stinear | 0m | YSA | white off white distinct | cocci | 424 | + |
| Stinear | 0m | YSA | Transparent Beige mucoid | rods | 425 | - |
| Stinear | 0m | TSA | Beige Mucoid Lumpy | rods | 426 | - |
| Stinear | 0m | TSA | Orange distinct | rods | 427 | - |
| Stinear | 0m | TSA | Beige Mucoid | rods | 428 | - |
| Stinear | 0m | TSA | Beige Mucoid | rods | 429 | - |
| Ace | 6m | TSA | Beige Mucoid Lumpy | rods | 43 | - |
| Ace | 0m | 1/2 | Off white mucoid | rods | 432 | - |
| Ace | 4m | YSA | off white beige mucoid distinct | rods | 433 | - |
| Pendant | 10m | 1/2 | off white distinct | rods | 434 | - |
| Stinear | 0m | TSA | Off White Distinct | cocci | 430 | + |
| Stinear | 0m | TSA | Beige Mucoid Lumpy | rods | 431 | - |
| Hand | 0m | SWA | Beige Mucoid Distinct | rods | 432 | - |
| Hand | 0m | 1/2 | Beige Mucoid Distinct | rods | 433 | - |
| Hand | 0m | TSA | Beige Mucoid Lumpy | rods | 434 | - |
| Hand | 0m | TSA | Off White Distinct | rods | 435 | - |
| Hand | 0m | TSA | Beige Mucoid Lumpy | rods | 436 | - |
| Hand | 0m | TSA | Beige Mucoid Lumpy | rods | 437 | - |
| Hand | 0m | TSA | Beige Mucoid Lumpy | rods | 438 | - |
| Hand | 0m | YSA | Beige Mucoid Lumpy | rods | 439 | - |
| Ace | 6m | TSA | Beige Mucoid Lumpy | rods | 44 | - |
| Ace | 0m | 1/2 | Off white distinct mucoid | rods | 442 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace | 4m | 1/2 | Off white distinct mucoid | rods | 443 | - |
| Ace | 8m | TSA | beige mucoid | rods | 444 | - |
| Hand | 0m | YSA | Beige Mucoid | rods | 440 | + |
| Hand | 0m | YSA | Beige Mucoid | rods | 441 | + |
| Dingle | 0m | SWA | Off White Distinct | rods | 442 | - |
| Dingle | 0m | 1/2 | Off White Distinct | rods | 443 | - |
| Dingle | 0m | TSA | Beige Mucoid Lumpy | rods | 444 | - |
| Dingle | 0m | TSA | Beige Mucoid Lumpy | rods | 445 | - |
| Dingle | 0m | TSA | Beige Mucoid Lumpy | rods | 446 | - |
| Dingle | 0m | TSA | Beige Mucoid Lumpy | rods | 447 | - |
| Dingle | 0m | YSA | Beige Mucoid Lumpy | rods | 448 | - |
| Dingle | 0m | YSA | Beige Mucoid Lumpy | rods | 449 | - |
| Ace | 6m | TSA | Beige Mucoid | rods | 45 | - |
| Ace | 0m | YSA | Beige Mucoid | short rods | 452 | - |
| Ace | 4m | 1/2 | Off white distinct mucoid | rods | 453 | - |
| Pendant | 0m | SWA | beige distinct | rods | 454 | - |
| Dingle | 0m | YSA | Beige Transparent Mucoid | rods | 450 | - |
| Dingle | 0m | YSA | Beige Transparent Mucoid | rods | 451 | - |
| Shield | 0m | YSA | Beige Transparent Mucoid | rods | 452 | - |
| Shield | 0m | YSA | Beige Transparent Mucoid | rods | 453 | - |
| Shield | 0m | YSA | Pink mucoid | cocci | 454 | + |
| Shield | 0m | YSA | Pink mucoid | cocci | 455 | + |
| Shield | 0m | YSA | Beige Mucoid | rods | 456 | - |
| Shield | 0m | TSA | Beige Mucoid | rods | 457 | - |
| Shield | 0m | TSA | Beige Mucoid | rods | 458 | - |
| Shield | 0m | TSA | Orange rusty mucoid distinct | cocci | 459 | + |
| Ace | 6m | YSA | White pink fungus | na | 46 | na |
| Ace | 0m | TSA | Beige Mucoid Distinct | rods | 462 | - |
| Deep | 0m | 1/2 | off white beige distinct | rods | 463 | - |
| Pendant | 0m | 1/2 | yellow distinct | short rods | 464 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Shield | 0m | TSA | Yellow white distinct | cocci | 460 | + |
| Shield | 0m | TSA | Beige Mucoid Lumpy | rods | 461 | - |
| Shield | 0m | SWA | Off white distinct | rods | 462 | - |
| Shield | 0m | 1/2 | Off white distinct | rods | 463 | - |
| Oval | 0m | SWA | Off white distinct | rods | 464 | - |
| Oval | 0m | SWA | Yellow white distinct | cocci | 465 | + |
| Oval | 0m | SWA | White fine distinct | rods | 466 | - |
| Oval | 0m | 1/2 | Off White Distinct | rods | 467 | - |
| Oval | 0m | YSA | Pink mucoid | cocci | 468 | + |
| Oval | 0m | YSA | Beige transparent mucoid | rods | 469 | - |
| Ace | 6m | YSA | Beige Mucoid Distinct | rods | 47 | - |
| Ace | 0m | TSA | Beige Mucoid | thin rods | 472 | - |
| Triple | 2m | SWA | Off white beige distinct | rods | 473 | - |
| Pendant | 0m | 1/2 | orange distinct | rods | 474 | - |
| Oval | 0m | YSA | Beige Mucoid Lumpy | rods | 470 | - |
| Oval | 0m | YSA | Beige Mucoid Lumpy | rods | 471 | - |
| Oval | 0m | TSA | Yellow white distinct | cocci | 472 | + |
| Oval | 0m | TSA | Beige Mucoid Lumpy | rods | 473 | - |
| Oval | 0m | TSA | Beige Mucoid | rods | 474 | - |
| Oval | 0m | TSA | Beige Mucoid Lumpy | rods | 475 | - |
| Jabs | 0m | 1/2 | Off white distinct mucoid | rods | 476 | - |
| Jabs | 0m | SWA | Off white mucoid | rods | 477 | - |
| Jabs | 0m | YSA | Transparent beige mucoid | rods | 478 | - |
| Jabs | 0m | YSA | Beige Mucoid | rods | 479 | - |
| Ace | 6m | YSA | Beige Mucoid Lumpy | rod | 48 | - |
| Ace | 0m | TSA | Off white distinct mucoid | cocci | 482 | - |
| Triple | 2m | TSA | Beige Mucoid | rods | 483 | - |
| Pendant | 0m | 1/2 | off white distinct | rods | 484 | - |
| Jabs | 0m | YSA | Transparent beige mucoid | rods | 480 | - |
| Jabs | 0m | YSA | Transparent beige | rods | 481 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Jabs | 0m | YSA | Beige Mucoid | rods | 482 | - |
| Jabs | 0m | TSA | Beige Mucoid | rods | 483 | - |
| Jabs | 0m | TSA | Beige Mucoid | rods | 484 | - |
| Jabs | 0m | TSA | Beige Mucoid Lumpy | rods | 485 | - |
| Jabs | 0m | TSA | Beige Mucoid Lumpy | rods | 486 | - |
| Jabs | 0m | TSA | Yellow Orange mucoid distinct | cocci | 487 | + |
| Jabs | 0m | TSA | Beige Mucoid Lumpy | rods | 488 | - |
| Deep | 0m | 1/2 | Beige Mucoid Distinct | rods | 489 | - |
| Ace | 6m | YSA | white mucoid distinct | rods | 49 | + |
| Ace | 0m | TSA | beige Orange Mucoid distinct | rods | 492 | - |
| Triple | 2m | TSA | Beige mucoid distinct | rods | 493 | - |
| Pendant | 0m | YSA | Dark Yellow/Orange Mucoid | rods | 494 | - |
| Deep | 0m | SWA | Off White Distinct | rods | 490 | - |
| Deep | 0m | YSA | Beige Mucoid Transparent | rods | 491 | - |
| Deep | 0m | YSA | pink white fungus | na | 492 | na |
| Deep | 0m | YSA | Bright Pink mucoid | cocci | 493 | + |
| Deep | 0m | YSA | Beige Mucoid Lumpy | rods | 494 | - |
| Deep | 0m | YSA | Beige Transparent Mucoid | rods | 495 | - |
| Deep | 0m | TSA | Beige Mucoid | rods | 496 | - |
| Deep | 0m | TSA | Beige Mucoid | rods | 497 | - |
| Deep | 0m | TSA | White mucoid distinct | cocci | 498 | + |
| Deep | 0m | TSA | Rusty Orange Mucoid lumpy | cocci | 499 | + |
| Ace | 0m | 1/2 | Off White Distinct | rod | 5 | - |
| Pendant | 10m | 1/2 | Off white distinct mucoid | Cocco bacilli | 52 | - |
| Triple | 8m | SWA | beige off white mucoid distinct | small rods | 53 | - |
| Pendant | 4m | 1/2 | Cream Distinct | Rods | 54 | - |
| Pendant Ice | 50cm | 1/2 | Off white Distinct | thin rods | 5ice3 | - |
| Ace | 6m | YSA | Beige Mucoid | rod | 50 | - |
| Ace | 2m | 1/2 | Beige Yellow distinct | small rods | 502 | - |
| Triple | 2m | TSA | Beige Mucoid Lumpy | long thin | 503 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | rods | | |
| Ace | 4m | SWA | off white distinct | rods | 504 | - |
| Beaver | 0m | SWA | Off White Distinct | rods | 500 | - |
| Beaver | 0m | 1/2 | Off White distinct Mucoid | rods | 501 | - |
| Beaver | 0m | 1/2 | Cream/white distinct mucoid | rods | 502 | - |
| Beaver | 0m | YSA | Beige Mucoid Lumpy | rods | 503 | - |
| Beaver | 0m | YSA | Beige Mucoid | rods | 504 | + |
| Beaver | 0m | YSA | White mucoid distinct | cocci | 505 | + |
| Beaver | 0m | YSA | Yellow Mucoid distinct | rods | 506 | - |
| Beaver | 0m | YSA | Beige Mucoid Lumpy | rods | 507 | - |
| Beaver | 0m | TSA | Beige Mucoid | rods | 508 | - |
| Beaver | 0m | TSA | Beige Mucoid | rods | 509 | - |
| Ace | 6m | SWA | Off White Distinct | rod | 51 | + |
| Ace | 2m | 1/2 | Yellow Mucoid distinct | rods | 512 | - |
| Triple | 2m | YSA | Beige Mucoid Lumpy | rods | 513 | - |
| Ace | 4m | 1/2 | off white distinct | rods | 514 | - |
| Beaver | 0m | TSA | Beige Mucoid Lumpy | rods | 510 | - |
| Beaver | 0m | TSA | Beige Mucoid Lumpy | rods | 511 | - |
| Crooked | 0m | YSA | Off White Distinct | rods | 512 | - |
| Crooked | 0m | YSA | White mucoid distinct | cocci | 513 | + |
| Crooked | 0m | YSA | Off white mucoid | rods | 514 | - |
| Crooked | 0m | YSA | Off white mucoid | cocci | 515 | + |
| Crooked | 0m | YSA | Orange Mucoid | rods | 516 | - |
| Crooked | 0m | YSA | Yellow Mucoid | rods | 517 | + |
| Crooked | 0m | SWA | White v fine | rods | 519 | - |
| Ace | 6m | SWA | Off White Distinct | rod | 52 | - |
| Ace | 2m | 1/2 | Off White Distinct | cocci | 522 | - |
| Triple | 2m | YSA | off white beige mucoid lumpy | long rods | 523 | - |
| Triple | 8m | SWA | orange red distinct | rods | 524 | - |
| Crooked | 0m | 1/2 | Off White Distinct | rods | 520 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Crooked | 0m | 1/2 | Off White Distinct | rods | 521 | - |
| Crooked | 0m | 1/2 | Off White Distinct | rods | 522 | - |
| Crooked | 0m | 1/2 | Off White Distinct | rods | 523 | - |
| Crooked | 0m | 1/2 | Beige Mucoid | rods | 524 | - |
| Crooked | 0m | TSA | Orange Mucoid distinct | rods | 525 | - |
| Crooked | 0m | TSA | Beige Mucoid | rods | 525 | - |
| Crooked | 0m | TSA | Beige Mucoid Lumpy | rods | 526 | - |
| Crooked | 0m | TSA | Beige Mucoid | rods | 526 | - |
| Crooked | 0m | TSA | Beige Mucoid | rods | 527 | + |
| Crooked | 0m | TSA | Orange Mucoid | rods | 528 | - |
| Crooked | 0m | TSA | Beige Distinct | rods | 529 | - |
| Ace | 2m | SWA | Off white yellow distinct | small rods | 532 | - |
| Triple | 2m | YSA | Beige Mucoid Lumpy | long rods | 533 | - |
| Triple | 8m | SWA | white distinct | rods | 534 | - |
| Crooked | 0m | TSA | white distinct | rods | 530 | + |
| Crooked | 0m | TSA | Off White Distinct | rods | 531 | - |
| Crooked | 0m | TSA | Orange distinct mucoid | cocci | 532 | + |
| Lebed | 0m | 1/2 | Off White Distinct | rods | 533 | - |
| Lebed | 0m | 1/2 | Off White Distinct | rods | 534 | - |
| Lebed | 0m | TSA | Beige Mucoid Distinct | rods | 535 | - |
| Lebed | 0m | TSA | Beige Mucoid Distinct | rods | 536 | - |
| Lebed | 0m | TSA | White mucoid distinct | cocci | 537 | + |
| Lebed | 0m | YSA | white Beige Mucoid Distinct | rods | 538 | - |
| Lebed | 0m | YSA | White mucoid distinct | rods | 539 | - |
| Ace | 6m | 1/2 | Off white beige mucoid distinct | rod | 54 | - |
| Ace | 2m | YSA | off white mucoid distinct | rods | 542 | - |
| Triple | 2m | 1/2 | Off White Distinct | short rods | 543 | - |
| Triple | 8m | TSA | beige mucoid | rods | 544 | - |
| Lebed | 0m | YSA | Pink Mucoid | cocci | 540 | + |
| Nicholson | 0m | TSA | Off White Distinct | cocci | 541 | + |

| | | | | | | |
|---|---|---|---|---|---|---|
| Nicholson | 0m | TSA | Beige Mucoid Distinct | rods | 542 | - |
| Nicholson | 0m | TSA | Beige Mucoid Distinct | rods | 543 | - |
| Nicholson | 0m | TSA | Yellow Orange mucoid smooth | cocci | 544 | + |
| Nicholson | 0m | YSA | White mucoid distinct | rods | 545 | - |
| Oblong | 0m | TSA | Beige Mucoid | rods | 546 | - |
| Oblong | 0m | TSA | Beige Mucoid | rods | 547 | - |
| Oblong | 0m | YSA | Orange Mucoid Lumpy | rods | 548 | + |
| Anderson | 0m | TSA | White mucoid distinct | rods | 549 | + |
| Ace | 8m | TSA | Beige Mucoid Distinct | rod | 55 | - |
| Ace | 2m | TSA | Off White Distinct | cocci | 552 | - |
| Triple | 2m | 1/2 | Off White Distinct | v short rods | 553 | - |
| Triple | 2m | TSA | beige mucoid | rods | 554 | - |
| Anderson | 0m | TSA | Beige Mucoid Lumpy | rods | 550 | - |
| Anderson | 0m | YSA | Beige Mucoid Lumpy | rods | 551 | - |
| Anderson | 0m | YSA | Orange Mucoid Lumpy | rods | 552 | - |
| Watts | 0m | 1/2 | Beige Mucoid Lumpy | rods | 553 | - |
| Watts | 0m | TSA | Beige Mucoid Lumpy | rods | 554 | - |
| Watts | 0m | TSA | Beige Mucoid | rods | 555 | - |
| Watts | 0m | TSA | Beige Mucoid | rods | 556 | - |
| Watts | 0m | YSA | Off white beige mucoid | rods | 557 | - |
| Watts | 0m | YSA | White mucoid Lumpy | rods | 558 | - |
| Watts | 0m | YSA | Beige Transparent mucoid | rods | 559 | - |
| Ace | 8m | TSA | Clear fine | cocci | 56 | + |
| Ace | 2m | TSA | beige distinct | small rods | 562 | - |
| Triple | 2m | 1/2 | Yellow distinct | short rods | 563 | - |
| Triple | 2m | 1/2 | orange mucoid distinct | small rods | 564 | - |
| Braunsteffer | 0m | 1/2 | Off White Distinct | cocci | 560 | + |
| Braunsteffer | 0m | YSA | fine white distinct | rods | 561 | - |
| Braunsteffer | 0m | TSA | White distinct | cocci | 562 | + |
| Braunsteffer | 0m | TSA | beige distinct | small rods | 563 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pauk | 0m | SWA | Off White Distinct | cocci | 564 | + |
| Pauk | 0m | YSA | beige distinct | rods | 565 | - |
| Pauk | 0m | TSA | Beige Mucoid Distinct | rods | 566 | - |
| Pauk | 0m | TSA | White cream distinct odd | cocci | 567 | + |
| Scale | 0m | 1/2 | Off White Distinct | rods | 568 | - |
| Scale | 0m | 1/2 | Off White Distinct | rods | 568 | - |
| Scale | 0m | TSA | Beige Mucoid | rods | 569 | - |
| Scale | 0m | TSA | Beige Mucoid | rods | 569 | - |
| Ace | 8m | TSA | Beige Mucoid | rods | 57 | - |
| Ace | 2m | TSA | Yellow Orange mucoid distinct | small rods | 572 | - |
| Triple | 0m | SWA | Off white beige distinct mucoid | rods | 573 | - |
| Triple | 2m | 1/2 | orange red distinct | rods | 574 | - |
| Scale | 0m | T SA | Beige Transparent mucoid | rods | 570 | - |
| Scale | 0m | TSA | Beige Transparent mucoid | rods | 570 | - |
| Scale | 0m | TSA | Beige Clear distinct | rods | 571 | - |
| Scale | 0m | TSA | Beige Clear distinct | rods | 571 | - |
| Scale | 0m | TSA | Beige Mucoid Distinct | rods | 572 | - |
| Scale | 0m | TSA | Beige Mucoid Distinct | rods | 572 | - |
| Scale | 0m | YSA | Beige Mucoid Distinct | rods | 573 | - |
| Scale | 0m | YSA | Beige Mucoid Distinct | rods | 573 | - |
| Scale | 0m | YSA | Orange Mucoid | rods | 574 | - |
| Scale | 0m | YSA | Orange Mucoid | rods | 574 | - |
| Scale | 0m | YSA | Beige Mucoid Distinct | rods | 575 | - |
| Scale | 0m | YSA | Beige Mucoid Distinct | rods | 575 | - |
| Zvezda | 0m | 1/2 | White distinct | rods | 576 | - |
| Zvezda | 0m | YSA | White distinct | cocci | 577 | + |
| Zvezda | 0m | YSA | White distinct | rods | 578 | - |
| Zvezda | 0m | TSA | White mucoid distinct | rods | 579 | - |
| Ace | 8m | TSA | Beige Mucoid Lumpy | rods | 58 | - |
| Ace | 2m | TSA | Beige Mucoid Distinct | small rods | 582 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Triple | 0m | SWA | Off white beige distinct mucoid | rods | 583 | - |
| Triple | 0m | SWA | beige distinct | rods | 584 | - |
| Zvezda | 0m | TSA | Beige Mucoid Distinct | rods | 580 | - |
| Zvezda | 0m | TSA | Beige Mucoid Distinct | rods | 581 | - |
| Collerson | 0m | 1/2 | Yellow White distinct | cocci | 582 | + |
| Collerson | 0m | 1/2 | White fine distinct | rods | 583 | - |
| Collerson | 0m | 1/2 | Off white distinct mucoid | rods | 584 | - |
| Collerson | 0m | SWA | Yellow White distinct | cocci | 585 | + |
| Collerson | 0m | SWA | White distinct | rods | 586 | + |
| Collerson | 0m | SWA | White distinct | rods | 587 | - |
| Collerson | 0m | TSA | Beige Mucoid Distinct | rods | 588 | - |
| Collerson | 0m | TSA | White mucoid distinct | rods | 589 | - |
| Ace | 8m | YSA | Beige Mucoid | rods | 59 | - |
| Ace | 4m | SWA | Off White Distinct | curved rods | 592 | - |
| Triple | 0m | TSA | Beige Mucoid | rods | 593 | - |
| Triple | 0m | 1/2 | clear distinct | rods | 594 | - |
| Collerson | 0m | TSA | Yellow Beige Mucoid | rods | 590 | - |
| Collerson | 0m | YSA | Orange distinct | rods | 591 | - |
| Collerson | 0m | YSA | Beige Mucoid Distinct | rods | 592 | - |
| Collerson | 0m | YSA | White distinct | cocci | 593 | + |
| Medusa | 0m | 1/2 | White distinct | cocci | 594 | + |
| Medusa | 0m | 1/2 | White distinct | cocci | 595 | + |
| Medusa | 0m | 1/2 | White distinct | cocci | 596 | + |
| Medusa | 0m | 1/2 | White distinct | rods | 597 | - |
| Medusa | 0m | 1/2 | Off White Distinct | cocci | 598 | + |
| Medusa | 0m | SWA | Transparent white distinct | rods | 599 | - |
| Ace | 0m | YSA | Beige Mucoid | rods | 6 | - |
| Pendant | 10m | YSA | Beige Mucoid Distinct | thin rods | 62 | - |
| Triple | 8m | TSA | beige mucoid distinct | rods | 63 | - |
| Pendant | 4m | SWA | Beige Cream mucoid distinct | rods | 64 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace | 8m | YSA | White mucoid Lumpy | rod | 60 | + |
| Ace | 4m | 1/2 | Yellow Distinct | cocci | 602 | - |
| Triple | 0m | TSA | Off white beige distinct mucoid | rods | 603 | - |
| Triple | 0m | 1/2 | orange dark mucoid | rods | 604 | - |
| Medusa | 0m | SWA | Yellow White distinct | cocci | 600 | + |
| Medusa | 0m | SWA | Off White Distinct | rods | 601 | - |
| Medusa | 0m | TSA | Beige Distinct | cocci | 602 | + |
| Medusa | 0m | TSA | Beige Mucoid Distinct | rods | 603 | - |
| Medusa | 0m | TSA | Yellow/Orange Distinct | cocci | 604 | + |
| Medusa | 0m | TSA | White mucoid distinct | cocci | 605 | + |
| Medusa | 0m | YSA | Beige fine distinct | rods | 606 | - |
| Medusa | 0m | YSA | Beige fine distinct | rods | 607 | - |
| Medusa | 0m | YSA | Clear white fine distinct | rods | 608 | - |
| Organic | 0m | SWA | White Beige distinct fine | cocci | 609 | + |
| Ace | 4m | TSA | Beige Mucoid | small rods | 612 | - |
| Triple | 0m | TSA | cream mucoid | rods | 613 | - |
| Triple | 0m | TSA | cream mucoid | cocci | 614 | + |
| Organic | 0m | YSA | off white distinct | rods | 610 | - |
| Organic | 0m | YSA | White mucoid distinct | rods | 611 | - |
| Organic | 0m | YSA | White mucoid distinct | rods | 612 | - |
| Organic | 0m | TSA | Beige Mucoid Lumpy | rods | 613 | - |
| Organic | 0m | TSA | Off White Distinct | rods | 614 | + |
| Organic | 0m | 1/2 | Beige Mucoid Lumpy | rods | 615 | - |
| Abraxas | 0m | 1/2 | Off White Distinct | rods | 616 | - |
| Abraxas | 0m | TSA | Beige Mucoid Lumpy | rods | 617 | - |
| Abraxas | 0m | TSA | Beige Mucoid Distinct | rods | 618 | - |
| Abraxas | 0m | TSA | Clear Fine | cocci | 619 | + |
| Ace | 8m | YSA | Beige Mucoid Lumpy | rods | 62 | - |
| Ace | 4m | YSA | Yellow Orange fine distinct | thin rods | 622 | - |
| Triple | 0m | YSA | off white beige distinct | rods | 623 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Triple | 0m | TSA | white fungi | na | 624 | na |
| Abraxas | 0m | TSA | Beige Mucoid Distinct | rods | 620 | - |
| Abraxas | 0m | YSA | Pink White Green Fungus | na | 621 | na |
| Abraxas | 0m | YSA | White distinct mucoid | rods | 622 | - |
| Abraxas | 0m | YSA | White Mucoid distinct | rods | 623 | - |
| Abraxas | 0m | YSA | Off white distinct mucoid | rods | 624 | - |
| Abraxas | 0m | SWA | Clear Fine | cocci | 625 | + |
| Pendant | 0m | YSA | Beige Mucoid Lumpy | rods | 626 | - |
| Pendant | 0m | YSA | Beige Mucoid Lumpy | rods | 627 | - |
| Pendant | 0m | YSA | cream white mucoid distinct | rods | 628 | - |
| Pendant | 0m | YSA | Beige Mucoid Distinct | rods | 629 | - |
| Ace | 8m | SWA | Clear Fine | cocci | 63 | + |
| Ace | 8m | SWA | Off White Distinct | rods | 632 | - |
| Triple | 0m | YSA | Beige Mucoid Lumpy | rods | 633 | - |
| Deep | 0m | 1/2 | orange mucoid distinct | rods | 634 | - |
| Pendant | 0m | YSA | orange distinct mucoid | cocci | 630 | + |
| Pendant | 0m | TSA | Orange Mucoid distinct | cocci | 631 | + |
| Pendant | 0m | TSA | Beige Mucoid Lumpy | rods | 632 | - |
| Pendant | 0m | TSA | Pale orange mucoid distinct | rods | 633 | - |
| Pendant | 0m | TSA | Beige Mucoid Lumpy | rods | 634 | - |
| Pendant | 0m | TSA | Beige Mucoid Lumpy | rods | 635 | - |
| Pendant | 0m | TSA | Beige Mucoid Lumpy | rods | 636 | - |
| Pendant | 0m | 1/2 | Off White Distinct | rods | 637 | - |
| Pendant | 0m | 1/2 | Beige Mucoid Lumpy | rods | 638 | - |
| Pendant | 0m | 1/2 | Beige Mucoid Lumpy | rods | 639 | - |
| Ace | 8m | 1/2 | Yellow Distinct | long rods | 642 | - |
| Triple | 0m | 1/2 | Off white distinct mucoid | small rods | 643 | - |
| Deep | 0m | 1/2 | clear mucoid distinct | rods | 644 | - |
| Pendant | 0m | 1/2 | Off White Distinct | rods | 640 | - |
| Pendant | 2m | YSA | Beige Mucoid | rods | 641 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pendant | 2m | YSA | Orange Mucoid distinct | rods | 642 | - |
| Pendant | 2m | YSA | Beige Mucoid | rods | 643 | - |
| Pendant | 2m | YSA | Orange Mucoid distinct | cocci | 644 | + |
| Pendant | 2m | YSA | Cream Beige Mucoid Lumpy | rods | 645 | - |
| Pendant | 2m | TSA | Orange Mucoid distinct | cocci | 646 | + |
| Pendant | 2m | TSA | Beige Mucoid | rods | 647 | - |
| Pendant | 2m | TSA | Pale Orange mucoid lumpy | rods | 648 | - |
| Pendant | 2m | TSA | yellow distinct | rods | 649 | - |
| Ace | 8m | 1/2 | Off white distinct mucoid | rods | 65 | - |
| Ace | 8m | 1/2 | Off white mucoid distinct | rods | 652 | - |
| Triple | 0m | 1/2 | yellow distinct | rods | 653 | - |
| Deep | 0m | 1/2 | yellow distinct | long rods | 654 | - |
| Pendant | 2m | TSA | Beige Mucoid | rods | 650 | - |
| Pendant | 2m | TSA | Pale yellow distinct | cocci | 651 | + |
| Pendant | 2m | 1/2 | Off White Distinct | rods | 652 | - |
| Pendant | 2m | 1/2 | Pale yellow distinct | cocci | 653 | + |
| Pendant | 2m | 1/2 | Off White Distinct | rods | 654 | - |
| Pendant | 8m | YSA | Beige/off white mucoid | rods | 655 | - |
| Pendant | 8m | YSA | Clear/off white distinct fine | cocci | 656 | + |
| Pendant | 8m | YSA | white distinct | rods | 657 | - |
| Pendant | 8m | YSA | Beige/off white mucoid lumpy | rods | 658 | - |
| Pendant | 8m | TSA | Rusty orange distinct mucoid | rods | 659 | - |
| Ace | 8m | 1/2 | Off White Distinct | rods | 662 | - |
| Triple | 0m | 1/2 | Off White Distinct | rods | 663 | - |
| Club | 0m | 1/2 | orange distinct | rods | 664 | - |
| Pendant | 8m | TSA | Beige/off white mucoid distinct | rods | 660 | - |
| Pendant | 8m | TSA | Rusty orange distinct | rods | 661 | - |
| Pendant | 8m | TSA | Beige/off white mucoid distinct | rods | 662 | - |
| Pendant | 8m | TSA | Beige Mucoid Lumpy | rods | 663 | - |
| Pendant | 8m | TSA | Beige Mucoid Distinct | rods | 664 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pendant | 8m | 1/2 | Clear /off white distinct | rods | 665 | - |
| Pendant | 8m | 1/2 | Clear/off white distinct fine | rods | 666 | - |
| Pendant | 8m | 1/2 | Off white distinct mucoid | rods | 667 | - |
| LH73 | 0m | SWA | Clear/off white distinct fine | rods | 668 | - |
| LH73 | 0m | SWA | Clear/off white distinct fine | rods | 669 | + |
| Ace | 10m | TSA | Yellow white mucoid lumpy | rods | 67 | + |
| Ace | 8m | 1/2 | Yellow Distinct | rods | 672 | - |
| Pendant | 4m | YSA | Beige distinct circles | rods | 673 | - |
| Triple | 4m | YSA | white/pink fungus | na | 674 | na |
| LH73 | 0m | SWA | Beige Mucoid | rods | 670 | - |
| LH73 | 0m | TSA | Beige Mucoid | rods | 671 | - |
| LH73 | 0m | TSA | Beige Mucoid | rods | 672 | - |
| LH73 | 0m | TSA | Beige Mucoid | rods | 673 | + |
| LH73 | 0m | TSA | Beige Mucoid Distinct | rods | 674 | - |
| LH73 | 0m | YSA | Beige Mucoid Distinct | rods | 675 | - |
| LH73 | 0m | 1/2 | Off White Distinct | rods | 676 | - |
| Reid | 0m | TSA | Beige Distinct | rods | 677 | - |
| Reid | 0m | 1/2 | Beige Mucoid | rods | 678 | - |
| Reid | 0m | 1/2 | Yellow off-white distinct | rods | 679 | - |
| Ace | 10m | TSA | Beige Mucoid Distinct | rods | 68 | - |
| Ace | 8m | YSA | Off white mucoid distinct | bone shaped rods | 682 | - |
| Pendant | 4m | YSA | Beige mucoid distinct | rods | 683 | - |
| Ace | 0m | SWA | clear off white distinct | rods | 684 | - |
| Reid | 0m | YSA | Beige mucoid distinct | rods | 680 | - |
| Angel2 | 0m | 1/2 | White distinct | cocci | 681 | + |
| Angel2 | 0m | 1/2 | Off White Distinct | rods | 682 | - |
| Angel2 | 0m | SWA | White distinct | cocci | 683 | + |
| Angel2 | 0m | YSA | Off White Distinct | rods | 684 | + |
| Angel2 | 0m | YSA | beige | rods | 685 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Angel2 | 0m | TSA | Off white very fine distinct | rods | 686 | + |
| Angel2 | 0m | TSA | White mucoid distinct | rods | 687 | - |
| Laternula | 0m | 1/2 | Off White Distinct | rods | 688 | - |
| Laternula | 0m | 1/2 | Off White Distinct | rods | 689 | - |
| Ace | 10m | TSA | Beige Mucoid | rods | 69 | - |
| Ace | 8m | TSA | Beige Orange mucoid Distinct | thin rods | 692 | - |
| Pendant | 4m | TSA | Beige mucoid | rods | 693 | - |
| Ace | 2m | TSA | beige mucoid distinct | rods | 694 | - |
| Laternula | 0m | SWA | Off White Distinct | rods | 690 | - |
| Laternula | 0m | YSA | Beige Mucoid | rods | 691 | - |
| Laternula | 0m | YSA | Beige Mucoid | rods | 692 | - |
| Laternula | 0m | YSA | Beige Mucoid Distinct | rods | 693 | - |
| Laternula | 0m | TSA | Beige Mucoid Lumpy | rods | 694 | - |
| Laternula | 0m | TSA | Beige Mucoid Lumpy | rods | 695 | - |
| Laternula | 0m | TSA | Beige Mucoid Distinct | rods | 696 | - |
| Cemetery | 0m | 1/2 | Beige Distinct | rods | 697 | - |
| Cemetery | 0m | 1/2 | Beige Mucoid Distinct | rods | 698 | - |
| Cemetery | 0m | SWA | Off white distinct mucoid | rods | 699 | - |
| Pedant | 0m | 1/2 | Off White Distinct | rods | 72 | - |
| Triple | 8m | TSA | Beige mucoid lumpy | long rods | 73 | - |
| Pendant | 10m | YSA | White Mucoid Distinct | cocci | 74 | - |
| Ace | 10m | YSA | White mucoid distinct | rods | 70 | + |
| Ace | 8m | TSA | Beige Mucoid Distinct | v fine rods | 702 | - |
| Pendant | 4m | TSA | cream beige mucoid | rods | 703 | - |
| Ace ice | I-W | TSA | beige mucoid | small rods | 704 | - |
| Cemetery | 0m | TSA | beige mucoid | rods | 700 | - |
| Ace 2 | 10m | TSA | Beige Mucoid | rods | 701 | - |
| Ace 2 | 10m | TSA | Beige Mucoid | rods | 702 | + |
| Ace 2 | 10m | TSA | Yellow white mucoid | cocci | 703 | + |
| Ace 2 | 10m | TSA | Beige Distinct | rods | 704 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace 2 | 10m | YSA | Rusty beige fine distinct | rods | 705 | - |
| Ace 2 | 10m | YSA | Beige Mucoid | rods | 706 | - |
| Ace 2 | 10m | YSA | Orange Mucoid | rods | 707 | - |
| Ace 2 | 10m | YSA | Yellow Orange Mucoid Lumpy | rods | 708 | - |
| Ace 2 | 10m | YSA | Beige Mucoid Lumpy | rods | 709 | - |
| Ace | 10m | YSA | Beige Mucoid Lumpy | rod | 71 | - |
| Ace | 10m | SWA | White mucoid distinct | rods | 712 | - |
| Pendant | 4m | 1/2 | off white beige mucoid distinct | rods | 713 | - |
| Ace ice | I-W | 1/2 | off white fine distinct | rods | 714 | - |
| Ace 2 | 10m | 1/2 | Off White Distinct | rods | 710 | - |
| Ace 2 | 10m | 1/2 | Beige Distinct | rods | 711 | - |
| Ace 2 | 10m | SWA | Beige Mucoid Distinct | rods | 712 | - |
| Ace 2 | 8m | TSA | Beige Mucoid Lumpy | rods | 713 | - |
| Ace 2 | 8m | TSA | Yellow beige Mucoid distinct | rods | 714 | - |
| Ace 2 | 8m | TSA | Cream Beige Mucoid | rods | 715 | - |
| Ace 2 | 8m | YSA | Beige Mucoid | rods | 716 | - |
| Ace 2 | 8m | YSA | white Beige Mucoid Distinct | rods | 717 | - |
| Ace 2 | 8m | YSA | Orange Mucoid Lumpy | rods | 718 | - |
| Ace 2 | 8m | 1/2 | Off white distinct mucoid | rods | 719 | - |
| Ace | 10m | 1/2 | Off white mucoid distinct | cocci | 722 | - |
| Pendant | 4m | 1/2 | off white fine distinct | rods | 723 | - |
| Ace ice | I-W | SWA | beige fine distinct | rods | 724 | - |
| Ace 2 | 8m | 1/2 | Yellow off-white distinct | rods | 720 | - |
| Ace 2 | 8m | 1/2 | Off White Distinct | rods | 721 | - |
| Ace 2 | 8m | SWA | Off White Distinct | rods | 722 | - |
| Ace 2 | 0m | TSA | Beige Mucoid | rods | 723 | - |
| Ace 2 | 0m | YSA | Off white beige mucoid | rods | 724 | - |
| Ace 2 | 0m | YSA | Beige Mucoid | rods | 725 | - |
| Ace 2 | 0m | 1/2 | Beige White distinct | rods | 726 | - |
| Ace 2 | 0m | 1/2 | Beige White distinct | rods | 727 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace 2 | 0m | SWA | Off White Distinct | rods | 728 | - |
| Ace 2 | 2m | TSA | Beige Mucoid | rods | 729 | - |
| Ace | 10m | SWA | Off White Distinct | rod | 73 | - |
| Ace | 10m | 1/2 | Off White Distinct | rods | 732 | - |
| Pendant | 0m | SWA | Yellow distinct | rods | 733 | - |
| Pendant ice | 50cm | TSA | beige mucoid | cocci | 734 | - |
| Ace 2 | 2m | TSA | Beige Mucoid | rods | 730 | - |
| Ace 2 | 2m | YSA | Beige Mucoid Lumpy | rods | 731 | - |
| Ace 2 | 2m | YSA | White mucoid distinct | rods | 732 | - |
| Ace 2 | 2m | 1/2 | Off white distinct mucoid | rods | 733 | - |
| Ace 2 | 2m | 1/2 | Cream beige mucoid distinct | rods | 734 | - |
| Ace 2 | 2m | SWA | Cream Beige distinct | rods | 735 | - |
| Ace | 10m | 1/2 | Off white distinct mucoid | small rods | 742 | - |
| Pendant | 0m | 1/2 | off white mucoid distinct | Cocco bacilli | 743 | - |
| Pendant ice | 50cm | SWA | beige fine | rods | 744 | - |
| Ace | 10m | 1/2 | Off White Distinct | rod | 75 | - |
| Ace | 10m | TSA | Off white distinct clear | rods | 752 | - |
| Pendant | 0m | 1/2 | Yellow distinct | rods | 753 | - |
| Pendant ice | I-W | YSA | yellow mucoid | rods | 754 | - |
| Ace | 10m | YSA | Beige Mucoid Distinct | rods | 762 | - |
| Pendant | 0m | TSA | beige mucoid distinct | long rods | 763 | - |
| Pendant ice | I-W | YSA | orange mucoid | Long rods | 764 | - |
| Ace | 0m | YSA | Beige Mucoid Distinct | rod | 77 | - |
| Pendant | 0m | YSA | cream beige mucoid distinct | rods | 773 | - |
| Pendant ice | I-W | TSA | beige mucoid | rods | 774 | - |
| Ace | 0m | TSA | Beige Mucoid Lumpy | rod | 78 | - |
| Pendant | 10m | SWA | Clear off white fine distinct | rods | 783 | - |
| Pendant ice | I-W | SWA | beige/off white distinct | rods | 784 | - |
| Ace | 0m | 1/2 | Off white distinct mucoid | rod | 79 | - |
| Pendant | 10m | YSA | Beige off white mucoid distinct | long rods | 793 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pendant ice | I-W | 1/2 | off white distinct | rods | 794 | - |
| Pendant | 0m | SWA | Off White Distinct | rods | 82 | - |
| Triple | 8m | YSA | beige mucoid lumpy distinct | rods | 83 | - |
| Pendant | 8m | 1/2 | Beige Fine Distinct | Small Rods | 84 | - |
| Ace | 0m | SWA | Beige Mucoid Distinct | rod | 80 | - |
| Club | 0m | 1/2 | Off white distinct mucoid | rods | 802 | - |
| Pendant | 10m | TSA | Beige mucoid lumpy | rods | 803 | - |
| Ace ice | 50cm | TSA | beige mucoid | cocci | 804 | - |
| Ace | 2m | TSA | Beige Mucoid Lumpy | rod | 81 | - |
| Club | 0m | 1/2 | Off white distinct mucoid | rods | 812 | - |
| Pendant | 8m | TSA | Orange beige mucoid distinct | rods | 813 | - |
| Ace ice | 50cm | 1/2 | off white distinct | small rods | 814 | - |
| Ace | 2m | YSA | Beige Mucoid Lumpy | rod | 82 | - |
| Club | 0m | YSA | Beige Mucoid Distinct | rods | 822 | - |
| Pendant | 8m | SWA | clear off white very fine distinct | rods | 823 | - |
| Ace | 2m | SWA | off white mucoid | rod | 83 | - |
| Pendant | 8m | 1/2 | Beige off white mucoid distinct | rods | 833 | - |
| Ace | 2m | 1/2 | Beige Mucoid Lumpy | rod | 84 | - |
| Pendant | 2m | YSA | Beige mucoid distinct off white | large rods | 843 | - |
| Ace | 2m | 1/2 | Off White Distinct | rod | 85 | - |
| Pendant | 2m | 1/2 | Off white distinct mucoid | small rods | 853 | - |
| Ace | 4m | TSA | Beige Mucoid | rod | 86 | - |
| Pendant | 2m | SWA | Off White Distinct | long rods | 863 | - |
| Ace | 4m | YSA | Beige Mucoid Distinct | rod | 87 | - |
| Ace | 4m | SWA | Beige Mucoid | rod | 88 | - |
| Ace | 4m | 1/2 | Off White Distinct | rod | 89 | - |
| Pendant | 8m | TSA | beige mucoid distinct | v thin rods | 92 | - |
| Triple | 8m | YSA | yellow white vv fine distinct | short rods | 93 | - |
| Pendant | 8m | TSA | Beige Mucoid Lumpy | Rods | 94 | - |
| Ace | 6m | TSA | Beige Mucoid | rod | 90 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ace | 6m | YSA | Beige Mucoid Lumpy | rod | 91 | - |
| Ace | 6m | 1/2 | Off White Distinct | rod | 93 | - |
| Ace | 8m | TSA | Beige Mucoid | rod | 94 | - |
| Ace | 8m | YSA | Beige Mucoid | rod | 95 | - |
| Ace | 8m | SWA | Off White Distinct | rod | 96 | - |
| Ace | 10m | TSA | Beige Mucoid Lumpy | rod | 98 | - |
| Ace | 10m | YSA | Beige Mucoid | rod | 99 | - |

**B2 – Similarity matrices calculated from the Dice coefficient for the (A) *Hpa* II and (B) *Alu* I restriction digest patterns for the ARDRA analysis (Section 5.2.1.) *mp* = *Marinomonas protea*, *mp rep* = *M. protea* replicate.**

**(A)– *Hpa*II similarity matrix**

| Isolates | 466 | 47 | mp | 154 | 124 | 5ice3 | 744 | 794 | 214 | 54 | 494 | 583 | 213 | 302 | 33 | 492 | 53 | 732 | 39 | 86 | Mp rep |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 466 | 1 | | | | | | | | | | | | | | | | | | | | |
| 47 | 0.4 | 1 | | | | | | | | | | | | | | | | | | | |
| Mp | 0 | 0.5 | 1 | | | | | | | | | | | | | | | | | | |
| 154 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | |
| 124 | 0 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | |
| 5ice3 | 0.4 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | |
| 744 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | |
| 794 | 0 | 0.5 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | |
| 214 | 0 | 0.5 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | |
| 54 | 0 | 0.5 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | |
| 494 | 0 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 1 | | | | | | | | | | |
| 583 | 0 | 0 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0 | 1 | | | | | | | | | |
| 213 | 0 | 0 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.66 | 1 | | | | | | | | |
| 302 | 0 | 0 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0 | 0.66 | 0.66 | 1 | | | | | | | |
| 33 | 0 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0 | 0.66 | 0.33 | 1 | 1 | | | | | | |
| 492 | 0.4 | 1 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0 | 0 | 0 | 0 | 1 | | | | | |
| 53 | 0 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0 | 0.33 | 0.33 | 0.66 | 0.66 | 0 | 1 | | | | |
| 732 | 0.4 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0 | 0.4 | 0.4 | 0.8 | 0.8 | 0 | 0.8 | 1 | | | |
| 39 | 0.8 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0 | 0 | 0 | 0.4 | 0.4 | 0 | 0.4 | 0.5 | 1 | | |
| 86 | 0.4 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0 | 0 | 0 | 0.4 | 0.4 | 0 | 0.4 | 0.5 | 1 | 1 | |
| Mp rep | 0.4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.5 | 0 | 0 | 0.4 | 0.4 | 0.5 | 0.4 | 0.5 | 0.5 | 0.5 | 1 |

**(B) – *Alu*I similarity matrix.**

| Isolates | 466 | 47 | Mp | 154 | 124 | 5ice3 | 744 | 794 | 214 | 54 | 494 | 583 | 213 | 302 | 33 | 492 | 53 | 732 | 39 | 86 | mprep |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 466 | 1 | | | | | | | | | | | | | | | | | | | | |
| 47 | 0.25 | 1 | | | | | | | | | | | | | | | | | | | |
| Mp | 0.57 | 0.28 | 1 | | | | | | | | | | | | | | | | | | |
| 154 | 0.57 | 0.57 | 1 | 1 | | | | | | | | | | | | | | | | | |
| 124 | 0.57 | 0.57 | 1 | 1 | 1 | | | | | | | | | | | | | | | | |
| 5ice3 | 0.85 | 0.57 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | |
| 744 | 0.85 | 0.57 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | |
| 794 | 0.85 | 0.57 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | |
| 214 | 0.85 | 0.57 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | |
| 54 | 0.85 | 0.28 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | |
| 494 | 0.4 | 0.4 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 1 | | | | | | | | | | |
| 583 | 0.25 | 0.25 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.4 | 1 | | | | | | | | | |
| 213 | 0.57 | 0.28 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.5 | 0.85 | 1 | | | | | | | | |
| 302 | 0.57 | 0 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.5 | 0.57 | 0.66 | 1 | | | | | | | |
| 33 | 0.57 | 0 | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 | 0.5 | 0.28 | 0.33 | 0.66 | 1 | | | | | | |
| 492 | 0.25 | 0.75 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.4 | 0.5 | 0.28 | 0.28 | 0.28 | 1 | | | | | |
| 53 | 0.5 | 0.25 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.4 | 0.75 | 0.85 | 0.57 | 0.28 | 0.5 | 1 | | | | |
| 732 | 0.88 | 0.44 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.33 | 0.88 | 0.75 | 0.5 | 0.25 | 0.66 | 0.88 | 1 | | | |
| 39 | 0.25 | 0.75 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.4 | 0.75 | 0.57 | 0.28 | 0.28 | 0.75 | 0.75 | 0.25 | 1 | | |
| 86 | 0.25 | 0.5 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 | 0.4 | 0.75 | 0.57 | 0.28 | 0.28 | 0.75 | 0.75 | 0.25 | 1 | 1 | |
| mprep | 0.85 | 0.28 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.5 | 0.33 | 0.33 | 0.33 | 0.66 | 0.57 | 0.57 | 0.5 | 0.57 | 0.57 | 1 |