Characterization of the bacterioplankton communities in the melt-water ponds of Bratina Island, Victoria Land, Antarctica

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Abstract

Antarctic ecosystems (such as the ponds by Bratina Island, Antarctica) provide an excellent opportunity to examine organisms that can live in one of the most extreme and geochemically varied environments in the world. These ponds are of interest as each one can vary greatly in size, depth, and age as well as profiles of dissolved oxygen, metal concentrations, pH and salinity. Even within ponds geochemically distinct stratified layers can form which can greatly influence their microbial communities. There are a number of studies which indicate that microbial populations found in Antarctic ponds will be highly diverse and variable due to the uniqueness of the environment. This study aims to increase our knowledge of microbial biodiversity and the environmental factors which structure them, in particular the stratification transition zones within ponds water columns. A thorough set of biological samples were taken from five selected ponds during mid-summer in the 09-10 season to complement those taken during the winter freeze-up in the 07-08 extended season by Hawes and co-workers. Oxygen concentration, pH, conductivity and temperature of each pond water sample were measured in the field and water samples were taken back to the University of Waikato for further analysis. This research primarily used the DNA fingerprinting technique ARISA, matched with geochemistry to identify and characterise the resident and functional members of the microbial community and understand how the community is structured in relation to environmental conditions. We found that the planktonic populations of the Bratina Island ponds do vary between ponds, that each pond has its own chemical signature and that populations do change with depth. One of the studied ponds, Egg, was found to

have an extreme chemical stratification leading to significantly different populations at each depth. Data analysis using BEST analysis determined that the changes in the bacterial populations in Egg are primarily in relation to the pH and conductivity at each depth which changes dramatically in the lower depths.

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List of Abbreviations

ARISA – Automated Ribosomal Intergenic Spacer Analysis

AFL – Automated ribosomal intergenic spacer analysis fragment length

Bp – Base pairs

BSA – Bovine Serum albumin

CTAB - cetyltrimethylammonium bromide-polyvinylpyrrolidone-β-mecaptoethanol

DGGE - Denaturing Gradient Gel Electrophoresis

ICPMS - inductively coupled plasma mass spectrometry

ITS – Internal Transcribed Spacer

MDS – Multi Dimentional Scaling

MIS – McMurdo Ice Shelf

M-TRFLP - Multiplex Terminal Restriction Fragment Length Polymorphism

Mya – million years ago

PCR - Polymerase Chain Reaction

q-PCR - quantitative Polymerase Chain Reaction

TRFLP – Terminal Restriction Fragment Length Polymorphism

rRNA – Ribosomal ribonucleic acid

Chapter 1: Literature Review

One of the fundamental challenges in biology today is to understand how organisms respond and evolve in relation to changing environments (Kussell et al., 2005). The ponds on Bratina Island (78° 01' S latitude, 165° 32' E longitude on the McMurdo ice shelf (MIS) in the Ross Sea area of Antarctica) provide an extraordinary and tractable opportunity to examine metabolic functioning and the adaptations that allow microbial communities to thrive under the extreme and diverse conditions experienced in Antarctica. However even after more than 100 years of Antarctic exploration we still know little about the microbial biodiversity and processes that occur in Antarctic ecosystems and the environmental factors that structure them (Wait et al., 2006). Trapped in this harsh but beautiful continent is an amazing set of ecosystems utilising a dynamic range of environments and thriving with limited impact incurred from mankind. It is the role of scientists to understand Antarctica in its entirety and to exhibit its wonders for everyone to see (Lucas, 1982).

1.1 The Physical Environment of Antarctica

The Antarctic continent is one of the harshest and most demanding environments in the world due to its broad range of extreme conditions (Cowan and Tow, 2004). It is Antarctica's combination of extremes that make each

environment within it unlike any other place on Earth, these extremes include: isolation; selection pressure from sub-zero temperatures and water stress (Bergstrom et al., 2006); solar UV radiation (Hughes et al., 2006); long periods of complete seasonal darkness (Cowan and Tow, 2004); and strong, dry katabatic winds (Hawes and Howard-Williams, 2007). As a result the continent is believed to have a highly reduced biodiversity (Bergstrom et al., 2006); in fact in 1903 when Captain Scott's party first discovered the dry valleys he considered them to be a barren land absent of any kind of life. Today we now know that this harsh landscape harbours a variety of diverse and productive ecosystems, each with its characteristic community of organisms (Sjoling and Cowan, 2003).

Antarctica has a variety of different environments, with each one containing its own environmental pressures dictating what inhabitants it contains. Sea ice and the marine systems (Figure 1) can be home to a broad range of microbial life (Becquevort et al., 2009; Priscu et al., 1998). Permanently ice free areas in Antarctica (Figure 2), the McMurdo Dry Valleys being the largest (less than 0.3% of the continent is ice free (Treonis et al., 1999)) contain a surprisingly diverse array of microbial life along with mosses, lichens and a few species of invertebrates (Cary et al., 2010). Vast areas of the Antarctic continent are permanently covered in thick ice and are largely absent of life (Figure 3) other than the researchers and explorers who temporarily stay there (Warren and Hudson, 2003). The other iconic Antarctic landscape are the animal/bird colonies (Figure 4), some are home to thousands of penguins/seals/birds (Joiris, 1991).

Figure 1: Ross ice shelf sea Ice Photographed by Stephen Archer

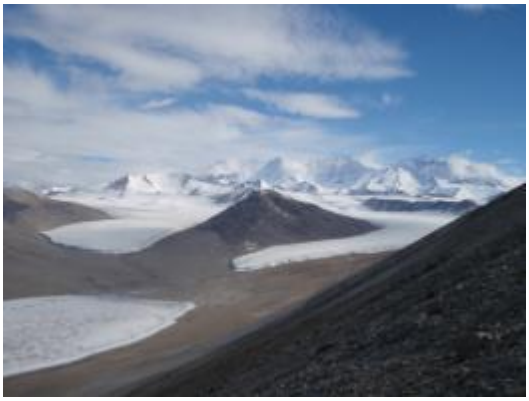

Figure 2: The Miers dry Valley Photographed by Stephen Archer

Figure 3: Looking out over the Antarctic Continent (Wang, 2008)

Figure 4: Penguins in the South Shetland Islands(George)

1.1.2 Why there is interest in Antarctica

Historically the majority of interest in Antarctic organisms has been in the multicellular marine life, including whales and seals (Yang et al.). However the majority of life on the Antarctic continent is in fact microbial, due to their ability to survive in extreme environments (Torsvik and Ovreas, 2008). Microorganisms in these extremes have the ability to answer a broad range of biological questions. Scientists may be able to look at the extreme cold, nutrient poor, water stressed environments in Antarctica and postulate what life may be like on other planets due to the similarities of some Antarctic habitats with Mars and Europa (Deming, 2002; Mahaney et al., 2009; Wallis et al., 2009). Some Antarctic microorganisms are highly sensitive to a range of environmental pressures which can be used to monitor how ecosystems function and how climate changes over time may affect larger ecosystems (Chattopadhyay, 2006; Maxwell and Barrie, 1989; Wynn-Williams, 1996). Antarctica also holds ecosystems which have been isolated from the rest of the world for thousands of years giving them special evolutionary value. They have been removed from outside influences experienced by other areas of the planet such as environmental fluctuations, competition by other organisms and gene flow (Vincent, 1999). The above are examples of pure science driven by understanding, however there are many unique biological products to be commercialised from Antarctica for a range of industrial applications (Nichols et al., 1999) including cleaning agents, food processing, molecular biology relevant enzymes, and hydrocarbon spill cleanup (Delille et al., 1997, 1998; Madigan and Martinko, 2006). We must understand this environment before it is irreparably damaged, by improving the understanding of Antarctic microbial ecosystem we

will be one step closer to protecting and harnessing the genetic resources of the continent (Vincent, 1999).

1.1.3 The Microorganisms in Antarctica

Microorganisms are fundamental to the functioning of Antarctic (and in fact all) ecosystems (Franzmann et al., 1997; Vakhlu et al., 2008). However unlike organisms in more temperate regions there are more restrictions put on growth rates and biodiversity in Antarctica (Wynn-Williams, 1996). Regardless of these restrictions there is still a massive microbial biodiversity found on the Antarctic continent able to accumulate a large volume of biomass (Hawes et al., 1999). One universal environmental characteristic from all microorganisms on the continent is their constant exposure to cold temperatures making them psychrophiles. A psychrophile is "an organism with an optimal growth temperature of 15 °C or lower, a maximum growth temperature below 20 °C and a minimum growth temperature at 0 °C or lower" (Bowman et al., 1997a; Madigan and Martinko, 2006). It is believed that planet Earth has gone through several ice ages where the entire planet would have resembled Antarctica. Thus cold tolerance is an essential skill historically for many microorganism's to have (Howard-Williams and Hawes, 2005). To grow psychrophiles have evolved a range of unique mechanisms that allow them to survive in freezing conditions (Deming, 2002). To stop ice crystals forming within their cell membranes and destroying their structural integrity some bacteria produce anti-freeze proteins which inhibit the formation of ice crystals (Gilbert et al., 2004). Generally the

most successful organisms have the ability to remain active over the winter period (Laybourn-Parry et al., 2002), however most bacteria are believed to go into inactive states when the ice is frozen to protect themselves during extreme cold (Chattopadhyay, 2000) then rapidly reactivate when conditions become more favourable. The organisms exhibiting the best survival mechanisms will ultimately colonise the continent easily and be widely distributed.

1.1.3 Colonisation and Distribution of Antarctic Organisms

Antarctica is unique in that it has been relatively isolated from the rest of the world since its separation from Gondwanaland more than 10 million years ago (Vincent, 1999). It also had the majority of its indigenous life removed from it during periods of climatic cooling and major glacial advances which occurred between 32-5 mya. This means that most life on Antarctica is the result of relatively recent (Ellis-Evans and Walton, 1990) and continuous colonization by invading organisms (Wynn-Williams, 1990) the most common invaders being prokaryotes (Franzmann et al., 1997). Microbial colonisation in Antarctica is generally carried out by air or carried by birds and mammals (especially humans) (Pearce et al., 2010; Wynn-Williams, 1990). It is thought by some that most microbes harbour the ability to live in the extreme Antarctic climate allowing them to invade and colonise Antarctica (Hughes et al., 2006). However there have been studies which have identified multiple phylotypes in Antarctica which are not found anywhere else (Jungblut et al., 2005).

The question of how microbes are distributed in time and space has been argued for decades (whether the current climate or historical events have the greatest impact on population structure (Martiny et al., 2006)). In 1934 Baas Becking summarised previous thoughts about biogeography in the famous microbiological tenet "Everything is everywhere, but the environment selects" meaning that organisms will be found wherever their ecological requirements are met. This means that communities will be structured based on the geochemical conditions of their environment (Baas-Becking, 1934). There are a number of studies which support this idea (Gray et al., 2007; Miletto et al., 2008; Sommaruga and Casamayor, 2009). However there are also those which prove that contemporary environmental conditions can be overridden by a long historical legacy (Foissner, 2008; Papke et al., 2003; Whitaker et al., 2003) or where suitable habitats are not colonised by all organisms which can colonise them (Foissner, 2008; Smith and Wilkinson, 2007). This means that populations in environments with the same chemistry will be different over distance (Martiny et al., 2006). An excellent site to study biogeography is on the meltwater ponds of Bratina Island due to their extreme geochemical heterogeneity in an extremely restricted area (DeMora et al., 1996).

1.1.4 Antarctic Ponds as a microbial environment

Most Antarctic environments are constantly under extreme water stress due to the low humidity, high winds and cold temperatures freezing most liquid water (Cary et al., 2010) however during the peak of the Antarctic summer some

bodies of frozen water will thaw giving a unique and rapidly changing environment (Hawes and Howard-Williams, 2007). During the peak of the Antarctic summer up to 20 percent of the McMurdo ice shelf can be covered by meltwater ponds some up to 10 Hectares in surface area making them a dominant environment in this area (Figure 5 shows an aerial view of the MIS ponds) (Demora et al., 1994). Antarctic ponds are unique because they are stable bodies of water that may be present for years and thaw to the bottom each summer temporarily giving a stable and less extreme environment only to freeze solid again during the long winter (Hawes et al., 1999). Although liquid water is not a limiting factor in Antarctic ponds (during summer) they are still exposed to some of the harshest conditions known in the world but they still contain and maintain a diverse microbial population (Howard-Williams and Hawes, 2005). The bottoms of the ponds are often dominated by dense cyanobacteria mats (as seen in Figure 6) although there is a significant planktonic and sediment population as well. To survive stresses such as yearly freeze thaw cycles the microbes in these ponds have developed various mechanisms to survive, including formation of a resting state during freezing (Heywood, 1984) and returning to activity when the ice melts (Wynn-Williams, 1996), or the production of large amounts of starch bodies within their cells for energy storage (Bell and Laybourn-Parry, 1999). Another unusual stress is the highly seasonal light regime which means that primary producers within these environments can only photosynthesize for a limited period of the year leading to a feast or famine situation. The various stresses on these organisms leads to a very slow net growth rate, but explosive growth when it does occur (Hawes and Howard-Williams, 2007). A well studied cluster of ponds in Antarctica can be found on the McMurdo Ice Shelf close to New

Zealand's Scott Base and America's McMurdo Station. These meltwater ponds offer an extremely useful set of diverse, adjacent ecosystems for study (Hawes and Howard-Williams, 2007).

Figure 5: An aerial view of Bratina Island and the adjacent ponds

Figure 6: Pond P70E on Bratina Island, Antarctica

1.2. Bratina Island Ponds

Near the northern tip of Brown peninsula Latitude 78°01'S, Longitude 165°32'E lies three huts establised as a base from which the adjacent Bratina Island ponds can be studied (seen Figure 7/figure 8). Bratina Island Ponds experience a unique, extreme and fluctuating climate which makes it difficult for pond systems to establish themselves and survive (Cowan and Tow, 2004). Their existence is driven by the short period of the year when liquid water is available to collect in the depressions of the undulating landscape of the McMurdo Ice Shelf (MIS) near to Bratina Island (Victoria Land, Antarctica) during the peak of summer (Howard-Williams and Hawes, 2005). The ponds are actually sitting on the top of a floating ice shelf which is covered in gravel and sand (the source of a range of nutrients and metals) (see figure 8). The unique surface originates from the marine sediment below the ice shelf coming into contact with the ice and getting trapped within it. The sediment is moved through the ice via a process of evaporation and sublimation at the top and freezing at the bottom resulting in sediment covering the landscape (Hawes and Howard-Williams, 2007). The depressions on the surface can mean ponds are linked to one another or separated through changes in the landscape or different levels of water input making each ponds level rise and fall. The balance between water supply and evaporation means that each pond will have a broad physiochemical range that can rapidly change (Laybourn-Parry et al., 2002). Ponds within metres of each other can vary greatly in size, depth, and age (Gibson et al., 2006) as well as chemical evolution and profiles of dissolved oxygen (Koob and Leister, 1972), metal concentrations,

pH and salinity (Matsumoto et al., 1992; Schmidt et al., 1991) which all affect the communities within them (Jungblut et al., 2005). Even within ponds there can be stratified layers within the water column with characteristic geochemistry provided the pond is not thoroughly mixed by the wind (Wait et al., 2006).

Figure 7: Map showing the location of Bratina Island http://www.niwa.co.nz/__data/assets/image/0018/52209/bar6_large.jpg

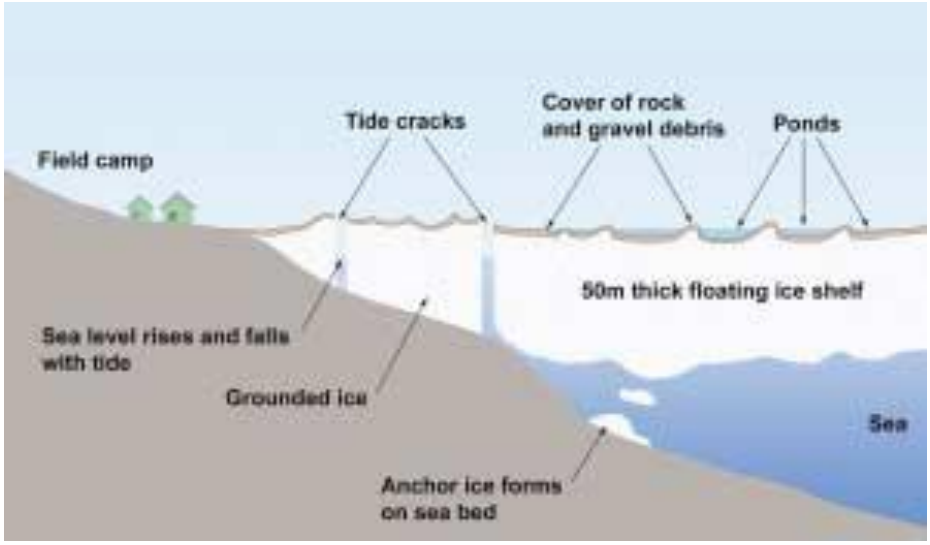

Figure 8: Bratina Island ponds created on the undulating surface of the McMurdo Ice Shelf (Hawes and Howard-Williams, 2007)

1.2.1 Pond physical-chemical characteristics

Antarctic ponds have a broad variety of physiochemical characteristics each interdependent on one another and resulting in highly different environments. This can be clearly seen in that two ponds viewed on the same day one is completely thawed while the other completely frozen due to the elevated levels of dissolved ions suppressing the freezing point in the liquid pond (see Figure 9). Trace metals can vary greatly between ponds depending on the depth, size of pool, age, and proximity to the major sources of metals. The metals originate from the sea, atmospheric salt fallout and the weathering of rocks and soils (input via meltwater) (Matsumoto et al., 1992). A combination of these factors makes the ponds physical and chemical characteristics highly varied between ponds and also temporally. To date the majority of research on Antarctic aquatic systems has focused on lake physical, chemical and biological processes (James et al., 1994; Miller and Aiken, 1996; Pearce, 2003; Priscu et al., 1998; Tyler et al., 1998), not on meltwater ponds. Lakes in Antarctica, unlike ponds, don't necessarily freeze to the bottom each year (Hawes et al., 1999), they can maintain a pool of liquid water under a protective ice layer, and frequently do not completely thaw in summer (Howard-Williams and Hawes, 2005). This markedly changes the environment in lakes compared to ponds throughout the year (Hawes et al., 1999). When ponds freeze in the winter it is from the top down, which means that there is a time when there is a protective ice-cover with a liquid water core as in most lakes (Hawes et al., 1999) changing the physical and biological processes occurring in the water column (Parker and Simmons, 1985). The ice forms a cap

but still allows light in for photosynthesis potentially leading to superstauration of oxygen within the water (Craig et al., 1992; Lyons et al., 2006). Another feature of perennial pond freezing is the concentration to dissolved salts in the bottom of the pond. When the ice freezes pure water freezes first, leaving the dissolved salts in solution making the unfrozen depths of the ponds hypersaline. The level of stratification is based on the concentration of the solutes in the pond and the size and depth of the pond. Deeper ponds have a greater ability to retain stratification due to the wind having less of an effect on pond mixing (Wait et al., 2006).

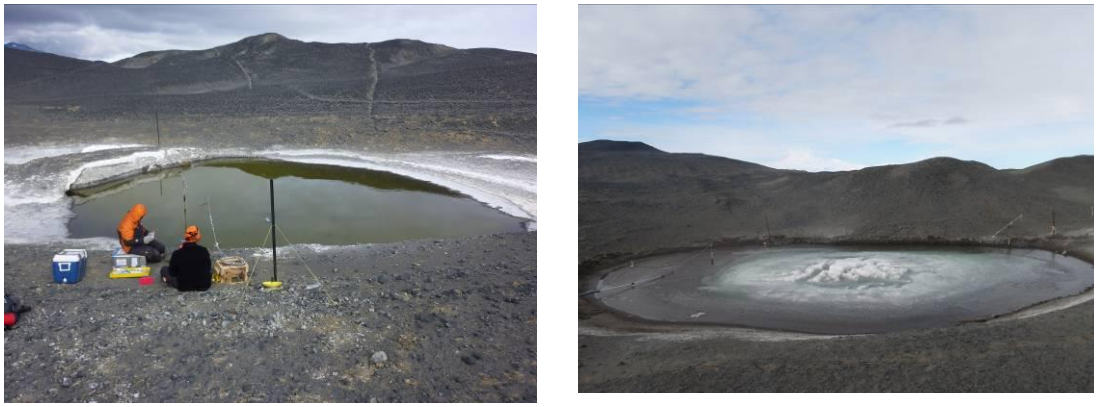

Figure 9: Left salt pond (hypersaline) completely thawed Right Legin Pond (top layer fresh water) still partially frozen.

1.2.2 Pond environment during freezing

Few studies have focussed on how meltwater ponds change during an Antarctic winter (Hawes et al., 1999). An early record of these ponds states that "Generally ice grows downward throughout February and concentrates dissolved material below it through a freezing out process" (Goldman et al., 1972), this was

also found to be true in a more recent study (Wait et al., 2006). It has also been established that high dissolved oxygen levels are present in the ponds from the middle of November to the middle of January, which is thought to occur due to the high levels of photosynthesis during this time resulting in high oxygen production, with the highest levels in early December (Koob and Leister, 1972; Lyons et al., 2006). Recently an extensive study between January 1997 and January 1998 followed the environmental conditions in and around three shallow ponds on the McMurdo Ice Shelf (Hawes et al., 1999). The ponds melted during summer when air temperature rose above 0 °C. Then between late February and early March the shallow "shores" began to freeze with the bottom of the lake remaining unfrozen until early June. This study also showed that the microbial mats remained photosynthetically active as long as there was still light and liquid water, so deeper ponds shield microbial mats from the elements for longer so remain active for longer (Hawes et al., 1999).

1.2.3 Community Structure and Biodiversity

Community structures in Antarctic lakes are highly dependent on the season. During summer there is light for photosynthesis and liquid water from melted ice resulting in an explosion of life from photosynthetic organisms such as dinoflagellates and phytoflagellates making them the dominant life in the ponds during this time. However these species are outcompeted throughout winter, autumn and spring (Bell and Laybourn-Parry, 1999) by populations that can grow heterotrophically (Laybourn-Parry et al., 2002). Almost all Antarctic ponds have a

dense microbial mat at the bottom (Hawes and Howard-Williams, 2007) (see figure 6), which consists largely of cyanobacteria. Cyanobacteria are an essential part of the ecosystem; they make up a substantial portion of benthic mats and planktonic populations, and are the major primary producer (Gibson et al., 2006). The sediment populations underlying the microbial mats often have an intimate relationship with the mats lining the pond carrying out important anaerobic reactions (Mountfort et al., 2003; Mountfort et al., 1999). Previous research has relied primarily on microscopic identification of microorganisms which identified low biodiversity (de los Rios et al., 2004; Sabbe et al., 2004; Vincent and James, 1996). Recent advances in culture-independent molecular ecology have changed the historical view that biodiversity in Antarctica is low (Hughes et al., 2006). Cowan and Sjoling (2003) investigated the microbial diversity of pond sediments from Bratina Island using 16S rRNA-dependent molecular phylogeny. They showed that there was a highly diverse bacterial population (7 major lineages) and a relatively low Archaeal diversity (only Crenarchaeota) in these ponds (Sjoling and Cowan, 2003). However not all samples may show the same level of biodiversity, another study found that biodiversity in pond sediments can be highly variable between ponds (Kemp and Aller, 2004).

1.2.4 Previous work

The Bratina Island huts were established in 1989 and provide an excellent base to do work on the adjacent ponds. On the wall in the cooking hut (see Figure 10) there are inscriptions from some of the researchers who have worked on the

ponds throughout the years. These ponds have had a number of extensive studies performed on them due to extreme heterogeneity in environments each once provides within easy walking distance from one another. The studies have included a number of descriptive studies on the physio-chemical environment (Demora et al., 1994; Matsumoto et al., 1992; Wait et al., 2006) and of the microorganisms within and between ponds. Although there is a substantial planktonic population in most lakes (Hawes and Howard-Williams, 2007), the majority of past microbial research carried out in Antarctic limnetic systems has focused on the dense microbial mats on pond and lake floors. These studies include: N2 fixation in the cyanobacterial mats lining the ponds on the MIS (Fernandez-Valiente et al., 2001); biomass accumulation and benthic mat dynamics in perennially ice covered lakes (Hawes et al., 2001); the functional and ecological role of cyanobacterial toxin production in MIS ponds (Hitzfeld et al., 2000); light availability and mat productivity dynamics in a Subantarctic lake (Moorhead et al., 1997); mat biodiversity across Eastern Antarctic lakes (Taton et al., 2006) and a detailed study of a continental lake and artificially derived mat population by phenotypic and genotypic means (Taton et al., 2003). There have also been studies of sediments underneath the benthic cyanobacterial mats including: biodiversity and community structure within anoxic lake sediments (Bowman et al., 2000); molecular diversity in MIS meltwater pond sediments (Sjoling and Cowan, 2003); anaerobic microbial dehalonation in MIS sediments(Griffin and Tiedje, 2007); biogeography of sediment populations in freshwater ponds (Gray et al., 2007) and specific studies on microbes isolated from MIS pond sediments (Mountfort et al., 1997; Mountfort et al., 1998). Given the fraction of Antarctica made of limnetic systems there has been a large amount

of work done of these environments however it was not until 07-08 that Hawes and Collegues collected water column samples with the intent of having the microbial populations elucidated from them. This is an important step to giving a more representative view of the bulk of the communities within these ponds instead of sample collection primarily from the edges of the pond (Wait et al., 2006).

Figure 10: The wall of the cooking hut on Bratina Island inscribed with previous researchers names, dates and projects since 1989

1.3 Antarctic Molecular Ecology

Advances in molecular ecology have provided the necessary tools to understand how organisms interact with their environment (Johnson et al., 2009). However, after decades of research on Antarctic lakes there is still little molecular work on the bacteria within the water column (Laybourn-Parry et al., 2002). When determining an environment's biodiversity you must first be able to isolate the organisms present and identify them either physiologically or genetically (Madigan and Martinko, 2006). The method used for organism isolation and identification depends on what you want to know, how much of each sample and how many samples you have, the time to analyse them, (Casamayor et al., 2002) and the funding available for the project.

1.3.1 Culture dependant techniques

Culture dependent techniques refer to the use of media to grow microorganisms. These are central to a range of biological investigations and are useful for determining the physiological properties of organisms in the environment. However, culture dependent techniques do have a range of limitations that culture independent techniques do not have. The biggest disadvantage is that a single type of culture media will not grow all microbes from an environmental sample (Sjoling and Cowan, 2003). This can be due to a lack of symbionts, substrates, inhibition by waste product build up, or the wrong

combination of physical and chemical parameters (Vakhlu et al., 2008). Also any difference in the culture conditions from those in the environment will put new selective pressures on the sample, potentially changing its community structure (Wagner et al., 1993). This is why investigators do not rely on culture dependent methods for characterizing microbial community composition (Pearce, 2003).

1.3.2 Culture independent techniques

Culture independent techniques get around the need to isolate and culture microorganisms which can be difficult and does not give a representative view of biodiversity (Ranjard et al., 2000). The development of culture-independent molecular techniques over the past few decades have allowed many unculturable organisms to be identified (Bowman et al., 1997b; Christen, 2008; Davidov et al., 2006; Hugenholtz et al., 1998; Pearce, 2003; Vakhlu et al., 2008). It is hard to evaluate the different approaches used as the most appropriate often depends on the environment being studied and desired level of resolution of populations. Typically 16S rRNA gene sequences are used to study bacterial communities as they are found in all bacteria, have conserved primer binding sites and have hypervariable regions which have been found to identify more diversity that culture dependent studies (Brambilla et al., 2001). Culture dependent studies on the other hand generally reveal a different set of organisms (Pearce, 2003), often isolating organisms which are only minor components of a population but can grow well on the selected media and not identifying the numerous unculturable organisms in an environment.

There are a broad range of culture independent techniques used to answer a number of microbiological questions with many being used simultaneously in a single study. These techniques include: Community cloning and 16S rRNA gene sequencing (either using sanger or next generation sequencing) to get a high resolution view of community diversity (Poitelon et al.; Swan et al.); quantitative Polymerase Chain Reaction (qPCR) where individual abundance can be determined (Zozaya-Hinchliffe et al., 2010); functional gene detection allowing specific investigation into genes coding for processes (Pereyra et al.); metagenomics where the entire genomes of the community population are sequenced allowing a thorough view of what microbes are there and what they are capable of doing (Tyson et al., 2004); and DNA fingerprinting techniques to investigate community diversity: Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Wood et al., 2008); Terminal Restriction Fragment Length Polymorphisms (tRFLP) (Dunbar et al., 2001); Denaturing Gradient Gel Electrophoresis (DGGE) (Kao et al., 2010) and Multiplex Terminal Restriction Fragment Length Polymorphism (MTRLP) giving the ability to simultaneously identify a number of different groups at once (Macdonald et al., 2008; Singh and Thomas, 2006).

Genetic fingerprinting theoretically gives a more representative view of the community diversity and has the advantage of generally being a faster option than culture dependant techniques (Hughes et al., 2002). Fingerprinting techniques such as ARISA (Wood et al., 2008) and tRFLP (Dunbar et al., 2001) are increasingly being used for high resolution fingerprinting. These techniques utilise fluorescently labelled PCR primers to amplify variable DNA sequences within the community's respective genomes. When run through a DNA sequencer

a ‘fingerprint’ is formed due to differential migration of DNA fragments, each size of fragment potentially represents a different species within a community (Ranjard et al., 2000). These techniques can be used to tentatively assign microbial biodiversity and species abundance. From the detailed fingerprinting analysis representative sites can be selected for clone library construction and DNA sequencing which increases the accuracy of the taxonomic assignment (Wood et al., 2008).

1.3.4. ARISA

ARISA is a high resolution DNA fingerprinting technique developed by Fisher and Triplette (1999) to rapidly estimate a population’s diversity so that it can be compared to other communities Wood et al., (2008). An outline of the technique can be seen in Figure 11. It involves the amplification of the community hypervariable Intergenic spacer region between the 16S rRNA and 23S rRNA genes found in all bacteria to determine the overall diversity within a population. ARISA is a reproducible, cheap, sensitive, fast, semi-quantitative fingerprinting method (Lear and Lewis, 2009) which has been optimised to be used in a multitude of different ways in a range of environments including: investigating how the thermophillic communities on Mount Erebus changes along a temperature gradient (Soo et al., 2009); determining whether the

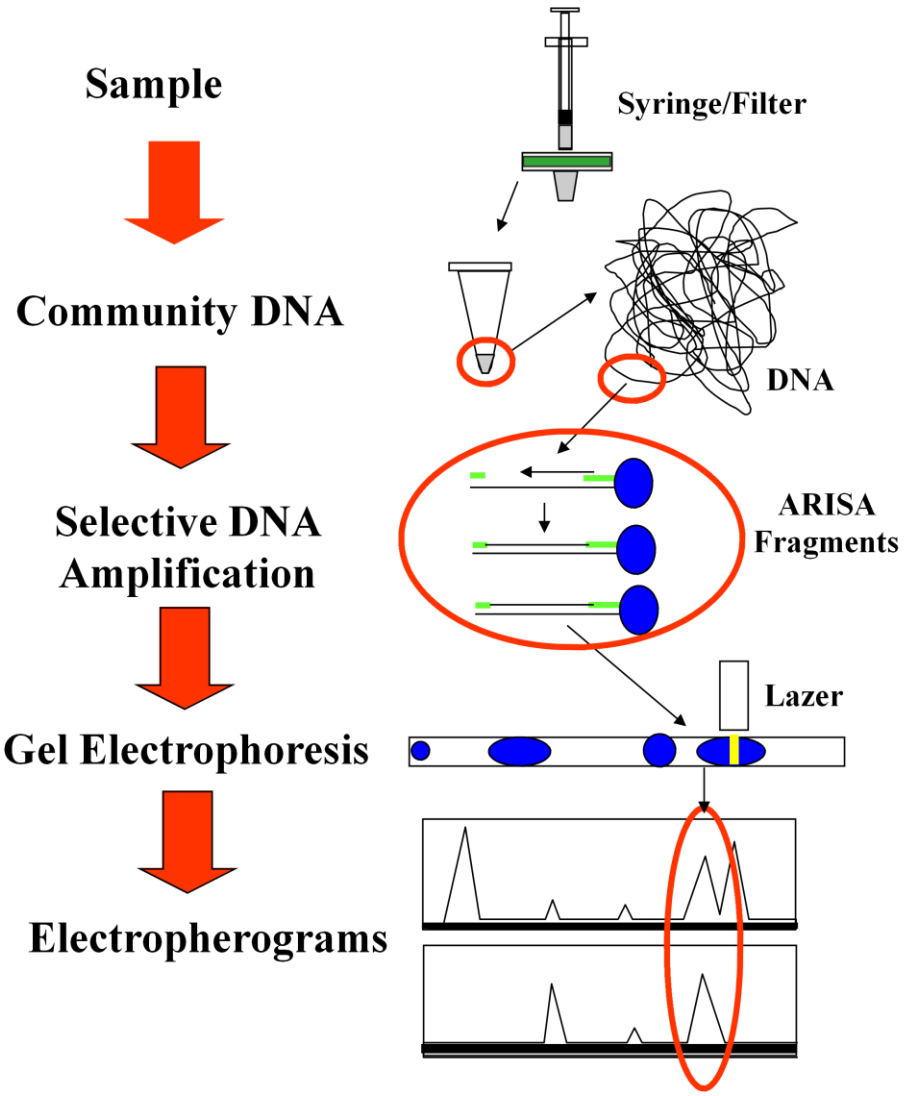

Figure 11: Flow diagram of ARISA genotyping of community samples

populations of Adelie penguin faecal flora is based on the environment or heredity of the penguin (Banks et al., 2009); or to determine how gut populations change with various gastrointestinal diseases (Roesch et al., 2009).

Due to the hypervariable ITS region there is good resolution between organisms and the highly conserved regions around the ITS region mean that universal primers can be used. However there can be some run to run variation, it requires more specialised, expensive equipment and can under/over estimate diversity based on the 16S rRNA gene bias leading to an incomplete view of how

the community is structured, what is there and the abundance each individual (Anderson and Cairney, 2004). ARISA can over estimate diversity by representing the same organisms with slightly different ITS regions as different organisms or the fact that one organism can contain more than one single type of 16S rRNA gene. It can underestimate diversity by incomplete lysis of all microbial cells before amplification, or the "universal" primers not amplifying all of a communities DNA (Popa et al., 2009). There are problems with any technique, however ARISA allows for a rapid and relatively high resolution and accurate comparison between two (or more) microbial populations (sites)(Ramette, 2009). Once run through a sequencer the differences can be visualised through the use of statistical software such as PRIMER 6.

1.3.5 Phylogenetic analysis

Due to the complexity in the interpretation of multiple community compositions (which can involve 100's of different species in each), it is necessary to use computer software to process the data into an output that is easily interpreted. The raw data that originates from high throughput fluorescently labelled fingerprinting techniques such as ARISA and TRFLP is best described as a histogram containing up to 1000's of peaks (each peak potentially representing a different species) for each community sample which has been run. The first step necessary is to eliminate all those which are considered to be ambiguous peaks or "noise". Initially peaks found below a threshold fluorescence reading (meaning that the peak may just be noise) are removed along with peaks which represent

DNA sequences that are too small or too big (which relies on knowing what sequence you are looking for). After this is done the data can be imported into statistical software to examine similarities and differences between the different populations peak abundance. PRIMER 6 is one such programme which allows you to process this data and compare the communities graphically in two main ways, through the use of phylogenetic trees where earlier branching infers lower similarity between two communities and the creation of 2D or 3D MDS plots (as seen in Figure 12). In this example the greater the difference between the samples the greater the difference in the populations. PRIMER 6 can also be used to link the differences between populations and their physical environment. To do this geochemical data is brought into PRIMER 6 where it is processed and run through a BEST analysis which will give a percentage of difference which is explained by each, or a combination of, environmental variables. Overall the use of this software allows researchers to visualise data and to compare different samples so that biological questions can be answered.

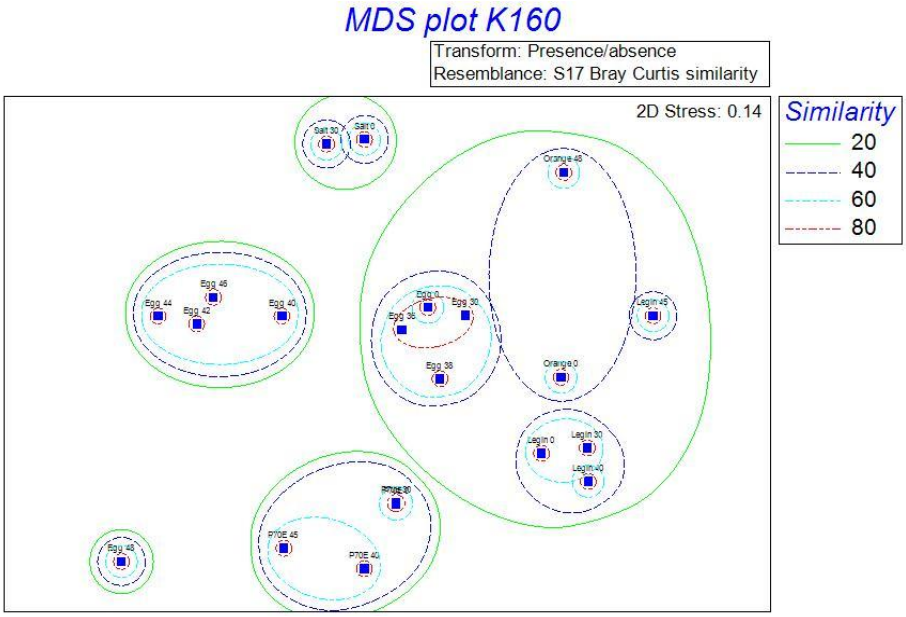

Figure 12: Example of an MDS plot showing community similarities

1.4 Aims, approach and outline of the thesis

This study aimed to identify the microbial communities present in the water column of Bratina Island meltwater ponds during the mid-summer stratification and to correlate this data to the geochemistry of the pond. This project focused on the transitions between stratified layers using a detailed study of microbiology coupled with extensive geochemistry in the field and back at the University of Waikato. The central question to be answered was what is driving the community composition and structure? Is there a single keystone parameter or are there multiple parameters that structure the communities in such a way that each pond is unique? To study these questions a sampling rig was designed allowing water samples to be collected from the centre of the MIS ponds while measuring the ponds oxygen concentration, pH, conductivity and temperature. Biomass was collected by filtration then preserved using cetyltrimethylammonium bromide-polyvinylpyrrolidone-β-mecaptoethanol (CTAB) and the filtered water was taken back to the University of Waikato for inductively coupled plasma mass spectrometry (ICP-MS) and nutrient analysis. DNA samples were extracted at the University of Waikato then community profiles screened for microbial community similarity using ARISA. The results of these procedures are presented in Chapter 2 of this thesis and show the differences between and within the ponds sampled and potential environmental drivers were determined.

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Chapter 2 - Characterization of the bacterioplankton communities in the melt-water ponds of Bratina Island, Victoria Land, Antarctica

2.1 Abstract

A unique collection of Antarctic aquatic environments (meltwater ponds) lie, within a limited area, on the rock and sediment covered undulating surface of the McMurdo Ice Shelf near Bratina Island (Victoria Land, Antarctica). Many of these ponds are naturally isolated from other ponds and show striking physiochemical heterogeneity coupled with steep vertical gradients and stratification of pH (from 9.46 to 8.11) and conductivity (from 8.2 to 93.6 mS/cm). During the 2009-10 mid-austral summer, using a specially designed micromanipulator sampling system, sets of discrete water samples were collected across the geochemical gradients of five selected ponds (Egg, P70E, Legin, Salt, and Orange) for biological and geochemical analysis. Bacterial DNA fingerprints of the ponds (utilising Automated Ribosomal Intergenic Spacer Analysis) appear to separate populations within each pond into the same group (based on a 2D MDS plot) as predicted. However one highly stratified pond (Egg) had two distinct bacterial communities representing the upper and lower part of the water column. A BEST analysis, which attempts to find links between population structure and the geochemistry of a pond, has shown that specific geochemical parameters (conductivity, pH, Ag^{109}, NO_2 and V^{51}) appear to influence the structure of microbial populations in these ponds by as much as 92.7%.

2.2 Introduction

One of the fundamental challenges in biology today is to understand how organisms respond and evolve in relation to changing environments (Kussell et al., 2005). The Antarctic aquatic ecosystems (lakes and ponds) scattered throughout the Antarctic continent and surrounding sea ice provide an extraordinary and tractable opportunity to examine metabolic functioning and the adaptations that allow microbial communities to thrive under these extreme conditions. Of the thousands of lakes and ponds throughout Antarctica the majority of research has been conducted on lakes, due to their tractable and stable nature (Bell and Laybourn-Parry, 1999; Bowman et al., 2000; Craig et al., 1992; Pearce, 2003; Taton et al., 2003). However, due to their sensitive nature ponds have the ability to be more affected by seasonal (and global) environmental differences, they are easily sampled with the correct equipment and due to their small size it is far easier to gain a representative view of the entire pond system environment.

During the peak of the Antarctic summer up to 20 percent of the McMurdo ice shelf can be covered by meltwater ponds of various sizes, making them a dominant environment in this area (Demora et al., 1994). The ponds of Bratina Island (78° 01' S latitude, 165° 32' E longitude on the McMurdo ice shelf (MIS) in the Ross Sea area of Antarctica, (Figure 14)) contain a collection of some of the most variable ponds, both chemically and biologically. Bratina Island Ponds experience a unique, extreme and fluctuating climate which makes it difficult for pond systems to establish themselves and survive (Cowan and Tow, 2004). Their existence is driven by the short period of the year during the peak of summer

when liquid water is available to collect in the depressions of the undulating landscape of the McMurdo Ice Shelf (MIS) (Howard-Williams and Hawes, 2005). The balance between water supply and evaporation means that each pond will have a broad range of physiochemical factors that can rapidly change (Laybourn-Parry et al., 2002). Ponds within metres of each other can vary greatly in size, depth, and age (Gibson et al., 2006) as well as chemical evolution and profiles of dissolved oxygen (Koob and Leister, 1972), metal concentrations, pH and salinity (Matsumoto et al., 1992; Schmidt et al., 1991). Even within ponds there can be stratified layers within the water column (Figure 13) with characteristic geochemistry derived from the freeze concentration of the dissolved salts and freshwater input floating on top of the denser brine. The maintenance of the stratification of a meltwater pond can be linked to two primary factors; the level of exposure to the wind, which causes the layers to mix, and the average conductivity in the pond which needs to be high to establish a density gradient with freshwater input (James et al., 1995; Wait et al., 2006).

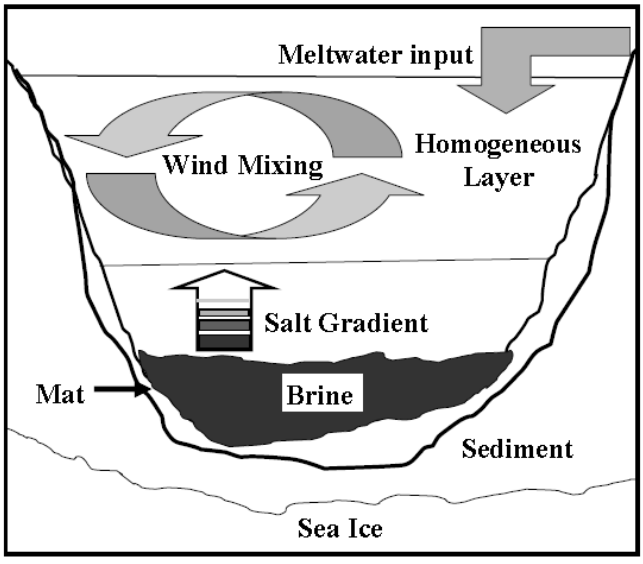

Figure 13: Diagrammatical representation of a stratified pond as would be found on the McMurdo Ice shelf near Bratina Island (such as Egg pond)

A large number of studies on the ponds near Bratina Island, and of Antarctic lakes, have provided insight into how some facets of these extreme communities function. These studies primarily focused on the mat and sediment samples lining the ponds (Fernandez-Valiente et al., 2001; Griffin and Tiedje, 2007) or the geochemistry of the water column (Demora et al., 1994; Matsumoto et al., 1992; Wait et al., 2006). This project is the first in-depth genetic study of water column microbial populations of Bratina Island ponds and attempts to identify key environmental drivers structuring the resident microbial communities. Previous research has relied primarily on microscopic identification of microorganisms which identified low biodiversity (de los Rios et al., 2004; Sabbe et al., 2004; Vincent and James, 1996) probably due to the presence of cryptic species. Recent advances in culture-independent molecular genetic tools have changed the historical view that biodiversity in Antarctica is low (Brambilla et al., 2001; Cary et al., 2010; Hughes et al., 2006; Sjoling and Cowan, 2003; Vincent and James, 1996). Cowan and Sjoling (2003) investigated the microbial diversity of the Bratina pond sediment from a single meltwater pond using 16S rRNA molecular phylogeny. They later showed that there was a highly diverse bacterial population (7 major lineages), and relatively low Archaeal diversity (only Crenarchaeota - (Sjoling and Cowan, 2003). A later study found that biodiversity in pond sediments was highly variable between ponds (Kemp and Aller, 2004) but what compounding environmental factors drive these differences was not determined.

Innovative molecular DNA technologies provide a mechanism to obtain a rapid and more comprehensive view of microbial populations than using culture-

dependent approaches (Hughes et al., 2002). One such technique is DNA fingerprinting which utilizes fluorescently labelled PCR primers to amplify a variable region of 16s rDNA from each organism in the community. When processed using a DNA sequencer a 'fingerprint' is formed due to differential migration of DNA fragments of different size, each fragment size potentially representing a different species within a community (Ranjard et al., 2000). These techniques can be used to assign a relative microbial diversity and structure of the community for comparative analysis. However, there are some potential limitations with culture-independent techniques which may result in a less representative view of the population, by either over or underestimating the species abundance (Hughes et al., 2002). When compared to other techniques (such as cloning and massively parallel sequencing (Brambilla et al., 2001; Shendure and Ji, 2008)) DNA fingerprinting is an economical option, which allows more samples within a study to be examined, from which representatives can be selected (if necessary) for further, more detailed analysis (Wood et al., 2008).

The aim of this study was to investigate the geochemical differences between and within the water column of 5 spatially distinct Bratina ponds (Figure 14 (Appendix 2)) and to understand how these differences affect the microbial populations within the water column. Due to their sensitivity and massive heterogeneity these ponds represent a fantastic biological resource for a range of future studies.

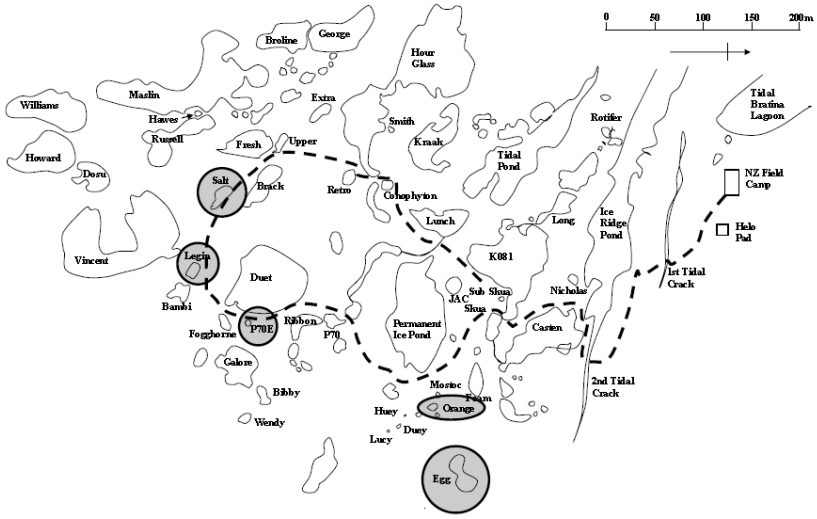

Figure 14: Map of the McMurdo Ice Shelf Ponds, based on the original New Zealand Department of Land and Surveys, 1991. Ponds circled were used in this study (K-160 ponds).

2.3 Materials and Methods

2.3.1 Field Sampling Strategy

Samples were collected using a specially designed micromanipulator (Figure 15) that had the ability to lower a small bore sampling tube (TYGON® R-3603 (Appendix 3)) down through the centre of the pond with great accuracy. The sampling tube was suspended over the pond, across two heavy duty metal stakes (waratahs), using a high tensile steel cable and wire strainer. A pulley system was employed to position the sample tube over the centre of the pond. To ensure that the tube went straight down, a 1.8kg weight was attached near the end of the sampling tube. The oxygen concentration and temperature of the ponds was measured in-situ using probes attached to the tip of the sampling tubing. The entire rig was lowered into the water by a micro-manipulator capable of fine scale movements (+/- 0.25mm), allowing a stable reading and sample to be taken at

each depth. Once in position, water was drawn from the pond through the heated (heat produced with a nicrome wire heating tape controlled via a rheostat) and insulated sampling tube using a peristaltic pump. Two times the void volume (428mL) was disposed of before sampling began to flush any existing water remaining in the tube from previous sampling. Water collected for biological samples was immediately filtered through a syringe mounted 0.22 micron filters (Whatman International Ltd, Kent, UK) until the filter clogged. A small amount of air was pushed through the filter to remove excess water, then gently flooded with a nucleic acid preservative buffer (CTAB - cetyltrimethylammonium bromide-polyvinylpyrrolidone-β-mecaptoethanol) and frozen for transportation back to the laboratory. 15mL of the filtrate was collected in 15mL falcon tubes (BD Biosciences, Franklin Lakes, New Jersey, USA), frozen immediately and returned to NZ for geochemical analysis. In addition; a 30mL non-filtered sample was taken in 50mL falcon tubes back to the field camp laboratory for immediate pH and conductivity measurements.

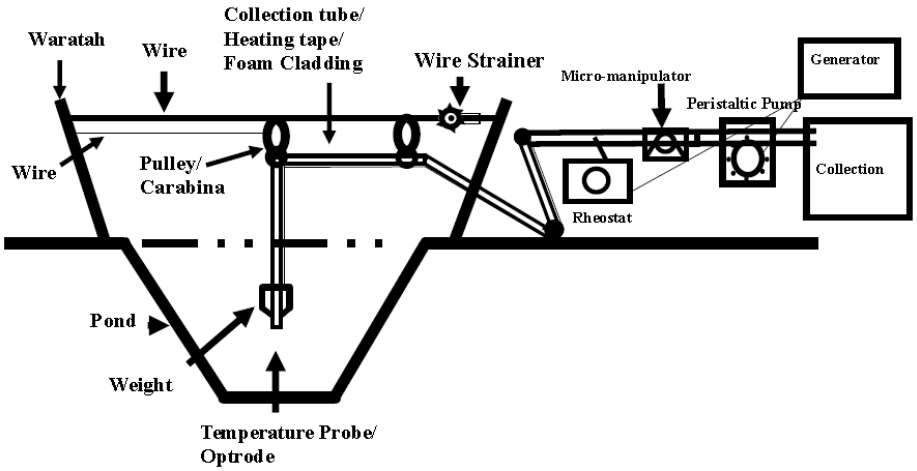

Figure 15: Sampling Rig used for fine scale water column sampling of Bratina Island Ponds.

2.3.2 Physiochemical analysis

In-field measurements (as previously discussed) included real time measurement of oxygen using a Fibox 3 LCD trace minisensor oxygen meter with data-logger (PreSens Precision sensing, Regensburg, Germany) and temperature using a Hobo temperature data logger (Onset Computer Corporation, United States) at the entrance of the sampling tube.

NH_4, NO_2 and phosphate measurements were carried out on the filtrate from each sample using an Aquakem 200CD (Thermo Fisher Scientific, Waltham, USA). Elemental analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS) using a Mass Spectrometer ELAN® DRC II (PerkinElmer Inc., Münster, Germany). To prepare the samples, 0.22 micron prefiltered pond water was added to 15 mL falcon tubes and diluted 1:50 with Milli-Q water (Millipore, Billerica, MA, USA). Six samples had to be diluted 1:1000 due to excessive salt concentrations. Once diluted, samples were acidified with 2% $HN0_3$ (Extra pure Nitric Acid, Ajax Finechem, NSW, Australia) to match the ICP-MS matrix. Elements analysed were: Li^{7}, B^{10}, Na^{23}, Mg^{24}, Al^{27}, S^{34}, K^{39}, Ca^{43}, V^{51}, Cr^{52}, Fe^{54}, Mn^{55}, Co^{59}, Ni^{60}, Cu^{65}, Zn^{68}, As^{75}, Se^{82}, Sr^{88}, Ag^{109}, Cd^{111}, In^{115}, Ba^{137}, Tl^{205}, Pb^{207}, Bi^{209}, U^{238}.

2.3.3 DNA Extraction of samples

DNA was extracted from the filtered water samples using a modified CTAB extraction protocol (Dempster et al., 1999). Frozen sealed 0.22 micron sample filters were first thawed on ice, and then connected to a syringe containing 1 mL of fresh CTAB. The exposed filter nipple was parafilmed and the entire assembly incubated in a Ratek Orbital mixer at 150 rpm and 65°C for 30 minutes. The filter assembly was allowed to cool then 0.5 mL of the CTAB in the syringe was pushed through the filter assembly to evacuate the lysate in an eppendorf tube. An equal volume of chloroform/isoamyl alcohol (24:1) was then added to the lysate and mixed on the orbital mixer at 150 rpm and 65°C for a further 30 minutes. The eppendorf tube was then centrifuged for 15 minutes at 12,500 rpm, and the aqueous phase transferred to a new tube. To initiate nucleic acid precipitation 1 volume of isopropanol and 0.5 volume of NaCl was added and then incubated at -80°C for at least one hour. The tube was then centrifuged at 15,500 rpm for 30 minutes, the supernatant discarded, the DNA pellet washed with 0.5 mL of 70% EtOH and then centrifuged at 15,500 rpm for 5 seconds. The pellet was dried and resuspended in 10-50 µL of sterile milliQ H_2O. The extracted DNA was Quantified using a Nanodrop ND-1000 at 260nm (NanoDrop Technologies, Montchanin, DE).

2.3.4 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA DNA fingerprinting was utilised to identify the community structure and relative diversity within bacterial and cyanobacterial populations in selected samples. From each of the samples the bacterial ITS (intergenic spacer region) in the ribosomal rDNA operon was amplified using PCR. Triplicate 30 µL reaction volumes were run for each sample, each reaction containing 0.3 mM of each primer (hexachlorofluorescein (HEX) fluorescent dye labelled ITSReub-Hex (GCCAAGGCATCCACC) and ITSF (GTCGTAACAAGCTAGCCGTA) (Cardinale et al., 2004)), 1x PCR buffer (Invitrogen Ltd, New Zealand), 0.2 mM dNTPs (Roche Diagnostics, New Zealand), 0.02 mg/mL Bovine serum albumin (BSA), 1 U of Platinum Taq (Invitrogen Ltd), 0.5 µM $MgCl_2$, 20 ng of genomic DNA and the reaction was made up to 30 µL with milliQ H_2O. Thermal cycling conditions were: 94°C for 5 min, then 30 cycles of 94°C for 45 sec, 55°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 7 min.

Cyanobacterial ARISA was also completed on samples selected for further analysis. Triplicate 25 µL reactions were run for each sample with each reaction containing 0.5 µM of each primer (6-carboxy-flurescine (FAM) fluorescent dye labelled CY-ARISA-F (GYCAYRCCCGAAGTCRTTAC) and 23S30R (CHTCGCCTCTGTGTGCCWAGGT) (Wood et al., 2008)), 1x PCR buffer (Invitrogen Ltd), 0.2 mM dNTPs (Roche Diagnostics), 1 U of Platinum Taq, 5 mM $MgCl_2$, 0.032 mg/mL BSA, 20 ng of template DNA and the entire reaction was made up to 25 µL with milliQ H_2O. Thermal cycling conditions were: 94°C

for 2 minutes then 35 cycles of 95°C for 20 sec, 55°C for 15 sec and 72°C for 1.5 min, followed by 7 min at 72°C.

All PCR reactions were run on a Bio-Rad DNA Engine® (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA). Once amplified all triplicate PCR reactions were combined, run on a 1% agarose gel to ensure amplification success and then purified using a 5M Quickclean PCR Purification Kit (Genscript Corporation, New Jersey, USA). The DNA concentrations of purified products were quantified using a NanoDrop™ 1000 Spectrophotometer, diluted to 5 ng/µL using Milli-Q H_2O and then sequenced at the University of Waikato DNA Sequencing Facility.

2.3.5 Data processing

To visualise, compare and interpret the ARISA fingerprints from different samples the sequencing output was run through an informatics pipeline (Abdo et al., 2006) producing text files which were analysed using Primer 6 software (Clarke and Gorley, 2006). This produced a visual representation of community structure which was able to be linked geochemical variations between samples.

The informatics pipeline produced a number of statistical grouping methods all of which were evaluated. An alpha value 0.001 was selected for both bacteria and cyanobacteria. This alpha value means that 0.001% of the peaks included in the analysis were statistically likely to be noise. Peaks that differed by less than 2 base pairs in length were considered to be identical and so were

"binned" together (ie. considered to be the same peak (Wood et al., 2008)). A noisemax cut-off of 250 fluorescence units was implemented ensuring that any peaks less than this intensity were eliminated as noise immediately. Fragment lengths less than 100 bp for bacteria and 150 bp for cyanobacteria were removed from analysis as these were considered to be too small to be ITS fragments (Cardinale et al., 2004; Wood et al., 2008). The processed text file from each sample was then imported into PRIMER 6.

In Primer 6, the peaks were transposed to presence/absence data for further analysis, the total number of peaks was totalled from each sample to be used as a proxy for biodiversity and a resemblance matrix was created based on the Bray Curtis similarity index. From the resemblance matrix a hierarchical clustering analysis was performed producing a relational dendrogram. Nonmetric multidimensional scaling (MDS) was performed on the resemblance matrix again in Primer 6, as it is an excellent visualisation that displays relative similarities between populations as distance (the closer two samples are the more similar the population). 2D MDS plots with a stress value of less than 0.2 were used as they were considered to have accurate information. The information gained from the dendrogram of the communities was overlaid onto the 2D MDS plots which provide percentage similarity levels at 20, 40, 60 and 80% assisting in the evaluation of community structure between samples.

The influence of a pond's geochemistry on its populations was determined using a BEST analysis to find the 'best match' in PRIMER 6. Geochemical data were normalised, a Draftsman plot created to view interrelations between the different variables so the data could be simplified and then Spearman's rank

correlation coefficient were calculated resulting in a probability (p) of the community changes being explained by the changes in the geochemistry changes. The combination of geochemical variables whose Euclidean distance matrix gives the highest p value were considered the most likely drivers of community dynamics.

2.4 Results

2.4.1 Physiochemical analysis

Inter and intra pond physical and chemical properties were found to be extremely variable with conductivities ranging from 0.43 to 110.9 ms, pH values ranging from 7.3 to 10.11, dissolved oxygen concentrations from 4.36 to 27.3 mg/L and temperatures ranging from -3.53 to 5.11°C (Appendix 1). Temperature was lowest at the bottom of Orange pond (-3.53°C) and highest at the bottom of Salt pond (5.11°C). A geochemical gradient was identified throughout the water column of most of the ponds. Oxygen concentration varied throughout the water column of each pond but generally it was at its highest at the deepest point (for example increasing from 9.71 at the top to 17.02 ppm in the bottom of Legin pond). Conductivity was also found to increase with depth in each pond (for example from 1.084 mS/cm at the top to 5.43 mS/cm at the bottom of Orange pond). The determined relative similarities in geochemistries between the ponds surveyed (Figures 16 and 17) show clearly that the ponds are geochemical distinct.

NH_4 was only detected throughout the water column in Egg and Salt ponds (which were found to have the greatest conductivities), with only traces at the top of Orange pond and the bottom of P70E. No NH_4 was detected in Legin pond. NO_2 was found at extremely low concentrations in all ponds except P70E where it was 10 times higher (0.032 ppm compared to 0.002 in most other pond depths) in the upper region. Co^{59} was only found in the bottom of Egg (46-48 cm) and throughout Salt pond. Ni^{60} was found to be below the detection limit in Legin and Orange ponds and the first (from the top) 38 cm of Egg. Legin pond also had a reduced amount of Cu^{65} and Mn^{55} compared to other ponds, and Orange had a reduced level of Mn^{55}.

Egg pond exhibits the most extreme physiochemical stratification observed (Figure 18). The pH of Egg remained stable between 9.41-9.47 in the top 38 cm dropping rapidly to 8.75 at 40 cm and to 8.11 at 48 cm. The conductivity of Egg changed from 8.2 mS at 0 cm, moderately increasing to 10.35 mS at 38 cm with a steep increase from 40 cm to 46 cm, ending in 93.6 mS at 48 cm depth. The dissolved oxygen content of Egg (appendix 1) ranged from 11.52 mg/L at Egg 0 cm, moderately increasing down the depth profile with peaks at 36 cm (17.06 mg/L), and 44 cm (27.3 mg/L). The temperature profile of Egg was relatively stable, decreasing from 1.55 °C at 0 cm to -1.06 ^{o}C at 46 cm with a slight increase at the bottom (48 cm) to 0.62 °C.

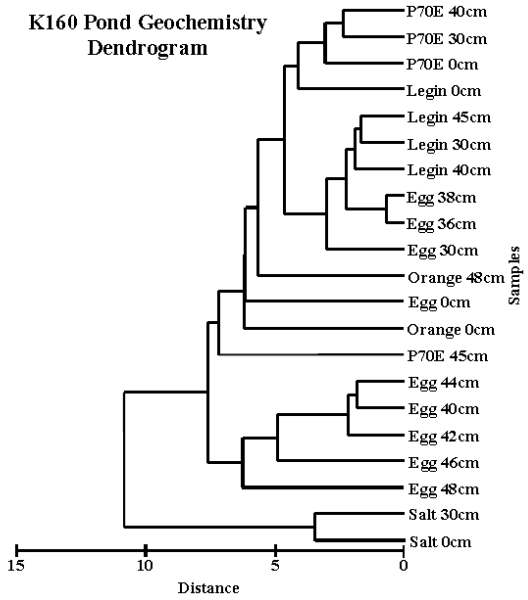

Figure 16: Dendrogram comparing relative geochemical differences between ponds and pond depths. Data normalised, converted to presence or absence and subjected to Euclidean distance resemblance matrix.

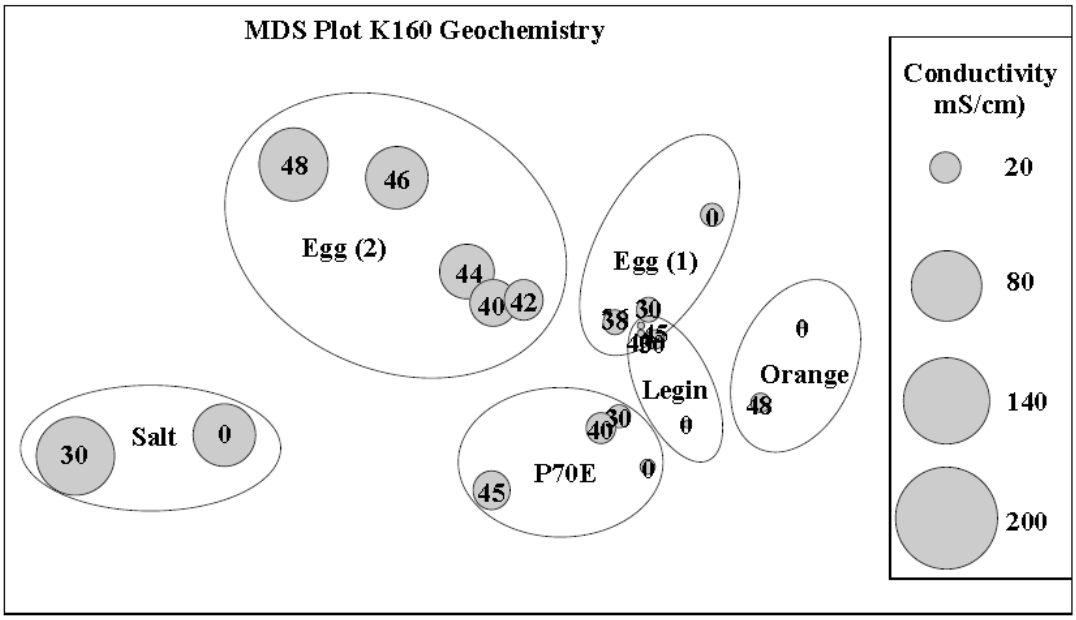

Figure 17: MDS plot of Geochemistry comparing all K-160 ponds. The greater distance between two points, the greater the difference between their geochemistries. Circles surrounding samples represent relative conductivity concentration (the larger the circle the higher the conductivity). MDS plot 2D stress: 0.08

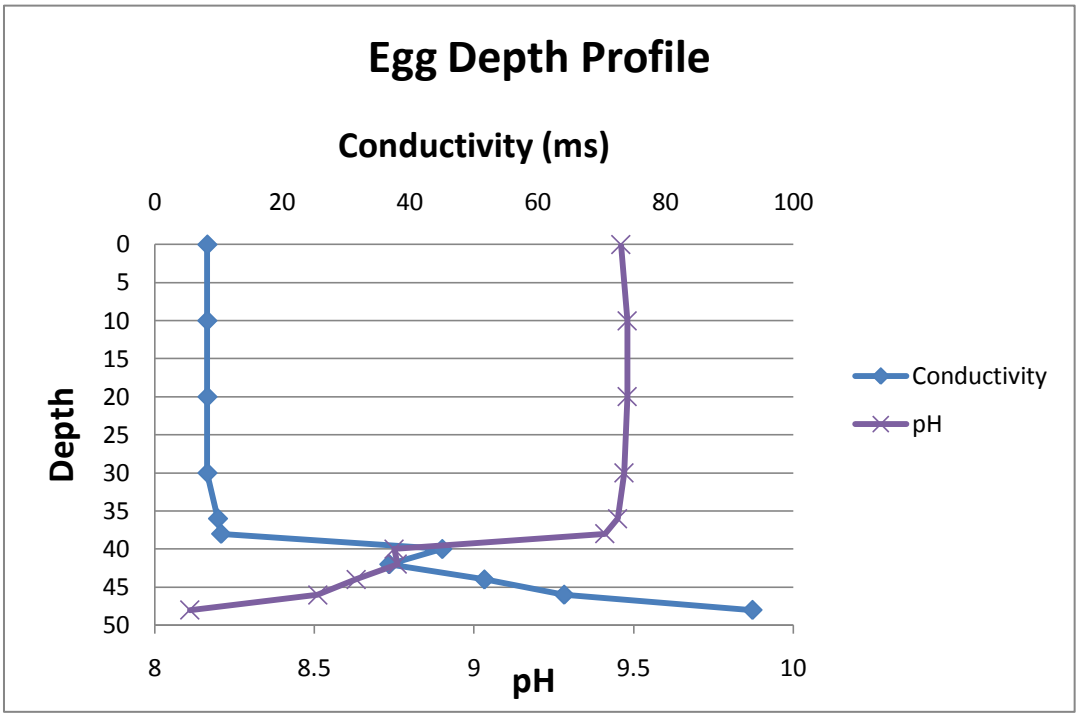

Figure 18: Egg pond geochemistry depth profile showing changes in Conductivity and pH (sampled 11.12.09)

2.4.2. DNA Fingerprinting

Using the number of specific peaks generated by ARISA it is possible to obtain a relative measure of bacterial and cyanobacterial diversity between samples (Table 1). Overall the ARISA fragment length (AFL) totals were similar between samples however there are several noteworthy outliers. Cyanobacterial diversity was found to be greater in P70E than any other pond (P70E 0 had 29 AFL's P70E 45 had 39 compared to a 16.6 mean) and in the bacterial analysis the diversity in Egg 0 was greater and in Salt 0 was lesser than in any other sample (46 AFLs in Egg 0 and 12 AFLs in Salt 0 compared to a 28.4 mean). Examination of the individual peaks in the bottom level of Egg pond identified a significantly different population when compared to any other depth in that pond.

Table 1: Summary of AFL number from each sample

CyanoARISA		BacARISA	
Sample Name	**AFL Number**	**Sample Name**	**AFL Number**
Egg 0	15	Egg 0	46
Egg 30	12	Egg 30	38
Egg 36	15	Egg 36	29
Egg 38	13	Egg 38	28
Egg 40	19	Egg 40	25
Egg 42	18	Egg 42	40
Egg 44	14	Egg 44	29
Egg 46	13	Egg 46	20
Egg 48	12	Egg 48	36
Legin 0	12	Legin 0	27
Legin 30	13	Legin 30	26
Legin 40	11	Legin 40	23
Legin 45	14	Legin 45	23
P70E 0	29	P70E 0	20
P70E 45	39	P70E 45	38
		P70E 30	25
		P70E 40	33
		Orange 0	30
		Orange 48	24
		Salt 0	12
		Salt 30	25

The clustering patterns produced in 2D MDS plots showed that the bacterial and cyanobacterial communities at different depths within each pond were distinct (at 40% similarity) according to pond sampled (Figure 20). Bacterial ARISA community comparisons once processed through informatics pipeline were visualised using a Bray-Curtis similarity dendrogram (Figure 19) and 2D MDS plot (Figure 20) below according to Bray-Curtis similarity based upon

presence/absence. There is a very clear clustering within pond communities in all but Egg Pond (Figure 19). Egg pond communities have 2 major clusters based on depth. The first (Egg (1)) is from the top depths 0-38 cm, the other (Egg (2)) is from lower depths (40-48 cm). Salt pond stands out as being extremely unique, exhibiting <20% similarity to all ponds sampled.

A reduced number of samples were analysed using cyanobacterial ARISA as this method was only used to look more specifically at the cyanobacterial structure in the Egg depth profile, with two other ponds (P70E and Legin) used for comparison. Cyanobacterial ARISA results are represented as a dendrogram (Figure 21) and a MDS plot (Figure 22). The dendrogram reveals that the cyanobacterial communities from each pond are unique from other pond communities. The cyanobacterial MDS Plot (Figure 22) shows that at the 40% similarity, each pond groups with itself (as in the bacterial ARISA). Unlike in the bacterial ARISA MDS plot all depths for the Egg pond profile clustered together at a 40% level of similarity whereas at the same level they formed distinct groups in the bacterial ARISA. However at 60% similarity the Egg (1) and Egg (2) clusters idetified in the bacterial ARISA separate from one another.

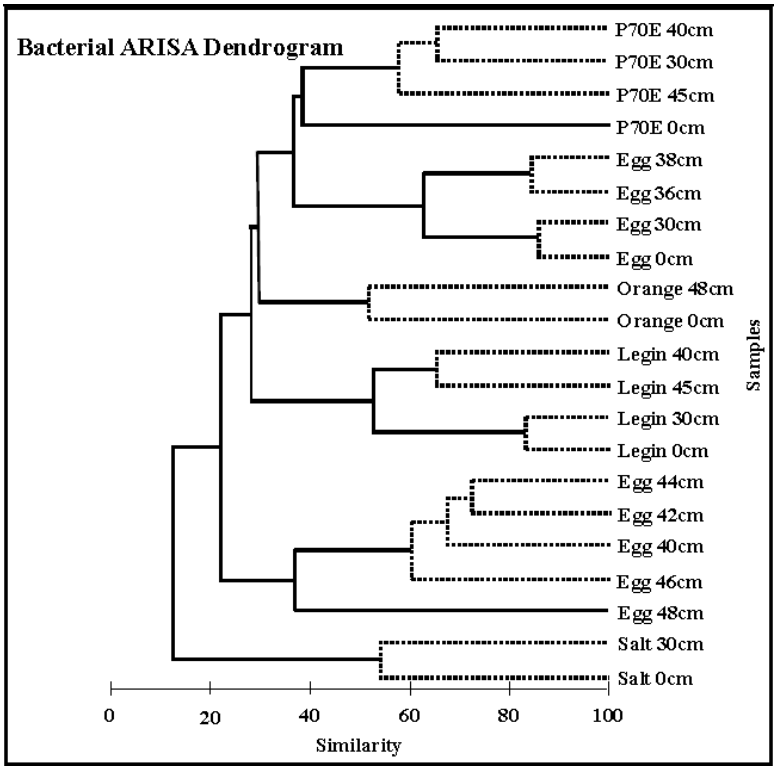

Figure 19: Dendrogram of K-160 Bacterial ARISA Communities. The dendrogram resolves distinct samples and dashed lines denote clusters that are statistically not resolved.

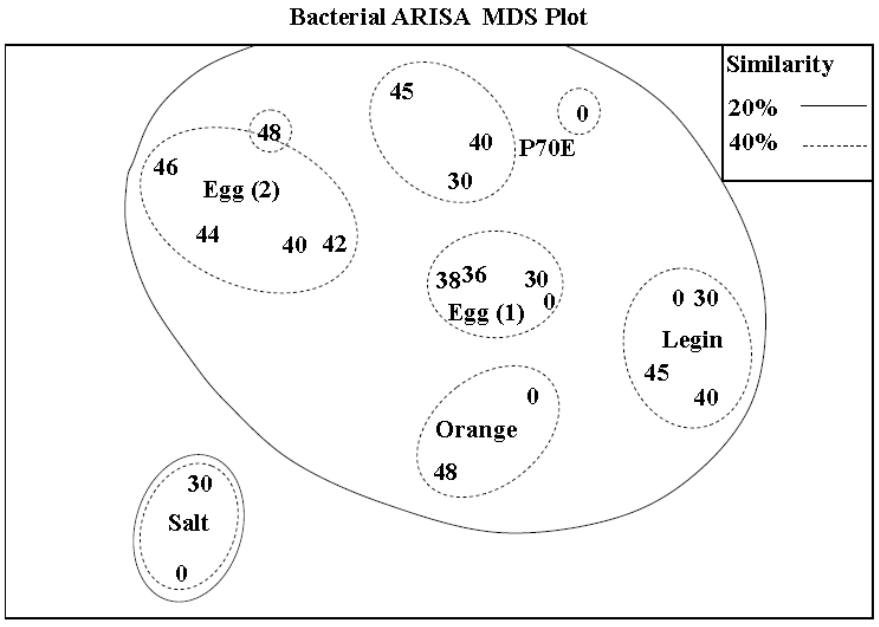

Figure 20: MDS Plot showing percentage similarities of K-160 Bacterial ARISA. Data normalised, converted to presence or absence points and subjected to a Bray Curtis similarity matrix, MDS plot 2D stress 0.15.

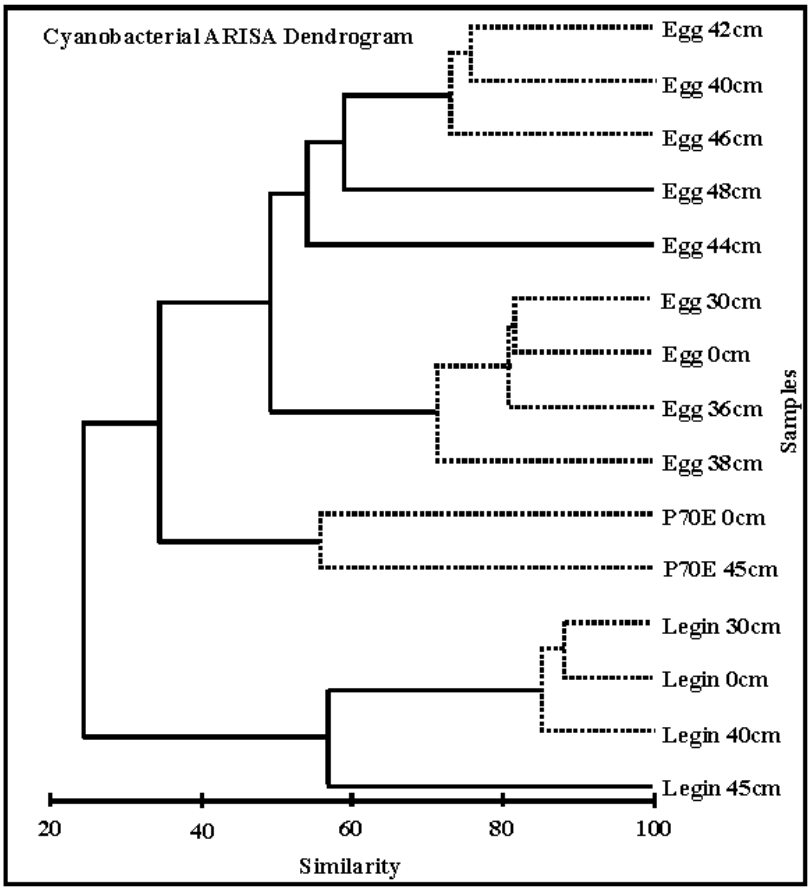

Figure 21: Dendrogram of K-160 Cyanobacterial ARISA Communities

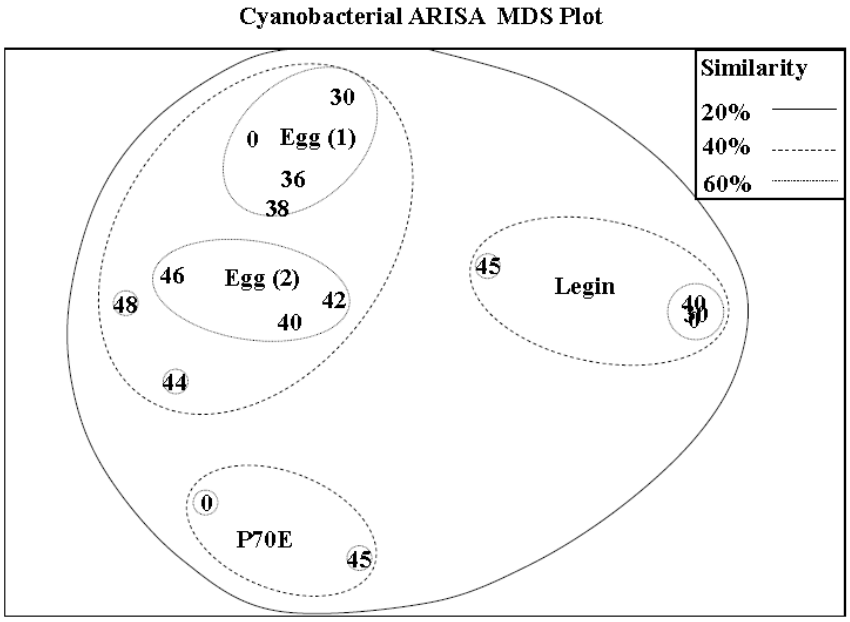

Figure 22: MDS Plot showing percentage similarities of K-160 Cyanobacterial ARISA. Data normalised, converted to presence or absence points and subjected to a Bray Curtis similarity matrix, MDS plot 2D stress 0.09

BEST analysis was conducted using PRIMER 6 software (Table 1) to examine how differences between the bacterial or cyanobacterial populations correlate to differences in the geochemical profiles. Both cyanobacterial and bacterial community structure differences appear well correlated to conductivity as a major contributing factor throughout the ponds studied: conductivity and Ag^{109} were the best explanatory variables for cyanobacterial populations (Spearman's $\rho = 0.662$); The same analysis revealed temperature, conductivity and V^{51} in as major explanatory variables in bacterial populations (Spearman's $\rho = 0.698$). If Egg Pond is separated from the other ponds and analysed on its own, higher probabilities are achieved for community linkages to geochemical variables within Egg pond. The Egg cyanobacterial population appears moderately influenced by pH, which has the highest probability as a single driver of diversity (Spearman's $\rho = 0.732$). For Egg bacterial populations both pH and conductivity were determined to be strongly correlated to bacterial diversity changes, a Spearman's ρ of 0.927 for pH and 0.926 for conductivity.

Table 2: BEST analysis results cyano/bacterial ARISA. A Spearmans rank correlation of 1.0 would indicate a 100% linkage between the variable/s selected and the differences in populations.

	BEST Analysis Results	
Variables		
1	Depth	
2	Temp (C + 10)	
3	DO (mg/L)	
4	Conductivity (mS/cm)	
5	pH	
6	Phosphate (ppm)	
7	NO_2 (ppm)	
8	NH_4 marine (ppm)	
9	Al^{27}	
10	V^{51}	
11	Ag^{109}	

Cyanobacterial ARISA			Bacterial ARISA		
	Correlation	**Variable**		**Correlation**	**Variable**
All Ponds	0.662	4,11	**All Ponds**	0.698	2,4,10
	0.639	2,4,11		0.697	2,4
	0.635	4,7,11		0.675	4,10
	0.623	2,4,7,11		0.654	4
	0.591	4,5,11		0.652	2-4,10
Only Egg	0.734	3,11	**Only Egg**	0.927	5
	0.732	5		0.927	5,7
	0.732	5,7		0.926	4
	0.730	4,11		0.926	4,7
	0.714	3,5		0.925	4,5

2.5 Discussion

Bratina Island meltwater ponds are excellent model ecosystems allowing an extensive study of highly variable environments, both geochemically and biologically, within a localised area (DeMora et al., 1996). The focus of this study was to determine if the unique geochemistry previously reported in the Bratina ponds support unique microbial communities.

As other studies have shown, Bratina Island ponds have their own unique geochemistries . Most of these geochemical variables have the ability to change rapidly during and between seasons, as seen when comparing a number of studies (Hitzfeld et al., 2000; Jungblut et al., 2005; Schmidt et al., 1991). However, comparison with previous work reveals that the geochemistry of certain parameters can be stable over considerable time scales (James et al., 1995; Wait et al., 2006). For example, the pH in Salt pond was within the range seen by Wait et al. (2006) and approximately the same value as found by James et al, (1995). Oxygen concentration varied between ponds, generally increasing with depth, as seen in a number of studies (Wait et al, 2006; (Matsumoto et al., 1992). The NH_4

levels in this study compare to those observed previously (James et al., 1995), with none detected in Legin or P70E, and similar levels in Salt. These studies indicate that there is some geochemical stability within ponds, however seasonal changes and variations in annual meltwater input can dramatically influence pond geochemistry which can in turn alter the microbial populations within an environment, but to what extent?

Analysis of ARISA fragments has allowed comparison and contrasting of community structure differences within and between ponds and through statistical analysis these differences can be realted to their geochemical environment. There are a number of limitations to using ARISA to compare population structure between communities. These include run-to-run variation, and the under or over estimation of microbial biodiversity leading to an incomplete view of community structure (Anderson and Cairney, 2004). ARISA can overestimate diversity due to single organisms with slightly different ITS regions being represented as different organisms, or the fact that one organism can contain more than one 16S rRNA gene. It can underestimate diversity by incomplete lysis of all community organisms before amplification, because "universal" primers do not amplify all target genes in a community, or because two functionally different species have very similar 16S rRNA genes (Popa et al., 2009). Regardless, ARISA is a reliable method which allows investigation of population differences (Soo et al., 2009; Wood et al., 2008) and has yielded a number of intriguing results in this study.

The AFL numbers gained from the ARISA data indicates that the relative diversity level between samples is similar. The highest level of diversity was seen in pond P70E (one of the least geochemically extreme ponds) and the lowest

diversity in Salt Pond, the most geochemically extreme. However, these levels of diversity were not consistent between all samples. For example the lowest level of Egg pond contained a similar conductivity level to Salt pond however had a diversity level above the mean level for all ponds. Although it is generally thought that extreme environments do limit biodiversity (Hacene et al., 2004) a study by Jiang et al, 2010 did find a positive correlation between salinity and diversity across hypersaline lakes on the Tibetan Plateau.

Although diversity levels between ponds were relatively similar the structure of these populations identified through the differences in Bacterial ARISA AFL patterns was highly variable and surprisingly, pond specific. Community profiles from different depths within a pond grouped closer to one another than to ARISA profiles from other ponds, and the clustering was congruent with clustering seen in the MDS plot of geochemistry. The two depth related groupings of Egg indicated that variations within community structure are driven by the distinct geochemical changes in the water column. The ARISA profile of the deepest point in Egg pond exemplified this observation containing a different ARISA profile as well as the highest conductivity and lowest pH within Egg pond, both which have been found to be drivers of population shifts in other studies (de Figueiredo et al., 2010; Sabbe et al., 2004). Overall these results show that bacterial populations are clearly different between and within ponds. The separate clusters of bacterial ARISA profiles combined with geochemical stratification of the water column indicated that Egg pond was a good candidate for a further study of cyanobacterial populations.

Cyanobacterial ARISA fingerprints indicated similar clustering of pond depths (at 40% similarity), however Egg showed greater similarity between depths than in the bacterial ARISAs at 40% similarity. At 60% similarity, patterns emerged for cyanobacterial ARISAs as was seen with the bacterial ARISAs, with Egg forming two groups for different depths. This provides further support for differences in populations between ponds. The groupings determined by ARISA in this study are similar to those found by microscopic investigation of depth integrated samples collected from the same ponds in a previous study (James et al., 1995). This microscopic analysis of the planktonic populations classified three groups of ponds based on size, conductivity and the presence and abundance of various planktonic groups. Each of the three groupings included ponds sampled in this study, P70E, Legin and Salt which at 40% similarity in bacterial ARISA also separate into the three groups identified by James et al., 1995. This demonstrates how these ponds consistently harbour a different geochemical environment and biological population over large time scales, however, determination of what factor drives the unique population has not been completed.

BEST analysis is a statistical technique which has been successfully utilised to link geochemical variability to changes in the biological community profiles gained from fingerprinting data (Smith et al., 2010; Soo et al., 2009; Wood et al., 2008). In this study BEST analysis of both ARISA studies indicated a correlation between differences in conductivity contributing to cyanobacterial and bacterial community structure changes for all of five of the ponds studied. When Egg was analysed on its own it was discovered that cyanobacterial community structure was significantly correlated to changes in pH as a single

driver, and bacterial populations were driven by conductivity as the major single driver. These results indicate that although Egg pond pH and conductivity may have a significant influence on microbial communities these is not necessarily the driving factors in all five ponds. pH and conductivity have previously been identified as drivers of community structure by numerous studies in aquatic environments (de Figueiredo et al., 2010; Jiang et al., 2010; Kaartokallio et al., 2005; Laque et al., 2010; Sabbe et al., 2004), and a microscopic study of the same Bratina ponds also noted some differences in populations possibly linked to conductivity (James et al., 1995).

An important consideration is that the time of year in which pond samples are taken dramatically influences geochemistry (Hawes et al., 1999; James et al., 1995; Matsumoto et al., 1992; Wait et al., 2006), which will in turn influence the resident microbiology. Samples for this study were collected in early December 2009, this was early in the season so ponds were not at their maximal biological activity compared to those previously collected in January (James et al., 1995; Wait et al., 2006). Since the ponds are meltwater fed, the amount of ice and snow input each year is important to the overall soluble ion concentration of the ponds. In 2009 the ponds were significantly shallower than in other study years, Pond P70E was 0.85 m deep in January 1992 (James et al., 1995; Wait et al., 2006), and was at least 0.8 m deep in October 2003 and at least 1 m deep in January 2004 (Wait et al., 2006), compared to a significantly shallower 0.45 m in this study. In 1991/92 Salt pond depth was measured to be 25 cm (James et al., 1995), and was at least 30 cm in October 2003 and 50 cm in January 2004 (Wait et al., 2006), compared to 30 cm in this study. Furthermore Legin Pond was not completely

thawed, as was found at a similar time of year in a previous study (James et al., 1995). It still had an ice cap and mushroom shaped ice centre, which meant that the Legin samples were not taken from the centre of the pond but from the leading edge of the ice centre. Considering these conditions, comparative analysis between samples in this study are meaningful, but caution should be used when comparing these results to those taken from the same pond at another time point.

Even though the meltwater ponds near Bratina Island are of marine origin many of them share a number of characteristics with the ponds and lakes scattered through the Antarctic terrestrial ecosystem. Stratification within the water column (Matsumoto et al., 1992) and microbial changes down the water column (Bell and Laybourn-Parry, 1999) are common to both systems (Matsumoto et al., 1992). To date more research has been conducted on lakes as their biology is generally more interesting, sampling through the centre of the lake is easier and they remain more stable from year to year (Fernandez-Valiente et al., 2001; Laybourn-Parry et al., 2002; Mikell et al., 1986). However, due to this stability the immediate effects of seasonality and long term effects of climate change are difficult to assess in the larger systems of the lakes. Due to their small size Bratina Island ponds are far more sensitive to these changes and more tractable to study.

2.6 Conclusion

The water column of five Bratina Island meltwater ponds were examined to understand how local geochemistry effects the structure of the resident

microbial community within the ponds, using fine scale biological and geochemical sampling. The biological samples were genotyped using a DNA fingerprinting method (ARISA), which allowed the systematic comparison of bacterial and cyanobacterial populations supported in each pond. Significant differences between and within pond geochemistry and microbial diversity were detected. Specifically, when examining Egg pond, a clear linkage between the cyanobacterial community and pH, and the bacterial community with conductivity, were identified as single variables that appear to influence microbial diversity. This reinforces the belief that geochemistry can be a driver of community structure. However, the microbial populations of a single Bratina Island pond may be found to have individual drivers, different to those of other ponds. This makes these ponds extremely valuable as a scientific resource as they can be considered to be completely isolated extreme aquatic ecosystems sharing the same climate, but representative of the geochemical diversity of aquatic ecosystems (both ponds and lakes) scattered across the Antarctic continent.

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Chapter 3: General Conclusions

This chapter is intended to review and conclude the preceding chapters and detail future work that should be conducted on the microbial ecology of these meltwater ponds. The main objective of this study was to identify and describe the biological, physical and geochemical differences between and within five ponds found on the McMurdo Ice Shelf near Bratina Island, Antarctica and to identify if the environmental conditions within each pond drive the structure of the microbial populations found within them.

Antarctica offers an untouched laboratory containing some of the most extreme and diverse environments in the world each with its own unique physicochemical properties. Bratina Island meltwater ponds are excellent model ecosystems for biogeochemical studies due to their highly variable environments both geochemically and biologically (DeMora et al., 1996). The mat and sediments of many of these ponds have been extensively studied (Fernandez-Valiente et al., 2001; Hawes et al., 2001; Sjoling and Cowan, 2003), as well as the geochemistry of the water column (James et al., 1994; Matsumoto et al., 1992; Wait et al., 2006). However, this project aimed to address the significant lack of biological information on the planktonic populations found within ponds water columns.

DNA fingerprints were successfully produced from spatial high resolution samples collected from the Bratina Island ponds. When combined with complementary physical and geochemical data, the differences between and

within ponds were determined. When examining Egg pond separately, a clear linkage of cyanobacterial abundance to pH, and bacterial abundance to conductivity, was resolved as single variables that appear to influence microbial community structure. This does not occur when the five ponds are analysed as a group. These findings indicate that each pond has different environmental drivers but that extreme gradients can cause changes in the microbial populations. These finding are supported by numerous studies which have identified pH and conductivity as drivers of bacterial community dynamics in a number of other aquatic environments (de Figueiredo et al., 2010; Jiang et al., 2010; Kaartokallio et al., 2005; Laque et al., 2010; Sabbe et al., 2004).

To the best of our knowledge this project was the first molecular study of the water column of these ponds. Although this study succeeded in an identification of the physical and geochemical conditions within the water columns of the five ponds studied and linked these variables with the relative differences between the pond/depth microbial populations, due to the restricted timeframe of a masters thesis there were a number of areas that would have been desirable to investigate.

It would have been beneficial to have taken more samples per pond to draw on for analysis so that each pond may have been analysed separately, similar to Egg pond, to definitively determine if each pond has its own unique driver. In addition, a greater range of geochemical variables from each sample could have been included in the study, such as the C and N concentration. Regrettably, due to the hypersalinity of some ponds, the samples had to be diluted 1000 fold which

may have lost data from some potentially important lower concentration metal ions from the analysis.

This study provides a foundation of work to expand into a much larger study which I will be conducting through my PhD. This will include some or all of the following:

1. The dynamics between the physical/geochemical/biological properties within the ponds at different times of the year.
2. Conducting next generation sequencing on pond samples to allow for specific identification of what organisms are present and in what abundance.
3. The microbial changes down the water column at higher resolution sampling intervals (mm intervals).
4. The differences between microbial populations in more ponds of different geochemistry (other ponds within this pond cluster).
5. The differences between those ponds found on the McMurdo ice shelf and on continental Antarctica.
6. The metabolic changes occurring to these microbes and also to examine the water column communities as well as that of mat and sediment samples to allow a thorough view of the entire ecosystem.

It is unfortunate that these areas of research could not be explored with the limited time frame given for a master's thesis. It is our intention to explore these areas through a proposed project recently submitted to Antarctica New Zealand by the Thermophile Research Unit.

Overall this project showed that the Bratina ponds have a impressive range of geochemical conditions that directly influence the microbial populations

residing within them, and that in the water column of stratified ponds populations can rapidly change as the geochemistry changes. Despite their close proximity to one another these ponds are, in effect, separate ecosystems that have unique environmental drivers making them a valuable resource for future studies.

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Appendix 1: K160 Geochemistry

	K160 (09-10 Season) Geochemistry									
	Depth	**Temp (C)**	**DO (mg/L)**	**Conductivity (mS/cm)**	**pH**	**Phosphate (ppm)**	**NO2 (ppm)**	**NH4 marine (ppm)**	**Li 7**	**Na 23**
Egg 0	0	1.55	11.52	8.2	9.46	1.83374	0.00192	0.01218	26.55	608578
Egg 30	30	0.75	11.65	8.21	9.47	0.01139	0.00195	0.00162	25.3	1042225
Egg 36	36	-0.92	17.06	9.86	9.45	0.01313	0.002	0.00684	25.2	1092516
Egg 38	38	-0.78	14.65	10.35	9.41	0.00396	0.00195	0.00427	33.1	1085431
Egg 40	40	0.17	19.62	45	8.75	0.0175	0.00191	0.02708	69.85	7844427
Egg 42	42	-0.27	25.11	36.7	8.76	0.01045	0.00202	0.01887	63	6081635
Egg 44	44	-0.78	27.3	51.6	8.63	0.01978	0.0018	0.02802	84.95	8010980
Egg 46	46	-1.06	23.48	64.1	8.51	0.01302	0.002	0.03422	98.85	10265408
Egg 48	48	0.62	24.46	93.6	8.11	0.23069	0.00202	1.38791	124.7	15648235
Legin 0	0	0.81	9.71	1.99	6.35	0.01725	0.00522	0	15.85	52815.65
Legin 30	30	1.11	11.84	0.43	10.11	0.0117	0.00482	0	15.6	53272
Legin 40	40	0.12	14.64	3.26	8.52	0.00436	0.00212	0	18.95	196895.7
Legin 45	45	0.2	17.02	2.8	9.92	0.00165	0.0021	0	22.7	316490.5
P70E 0	0	3.29	9.64	7.03	8.09	0.00167	0.03246	0	28.55	665577.4
P70E 30	30	3.76	11.93	10.78	7.3	0.0355	0.01846	0	30.55	501026.2
P70E 40	40	2.06	7.42	18.41	8.16	0.00489	0.02458	0	29.45	986136
P70E 45	45	1.98	4.36	21.63	8.02	0.00916	0.00317	0.00911	37.65	2095524
Salt 0	0	4.39	11.31	79.1	8.8	0.03173	0.00203	0.03804	93.85	24289453
Salt 30	30	5.11	9.74	110.9	9.02	0.06473	0.00234	0.07429	109.8	33450433
Orange 0	0	-1.97	10.15	1.084	9.6	0.42872	0.00298	0.10008	19	271116.3
Orange 48	48	-3.53	11	5.42	9.55	0.0572	0.00237	0	25.6	754638.4

	K160 (09-10 Season) Geochemistry									
	S 34	**K 39**	**Ca 43**	**V 51**	**Cr 52**	**Fe 54**	**Mn 55**	**Co 59**	**Ni 60**	**Zn 68**
Egg 0	33382.97	29768.1	19223.91	6.05	0	0	12	0	0	0
Egg 30	50442.12	49704.7	25379.47	7.77	0	0	19.4	0	0	0
Egg 36	51350.4	55175.35	26481.15	6.17	0	105.16	27.06	0	0	0
Egg 38	43723.12	53158.85	25225.55	5.3	0	0	25.86	0	0	0
Egg 40	256300.6	229619.9	65775.77	0.37	0	0	385.7	0	2.92	0
Egg 42	191391.1	186786.7	57097.3	6.27	0	0	285.55	0	2.09	0
Egg 44	306792.9	248265.4	75073.09	0.58	0	0	462.03	0	3.93	0
Egg 46	374317.9	326471.7	78247.37	1.84	0	0	539.15	0.51	4.67	0
Egg 48	460123.9	396130.5	94337.38	2.21	0	0	498.79	1.03	6.63	0
Legin 0	0	2362.95	3532.93	4.87	0	0	0.2	0	0	0
Legin 30	9235.11	2700.8	3966.04	4.42	0	0	0.35	0	0	0
Legin 40	39578.17	7943.5	12217.56	5.82	0	0	0.86	0	0	0
Legin 45	58851.86	13941.3	19189.18	8.51	0	0	2.79	0	0	0
P70E 0	64381.33	29185.8	81021	3.25	0	0	56.37	0	0.41	0
P70E 30	76305	21579.6	80882.24	2.44	0	139.97	82.61	0	1.17	0
P70E 40	103098.8	41435.7	130098.4	1.73	0	0	262.38	0	3.01	10.05
P70E 45	225877.6	78003.4	292081	0.26	0	0	1091.97	0	9.87	0
Salt 0	10826266	500161.9	196405.1	11.7	0	0	111.23	1.13	14.59	0
Salt 30	17512464	542671.9	241616	18.16	0	0	281.31	1.49	14.2	0
Orange 0	22853.12	12013.8	9349.7	18.77	24.93	0	7.33	0	0	0
Orange 48	60368.17	29642.5	7461.44	13.4	0	0	6.18	0	0	0

	K160 (09-10 Season) Geochemistry								
	Sr 88	**Ag 109**	**Cd 111**	**In 115**	**Ba 137**	**Tl 205**	**Pb 207**	**Bi 209**	**U 238**
Egg 0	297.14	0	0.25	0	12.25	0	0	0	0.43
Egg 30	437.15	0.05	0.44	0	5.75	0	0	0	0.67
Egg 36	460.87	0.2	0	0	11	0	0	0	0.76
Egg 38	489.5	0	0	0	24.05	0	0	0	0.62
Egg 40	2273.61	0.34	0	0	48.4	0	0	0	1.76
Egg 42	1818.62	0	0	0	39.95	0	0	0	1.47
Egg 44	2535.48	0.98	0.05	0	51.4	0	0	0	2.08
Egg 46	2916.47	0.54	0	0	83.3	0	0	0	2.74
Egg 48	3774.11	0.88	0	0	65.1	0	0	0	3.06
Legin 0	60.18	1.04	0	0	3.4	0	0	0	0.05
Legin 30	61.86	0.94	0	0	8.25	0	0	0	0.05
Legin 40	283.08	0.59	0	0	12.15	0	0	0	0.24
Legin 45	483.08	0.34	0	0	5.85	0	0	0	0.43
P70E 0	1681.89	1.74	0.2	0	16.45	0	0	0	1.71
P70E 30	1544.86	0.35	0	0	11.45	0	0	0	1.22
P70E 40	3102.28	1.44	0	0	20.25	0	0	0	2.19
P70E 45	7749.36	1.93	0.2	0	31.75	0	0	0	3.5
Salt 0	14918.7	0	0.05	0	117.2	0	0	0	13.29
Salt 30	16489.04	0	0	0	156.5	0	0	0	12.33
Orange 0	174.9	1.44	0.8	0.35	12.1	0.7	0	1.19	1.04
Orange 48	230.09	3.83	0	0.05	16.95	0	0	0.65	1.92

Appendix 2: Ponds sampled

Orange Pond

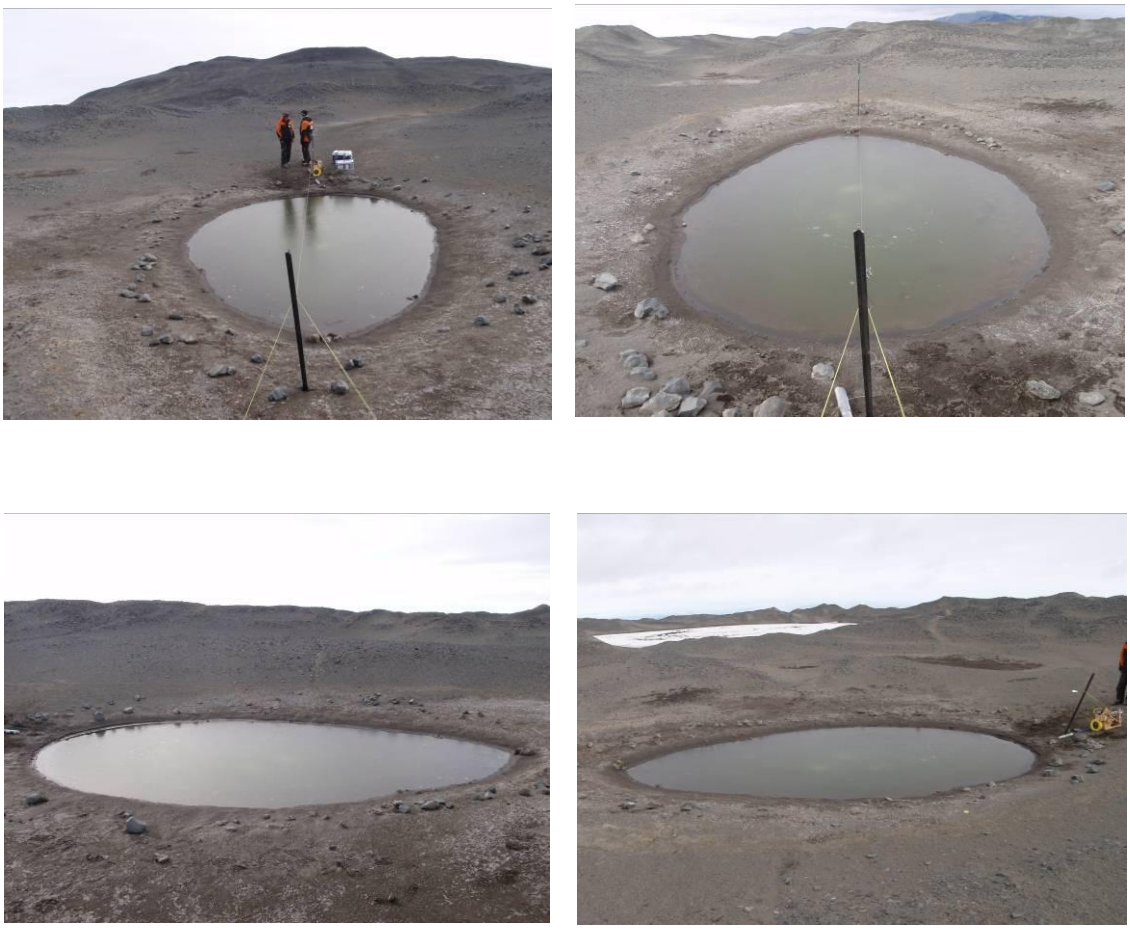

Figure 23: View from all sides of Orange Pond 9.12.09. Photographed by Stephen Archer

Legin Pond

Figure 24: View from all sides of Legin Pond 10.12.09. Photographed by Stephen Archer

Egg Pond

Figure 25: View from all sides of Egg Pond 11.12.09. Photographed by Stephen Archer

Salt Pond

Figure 26: View from all sides of Salt Pond 11.12.09. Photographed by Ian McDonald

Pond P70E

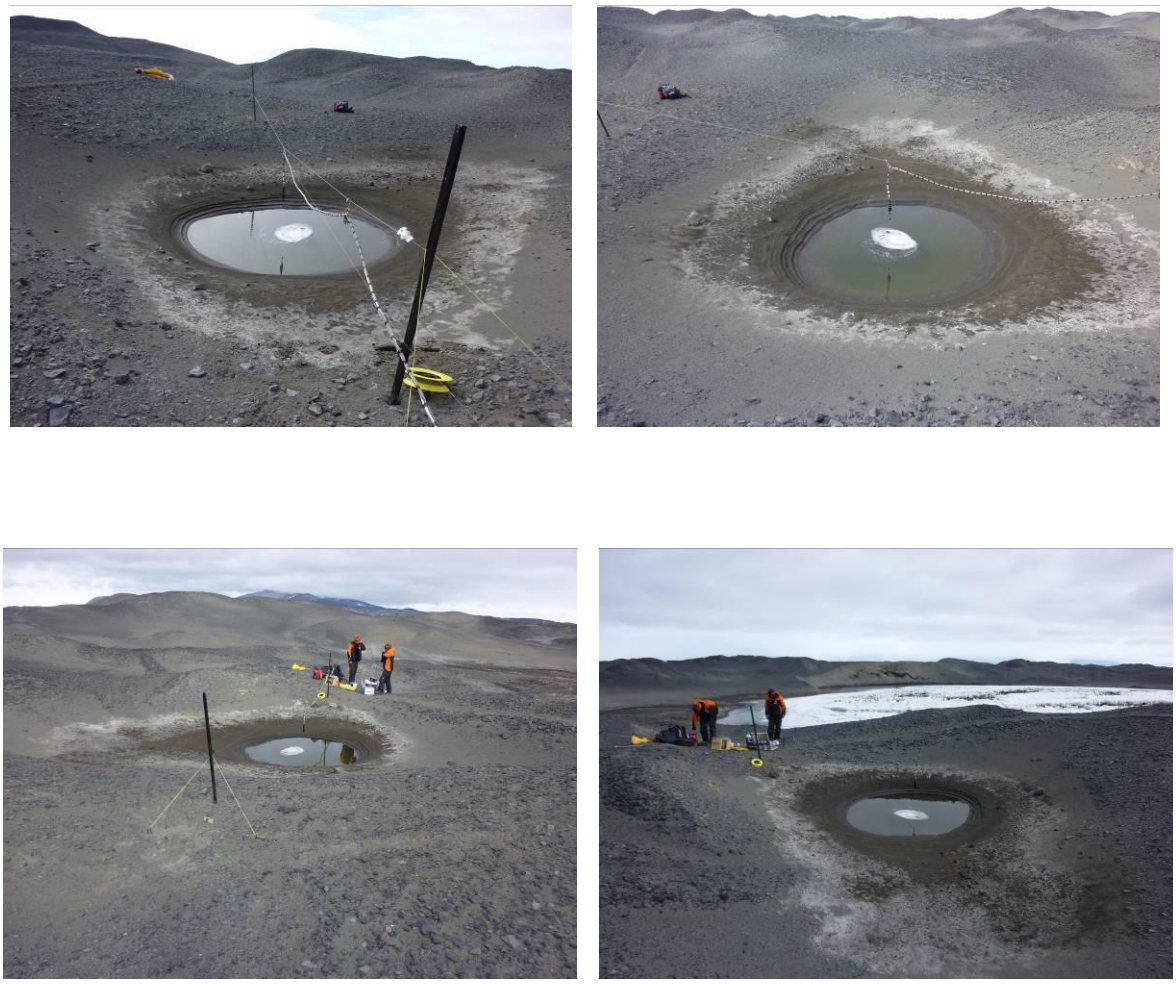

Figure 27: View from all sides of Pond P70E 10.12.09. Photographed by Ian McDonald

Appendix 3 – Tygon Tubing specification sheet

TYGON® R-3603 Laboratory and Vacuum Tubing

The most consistently reliable tubing for the transfer of liquids and gases, Tygon® Laboratory Tubing handles virtually all inorganic chemicals found in today's laboratories.

Provides Versatility for Today's Laboratories

Crystal clear and flexible, Tygon® Laboratory Tubing handles virtually all inorganic chemicals found in the lab. It is non-oxidizing and non-contaminating.

Long-lasting and crack-resistant, Tygon® Laboratory Tubing is less permeable than rubber tubing. The glassy-smooth inner bore helps prevent buildup so that cleaning is facilitated. Coils are marked at 3-foot intervals for easy measuring.

Tygon® Laboratory Tubing may be safely sterilized by coiling loosely in surgical muslin and autoclaving at 15 psi of steam for 30 minutes. Following initial sterilization, a shrinkage of 2%-4% may be noted. However, no further shrinkage should occur with subsequent sterilizations.

Due to its excellent chemical resistance, Tygon® Laboratory Tubing may also be sterilized chemically or by gas (ethylene oxide). Coil tubing and wrap loosely in muslin or linen and follow the directions of your sterilization equipment manufacturer. Where aeration equipment such as vacuum and drying is required, follow the recommended times for degassing to ensure against gas retention and to reduce any residual concentration below known safety limits.

Secure Fitting Attachment

Tygon® Laboratory Tubing has a Shore A Durometer of 55, which enables it to be slipped over fittings quickly and easily, and to grip securely. Its ability to bend readily to sharp radius curves speeds and simplifies laboratory set-ups.

Tygon® Laboratory Tubing is specially formulated for resistance to flex-fatigue and abrasion. In many peristaltic pump applications, it will outlast its nearest competitor by at least 2 to 1. As a tubing for instrumentation connection, vent, drain, and other general laboratory applications, Tygon® Laboratory Tubing offers superior life, which minimizes the labor and expense of replacement.

Available in Vacuum Tubing Sizes

Tygon® Vacuum Tubing has extra heavy walls that will withstand a full vacuum at room temperature (29.9" [759mm] of mercury at 73°F [23°C] and up to 27" [686 mm] of mercury at 140°F [60°C]). Like standard Tygon® Laboratory Tubing, Tygon® Vacuum Tubing resists most inorganic chemicals and can be used in corrosive atmospheres. Clarity allows monitoring for possible backups, which could result in equipment damage. Tygon® Vacuum Tubing will not swell closed if vacuum pump oil should happen to back into it. Tygon® Vacuum Tubing has a low vapor pressure – less than 3×10^{-2} mm Hg at 140°F.

Consistent performance for reliable analysis

Features/Benefits

- Outstanding chemical resistance
- Lot-to-lot consistency for reproducible results
- Increases productivity in peristaltic pumps – outlasts other clear tubing 2 to 1
- Standard sizes available to hold full vacuum at room temperature
- Smooth, polished inner wall
- Non-aging and non-oxidizing
- Meets FDA CFR part 175.300 criteria

Typical Applications

- General laboratory
- Analytical instruments
- Peristaltic and vacuum pumps
- Ideal for condensers, incubators, desiccators, gas lines and drain lines

SAINT-GOBAIN
PERFORMANCE PLASTICS

TYGON® R-3603 Tubing Inventoried Sizes

Saint-Gobain Part Number	I.D. (inches)	O.D. (inches)	Wall Thickness (inches)	Length (Feet)	Minimum Bend Radius (inches)	Maximum Working Pressure at 73°F (psi)*	Vacuum Rating, in. of Mercury at 73°F
AAC00001	1/32	3/32	1/32	50	1/8	60	29.9
AAC00002	1/16	1/8	1/32	50	1/4	45	29.9
AAC02002	1/16	1/8	1/16	100	1/8	45	29.9
AAC04002	1/16	1/8	1/16	50-0	1/8	45	29.9
AAC00003	1/16	1/8	1/16	50	1/8	35	29.9
AAC00004	1/32	5/32	1/32	50	3/8	10	29.9
AAC02004	1/32	5/32	1/32	100	3/8	10	29.9
AAC00005	1/32	7/32	1/16	50	1/4	15	29.9
AAC00006	1/8	1/16	1/32	50	1/2	25	20
AAC04006	1/8	1/16	1/32	50-0	1/2	25	20
AAC00007	1/8	1/4	1/16	50	3/8	45	29.9
AAC02007	1/8	1/4	1/16	100	3/8	45	29.9
AAC04007	1/8	1/4	1/16	50-0	3/8	45	29.9
AAC00009	3/32	7/32	1/32	50	3/8	20	12
AAC00010	3/32	9/32	1/16	50	1/2	15	29.9
AAC02010	3/32	9/32	1/16	100	1/2	15	29.9
AAC00011	1/16	1/4	1/32	50	1	20	9
AAC00012	1/16	5/16	1/16	50	5/8	10	29.9
AAC02012	1/16	5/16	1/16	100	5/8	10	29.9
AAC04012	1/16	5/16	1/16	50-0	5/8	10	29.9
AAC00013	1/16	3/8	1/32	50	1/2	40	29.9
AAC00014	1/16	3/16	1/8	50	3/8	35	29.9
AAC00016	1/4	5/16	1/32	50	1-1/2	15	5
AAC00017	1/4	3/8	1/16	50	1	25	20
AAC02017	1/4	3/8	1/16	100	1	25	20
AAC04017	1/4	3/8	1/16	50-0	1	25	20
AAC00019	1/4	3/16	1/32	50	3/4	15	29.9
AAC00019	1/4	1/2	1/8	50	1/2	40	29.9
AAC02019	1/4	1/2	1/8	100	1/2	40	29.9
AAC00022	5/16	7/16	1/16	50	1-1/2	20	13
AAC00023	5/16	1/2	3/32	50	1	30	29.9
AAC00024	5/16	9/16	1/8	50	3/8	15	29.9
AAC00025	5/16	5/8	5/32	50	3/4	45	29.9
AAC00027	3/8	1/2	1/16	50	1-1/2	20	9
AAC00029	3/8	9/16	3/32	50	1-1/8	25	21
AAC00029	3/8	5/8	1/8	50	1-1/8	30	29.9
AAC00032	7/16	9/16	1/16	50	3/4-4	15	7
AAC00033	7/16	5/8	3/32	50	1-1/4	20	15
AAC00034	7/16	11/16	1/8	50	1-1/8	25	29
AAC00036	1/2	5/8	1/16	50	1-3/8	10	5
AAC00037	1/2	11/16	3/32	50	3/4-4	20	12
AAC02037	1/2	11/16	3/32	100	3/4-4	20	12
AAC00039	1/2	3/4	1/8	50	1-1/2	25	21
AAC00039	1/2	13/16	5/32	50	1-1/2	30	29.9
AAC00041	5/16	3/4	3/32	50	2-1/2	15	9
AAC00042	5/16	13/16	1/8	50	2	20	17
AAC00043	5/8	13/16	3/32	50	1	15	7
AAC00046	5/8	7/8	1/8	50	2-1/8	20	13
AAC00047	5/8	15/16	5/32	50	2	25	21
AAC00050	11/16	7/8	3/32	50	3-1/2	15	6
AAC00053	3/4	1	1/8	50	3-1/4	19	9
AAC00054	3/4	1-1/16	5/32	50	2-3/4	20	15
AAC00055	3/4	1-1/8	3/16	50	2-1/8	25	21
AAC00057	3/4	1-1/4	1/4	50	2	30	29.9
AAC00059	7/8	1-1/8	1/8	50	4-1/8	15	7
AAC00060	7/8	1-3/16	5/32	50	3-1/2	20	11
AAC00062	1	1-1/4	1/8	50	4-3/4	15	5
AAC00064	1	1-3/8	3/16	50	4	20	12
AAC00065	1	1-1/2	1/4	50	3	25	21
AAC00069	1-1/8	1-1/2	3/16	50	4-1/2	19	9
AAC00069	1-1/4	1-1/2	1/8	50	7-3/8	12	3
AAC00070	1-1/4	1-5/8	3/16	50	5-1/2	15	7
AAC00071	1-1/4	1-3/4	1/4	50	4-3/8	20	19
AAC00073	1-1/2	1-3/8	3/16	50	3-1/4	15	5
AAC00074	1-1/2	2	1/4	50	5-3/8	19	9
AAC00076	1-3/4	2-1/4	1/4	50	3-1/2	16	7
AAC00079	2	2-1/2	1/4	50	3-3/8	15	5
AAC05079	2	2-1/2	1/4	20	3-3/8	15	5

*Working pressures are calculated at a 3.5 ratio relative to burst pressure using ASTM D1599.

The values listed for working and burst pressures are derived from tests conducted under controlled laboratory conditions. Many factors will reduce the tubing's ability to withstand pressures including temperature, chemical attack, stress, pulsation and the attachment to fittings. It is imperative that the user conduct tests simulating the conditions of the application prior to specifying the tubing for use.

TYGON® is a registered trademark.

TYGON® R-3603 Tubing Inventoried Sizes

Saint-Gobain Part Number	I.D. (inches)	O.D. (inches)	Wall Thickness (inches)	Length (Feet)	Minimum Bend Radius (inches)	Maximum Working Pressure at 73°F (psi)*	Vacuum Rating, in. of Mercury at 73°F
Vacuum Sizes							
AAC00035	1/16	3/16	1/16	50	1/4	60	29.9
AAC00020	1/4	5/8	1/16	50	1/2	30	29.9
AAC00030	1/8	3/8	1/4	50	5/8	30	29.9
AAC00040	1/2	1-1/8	5/16	50	3/8	45	29.9
AAC00060	3/4	1-3/8	1/8	50	1	40	29.9
AAC00050	3/4	1-1/2	1/8	50	1-1/2	15	29.9
AAC00065	1	2	1/2	50	1-3/8	15	29.9
AAC05065	1	2	1/2	20	1-3/8	15	29.9
Unipaks (10/10' long these per carton)							
AACUN007	1/8	1/4	1/16	10/10'	1/8	45	29.9
AACUN012	1/16	5/16	1/16	10/10'	5/8	10	29.9
AACUN017	1/4	3/8	1/16	10/10'	1	25	20
AACUN019	1/4	1/2	1/8	10/10'	1/2	40	29.9
AACUN023	1/8	1/2	1/16	10/10'	1-1/2	10	9
AACUN035	1/2	5/8	1/16	10/10'	2-1/8	10	5
AACUN037	1/4	1	1/8	10/10'	3-1/4	19	9
AACUN062	1	1-1/4	1/8	10/10'	4-3/4	15	5
Vacuum Unipaks (10/10' or 4/10' long these per carton as shown below)							
AACUN015	1/16	3/16	1/16	10/10'	1/4	60	29.9
AACUN020	1/4	5/8	1/16	10/10'	1/2	30	29.9
AACUN030	1/8	3/8	1/4	10/10'	5/8	30	29.9
AACUN040	1/2	1-1/8	5/16	10/10'	3/8	45	29.9
AACUN060	3/4	1-3/8	1/8	10/10'	1	40	29.9
AACUN059	3/4	1-1/2	1/8	10/10'	1-1/2	15	29.9
AACUN062	1	2	1/2	4/10'	1-3/8	15	29.9
Metric Sizes							
Saint-Gobain Part Number	I.D. (mm)	O.D. (mm)	Wall Thickness (mm)	Length (meters)	Minimum Bend Radius (mm)	Maximum Working Pressure at 73°F (psi)*	Vacuum Rating, in. of Mercury at 73°F
AAC05002	2	4	1	15	7	40	29.9
AAC05003	3	5	1	15	13	30	29.9
AAC05004	4	6	1	15	16	25	210
AAC05005	5	9	1.5	15	19	20	29.9
AAC05006	6	9	1.5	15	22	25	220
AAC05008	7	10	1.5	15	29	20	150
AAC05006	9	12	2	15	29	25	210
AAC05010	10	14	2	15	42	20	140

*Working pressures are calculated at a 3.5 ratio relative to burst pressure using ASTM D1599.

TYGON® R-3603 Tubing Typical Physical Properties

Property	ASTM Method	Value or Rating
Durometer Hardness Shore A, 15 Sec	D2240-02	55
Color	-	clear
Tensile Strength, psi (MPa)	D412-98	1650 (11.4)
Ultimate Elongation, %	D412-98	480
Tear Resistance, lb/inch (kN/m)	D1004-94	125 (22)
Specific Gravity	D792-00	1.19
Water Absorption, % 24hrs.@ 23°C	D570-98	0.24
Compression Set Constant Deflection, % @ 158°F (70°C) for 22 hr.	D395-01 Method B	64
Brittleness By Impact Temp, °F (°C)	D746-98	-58 (-50)
Maximum Recommended Operating Temp, °F (°C)	-	165 (74)
Dielectric Strength, v/mil (kV/mm)	D149-97	600 (239)
Tensile Modulus, @ 100 % Elongation, psi (MPa)	D412-98	650 (4.5)
Tensile Set, %	D412-98	100

Unless otherwise noted, all tests were conducted at room temperature (73°F). Values shown were determined on 0.075" thick extruded strip or 0.075" thick molded ASTM plaques or molded ASTM durometer buttons.

TYGON® Tubing is not intended for use as an implant material.

Saint-Gobain Performance Plastics
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Akron, OH 44305
Tel: 800-758-1554
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SAINT-GOBAIN
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